Effects of sensory ablation on the young and aged ventriculo-olfactory neurogenic system

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Submitted in fulfilment of the requirements of the degree of
Doctor of Philosophy
January 2013
Abstract

Adult neurogenesis is a dynamic field of study, with potential for generating therapies for neurodegenerative disorders. The demonstration of adult brain neurogenesis strengthens the rationale for regenerative therapies for neurodegenerative disorders. The ‘ventriculo-olfactory neurogenic system’ (VONS) is used in the study neurogenesis, migration and differentiation of neural stem cells. Importantly, subventricular zone (SVZ) neuroblasts recapitulate a population of dopaminergic neurons within the olfactory bulb (OB), possibly the only population of dopaminergic interneurons, generated throughout adult life. This study aims to simulate a pathophysiological model for olfactory epithelial cell turnover to demonstrate a link between the olfactory epithelium (OE) and cell proliferation in the SVZ.

In order to examine the relationship between the OE and the SVZ, an ablation protocol was generated. Using a double dose of methimazole, the OE was completely ablated leading to denervation of the olfactory bulb. This denervation resulted in an increase of astrocyte and microglial activity on day 11 which remained elevated up to day 21. This was accompanied by a significant decrease of tyrosine hydroxylase immunoreactivity in type I periglomerular cells on day 17. While there was a decrease in the type II periglomerular cells on day 17, this was shown to be not significant.

Edu and cleaved caspase-3 were used to examine whether the changes in the OB had an effect on the rates of proliferation or cell death in the SVZ. There were no changes seen in the rates of cell death seen. However, cell counts revealed that treated animals exhibited an increase of proliferation in the SVZ on day 11. The ablation protocol was then modified for an aged population to examine the effects of aging on the VONS. After confirming ablation of the OE and denervation of the OB, it was shown that treated animals from the day 14 group exhibited an increase in the number of newly generated cells in the SVZ. This showed that while there was a delay and decrease in overall proliferation between young and aged animals, the aged animals still retain the capability to increase neurogenesis in response to injury.
The migration of cells, generated during the pulse of neurogenesis seen in the SVZ, was then tracked. It was shown that neither sensory ablation nor age affect time taken for cells to migrate from the SVZ to the olfactory bulb. However, the treated animals (young and aged) showed an increase in the number of cells that were recruited to the glomerular layer. When analysed, both the young and aged methimazole treatment animals had an increase in the differentiation rate of TH and calbindin positive interneurons suggesting that sensory ablation may influence the generation of periglomerular subtypes.
Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

_____________________________
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### Abbreviations

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<tr>
<td>AMPA</td>
<td>2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CYP2A5</td>
<td>Cytochrome P450 2A5</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole 2HCl</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
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<tr>
<td>EdU</td>
<td>5-ethyl-2’-deoxyuridine</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EPL</td>
<td>External plexiform layer</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GCL</td>
<td>Granule cell layer</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GL</td>
<td>Glomerular layer</td>
</tr>
<tr>
<td>IPL</td>
<td>Internal plexiform layer</td>
</tr>
<tr>
<td>LP</td>
<td>Lamina propria</td>
</tr>
<tr>
<td>MC</td>
<td>Mitral cell layer</td>
</tr>
<tr>
<td>MCM2</td>
<td>Minichromosome marker-2</td>
</tr>
<tr>
<td>Abbreviation</td>
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<tr>
<td>mGluR5</td>
<td>Metabotropic glutamate receptors</td>
</tr>
<tr>
<td>NCAM</td>
<td>Neural cell adhesion molecule</td>
</tr>
<tr>
<td>NDS</td>
<td>Normal donkey serum</td>
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<tr>
<td>NFL</td>
<td>Nerve fibre layer</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NRTN</td>
<td>Neurturin</td>
</tr>
<tr>
<td>OB</td>
<td>Olfactory bulb</td>
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<td>OE</td>
<td>Olfactory epithelium</td>
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<tr>
<td>OMP</td>
<td>Olfactory marker protein</td>
</tr>
<tr>
<td>ORN</td>
<td>Olfactory receptor neuron</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PG</td>
<td>Periglomerular</td>
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<tr>
<td>PSA-NCAM</td>
<td>Polysialylated neural cell adhesion molecule</td>
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<td>RMS</td>
<td>Rostral migratory stream</td>
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<td>SVZ</td>
<td>Subventricular zone</td>
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<td>TH</td>
<td>Tyrosine hydroxylase</td>
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<td>VONS</td>
<td>Ventriculo-olfactory system</td>
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Acknowledgements

This research was carried out at Eskitis Institute for Cell and Molecular Therapies, Griffith University in association with the School of Biomolecular and Physical Sciences, Faculty of Science, Environment, Engineering and Technology, Griffith University. The project was funded by a National Centre for Adult Stem Cell Research Postgraduate Research Scholarship.

I would like to thank my previous supervisor Dr. Asanka Karunaratne for helping me start my scientific career. To my current supervisor Dr. Adrian Meedeniya, who adopted me into his group, I would have never been able to finish without your guidance, encouragement, knowledge and support. Thank you also to my associate supervisor Dr. Stephen Wood for his help with thesis corrections.

A big thank you everyone in my lab group, I would have not been able to complete my project without your help. A special thank you to Patrick Dywer, whose pilot study was the starting point for my project. To Brenton Cavanagh for his help with the microscope and giving me a crash course on how to use Photoshop and Illustrator, to Gervase Tuxworth for his help the densitometry work and general computer knowledge and to Tavia Goodison for teaching me about the joys of animal work. I would also like to thank Mandy Scheibe for eating chocolate with me when I was stressed and Charlotte Dickson for keeping me company in the lab.

Of course thanks to all my friends at Eskitis, you have been like my second family and made coming to work enjoyable. To Bernadette Bellette thank you for not only being an amazing friend outside work but for all the advice, support and encouragement you have given me in my PhD. Thank you to Jeremy Newman for always being there, whether it was to help me with statistics or to have a tea break with. Thanks to Colm Cahill for always having an answer to every question no matter how small or annoying it was.

Thank you to Fenil Shah for always believing in me, even when I didn’t believe in myself, but most of all thank you for always listening. To My Mum, Dad and Brother thank you for always encouraging me to achieve my goals and for putting up with me.
Chapter 1

Introduction
1.1 General introduction

The discovery of adult neurogenesis in the brain allows for a dynamic field of study with potential for generating therapies for neurodegenerative disorders. Multipotent neural ‘stem cells’ with the capacity for self-renewal have been well studied in the adult hippocampus and subventricular zone of the mammalian species, including humans (Eriksson et al., 1998; Gage, 2000). As the largest area of neurogenesis in the adult mammalian brain the subventricular zone (SVZ) has become one of the predominant areas to study neural stem cells in terms of neurogenesis, migration, differentiation and death (Alvarez-Buylla and Garcia-Verdugo, 2002). It is now known that amongst other roles, one of the primary functions of the SVZ is to supply neuronal progenitors to recapitulate the constantly changing circuitry of the olfactory bulb (OB). Of particular interest is the ability of the SVZ to replenish dopaminergic neurons present within the glomeruli of the OB. These dopaminergic neurons are perhaps the only dopaminergic population that undergoes genesis throughout adult life.

The system of neurogenesis and migration of progenitor cells from the SVZ to the OB is termed the ventriculo-olfactory neurogenic system (VONS). This study will extend this term to include the olfactory epithelium (OE), an area of constant neurogenic activity, where olfactory receptor neurons (ORNs) that synapse within the OB are replaced throughout life (Mackay-Sim and Kittel, 1991). Allowing for examination into whether cell turnover in the OE influences neurogenesis in the SVZ. This will be accomplished through stimulating large-scale cell turnover in the OE of a mouse model, and studying the rate of neurogenesis in the SVZ in response, as well as changes in periglomerular interneuron. This will further our understanding of the signalling that stimulates cell proliferation and differentiation in the SVZ, OB and olfactory epithelium. The specific aims and methods by which this will be accomplished are discussed later in this chapter (Section 1.6 – 1.8).
1.2 Adult neurogenesis

The notion that neurons are not generated in the adult brain was regarded as dogma by the scientific community up until the late 20th century. Today, what once was the ‘central dogma of neurobiology’ has collapsed, and it is now widely accepted that neurogenesis occurs at multiple locations in the adult brain (Altman, 1962; Alvarez-Buylla and Garcia-Verdugo, 2002; Corotto et al., 1993; Luskin, 1993; Seki and Arai, 1993). The first evidence that neurogenesis occurs in certain regions of the adult mammalian brain came from 3H-thymidine labelling studies conducted by Altman and Das (Altman and Das, 1965a). Altman (Altman and Das, 1965a) found cells labelled within the granule cell layer of the dentate gyrus, ependymal and sub-ependymal layers of the third and lateral ventricles, and later OB of adult rodents (Altman, 1969; Altman and Das, 1965b). In the early 1990s new methods for studying neurogenesis in the adult central nervous system (CNS) were introduced. Studies using retroviral vectors and bromodeoxyuridine (BrdU) confirmed that not only did neurogenesis occur in the adult mammalian brain but it occurs continuously in some niches of the adult CNS (Corotto et al., 1993; Luskin, 1993; Seki and Arai, 1993)
1.2.1 Neurogenic niches

Neurogenic niches are defined as microenvironments that anatomically house stem cells and functionally control their development \textit{in vivo}. Significant progress has been made in describing stem cell niches at cellular, molecular, and functional level. In the adult mammalian brain, active neurogenesis has been shown to be restricted to two discrete regions in the forebrain (Altman and Das, 1965a; Alvarez-Buylla and Garcia-Verdugo, 2002; Ihrie and Alvarez-Buylla, 2011; Riquelme et al., 2008). Neural stem or progenitor cells have been shown to reside in periventricular tissue, known as the SVZ of the lateral ventricle, and in the sub-granular zone of the hippocampal dentate gyrus (Figure 1.1). While the niches for hippocampal and SVZ exhibit some similarities, there are many clear differences.

![Figure 1.1: Germinal centres in the adult brain.](image)

\textbf{Figure 1.1: Germinal centres in the adult brain.} Neurogenesis in the adult brain is largely confined to two germinal centres. EdU (red) shows dividing cells in the dentate gyrus of the hippocampus (yellow arrows) and the SVZ (white arrows). Cell nucleus shown in blue (DAPI).
Hippocampal neurogenesis is physically localized to the dentate gyrus which is enriched by different nerve terminals and regulated through neurotransmitters. In the adult, proliferating radial and non-radial precursors within the hippocampal dentate gyrus give rise to intermediate progenitors, which in turn generate neuroblasts (Seri et al., 2001). Neuroblasts then migrate into the inner granular cell layer and differentiate into dentate granule cells in the hippocampus (Eriksson et al., 1998; Venere et al., 2012). These newly born neurons extend dendrites toward the molecular layer and project axons through the hilus toward the CA3 region before integrating synaptically into the existing circuitry.

In contrast, the area of neurogenesis in the SVZ is physically separate from the site of integration; the OB. Proliferating radial glia-like cells give rise to transient amplifying cells, which in turn generate neuroblasts. Neuroblasts form a chain and migrate through a tube of astrocytes, known as the RMS, toward the OB (Lois et al., 1996). Upon reaching the centre of the OB, immature neurons detach from the RMS and migrate radially toward glomeruli, where they differentiate into different subtypes of mature interneurons (Lledo and Saghatelyan, 2005). The largest percentage becomes granule neurons, which lack axons and form dendro-dendritic synapses with mitral and tufted cells (Lledo et al., 2008). The remaining become periglomerular neurons, a small percentage of which are dopaminergic (Kohwi et al., 2005; Kosaka et al., 1998; Panzanelli et al., 2007).

The identity and biology of neural progenitors in the adult brain are of considerable interest, reflecting their possible roles in health and disease and their potential for treating neurological illness. The progenitors in the VONS are of special interest because they contain a population of dopaminergic neurons similar to the ones lost in Parkinson’s disease.
1.2.2 The ventriculo-olfactory neurogenic system

In vertebrates, interneurons of the OB are generated postnatally throughout life at the SVZ. In animal models, neurogenic cells from the SVZ form a constitutively migrating pathway of neuroblasts, the RMS, destined for the OB (Figure 1.2) (Altman, 1969; Doetsch et al., 1997). The collective term for the SVZ together with the RMS is ‘ventriculo-olfactory neurogenic system’ (VONS) (Alvarez-Buylla and Garcia-Verdugo, 2002; Curtis et al., 2007). As stated previously this study will extend this term to include the olfactory epithelium.

![Figure 1.2: Schematic sagittal view of the adult rodent brain showing the SVZ–OB system.](image)

The SVZ lines lateral wall of the lateral ventricle (LV, blue) and produces new neurons throughout adult life. These neurons migrate along the RMS (red lines) into the core of the OB where they disperse radially (dotted lines) and differentiation into granule and periglomerular interneurons. OB- olfactory bulb, RMS- Rostral migratory stream LV-lateral ventricle, CC -corpus callosum, NC – Neocortex, CB- Cerebellum.(Alvarez-Buylla and Garcia-Verdugo, 2002)
1.3 The mammalian olfactory system

In mammals the olfactory system enables odour detection and discrimination. It also regulates a wide range of functions, including integrative functions, reproductive function and social behaviours. Humans have a less sophisticated olfactory system when compared to other mammals, but none the less are still able to detect and discriminate between 400000 different odorants. The olfactory system is composed of two anatomically and functionally distinct chemosensory systems. The primary olfactory system is involved in detection of odours and consists of the OE and the OB (Bear et al., 2006).

1.3.1 Olfactory epithelium

The OE consists of basal cells, sustentacular cells, and the ORNs. It is located on the lamina propria, which is a loose connective tissue made up of Bowman’s glands, blood vessels and nerve bundles (Purves and Augustine, 2004). Together, these two regions encompass the olfactory mucosa (Figure 1.3). Odours are detected in the nasal cavity by ORNs, which project cilia into the cavity from the apical surface OE. The distal dendrites of the olfactory sensory neurons project into the OB, terminating within a neuropil termed olfactory glomeruli.
1.3.1.1 Development of the olfactory epithelium

In mice the OE is first formed at embryonic day 8 (E8) from ectodermal thickenings called olfactory placodes which contain OE precursors (Bhattacharyya and Bronner-Fraser, 2008; Cornesse et al., 2005; Schlosser and Ahrens, 2004). Based on the proximity to the neural tube, the olfactory placode can be subdivided into the epiblast and nervous layers. The epiblast gives rise to basal cells and sustentacular cells, while the nervous layer will proliferate and differentiate into ORNs. Cellular proliferation at E10.5 causes the formation of depressions called olfactory pits (Bhattacharyya and Bronner-Fraser, 2008; Cornesse et al., 2005; Schlosser and Ahrens, 2004). Each olfactory pit has an outermost layer of pseudostratified columnar epithelial cells, which form ridges consisting of lateral and medial nasal processes. Both nasal processes continue proliferating; the median process forms the nasal septum. Continued proliferation in the lateral nasal process forms the turbinates, which increase the surface area of the OE (Cornesse et al., 2005; Schlosser and Ahrens, 2004).

1.3.1.2 Structure of the olfactory epithelium

In the adult, the OE is a pseudostratified columnar epithelium divided into three major compartments (Figure 1.3). An apical compartment, which contains the soma (cell body) of the supporting cells (Hempstead and Morgan, 1983) the basal compartment and an intermediate compartment, which contains ORNs (Mackay-Sim and Kittel, 1991; Morrison and Costanzo, 1990). Underlying the OE is the lamina propria, which contains blood vessels, special glial cells called olfactory ensheathing cells, small diameter bundles of axons originating from the ORN cell bodies and Bowman’s gland (Raisman, 1985).
Figure 1.3: Cell types in the olfactory epithelium. (A) A schematic representation of the OE is populated by sustentacular support cells (Sus), mature and immature ORNs (ORNm and ORNi, respectively), OECs, cells of Bowman’s gland (BG) and two types of progenitors (GBCs and HBCs) (Duggan and Ngai, 2007). (B) Immunofluorescence image of the olfactory epithelium, mature (green) and immature ORNs (red) and a basal cell layer consisting of horizontal basal cells and globose basal cells. Below the epithelium is the lamina propria, which contains nerve bundles (green and red ovoid shapes) and Bowman’s glands.
The sustentacular cells and Bowman’s glands play a supporting role in the olfactory epithelium. While the Bowman’s glands generate immunoglobulin-containing mucus to lubricate and protect the OE, the sustentacular cells provide trophic, metabolic, and mechanical support for ORNs (Vogalis et al., 2005). In addition, both Bowman’s glands and sustentacular cells express cytochrome P450 enzymes for breaking down harmful toxins introduced into olfactory system (Purves and Augustine, 2004). As such, the integrity of the OE is maintained by the sustentacular cells and Bowman’s glands. (Kandel and Squire, 2000).

ORNs are bipolar nerve cells, from the apical side a single dendrite extends to the epithelial surface. At the epithelium surface the dendrite forms a knob that is covered by cilia, that protrudes through the mucous covering the epithelium. On each ORN the cilia express one of about a thousand olfactory receptors (Wang et al., 1998). From the other pole of the ORNs projected an axon through the lamina propria.

1.3.1.3 Neurogenesis in the olfactory epithelium

The peripheral mammalian OE is constantly exposed to the external environment. As a result the OE is directly exposed to harmful substances such as toxins, pathogens and corrosive chemicals. Because of this, there is the requirement for a cycle of degeneration and regeneration of ORNs throughout life (Mackay-Sim and Kittel, 1991). Therefore, new populations of olfactory cells must be generated in order to maintain olfactory function (Murdoch and Roskams, 2007). After significant damage to the olfactory mucosa, neurogenesis commences next to the basal section of the OE (Calof et al., 1999; Schwob, 2002). It is thought that horizontal basal cells are the stem cell as studies have shown that they can generate olfactory and non-olfactory neural lineages as well as glial cells and globular basal cells (Carter et al., 2004; Duggan and Ngai, 2007; Iwai et al., 2008; Leung et al., 2007)(Figure 1.4). They also have close antigenic resemblance to non-nervous system epithelial stem cells.
Figure 1. 4: Neurogenesis in the olfactory epithelium. (A) GBCs have the capacity to regenerate the ORN population under normal conditions. (B) Under ablation the loss of both neuronal and non-neuronal cells lead to the recruitment of HBCs, which proliferate and differentiate into all of the mature cell types of the olfactory epithelium. (HBC = horizontal basal cell, GBC = globular basal cell, Sus = sustentacular support cells, ORNm = mature olfactory receptor, ORNi = immature olfactory receptor, OEC = olfactory ensheathing cell, BG = Bowman's gland. (Duggan and Ngai, 2007)

In mice it has been shown that olfactory stem cells undergo slow, asymmetric cell division every 50 days. This results in both the maintenance of the stem cell population and the generation of transitory amplifying progenitors, which are committed to differentiate into neurons. The balance of cell death and regeneration is regulated by autocrine and paracrine signals controlling proliferation, differentiation and survival (Mackay-Sima and Chuahb, 2000; Newman et al., 2000). Although the OSNs are continuously replaced, the spatial map of the glomerular innervation remains the same, even after extensive injury.

1.3.2 The Olfactory Bulb

The OB lies within the vertebrate forebrain and is the first site of processing signals from activated ORNs in the OE. The exact function of the OB is not yet defined, but it has been suggest that it is responsible for enhancing discrimination of odours and the sensitivity of odour detection. After processing in the bulb, sensory information is passed on to higher cortices in the CNS (Bear et al., 2006).
1.3.2.1 Development of the olfactory bulb

The OB is a late maturing structure with most of its interneuron population generated postnatally. In the mouse embryo, the structure of the OB can be visibly differentiated from the forebrain at E12. Mitral cells arise first from between E11 and E13, followed by external tufted cells, which appear from E13 to E18 (Hinds, 1968a, b). Both of these cell types are derived from a proliferative region known as the ventricular zone. Consequently, the production of mitral and tufted cells is confined to the developmental stages of the brain, whereas granule and periglomerular cells are produced in the SVZ throughout life.

