The Cell Cycle Dynamics & Proliferation in the Development of the Sympathetic Nervous System

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Abstract

This thesis is focused at characterising the cell cycle dynamics and proliferation of neural crest cell derivatives that give rise to the sympathetic nervous system. The dynamics of cell proliferation during nervous system development are poorly understood outside the mouse neocortex. During normal development, extensive, controlled proliferation of neural crest cells and their derivatives is essential to produce the sympathetic ganglia. However, nothing is known about their in vivo cell cycle dynamics during normal embryonic development, although the developing sympathetic ganglion has long been an easily accessible and popular model tissue for studying neuronal differentiation. Identifying the cell cycle dynamics involved in the production of the sympathetic nervous system will ultimately lead to an improved understanding of how different factors can influence cell cycle dynamics and proliferation overall.

Chapter 1: The literature review initially provides a description of the anatomical arrangement of the various components of the autonomic nervous system, followed by a brief history of the neural crest. In the main part of the literature review, I summarise the current information on the differentiation of cells in the sympathetic ganglion into noradrenergic neurons. This section concentrates on the role of the signals and transcription factors that cause neural crest cells to differentiate as sympathetic neurons. Following this, I discuss what is known about proliferation in the sympathetic nervous system, focusing on the molecular control of proliferation in the developing sympathetic nervous system. This discussion considers many of the same signals and transcription factors that are involved in differentiation of sympathetic neurons.

Chapter 2: Is the materials and methods chapter detailing all of the tools and techniques that I have employed to answer questions about proliferation and cell cycle dynamics.

Chapter 3: This chapter first identifies the changing phenotype and relative proportions of cells in the mouse stellate ganglion as it develops, using multiple label immunofluorescence to identify neural crest cells, neuroblasts and glial
progenitors. Next, I use BrdU pulse labelling, the most widely used technique to measure changes in proliferation, to look at the development of the sympathetic ganglion. BrdU, a thymidine analogue, labels cells in S-phase that are actively synthesising DNA. This chapter illustrates the significant limitations of using a single pulse of BrdU to measure proliferation. I conclude this chapter with an extensive consideration of the available methods that can be used to measure proliferation, and finally discuss the most appropriate approach for investigating cell cycle dynamics and proliferation in the developing sympathetic nervous system. This approach uses dual labelling with S-phase markers (BrdU and EdU) in combination, for the first time, with multiple label immunofluorescence for cell specific markers. It is adapted from previously published techniques developed to measure cell cycle parameters in the developing murine neocortex.

Chapter 4: In this chapter, using the new approach, I demonstrate that in vivo cell cycle dynamics can be measured in the developing sympathetic nervous system. This approach yields a complete characterisation of the cell cycle dynamics for neural crest cells and their derivatives (neuroblasts and glial progenitors) over the course of sympathetic nervous system development and led to a number of novel insights. I identified that neural crest cells withdraw from the cell cycle as they differentiate into neuroblasts and that they re-enter the cell cycle within 24h to continue dividing and allow the ganglion to grow. In addition, the cell cycle length of differentiating neural crest cells increases dramatically before differentiation, consistent with the lengthening being the trigger for differentiation. Finally a mathematical model was generated that validated the experimental data and which can be used to test future hypothesis about how proliferation may be altered if the developmental program is perturbed.

Chapter 5: Here I test the validity of my newly developed approach by applying this method to situations where normal development has been perturbed. In the first example, I have identified that the removal of the glial family ligand co-receptor, Ret results in a 5 fold increase in the cell cycle length of neuroblasts late in development. The effect of Ret knockout has been examined previously, but this dramatic effect was not recognised. This validates the power of my newly
developed techniques. I then showed that this cell cycle defect could be rescued by administration of erythropoietin, identifying this hormone as a novel mitogen that may play a key role in SNS development and suggesting that the effect of lesioning Ret may be indirect, due to loss of the kidney, the normal source of erythropoietin. Finally, I show that in a preliminary study, conditional expression of stable β-catenin results in a reduction in the proliferating pool of neuroblasts in the sympathetic ganglion.

Chapter 6: In the general discussion, I re-examine the similarities and differences between the development of the SNS compared to development in other parts of the nervous system, in light of my findings. I discuss possible mechanisms for the actions of both erythropoietin and β-catenin on cell cycle dynamics. In addition to this, I discuss the strengths and limitations of the method that I have used to examine proliferation and also highlight potential alternative methods. I conclude with consideration of future experiments that will grow out of my approach. Finally, I briefly discuss the significance of my results in light of the existence of neuroblastoma, an often aggressive and difficult to treat childhood cancer. As this disease is a result of uncontrolled cell proliferation of the neural crest cell derivatives that generate the sympathetic ganglia, my approach may well shed light on the genesis of this disease.
Author’s Declaration

The investigation conducted in this thesis is entirely my own work unless stated otherwise (see the preface to chapter 4). Materials and work from other sources and publications have been appropriately acknowledged.

This thesis is less than 100,000 words in length, exclusive of figures, tables, maps bibliographies and appendices.

David Gonsalvez
Acknowledgements

Supervisors and members of the Anderson lab

First of all, I would like to thank Colin Anderson. The first time I met Colin was actually quite serendipitous. In 2006, I was actually trying to regain a spot back into the BSc course on the back of paying up front full fees for about a year’s worth of subjects. After getting my results, I promptly went to the science faculty to re-enroll and be admitted in a government supported place, however, it was mid year and I was told that I had to wait until the next year to re-enroll. I was a quite disappointed that I could not get back in to uni and was wondering what to do with my up-coming 6 months of ‘free time’. So, I went up to level seven to see if I could find some work in the anatomy department. I happened to run into Colin. After some interrogation as to what I was doing suspiciously lurking around on Level 7, I found myself in Colin’s office talking about research projects. I was actually very intrigued because the concept of doing research looked pretty cool, and I did not know that this side of the uni even existed. I don’t remember the exact details, but shortly after that conversation I was back, re-enrolled in a government supported place in the BSc program. The rest is history. Colin has patiently (very patiently) supported my academic progress since that day I ran into him on L7. His open door policy and willingness to talk though complex ideas is a style that really complements the way I learn. Colin you have been fantastic supervisor and a great mentor. I honestly believe your support has been the key factor in academic my progress to this point. For that I will always be grateful.

Kylie Cane, it has been great getting to know you over the last 4 years. Thank you for all your support, particularly in introducing me to the world of tissue culture and molecular biology. I have enjoyed working along side you in the lab and I really appreciate the help and guidance you have given me during my PhD, many thanks.

Jan Morgan, I have learned so much from you, even when are not directly showing me how to do something. You have been the person who looks after me most in the lab, you’re a fantastic person and thanks for all the help.
I would also like to thank the past members of the Anderson lab that have helped me and shared in this experience; Linda Chuang, Michael Dickinson, Sally Gordon and Bowen Fung.

Members/associates of the young lab past and present

Heather Young, I really appreciate all the advice, support and input that you have given me, I really appreciate your generosity, thank you. Richard Anderson, Lincoln Stamp, Sonya McKeown, Adam Wallace and Alan Lomax, thanks for all the input in lab meetings, tips regarding talks and general advice. A special mention to Annette Bergner who helped me on countless occasions with mouse work, for training me on the confocal and generally for being pretty cool all round.

The department of Anatomy and Neuroscience and the people that make it

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PhD students that are/have walked the walk with me

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To my other PhD comrades past and present; Ryo Hotta, Alice Ly, Adrian Monk, Atta Ali, Abrez Housain, Linette Tan, Leni Reva, Nicki Cranna, Manda Lynx, Linda Gallo, Ben Wheaton, Shane Liddlow, Jordanna Masters, Kate Hatzopolous, Michelle le Roux, Niloufer Johansen and Rowena Sannang it has been a real blast.

*The people who have taught me to teach*

After all, the title Doctor is really just an agentive noun that comes from the Latin verb *docēre*, meaning ‘to teach’. So, it is only fitting that I thank the people who have actively helped me develop my teaching skills along the way, Chris Briggs, Jason Ivanusic, Jenny Hayes, Vasha Pilbrow, Simon Murray, Priscilla Barker and Tony Shafton. Thank you for your advice, help and example.

*My family and friends*

I think you guys are the real heroes, you have supported all that I have done particularly when it has been tough. The knowledge that you can lean on people really makes any task seem achievable, going through this has emphasised the importance of providing support. Thank you for your understanding and selflessness. In particular I would like to thank Mum and Dad. Mario, thanks for always being there, always supporting with sound logical advice and for all the philosophical conversations, you are a real inspiration.

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I will close this little bit with a quote, and when necessary please remind me of it;

*“Action expresses priorities”*  
Mohandas Karamchand Gandhi
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ALK</td>
<td>Anaplastic lymphoma receptor tyrosine kinase</td>
</tr>
<tr>
<td>Alk3</td>
<td>Activin receptor-like kinase 3</td>
</tr>
<tr>
<td>ANS</td>
<td>Autonomic Nervous System</td>
</tr>
<tr>
<td>AP-2</td>
<td>Activating enhancer binding protein 2</td>
</tr>
<tr>
<td>ARTN</td>
<td>Artemin</td>
</tr>
<tr>
<td>Ascl1</td>
<td>Achaete-scute homolog 1</td>
</tr>
<tr>
<td>BFABP</td>
<td>Brain fatty acid binding protein</td>
</tr>
<tr>
<td>BHLH</td>
<td>Basic helix loop helix</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-Bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>CBF1</td>
<td>Notch effector C-promoter binding factor 1</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre-recombinase</td>
</tr>
<tr>
<td>DβH</td>
<td>Dopamine beta hydroxylase</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglia</td>
</tr>
<tr>
<td>EdU</td>
<td>5-Ethyl-2-deoxyuridine</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchyme transition</td>
</tr>
<tr>
<td>ENS</td>
<td>Enteric nervous system</td>
</tr>
<tr>
<td>Epo</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>EpoR</td>
<td>Erythropoietin receptor</td>
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<tr>
<td>Fz3</td>
<td>Frizzled3</td>
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<tr>
<td>G1</td>
<td>Gap1 - phase</td>
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<tr>
<td>G2</td>
<td>Gap2 - phase</td>
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<tr>
<td>GDNF</td>
<td>Glial cell line-derived neurotropic factor</td>
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<tr>
<td>GF</td>
<td>Growth fraction</td>
</tr>
<tr>
<td>GFL</td>
<td>GDNF family ligand</td>
</tr>
<tr>
<td>GFRα</td>
<td>GDNF family receptor alpha</td>
</tr>
<tr>
<td>GOF</td>
<td>Gain of function</td>
</tr>
<tr>
<td>Hand2</td>
<td>Heart and neural crest derivatives-expressed protein 2</td>
</tr>
<tr>
<td>HH</td>
<td>Hamburger &amp; Hamilton</td>
</tr>
<tr>
<td>Hu</td>
<td>Anti-neuronal nuclear antibody 1</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
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<tr>
<td>Insm</td>
<td>Insulinoma-associated</td>
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<tr>
<td>LI</td>
<td>Labelling index</td>
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<tr>
<td>LOF</td>
<td>Loss of function</td>
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<tr>
<td>Mk</td>
<td>Midkine</td>
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<tr>
<td>NC</td>
<td>Neural crest</td>
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<td>Neural crest cell</td>
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<tr>
<td>NCCs</td>
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<td>NE</td>
<td>Neuroepithelium</td>
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</table>
NECs  Neuroepithelial cells
NI   Neuronogenetic interval
NICD Notch intracellular domain
NRTN Neurturin
Phox2 Paired-like homeobox-2
PGP9.5 Protein gene product 9.5
PKA  Protein kinase A
PNS  Peripheral nervous system
PSPN Persephin
Ptn  Pleitrophin
PVE  Proliferative pseudostratified epithelium
Q    Quiescent fraction
Rbpj Recombining binding protein suppressor of hairless (=CBF1)
Ret  Rearranged in transfection
RNA  Ribonucleic acid
SCG  Superior cervical ganglion
SCG10 Superior cervical ganglion 10, a stathmin-family protein
SG   Sympathetic ganglia
Shh  Sonic hedgehog
shRNA Short hairpin ribonucleic acid
SNS  Sympathetic nervous system
StG  Stellate ganglion
SVZ  Subventricular zone
Tc   Cell cycle length
TH   Tyrosine hydroxylase
Ts   S-phase length
VZ   Ventricular zone
The cover image:

This is an illustration by Santiago Ramón y Cajal of the peripheral nervous system, with the paravertebral sympathetic ganglia as a focus. It comes from Textura del Sistema Nervioso del Hombre y de los Vertebrados, 1899 -1904 (Garcia-Lopez et al., 2009)
Chapter 1

1 Literature Review
1.1 The autonomic nervous system

The nervous system is constantly receiving and responding to external stimuli, and simultaneously, it needs to monitor and coordinate the organism’s own internal environment in such a way that all of the cells in the body can perform their functions optimally (Fig. 1B). The peripheral effectors of the nervous system that are chiefly concerned with maintaining an optimal internal milieu are collectively termed the autonomic nervous system (ANS). These effectors are classified into three components, parasympathetic (PNS), sympathetic (SNS) and enteric (ENS) systems. The efferent neurons that constitute these components lie in discrete locations within the central and peripheral nervous systems (CNS and PNS – Fig. 1A). The cells deemed parasympathetic represent cranial and sacral outflows of the ANS, cells deemed enteric are those that are contained within the walls of the alimentary canal and cells deemed to be sympathetic represent thoracolumbar outflows (Fig. 1A). It is important to acknowledge that the original intention for such a classification was not to consider the components of the ANS as autonomously operating systems aside from the CNS: “the word autonomic does suggest a much greater degree of independence from the central nervous system than in fact exists” (Langley, 1921). Rather, this classification provides a means for identification so that the components of the ANS can be studied. In actual fact the central processors for cognition, sensory information and behavioral states are all integrated to produce behavior (Figure 1B - Janig, 2006).

1.2 Anatomy of the sympathetic nervous system

The sympathetic nervous system consists of preganglionic neurons with cell bodies that lie in the intermediolateral column of the spinal cord between spinal segments C8 and L2 in a rodent or human (Fig. 1A). The postganglionic neurons and chromaffin cells, on which the preganglionics terminate, are found in paravertebral ganglia, prevertebral ganglia and the adrenal medulla (Fig. 1A). It is interesting to note that all paravertebral and prevertebral ganglia are found
juxtaposed to primary branches of the aorta, which in part reflects their developmental locations. Sympathetic ganglia and the adrenal medulla are not solely occupied by postganglionic neurons and adrenal chromaffin cells. They are also comprised of satellite glia and sustentacular cells respectively. These cells are crucial to the proper functioning of both the neurons and the chromaffin cells. All of the cells that make up the peripheral part of the SNS have a common developmental history. They are derived from the neural crest (NC) caudal to the level of somite five (LeDouarin, 1986, Durbec et al., 1996).

The NC is a transient, multipotent group of embryonic cells that undergoes an epithelial to mesenchymal transition (EMT), delaminates from the dorsal neural tube, then migrates extensively throughout the developing embryo, proliferating and differentiating to give rise to a vast array of cell types, tissues and organs. Considered a vertebrate synapomorphy and sometimes referred to as the fourth germ layer, the NC provides a unique model for studying embryonic induction, specification, tissue commitment, migratory potential, cell fate determination and proliferation (Sauka-Spengler and Bronner-Fraser, 2008). The multipotent stem cell characteristics of NCCs make them viable candidates for many stem cell based therapies (Hotta et al., 2010). Also, the principal features of their developmental history (EMT, migration and proliferation) are the key features shared by all cancer pathologies. It is therefore not surprising that this unique group of cells has, for more than a century, drawn the attention of many scientists and continues to do so.

1.3 A short historical account of the neural crest

In 1868 Wilhelm His Sr. described a specific group of cells in a groove (Zwischenrinne) between the neural plate and the ectoderm in the early stages of chick embryonic development. In later stages of development, he identified a group of cells that looked like a strand or cord between (Zwischenstrang) the neural tube and the ectoderm. He went on to postulate that the Zwischenrinne cells gave rise to the Zwischenstrang and noted that this strand of cells was the
source of spinal and cranial ganglia in chicken embryos (Hall, 1999, 2000, 2009). The name neural crest was actually coined by Arthur Milnes Marshal (Marshall, 1878, 1879). In contrast to the description of His, Marshal stated that the neural crest was in fact part of the dorsal aspect of the neural tube once the tube had closed and identified that it was separate from the overlying ectoderm (Marshall, 1878, 1879). Marshal also gave the term “neural ridges” to the boundary between the neural and non-neural ectoderm prior to tube closure (Marshall, 1879). Both His and Marshall agreed that these cells gave rise to the peripheral nervous system, including cranial and sensory ganglia.

In 1893, Julia Platt was the first person to challenge the germ layer theory and introduce the idea that the cartilages of the craniofacial and pharyngeal arch skeletons, together with dentine-producing cells of the teeth (odontoblasts) arose from the ectoderm adjacent to the neural tube (Hall, 1999, 2009). These radical ideas were in striking contrast to the existing germ layer theory that only cells from the mesoderm could give rise to skeletal structures and, although supported by many, the greater scientific community rejected Platt’s ideas (Hall 1999). It was not until the 1940's, nearly fifty years later that Platt’s ideas were confirmed and accepted. The 1940's were a seminal period regarding our knowledge about the NC. Many studies had shifted their focus to understanding the potential of these remarkable cells and at the same time many people were also identifying the inductive signals capable of promoting NC formation. Over the past several decades the availability of advanced imaging, molecular and genetic techniques has resulted in a far better understanding of the development of these cells, however, much is yet to be understood about the processes governing the rapid tissue growth fueled by proliferation of NCCs and their progeny.
1.4 Neural crest cell migration

Sympathetic ganglia are populated by cells that emigrate from both the vagal (somites 1-7) and trunk (somites caudal to 7) NC (Durbec et al., 1996, LeDouarin and Kalchiem, 1999). NCCs caudal to somite 5 give rise to components of the enteric nervous system, adrenal chromaffin cells, sympathetic ganglia, dorsal root ganglia (DRG) and melanocytes (Kulesa et al., 2009). The superior cervical ganglion (SCG) is the most rostral of all sympathetic ganglia and is generated by cells from both vagal and trunk NC (Durbec et al., 1996, LeDouarin and Kalchiem, 1999). The stellate, or first thoracic ganglion (StG) and all ganglia caudal to it, as well as the chromaffin cells of the adrenal medulla, are populated by trunk NCCs only (Durbec et al., 1996). NCCs that gives rise to the DRG, sympathetic ganglia and adrenal chromaffin cells migrate through the rostral portion of each somite, and is called the rostral migratory stream (Kulesa et al., 2009). Prior to division of the somite into the dermomyotome and sclerotome, NCCs migrate ventrally in the intersomitic furrow toward the dorsal aorta (Kulesa et al., 2009). These ventrally migrating NCCs follow the intersomitic blood vessels and will give rise to sympathetic ganglia and the adrenal chromaffin cells (Schwarz et al., 2009a, Schwarz et al., 2009b, Schwarz and Ruhrberg, 2010). Once the somite has divided, the vast majority of NCCs enter the somite and either arrest next to the neural tube, where these cells will give rise to the DRG, or continue on to populate the sympathetic ganglia alongside the dorsal aorta.

Trunk NCCs in the murine SNS emigrate from the neural tube between E8.5-E10 (Serbedzija et al., 1990). There is a strong correlation between the time that a cell leaves the dorsal neural tube and the final tissue that it will generate (Serbedzija et al., 1990, Krispin et al., 2010). NCCs that migrate out of the neural tube early tend to give rise to sympathetic ganglia, while cells that migrate out later in development give rise to DRG (Serbedzija et al., 1990, Krispin et al., 2010). In the mouse, NCCs that give rise to sympathetic ganglia emigrate out of the neural tube between E8.5 and E9.5 (Serbedzija et al., 1990). Injections of vital dye into the dorsal neural tube prior to E9.5 labels cells that are found, either 12
or 24 h later, in developing sympathetic ganglia (Serbedzija et al., 1990). Vital dye injections at E9.5 or later, fail to label any cells in sympathetic ganglia harvested either 12h or 24h after the injection. Injections on or after E10.5, do not label migrating cells and marks only cells that remain located in the dorsal neural tube (Serbedzija et al., 1990). So in the mouse, the NCCs that form the StG primordia appear to arrive around E9.0-E9.5. Migration then ceases and the cells differentiate and proliferate to ultimately give rise to the mature sympathetic ganglion (Serbedzija et al., 1990).

1.5 Differentiation

1.5.1 From progenitors to neurons and glia

As NCCs coalesce into the ganglion primordia, their fate becomes restricted and they adopt either a neuronal or a glial phenotype. At present, the mechanisms that govern the decision to adopt either a neuronal or glial phenotype are not understood. At E9.5 in a mouse embryo, trunk NCCs positive for the expression of Sox10 up-regulate the expression of the homeobox transcription factor Phox2b, a transcription event that is one of the first signs of differentiation from a multipotent migrating NCCs to an early sympathetic progenitor (Rohrer, 2011). At E10.5, the vast majority of cells in the sympathetic ganglion primordia have up-regulated the expression of Phox2b leading to three phenotypically distinct populations of cells; 1) cells that express Sox10 only (Sox10+/Phox2b-); 2) cells that express Sox10 and Phox2b (Sox10+/Phox2b+); and 3) cells that express Phox2b only (Sox10-/Phox2B+ - Callahan et al., 2008). In the mouse, Phox2b is strongly expressed by NCCs that adopt a neuronal phenotype, but is also expressed at low levels in Sox10+ NC-derived glial cells (Corpening et al., 2008, Sasselli et al., 2012). Sox10 expression has been shown to maintain multipotency and is absent from cells that adopt a neuronal phenotype (Kim et al., 2003, Callahan et al., 2008, Tsarovina et al., 2008, Rohrer, 2011). So, at E10.5 in the mouse, the Sox10-/Phox2b+ population represents cells that have
committed to a neuronal lineage, however, we really cannot predict the fate of a cell that is either Sox10+/Phox2b- or Sox10+/Phox2b+ at this time.