1.3.2.2 Structure of the olfactory bulb

The OB is a highly organised structure composed of several distinct layers which are responsible for different synaptic specializations. The layers are the glomerular layer, external plexiform layer, mitral cell layer, internal plexiform layer and the granule cell layer. The glomerular layer is the most distinctive area of the OB and it is here that the ORN axons synapse at structures known as the glomeruli (Figure 1.5). Each glomerulus consists of mitral cell dendritic arborisations, olfactory nerve fibres, and periglomerular cells. The axons from the OE projected individually until the lamina propria where they coalesce into branches of the olfactory nerve that crosses the cribriform plate. The olfactory bundles generally remain heterotopic until they reach the olfactory nerve layer. In the bulb the ORNs synapse within 2 of the 1800 glomeruli (Ressler et al., 1993; Vassar et al., 1993) forming a mirror representation of the receptors: one in each hemisphere of the bulb (Nagao et al., 2000). Factors underlying this highly specific, yet complex mechanism remain to be elucidated (Bulfone et al., 1998; Mombaerts et al., 1996; Wang et al., 1998). The ORNs form glutamatergic synapses with mitral, tufted and periglomerular cells (Cleland and Sethupathy, 2006; Ennis et al., 1996; Pinching and Powell, 1971; Shipley and Ennis, 1996) which then project from the OB and relay sensory information to the higher cortices. However, before sensory information is relayed to other areas of the brain it is first processed in the OB (Aungst et al., 2003).
Figure 1.5 The structure of OB. The OB is composed of several distinct layers which from superficial to deep are as follows: nerve fibre layer (NFL), glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MC), internal plexiform layer (IPL) and granule cell layers (GCL). Image taken by Daniel Amaya.
1.3.2.3 Circuitry in the bulb

Each ORN expresses a single odorant receptor, which is randomly dispersed within epithelium (Ressler et al., 1993; Strotmann et al., 1992; Vassar et al., 1993). These receptors are seven-transmembrane domain G protein coupled receptors that activate adenyl-cyclase thereby increasing levels of cyclic adenosine monophosphate (cAMP). The increase levels of cAMP causes the opening of cyclic nucleotide-gated cation channels, depolarizing the neuronal membrane and generating an action potential (Dash et al., 1991). ORNs then synapse with mitral cells, tufted cells and periglomerular neurons in the OB (Nedelec et al., 2005). Mitral and tufted (hereafter referred to as mitral cells) cells are the principal output neurons of the olfactory system. They synapse directly with the ORNs and relay sensory information to higher cortical areas (Figure 1.6). The mitral cells also form excitatory synapses with periglomerular neurons that presynapticaly inhibit ORNs output (Aungst et al., 2003; Hayar et al., 2004a; Hayar et al., 2004b; Murphy et al., 2005). Periglomerular neurons have been shown to form dendro-dendritic reciprocal synapses with mitral cells, which play an important role in the temporal patterning (Spors and Grinvald, 2002). Mitral cells excite the periglomerular neuron dendrites which in turn can inhibit mitral cell primary dendrites via graded inhibition (Cleland and Sethupathy, 2006; Ennis et al., 1996; Shipley and Ennis, 1996). Periglomerular neurons are also able to modulate neuron–mitral cell transmission presynaptically. The second site of odour processing is in the external plexiform layer where lateral interactions between mitral and tufted output cells are mediated by inhibitory granule cells (Aungst et al., 2003) (Figure 1.6). Interestingly, the periglomerular and granule neurons are turned over throughout life in the adult OB.
Figure 1.6: Circuit diagram of the mammalian OB. ORNs expressing the same odorant receptor type converge together as they cross the cribriform plate and arborize together to form glomeruli (shaded ovals) in the glomerular layer (GL). In the glomeruli ORNs form excitatory synapses with dendrites of mitral (Mi), periglomerular (PG) and external tufted (ET) cells; which activate short-axon (SA) and local PG cells. PG cells synapse with mitral cell primary dendrites; the mitral cells excite the PG dendrites which inhibit mitral cells via graded inhibition. Mitral cells are the primary output neuron of the OB, projecting axons to several cortical and subcortical target regions. (OE = olfactory epithelium, EPL = external plexiform layer, MCL = mitral cell layer, IPL = internal plexiform layer, GCL = granule cell layer. Filled triangles denote excitatory (glutamatergic) synapses; open circles denote inhibitory (GABAergic) synapses. (Cleland, 2010)
1.3.2.4 Neurogenesis in the olfactory bulb

It has been shown that adult OB neurogenesis is required in maintaining the long term structure of the OB, as well as short-term olfactory memory, olfactory fear conditioning, and long-term associative olfactory memory involving active learning (Lazarini and Lledo, 2011). In addition, OB neurogenesis may regulate pheromone-related behaviours, such as mating and social recognition (Feierstein et al., 2010). While mitral and tuft cells are generated only in the developing embryonic CNS, granular and periglomerular neurons are continuously produced throughout the lifetime in the SVZ. The neurogenesis, migration, maturation and final integration will be discussed in depth in the following sections.

1.3.2.5 Adult born olfactory interneurons

Adult-born OB interneurons can be divided into two types: granule cells found in the granule cell layer, and periglomerular cells located in the glomerular layer. Adult born granule cells can be further subdivided into two distinct populations by morphological criteria. Superficial granule cells establish synapses with tufted cells in the superficial lamina of the external plexiform layer (Shepherd, 2004) and deep granule cells contact dendrites of mitral cells in the deep lamina of the external plexiform layer (Mori et al., 1983; Orona et al., 1983; Shepherd, 2004). Adult born periglomerular cells are a population of interneurons that surround the olfactory glomerulus and projects processes into the glomeruli (Pinching and Powell, 1971). Periglomerular cell dendrites form dendrodendritic synapses with mitral, tufted and other periglomerular cells within the glomeruli and neighboring glomerulus (Pinching and Powell, 1971). In 1995 Kosaka (Kosaka et al., 1995) categorised periglomerular cells into two types that contained 3 distinct groups, based on their activity and expression of tyrosine hydroxylase (TH), calbindin and calretinin. Type I periglomerular cells are TH positive and innervate the ORN zones, where they synapse directly with ORNs (Kasowski et al., 1999; Kosaka and Kosaka, 2005; Toida et al., 2000) (Figure 1.7). In contrast, Type II periglomerular cells send their dendrites only to the non-ORN zone, receiving few or
no axons from the ORNs (Gutierrez-Mecinas et al., 2005b; Kosaka and Kosaka, 2005). They are further divided into two different groups identifiable by the expression of calcium binding proteins, calretinin or Calbindin D-28k (Kosaka et al., 2001; Kosaka et al., 1997a; Kosaka et al., 1997b).

In mice, OB interneuron production is highest in the first postnatal week (Batista-Brito et al., 2008; Lim et al., 1997). It has been suggested that different interneuron subtypes are preferentially produced at different ages. Lemasson (Lemasson et al., 2005) suggested that cells born early after birth were more likely to integrate and survive in the superficial granule cell layer, when compared to cells born later. Another study suggested that periglomerular cells were more likely to be calbindin-positive if derived from the neonate, and more likely to be calretinin-positive or TH-positive if derived from the adult-labelled SVZ (Young et al., 2007). They also showed that embryonic or neonatal cells grafted into adult brain produced a higher percentage of calbindin positive cells and a lower percentage of calretinin positive cells than older tissues. However, a recent study in which OB interneurons were labelled at different time points found that TH positive cells are produced in greater proportions at early time points rather than later in development (Batista-Brito et al., 2008). This suggests an age dependent regulation, whether it is changes in SVZ or the OB, can influence the final maturation of newly born neurons.

However, progenitor patterning is not solely reliant on temporal regulation, as it also seems to require some form of spatial regulation. Heterotopically cell grafts have shown that phenotypes are acquired based on origin, which shows that mature identities are primarily determined by intrinsic properties (De Marchis et al., 2007; Kohwi et al., 2007; Merkle et al., 2007). This specification is even maintained after progenitor cells have been cultured in vitro and then grafted. These observations suggest that progenitors are pre-programmed at birth, to generate different subsets of OB interneurons, based on the domains within of the SVZ. The same transplantation experiments indicate that intrinsic factors are major determines in dendritic arborisation of postnatally generated GCs (Kelsch et al., 2007). This data points, to the fact that postnatal SVZ stem cells are heterogeneous and programmed during embryonic development.
Figure 1. 7: Locations and connections of interneurons in the OB. Schematic representation of subpopulations of olfactory interneurons distributed throughout the adult mouse OB. Type I periglomerular neurons express Tyrosine hydroxylase (TH, pink). These cells synapse directly with ORNs (O, green), mitral cells (M, blue). Type II periglomerular neurons express calretinin (Crt, orange) or calbindin (Cb, green) and synapse on the mitral cell axons. Granule cells (G) in red, synapse on the mitral cell axons in the granule cell layer (GCL). (Dcx = new neuroblasts, RMS = rostral migratory stream, GCL = granule cell layer, MCL = mitral cell layer, EPL = external plexiform layer, GL = glomerular layer, PV = parvalbumin). (Young et al., 2007).

1.3.2.5.1 Dopaminergic neurons in the olfactory bulb

Type I periglomerular interneurons are of special interest in regenerative therapies as they are thought to be the only population of regenerating dopaminergic cells in the adult brain. These dopaminergic periglomerular cells are the most characterized of all the periglomerular cells; however they still require greater detail of study. The dopamine receptors are located in the terminal of olfactory sensory neurons, dendrites of tufted, mitral cells and dopaminergic...
periglomerular cells (Gutierrez-Mecinas et al., 2005a). This allows them to modulation of inputs in the OB via depression of the synaptic transmission between ORNs and mitral cells (Berkowicz and Trombley, 2000; Hsia et al., 1999). It also indicates the ability of self-regulation, regulation of glutamatergic synapses of mitral cells and tufted cells and also the regulation of other dopaminergic periglomerular neurons.

1.4 Subventricular zone

The SVZ is a narrow zone of tissue located throughout the lateral walls of the lateral ventricles of the brain and gives rise to new neurons destined for the neocortical associated areas of the adult mammalian forebrain (Lim et al., 1997; Lois and Alvarez-Buylla, 1993; Mirzadeh et al., 2010). It is divided into four main layers which contains four cells types; neuroblasts (type A), astrocytes (type B1 and B2), undifferentiated cells (type C) and ependymal cells (type E). Throughout adult life the SVZ produces a population of progenitor cells which migrate along the RMS to the OB (Quinones-Hinojosa et al., 2006; Sawamoto et al., 2011).

1.4.1 Development of the SVZ

It has been theorised that the adult SVZ is originally derived from the lateral ganglionic eminence of the embryonic telencephalon (Wichterle et al., 2001). Studies have shown that transplanted lateral ganglionic eminence progenitors in the adult SVZ produce neuroblasts that migrate to the OB. These neuoblasts mature into interneurons similar to those produced from grafting adult SVZ derived cells to the adult SVZ or those natural occurring adult brain (Lois and Alvarez-Buylla, 1994). This has been corroborated by genetic evidence which links the embryonic lateral ganglionic eminence to adult SVZ.
The SVZ, arises in the vicinity of the bulb between E14 and E17 (Brazel et al., 2003) and produces a population of progenitors which migrate to the OB to form interneurons. In the E16 mouse, over 90% of the SVZ cell population is dividing, whereas the majority of the cells in the ventricular zone (VZ) are leaving the cell cycle (Caviness and Takahashi, 1995). As neurogenesis in the VZ halts, the SVZ continues to produce progenitors, the rate of production peaking during early postnatal development. After this initial period, neurogenesis in the SVZ continues on throughout the adult life (Bayer, 1983; Nelson et al., 1974).

During the later stages of foetal development, the ventricular system begins to decrease in size such that by birth it is no longer readily identified (Bayer et al., 1991). The neural stem cells that line the embryonic ventricular system are depleted from this region as a layer of postmitotic ependymal cells replaces the VZ. As the VZ contracts, the SVZ continues to expand during the perinatal period (Thomaidou et al., 1997) (Figure 1.8). Neural stem cells persist only in restricted regions and as the brain matures, these stem cells differentiate from radial glial cells into astrocytic SVZ type B cells (Merkle et al., 2004) Although the SVZ is a continuous proliferative zone surrounding the ventricles, the four distinct divisions can be distinguished during foetal development based on morphology, size, cell composition, and date of embryonic emergence as well as differential expression of several transcription factors and homeobox genes.

**Figure 1.8:** Depiction of the proliferative ventricular zone and SVZ through development and into adulthood. The SVZ in the adult is composed of migrating neuroblasts (A cells), astrocytes (B cells), and transitory amplifying progenitor cells (C cells) separated from the ventricle (V) by a monolayer of ependymal cells (E cells) (Conover and Allen, 2002)
1.4.2 Structure of SVZ

The SVZ is a thin region that spans the entire lateral wall of the lateral ventricle and consists of four main cell types. Neuroblasts or (Type A cells), that express doublecortin, β-III tubulin, and PSA-NCAM (Bonfanti and Theodosis, 1994; Nacher et al., 2001; Rousselot et al., 1995). Astrocyte like cells (Type B), which express astrocytic markers such as glial fibrillary acidic protein (GFAP) and glutamate–aspartate transporter (GLAST) (Bolteus and Bordey, 2004; Platel et al., 2009). Transit-amplifying cells (Type C cells) express epidermal growth factor (EGF) receptor (Cesetti et al., 2009; Pastrana et al., 2009; Platel et al., 2009), dlx2, and mash1 (Cesetti et al., 2009; Pastrana et al., 2009; Platel et al., 2009) and finally Type E, ependymal cells which are crucial in cell signalling.

The SVZ is organized into tubes containing 4–5 neuroblasts, also called chains of neuroblasts. Each chain of neuroblasts is ensheathed by processes of astrocyte-like cells, which form the wall of the “tubes” (Doetsch and Alvarez-Buylla, 1996; Lois and Alvarez-Buylla, 1994; Peretto et al., 2004). A subset of the astrocyte-like cells contacts both the ventricle and the blood vessels and these ‘SVZ astrocytes’ are thought to be the neural progenitors (Lacar et al., 2012; Mirzadeh et al., 2008; Tavazoie et al., 2008) Astrocyte-like cells are scattered along the SVZ and RMS (Doetsch et al., 1999; Gritti et al., 2002). They self-renew and generate transit-amplifying cells that also asymmetrically divide to give birth to neuroblasts (Figure 1.9). Neuroblasts remain proliferative along the SVZ and migrate to the OB via the RMS (Altman, 1969; Lois and Alvarez-Buylla, 1994; Luskin et al., 1993; Peretto et al., 2004) Once in the OB, they leave the RMS and migrate radially to the different neuronal layers.
Figure 1. 9: **Cell types and anatomy of the adult SVZ niche.** Schematic of a coronal section of the adult mouse brain showing the SVZ (orange) lining the lateral ventricle (LV). Multi-ciliated ependymal cells (E, gray) line the walls of the lateral ventricle. Chains of neuroblasts (A) ensheathed by processes of astrocyte cells form “tubes” (B). Transit-amplifying cells (C) are found in small clusters adjacent to the chains. Signals released from axons (pink) regulate proliferation and survival in this region. A specialized basal lamina (BL, black), endothelial cells, blood vessels (BV) and the basal lamina are all likely key components of the niche. (Sandeman et al., 2011)

1.4.3 Neurogenesis in the SVZ

The progenitors of the OB interneurons (granule and periglomerular interneurons) are located within a discrete region of the anterior part of the SVZ (Luskin, 1993) As most of the interneurons of the OB are generated postnatally and throughout the entire lifetime of mammals, cells within the SVZ divide relatively rapidly before leaving the SVZ (Betarbet et al., 1996a; Kishi, 1987; Lois and Alvarez-Buylla, 1994; Luskin, 1993). New cells migrate away from the SVZ before they undergo differentiation and express neuronal markers (Eriksson et al., 1998). Progenitors for the OB migrate along a well-defined pathway (the RMS) and then reorient to reach their correct target layers and differentiate into interneurons.
Neuroblasts arise from GFAP expressing astrocytes. While astrocytes can be found throughout the SVZ, those that act as stem cells are found in the ependymal layer, directly exposed to the cerebrospinal fluid (Doetsch et al., 1999). Cell signalling and transcription factor expression controls whether precursors give rise to glial cells or neuroblasts. In contrast to other immature neurons in the CNS, neuroblasts continue to divide as they migrate from their site of generation despite having the morphological appearance of migrating neurons and antigenic markers of differentiated neurons (Menezes et al., 1995). There may be regional differences in the fate of descendants of proliferating cells in the SVZ and migratory pathway. Studies have shown that cells that divide within the migratory pathway make more periglomerular cells than granule cells. While cells dividing within the SVZ make more granule cells than periglomerular cells (Luskin, 1993; Zigova et al., 1996). The signalling mechanisms that control the proliferation and migration of these neuroblasts are only just beginning to emerge.

1.4.4 Cell signalling in the SVZ

Signalling within the SVZ is highly complex with intricate communication between the different cell types and has been attributed to various mechanisms. The main pathways that are well understood are bone morphogenic proteins (BMPs), Notch signalling and the Wnt pathway. The BMP-binding protein Noggin is expressed in ependymal cells and blocks BMP signals, allowing neurogenesis to occur; it is hypothesised that the absence of Noggin would lead to glial formation (Figure 1.10 A)(Lim et al., 2000; Peretto et al., 2004). Notch signalling has been shown to maintain stem cells by inhibiting neurogenesis. It has also been proposed to produce neurons or cells of a glial fate (Lardelli et al., 1996)(Figure 1.10 B). The Wnt/β-Catenin pathway is important in the regulation of proliferation and differentiation of neural progenitor cells (Hirabayashi and Gotoh, 2005; Patapoutian and Reichardt, 2000). Adachi, 2007(Adachi et al., 2007) has demonstrated that β-Catenin signalling plays an integral role in the proliferation of progenitor cells in the SVZ of the adult mouse brain. There are also many other less well understood extrinsic signals such as dopamine, serotonin, γ-aminobutyric acid (GABA) and glutamate that have an effect on neurogenesis in the SVZ.
Dopamine is thought to up regulate neurogenesis. Studies have shown that in individuals where dopaminergic signalling is disturbed; proliferation in the SVZ is decreased (Hoglinger et al., 2004; Huisman et al., 2004; Sui et al., 2012). This is consistent with findings by Baker, Hoglinger, Yang and Sui (Baker et al., 2009; Hoglinger et al., 2004; Sui et al., 2012; Yang et al., 2008) which show that dopaminergic antagonists, Parkinson’s disease and removal of the dopaminergic projections decreases proliferation in the SVZ in mouse models. In the embryonic and adult SVZ D2-like dopamine receptors have been found on transit-amplifying cells suggesting that increased neurogenesis could be activated by the EGF receptor in conjunction with release of EGF in a PKC-dependent manner (Coronas et al., 2004) or via a ciliary neurotrophic factor-dependent mechanism (Yang et al., 2008). However, these finding are heavily dependent on the strain of mice used making comparison from different studies difficult (Baker et al., 2009).

Serotonin transporters are present on neuroblasts or 5-HT fibres entering the SVZ (Brezun and Daszuta, 1999a; Shibui et al., 2009) but studies are required to further understand the pattern of serotonin transporter expression in the SVZ. Overall, serotonin is considered as a
positive regulator of adult neurogenesis in the SVZ. Activation of 5-HT\textsubscript{1A} and 5-HT\textsubscript{2C} receptors \textit{in vivo} increased proliferation in the SVZ (Banasr et al., 2004). While inhibition of serotonin synthesis or lesioning of the serotoninergic raphe nucleus reduces neurogenesis by approximately 60% (Brezun and Daszuta, 1999b) Although the effects of serotonin seem to be mediated through stimulation of bone derived neurotrophic factor (BDNF) expression more studies are required to ascertain the exact pathway (Mattson et al., 2004).

GABA type A receptors are functionally expressed on both neuroblasts and astrocyte-like cells. The function of GABA receptors on transit-amplifying cells has not been explored, although changes in their proliferation could affect the number of adult-born neuroblasts. (Bolteus and Bordey, 2004; Nguyen et al., 2003; Wang et al., 2003) Neuroblasts synthesize and release GABA in both the SVZ and RMS (Bolteus and Bordey, 2004; Wang et al., 2003) which was found to reduce the speed of neuroblast migration in acute sagittal slices (Bolteus and Bordey, 2004; Platel et al., 2007a). GABA also been shown to increase the dendritic growth of neuroblasts, which were integrating in the OB (Gascon et al., 2006). Together these studies suggest that GABA controls some of the early and late stages of cell development.

Glutamate has also a role in cell signalling in the SVZ, with the presence of metabotropic glutamate receptors (mGluR5) and N-Methyl-D-aspartic acid (NMDA), 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (AMPA), and kainate receptors observed in cultured SVZ cells (Brazel et al., 2005). Calcium imaging and electrophysiological recordings in slice cultures found that neuroblasts express AMPA, kainate, and NMDA receptors and mGluR5 (Platel et al., 2008; Platel et al., 2010; Platel et al., 2007a). While each neuroblast expresses a mosaic of glutamate receptors, an individual neuroblast does not express all four receptor types, a phenomenon which may affect generation different populations. The characterization of glutamate receptor expression in other SVZ cell type is less clear. It is thought astrocyte-like cells released glutamate in a calcium-dependent manner onto neuroblasts given that they did not display any NMDA or AMPA-induced currents or calcium increases even in the presence of an inhibitor of AMPA receptor desensitization (Platel et al., 2010).
1.5 Rostral migratory stream

Proper development and function of the mammalian brain requires migration of post mitotic neurons from their site of origin to their ultimate destination in the brain. Altman and Das (Altman and Das, 1965a), through $^3$H-thymidine injections, showed that periglomerular and granular cells present in the adult OB were still forming after birth and hypothesised that proliferating cells in the SVZ were migrating to the OB. This has been verified by more recent studies using $^3$H-thymidine, electron microscopy and replication-deficient retroviruses combined with immunocytochemistry (Zhaoa and Namb, 2007). These showed neuronal precursors migrating from the forebrain using a well-defined pathway called the RMS (Figure 1.11) (Chazal et al., 2000; Mendoza-Torreblanca et al., 2008; Peretto et al., 1997).

1.5.1 Development of the RMS

The RMS first starts to develop at E14 as a collection of neuronal progenitor cells in the rhinencephalic epithelium (Pencea and Luskin, 2003). The neuronal progenitor cells exhibit a neuronal phenotype but retain have the ability to divide. This distinguishes them from the majority of neural progenitors of the forebrain, which stop dividing when they begin to differentiate (Pencea et al., 2001). These neuronal progenitor cells aggregate into a patch within the epithelium in the vicinity of the OB. Followed by the initial formation of the RMS where the cells bordering the patch extend into the OB creating the nonpatch portion of the RMS (Pencea and Luskin, 2003). The RMS then enlarges by the production and/or acquisition of patch and nonpatch cells from the SVZ. During the final development which occurs postnatally in the rodent, the distinction between patch and nonpatch regions of the RMS is lost (Pencea and Luskin, 2003).
1.5.2 Structure of RMS

In the adult mouse brain, the RMS is a pathway of precursor cells, arranged around a vestigial lumen and surrounded by a glial tube, which connects the lateral ventricle to the OB (Figure 1.11). Neuroblasts migrate from the anterior region of the SVZ ventrally and laterally, between the striatum and the anterior forcep of the corpus callosum, forming the vertical arm (VA). Cells continue to migrate rostrally, becoming concentrated within the olfactory tract, forming the horizontal arm (HA) before reaching the OB (Bonfanti and Peretto, 2007; Bonfanti and Ponti, 2008; Peretto et al., 2005; Peretto et al., 1997, 1999; Ponti et al., 2006)(Figure 1.11). After the neuroblasts arrive at the OB, they disperse into layers within the OB and eventually mature into interneurons (Lois and Alvarez-Buylla, 1994; Peretto et al., 2005). The RMS was recently discovered in the adult human forebrain (Curtis et al., 2007). In humans the RMS also comprises a large number of proliferating cells that form a continuous stream from the SVZ towards the OB. The RMS first extends ventro-caudally from the lateral ventricle immediately adjacent to the caudate nucleus to form a long descending limb before extending ventro-rostrally to form a shorter rostral limb that enters the olfactory tract towards the OB (Curtis et al., 2007 6373). In brains that have suffered neurological disease, neuroblasts have the capability to exit from the RMS and migrate to distant areas of neuronal death. This makes the RMS an important region of study for the future development of endogenous neuronal replacement strategies.

Figure 1.11: The rostral migratory stream. RMS is pathway of neuroblasts (red) surrounded by a glial tube (green) that connects the lateral ventricle to the OB. Neuroblasts migrate from the anterior region of the SVZ ventrally and laterally forming the vertical arm (VA). Cells continue to migrate rostrally, becoming concentrated within the olfactory tract, forming the horizontal arm (HA) before reaching the OB.
1.5.3 Migration in the RMS

After leaving the SVZ the neuroblasts migrate through the rostral-migratory stream in tightly apposed chains, or ‘chain-migration’ (Doetsch and Alvarez-Buylla, 1996). Several permissive cell surface adhesion molecules also have been shown to be important for chain migration. One of the better studied is neural cell adhesion molecule (NCAM) and its polysialylated form (PSA-NCAM). NCAM and PSA-NCAM have been shown to play a role in the migration of SVZ-derived cells (Bonfanti and Theodosis, 1994; Chazal et al., 2000). In transgenic mice devoid of all isoforms of NCAM and/or PSA-NCAM, there is a decrease in the number of cells migrating along a morphologically altered RMS (Chazal et al., 2000; Cremer et al., 1994; Tomasiewicz et al., 1993). Staining of adult sagittal brain sections with a monoclonal antibody that recognizes PSA-NCAM revealed an immunoreactive pathway connecting the SVZ with the core of the OB. At high magnification, PSA-NCAM immunoreactivity surrounds long chains of cells oriented in the direction of migration (Rousselot et al., 1995). \(^3\)H -thymidine labelling revealed that the proliferating cells in the RMS were either PSA-NCAM positive or closely associated with PSA-NCAM immunoreactive regions (Chazal et al., 2000; Cremer et al., 1994; Tomasiewicz et al., 1993). Similar studies using DiI labelling, as well as studies that trace migration using replication-deficient retroviruses, also show that migrating cells in the RMS are organized as long chains of cells streaming towards the OB (Figure 1.12). These PSA-NCAM-positive cells possess a migratory morphology with elongated cell bodies, unipolar or bipolar processes, with the occasional appearance of cone-like end-feet. Furthermore, these cells are often arranged in parallel to the direction of the RMS (Kam et al., 2009). Some of the proliferating cells found in the adult human RMS show co-expression with the migratory neuroblast marker PSA-NCAM (Curtis et al., 2007; Kam et al., 2009). This suggests that they are type A migratory neuroblasts that are not yet neuronally committed. Studies have also shown that \(\alpha 6\beta1\) integrin and tenascin and chondroitin sulfate-containing proteoglycans appears to be required for chain migration of type A cells (Jacques et al., 1998).

In contrast to migrating cells elsewhere in the forebrain, markers for radial glial cells are not found in the RMS nor are there axonal processes in the RMS. These observations suggest that the chains of cells migrating through the RMS are not guided by radial glia or axons.
However, staining for GFAP reveals a meshwork of GFAP-positive cells encapsulating the PSA-NCAM-positive chains (Jankovski and Sotelo, 1996; Lois et al., 1996; Peretto et al., 1997) It has been shown that while astrocytes are not essential for chain migration (Wichterle et al., 1997), factors secreted by astrocytes enhance the migration of SVZ neuroblasts (Mason et al., 2001). Astrocytes may also support survival, provide directional information and/or prevent type A cells from escaping normal migratory routes. It has been hypothesised that the OB secretes a chemo-attractant that controls the movement of cells in the RMS. However progenitors still migrate to the bulb in the case of bullectomy (Kirschenbaum et al., 1999). Therefore, if the OB does secrete chemoattractants, these factors are nonessential for the rostral migration. It has been also been suggested that chemorepulsion is mediated by Slit-Robo signaling (Hu, 1999; Wu et al., 1999) gradients, but this brings up complications on how repulsive agents can establish complex migratory route. Slits, instead may serve as general inhibitors of migration preventing SVZ neuroblasts from migrating into nonspecific regions of the brain (Mason et al., 2001). Once the migrating neuroblasts reach the OB, they leave the RMS and migrate radially to different layers, and differentiate into granule and periglomerular neurons (Lois et al., 1996; Luskin, 1993). There is nothing currently known about the signals within the OB which induce their exit from the RMS or the mechanism of radial migration.

![Figure 1.12: Chain migration in the rostral migratory stream.](image)

Newly formed neurons leave the SVZ and in the RMS migrate along each other to form long chains. The meshwork of glial cells (green) surrounds the chains of migrating cells (red).
1.6 Methimazole

Methimazole was originally developed as a drug to treat hyperthyroidism (Shibui, 2012). Patients experienced symptoms of anosmia (loss of smell) and hypogeusia (loss of taste) and studies revealed that the chemical specifically targets and destroys discreet nasal cell populations. Since then, methimazole has been developed as a highly effective method for chemical ablation of the OE (Bergman and Brittebo, 1999; Bergstrom et al., 2003; Brittebo, 1994).

1.6.1 Mechanisms of methimazole toxicity

Methimazole toxicity occurs in cells expressing cytochrome P450 2A5 (CYP2A5), which metabolises methimazole into reactive intermediates. It has also been found to act as an inhibitor of the flavin-containing monoxygenases and is known to cause tissue-selective toxicity in rodent olfactory mucosa (Bergman and Brittebo, 1999; Bergstrom et al., 2003; Brittebo, 1994; Casallo Blanco et al., 2007; Genter et al., 1995). Studies in rodents has revealed wide spread cell death throughout the olfactory region caused by CYP2A5 - dependant metabolism of the compound into reactive intermediates (Brittebo, 1995; Xie et al., 2011). These intermediates bind covalently to the tissue, causing cell death. Methimazole-induced cell death in rats is predominately apoptotic, triggered through the mitochondrial cytochrome c-mediated caspase-3 activation pathway (Sakamoto et al., 2007). Members of this cytochrome subfamily are expressed in a few areas around the body, including the liver, respiratory epithelium of the lungs, tracheal lining and olfactory mucosa.
1.6.2 Methimazole in the olfactory epithelium

Metabolites are found bound to the bowman’s glands and sustentacular cells which are responsible for maintaining structure in the OE. As a result the OE structure breaks down and results in the subsequent death of all cells residing in the OE (Brittebo, 1995; Mizutani et al., 1999) Despite this, methimazole is considered a mild treatment compared to other chemical ablation methods, as it discreetly targets and destroys the OE structure in a manner that allows quick regeneration and has no other serious effects on the animal (Bergman and Brittebo, 1999; Bergstrom et al., 2003; Brittebo, 1995)

1.6.3 Impacts on the olfactory bulb

The OB is innervated by axons extending from the OE. Mitral, tufted and periglomerular cell populations rely on this signalling in order to process sensory information and transmit to higher cortices. Two weeks after injection of methimazole increased expression of GFAP in the OB could be seen, and is considered to be a general marker of ‘neurotoxic insult’ (Bergman and Brittebo, 1999; O'Callaghan, 1994). Bergman 1999 (Bergman and Brittebo, 1999) hypothesised this may occur as the OB depends on trophic support from projections of the ORNs.

1.7 Identifying sites of cell proliferation

The ability to track dividing cells in the adult brain has led to the major discoveries relating to neurogenesis. With the advent of each new method, new findings and evidence have been produced resulting in breakthroughs in the field (Cavanagh et al., 2011). The most powerful method for identifying proliferative cells is using labelled thymidine molecules.
1.7.1 Previous methods

Detection of neurogenesis and cell proliferation in the adult rodent brain has become commonplace in stem cell biology today. Tritiated thymidine autoradiography is one of the earliest techniques for identifying differentiating cells in the CNS. It involved the use of a radioactively labelled thymidine molecule which was detected using autoradiographical techniques (Altman, 1963). However, the method was also expensive, time-consuming and required a radiolabeled substrate (Taupin, 2007). The invention of BrdU was an important step forward for adult neurogenesis research. The advantage of BrdU labelling is that it does not require the use of autoradiography as it can be detected with immunohistochemistry. Using antibodies specific for the anti-BrdU antibody, the site of incorporation can be visualised using microscopy. However, it is a toxic chemical that can trigger cell death in treated specimens and detection requires that the DNA is denatured, which requires harsh treatment of tissue (Taupin, 2007). Other studies use proliferating cell nuclear antigen (PCNA), Ki67, phospho-histone H3 (Ph3) and minichromosome marker-2 (MCM2). The advantage of these markers over BrdU labelling is that they are endogenously-produced cell cycle markers made by dividing cells. The major disadvantages arise from the fact that the cell cycle is very tightly controlled, so the cell cycle proteins are only expressed for a very short period of time and are then rapidly degraded, and so the future fate of the cell cannot be determined.

1.7.2 5-ethyl-2’-deoxyuridine (EdU)

A new cell proliferation marker, EdU has recently been developed by Invitrogen (Invitrogen, 2007). EdU works in the same manner as BrdU, however the fluorescence labelling reaction is relatively quick and gentle on tissue (Cavanagh et al., 2011; Chehrehasa et al., 2009).
1.7.2.1 EdU Chemistry

Detection of DNA synthesis in proliferating cells relies on the incorporation of labelled DNA precursors into cellular DNA during the S phase of the cell cycle. EdU has been shown to robustly label dividing cells \textit{in vitro} and \textit{in vivo} (Cavanagh et al., 2011; Chehrehasa et al., 2009). It uses a thymidine analogue in which a terminal alkyne group replaces the methyl group in the 5 position, which is readily incorporated into cellular DNA during DNA replication. When the presence of EdU, the terminal alkyne group is then detected through its reaction with fluorescent azides, in a Cu(I)-catalysed cycloaddition ("click" chemistry) (Invitrogen, 2007; Patton, 2004) (Figure 1.13). EdU eliminates steps required by traditional BrdU assays that require DNA denaturation and harsh permeabilization which can damage sample morphology and integrity. The EdU protocol has been shown to maintained structural and antigenic integrity of neural tissues to accommodate high resolution, multiple-fluorescence microscopy with antibodies to neuronal markers and is also compatible with the DNA intercalating dye, DAPI (Chehrehasa et al., 2009; Invitrogen, 2007; Salic and Mitchison, 2008).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{click_chemistry.png}
\caption{Click chemistry. A covalent bond being formed between an azide and an alkyne (Patton, 2004).}
\end{figure}
1.8 Aims

Although there has been extensive research into each part of the ventriculo-olfactory system, there have been relatively few attempts to relate neurogenesis in the OE to that in the SVZ. To address this, this study aims to determine whether cell turnover in the OE results in up-regulation of neurogenesis in the SVZ. The idea behind this is to generate a model based on the natural turnover of ORNs and propose a reason for the continued production of neuroblasts in the SVZ throughout the adult lifetime.

To achieve this, immunofluorescence microscopy will be carried out on an experimental group of mice that have been treated with methimazole (1.6), to ablate the OE, and incorporated with EdU (1.7), a thymidine analogue to track proliferating cells.

This study has been developed to address these findings, and to study the effects of the olfactory lesion model on aged and young mice. This study’s aims are as follows:

- Establish the relationship between the growth of new nerve cells underlying the sense of smell (olfactory epithelial neurogenesis) and those in a brain region (SVZ neurogenesis)

- Examine the rate of cell proliferation in the SVZ in response to a focal ablation of the olfactory epithelium.

- Elucidate the mode of communication for driving the differentiation of periglomerular neuronal progenitors with emphasis on dopaminergic neurons.
1.9 Hypothesis

This study proposes that turnover of ORNs impacts on periglomerular neurons in the OB (specifically Type I periglomerular cells), which then stimulates neurogenesis in the SVZ. In order to assess this, experiments will be carried out to establish the temporal relationship between neurogenesis in the OE and neurogenesis in the SVZ as well as to examine the rate of cell proliferation in a specific area of the VONS, namely, the rostro-caudal arm of the SVZ in response to a focal ablation of the OE.

1.10 Relevance of the study

This study will investigate the regulatory mechanisms underlying cell proliferation in the neurogenic sites of the brain and periphery within the VONS. This region demonstrates not only a site of adult neurogenesis, migration, maturation; it also contains possible to only population of adult born dopaminergic neurons. The study is designed to further our understanding of the signalling that stimulates cell proliferation and differentiation in the SVZ, OB and OE. This will provide novel data on dopaminergic cell genesis in adult brain and regulatory mechanisms underlying neurogenesis in the VONS. We will also obtain data on mechanisms underlying olfactory neurogenesis and circuit formation. As well as data on the differentiation of progenitor cells to functional dopaminergic neurons this is of significance for research on Parkinson’s disease therapy. It will provide data to allow comparison to existing data on cell proliferation and signalling mechanisms within the olfactory system, and allow for future experiments, to examine the roles of transcription factor and neurotrophic factor in the genesis and development of dopaminergic cells.
Chapter 2

Materials and Methods
2.1 Materials

2.1.1 Animals

Adult male C57/Bl6 mice aged 3 months (young) were obtained from the ARC. Geriatric male C57/Bl6 mice aged between 20 and 24 months (aged) were obtained in house. All animals were housed at the ESKITIS animal housing facility in N75, Griffith University Nathan Campus and were allowed access to standard rodent food and water ad libitum. All animals were obtained, housed and euthanized under the Griffith University Animal Ethics Committee approval (ESK/02/11/AEC and ESK/03/12AEC).

2.1.2 Chemicals, Reagents and Kits

Table 2.1: List of chemicals, reagents and kits.

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Click-iTTM EdU Cell Proliferation Assay Kit</td>
<td>Life Technologies, California, USA</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>Sigma Chemical Company, Missouri, USA</td>
</tr>
<tr>
<td>Ethanol (Ethyl Alcohol, C₂H₅OH)</td>
<td>Sigma Chemical Company, Missouri, USA</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid (EDTA),</td>
<td>Chem Supply, South Australia, Australia</td>
</tr>
<tr>
<td>Glycerol (CH₂OHCHOHCH₂OH)</td>
<td>Sigma Chemical Company, Missouri, USA</td>
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<tr>
<td>Hydrochloric acid (HCl)</td>
<td>Sigma Chemical Company, Missouri, USA</td>
</tr>
<tr>
<td>Ketamine</td>
<td>Troy Laboratories, New South Wales, Australia</td>
</tr>
<tr>
<td>Methimazole</td>
<td>Sigma Chemical Company, Missouri, USA</td>
</tr>
<tr>
<td>Normal donkey serum</td>
<td>Chemicon, Missouri, USA</td>
</tr>
<tr>
<td>O.C.T Compound</td>
<td>Supplier and Location</td>
</tr>
<tr>
<td>-----------------------------------------------------</td>
<td>--------------------------------------------</td>
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<tr>
<td>Paraformaldehyde (PFA)</td>
<td>Sigma Chemical Company, Missouri, USA</td>
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<tr>
<td>Picric acid (C₆H₃N₃O₇)</td>
<td>Sigma Chemical Company, Missouri, USA</td>
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<tr>
<td>Sodium Azide (NaN₃)</td>
<td>Chem Supply, South Australia, Australia</td>
</tr>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>Chem Supply, South Australia, Australia</td>
</tr>
<tr>
<td>Sodium Hydroxide (NaOH)</td>
<td>Merck, Victoria, Australia</td>
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<tr>
<td>Sodium Nitrite (NaNO₂)</td>
<td>Chem Supply, South Australia, Australia</td>
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<tr>
<td>Sodium Phosphate (NaH₂PO₄ + Na₂HPO₄)</td>
<td>Merck, Victoria, Australia</td>
</tr>
<tr>
<td>Sucrose (C₁₂H₂₂O₁₁)</td>
<td>Merck, Victoria, Australia</td>
</tr>
<tr>
<td>Triton X-100 (C₃₄H₆₂Oₓ)</td>
<td>Sigma Chemical Company, Missouri, USA</td>
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<tr>
<td>Vectashield DAPI (4′-diamidino-2-phenylindole 2HCl, Vector Labs) mounting media</td>
<td>Vector Laboratories, California, USA</td>
</tr>
<tr>
<td>Ilium Xylazil</td>
<td>Troy Laboratories, New South Wales, Australia</td>
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2.1.3 Antibodies

Table 2.2: List of primary antibodies. IF = Immunofluorescence

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<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Dilution</th>
<th>Application</th>
<th>Source (catalogue number)</th>
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<tbody>
<tr>
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<td>Rabbit</td>
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<td>IF</td>
<td>Swant (CB 38)</td>
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<td>Calbindin D</td>
<td>Mouse</td>
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<td>IF</td>
<td>Biosensis (M976-100)</td>
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<td>Calretinin</td>
<td>Goat</td>
<td>1:500</td>
<td>IF</td>
<td>Swant (CG1)</td>
</tr>
<tr>
<td>Cleaved Caspase-3</td>
<td>Rabbit</td>
<td>1:500</td>
<td>IF</td>
<td>Cell signalling(9661)</td>
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<tr>
<td>DOPA decarboxylase</td>
<td>Rabbit</td>
<td>1:500</td>
<td>IF</td>
<td>ABCAM (AB3905)</td>
</tr>
<tr>
<td>Glial fibrillary acidic protein (GFAP)</td>
<td>Mouse</td>
<td>1:1000</td>
<td>IF</td>
<td>Chemicon/Millipore (MAB3402)</td>
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<tr>
<td>Iba1</td>
<td>Rabbit</td>
<td>1:500</td>
<td>IF</td>
<td>WAKO (019-19741)</td>
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<tr>
<td>Neuronal Class III B-tubulin</td>
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<td>1:1000</td>
<td>IF</td>
<td>Covance (PRB-435P)</td>
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<tr>
<td>Olfactory marker protein (OMP)</td>
<td>Goat</td>
<td>1:1000</td>
<td>IF</td>
<td>WAKO (544-10001-WAKO)</td>
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<tr>
<td>Tyrosine hydroxylase (TH)</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>IF</td>
<td>Pel-freeze(P40101-0)</td>
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<tr>
<td>Tyrosine hydroxylase (TH)</td>
<td>Mouse</td>
<td>1:600</td>
<td>IF</td>
<td>Immunostar (22941)</td>
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Table 2.3: List of Secondary antibodies. IF = Immunofluorescence

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<th>Host</th>
<th>Dilution</th>
<th>Application</th>
<th>Source</th>
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</thead>
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<td>Donkey</td>
<td>1:600</td>
<td>IF</td>
<td>Invitrogen (A21206)</td>
</tr>
<tr>
<td>Rabbit IgG</td>
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<tr>
<td>Alexa Fluor® 594</td>
<td>Donkey</td>
<td>1:600</td>
<td>IF</td>
<td>Invitrogen (A21207)</td>
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<tr>
<td>Rabbit IgG</td>
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<tr>
<td>Alexa Fluor® 647</td>
<td>Donkey</td>
<td>1:600</td>
<td>IF</td>
<td>Invitrogen (A-21447)</td>
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<tr>
<td>Rabbit IgG</td>
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<td>Donkey</td>
<td>1:600</td>
<td>IF</td>
<td>Invitrogen (IVA21202)</td>
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<td>Mouse IgG</td>
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<td>IF</td>
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<td>Mouse IgG</td>
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<td>Alexa Fluor® 488</td>
<td>Donkey</td>
<td>1:600</td>
<td>IF</td>
<td>Invitrogen (A-11055)</td>
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<td>Goat IgG</td>
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<td>Donkey</td>
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<td>IF</td>
<td>Invitrogen (A21447)</td>
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<td>Goat IgG</td>
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<td>Alexa Fluor® 488</td>
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<td>1:200</td>
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<td>Invitrogen (A10266)</td>
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<td>Azide</td>
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<td>Donkey</td>
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<td>Alexa Fluor® 647</td>
<td>Donkey</td>
<td>1:200</td>
<td>EdU Click it</td>
<td>Invitrogen (A10270)</td>
</tr>
</tbody>
</table>
2.1.4 Solutions

PBS (10 × Stock)

1.36 M NaCl

27 mM KCl

100 mM Na₂HPO₄

18 mM KH₂PO₄

1x Phosphate buffered saline solution

0.262 g/L NaH₂PO₄·H₂O

1.4417 g/L Na₂HPO₄·2H₂O

9g/L NaCl

1x Phosphate buffered saline with 0.1% Triton (PBS-Triton)

100ml/L 10x PBS

1ml/L Triton X-100 0.1% (C₃₄H₆₂Oₓ)

1x Phosphate buffered saline with 0.1% Azide (PBS-Azide)

100ml/L 10x PBS

1g/L Sodium Azide (NaN₃)

Phosphate buffered saline with 30% sucrose

1x PBS

300g/L sucrose
0.1M Phosphate buffered saline with 0.5% sodium nitrite

1x PBS
5g/L Sodium Nitrite

Modified Zambonies fixative

20g/L paraformaldehyde
500m/L distilled water
400ml/L Part B (28.39g Na₂HPO₄ in 1L)
100ml/L Part A (31.2g NaH₂PO₄·2H₂O in 1L)
2ml/L saturated picric acid

Methimazole solution

5ug/μl Methimazole (C₄H₆N₂S)

20% Ethylenediaminetetra acetic acid (EDTA)

200g/L EDTA
25g NaOH
11g/L Na₂HPO₄
3.5g/L NaH₂PO₄

5-ethynyl-2'-deoxyuridine (EdU) solution for injection

1.5625mg/ml EdU (C₁₁H₁₂N₂O₅)

Terminal anaesthetic at 4:1 ratio, dosage of 0.4ml per animal

1000mg/ml Ketamine
20mg/ml Xylazil
2.2 Methods

2.2.1 Methimazole Treatment

Ablation of the olfactory epithelium in the young C57/Bl6 mice.