In the chick, it has been shown that the NC progenitors in putative sympathetic ganglia are prevented from precociously differentiating by negative feedback regulation (Tsarovina et al., 2008). This involves the expression of Delta1 on cells that already express the neural marker SCG10 (a stathmin-family protein and marker of the neuronal lineage), and which have lost the expression of Sox10 (Tsarovina et al., 2008). The Sox10-/SCG10+ cells are analogous to the Sox10-/Phox2b- cells described above and have committed to a neuronal phenotype. In the chick, Delta1 expressed on SCG10+ cells activates Notch signaling in the Sox10+ NC progenitors (these cells are analogous to the Sox10+/Phox2b- or Sox10+/Phox2b+ cells observed in the mouse at E10.5) and prevents their differentiation (Tsarovina et al., 2008, Rohrer, 2011). In the chick, in vivo inhibition of Notch signalling depletes the progenitor pool and leads to an increase in the proportion of SCG10 cells (Tsarovina et al., 2008). In contrast, over-expression of Notch results in the maintenance of undifferentiated NC progenitors at the expense of the neuronal SCG10+ population (Tsarovina et al., 2008). It would appear that Notch/Delta signaling controls the balance between progenitor cell maintenance and differentiation that leads to a neuronal phenotype (Tsarovina et al., 2008).

The SNS is unlike the CNS because the production of neuronal and glial cells occurs simultaneously from the onset of SNS neurogenesis (Nishino et al., 2010). However, there is virtually nothing known about the mechanisms governing fate restriction and it is not entirely clear at what point NCCs are restricted to either a neuronal or a glial lineage. The first sign of commitment to a neuronal phenotype in the murine SNS is by E10.5 (E3 in the chick). This is marked by an up-regulation of the expression of neuronal genes responsible for the production of enzymes such as; tyrosine hydroxylase (TH), the enzyme responsible for catalyzing the rate-limiting step in catecholamine biosynthesis, dopamine-β-hydroxylase (DBH), the enzyme responsible for the conversion of dopamine to noradrenaline and a host of pan neuronal markers, including
PGP9.5, SCG10, tuj1, Hu and neurofilament (Groves et al., 1995, Shah et al., 1996, Callahan et al., 2008, Wildner et al., 2008, Danesh et al., 2009, Rohrer, 2011). However, in the developing murine SNS at E10.5, many of the cells in ganglion primordia are also positive for the expression of Sox10 (Callahan et al., 2008). Sox10 has been shown to maintain multipotency in NCCs (Kim et al. 2003). So, it appears that there is an overlap between Sox10 expression and expression of pan-neuronal markers at E10.5 in the mouse. At E11.5 (E3 in the chick) Sox10 is now absent from cells that are positive for the expression of TH (Callahan et al., 2008). Although the potential of the Sox10-/TH+ cells is yet to be confirmed, given their lack of Sox10 expression I assume that cells positive only for TH are unipotent and will give rise only to neurons or neuronal precursors. On this basis, I refer to cells expressing a neuronal marker, such as TH and lacking Sox10 as neuronal precursors, based on an assumption that they are unipotent. Note that there are currently no markers to distinguish post-mitotic neurons from mitotic neural precursors, and it is not known when neuronal precursors first adopt the functional physiological capabilities of a mature SNS neuron.

At E11.5 almost all of the cells that are Sox10+/TH- also co-express brain fatty acid binding protein (BFABP). BFABP is expressed in all glial cells of the PNS and BFABP expression never overlaps with the expression of TH (see chapter 3 Fig. 3.3 and Callahan et al., 2008). Cells that are Sox10+/BFABP+ are considered to have adopted a glial phenotype (Callahan et al., 2008). Although the potential (what cells they can give rise to) of the Sox10+/BFABP+ cells has not been examined in the SNS, I will refer to these as glial progenitors and not precursors, based on the fact that Sox10 has been shown to maintain multipotency in NC derived cells (Kim et al., 2003).

1.5.2 Inductive signals that initiate differentiation

The conversion from a multipotent NC stem cell to a differentiated noradrenergic sympathetic neuronal precursor, positive for the expression of pan-neuronal and noradrenergic markers, is achieved by the actions of a network of transcription factors that includes the basic helix-loop-helix (BHLH) transcription factors Ascl1 (previously termed Mash1) and Hand2, the Paired-

Expression of the transcription factors depends on signals from the dorsal aorta, as well as the notochord and floor plate of the neural tube (Groves et al., 1995, Shah et al., 1996, Mehler et al., 1997). The floor plate/notochord-derived signals responsible for differentiation of NCCS are yet to be fully characterised. However, an identified floor plate/notochord signal that either directly or indirectly influences NC differentiation is sonic hedgehog (Shh - Jessell, 2000, Morikawa et al., 2009a). Shh is released by the floor plate and notochord and in Shh null mutant mice, neuronal differentiation of sympathetic trunk NC precursors is delayed and patterning of trunk ganglia is disrupted (Jessell, 2000, Morikawa et al., 2009a).

The dorsal aorta-derived signals have been identified as members of the bone morphogenetic protein (BMP) family (BMPs - Shah et al., 1996). In the mouse and chick, the endothelial cells of the dorsal aorta have been shown to express BMPs 2, 4 and 7, and development of sympathetic ganglia is crucially dependent on BMP signaling (Reissmann et al., 1996, Shah et al., 1996, Varley and Maxwell, 1996, Pimanda et al., 2007, Danesh et al., 2009, Rohrer, 2011). Conditional removal of the type 1 BMP receptor, Alk3, was achieved by crossing mice that express Cre-recombinase driven by the Wnt1 promoter to mice in which the Alk3 gene was sandwiched between two loxP sites (Wnt1-Cre:Alk3<sup>flox/flox</sup>). This results in a complete loss of the sympathetic ganglia (Morikawa et al., 2009b). NCCs migration to the dorsal aorta is unaffected in Wnt1-Cre:Alk3<sup>flox/flox</sup> mice, however, there is a significant increase in cell death in the region lateral and dorsal to the dorsal aorta and NCCs fail to form the primitive ganglion (Morikawa et al., 2009b).
BMPs have been shown to induce expression of the transcription factors that are necessary for sympathetic noradrenergic neuronal differentiation in vitro and in vivo. Application of the BMP antagonist, noggin, completely prevents the expression of transcription factors necessary for noradrenergic differentiation in the chick embryo (Reissmann et al., 1996, Schneider et al., 1999). Although it is known that BMP signaling is required for the expression of transcription factors that lead to noradrenergic differentiation and that signaling via the Alk3 receptor is necessary for the development of the sympathetic nervous system, the intracellular pathway(s) that subserve these functions are yet to be elucidated. In the mouse, removal of the canonical BMP-signaling pathway via conditional deletion of Smad4 does not affect noradrenergic differentiation (Morikawa et al., 2009b). Instead, these conditional knockouts are associated with proliferation defects (Morikawa et al., 2009b). In addition to this, conditional deletion of Tac1, a prominent downstream target of BMP receptor activation that does not depend on the activation of Smad4 (Zhang, 2009), does not have any effect on SNS differentiation (Morikawa et al., 2009b). So, the key BMP mediated down steam signaling pathways necessary for in vivo SNS differentiation are it is still unknown. One possible candidate pathway has been demonstrated in vitro, where it has been shown that BMP4 can activate PKA via a non-canonical signaling pathway that is independent of cAMP (Liu et al., 2005). BMP signaling via PKA is sufficient to elicit neurogenesis (Liu et al., 2005). This signaling pathway may provide an explanation as to why differentiation persists in the Smad4 and Tac1 conditional knockouts. However, no evidence exists to demonstrate the effect of this non-canonical signaling pathway in vivo.

1.5.3 Noradrenergic differentiation

The transcription factors Ascl1, Phox2b, Insm1, Hand2, Phox2a and Gata2/3 act in a complex network. However, there is an underlying sequence of transcriptional events that is almost identical for both the chick and the mouse (Rohrer, 2011). In the chick the process is drawn out over a longer timeframe, HH stage 16 to 25-27 (approximately 4-5 days), compared to the mouse, E9.5 to E10.5-E11.5 (approximately 1-2 days - Groves et al., 1995, Rohrer, 2011). Our current understanding of the individual actions of these molecules, comes
primarily from either gain of function (GOF) or loss of function (LOF) experiments, but the ability of transcription factors to cross-regulate and functionally compensate for each other exacerbates the difficulty of understanding the differentiation program.

1.5.3.1 Ascl1, Phox2b and Insm1 in differentiation

The earliest member of the transcriptional network expressed by murine trunk sympathetic NC precursors is Ascl1 (Pattyn et al., 1999, Goridis and Rohrer, 2002, Wildner et al., 2008). Ascl1 mRNA transcripts are detectable from E9.5, in trunk NC sympathetic precursors, in the ganglion anlage next to the dorsal aorta (Guillemot and Joyner, 1993, Groves et al., 1995, Pattyn et al., 1999). Phox2b is expressed just after Ascl1 at the ganglion primordia and, in Phox2b null mutant mice, there is a complete absence of the entire sympathetic nervous system that is due to the death of all trunk NC precursors by E11.5 (Groves et al., 1995, Pattyn et al., 1999, Goridis and Rohrer, 2002). Phox2b is the only transcription factor that cannot be compensated for during the initial stages of differentiation, around E9.5 (Pattyn et al., 1999, Goridis and Rohrer, 2002). Conditional deletion of Phox2b, after E10.5, was achieved using the Cre-lox system where lox P sites were inserted either side of the Phox2b gene and Cre-recombinase was driven by the Islet1 promoter ([Islet1-Cre:Phox2bflox/flox] - Coppola et al., 2010). Islet1 is first expressed in the SNS at around E10-10.5, but its role in SNS development is yet to be examined (Coppola et al., 2010). In Islet1-Cre:Phox2b^{floxflox} mice, NCCs avoided cell death and were later observed to express markers for noradrenergic differentiation like TH and DβH (Coppola et al., 2010). These results demonstrate that, after the initial stages of development, other members of the transcriptional network can compensate for the functions of Phox2b and that the actions of Phox2b are only essential in the narrow window from E9.5-E10.5.

In Ascl1 null mutant mice (Ascl1^{-/-}), there is a delay in the onset of the expression of all other members in the transcriptional network with the exception of Phox2b (Guillemot et al., 1993, Pattyn et al., 2006, Wildner et al., 2008). In addition to this, there is a reduction in the fraction of proliferating cells in the Ascl1^{-/-} mice (Pattyn et al., 2006, Wildner et al., 2008). The combined
actions of Ascl1 and Phox2b results in the expression of Insm1 that is first detectable at E10-E10.5 (Wildner et al., 2008). The interactions between Ascl1, Phox2b and Insm1, leads to the rapid up-regulation of Phox2a, Gata2/3, and Hand2, so that by E11.5 the vast majority of cells in the ganglion are positive for a noradrenergic neuronal phenotype and express the pan-neuronal markers Tuj1, SCG10, neurofilament, PGP9.5 and Hu, and enzymes necessary for the biosynthesis of noradrenaline, TH and DBH (Groves et al., 1995, Shah et al., 1996, Callahan et al., 2008, Wildner et al., 2008, Danesh et al., 2009, Rohrer, 2011).

Similar to the phenotype observed in the Ascl1−/− mice, Insm1 null mutant mice (Insm1−/−) have a delay in the expression of all downstream transcription factors (Wildner et al., 2008). Perhaps most interesting is that the relative levels of both Ascl1 and Phox2b are altered in Insm1−/− mice (Wildner et al., 2008). Removal of Insm1 results in a 2.6 fold increase in the amount of Ascl1, accompanied by a 1.9 fold decrease in the amount of Phox2b in sympatheo-adrenal cells (Wildner et al., 2008). So, either directly or indirectly, Insm1 plays a role in up-regulating expression of Ascl1 and a role in reducing the expression of Phox2b (Wildner et al., 2008). It is tempting to speculate that the delayed effects in the differentiation program, observed in the Insm1−/− mice, are due to the altered epistasis between Ascl1 and Phox2b. It is also tempting to speculate that an ideal ratio of Ascl1 to Phox2b is essential to proper differentiation and when this is disrupted, differentiation is perturbed.

1.5.3.2 Phox2a, Hand2 and Gata2/3 in differentiation

By E10.5, Phox2a, Hand2 and Gata2/3, are all expressed as a result of the combined actions of Ascl1, Phox2b and Insm1 (Guillemot and Joyner, 1993, Tiveron et al., 1996, Hirsch et al., 1998, Howard et al., 2000, Goridis and Rohrer, 2002, Tsarovina et al., 2004, Howard, 2005, Sarkar and Howard, 2006, Wildner et al., 2008, Morikawa et al., 2009b, Coppola et al., 2010, Rohrer, 2011). In the chick, the order of the onset of some transcription factors can be identified, however, in the mouse, differentiation is temporally condensed making it difficult to ascertain the specific order, if any, in which transcription events occur (Groves et al., 1995, Rohrer, 2011).
Phox2a is another paired-like homeobox-2 transcription factor that is initially expressed in the sympathetic nervous system from approximately E10.5 (Hirsch et al., 1998). In sympathetic ganglia, mice carrying a null mutation for Phox2a (Phox2a\(^{-/-}\)) do not appear to have any major defects in noradrenergic differentiation (Morin et al., 1997). This is in contrast to other parts of the nervous system, such as the locus coeruleus, where removal of Phox2a results in the loss of the noradrenergic phenotype (Morin et al., 1997). The parasympathetic, sphenopalatine and otic ganglia are absent in Phox2a\(^{-/-}\) mice, and the distal aspects of the IX\(^{th}\) and the X\(^{th}\) cranial ganglia are severely atrophic in the absence of Phox2a (Morin et al., 1997).

In sympathetic ganglia, the loss of Phox2a is compensated for by Phox2b after E10.5, hence the reason for the lack of defects (Morin et al., 1997). It has also been shown that Phox2a can compensate for some of the functions of Phox2b (Coppola et al., 2010) in that in the Islet1-Cre:Phox2b\(^{lox/lox}\) mice, noradrenergic neurons are still produced (Coppola et al., 2010). In double conditional knockouts, where both Phox2a and Phox2b have been removed under the Islet1 promoter using the Cre-loxP system (Islet1-Cre:Phox2a/b\(^{lox/lox}\)), there is virtually a complete absence of the noradrenergic phenotype (Coppola et al., 2010).

Hand2 is a member of the twist family of BHLH transcription factors that is restricted to the ANS during neural development (Srivastava et al., 1995, Howard et al., 1999, Dai and Cserjesi, 2002). Hand2 contains a strong transcriptional domain in the amino terminal third of the protein and functions by forming heterodimers with other BHLH transcription factors (Dai and Cserjesi, 2002). In the mouse, elimination of Hand2 did not affect the expression of Ascl1, Phox2a/b or Gata2/3, but there was a significant loss in the expression of TH and D\(\beta\)H (Howard, 2005, Sarkar and Howard, 2006, Morikawa et al., 2007, Hendershot et al., 2008). In addition, conditional removal of Hand2 results in severely hypoplastic ganglia, suggesting that Hand2 is also important for proliferation (Hendershot et al., 2008).

Gata3 is another zinc finger transcription factor that is important for sympathetic neural development (Lim et al., 2000, Tsarovina et al., 2004,
Moriguchi et al., 2006, Hendershot et al., 2008, Tsarovina et al., 2010). At E10.5 in *Gata3* null mutants (*Gata3<sup>-/-</sup>), the sympathetic ganglion primordia appear to have normal expression of Ascl1, Phox2b, and Hand2, however, the expression level of TH is greatly decreased and there is a complete loss of Gata2 expression (Tsarovina et al., 2004). *Gata3<sup>-/-</sup>* mice fail to acquire a noradrenergic phenotype and there is a considerable amount of cell death early in development. When *Gata3* is conditionally removed under the DβH promoter (*DβH-Cre:Gata3<sup>flox/flox</sup>), cells successfully acquire a noradrenergic phenotype which indicates that Gata3 expression, prior to the expression of DβH, is necessary to elicit noradrenergic differentiation (Tsarovina et al., 2010). The level of cell death seen in the *DβH-Cre:Gata3<sup>flox/flox</sup>* mice is similar to that of the *Gata3<sup>-/-</sup>* mice suggesting Gata3 is needed after the initial stages of development to avoid cell death (Tsarovina et al., 2010).

### 1.5.3.3 Summary of Noradrenergic Differentiation

In the mouse, it takes only about two days to complete the transcription events necessary to convert multipotent murine trunk NCCs, into sympathetic neuroblasts that express the genes necessary for the production of noradrenaline. In the initial stages of development it would appear that the ratio of Ascl1 to Phox2b expression is essential for driving the rapid up regulation of subsequent transcription factors (Rohrer, 2011). Insm1 is expressed soon after both Ascl1 and Phox2b, and either directly or indirectly, Insm1 then plays a role regulating the ratio of Ascl1 to Phox2b expression (Wildner et al., 2008). Insm1 negatively regulates the expression of Ascl1 (Fig. 1.2) and positively regulates the expression of Phox2b (Wildner et al., 2008). Together the combined expression of Ascl1, Phox2b and Insm1, results in the rapid up-regulation of Phox2a, Hand2 and Gata2/3. These transcription factors participate in complex interactions and it is this network that drives the expression of TH and DβH (Fig. 1.2).
1.6 Proliferation and the cell cycle

The production of new cells requires existing cells to undergo cell division. For this to occur, a cell needs to pass through a highly regulated program known as the cell cycle (Fig. 1.3). The cell cycle coordinates the replication of the genome, and subsequent segregation of each genomic replicate into the new daughter cells (Coffman, 2004). In the adult, the vast majority of cells are said to be quiescent, that is, they have either temporarily or permanently withdrawn from the cell cycle. Cells that are quiescent, or withdrawn from the cell cycle, are in a state called G0 (or G1/G0). Cell division does not occur in G0 and the cell cycle machinery is disassembled (Coffman, 2004). G0 is a phase outside the ‘active’ phases of the cell cycle, which itself is composed of four discrete phases, all of which are necessary for successful cell division: Gap 1 (G1) is the interphase between the mitosis (M-phase) of the last cycle and DNA synthesis (S-phase) of the new cycle (Israels and Israels, 2000). All new cells are born into G1 and it is also the phase in which cells can withdraw from the cell cycle and move into G0 (Fig. 1.3). In G1, cells undergo protein synthesis and growth and they are subject to extracellular signals including mitogens and growth factors. In response to these stimuli, cells will either; 1) withdraw and move into G0, or, 2) pass from G1 into S-phase and continue to divide (Israels and Israels, 2000). If cells pass into S-phase, the entire genome is replicated. DNA replication is a semi-conservative process in which the double stranded helix is unraveled at sites called origins into two single strands of DNA, forming the replication fork (Shirahige et al., 1998, Bell and Dutta, 2002). The new DNA is added to the existing strands through a complex series of enzyme-dependent reactions (Bell and Dutta, 2002). The entire replication process is completed during S-phase and usually takes between 2-8h (Nowakowski et al., 1989a, Caviness et al., 1995, Israels and Israels, 2000).

Since the late 50’s, investigators have taken advantage of S-phase, using it to identify cycling cells in vivo and in vitro (Bessman et al., 1958, Eidinoff et al., 1959, Stockdale et al., 1964). Radioactive thymidine, or, more recently, analogues of thymidine such as 5-bromo-2-deoxyuridine (BrdU), can be taken up by cells
that are in S-phase and become incorporated into the new strand of DNA (Bessman et al., 1958). When delivered at an adequate concentration, BrdU and other analogues are randomly substituted for thymidine in the replication process and can be detected using histological methods to mark cycling cells (Bessman et al., 1958, Eidinoff et al., 1959, Stockdale et al., 1964, Rothman et al., 1978, Gratzner, 1982, Nowakowski et al., 1989b). Labelling with thymidine analogues is the most popular way to identify proliferation in vivo.

Once DNA synthesis is complete, cells progress into the phase Gap2 (G2). G2 is the second interphase between M and S phases and is the period where DNA is checked to ensure the genome has been copied properly. Cells move from G2 into M-phase where the genetic material is ‘packed and moved’, this is marked by the generation of bipolar mitotic spindles, segregation of sister chromatids and cell division itself (Israels and Israels, 2000, Coffman, 2004). All of the cell cycle phases (G1, S, G2 and M) are crucial for proper cell division. The regulation of these processes must ensure that all cells do not move into the next phase until all events have been properly completed. Cyclins and Cyclin dependent kinases (Cdk's), regulated by a myriad of intracellular signaling pathways, serve as the checkpoints, or controls, that exist between the various phases of the cell cycle. The role of the Cdk's ensures that the propagation of mutated or damaged cells does not occur and is critical to all life (Israels and Israels, 2000). The cell cycle is one of the most important processes in biology. Its continued reiteration leads to exponential proliferation and it is the sole driving force behind the production of all tissues and organs, including the nervous system.

1.7 Neurogenesis

There are fundamental differences between the way new neurons are generated (neurogenesis) in the CNS and parts of the ANS, versus the way they develop in sympathetic ganglia and these are discussed below.
1.7.1 Neurogenesis in the CNS

The proliferation of stem cells and precursor cells whose progeny give rise to neurons in the CNS is classically understood to be the process of neurogenesis (Altman, 1969a, b, Caviness et al., 1995). In neurogenesis in the CNS, stem and neural precursor cells divide to generate more stem and neural precursor cells as well as cells that withdraw from the cell cycle and differentiate as neurons. Cell division occurs in the ventricular zone (VZ) and subventricular zone (SVZ) of the developing CNS, while differentiating cells move into more superficial layers. The number of neurons generated depends on 3 main criteria; 1) The length of the neuronagenetic interval (NI), 2) the proportion of cells that are proliferating during the NI, and 3) the cell cycle lengths (Tc) of the dividing cells (Nowakowski et al., 1989b, Caviness et al., 1995). The NI begins with the proliferation of precursors that give rise to the first postmitotic neurons and continues until the last neurons are generated (Caviness et al., 1995, Rohrer, 2011). The length of NI depends on the time that it takes for all cells in the proliferating population of cells to withdraw from of the cell cycle and move into G0 (Caviness et al., 2003). At any point during the NI, there will be a fraction of cells that are proliferating, the growth fraction (GF), and a fraction that have differentiated as neurons and withdrawn from the cell cycle, known as the Q fraction (Q for quiescent). At all times GF+Q must equal 100% of the total cells, and the NI ends when Q = 100% (GF = 0%). This means, the faster the decline in the GF, the shorter the NI (Caviness et al., 2003). In the CNS, the length of the NI varies and in some places, like the dentate gyrus of the hippocampus, it continues through adult life due to the presence of adult neural stem cells (Altman, 1969b, van Praag et al., 2002).