Methimazole was delivered via two intraperitoneal injections using a 25 gauge needle, three days apart at a dose of 50mg/kg body weight in a solution of 5mg/ml PBS (pH7.35). Control animals received intraperitoneal injections of sterile 0.1M PBS.

Ablation of the olfactory epithelium in the aged C57/Bl6 mice.

Methimazole was delivered via one intraperitoneal injection using a 25 gauge needle at a dose of 50mg/kg body weight in a solution of 5mg/ml PBS (pH7.35). Control animals received intraperitoneal injections of sterile 0.1M PBS.

2.2.2 EdU Labelling

To label dividing cells over time in vivo, EdU was delivered via intraperitoneal injections using a 25 gauge needle three consecutive days before sacrifice, at a dose of 30mg/kg body weight in a solution of 10mg/ml PBS (pH7.35).
2.2.3 Tissue harvest

2.2.3.1 Perfusions and fixation

At the time of sacrifice, animals were injected with an overdose of ketamine and xylazil (400µl concentration 4:1). On the absence of the foot-retraction reflex, an abdominal incision was made to expose the diaphragm. The thoracic cavity was accessed to reveal the heart using two lateral incisions, allowing the rib-cage to be retracted. A cannula made from a 23 gauge hypodermic needle attached to a perfusion pump was inserted in the aorta via the left ventricle. The right atrium was cut allowing clearing of the blood from the circulation. A perfusion pump set at 1ml/minute delivered PBS containing 0.1M sodium nitrite, a vasodilator, until the perfusate showed no trace of blood (approximately 2-3ml) after which the perfusate was switched to Modified Zambonie’s fixative until the animal had stiffened (approximately 3-4ml).

2.2.3.2 Dissection

Following fixation, the spine was severed and the head removed. Skin, eyes and musculature was cleared from the cranium and the lower mandible removed. The skull was then carefully dissected off, and the brain and nose separated. Tissues were then drop-fixed in Modified Zambonie’s fixative overnight in vacuo.
2.2.3.3 Processing

Tissue was removed from modified Zambonie’s fixative, washed twice in 1xPBS for 30min and permeabilised in DMSO for one hour followed by two washes in 1x PBS. The specimen was then place in 30% sucrose in PBS azide and placed at 4°C overnight before being placed in a series of OCT solutions of increasing concentrations (20%, 50% and 70% in 30% sucrose in PBS with azide) for 1 hour each. The tissue was then mounted in 100% OCT and stored at -80°C until sectioning. All washes and incubations are done in vacuo at room temperature unless otherwise stated.

Specimens containing bone were first treated for two weeks in 20% EDTA to decalcify bone (EDTA was changed daily and the specimen placed in vacuo for one hour each day). After decalcification the tissue was then washed twice in 0.1M PBS and put through standard processing.

2.2.4 Sectioning

All tissue was sectioned using a Leica CM 3050S cryostat (Leica Microsystems, Germany) set at approximately -18°C at a thickness of 40µM and stored either at 4°C in a 24-well plate containing PBS-azide as free-floating sections or at -80°C as adherent sections.
2.2.5 EdU detection and Immunohistochemistry

Adherent sections were removed from the freezer and dried in a vacuum oven at 49°C for 5 minutes and then sealed with a Pap-pen. Free floating section were removed from PBS-azide and placed in a fresh 24 well plate containing 1x PBS. From this point, both adherent and free floating section received the same treatment. Sections were washed in 1x PBS-Triton for 5 minutes and then permeablised with DMSO (neat) for 20 minutes and washed twice in 1x PBS. After permeablisation, sections were then processed via EdU detection and/or Immunohistochemistry. All step where performed in vacuo unless otherwise stated.

2.2.5.1 Edu Detection

**EdU labelled cells were detected using the Click-iT™ EdU Cell Proliferation Assay Kit (Invitrogen, 2007).**

The Click-iT™ EdU Cell Proliferation Assay Kit contains 12 reagents named component A to component L. For this part of the experiment components B, E, G, H and I were required. Although Invitrogen did not supply the entire ingredient list for of each of these reagents, information was given about the active compounds of each. Component B contains the Alexa Fluor® azide, E is a saponin-based permabilisation and wash reagent, G is a reaction buffer, H contains copper sulphate and I is a buffer additive.

Tissue sections were incubated in component E for 30 min on a rocker, followed by incubation with the reaction cocktail for 1 hour at room temperature, protected from light. 1500 μl of reaction cocktail contained 7.5 μl of component B, 30 μl of component H, 1313 μl of component G and 150 μl of component I. The sections were then incubated for 30min in component E and washed for three time 5 min in PBS. They were then either cover slipped using Vectashield DAPI mounting media or continued to immunohistochemistry.
2.2.5.2 Immunohistochemistry

Tissue sections were blocked using 10% Normal Donkey Serum in PBS-Triton 0.1% for approximately 1 hour in a humidified chamber. Primary antibodies were diluted in 10% NDS in PBS-Triton and applied overnight in a humidified chamber at room temperature. Sections were washed three times in 1x PBS for five minutes before adding secondary antibodies. Secondary antibodies were diluted in 10% NDS in PBS-Triton (0.1%), added to the section and left to incubate for 4 hours. Specimens were then washed three times in 1x PBS for 5 minutes and cover slipped using Vectashield DAPI mounting media.

2.2.6 Image capture and image preparation

Images were captured using an Axio Imager Z1 epi-fluorescence microscope with Apotome and an Axiocam Mrm camera (Carl Zeiss, Germany). Serial optical sections were captured using AxioVision software (Carl Zeiss, Germany) and projected to provide two-dimensional maximum brightness images. Figures were compiled in Adobe Photoshop 7.0 and Adobe Illustrator 10.0 (Adobe Systems Incorporated). Scale bars present on merged image represents the scale in all single channel images. In the case of large image panels the scale bar present in the last image represents the scale in all images.
2.2.7 Quantification

2.2.7.1 Densitometry

**Densitometry measurements of OB GFAP and Iba1 immunoreactivity**

Three optical sections (at 5 μm, 20 μm, and 35 μm) from a series of optical sections of GFAP and Iba1 immunoreactive cells from four preselected regions of the OB and captured using a high-resolution objective (63x oil immersion objective). Sections were chosen for analysis from the lateral interaural 0.96, from figure 109 in the Paxinos Mouse Brain Atlas 2nd Edition (Figure 2.1). The sections were all processed at the same time, and the exposure was set to the brightest intensity to circumvent overexposure artefact, and background was set by imaging a region devoid of immunoreactive cells. The images were converted to grayscale for analysing the fluorescence intensity. Quantitative densitometric data were generated as a pixel density measure using a custom MATLAB (The Mathworks) Software Programme. The experimenter was blinded to all treatment groups.

2.2.7.2 Cell counts

**Cell counts were carried out on immunohistochemically labelled cell populations within the glomeruli of the OB and SVZ.**

In the OB, the single Z slices from glomeruli in of sagittally sectioned OBs were analysed. Sections were chosen for analysis from the intraneural 8.08, from Figure 1 and lateral intraneural 0.96 (Figure A), from Figure 109 in the Paxinos Mouse Brain Atlas 2nd Edition (Figure 2.1B). Cell counts were carried out on cells expressing combinations of the following markers TH, Calbindin, Calretinin, GFAP, cleaved caspase-3 and DAPI from single glomeruli chosen from the dorsal, rostral and ventral region of each bulb. Counts were
carefully carried out to include cells associating with the chosen glomeruli as defined by DAPI staining and their position to the neuropil.

In the SVZ 15µm high Z-series were captured from 2 predefined regions of the lateral ventricle and beginning of the RMS. Sections were chosen for analysis from the lateral intraneural 0.96, from figure 109 in the Paxinos Mouse Brain Atlas 2\textsuperscript{nd} Edition (Figure 2.1). All cells showing a cell nucleus which co localised EdU and DAPI were counted. (Norazit et al., 2010).

Figure 2.1: Reference images from Paxinos Mouse Brain Atlas 2\textsuperscript{nd} Edition. Sections were chosen for analysis from the (A) Interaural 8.08, from Figure 1 (B) lateral Interaural 0.96, from figure 109 in the Paxinos Mouse Brain Atlas 2\textsuperscript{nd} Edition.
2.2.8 Statistical analysis

Using SPSS version 15 one-way analysis of variance (ANOVA) or Student T test were performed to analyse the data sets. Once analysis claimed the existence of significant difference in the data, post hoc Tukey’s honest significance difference was used identify significant change. All graphs were generated in Microsoft Excel and expressed as “mean ± SE”.
Chapter 3

Cellular dynamics in the primary olfactory system in response to methimazole treatment
3.1 Introduction

The mammalian OE is constantly exposed to the external environment. As a result, the cells in the OE are susceptible to damage from toxins, infectious agents or trauma (Breer et al., 2006). Therefore, they are constantly undergoing a cycle of degeneration and regeneration. One of the major cellular components in the OE is the ORNs. These neurons detect odours at the OE and relay this information via projecting axons into the OB. Once in the bulb these receptor neurons terminate in the glomeruli and interact with interneurons of the OB. By forming glutamatergic synapses with mitral and periglomerular cells (Cleland and Sethupathy, 2006; Ennis et al., 1996; Pinching and Powell, 1971). The mitral cells relay sensory information to the higher cortices; however, before sensory information is relayed to other areas of the brain it is first processed in the OB by the periglomerular neurons (Aungst et al., 2003).

Periglomerular neurons can be categorised into two groups Type I periglomerular cells are GABAergic neurons, that predominately express tyrosine-hydroxylase (TH) and synapse directly with ORNs (Baker, 1990; Crespo et al., 2003; Kosaka and Kosaka, 2007; Toida et al., 2000). Type I neurons are thought to be the only subset of TH expressing cells that are repopulated in adult life. In contrast, Type II periglomerular cells are GABA and glutamic acid reactive, and do not synapse directly with ORNs but act by inhibiting mitral cell axons. These cells can be identified by the expression of the calcium binding proteins; calretinin or calbindin (Kosaka et al., 1997a). This study hypothesises that death and regeneration of ORNs triggers turnover of periglomerular neurons in the bulb.

A pathophysiological model for olfactory epithelial cell turnover is used to cause a denervation of the OB. Specifically; methimazole is used to chemically ablate the olfactory epithelium. Methimazole was originally developed as a drug to treat hyperthyroidism in humans (Cooper et al., 1984). However, patients experienced symptoms of anosmia (loss of smell) and hypogeusia (loss of taste) resulting in alter sensory perception. Studies revealed that the chemical specifically targets discreet nasal cell populations. Since then, methimazole has been developed as a highly effective method for chemical ablation of the OE as it mimics the toxic environmental stimuli (Bergman and Brittebo, 1999; Bergstrom et al., 2003;
Brittebo, 1994). Methimazole toxicity occurs in cells expressing CYP2A5, which metabolise methimazole into reactive intermediates. Studies in rodents have revealed widespread apoptosis throughout the olfactory region caused by these reactive intermediates binding covalently to the tissue (Xie et al., 2011). Metabolites are found bound to the bowman’s glands and sustentacular cells, which are responsible for maintaining structure in the OE. Once bound, cell death is triggered through a mitochondrial, cytochrome c-mediated caspase-3 activation pathway (Sakamoto et al., 2007). As a result the olfactory epithelial structure breaks down resulting in the subsequent death of all cells residing in the epithelium (Brittebo, 1995; Mizutani et al., 1999) Despite this, methimazole is considered a mild treatment compared to other chemical ablation methods, as it discreetly targets and destroys the structure of the OE in a manner that allows rapid regeneration and has no other serious effects on the animal (Bergman and Brittebo, 1999; Bergstrom et al., 2003; Brittebo, 1994)

Summary of the aims undertaken in this chapter:

- To generate a reproducible pathophysiological model that ablates the OE and denervates the OB.

- To quantify the effects denervation has on the periglomerular cells of the OB.

- To quantify glial activation after denervation the OB.
3.2 Results

The temporal effects of methimazole on the olfactory system needed to be determined. This involved quantifying the duration of ORN death, post-exposure to the toxin, and duration of regeneration of the OE. To demonstrate the effects of methimazole a cohort of 50 animals was divided into a control and experimental group and treated as shown below.

**Day 1**

Control animals
- 25 mice were injected with PBS via intraperitoneal injection.

Treated animals
- 25 mice were injected with methimazole via intraperitoneal injection.

**Day 3**

Control animals
- The 25 mice from day 1 were once again injected with PBS via intraperitoneal injection.

Treated animals
- The 25 mice from day 1 were once again injected with PBS via intraperitoneal injection.

The control and treated animals were divided into five harvest groups each containing five animals in each. Each harvest group had a 3 day exposure to EdU at 30mg/kg body weight via intra peritoneal injection and were sacrificed (2.2.3) on the fourth day, as shown in the table below (Table 3.1). EdU treatment was used to mark proliferating cells.
Table 3.1: Harvest groups with corresponding EdU injection and sacrifice days for olfactory epithelium ablation.

<table>
<thead>
<tr>
<th>Harvest group</th>
<th>EdU injection day</th>
<th>Day Sacrificed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>3,4,5</td>
<td>6</td>
</tr>
<tr>
<td>Group 2</td>
<td>8,9,10</td>
<td>11</td>
</tr>
<tr>
<td>Group 3</td>
<td>11,12,13</td>
<td>14</td>
</tr>
<tr>
<td>Group 4</td>
<td>14,15,16</td>
<td>17</td>
</tr>
<tr>
<td>Group 5</td>
<td>17,18,19</td>
<td>20</td>
</tr>
</tbody>
</table>

After fixation and harvesting the brains and noses were dissected, sectioned and probed with a panel of immunohistochemical markers.

3.2.1 Olfactory Epithelial cell dynamics

3.2.1.1 Degeneration and regeneration of the young olfactory epithelium

The degeneration and subsequent regeneration of the OE was analysed through immunohistochemical analysis for (olfactory marker protein) OMP and β-3 tubulin, markers which are expressed in mature and immature ORNs of the olfactory epithelium, as well as DAPI to labelled cell nuclei, cleaved caspase 3 to mark apoptosis and EdU to identify new cells born three days prior to harvest (2.2.5.2). Animals were divided into groups (Tables 3.1), and control and methimazole treated animals were compared at all-time points. Four equivalent sections were selected and examined from each animal and repeated for a total of three animals.

In comparison to the control animals (Figure 3.1A), the methimazole injected animals showed a distinct lack of olfactory marker protein (OMP) (Figure 3.1B), which revealed the extent of lesioning in neuroepithelium. Using large scale mosaic images (2.2.6), it was seen
that the majority of the OMP had degenerated by day 6, leaving only the horizontal basal cell layer and basal lamina, (Figure 3.1B). The nerve fibres bundles within the lamina propria were also showed reduced in OMP staining.

**Figure 3.1: Coronally sectioned olfactory epithelium.** (A) Region of the dorsal OE from control animal, exhibiting strong OMP signal in the LP and OE. (B) Region of the dorsal OE on day 6 after methimazole injection, showing the complete loss of OMP signal in the OE (white arrow), leaving reduced staining in the LP. (OMP shown in red LP=Lamina propria, NC=Nasal cavity, OE=olfactory epithelium). Images taken at 100x magnification, Scale bar = 100µm.

Using high magnification imaging (2.2.6) OMP expression in the epithelium was monitored over time in conjunction with cleaved caspase-3 (Figure 3.2). A marked loss of OMP staining was seen in the epithelial layer 6 days post methimazole injection, (Figure 3.2 E, white arrow) while the basal layer was relatively unaffected (Figure 3.2 H, white arrow). This corresponded with an increase of cleaved caspase-3 staining within the epithelial layer of the OE (Figure 3.2 F, white arrow). However, cleaved caspase-3 was not expressed the nerve fibre bundles in the lamina propria (Figure 3.2 H, white arrow). By day 11, OMP staining was severely reduced (Figure 3.2 I) and cleaved caspase-3 was located in the nerve fibre bundles (Figure 3.2 J, white arrow), co-localising with the remaining OMP positive fibres (Figure 3.2 L, white arrow). Day 14 showed an increase in OMP staining in the axons within nerve bundles and the epithelium (Figure 3.2 M, white arrow) and cleaved caspase-3 staining had reduced in level and is absent from the nerve bundle (Figure 3.2 N). At day 17 the OMP immunoreactivity was fully restored showing extensive localisation within the epithelial cells and nerve fibre bundles in the lamina propria (Figure 3.2 Q). Cleaved caspase-3 levels in the regenerated epithelium were comparable to the control animals (Figure 3.2 R).
Figure 3.2: OMP and cleaved caspase-3 expression in the olfactory epithelium. Distribution mature ORNs and cleaved caspase-3 in control OE (A-D). Day 6 (E-H) OE was undergoing cell death as seen by the increase of cleaved caspase-3 (F, white arrow) and reduction of OMP (E, white arrow). Day 11(I-L) OMP staining was severely reduced (I) and cleaved caspase-3 was located throughout the nerve bundle (J and L, white arrow). Day 14 (M-P) shows regeneration with an increase in OMP in the OE, as well as increased axons within nerve bundles (M, white arrow). Cleaved caspase-3 had decreased both within the epithelium and nerve bundles (N). By Day 17 (Q-T) Cleaved caspase-3 had return to normal levels (R) and OMP was showing extensive localisation of epithelial cells and within nerve fibre bundles in the lamina propria (Q), (OMP shown in red, β-3 Tubulin in green, DAPI in blue). Images taken at 400x magnification, Scale bar =100µm.

The loss of OMP expression in the epithelium was also monitored in conjunction with βIII tubulin and EdU (Figure 3.3). Using high magnification imaging (2.2.6) a marked loss of βIII tubulin and OMP staining was seen 6 days post methimazole injection (Figure 3.3 F, G). This corresponded with a large number of EdU-positive cells along the basement membrane (Figure 3.3 H, white arrow). By day 11 the OE showed expression of βIII tubulin (Figure 3.3 L white arrow) and a few OMP-positive cells (Figure 3.3 K). EdU positive cell were now located throughout the epithelium, with some co-localising with βIII tubulin-positive cells (Figure 3.3 O). Day 14 showed an increase in both of βIII tubulin (Figure 3.3 Q) and OMP-positive cells (Figure 3.3 P, white arrow) and EdU-positive cell remained located throughout the epithelium (Figure 3.3 R, white arrow). At day 17 the OE was in late stages of regeneration, showing extensive localisation of both of βIII tubulin (Figure 3.3 V) and OMP
positive cells (Figure 3.3 U). EdU positive cells had moved more towards the apical surface with some co-localising with OMP positive cells (Figure 3.3 W, white arrow).