The vast majority of neocortical neurons arise from a proliferative pseudostratified epithelium (PVE) at the margin of the embryonic cerebral ventricles (Takahashi et al., 1993). In a number of studies unique methods have been developed, that can determine the key parameters of proliferation in the cerebral cortex (Gratzner, 1982, Nowakowski et al., 1989b, Takahashi et al., 1993, 1995, Hayes and Nowakowski, 2000a). We know now that the output from the PVE is sufficient to produce the mouse neocortex over a 6 day NI from E11 to E17 (Takahashi et al., 1995). In this period, cells go through a total of 11 cell cycles.
and neurons are produced in a temporal sequence that generates the cortical laminae sequentially (Caviness et al., 2003). The $T_c$ in the proliferating pool increases as development proceeds, varying from ~8h to 18h (Nowakowski et al., 1989b, Caviness et al., 1995, Takahashi et al., 1995).

Many of the changes in proliferation seen during the NI can be correlated to the changes in the types of divisions cells undergo. Early in the NI, stem cells divide by symmetrical divisions that lead to the expansion of the neural stem cell pool, the cell cycle lengths are shorter and the GF > Q (Caviness et al., 1995, Caviness et al., 2003, Gotz and Huttner, 2005). The extent of the expansion in the neural stem cell pool is largely dependent on the founding number of cells (Caviness et al., 2003). In the mid-phase of the NI, the initial production of neurons occurs by asymmetric divisions of precursors where one of the daughter cells remains in the pool of precursors and the other differentiates into a neuron (Gotz and Huttner, 2005). During this part of the NI, the GF decreases and the length of $T_c$ increases (Caviness et al., 2003, Guillemot, 2007). The last phase of the NI, depletes the proliferating pool and results in the GF approaching 0% (or Q approaching 100%). This occurs with neural progenitors divide symmetrically, such that both daughter cells will become terminally differentiated neurons (Gotz and Huttner, 2005, Guillemot, 2007).

It is important to note that over the course of CNS neurogenesis, differentiation and proliferation appear to have inverse affects on each other. As the rate of differentiation increases, GF decreases and $T_c$ increases. The increase in $T_c$ is due to the fact that cells spend more time in $G_1$, which seems to be the only phase of cell cycle affected, increasing in length as cells differentiate (Caviness et al., 1995, Caviness et al., 2003, Dehay and Kennedy, 2007). The amount of time a cell spends in S and G2+M remains constant regardless of the temporal progression through the NI (Takahashi et al., 1995). However, recent studies show that some progenitors may experience a lengthening of S-phase or G2 (Arai et al., 2011). Proliferation ends as cells withdraw from the cell cycle, these cells differentiate to express neuronal markers, so during CNS development
cells expressing neuronal markers have withdrawn from the cell cycle and do not divide (Dehay and Kennedy, 2007, Guillemot, 2007, Rohrer, 2011).

In the CNS, it is generally accepted that neurogenesis precedes gliogenesis but there is overlap in several regions of the brain (Jacobson, 1991). The production of glia persists long after the NI and is continued throughout life (Altman, 1966, Sturrock, 1982, Lee et al., 2000). The first population of glial cells produced is the radial glia, which act as stem cells with processes spanning the entire thickness of the developing cerebral wall. These are followed by oligodendrocyte precursors, astrocytes and then oligodendrocytes themselves (Rakic and Nowakowski, 1981, Lee et al., 2000). The oligodendrocyte lineage originates primarily from the ventral regions of the developing neural tube and astrocytes are generated from the dorsal regions of the neural tube (Rakic and Nowakowski, 1981, Timsit et al., 1995, Pringle et al., 1998, Miyata et al., 2001, Noctor et al., 2001). Proliferation in CNS glial precursors can occur during migration and due to the lack of anatomical predictability, the cell cycle kinetics involved in gliogenesis is not well-understood (Lee et al., 2000).

1.7.2 Neurogenesis in the ANS

Neurogenesis in the ANS is quite different to that in the CNS, with fundamental differences in the generation of the cells that make up the ANS. Autonomic ganglia originate from either: 1) NC stem cells that have detached from the ventricular epithelium of the dorsal neural tube and migrated a long way through the body to the final site of the ganglion that they form; or 2) are produced directly from ectodermal cells, called placodes, that are close to the final site of the ganglia that they generate. Only parasympathetic ganglia form from placodes. Common to all autonomic ganglia is the fact that NC or placode-derived precursor cells, and differentiated neurons and glia, are all intermingled in the developing ganglia and unlike the CNS, in the ANS there does not appear to be any identified layered anatomical arrangement of proliferating cells. The process of neurogenesis in different parts of the autonomic nervous system can differ and will each will be discussed.
1.7.2.1 Neurogenesis in parasympathetic ganglia

Sox10 positive NC cells that have migrated out of the dorsal neural tube are the founding cells for the vast majority of parasympathetic ganglia, with ectodermal placodes providing the remainder of the cells. Sox10+ NCCs serve as the proliferating pool of precursors that will give rise to all sensory and parasympathetic neurons respectively. In parasympathetic ganglia, withdrawal from the proliferating pool coincides with the expression of neuron specific markers like neurofilament, SCG10, Q211 and Islet1 (Rohrer and Thoenen, 1987, Francis and Landis, 1999, George et al., 2010, Rohrer, 2011). Overall, neurogenesis in the parasympathetic ganglia proceeds in a similar fashion to that of the DRG and CNS – proliferation of an undifferentiated precursor pool, followed by neuronal differentiation that is accompanied by withdrawal from the cell cycle.

However, there is one identified exception that is unlike neurogenesis in the CNS: In the ciliary ganglia of the chick, there is a population of neuronal progenitors that withdraw from the cell cycle as undifferentiated neuronal precursors (Lee et al., 2002). These undifferentiated precursors remain in a quiescent state (in G₀) and later differentiate into neurons, so in this case, withdrawal from the cell cycle is not linked immediately to terminal differentiation (Lee et al., 2002).

1.7.2.2 Neurogenesis in the ENS and SNS

Neurogenesis in the ENS and SNS, is understood in even less detail than that of the CNS and other parts of the ANS. Little is known about the patterns of proliferation of the cells that give rise to neurons and glia, and hardly anything is known about their cell cycle kinetics. In addition to this, there are key differences in the neurogenesis of the SNS and ENS, compared to the CNS and the other parts of the PNS. The most striking of these is the fact that withdrawal from the cell cycle does not appear to be strictly linked to differentiation (Rohrer and Thoenen, 1987, Hendershot et al., 2007, Sasselli et al., 2012). In the ENS, some of the proliferating neural precursors express pan-neuronal markers, and in the SNS almost all proliferating cells express genes necessary for the production of
noradrenaline (Groves et al., 1995, Coppola et al., 2010, Rohrer, 2011, Sasselli et al., 2012).

The two major differences between proliferation in the ENS versus proliferation in the SNS is the fact that, in the ENS, proliferating cells are also migrating caudally to colonise the gut (Sasselli et al., 2012). In the SNS, NC precursors reach the ganglion primordia and then adopt a noradrenergic phenotype and are positive for the expression of TH and DβH. Also in the SNS, the bulk of proliferation takes place by cells that have stopped migrating and acquired their final neurotransmitter phenotype (Groves et al., 1995, Glebova and Ginty, 2005, Rohrer, 2011).

1.7.3 SNS neurogenesis in the mouse and chick

In both the mouse and the chick, proliferation of cells that give rise to the SNS occurs in two phases. The first phase is proliferation of progenitors that do not have a phenotypic expression profile that identifies them as committed to either the neuronal or glial lineage. In both mouse and chick, at least 2 populations of early progenitors have been identified; 1) cells that are Sox10+/Phox2b-; and 2) Sox10+/Phox2b+ cells, which are Sox10+ cells that have initiated differentiation and up-regulated the expression of Phox2b (Callahan et al., 2008, Tsarovina et al., 2008). The second phase is proliferation by cells that have differentiated into neuronal precursors or glial progenitors. Earlier, we defined neuronal precursors, in either the mouse or the chick, as cells that lack the expression of Sox10 and which are positive for the expression of pan-neuronal/noradrenergic markers (pan-neuronal; SCG10, Tuj1, neurofilament, PGP9.5 and Hu, and noradrenergic; TH and DβH). I also earlier classified glial progenitors as cells that maintain the expression of Sox10 but co-express the glial marker BFABP. It is important to note that there is no data on the phenotypic identification of the glial lineage in the chick, however, the glial progenitors are believed to be represented by the small population of Sox10+ cells observed at E7 (Tsarovina et al., 2008).

There is virtually no information about proliferation in either of the Sox10+/Phox2b- or Sox10+/Phox2b+ cells, but there is a difference in the
relative proportions of these cells at equivalent development stages in both the mouse and the chick (Fig. 1.4). In the chick sympathetic ganglion, the early phase of proliferation by the uncommitted progenitors is drawn out for an extra 48h (from E3-E7) compared to the mouse ((E9.5-E11.5) - Tsarovina et al., 2008). In the chick, during the early phase of proliferation from E3-E5, there is no change in the proportion of multipotent Sox10+/Phox2b- NCC progenitors (Fig. 1.4 - Tsarovina et al., 2008). In the mouse (Fig. 1.4), this progenitor pool is depleted rapidly and almost all of the Sox10+/Phox2b- cells have differentiated into the Sox10+/Phox2b+ population by E10.5 (figure 1.4 - Callahan et al., 2008). Thus, common to the development of both mouse and chick SNS, is the existence of NCC progenitors that are Sox10+/Phox2b- that will soon adopt a Sox10+/Phox2b+ phenotype. However, up-regulation of Phox2b appears to occur more rapidly in the mouse compared to the chick ((Fig. 1.4) - Callahan et al., 2008, Tsarovina et al., 2008).

In the chick, perturbing the Notch/Delta signalling pathway changes the relative proportion of Sox10+ precursors relative to the proportion of cells that lack Sox10 expression and have up-regulated the expression of neural markers such as SCG10 (Tsarovina et al., 2008). In vivo inhibition of Notch more rapidly depletes the pool of Sox10+ precursors by increasing the number of SCG10+ neural precursors (Tsarovina et al., 2008). We may speculate that, in the chick, the window for Notch/Delta signalling is drawn out over a longer time frame when compared to the mouse and that this is the reason why the differentiation in the mouse occurs more quickly. However, the extent to which Notch-Delta signaling affects development in the mouse sympathetic ganglion is yet to be determined.

Labelling studies in the chick, using the thymidine analogue H3thymidine, have visualised proliferation of the catecholamine-positive precursor cells (expressing Phox2b+/TH+ and pan-neuronal markers). Results from these cumulative labelling studies have shown that these cells have the capacity to divide at least three times over a three day period, irrespective of the developmental stage examined i.e. E3-E6, E6-E9 or E9-E12 (Rothman et al.,
This suggests that at least some of the proliferating catecholaminergic precursors must have cell cycle lengths between 18-24h (Rothman et al., 1978). In a separate set of cumulative labelling studies, it was also shown that some cells in the chick sympathetic ganglion exit the cell cycle between E2 and E3 (Rothman et al., 1978) at a time when there are very few Sox10-/TH+ neuroblasts (Tsarovina et al., 2008). It is possible that these cells represent the earliest neuroblasts to exit from the cell cycle. Alternatively, they may be leaving the proliferating pool as undifferentiated progenitors, analogous to the situation in the chick ciliary ganglion, where similar cells exit early and differentiate into neuroblasts without further division later in development (Lee et al., 2002). Other than these findings, there is no information at present about the cell cycle kinetics of the SNS precursors and a detailed description of proliferation does not exist for the mouse.