Figure 3. 3: OMP, β-3 tubulin and EdU localisation in the olfactory epithelium. Distribution of immature neurons (B), mature ORNs (A) and proliferative cells (C) in the control OE. Day 6 (F-J) OE shows staining of ORN (F, white arrow) and βIII tubulin (G) only in the nerve bundles of the OE. EdU was seen in a large number of cells in the basal membrane (H, white arrow). Day 11 (K-O) the OE was undergoing regeneration with βIII tubulin positive cells within the neuroepithelium(L, white arrow), OMP was seen within the nerve bundles (K) and EdU positive cells had begun to migrate apically (M). Day 14 (P-T) few OMP positive cells were detected in regions of regeneration (P, white arrow), βIII tubulin levels had returned to control levels (Q) and EdU positive cells remained evenly distributed within the OE (R, white arrow). Day 17 (U-Y) both OMP (U) and βIII tubulin (V) showed extensive localisation within epithelial cells. EdU positive cell had moved closer toward the apical surface (W, white arrow). (OMP shown in red, β-III tubulin in green, EdU in white, DAPI in blue). Images at 400x magnification, Scale bar = 50μm.
3.2.2 Olfactory bulb cell dynamics in response to sensory ablation

After successfully developing a protocol for ablating the OE the downstream effects of sensory ablation on the OB were examined. This was done by first quantifying the extent and duration of the loss of ORN axon terminals in the glomeruli. After the timeframe for denervation was established, immune response and specific periglomerular interneuron response were examined.

3.2.2.1 Denervation of the glomerular layer in the olfactory bulb
OMP is expressed in the axon terminals of ORNs that extend into the OB and terminate within the glomeruli. Thus, the glomeruli were qualitatively analysed for the amount of OMP-positive fibres at different time points following methimazole injection. When compared to the control animals (Figure 3.4 A), methimazole injected animals showed a distinct lack of OMP within the glomeruli of the OB at day 17 (Figure 3.4 B). OMP staining in the accessory bulb remained unaffected at all-time points (Figure 3.4 white arrows). Using large scale mosaic images (2.2.6) it was seen that the majority of the OMP staining had decreased by day 17, this value was generated through qualitative analysis, and is not an exact figure, merely an indication of the degree of ORN loss.

![Figure 3.4: Sagittal sectioned OB. (A) OB from control animal, exhibiting strong OMP signal in the glomerular layer and accessory bulb (with arrow). (B) OB on day 17 showing the loss of OMP staining in the external nerve fibre and the glomerular layer and the retention of OMP staining in the accessory bulb (white arrow). (OMP shown in red, DAPI in Blue). Image taken at 200x magnification, Scale bar = 200 µm.](image)

Using high magnification images (2.2.6) the loss of OMP staining in the bulb was monitored over time (Figure 3.5). Extensive OMP staining defined the glomeruli in the control animals. At day 6 though to 14 there was a gradual decrease in OMP expression in the glomeruli. By day 17 the external nerve fibre layer glomeruli and nerve fibre layer was almost completely devoid of OMP staining. ORN axons had reinnervated the OB by day 21, as demonstrated by strong OMP staining in glomeruli.
Figure 3.5: OMP expression in glomeruli after methimazole treatment. Control animals show extensive OMP expression defined the neuropil of the glomeruli. Day 6 to day 14 there is a gradual decrease in ORN axon terminal density in glomeruli. Day 17 the glomeruli and nerve fibre layer is almost completely devoid of OMP staining. Day 21 ORN axons have reinnervated the OB, exhibited by strong OMP staining in glomeruli (OMP shown in red, DAPI in Blue). Mosaic images at 630x magnification, Scale bar = 10 µm

3.2.2.2 Glial response in to sensory denervation

The OB contains large numbers of astrocytes and microglial which may be important for normal development and changes seen after olfactory deprivation. To measure glial response within the OB, densitometry was used to measure GFAP (astrocyte) and Iba1 (microglia)
immunoreactive cells (2.2.7.1). Images were captured using a high-resolution objective (63× oil immersion objective) from two glomeruli (Figure 3.6) and the measurements were undertaken on 3 animals from each harvest group (Table 3.1).

Figure 3. 6: GFAP and IBA1 expression in the glomeruli of the OB. An example of an image used for densitometry of Iba1 and GFAP positive cells in the glomeruli. (GFAP shown in green, Iba1 in red, DAPI in Blue). Mosaic images at 100x magnification. Scale bar = 10 µm.

GFAP showed an initial percentage 1.2 ± 0.12 followed by a gradual increase in density after methimazole injection which peaked on day 14 at 12.88% ± 1.05 followed by a decrease at day 17 to 11.72% ± 1.19 and at day 20 to 10.635 ± 0.62 (Figure 3.7). One way ANOVA (2.2.8) revealed a significant increase for GFAP density within the glomeruli with a P value of < 0.001. Post hoc Tukey tests indicated that the percentage of GFAP expression was significantly increased at days 11, 14, 17 and 20 (p < 0.001) when compared to controls (day 0) (3.7*).
Figure 3.7: Response of astrocytes to methimazole exposure. GFAP density is significantly increased in the glomeruli at 11, 14, 17 and 20 days following methimazole exposure when compared to the control (day 0). (Significant values of p<0.001 denoted by asterisk). Data presented as mean ± SE.

Iba1 followed a similar trend to GFAP, with an initial percentage 0.5 ± 0.13 followed by gradual increase peaking at day 14 at 11.17% ± 1.12 then declining in density over day 17 and 20 to 10.34% ± 0.84 and 10.68% ± 0.62 respectively (Figure 3.8). Statistical analysis (one way ANOVA) revealed there was a significant increase for Iba1 density within the glomeruli with a p value of < 0.001. Post hoc Tukey tests indicated that the percentage of Iba1 expression was significantly increase at days 11, 14, 17 and 20 (p < 0.001), compared to controls (day 0).
3.2.2.3 Effects of olfactory receptor neuron denervation on periglomerular neurons

The glomerular layer of the OB contains periglomerular cells which are categorised as Type I and Type II. Type I periglomerular cells predominantly express TH whereas Type II periglomerular cells are identified by either calbindin or calretinin expression (Figure 3.9). Immunohistochemistry (2.2.5.2) and high magnification images (2.2.6) were used to examine the effect of methimazole-induced lesioning on the periglomerular interneurons. These changes were quantified by conducting cell counts on individual periglomerular cells types as a percentage of total cells (2.2.7.2).

**Figure 3. 8: Response of microglia to methimazole exposure.** Iba1 density is significantly increased in the glomeruli at 11, 14, 17 and 20 days following methimazole exposure when compared to the control (day 0). (Significant values of p<0.001 denoted by asterisk). Data presented as mean ± SE.
Figure 3. 9: TH, calbindin and calretinin expression in the glomeruli of the OB. TH immunoreactive neurons form the majority of the Type I class of neurons and calbindin and calretinin immunoreactive neurons form the majority of the Type II class of neurons. The remaining DAPI stained nuclei in the periglomerular cell layer would primarily be glial cells (astrocytes and microglia). (TH, Calbindin, Calretinin and Merged image includes DAPI nuclei staining in blue, PG=periglomerular). Images taken at 200x magnification, Scale bar = 20 µm.

3.2.2.3.1 Effects of olfactory receptor neurons denervation on Type I cells
The effect and temporal dynamics on the Type I periglomerular interneurons after denervation of the OB was determined. This involved immunohistochemical analysis for OMP to mark denervation and dopamine markers; TH and DOPA decarboxylase to label Type I periglomerular neurons. Co-labelling with DAPI was used to identify cell nuclei and cleaved caspase 3 to identify apoptosis (2.2.5.2). Animals were divided into groups (Tables 3.1) and control and methimazole were compared at all-time points.

Initially, a combination of TH and DOPA decarboxylase was used to quantify the effects of denervation on Type I periglomerular neurons. Using the substantia nigra as an internal control, a 100% co-localisation of the markers within the nerve cell bodies was shown (Figure 3.10 White *). However within the OB, co-localisation between TH and DOPA decarboxylase was not seen (Figure 3.10, white arrows). Therefore, a combination of TH and cleaved caspase-3 was used to determine the fates of Type I periglomerular neurons within the OB.
Figure 3. 10: TH and DOPA decarboxylase expression in the substantia nigra and OB. Substantia nigra shows co-localisation (white *) of TH (green) and DOPA (red) in the nerve cell bodies. TH (green) and DOPA (red) in the OB shows no co-localisation between TH and DOPA decarboxylase was not seen (white arrows). Images taken at 630x magnification, Scale bar = 10 µm.
Using large mosaic images (2.2.6), TH and OMP expression in the bulb was monitored over a 20 day period (Figure 3.11). Extensive TH and OMP expression in the glomeruli layer was seen in the control animals (Figure 3.11 control). At day 17 post-lesion there was a drastic decrease in TH expression of the glomeruli (Figure 3.11 treated) and OMP expression was mainly confined to the nerve fibre layer.
Figure 3. TH and OMP expression in the OB before and after methimazole treatment. The control exhibits dense TH (green) and OMP (red) cell dendritic arbour in the glomeruli layer. In contrast the methimazole treated animals exhibit a loss of TH (green) and OMP (red) in the glomeruli layer. Images taken at 200x magnification. Scale bar = 400µm
To quantify the TH loss, cell counts were performed on glomeruli from the dorsal, rostral, and ventral regions of OB on sagittal sections. Single Z slices from three glomeruli (Figure 3.12 A) were chosen from each area and the counts were undertaken on 3 animals from each harvest group (Table 3.1). Cell counts were carefully carried out to only include cells the stained positive for both TH and DAPI and were associated with the chosen glomeruli as defined by DAPI. Statistical analysis revealed that in the OB, there was a significant decrease in TH positive cells \( (p=0.009) \). Post hoc Tukey tests indicated that the percentage of TH positive cells was significantly reduced at day 14 \( (p=0.027) \) and 18 \( (p=0.017) \), compared to controls (Figure 3.12 B). Cell counts were performed with the help of Patrick Dwyer.
Figure 3. 12: Response of Type I periglomerular interneurons to methimazole treatment. (A) An example of an image used for counting Type I cells using TH (green) and DAPI (blue) positive cells in the glomeruli. OMP (red) was used as an indicator of sensory denervation. Images taken at 630x magnification, Scale bar = 10 µm. (B) Line graph showing significant decrease in the percentage of TH positive cells in glomeruli at 14 and 17 days following methimazole exposure when compared to the control (day 0). (Significant values of p<0.05 denoted by asterisk) Data presented as mean ± SE.
To determine if the reduction in cell number was due to cell death or down-regulation of TH, cleaved caspase-3 was used as an apoptosis marker. Although there was increase in cleaved caspase-3 levels within axons of the glomerular layer 17 days after methimazole treatment (Figure 3.13A), only a few cells showed cleaved caspase-3 expression in the nucleus (Figure 3.13B).

**Figure 3.13**: Cleaved caspase-3 and TH expression in the OB after methimazole treatment. (A) Increased levels of cleaved caspase-3 (red) were seen within axons of the glomerular layer 17 days after methimazole treatment. (B) Co-localisation of cleaved caspase-3 (red) and TH (green) expression in the nucleus of the cells (white *). DAPI labels cell nucleus shown in blue. Images taken at 630x magnification, Scale bar = 20 µm.
3.2.2.3.2 Effects of denervation on Type II cells

The effect of ORN denervation of the OB on the Type II periglomerular interneurons was determined. This involved immunohistochemical analysis for OMP to mark denervation and calcium binding proteins; calbindin and calretinin to label Type II periglomerular neurons. As well as DAPI to label cell nuclei and cleaved caspase 3 to identify apoptosis (2.2.5.2). Animals were divided into control a methimazole groups (Tables 3.1) and were compared at all-time points.

Using low magnification mosaic images (2.2.6), the loss of calbindin and calretinin expression in the bulb was monitored over time. There was no visible decease in Type II cell numbers or arbour in the five time points tested (data not presented). To quantify any change in calbindin and calretinin expression post-lesion, cell counts were performed on glomeruli from the dorsal, rostral, and ventral regions of OB on sagittal sections. Single Z slices from three glomeruli (Figure 3.14 A) were chosen from each area and the counts were undertaken on 3 animals from each harvest group (Table 3.1). Cell counts included only cells that stained positive for either calbindin or calretinin and DAPI and were associated with the chosen glomeruli as defined by DAPI staining. Cell counts were performed with the help of Patrick Dwyer. Although there was a decrease in cell number on day 14, statistical analyses revealed there was no significant change in number of calretinin and calretinin positive cells (Figure 3.13 B).
Figure 3.14: Response of Type II periglomerular interneurons to methimazole treatment. (A) An example of an image used for counting Type II cells using calretinin (green), calbindin (red) and DAPI (blue) positive cells in the glomeruli. Images taken at 630x magnification, Scale bar = 10 µm. (B) Line graph showing decrease in the percentage of Type II cells in glomeruli at 14 and 17 days following methimazole exposure when compared to the control (day 0). Data presented as mean ± SE.
3.3 Discussion

3.3.1 Overview and summary

The hypothesis tested in this study was that ORN turnover in the OE results in altered cell function or cell death within the OB. Methimazole was used to focally ablate the epithelium generating a pathophysiological model of olfactory epithelial cell turnover. Based on data obtained in a pilot study, a two sequential methimazole injection protocol were used to ablate the OE over a longer duration, causing a robust denervation of the OB. After methimazole or PBS injections animals were treated with EdU and sacrificed at 6, 11, 14, 17 and 20 days following the first injection. The degeneration and regeneration of the OE and bulb were analysed using a panel of immunohistochemical markers. These showed that greater than 90% of the OE was ablated 6 days post treatment and fully regenerated by day 20. The axons of the ORNs which terminate in the glomeruli had degenerated by day 17 and were regenerated completely by day 20. A reactive gliosis, as reflected by an increase in glial markers, was seen at day 11, peaking at day 14 and reduced in number but remained elevated at day 20 post treatment. Cell counts and subsequent statistical analyses revealed that at 14 and 17 post-lesioning, a significant reduction in the number of Type I cells when compared to the unlesioned control. There was no significant effect of the lesion on the Type II

3.3.2 Response of the olfactory epithelium after methimazole treatment

To determine the duration of ORN death, post-exposure to the methimazole, and the time required for regeneration of the OE was established. Whilst some estimate could be made from the literature, specific temporal data was essential for this study (Bergman and Brittebo, 1999; Bergstrom et al., 2003; Brittebo, 1995). Previous projects in our laboratory have shown that a single injection of methimazole does not completely ablate the OE nor denervate the
OB. However, these studies showed some epithelial ablation after 3 days after treatment. Given this, it was determined to maximise the duration of the denervation by using a second injection given 2 days the initial methimazole injection. The aim was to cause a near-complete and prolonged ablation of ORNs in the olfactory epithelium, together with their axon terminals in the glomeruli of the OB. Based on data from an early cohort, fifty animals were divided into two groups treated with either methimazole or PBS and sacrificed at a series of time points to characterize the degeneration and subsequent regeneration of the olfactory epithelium. Cleaved caspase -3 was used to determine the extent of apoptotic cell death. Expression of OMP was used to mark mature olfactory neurons as a baseline indicator of OE structure. The expression of EdU and βIII tubulin, were analysed to calculate the extent of regeneration of olfactory neurons within the epithelium.

3.3.2.1 Degeneration of the olfactory epithelium

The degeneration of the OE was confirmed by the expression of cleaved-caspase-3. The loss of OMP expression, together with the loss of DAPI stained nuclei, was seen on day 6, occurring simultaneously with an increase of cleaved caspase-3 expression in the epithelium (Figure 3.1). The loss of cells after methimazole treatment is cause by reactive intermediates triggering the mitochondrial cytochrome c-mediated caspase-3 activation pathway (Sakamoto et al., 2007). This data is further corroborated by Xie 2011, Brittebo 1995 and Mizutani 1999 (Brittebo, 1995; Mizutani et al., 1999; Xie et al., 2011) who all showed destruction the OE structure after methimazole treatment. By day 11 the OE showed maximum ablation, with cleaved caspase-3 expression also occurring in the nerve fibre bundles (Sakamoto et al., 2007; Xie et al., 2011). These nerve fibre bundles are responsible for transporting sensory information to the OB and it is their death that will lead to denervation of the OB. Day 14 showed the beginning of regeneration with an increase in OMP expression and a decrease in cleaved caspase-3 in the axons within nerve bundles and in the epithelium(Sakamoto et al., 2007; Xie et al., 2011). By day 20 the OE was fully regenerated with extensive OMP localisation within epithelial cells and nerve fibre bundles in the lamina propria (Brittebo, 1995; Mizutani et al., 1999; Xie et al., 2011). The expression levels of cleaved caspase-3 levels were comparably to the control animals (Figure 3.2).
3.3.2.2 Regeneration of the olfactory epithelium

The regeneration of the OE was further characterized by the expression of βIII tubulin which shows the growth of immature neurons and EdU to show regions of proliferation. Loss of OMP and βIII tubulin of staining was seen 6 days post methimazole injection. This corresponded with a large number of EdU positive cells along the horizontal basal cell layer, the putative location of the olfactory stem cells (Carter et al., 2004; Iwai et al., 2008; Leung et al., 2007). These cells expressed neither βIII tubulin, nor OMP. By day 11 the immature neurons (βIII tubulin) had been restored to the levels seen in the control animals. The EdU labelled cells continued to divide and some migrated towards the surface of the olfactory. Many of these EdU positive cells were also positive for βIII tubulin which infers that the dividing cells seen at day six were maturing. Day 14 showed an increase in both mature and immature neuron with an increase in the distribution of EdU positive cells. The OE was fully restored by day 20 showing extensive localisation of both of immature and mature ORNs within the OE and the nerve fibre bundle (Calof et al., 1996). EdU positive cells had migrated towards the apical surface and were seen to be co-localising with OMP cells as they developed into mature ORNs (Figure 3.3) (Graziadei and Graziadei, 1979). The speed of degradation and regeneration highlights that methimazole is a mild to chemical ablation method, as it discreetly targets and destroys the OE structure in a manner that allows quick regeneration and has no other serious effects on the animal (Bergman and Brittebo, 1999; Bergstrom et al., 2003; Brittebo, 1994).

3.3.3 Downstream response of sensory ablation in the olfactory bulb

Once the timeline of degeneration and regeneration was established the study focused on characterising the impact of the loss of olfactory sensory neurons, on the OB. The loss of ORN axon terminals within the glomeruli in the OB was defined through the expression of OMP. Cleaved caspase- 3, GFAP, Iba1, DOPA decarboxylase, TH, calbindin and calretinin were used to analysis immune response and the downstream effects on the periglomerular interneurons.
3.3.3.1 Denervation of the olfactory bulb

In control animals extensive OMP expression defined the glomeruli and the nerve fibre layer. At day 6 though to 14 there was gradual decrease in OMP expression in the glomeruli this corresponded with the degeneration of the OE (3.3.2). However maximum denervation of the glomeruli and nerve fibre layer did not occur until day 17, when the OE had shown to be fully regenerated (3.3.2). The temporal differences in the denervation of the OE and the OB correspond to the time required for the axons of the ORNs to degenerate. This is reflected in the time taken for cleaved caspase-3 to localise from the epithelium (day 6) to the nerve fibre bundles (day 11) (Figure 3.5). This process of degeneration delay would facilitate the fast re-innervation of the OB. Whilst the absence of OMP suggests the loss of the axons and terminals of the olfactory sensory neuron, the cellular remnants would potentially provide the required guidance cues for the newly projecting axons. This has been shown in other peripheral nerve regeneration studies (Carter et al., 2004; Iwai et al., 2008; Leung et al., 2007). Interestingly at no time point did the accessory bulb lose innervation, this is thought to be because it is innervated by the vomeronasal organ which is anatomically segregated from the OE and unaffected by methimazole treatment (Brunjes and Kishore, 1998; Dulac and Axel, 1995; Wagner et al., 2006).

3.3.3.2 Activation of glial cells after olfactory receptor neuron death

Astrocyte marker GFAP showed a gradual increase after methimazole injection peaking on day 14 followed by a decrease on day 17 and 20. Analysis via one way ANOVA showed that GFAP expression was significantly greater at days 11, 14, 17 and 20 (p < 0.001) when compared to controls (Figure 3.7). Microglia marker Iba1 followed a similar trend to GFAP with a gradual increase peaking at day 14 then declining in density over day 17 and 20. Analysis via one way ANOVA showed that Iba1 immunoreactivity was also significantly higher at days 11, 14, 17 and 20 (p < 0.001), compared to controls (Figure 3.8). Increased expression of GFAP and Iba1 are indicative of glial activation a process which occurs during
neurodegeneration (Eng et al., 1992; Fiske and Brunjes, 2001; Norazit et al., 2011; Reier et al., 1986). While the exact mechanisms for activation of glia are not yet fully understood in this case it can be attributed to the death of the ORN axons which terminate in the bulb and the concurrent impact of this denervation on the periglomerular cells (3.3.2.2). Astrocytes react to neuronal death leading to vigorous astrogliosis; they have also been shown to activate microglia through CA2+ waves (Schipke et al., 2002) and iNOS expression (Brahmachari et al., 2006; Murphy, 2000). Upon neuronal death microglia transforms to phagocytos neural debris (Ashwell, 1991; Lafarga et al., 1998; Rinaman et al., 1991). The time taken for the microglia to transform and remove the dead ORNs also offers a possibly explanation for the delay between OMP lose in the OE and OB. This pathophysiological model enables glial activation in the brain in the absence of direct lesioning and concurrent breaching of the blood brain barrier.

3.3.3.3 Presynaptic terminal loss affects periglomerular cells

With the successful denervation of the OB for an extended period of time the effect of presynaptic terminal loss on the postsynaptic interneuron populations was tested. The effects of OE ablation on periglomerular cell populations were examined at specific time points following methimazole exposure.

3.3.3.3.1 Loss of TH immunoreactivity in Type I periglomerular neurons

Initial analysis used TH and DOPA decarboxylase as markers to test effects of denervation on Type I periglomerular neurons. This was because the loss of TH immunoreactivity alone may be attributed to either down regulation of TH expression or cell death due the loss of innervation by ORNs. Several studies indicated the use of DOPA decarboxylase as a robust secondary marker to determine the fate of the Type I cells (Baker, 1990; Ljungdahl et al., 1977; Weihe et al., 2006). Unfortunately, while the dopaminergic cells of the substantia nigra showed 100% co-localisation of the markers within cell bodies, no co-localisation of TH and
DOPA decarboxylase within the OB was seen (Figure 3.10). Thus, DOPA decarboxylase could not be used to identify the dopaminergic neurons of the OB.

Using TH as a marker there is a drastic decrease in expression at days 14 and 17 post lesions of the glomeruli which corresponds to the maximum denervation as shown by OMP expression (Figure 3.11). Statistical analysis revealed that the percentage of Type I cells was significantly reduced at day 14 ($p=0.027$) and 17 ($p=0.017$), compared to controls (Figure 3.12B). These results are similar to data from studies ablating the OE in rodents using zinc sulphate or naris occlusion (Baker, 1990; Baker et al., 1983; Baker et al., 1993; Cho et al., 1996; Mandairon et al., 2003). These former studies proposed a down regulation of TH expression in the OB, rather than a loss of Type I cells.

An alternative hypothesis is that a reduction in TH positive cells could represent Type I periglomerular cell death due to the loss of synaptic input (Sawada et al., 2011). Death of Type I cells following ablation of the OE may represent a natural mechanism of cell turnover in the olfactory system, as some cell death has been recorded in this area following OE ablation (Mandairon et al., 2003; Sawada et al., 2011). To determine if the reduction in cell number was due to cell death or down regulation of TH, cleaved caspase-3 was used as an apoptosis marker (Gavrieli et al., 1992). Although there was increase in cleaved caspase-3 levels seen within the glomerular layer 17 days after methimazole treatment only a few cell showed expression in the nucleus (Figure 3.13). This could be because of a lack of cell death or time points observed after denervation. The expression of cleaved caspase-3 is restricted to a short duration during the apoptotic cascade (Narkilahti et al., 2003) and it is possible that the precise time point of caspase-3 expression was missed in this study. At day 17 there are minimal number of TH cells present makes comparison between the two markers difficult. Previous studies have shown in postnatal mice naris occlusion dramatically reduces OB size caused by cell death (Brunjes, 1994; Fiske and Brunjes, 2001; Najbauer and Leon, 1995). While adult animals demonstrate reduced response to naris occlusion (Brunjes, 1985; Brunjes and Borror, 1983), long periods of deprivation call still result in a reduction of size (Henegar and Maruniak, 1991; Maruniak et al., 1989). Taken together these results suggest that the reduction in TH could possibly be related to cell death.
3.3.3.2 Type II periglomerular neurons are unaffected by olfactory receptor neurons loss

Qualitative analysis revealed no gross morphological changes in the dendrites of the Type II neurons following extended absence of axon terminals of ORNs from the OB. Although quantitative analysis revealed a decrease in Type II cells 14 and 18 days following methimazole injection it was not statistically significant (Figure 3.14B). This trend coincides with a previously published report showing a 30% reduction in the number of calbindin cells following sensory deprivation in the developing rat OB (Philpot et al., 1997a). The less dramatic reduction, then that of the type 1 cells, could be because of the structure and circuitry within the bulb. Type II periglomerular neurons do not synapse directly with ORNs but to the mitral and tufted cells and have been shown not to require innervation from the OE for survival (Bastien-Dionne et al., 2010; Bovetti et al., 2009).
3.4 Conclusion

A focal, extended lesioning protocol was designed and demonstrated to nearly completely ablate the OE and sensory innervation of the OB. This resulted in an increase in glia activation and subsequent effects on periglomerular neurons. It was shown that ORN loss greatly affected TH expression in the Type I periglomerular cells, whether due to cell death or down-regulation of TH remains to be elucidated. The next chapter will discuss upstream effects of ORN ablation on cell proliferation in the SVZ.
Chapter 4

Neurogenesis in the Subventricular zone: response to pathophysiological alterations in the primary olfactory system and age
4.1 Introduction

As demonstrated (chapter 3), methimazole ablated the OE and fully denervate the ORN axon terminals in the glomeruli of the OB. This denervation leads to an increase in glial activity starting at day 6 and peaking at day 14. Significant loss TH immunoreactivity was also seen following the loss of the axon terminals within the glomeruli. The next aim investigates whether cell turnover in the OE results in up-regulation of neurogenesis in the SVZ and will establish a model to investigate mechanisms underlying adult neurogenesis.

The SVZ is a narrow layer of tissues located throughout the lateral walls of the lateral ventricles. The SVZ produces a population of OB interneuron progenitors throughout life (Alvarez-Buylla et al., 2000). New cells migrate away from the SVZ before they undergo differentiation and express neuronal markers (Alvarez-Buylla et al., 2000; Brazel et al., 2003; Kirschenbaum et al., 1999). The progenitors then migrate along a well-defined pathway (the RMS) and orient to reach their correct target layers and differentiate into interneurons.

Signalling within the SVZ is highly complex, with intricate communication between the different cell types and has been attributed to various mechanisms. There are many important intrinsic signalling pathways that affect neurogenesis within the SVZ such as BMPs, Notch, Wnt and β-Catenin pathways (Hirabayashi and Gotoh, 2005; Patapoutian and Reichardt, 2000). There are also a number of extrinsic signals that have an effect on neurogenesis in the SVZ such as dopamine, serotonin, GABA and glutamate (Baker et al., 1991; Brezun and Daszuta, 1999a; Castelo-Branco et al., 2006; Platel et al., 2007b). However, these signalling mechanisms are still not well understood. This study is designed to elucidate a mechanism that stimulates cell proliferation and differentiation in the SVZ, linking it to changes in the OE. By using EdU as a marker for newly divided cells (Cavanagh et al., 2011; Cheherehasa et al., 2009).
This chapter uses EdU to provide data on proliferation and differentiation in cells derived from the SVZ in the ageing brain, in response to focal chemical ablation of the neuroepithelium.

Summary of the aims undertaken in this chapter:

- Examine any changes in the SVZ in response to a focal ablation of the olfactory epithelium.

- Quantify the rate of cell proliferation in the SVZ in response to a focal ablation of the olfactory epithelium.

- Elucidate the effect of ageing on SVZ cell proliferation.
4.2 Results

Once the temporal effects of methimazole on the primary olfactory system were determined the effects of denervation on the SVZ were examined. To do this, a cohort of 50 animals were divided in to a control and experimental group and treated as shown below.

**Day 1**

Control animals
- 25 mice were injected with PBS via intraperitoneal injection.

Treated animals
- 25 mice were injected with methimazole via intraperitoneal injection.

**Day 3**

Control animals
- The 25 mice from day 1 were once again injected with PBS via intraperitoneal injection.

Treated animals
- The 25 mice from day 1 were once again injected with methimazole via intraperitoneal injection.

The control and treated animals were divided into five harvest groups containing five animals each. Each harvest group had a 3 day exposure to EdU at 30mg/kg body weight via intraperitoneal injection and were sacrificed (2.2.3) on the fourth day, as shown in the table below. EdU treatment was used to mark proliferating cells.
Table 4.1: Harvest groups with corresponding EdU injection and sacrifice days for SVZ counts.

<table>
<thead>
<tr>
<th>Harvest group</th>
<th>EdU injection day</th>
<th>Day Sacrificed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>3,4,5</td>
<td>6</td>
</tr>
<tr>
<td>Group 2</td>
<td>8,9,10</td>
<td>11</td>
</tr>
<tr>
<td>Group 3</td>
<td>11,12,13</td>
<td>14</td>
</tr>
<tr>
<td>Group 4</td>
<td>14,15,16</td>
<td>17</td>
</tr>
<tr>
<td>Group 5</td>
<td>17,18,19</td>
<td>20</td>
</tr>
</tbody>
</table>

After harvesting, the brains and noses were processed, sectioned and treated with a panel of immunohistochemical markers.

4.2.1 Cell genesis in the SVZ and response to olfactory sensory neuron ablation

The effects that ablation of the OE and subsequent denervation of the OB had on the SVZ were analysed through immunohistochemical analysis combined with the cell proliferation marker EdU. EdU was administered to assess cell proliferation and was co-labelled with DAPI for analysis of SVZ cell proliferation. Cleaved caspase-3 was used as a marker for cell death (2.2.5.2). Animals were divided into groups (Tables 4.1) and control and methimazole were compared at all-time points.

Using the Paxinos Mouse Brain Atlas 2nd Edition as a guide, the distance from midline in the sagittal plane and the characteristic anatomy was used to determine the precise lateral orientation. This was important to avoid variability in the quantitative analysis. Care was taken to keep the shape of the ventricle constant as cells proliferate at different rates depending on the region of the SVZ (Figure 4.1A). For proliferation studies images were
captured from two preselected areas as a 20μm high series of optical slices (Z-stack). From this stack three images were taken at 5 μm, 10 μm and 15 μm depths and used for cell counts (Figure 4.1 B, 1 and 2). All cell nuclei which co-localised EdU and DAPI were counted. All cell counts had time point matched controls to combat natural variation in a continuously dividing area. One section from each hemisphere of the brain was taken and 3 animals were used from each harvest group (Tables 4.1).

Figure 4.1: Sagitally-sectioned lateral ventricle and example of an image used for counting. (A) The characteristic anatomy of the lateral ventricle (LV) was used to avoid variability. Rectangles indicate regions where images were taken and cell counts were performed. Images taken at 200x magnification, Scale bar = 200 μm. (B) 1 and 2 are an example of an image used for counting dividing cells (Red) in the SVZ. Images taken at 400x magnification, Scale bar = 10 μm. (DAPI in blue, RMS = rostral migratory stream).
While there were some variations seen between groups, control and treated animals showed consistent cell counts of around 60 EdU positive cells on days 6, 14, 17 and 20. Importantly, there was a 59% increase in cell numbers seen between the control and treated groups on day 11. From 53± 5 EdU positive cells in the control to 82± 6.9 cells in the treated. Using one way ANOVA (2.2.8) revealed a significant increase in cell number in the SVZ after methimazole treatment with a P value of 0.03. Post hoc Tukey tests indicated that there was a significant increase in cell number between the treated animals on day 11 and the time match control (p 0.04) (Figure 4.2*).

Figure 4. 2: Cell proliferation in the SVZ in control verse methimazole treated animals. Cell counts of around 60 EdU positive cells were seen across days 6, 14, 17 and 20. However, a significant increase of 59% (from 53± 5 to 82± 6.9) was seen between the control and treated animals on day 11. (Significant values of p<0.05 denoted by asterisk). Data presented as mean ± SE.
4.2.2 Aged SVZ cell genesis in response to sensory ablation

The addition of an aged cohort allowed for the study of ageing on neurogenesis. Based on the time points which showed increased neurogenesis elucidate in the young animals an experimental cohort comprised of 2 year-old mice were used to determine the effect of increased age on SVZ cell genesis.

4.2.2.1 Degeneration and regeneration of the aged olfactory epithelium and bulb

The effects of methimazole on the aged OE and bulb were first determined. This was analysed using immunohistochemical labelling for OMP, which is expressed in mature ORNs, as well as DAPI to label cell nuclei (2.2.5.2). In comparison to the control animals, the methimazole injected animals showed a distinct lack of OMP staining in the OE on day 3 (Figure 4.3 A, B) and showed a return to control levels by day 20 (Figure 4.3 A, B, C). In the OB it was seen that the majority of the OMP staining was lacking on day 17 and had returned to control levels by day 20 (Figure 4.3 D, E, F).
Figure 4. 3: Degeneration and regeneration of the aged OE and bulb. Distribution of mature ORNs in control OE (A). Day 3 shows reduction of OMP after methimazole (B). Day 20 OMP staining showing the regeneration of the OE (C). Images taken at 400x magnification, Scale bar = 20µm. (OMP shown in red, DAPI in Blue). Control animal OB exhibits strong OMP signal in the glomerular layer (D). Day 17 shows the loss of OMP staining in the glomerular layer after methimazole treatment (E). Day 20 OMP staining showing the reinnervation of the OB (C). (OMP shown in red, DAPI in Blue). Image taken at 630x magnification, Scale bar = 10 µm.

4.2.2.2 Cell genesis in the aged SVZ response to olfactory sensory neuron ablation

Once the effects of methimazole on the aged epithelium were established, effects of denervation of the primary olfactory circuit on the aged SVZ were determined. Animals were divided into two groups, one following the timeline for the original pulse seen in young the animals. To check for a delayed pulse a second group, containing a control group and a methimazole group, will receive EdU at a later time point.
Day 1

Control animals
- 10 mice were injected with PBS via intraperitoneal injection.

Treated animals
- 10 mice were injected with methimazole via intraperitoneal injection.

The control and treated animals were then divided into two harvest groups containing three animals. Each harvest group had a 3 day exposure to EdU at 30mg/kg body weight via intraperitoneal injection and be sacrificed on the fourth day and sacrificed as shown in the table below.

Table 4.2: Aged animal harvest groups with corresponding EdU injection and sacrifice days for SVZ counts.

<table>
<thead>
<tr>
<th>Harvest group</th>
<th>EdU injection day</th>
<th>Day Sacrificed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>8,9,10</td>
<td>11</td>
</tr>
<tr>
<td>Group 2</td>
<td>11,12,13</td>
<td>14</td>
</tr>
</tbody>
</table>

The animals were perfusion fixed and brains and noses were dissected, processed, sectioned and treated with a panel of immunohistochemical markers to examine the effects of OE ablation on the SVZ. EdU was administered to assess cell proliferation and was targeted in conjunction with DAPI for analysis of SVZ cell proliferation (2.2.5). Animals were divided into groups (Table 4.2) and control and methimazole were compared at all-time points.

As before the Paxinos Mouse Brain Atlas 2nd Edition was used as a guide for anatomy. The cell counts were conducted as per the experiments on the young animals. All cell nuclei which co-localised EdU and DAPI were counted and cell counts had time point matched. One section from each hemisphere of the brain was taken and 3 animals were used from each harvest group (Tables 4.2).
On Day 11, control and treated animals appeared to have consistent cell count of approximately 20 ± 2 cells. By day 14 there was a 36% (22 ± 0.9 to 30 ± 1.8) increase in the number of Edu positive cells in the treated animals compared to the controls. Using one way ANOVA (2.2.8) revealed a significant difference in EdU labelled cells over time in the SVZ after methimazole treatment with a P value equal to 0.003. Post hoc Tukey tests indicated that there was a significant increase in cell number between treated animals on day 14 and the time match control (p 0.01) (Figure 4.5 *).

![Proliferation in response to methimazole treatment in aged animals](image)

**Figure 4.4: Cell proliferation in the aged SVZ following methimazole exposure.** When comparing the different time points a 36% increase was seen between treated animals on day 14 and the time match control. (Significant values of p<0.05 denoted by asterisk). Data presented as mean ± SE.

### 4.2.3 Age related changes in proliferation in the SVZ

Using the time points were increased neurogenesis occurred the two age groups were compared. The control young and aged control groups there was a 58% (53 ± 5 to 22 ± 0.9) fewer of EdU positive cells in the aged animals. There was a 63% (82 ± 6.9 to 30 ± 1.8)
decrease seen in the number of EdU positive cells in the aged cohort when comparing the treated animals. There was also a decrease in the percentage increase of cells between control and treated within each age groups (from 59% to 36%). Using one way ANOVA (2.2.8) revealed a significant difference in proliferation between young and aged SVZ after methimazole treatment with a P value of < 0.001. Post hoc Tukey tests indicated that there was a significant decrease in cell number between both young and aged controls (p < 0.001) and treated animals (P < 0.001) (Figure 4.6).

Figure 4.5: Difference in cell proliferation between young and aged SVZ following methimazole exposure. Significant decrease was seen in the number of EdU positive between the young and aged control and treated animals. There was a 58% (53 ± 5 to 22 ± 0.9) decrease between the controls, a 63% (82 ± 6.9 to 30 ± 1.8) decrease between the treated animals and a decrease in the percentage increase of cells between control and treated within aged groups (from 59% to 36%). (Significant values of p<0.05 denoted by asterisk, significant values of p<0.05 denoted by Δ). Data presented as mean ± SE.
4.3 Discussion

4.3.1 Overview and summary

It was hypothesised that OE ablation and the resultant denervation of the OB would trigger an up-regulation of cell proliferation in the SVZ. Following methimazole injections animals were treated with EdU, sacrificed at 6, 11, 14, 17 and 20 days. EdU together with immunohistochemical markers changes in proliferation and cell death to be established. Cell counts and subsequent statistical analyses revealed that treated animals from the day 11 group exhibited a 59% increase in the number of newly divided cells. Using these time points, an aged population was used to examine the effects of aging on neurogenesis. After confirming ablation of the OE and denervation of the bulb was sufficient with only one methimazole dose, animals were treated with EdU, sacrificed and immunohistochemical markers were used to examine changes in proliferation. The first was on day 11 to coincide with the pulse seen in young animals and the second was on day 14 to allow for a possible delay in response. Statistical analyses of cell counts revealed that treated animals from the day 14 group exhibited a 36% increase in the number of cells. Comparing these studies showed that there was a decrease in overall proliferation between young and aged animals; however the increase in neurogenesis after methimazole treatment was seen in both groups.
4.3.2 Olfactory epithelial ablation increases cellular proliferation in the SVZ

Previous studies have demonstrated a relationship between sensory input to the OB and the rate of proliferation in the SVZ (Corotto et al., 1994; Kirschenbaum et al., 1999). Many of these studies relied on surgical methods to elucidate the connection between sensory deprivation and SVZ proliferation rates (Graziadei et al., 1979; Lim and Brunjes, 1999; Mandairon et al., 2003). This study aimed to further these results by generating a model that focally ablates ORNs to reduce changes in the SVZ caused by a brain lesion-dependent mechanism (Rakic, 2002a). Using methimazole successful ablation of the OE and denervation the OB was achieved (Chapter 3). Any change in the proliferation or cell death within the SVZ was monitored with EdU and cleaved caspase -3, respectively. After ablation, a significant increase of 59% was seen in cell proliferation within the SVZ on day 11 (Figure 4.2). This follows the pattern seen in the study conducted by Mandairon et al., 2003 (Mandairon et al., 2003) who reported an increase in cell proliferation 8 days after surgical section of the ORNs. These results are contradicted by studies using naris occlusion (Brunjes, 1994; Brunjes and Kishore, 1998; Corotto et al., 1994) or by genetic knock-out of olfactory transduction (Petreanu and Alvarez-Buylla, 2002) which both report no change or a decrease in proliferation. However, olfactory ablation via methimazole and axotomy induces the death of ORNs preventing any afferent activity or trophic support from reaching the OB and therefore could affect cell proliferation via alternate mechanisms. The effect of sensory ablation had on cell death was then examined. Unlike Mandairon et al., 2003 (Mandairon et al., 2003), no cell death was evident within the SVZ at any time point after ablation. This may be due to the use of cleaved caspase-3 to mark apoptotic cells instead of TUNNEL. Cleaved caspase-3 will only label cells that have the caspase pathways activated while TUNNEL labels DNA fragments in cells which have undergone apoptosis (Narkilahti et al., 2003). Alternatively, it could also be a response to the type of method used to denervation of the OB. Mandairon (Mandairon et al., 2003) used axotomy which involves directly cutting the ORN axon bundles at the cribriform plate, which is a highly invasive acute method and could possible cause multiple local effects including cell death. Studies that relay on surgical methods such as bulbectomy have also seen cell death in the SVZ (Graziadei et al., 1979;
Lim and Brunjes, 1999) while sensory deprivation via by naris occlusion reports no cell death (Corotto et al., 1994). This suggests that the severity of the treatment can alter cell responses. The lack of apoptosis coupled with the increase in proliferation within the SVZ suggests that the mechanisms controlling neurogenesis is not limited to cell death adjusting the rate of neurogenesis (Bayer, 1983; Kaplan et al., 1985).