1.8 Molecular control of neurogenesis in the SNS

There are a number of extracellular mitogens and growth factors, as well as their cell surface receptors that have been shown to affect proliferation. Removal of any of these signalling components can result in a reduction of proliferation. In addition to this, LOF experiments resulting in the removal of any of the key differentiation transcription factors almost always lead to an apparent decrease in proliferation.

There are at least four ways in which proliferation can be affected by changes in either extracellular mitogen and growth factor signalling, or transcription factor expression:

1) Perturbing cell cycle length: alterations in cell cycle length will lead to either an increase or decrease in the number of cells generated;

2) Altering cell cycle withdrawal: this may lead to alterations in the fraction of dividing cells. If cells on average withdraw from the proliferating pool too early, this will result a reduction in the number of cells ultimately generated, however, if cells remain in the proliferating pool for longer and
do not withdraw, there will be an increase in the overall number of cells observed.

3) Disturbing cell cycle progression can lead to apoptosis: GOF or LOF experiments may affect transcription events necessary to pass cell cycle checkpoints, resulting in cell death by apoptosis; and

4) Disturbing a cell survival mechanism: Changes in survival factor availability, access or signalling may also result in apoptosis.

The next section will be a discussion of a number of experiments, in which comparisons are made between normal and perturbed SNS neurogenesis. It is important to note that the effects on proliferation described are often indirect and the assessment of proliferation relatively crude. The effects on proliferation are sometimes extrapolated from observed changes in either the total end number of cells, the relative changes in the per section area of the ganglion, changes in the proportion of cells incorporating thymidine analogues, or the fraction of cycling cells identified by antibodies to proliferation-specific antigens. Unlike the CNS, there does not exist a holistic description of proliferation in the SNS that accounts for the total number of cells generated, how fast they need to divide to generate this total, the number of divisions needed, or how many cells are dividing at any given time. This inherently limits our overall understanding of how proliferation is affected when the system is perturbed and must be kept in mind when trying to interpret experimental outcomes.

1.8.1 Mitogens and their signalling receptors

1.8.1.1 Insulin-like Growth Factor (IGF)

IGF-1 and IGF-2 were identified as having important roles for both proliferation and neurite outgrowth in the developing avian SNS (Zackenfels et al., 1995). The cellular sources of IGFs were identified in different cell populations of the developing chick SNS at E7 (Zackenfels et al., 1995). Neuronal cells were identified by the expression of Q211, glial cells were identified by the expression of O4 and a small number of remaining cells that were positive for neither neuronal or glial markers, but were positive for the expression of
fibronectin, were deemed to be fibroblasts (Rohrer, 1985, Zackenfels et al., 1995). All cells expressed IGF-2, but it was most strongly expressed in the neuronal population and only weakly expressed in the glial population (Zackenfels et al., 1995). IGF-1 was only expressed by fibroblasts and was not expressed by neuronal or glial cells (Zackenfels et al., 1995). In vivo application of both IGF-1 and IGF-2 was shown to significantly increase the proportion of dividing cells, measured by their efficacy in incorporating H³thymidine (Zackenfels et al., 1995). It was also shown that IGF-2 was strongly expressed in the liver at E7, implying that in addition to acting as an autocrine and paracrine signal, IGF-2 can reach the cells of the developing SNS as a circulating hormone (Zackenfels et al., 1995). However, as IGF-1 was only expressed by the fibroblasts, it can only have a paracrine role (Zackenfels et al., 1995). Both IGF-1 and IGF-2 signal through the IGF-1 receptor and have also been shown to increase proliferation in other parts of the nervous system (Sara and Hall, 1990, Aberg et al., 2000). In the adult rat hippocampus, IGF-1 increases proliferation in the dentate subgranular proliferative zone (Aberg et al., 2000). However, the in vivo functions of IGFs are yet to be examined in the developing mammalian SNS.

### 1.8.1.2 ALK and Midkine

The anaplastic lymphoma receptor tyrosine kinase (ALK), together with its ligands midkine (Mk) and pleiotrophin (Ptn), are all expressed in developing chick sympathetic ganglia (Reiff et al., 2011). An essential role for ALK signalling has been shown in vivo and in vitro for developing sympathetic precursors that already express markers for noradrenergic neuronal differentiation (Reiff et al., 2011). In cultures of immature neurons from E7 chick SG, ALK over-expression and forced expression of constitutively active ALK receptors (ALKF1174 and ALKr1275), significantly increased proliferation (Reiff et al., 2011). In addition to this, inhibition of ALK signalling using ALK-specific shRNA-mediated knockdown (shALK), or the selective inhibitor NPV-TAE684, resulted in a significant decrease in proliferation, measured by a reduction in the efficacy of cells to incorporate the thymidine analogue, EdU (Reiff et al., 2011). These reductions in proliferation were not nearly as severe when the inhibitor was applied to cells in which over-
expression or forced expression of constitutively active ALK was already introduced (Reiff et al., 2011).

In vivo experiments also showed that knockdown of endogenous Mk resulted in a massive reduction in ganglion size, measured by a significant decrease in the area of either TH or Phox2b staining (Reiff et al., 2011). The reduction in the area of either Phox2b or TH staining was not due to cell death and no change in TUNEL staining (a marker of apoptotic cell death) was observed between controls and embryos with reduced Mk (Reiff et al., 2011). However, a significant decrease in the proportion of cells that incorporated EdU was observed when Mk levels were reduced, reflecting a lack of proliferation that is likely to account for the reduction in the ganglion size (Reiff et al., 2011). In addition to this, ectopic expression of Mk resulted in a large increase in proliferation in the chick SG (Reiff et al., 2011). An increase in the proportion of EdU positive cells was observed from E4 to E7 in embryos exposed to ectopic Mk, however after E7, the chick embryos were growth retarded and the higher levels of proliferation did not continue. The reduced embryonic growth after E7 is likely due to the anti-angiogenic effects on the chorioallantoic blood vessel development when Mk is over-expressed (Reiff et al., 2011).

1.8.1.3 The GDNF-family ligand (GFL) receptors, Ret and GFR α1, 2 & 3

The GFLs include glial cell line-derived neurotropic factor (GDNF), neurturin (NRTN), artemin (ARTN) and persephin (PSPN). GFLs selectively bind GDNF-family receptor alpha (GFRα) co-receptors that dimerise to form a receptor-signalling complex with the tyrosine kinase receptor Ret ((Rearranged in transfection) - Baloh et al., 2000, Airaksinen and Saarma, 2002). Loss of function experiments have identified Ret, GDNF, GFRα1, GFRα3 and ARTN as all playing a role in SNS neurogenesis and proliferation (Moore et al., 1996, Pichel et al., 1996, Cacalano et al., 1998, Enomoto et al., 1998, Nishino et al., 1999, Rossi et al., 1999, Andres et al., 2001, Enomoto et al., 2001, Airaksinen and Saarma, 2002, Honma et al., 2002, Ledda et al., 2002, Callahan et al., 2008).

ARTN is a molecule that is transiently expressed by blood vessels in the developing embryo (Balogh et al., 1998, Honma et al., 2002). It has been shown to
act as an axon guidance molecule and signals via a receptor complex that is formed by the dimerisation of Ret and its co-receptor GFRα3 (Baloh et al., 1998, Andres et al., 2001, Airaksinen and Saarma, 2002, Honma et al., 2002). In mice lacking ARTN, GFRα3 or Ret (ARTN+/−, GFRα3+/−, or Ret+/− respectively), the proximal axon projections of sympathetic neural precursors are disrupted and vary greatly from control embryos (Nishino et al., 1999, Enomoto et al., 2001, Honma et al., 2002). In addition to the general axon projection defect, the development of the SCG is severely affected, with SCGs being displaced caudally and severely hypoplastic (Nishino et al., 1999, Enomoto et al., 2001, Honma et al., 2002). There is also reduction in the size of thoracic chain ganglia; however this is not as severe as the reduction in size noted for the SCG (Nishino et al., 1999, Enomoto et al., 2001, Honma et al., 2002). In ARTN+/−, GFRα3+/− and Ret+/− knockouts, there appears to be no significant difference in the proportion of cells that incorporate BrdU when compared to controls (Nishino et al., 1999, Enomoto et al., 2001, Honma et al., 2002). It has been proposed that the reductions in the size of ganglia in the ARTN+/−, GFRα3+/− and Ret+/− mice are due to cell death as a consequence of the incorrect axon projection phenotype. This is reflected by an increase in caspase 3 or TUNEL staining from E13.5 onward (Nishino et al., 1999, Enomoto et al., 2001). For the ARTN+/− mice, cell death was examined later in development and does not appear to affect the SCG after E16.5 (Honma et al., 2002). So, the reduction in cell number is in part due to cell death, however, the precise reason for the reduction in cell number remains unknown.

GDNF can bind to either the GFRα1 or GFRα2 receptor to activate downstream signal transduction via the Ret receptor (Airaksinen and Saarma, 2002). In both GDNF and GFRα1 null mutant mice (GDNF−/− and GFRα1−/− respectively), there is a subtle decrease in the size of all sympathetic ganglia below the level of the SCG (Moore et al., 1996, Pichel et al., 1996, Enomoto et al., 1998, Airaksinen et al., 1999, Airaksinen and Saarma, 2002). The SCG is only affected in GDNF−/− (not GFRα1−/−) mice, where there is a 35% decrease in the number of cells (Moore et al., 1996, Pichel et al., 1996, Enomoto et al., 1998, Airaksinen et al., 1999, Airaksinen and Saarma, 2002). This raises two interesting questions: 1) why is there no affect on SCG development when GFRα1 is removed (Nishino et al.,
1999); and 2) why, in the *GDNF* knockout mice, is the SCG the only ganglion severely affected? The answer to the first question may be explained by the fact that GDNF can also bind GFRα2, which is heavily expressed by cells in the SCG at E12.5 so, GFRα2 may be able to functionally compensate when GFRα1 is missing, (Airaksinen et al., 1999, Nishino et al., 1999, Rossi et al., 1999, Baloh et al., 2000, Airaksinen and Saarma, 2002). To answer the second question, GFRα1 is present on neuroblasts only in the SCG, not other sympathetic ganglia (Nishino et al., 1999, Callahan et al., 2008), so it is possible that GDNF has a role in the SCG, but not other sympathetic ganglia, which makes the SCG uniquely vulnerable to the absence of GDNF.