4.3.3 Reduction and delay in cellular proliferation in aged SVZ

It has been shown that there is an age-related decline in neurogenesis in the SVZ (Ahlenius et al., 2009; Maslov et al., 2004; Tropepe et al., 1997). To examine the effect of aging on the aged VONS, aged mice were treated with methimazole and the individual regions of the system were then examined. Aged mice showed both a delay in response time together with a reduced rate of neurogenesis in the SVZ system.

In some tissues with a high cell turnover the rate, cell differentiation becomes slower with age (Lorenzon et al., 2004; Suzukawa et al., 2011). Due to this the OE response to methimazole was the first area examined. In comparison to the control animals the methimazole injected animals showed a distinct lack of OMP by day 3 and were fully regenerated by day 20. This time span for axon regrowth reflects previous reports (Miragall and Monti Graziaidei, 1982; Schwob et al., 1992; Suzukawa et al., 2011). The OB was then examined to see the extent of denervation using high magnification images it was seen that when compared to controls the majority of the ORNs had degenerated by day 17 and was fully regenerated by day 21 when compared to the control animals (Figure 4.4). This corroborates with the time points seen with in the literature (Cummings et al., 2000; Slotnick et al., 2001; Suzukawa et al., 2011). These time points also match with previous studies within our laboratory where young animals were treated with a single dose of methimazole (unpublished data). These findings, combined with work done in this study, suggest that there is age-related decrease in the ability of OE to response to injury. However, there is minimal age-related decline in the capacity of OE to regenerate after injury. After establishing the effects of methimazole on an aged system the upstream effects of the SVZ were examined. A
significant increase of 36% was seen in cell proliferation on day 14 when treated animals were compared to time matched controls (Figure 4.5). The delay time seen between the young and aged animals suggests that there could be a reduction in signalling to the SVZ (Kondo et al., 2010; Lorenzon et al., 2004; Mattson et al., 2004; Suzukawa et al., 2011).

When comparing the rates of proliferation in the young animals to that of the aged animals, there was a significant decreases in proliferation between young and aged SVZ between both young and aged controls (58% from 53 ± 5 to 22 ± 0.9) P < 0.001 and treated (63% from 82 ± 6.9 to 30 ± 1.8) P < 0.001 animals (Figure 4.5). This correlates to studies by Morshead et al., 1994 and Shimazaki et al., 2001 (Morshead et al., 1994; Shimazaki et al., 2001) who saw a 75% decreased in SVZ proliferation in 24-month-old mice. As well as Tropepe et al., 1997 (Tropepe et al., 1997) who demonstrated that aging resulted in a twofold decline in neurogenesis, when measured by BrdU incorporation. This decrease is cause by a proportional decline in cell proliferation and progenitors survival as shown by (Stoeber et al., 2001) who used G1-phase cell cycle marker Mcm2 cells and nucleoside analogues 5-iodo-2-deoxyuridine and 5-chloro-2-deoxyuridine to mark proliferation. As well as the overall reduction in proliferation, the percentage increase of cells between control and treated within aged groups was also decreased (from 59% to 36%). This may be caused by the OB exerting some level of feedback control on proliferation of the SVZ (Jankovski et al., 1998; Kirschenbaum et al., 1999; Rochefort et al., 2002) which is affected by age allowing for a reduction in the regenerative capacity of the SVZ.
4.4 Conclusion

Using the focal, extended lesioning protocol designed and demonstrated in chapter 3 the OE and OB were completely ablate which resulted in an increase in proliferation in the SVZ of young animals. The ablation protocol was then modified for an aged population to examine the effects of aging on the ventriculo-olfactory system. This resulted in an increase in proliferation at a delayed time point. Suggesting that while the aged system has a decreased proliferation rate it still retains the capacity to respond injury. The next chapter will discuss the effects of ablation on migration recruitment and integration of the pulse of increased cell proliferation in the SVZ.
Chapter 5:

Communication between the primary olfactory system and the SVZ
5.1 Introduction

Using the methimazole model generated (chapter 3), links between the OE and cell proliferation in the SVZ were examined (chapter 4). Showing that ablation of the OE and OB increases the rate of proliferation in the SVZ at a discrete time point. This chapter will address the migration, recruitment and differentiation of this pulse of cells into the glomerular layer of the OB.

The OB is a highly organized structure composed of several distinct layers, responsible for different synaptic specializations. With the glomeruli being the most distinctive area and contains mitral cell dendritic arborisations, olfactory nerve fibres, and periglomerular interneurons (Pinching and Powell, 1971). This study is specifically interested in periglomerular cells, as they contain possibly the only population of regenerating dopaminergic cells. Periglomerular interneurons are generated at the SVZ throughout life (Belluzzi et al., 2003; Betarbet et al., 1996b; Kishi, 1987), before migrating along the RMS and to reach their correct target layers and differentiate (Eriksson et al., 1998). Within the OB, periglomerular interneurons contact multiple mitral cell dendrites and provide lateral inhibition of neighbouring glomeruli while allowing excitation of a specific mitral cell dendritic tree (Morrison and Costanzo, 1990; Shipley and Ennis, 1996). They can be categorised into two types, which contain 3 distinct groups based on their activity and expression of GABA, calbindin and calretinin (Kosaka et al., 1995). Type I periglomerular cells synapse directly with ORNs and are dopaminergic (TH-positive) (Toida et al., 2000). In contrast, Type II periglomerular cells are further divided into two different groups identifiable by the expression of calcium binding proteins calretinin or calbindin D(Kosaka et al., 2001 ; Kosaka et al., 1997a).

There have been studies into the development of different interneuron subtypes and their generation at different ages. Currently there is contradictory data available, some studies suggest that periglomerular cells were more likely to be calbindin positive if derived from the neonate, and more likely to be calretinin positive or TH positive if derived from the adult-labelled SVZ. While others have shown that TH positive cells are produced in greater
proportions at early time points rather than later in development (Batista-Brito et al., 2008). There is also a strong argument that progenitor patterning is not solely reliant on temporal regulation, rather it also seems to require some form of spatial regulation (De Marchis et al., 2007; Kohwi et al., 2007; Merkle et al., 2007). However, it has also been shown that different methods of cell tracking create a bias depending on the area within the SVZ that is labelled (Betarbet et al., 1996b; De Marchis et al., 2007; De Marchis et al., 2004; Lemasson et al., 2005; Luskin, 1993; Merkle et al., 2007; Smith and Luskin, 1998; Winner et al., 2006). This study will not only contribute data to the discussion of aged dependent production but also provide novel data on whether sensory ablation has an effect on subtype production.

Summary of the aims undertaken in this chapter:

- To characterise the migration and differentiation of the newly generated SVZ cells.

- To determine the subtype of cells produced in the SVZ following sensory ablation and whether these are incorporated into the OB

- To study the effects of age on olfactory periglomerular neuron fate specification.
5.2 Results

Having demonstrated an increase in neurogenesis within the SVZ, the subsequent migration, recruitment and differentiation of these cells was examined. Using EdU injected at the time of increased neurogenesis the cell were tracked at different time points. To do this a cohort of 60 animals was divided into a control and experimental group and treated as shown below.

**Day 1**

Control animals
- 30 mice were injected with PBS via intraperitoneal injection.

Treated animals
- 30 mice were injected with methimazole via intraperitoneal injection.

**Day 3**

Control animals
- The 30 mice from day 1 were once again injected with PBS via intraperitoneal injection.

Treated animals
- The 30 mice from day 1 were once again injected with methimazole via intraperitoneal injection.

The control and treated animals were divided into six harvest groups containing five animals. Each harvest group had a 3 day exposure to EdU at 30mg/kg body weight via intra peritoneal injection and were sacrificed (2.2.3) on the fourth day, as shown in the table below. The EdU treatment was used to mark proliferating cells.
Table 5.1: Young animal harvest groups with corresponding EdU injection and sacrifice days for differentiation studies.

<table>
<thead>
<tr>
<th>Harvest group</th>
<th>EdU injection day</th>
<th>Day Sacrificed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>8,9,10</td>
<td>16</td>
</tr>
<tr>
<td>Group 2</td>
<td>8,9,10</td>
<td>19</td>
</tr>
<tr>
<td>Group 3</td>
<td>8,9,10</td>
<td>22</td>
</tr>
<tr>
<td>Group 4</td>
<td>8,9,10</td>
<td>25</td>
</tr>
<tr>
<td>Group 5</td>
<td>8,9,10</td>
<td>28</td>
</tr>
<tr>
<td>Group 6</td>
<td>8,9,10</td>
<td>31</td>
</tr>
</tbody>
</table>

After harvesting, the brains and noses were processed, sectioned and treated with a panel of immunohistochemical markers.

5.2.1 Migration of newly generated cells from the young SVZ

The time taken for the newly proliferated cells (from the SVZ), to migrate and disperse within the OB was first established. Using time points based on data available in the current literature EdU positive cells were tracked (Table 5.1) by using large scale mosaic images (2.2.6) of coronally sectioned OBs. EdU positive cells were observed at the centre of the bulb at day 16, which then migrated radially between days 19 and 25. By day 31 EdU positive cells had migrated to the glomerular layer. There was no difference between the time taken for cells in the control or treated to reach the bulb. Based on these results day 31 was chosen for further analysis of cell recruitment and differentiation.
5.2.2 Recruitment and differentiation of newly generated cells in the young animal

To test whether sensory ablation influences the recruitment of newly divided cells into the glomerular layer, the total number of EdU positive cells was counted. Three sections from both control and methimazole group 6 (Tables 5.1) were selected using the Paxinos Mouse Brain Atlas 2nd Edition to avoid variability. Cell counts were performed on the entire glomerular layer in each section and repeated for a total of 3 animals from each group. Cell counts were carried out on cells the stained positive for both EdU and DAPI and were associated with periglomerular layer as defined by DAPI and mature periglomerular interneuron markers.

When comparing control and methimazole treated groups, a 42% increase was seen in the total number of EdU positive from an average of 48.2 ± 2.8 cells in the control group to 68.6 ± 4 cells in the treated (Figure 5.1). Independent Samples T-Test (2.2.8) revealed there was a significant increase in EdU positive cells within the glomerular layer of the methimazole treated animals, P value < 0.001 (Figure 5.1).

![Recruitment EdU positive cells in the glomerular layer](image)

**Figure 5.1: Recruitment of EdU positive cells in the glomerular layer.** A 42% (48.2 ± 2.8 to 68.6 ± 4) increase was seen in the number of EdU positive cells located to the glomerular layer 31 days after methimazole treatment in young animals. (Significant values of p<0.001 denoted by asterisk). Data presented as mean ± SE.
These results were then further explored by comparing changes in differentiation between the control and treated animals. Differentiation was measured through immunohistochemical analysis of markers which are expressed in mature Type I and Type II periglomerular interneurons. Cell counts were performed on EdU positive cells that co-localized with TH, calbindin or calretinin, as well as DAPI. Two sections from each control and treated groups were selected using the Paxinos Mouse Brain Atlas 2\textsuperscript{nd} Edition and cell counts were performed on the entire glomerular layer. Counts were repeated for a total of 3 animals from each group.
Tyrosine hydroxylase/ EdU

The control animals showed an differentiation of $1.2\% \pm 0.6$ for TH/EdU positive cells in the glomerular layer. In the treated animals, there was an increase in the rate of differentiation to $2.6\% \pm 0.77$ was seen (Figure 5.2). The data was then analysed by Independent Samples T-Test (2.2.8) and the changes were not significant.

Figure 5. 2: Differentiation of newly formed TH cells in the glomerular layer. (A) Representation TH (green) and EdU (red) co-localisation in the glomeruli. Cell nucleus labelled with DAPI (blue). Images taken at 630x magnification, Scale bar = 5 µm. (B) An increase to $2.6\% \pm 0.77$ was seen in the percentage of EdU positive cell co-localising with TH in methimazole treated animals.
Calbindin/EdU

For calbindin/EdU positive cells a 0.6% ± 0.6 differentiation rate was seen for the control animals which increased to 1.7% ± 0.1 in the treated animals (Figure 5.3). However, when analysed (Independent Samples T-Test) it was shown that the changes were not significant.

Figure 5. 3: Differentiation of newly formed calbindin cells in the glomerular layer. (A) Representation calbindin (green) and EdU (red) co-localisation in the glomeruli. Cell nucleus labelled with DAPI (blue). Images taken at 630x magnification, Scale bar = 5 µm. (B) An increase to 1.7% ± 0.1 was seen in the percentage of EdU positive cell co-localising with calbindin after methimazole treatment.
Calretinin/ EdU

The differentiation of calretinin/ EdU positive cells was similar with the control group showing 1.3% ± 0.6 and the treated animals 1.4 % ± 0.8 (Figure 5.4).

Figure 5.4: Differentiation of newly formed calretinin cells in the glomerular layer. (A) Representation calretinin (green) and EdU (red) co-localisation in the glomeruli. Cell nucleus labelled with DAPI (blue). Images taken at 630x magnification, Scale bar = 5 µm. (B) No change was seen in the percentage of EdU positive cell co-localising with calretinin after methimazole treatment.
5.2.3 Recruitment and differentiation of newly generated cells in the aged olfactory bulb

The additional cohort of aged animals allowed for the study of age related changes in differentiation. Based on the results acquired from the young animals, a series of experiments were designed and conducted on aged mice.

**Day 1**

Control animals

- 5 mice were injected with PBS via intra peritoneal injection.

Treated animals

- 5 mice were injected with methimazole via intra peritoneal injection.

The control and treated animals were then had a 3 day exposure to EdU at 30mg/kg body weight via intra peritoneal injection and was sacrificed (2.2.3) on the fourth day as shown in the table below. The EdU treatment was used to mark proliferating cells.

**Table 5. 2: Aged animal harvest group with corresponding EdU injection and sacrifice days for differentiation studies.**

<table>
<thead>
<tr>
<th>Harvest group</th>
<th>EdU injection day</th>
<th>Day Sacrificed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>11,12,13</td>
<td>30</td>
</tr>
</tbody>
</table>

After harvesting, the brains and noses were processed, sectioned and treated with a panel of immunohistochemical markers.
The same method developed to examine differentiation within the young OB was used to examine recruitment and differentiation within the aged OB (5.2.2). First, the recruitment of newly divided cells into the glomerular layer was tested. There was a 40% increase was seen in the total number of EdU positive cells within the glomeruli when comparing to control (20 ± 1.7) and methimazole (28 ± 2.6) treated groups. Statistical analysis (Independent Samples T-Test (2.2.8) revealed there was a significant increase in EdU positive cells present in the methimazole treated animals with a P value = 0.02 (Figure 5.5).

![Recruitment EdU positive cells in the aged glomerular layer](image)

**Figure 5. 5: Recruitment of EdU positive cells in the aged glomerular layer.** An increase of 40% was seen in the number of EdU positive cells located to the glomerular layer between control (20 ± 1.7) and methimazole treated (28 ± 2.6) animals. (Significant values of p<0.02 denoted by asterisk). Data presented as mean ± SE.

Any change in the in cell phenotype was again measured through immunohistochemical analysis for co-localization of EdU positive cells with TH, calbindin and calretinin as well as DAPI to label cell nuclei. As stated previously (5.2.2), two sections were selected to perform glomerular layer cell counts, and counts were repeated on a total of 3 animals from each group.
Tyrosine hydroxylase/EdU

TH/EdU positive cells showed a 1.3% ± 1.3 differentiation rate in the control animals which increased to 2.5% ± 1.3 in the treated animals (Figure 5.6). However, when analysed (Independent Samples T-Test) it was shown that the changes were not significant.

![Image](image_url)

*Figure 5.6: Differentiation of newly formed TH cells in the aged glomerular layer. (A) Representation TH (green) and EdU (red) co-localisation in the glomeruli. Cell nucleus labelled with DAPI (blue). Images taken at 630x magnification. Scale bar = 5 µm. (B) An increase from 1.3% ± 1.3 to 2.5% ± 1.3 was seen in the percentage of EdU positive cell co-localising with TH after methimazole treatment.*
Calbindin/ EdU

Control animals showed an differentiation of 0% ± 0 for TH/EdU positive cells in the glomerular layer. In treated animals the rate was seen to increase to 1.3% ± 1.3 (Figure 5.7). When the data was analysed (Independent Samples T-Test) the changes not significant.

Figure 5. 7: Differentiation of newly formed calbindin cells in the aged glomerular layer. (A) Representation calbindin (green) and EdU (red) co-localisation in the glomeruli. Cell nucleus labelled with DAPI (blue). Images taken at 630x magnification, Scale bar = 5 µm. (B) An increase from 0% ± 0 to 1.3% ± 1.3 was seen in the percentage of EdU positive cell co-localising with calbindin after methimazole treatment.
Calretinin/ EdU

There was a small decrease in calretinin/EdU positive cells from 1.4% ± 1.4 to 1.1% ± 1.4 when comparing control and treated animals (Figure 5.8). Using Independent Samples T-Test (2.2.8) it was shown that the decrease was not significant.

Figure 5. 8: Differentiation of newly formed calretinin cells in the aged glomerular layer. (A) Representation calretinin (green) and EdU (red) co-localisation in the glomeruli. Cell nucleus labelled with DAPI (blue). Images taken at 630x magnification, Scale bar = 5 µm. (B) A decrease from 1.4% ± 1.4 to 1.1% ± 1.4 was seen in the percentage of EdU positive cell co-localising with calretinin after methimazole treatment.
5.2.4 Age related changes in migration and differentiation in the bulb

The effect of age on the recruitment of new cells into the glomerular layer of the OB was then examined. There was a 58% reduction in the total number of EdU positive cells recruited to the glomerular layer in both the control animals (48 ± 2.8 to 20 ± 1.7) and in the treated (68 ± 4 to 28 ± 2.6) (Figure 5.9). The percentage increase between the control and treated aged match groups was similar at approximately 40%. Using one way ANOVA (2.2.8), it was revealed there was a significant decrease with age in recruitment of cells to the OB (P value < 0.001). Post hoc Tukey tests indicated that there was a significant decrease in cell number between both young and aged controls (p < 0.001) and treated animals (P < 0.001).

![EdU positive cells in the glomerular layer](image)

**Figure 5.9: Comparing recruitment of EdU positive cells in the young and aged glomerular layer.** A significant decrease of 58% was seen in the total number of cells recruited to glomerular layer in both the control (48 ± 2.8 to 20 ± 1.7) and treated (68 ± 4 to 28 ± 2.6) animals of the aged group. However, the increase seen after treatment was approximately 40% in both aged groups (Significant values of p<0.02 denoted by asterisk, Significant values of p<0.001 denoted by ∆). Data presented as mean ± SE.
The percentage of newly recruited cells to obtain periglomerular phenotypes was compared between the young and aged animals. It was seen that both groups showed a similar increase in the TH/EdU positive cells and in calbindin/EdU positive cells found within the glomerular layer after methimazole treatment. No change was seen in the number calretinin/EdU positive cells (Figure 5.10). However, using one way ANOVA (2.2.8) revealed there was no significant difference in the differentiation of cells to the glomerular layer between young and aged.

Figure 5.10: Comparing differentiation rates of EdU positive cells in glomerular layer of young and aged. No difference was seen in the differentiation rate between both the young and aged animals. Both age groups showed a similar increase of TH/EdU positive and of calbindin/EdU positive cells after methimazole treatment and no change in the number calretinin/EdU positive cells.
5.3 Discussion

5.3.1 Overview and summary

Following the increase of cell proliferation in the SVZ, the migration and differentiation of the EdU positive cells were quantified. It was hypothesised that the pulse of newly formed cells would trigger an increase in cell recruitment in the glomerular layer of the OB due to the decrease in periglomerular cells (chapter 3). Following methimazole injections animals were treated with EdU on days 8, 9, 10 for the young animals and on days 11, 12, 13 for the aged animals to coincide with the pulse of neurogenesis seen in the SVZ. Immunohistochemical markers, along with EdU, were used to check for any change in recruitment. Cell counts and subsequent statistical analyses revealed that treated animals from both the young and aged cohorts exhibited a significant increase in the number of EdU positive cells within the glomerular layer of the treated animals. The trend in differentiation between young and aged animals was similar, with TH/EdU and calbindin/EdU both increasing and calretinin staying the same. However, these results were shown not to be significant. By comparing this data it was shown that there was a decrease in overall recruitment in the aged animals; however the differentiation after methimazole treatment was similar in both groups.