In SNS development, loss of function experiments have identified different roles for ARTN/GFRα3, and GDNF/GFRα1 signalling even though both of these receptor ligand complexes activate intracellular signalling via the Ret receptor. This highlights the fact that the specific GFL/GFRα combinations that form signalling complexes with Ret must trigger a least some different intracellular pathways that are not currently well understood *in vivo*. In addition to this, only SCG are severely displaced in the ARTN, Ret or GFR knockout mice, also, in the GDNF knockout mice, only the SCGs experience large losses in the number of cells. There may exist some slight rostro-caudal differences in the exposure, dependency and expression profiles of GFLs and GFRα receptor types in the developing SNS. The rostro-caudal differences may be due, in part, to the fact that some cells that populate the SCG come from the vagal NC, while all other ganglia are populated by only trunk NC (LeDouarin and Teillet, 1973, LeDouarin, 1986, Durbec et al., 1996). It has been shown that the loss of GFL-signalling components, such as Ret and GDNF, results in severe migration and survival defects for vagal NCC derivatives (Moore et al., 1996, Pichel et al., 1996, Enomoto et al., 2001). In Ret−/− mice, vagal NC cells fail to fully colonise the gut resulting in aganglionosis distal to the stomach, and, in GDNF−/− mice, there is virtually complete aganglionosis of the entire gut (Moore et al., 1996, Pichel et al., 1996, Enomoto et al., 2001). The fact that the SCG is the only ganglion to be populated by cells from the vagal NC, may reflect a differential dependency on Ret and GDNF, compared to ganglia populated purely by trunk NC cells, however a
mechanism for these differential effects is yet to be demonstrated (LeDouarin and Teillet, 1973, LeDouarin, 1986, Durbec et al., 1996).

1.8.1.4 Frizzled3 and Wnt3a

Frizzled receptor-mediated Wnt-signalling is involved in many developmental processes including neural cell fate specification, cell proliferation, neuronal survival and synaptogenesis (Wang et al., 2002, Ciani and Salinas, 2005, Armstrong et al., 2011). Frizzled3 (Fz3) is expressed in the developing SCG during the period of neurogenesis (Armstrong et al., 2011). Compared to WT controls, SCGs of Fz3 knockout (Fz3/−) mice had a 29%, 39% and 69% decrease in the number of sympathetic precursor cells on E13.5, E14.5 and E16.5 respectively (Armstrong et al., 2011). In the Fz3/− mice, it was shown that fewer cells had incorporated the S-phase marker, EdU, when compared to controls (Armstrong et al., 2011). In addition, over the period from E12.5 to E13.5, a larger proportion of cells withdrew from the proliferating pool of precursors in the Fz3/− mice compared to WT controls, in conjunction with the lower proportion of EdU labelling. This may account for the reduction in cell numbers observed in the Fz3/− mice (Armstrong et al., 2011).

β-catenin, a down-stream target of canonical frizzled-mediated Wnt signalling, was examined to see if the effects seen in the Fz3/− mice were due to canonical Wnt-signaling. β-catenin floxed animals (β-cateninfloxflox) were crossed to mice that express Cre-recombinase under the TH promoter (TH-Cre) to delete β-catenin from tissues that express TH (TH-Cre:β-cateninfloxflox). TH-Cre:β-cateninfloxflox mice showed proliferative defects that mimicked the proliferative defects observed in the Fz3/− mice (Armstrong et al., 2011). The proliferative defects observed in the Fz3/− mice may well be due to the lack of canonical Wnt signalling via the actions of β-catenin (Armstrong et al., 2011).

In addition to the proliferation defects mentioned above, sympathetic axons failed to properly innervate some target tissues in the Fz3/− mice (Armstrong et al., 2011). However, these projections defects were not observed in the TH-Cre:β-cateninfloxflox mice, suggesting that Fz3 is involved in a non-canonical signalling pathway that is important for final target innervation (Armstrong et al., 2011).
Axon defects have also been observed in the CNS of Fz3\(^{-/-}\) mice (Wang et al., 2002). In the CNS, neural differentiation and target innervation occurs in cells that are no longer proliferating. It would appear that, in the CNS, Fz3 has a role only in axon guidance and does not appear to affect proliferation (Wang et al., 2002). The CNS projection defects, observed in the Fz3\(^{-/-}\) mice, are almost identical to the defects observed in Celsr3 conditional knockouts (Wang et al., 2002, Zhou et al., 2008, Berger-Muller and Suzuki, 2011). Celsr3 (flamingo in Drosophila) is a seven-pass trans-membrane receptor that overlaps in expression with Fz3 in the CNS and is also expressed in the murine SNS (Shima et al., 2002, Wang et al., 2002, Zhou et al., 2008). It has been shown that seven-pass trans-membrane receptors can interact as adhesion molecules and play a role in planar cell polarity (Berger-Muller and Suzuki, 2011). An interaction between Fz3 and Cscl3 may be important to axon guidance and final target innervation in the SNS. In summary, Fz3 would appear to be involved in proliferation, via canonical Wnt signaling through β-catenin, and final target innervation, by a signalling pathway that is yet to be determined.

### 1.8.2 BMP signalling in proliferation

BMPs have been long established as the key signals that initiate the transcriptional network that leads to a noradrenergic phenotype (Rohrer, 2011). Removal of the type 1 BMP receptor, Alk3 (not the anaplastic lymphoma kinase receptor mentioned above), results in the loss of the SNS due to death of NC precursors immediately after migration (Morikawa et al., 2009b). However, using Wnt1-conditional removal of Smad4 (Wnt1-Cre:Smad4\(^{flox/flox}\)), the canonical down-stream signalling component for the Alk3 receptor, did not result in cell death (Morikawa et al., 2009b). Instead, in Wnt1-Cre:Smad4\(^{flox/flox}\) mice there was a decrease in proliferation, noted by the reduction in the proportion of cells that incorporated BrdU (Morikawa et al., 2009b). In Wnt1-Cre:Smad4\(^{flox/flox}\) mice, the noradrenergic differentiation program was largely unaffected except for minor reductions in the expression of some transcription factors like Ascl1 (Morikawa et al., 2009b). This suggests that canonical BMP signalling via the Alk3 receptor is primarily necessary for proliferation, and that some unidentified, Alk3-mediated,
intracellular signalling mechanism is needed to avoid cell death (Morikawa et al., 2009b).

1.8.3 Members of the noradrenergic transcriptional network affecting proliferation

1.8.3.1 Ascl1 signalling in proliferation

Ascl1 is the earliest of all the transcription factors expressed by the NC precursors that will give rise to the SNS (Lo et al., 1991, Guillemot et al., 1993). The removal of Ascl1 (Ascl1	extsuperscript{−/−}), results in a decrease in the incorporation of BrdU, similar to what is observed in the Wnt1-Cre:Smad4	extsuperscript{flox/flox} mice (Parras et al., 2002, Pattyn et al., 2006, Morikawa et al., 2009b). In fact, the reduced levels of Ascl1 expression in Wnt1-Cre:Smad4	extsuperscript{flox/flox} mice may account for the proliferative defect observed in those animals (Morikawa et al., 2009b). In Ascl1	extsuperscript{−/−} mice, the reduction in proliferation occurs over the period from E11.5 to E15.5, with the end result being a significantly hypoplastic mature ganglion (Morikawa et al., 2009b). Differentiation into a neuronal noradrenergic phenotype is also affected, in Ascl1	extsuperscript{−/−} mice, with the expression of neural and noradrenergic markers reduced and delayed by up to 2 days. Cell death does not appear to play a role in the reduced number of cells (Parras et al., 2002, Pattyn et al., 2006, Morikawa et al., 2009b).

1.8.3.2 Phox2b and Phox2a in proliferation

Phox2b is expressed very soon after Ascl1 and Phox2b null mutants fail to generate a SNS, with cells in the ganglion dying by ~E11.5 (Pattyn et al., 1999). Islet1/2 expression appears after Phox2b, at about the same time as Phox2a expression appears in the developing SNS (Coppola et al., 2010). Islet1/2 expression then overlaps with both Phox2a and Phox2b expression in subsequent stages of development in sympathetic ganglia (Coppola et al., 2010). Conditional removal of Phox2b under the Islet1/2 promoter (Islet1/2-Cre:Phox2b	extsuperscript{flox/flox}) means the initial expression of Phox2b prior to ~E10 is unaffected, but after ~E10 Phox2b expression is removed (Coppola et al., 2010). Interestingly, in Islet1/2-Cre:Phox2b	extsuperscript{flox/flox} CKO mice, NC precursor cells survived
and went on to form sympathetic ganglia, so, Phox2b is needed in the early stages of ganglion formation, prior to E10, to prevent cells undergoing cell death (Coppola et al., 2010). At E16.5 in the Islet1/2-Cre:Phox2b\textsuperscript{flox/flox} mice, a 60% reduction in the size of the ganglia was observed compared to that of controls (Coppola et al., 2010). The reduction in ganglion size was not due to cell death, but a proliferative defect marked by a decrease in BrdU incorporation (Coppola et al., 2010). Also observed in the Islet1/2-Cre:Phox2b\textsuperscript{flox/flox} mice, was a decrease in the expression of noradrenergic markers at E16.5 (Coppola et al., 2010).

Collectively, these results suggest that Phox2b is required for the maintenance of a noradrenergic phenotype and for proliferation after the initial stages of development (Coppola et al., 2010).

The ability for Phox2a to compensate for both the proliferative and differentiation losses due to the conditional removal of Phox2b, under the Islet1 promoter has been examined (Coppola et al., 2010). Phox2a and Phox2b double conditional knockouts were generated, again using the Islet1/2 promoter (Islet1/2-Cre:Phox2a/b\textsuperscript{flox/flox}) - Coppola et al., 2010). It was shown that the proliferative defects in the Islet1/2-Cre:Phox2a/b\textsuperscript{flox/flox} mice were more substantial compared to the Islet1/2-Cre:Phox2b\textsuperscript{flox/flox} alone (Coppola et al., 2010). The ganglia of Phox2a/b\textsuperscript{Islet-CKO} mice were 77% smaller than that of the control ganglia and 17% smaller that the ganglia observed in Islet1/2-Cre:Phox2b\textsuperscript{flox/flox} mice (Coppola et al., 2010). Also, for the Islet1/2-Cre:Phox2a/b\textsuperscript{flox/flox} mice, fewer differentiated into a noradrenergic phenotype compared to the Islet1/2-Cre:Phox2b\textsuperscript{flox/flox} mice (Coppola et al., 2010). Phox2a appears to have the capacity to drive both proliferation and noradrenergic differentiation in the absence of Phox2b (Coppola et al., 2010).

The experiments with conditional removal of Phox2a/b show that transcription factors can play different roles at different stages of neurogenesis. It is clear that the lack of Phox2b, prior to E10.5, results in cell death that cannot be compensated for by Phox2a (Coppola et al., 2010). However, after E11.5 Phox2b has a key role in driving proliferation and maintaining noradrenergic differentiation that can be partly, but not completely be compensated for by the actions of Phox2a (Coppola et al., 2010).
1.8.3.3 **Insm1 signalling in proliferation**

Insm1 is expressed soon after both Ascl1 and Phox2b in the transcriptional network that leads to a noradrenergic neuronal phenotype (Wildner et al., 2008). In the *Insm1* null mutant mice (*Insm1*^lacZ/lacZ^), the expression of all subsequent members of the differentiation network is delayed and there is a reduction in the proportion of cells that incorporate BrdU over the period from E11.5 to E14.5 (Wildner et al., 2008). From E14.5 onward, proliferation is unaffected. The removal of *Insm1* almost identically phenocopies the proliferation defects seen in the *Ascl1^-/-* mice, however, in the *Insm1*^lacZ/lacZ^ mice, the intensity of Ascl1 staining is increased and persists for longer compared to controls (Wildner et al., 2008). The increase in Ascl1 expression, observed in *Insm1*^lacZ/lacZ^ mice, suggests that either directly or indirectly, Insm1 negatively regulates the level of Ascl1 expression (Wildner et al., 2008). If we consider the proliferation defects observed in the *Insm1*^lacZ/lacZ^ mice in light of the observations from *Ascl1^-/-* mice, we may speculate that a specific level of Ascl1 expression may be necessary for proliferation: If Ascl1 expression is above or below an ideal level, proliferation is reduced. Alternatively, Insm1 may directly affect proliferation in a way that cannot be compensated for by the higher levels of Ascl1, or Insm1 may be responsible for up regulating some unidentified factor that is required for Ascl1 to have an effect on proliferation.