5.3.2 Migration is unchanged after olfactory bulb denervation

The migration of cells from the SVZ to the OB has been well documented. Neuroblasts migrate 5–8 mm through the RMS towards the OB via chain migration at speeds reaching 80μm/h (Davenne et al., 2005; Kim et al., 2009). Because of this fast migration, new cells have been shown to reach the bulb as early as two to six days after leaving the SVZ. The results of this study reflect the literature findings, as EdU positive cells were first observed in bulb six days after Edu labelled neurogenic pulse (Figure5.1). Once in the bulb, immature
neurons migrated radially and were first seen in the glomerular layer 18 days after labelling, which corresponds with current studies (Belluzzi et al., 2003; Carleton et al., 2003; Doetsch and Alvarez-Buylla, 1996). No difference in the time taken for EdU positive cells to reach the OB was seen between the control and treated animals in either aged group. This suggests that neither sensory ablation nor age affects migration rates.

5.3.3 Newly generated cells are recruited into the periglomerular layer

In rodents, cells in the glomerular layer differentiate into dopaminergic, calbindin, or calretinin interneurons (Bagley et al., 2007; Kosaka et al., 1995; Kosaka et al., 1998; Kosaka and Kosaka, 2008; Whitman and Greer, 2007). It has been demonstrated that all these chemospecific populations of periglomerular cells can be produced during adulthood (Bagley et al., 2007; Batista-Brito et al., 2008; De Marchis et al., 2007; Whitman and Greer, 2007). There is conflicting data with regards to the ratio of newly generated interneurons postnatally, mainly due to methods bias (Batista-Brito et al., 2008; De Marchis et al., 2007). The method of cell labelling BrdU, retrovirus, flurogold or transplantation) has been shown to produce different ratios between granule cells and periglomerular cells depending on the area within the SVZ that is labelled (Betarbet et al., 1996b; De Marchis et al., 2004; Lemasson et al., 2005; Luskin, 1993; Merkle et al., 2007; Smith and Luskin, 1998; Winner et al., 2006). Due to this EdU, a thymidine analogue, was given via intra peritoneal injection to analyse differentiation of newly generated cells into the glomerular layer, as well as any changes caused by sensory ablation and age. There was a 58% decrease in the total to number of EdU positive cells recruited to the glomerular layer in the control (48 ± 2.8 to 20 ± 1.7) and treated (68 ± 4 to 28 ± 2.6) animals. Although the total recruitment of cells in the young animals was greater, the increase between control and treated within the same aged group was relative. With a 40% increase in both young (48 ± 2.8 to 68 ± 4) and aged animals (28 ± 2.6 to 20 ± 1.7) (P < 0.001) (Figure5.10).
5.3.3.1 Denervation not age influence interneuron subtype formation.

The percentage of the newly recruited cells to obtain periglomerular phenotypes was compared between the control and methimazole treated animals. Although there have been some studies that have examined ventriculo-olfactory neurogenic dynamics via sensory ablation, none have distinguished the effects on individual periglomerular interneuron subtypes (Mandairon et al., 2003; Sawada et al., 2011). The strength of this study is that changes in differentiation of individual subtypes are explored. In the young animals while there was an increase in the percentage of EdU positive cell co-localising with TH (from 1.2% ± 0.6 to 2.6% ± 0.7 (Figure 5.3)) and calbindin (0.6% ± 0.6 to 1.7% ± 0.1(Figure 5.4)) and calretinin remained the same at approximately 1.4% (Figure 5.5). The aged animals showed similar trends, with an increase in the percentage of both TH/EdU (1.3% ± 1.3 to 2.5% ± 1.3(Figure 5.6)) and calbindin/EdU positive cells (0% ± 0 to 1.3% ± 1.3(Figure 5.7)) and no difference in calretinin/EdU (1.4% ± 1.4 to 1.1% ± 1.4(Figure 5.8)) positive cells. When comparing the percentage increase across the difference subtypes in the young and aged animals showed similar differentiation percentages for all subtypes. It was seen that both groups showed similar trends with an increase of TH/EdU positive and of calbindin/EdU positive cells found within the glomerular layer and no change in the number calretinin/EdU positive cells (Figure 5.11). These trends could possibly suggest that sensory ablation, not age, affects interneuron subtype by increased differentiation of TH and calbindin positive interneurons. However, while there was a suggested trend the increases were shown to be not significant. This could be due to a large number of EdU positive, interneuron subtype negative cells that were seen in the glomerular layer. Cells lacking a phenotype marker could be a result of delayed neuronal differentiation in the glomerular layer and possible be caused by the additional time needed for migration to periphery of the OB or the requirement of additional stimuli from surrounding cells (Carleton et al., 2003; Sui et al., 2012). Carleton (Carleton et al., 2003) has seen the while differentiation in the glomerular layer occurs 1 month after BrdU labelling of SVZ cells, the peak of dopaminergic differentiation does not occur until 2 months of age. This suggests that a longer time for differentiation is needed perhaps in conjunction with neuroD1, a marker to show terminally differentiated cells (Boutin et al., 2010). By increasing differentiation time the number of TH positive interneurons would possibly increase in treated animals compared to controls. This is based
on studies that have shown that disruption in olfaction up regulates the expression of Pax6, a key molecular determinant of the dopaminergic fate (Brill et al., 2008; Dellovade et al., 1998; Hack et al., 2005; Kohwi et al., 2005). The lack of difference in differentiation rates between young and aged animals reflects studies that have shown unchanged levels of periglomerular immunoreactivity in the C57BL/6J strain over time (Mirich et al., 2002). Furthermore, the aged system to generate increase in total cell number during aging despite a reduction in neurogenesis (Jankovski et al., 1998; Kirschenbaum et al., 1999; Rochefort et al., 2002).
5.4 Conclusion

Using EdU as a marker of proliferation, the pulse of neurogenesis seen in the SVZ after ablation was tracked (Chapter 4). It was seen that neither sensory nor ablation nor age played an effect on time taken for cells to migrate from the SVZ to the OB. There was however a similar change in the differentiation rate seen after methimazole treatment in both age groups suggesting that sensory ablation affects TH and calbindin positive interneurons.
Chapter 6

Final Discussion
6.1 Summary of Results

The major results obtained from the studies presented in this thesis are outlined below:

- A methimazole injection protocol was developed that ablated the OE on day 11 and denervated the OB on day 17.

- The OB showed a significant increase in glial markers 11 days post treatment.

- A significant reduction in the number of Type I periglomerular cells was seen on day 14 thought to day 17.

- Ablation of the OE caused an increase of cell proliferation in the SVZ in both young and aged mice.

- The migration of cells from the SVZ to the OB was not affected by ablation or age.

- Ablation of the OE in both the young and aged mice exhibited a significant increase in recruitment of EdU positive cells within the glomerular layer.

- A suggested increase in differentiation of TH and calbindin positive cells in both young and aged mice.
6.2 Discussion

This projected aim of this thesis was to generate a novel methodology to examine the effects on OB denervation of the VONS. In order to generate this model, the effects of prolonged ablation on not only the OE but the OB needed to be determined. Using data obtained within our laboratory, a two methimazole injection protocol was generated. This method resulted in the subsequent death of ORNs via the cytochrome c-mediated caspase-3 activation pathway as shown by a cleaved caspase-3 marker (chapter 3) (Brittebo, 1995; Mizutani et al., 1999; Sakamoto et al., 2007; Xie et al., 2011). Ablation of ORNs in the OE leads to the loss of axon terminals within the glomeruli of the OB 17 days after the first methimazole injection. This is due to the fact that ORNs are responsible for activating neurons in the glomeruli of the OB (Cleland and Sethupathy, 2006; Ennis et al., 1996; Pinching and Powell, 1971; Shipley and Ennis, 1996). The effects of synaptic denervation of bulb on the periglomerular interneurons and glial response were then examined.

Periglomerular cells can be categorised into two groups, Type I periglomerular cells predominately express tyrosine-hydroxylase (TH) (Baker, 1990; Crespo et al., 2003; Kosaka and Kosaka, 2007; Toida et al., 2000). Whereas, Type II periglomerular cells express calcium binding proteins; calretinin or calbindin (Kosaka et al., 1997a; Kosaka et al., 1997b). There is some debate on the effects on sensory inputs on Type I cell types due to TH ability to down regulate. Many studies have used DOPA decarboxylase as a stable marker for Type I cells (Baker, 1990; Ljungdahl et al., 1977; Weihe et al., 2006). Based on their results it has been proposed that denervation reflects a down regulation of TH expression in the OB, rather than a loss of Type I cells (Baker, 1990; Baker et al., 1983; Baker et al., 1993; Cho et al., 1996; Mandairon et al., 2003). Unfortunately, in this study while the dopaminergic cells of the substantia nigra showed 100% co-localisation of the markers within cell bodies, no co-localisation of TH and DOPA decarboxylase within the OB was seen. Therefore a suggested alternative hypothesis is that a reduction seen in TH positive cells could represent Type I periglomerular cell death as a result of lost innervation from ORNs (Sawada et al., 2011). To determine if the reduction in cell number was due to cell death or down regulation of TH, cleaved caspase-3 was used as an apoptosis marker to confirm cell death. Cleaved caspase-3
has been previously recorded in this area following sensory ablation (Gavrieli et al., 1992; Mandairon et al., 2003; Sawada et al., 2011). Although there was increase in cleaved caspase-3, levels only a few cell showed expression in the nucleus. This meant that cleaved caspase-3 staining could be due to ORNs axonal death and not the death of the TH positive cells. However, due to the minimal time points available not enough data from this study has been gathered to substantiate either hypothesis.

This study also showed that while dopaminergic (Type I) neurons were affected by sensory deprivation, there was only a decreasing trend in the Type II periglomerular interneurons which was shown to be not significant. This decreasing trend could be due to a 30% reduction in the number of calbindin cells following sensory deprivation in the developing rat OB (Philpot et al., 1997a). The fact that the Type II interneuron are only marginally affected correlates to previous experiments, showing that Type II cells in the bulb are relatively stable, even after unilateral naris occlusion or bulbectomy (Lim and Brunjes, 1999; Philpot et al., 1997a; Philpot et al., 1997b). One reason for this could relate to the organization of these neurons within the bulb. Type I cells receive input from the olfactory nerve, while Type II periglomerular neurons do not synapse directly with ORNs but to the mitral and tufted cells (Bastien-Dionne et al., 2010; Bovetti et al., 2009; Kosaka and Kosaka, 2007). Therefore, it can be suggested that interneurons that are in direct contact with the sensory neurons might be more sensitive to sensory ablation.

To examine any immune response caused the death of the ORN, astrocyte and microglia markers GFAP and Iba1 were tested using densitometry. Astrocytes are the first to react to neuronal death causing astrogliosis, they have also been shown to activate microglia (Brahmachari et al., 2006; Murphy, 2000; Schipke et al., 2002). Both markers showed a gradual increase in density after methimazole injection peaking on day 14 followed by a decrease at day 17 and 20. Given the time (Day 11) at which the increased expression of GFAP and Iba1 may be attributed to the death of the ORNs axons that terminate in the bulb (3.3.2.2) and is indicative of neuroglia activation during degeneration (Eng et al., 1992; Fiske and Brunjes, 2000; Reier et al., 1986). The continuation of increase levels of microglia may be due to their function in neuroplasticity. Microglial involvement in synaptic stripping may help in the reforming of the synapses with the newly formed ORNs (Nakajima and Kohsaka,
Also the ability of microglia to express growth factors after neural degeneration may play a role in maintaining normal sensory activity (Heese et al., 1997; Heese et al., 1998; Leon, 1998).

To test the communication between the OB and SVZ, the proliferation within the SVZ was monitored with EdU and cleaved caspase -3. Previous studies have demonstrated a relationship between sensory input to the OB and the rate of proliferation in the SVZ (Corotto et al., 1994; Kirschenbaum et al., 1999; Mandairon et al., 2003). Ten days after sensory ablation a significant increase in cell proliferation was seen within the SVZ in both young and aged animals. These results coincide with a study conducted by Mandairon et al., 2003 (Mandairon et al., 2003) who saw an increase in cell proliferation 8 days after surgical section of the ORNs. When comparing with the data in chapter 3, the increase in neurogenesis and the increase in glia activity occur simultaneously. This indicates the changes in the SVZ could be caused by a brain lesion-dependent mechanism rather than olfactory deprivation. Previous studies have shown that brain injury (shown in this study by glial response) can cause an increase in SVZ neurogenesis (Rakic, 2002a, b; Tzeng and Wu, 1999; Weinstein et al., 1996). This theory is also supported by studies that reported no change or a decrease in proliferation in treatments that do not cause neuronal death such as naris occlusion (Brunjes, 1994; Brunjes and Kishore, 1998; Corotto et al., 1994) or by genetic knock-out of olfactory transduction (Petreanu and Alvarez-Buylla, 2002).

The pulse of neurogenesis was then labelled using EdU to monitor changes to migration and recruitment. There was no difference between the control and treated animals in the number of days taken for EdU positive cells to reach the OB or migrated radially (Belluzzi et al., 2003; Carleton et al., 2003; Doetsch and Alvarez-Buylla, 1996). This suggests that neither sensory ablation nor age play a part in migration rates. Due to the high speeds that cells migrate through the RMS to the OB, and the ability of cells to migrate after bulbectomy it was unlikely that sensory ablation would affect migration speed (Alvarez-Buylla, 1997; Davenne et al., 2005; Jankovski et al., 1998). After establishing a time frame for migration, recruitment of EdU positive cells into the glomerular layer was tested. A significant increase of EdU positive cells was seen in both the young and aged animals (Figure 5.2). While the total recruitment of cells in the young was greater than that of the aged, the increase between
the same age groups was relative. The increase in recruitment reflects the increased cell number or neurogenic pulse in see in chapter 4, suggesting that the newly generated cells in the SVZ successfully migrate to the olfactory bulb and do not undergo cell death.

It has been previously demonstrated that the OB responds to changes in environmental conditions to help maintain normal function (Guthrie et al., 1991; Hack et al., 2005; Leon, 1998; Waggener and Coppola, 2007). To examine the effects of sensory ablation on the ability of newly recruited cells to obtain periglomerular phenotypes, EdU positive cells were checked for co-localisation with interneuron markers. Percentages of EdU positive cells, rather than total cell number, allows for direct comparison between control and treated animals regardless of the difference in recruitment numbers. It was seen that both age groups showed similar trends with an increase of TH/EdU positive and in calbindin/EdU positive cells found within the glomerular layer and no change in the number of calretinin/EdU positive cells. The trend suggests that sensory ablation and not age could possibly affect interneuron sub-type by increasing differentiation of TH and calbindin positive interneurons. Previous data has linked disruption of dopamine pathways and TH positive cells in the bulb. Most recently Sui et al., 2012 (Sui et al., 2012) showed that disruption of dopamine circuitry in the substantial nigra resulted in a decrease of neurogenesis in the SVZ but an increase in TH–positive cells. It has also been shown that humans with Parkinson disease have an increased number of TH-positive cells in the OB. Within the OB itself reduction in TH expression is accompanied by an increase in the density of D2 receptors on the olfactory nerve terminals (Guthrie et al., 1991)and up regulates the expression of Pax6 a key transcription factor in dopaminergic fate (Beckervordersandforth et al., 2010; Brill et al., 2008; Dellovade et al., 1998; Hack et al., 2005; Kohwi et al., 2005; Ninkovic et al., 2010). This suggests that the disruption in TH levels by sensory ablation (chapter 3) could possible influence production, survival or differentiation of TH positive interneurons. The increase of calbindin positive cells may be linked to slight decrease seen in type II cells. This decreasing could be due to a reduction of calbindin positive cells as seen in the rat OB following sensory deprivation (Philpot et al., 1997a). However, the increase seen were shown to be not significant, and will require further study to clarify the exact response sensory ablation has on periglomerular subtype.
Studies have shown that in some tissues age affects cell proliferation and differentiation (Kalaydjiev et al., 2002; Kondo et al., 2010; Lorenzon et al., 2004; Petreanu and Alvarez-Buylla, 2002). The phenomenon also affects the ageing brain, making it more susceptible to neurodegenerative disorders (Grote and Hannan, 2007). Studying the response to injury in aged animals allows for greater understanding of the conditions in which degeneration occurs. This project showed that one methimazole injection was enough to obtain complete denervation of the OB in aged animals. With treated animals showing a lack of ORNs in the OE by day 3 and denervation of the OB by day 17, which matches the time points seen with in the literature (Cummings et al., 2000; Miragall and Monti Graziadei, 1982; Schwob et al., 1992; Suzukawa et al., 2011). After establishing the effects of methimazole on an aged OE and OB the upstream effects of the SVZ were examined showing a significant increase in cell proliferation. However, when compared to the young animals there was an overall decrease in the number of new cells in the treated and control aged animals. It has been shown that there is an age-related decline in neurogenesis in the SVZ (Ahlenius et al., 2009; Maslov et al., 2004; Tropepe et al., 1997). This correlates to studies by Morshead et al., 1994 and Shimazaki et al., 2001 (Morshead et al., 1994; Shimazaki et al., 2001) that saw up to 75% decrease in SVZ proliferation in 24-month-old mice. As well as Tropepe et al., 1997 (Tropepe et al., 1997) who demonstrated that aging, resulted in two fold decline in neurogenesis in both cell proliferation and survival (Wharton et al., 2001). This could be caused by many factors. There is evidence that age decreases EGFR signalling, which is critical in maintaining NSC numbers and, consequently, in the production of new neurons in the OB. (Gulli et al., 1996; Kamer et al., 2004; Purdom and Chen, 2003; Tikhomirov and Carpenter, 2004; Yoshimoto and Imoto, 2002). Despite the overall reduction in proliferation in the SVZ, the percentage increase in neurogenesis between control and treated within aged groups was similar. There was also no difference in differentiation rates between young and aged animals, which reflects studies that have shown unchanged immunoreactivity in the C57BL/6J strain over time (Mirich et al., 2002). Together these findings suggest that while the aged VONS is more vulnerable to toxic assault and displays a reduction in total SVZ neurogenesis it is robust enough to respond in the same fashion and the young adult system.
6.3 Conclusion

The data from this study suggests that olfactory sensory innervation plays a part in modulating cell genesis in the SVZ and effects the recapitulation of the periglomerular interneurons in the OB. The increase in astrocytes and microglial activity, caused by the death of the ORNs, may play a role in mediating the increase in proliferation seen in the SVZ. While the decrease in TH positive cells may play a role in regulating periglomerular interneuron subtype production. It was also shown that despite the decreases levels of proliferation in the aged VONS it retains the capacity to respond to injury. Understanding influences on neurogenesis allows for development of therapeutic strategies to compensate for neuronal loss.
6.4. Future Directions

The results achieved in this project suggest interesting future directions into the understanding of regeneration of dopaminergic neurons for neurodegenerative diseases and recovery from injury. Possible future directions are: elucidating changes in transcription factor pathways and investigating signalling molecules that influence production, survival or differentiation of TH positive interneurons after sensory ablation.

As mentioned earlier, decrease in OB TH up regulates the expression of Pax6 a key transcription factor in dopaminergic fate (Brill et al., 2008; Dellovade et al., 1998; Hack et al., 2005; Kohwi et al., 2005; Ninkovic et al., 2010). By looking at changes in Pax6 as well as other known dopaminergic transcription factors (Id2, ER81 and the homeodomain factor Meis2) after sensory ablation, will allow for understanding into the transcriptional response that triggers the production of new TH positive cells (Allen et al., 2007; Havrda et al., 2008; Kohwi et al., 2005; Long et al., 2007; Murray et al., 2003; Saino-Saito et al., 2007). The co-expression of Pax6, ER81 and Meis2 may also be useful in studies where identification of putative dopaminergic interneurons is needed independent of TH staining.

Other future work involves investigating the neurotropic roles of endogenous signalling mechanisms such as GDNF and neurturin (NRTN) on production, survival or differentiation of TH positive interneurons after sensory ablation. GDNF and NRTN expression in the olfactory mucosa is well established, with studies showing GDNF and NRTN mRNA expression in the olfactory mucosa (Buckland and Cunningham, 1999; Golden et al., 1998; Nosrat et al., 1996). Immunohistochemical Studies have shown that GDNF expression is widespread in mature and immature ORNs (Maroldt et al., 2005) whereas NRTN immunoreactivity is restricted to nerve fibers and terminals in the glomeruli of mature olfactory neurons (Maroldt et al., 2005). There is also data showing olfactory ensheathing cells expressing NRTN, GDNF and several other neurotrophic molecules (Woodhall et al., 2001). These expression patterns suggest an autocrine and paracrine role in the support of ORNs. The neurotrophic role played by these molecules is strengthened based on the
supportive role of ensheathing cells (Agrawal et al., 2004; Lipson et al., 2003; Pellitteri et al., 2007). Because of GDNFs presence in precursor cells and ensheathing cells, it is proposed that it play a guidance role in the OB (Cao et al., 2006; Paratcha et al., 2006). There are limited studies for a potential function for NRTN; however as it has a restricted expression in the olfactory system this suggests that it may also play a role in providing guidance ques. By using multiple tools such as antagonists and agonists to signalling pathways, gene silencing and transgenic approaches this would lead to further understanding of neurogenesis in the adult brain, and add to research in therapies for neurodegenerative diseases and recovery from injury.
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