1.8.3.4 **Hand2 and Gata 2/3 in proliferation**

Hand2 and Gata2/3 are expressed after, Mash1 Phox2b and Insm1 in the transcriptional network (Lim et al., 2000, Howard, 2005, Lucas et al., 2006, Moriguchi et al., 2006, Morikawa et al., 2007, Hendershot et al., 2008, Wildner et al., 2008). Insm1 has pleiotropic effects on proliferation and differentiation, possibly by influencing the timing of expression Hand2 and Gata3 in the murine sympathoadrenal lineage (Wildner et al., 2008, Schmidt et al., 2009). However, more severe effects are observed in LOF experiments in which either *Hand2* or *Gata3* have been deleted (*Hand2^-/-* *Gata3^-/-* respectively). Removal of either *Hand2* or *Gata3*, results in embryonic lethality due to the lack of catecholamine biosynthesis (Lim et al., 2000, Hendershot et al., 2008). In both knockout mice, lethality can be overcome by feeding pregnant dams with catecholamine.
intermediates, this allows the later effects of either Hand2 or Gata3 to be observed (Lim et al., 2000, Hendershot et al., 2008). Conditional removal of Hand2 under the Wnt1 promoter (Wnt1-Cre:Hand2^flox/flox), resulted in severely hypoplastic ganglia compared to WT littermates (Hendershot et al., 2008). This is also accompanied by a severe reduction in the expression of TH and DβH (Hendershot et al., 2008). In Wnt1-Cre:Hand2^flox/flox mice, the loss in cell number is due to the a reduction in fraction of cycling cells as measured by the expression of Ki67 (Hendershot et al., 2008). Ki67 is a nuclear, non-histone protein that is expressed in all phases of the cell cycle except G0 and therefore can be used to identify cycling cells (Eisch and Mandyam, 2007, Yerushalmi et al., 2010). In WT control mice at E10, 87% of the total cells are positive for Ki67 expression, however, in Wnt1-Cre:Hand2^floxflox mice only 8% of the total are Ki67 positive. The decrease in the percentage of dividing cells, seen at E10, results in severely hypoplastic ganglia in the Wnt1-Cre:Hand2^floxflox mice (Hendershot et al., 2008).

Earlier I mentioned that Gata3^-/- mice, which have been rescued from embryonic lethality by feeding pregnant dams catecholamine intermediates, fail to acquire a noradrenergic phenotype (Tsarovina et al., 2004). If Gata3 is conditionally removed using the DβH promoter (DβH-Cre:Gata^floxflox), sympathetic progenitors do express both TH and DβH and appear to differentiate normally, however, there is severe cell death that is similar to what is observed in the Gata3 null mutants (Tsarovina et al., 2010). Taken together, Gata3 appears to be necessary, for initial sympathoadrenal progenitor differentiation and then subsequently for avoiding cell death (Lim et al., 2000, Tsarovina et al., 2004, Tsarovina et al., 2010). In the DβH-Cre:Gata^floxflox mice, Tsarovina et al. (2010) showed a diminished expression of anti-apopotic genes (Bcl-2, Bcl-xL, and NFkB) accompanied by an increase in the expression of the pro-apopotic genes (Bik, Bok, and Bmf). The mechanisms connecting Gata3 to the regulation of these factors is unknown, however, it is known that the expression of Gata3 can also influence the expression Mash1, Phox2b and Hand2 (Moriguchi et al., 2006), which are all key regulators of proliferation and necessary to avoid cell death. So, the lack of Gata3 may directly and indirectly result in a lack of expression of factors needed to avoid cell death.
1.8.4 Other transcription factors and proliferation

In addition to extrinsic mitogens and growth factors and the members of the transcriptional network responsible for noradrenergic neuronal differentiation, it has been shown that proliferation of SNS precursors in vivo responds to a number of transcription factors that have not been identified as members of the transcription network that generates the noradrenergic phenotype in sympathetic neuroblasts.

1.8.4.1 AP-2 transcription factors

Members of the AP-2 (Activating enhancer binding Protein 2) family of transcriptions factors, AP-2α and AP-2β have been shown to effect SNS neurogenesis in vivo (Potzner et al., 2010, Schmidt et al., 2011). The AP-2 proteins form homo- or heterodimers with other AP-2 family members that bind specific DNA sequences and are thought to be involved in many processes including differentiation and cell cycle progression (Williams and Tjian, 1991, Hong et al., 2008).

At E9.5, AP-2α and AP-2β are both co-expressed in virtually all migrating NC and SNS precursors, the expression of AP-2α and AP-2β is lost by E13.5 and E16.5 respectively (Schmidt et al., 2011). In double-knockout mice, for both AP-2α and AP-2β (AP-2αΔKO), only a few cells remain in the sympathetic ganglia (Schmidt et al., 2011). This is due to widespread apoptosis observed in NC cells at E9.5, indicating that the combined functions of AP-2α and AP-2β are critical to SNS development (Schmidt et al., 2011). The fact that AP-2 proteins can form homo- or heterodimers means that functional redundancy is likely to exist for both molecules (Williams and Tjian, 1991, Hong et al., 2008, Schmidt et al., 2011).

In AP-2β null mutants (AP-2β−/−) the average per section area of developing sympathetic ganglia is 40% smaller compared to control ganglia at E10.5. This relative reduction in size persists and remained 40% smaller at E16.5 (Schmidt et al., 2011). In the AP-2α null mutants (AP-2α−/−), there is no effect on SNS neurogenesis (Schmidt et al., 2011). These results suggest that AP-2β can completely compensate for the loss of AP-2α and that AP-2α can compensate for 60% of the function of AP-2β (Schmidt et al., 2011). In addition to the expression
of AP-2α and Ap-2β, NC stem cells also express AP-2γ, the functions of which are yet to be determined for the SNS (Hong et al., 2008). AP-2γ may be able to compensate for some functional deficits in the single knockouts and may also explain the few cells that survive in the AP-2DKO mice.

Tissue specific deletion of AP-2α, in AP-2β−/− mice, was achieved by crossing loxP flanked AP-2α mice, to mice that express cre-recombinase under either the Ascl1 or DβH promoters (Ascl1-Cre:AP-2αfloxflox|AP-2β+/− and DβH-Cre:AP-2αfloxflox|AP-2β−/− respectively). Ascl1 is expressed by ~E9.5, so, in the Ascl1-Cre:AP-2αfloxflox|AP-2β−/− mice, it is possible to examine the effects of the double knockout after NC cells have migrated to the ganglion primordia. DβH expression occurs as NC precursors adopt a noradrenergic phenotype at ~E10.5, so, in the DβH-Cre:AP-2αfloxflox|AP-2β−/− it is possible to examine the effects the double knockout phenotype after initial noradrenergic differentiation. The decrease in the number of cells, due to the conditional double knockout phenotypes, was more severe in the Ascl1-Cre:AP-2αfloxflox|AP-2β−/− mice, compared to the DβH-Cre:AP-2αfloxflox|AP-2β−/− mice (Schmidt et al., 2011). This suggests that the earlier AP-2α is removed, the more severe the phenotype. This would be expected, given that AP-2α expression diminishes rapidly such that RNA transcripts cannot be detected after E16.5 (Schmidt et al., 2011).

1.8.4.2 SoxC proteins Sox4 and Sox11

The SoxC proteins, Sox4, Sox11 and Sox12, are broadly and dynamically expressed during embryogenesis (Schepers et al., 2002). The generation of null mutants for each of the SoxC (Sox4−/−, Sox11−/− and Sox12−/−) genes has shown that Sox4 and Sox11 are essential for development (Schilham et al., 1996, Sock et al., 2004, Wilson et al., 2005, Hoser et al., 2007, Nissen-Meyer et al., 2007, Wurm et al., 2008, Potzner et al., 2010). In mice, Sox4 is expressed in ~15% of all SNS precursors at E11.5, this percentage significantly increases to reach the peak of expression at E14.5 and then reduces, but a considerable number of cells are still positive for Sox4 expression at E18.5 (Potzner et al., 2010). In contrast, Sox11 is expressed in 46% of SNS precursors at E11.5, this fraction is unchanged at E12.5,
it then declines so that only a small proportion of cells are positive for Sox11 at E18.5 (Potzner et al., 2010).

In the Sox11 null mutant mice (Sox11−/−), there is a significant reduction in the proportion of cells that incorporate BrdU and the expression of Sox4 is reduced (Potzner et al., 2010). However, tissue specific deletion of Sox11 using the LoxP/Cre system, where Cre-recombinase under the Wnt1 promoter (Wnt1-Cre:Sox11<sub>flox/flox</sub>), did not significantly alter proliferation or the expression of Sox4 in the developing SNS (Potzner et al., 2010). Sox11 positive non-NC cells are found in the tissue surrounding the primitive ganglion and in the dorsal aorta itself (Potzner et al., 2010). The signals from the tissue close to the developing sympathetic ganglia, particularly the dorsal aorta, are critical in the development of the SNS (Groves et al., 1995, Shah et al., 1996, Mehler et al., 1997, Potzner et al., 2010). The cells producing these signals will be altered in the Sox11−/− mice, but unaffected in the Wnt1-Cre:Sox11<sub>flox/flox</sub> mice and this may account for the different effects when Sox11 is conditionally removed compared to the null mutants (Potzner et al., 2010). Tissue specific deletion of Sox4, under the DβH promoter (DβH-Cre:Sox4<sub>flox/flox</sub>), did not significantly alter the proportion of SNS precursor cells that incorporated BrdU at E11.5, E14.5 and E16.5 (Potzner et al., 2010). However, there was an increase in cell death at both E14.5 and E16.5 and this may account for the reduction in the size of the ganglion.

### 1.9 Cell Death

Cell death is a critical feature of normal neural development and is the proposed mechanism by which neuron number is matched to the total amount of target tissue (Oppenheim, 1991). That is, neurons are overproduced, target tissues release survival factors and neurons that fail to compete successfully for these target-derived survival factors die by programmed cell death (Oppenheim, 1991, Glebova and Ginty, 2005, Deppmann et al., 2008). It is important to make a distinction between cell death due to failure to obtain a survival factor, like nerve growth factor (NGF), and cell death as a consequence of failing a cell cycle check.
point or being unable to advance through the cell cycle (Israels and Israels, 2000, Coffman, 2004). Hence, in the many examples where perturbing the normal developmental program results in cell death, apoptosis may well be due to disturbances in cell cycle progression, rather than the absence of access to an NGF-like survival factor (Tsarovina et al., 2010, Schmidt et al., 2011). This is most likely early in development when neuroblasts have no axons and SNS precursors do not depend on target-derived trophic support from molecules such as NGF (Glebova and Ginty, 2005). Under these circumstances, the title of survival factor that is bestowed upon many transcription factors and other proteins that results in cell death upon their removal through mutagenesis, is likely a misnomer.

1.10 Summary

Many transcription factors, receptors and extracellular signals are involved in the control of proliferation that drives growth in the SNS. Through various GOF and LOF experiments, much has been learned about factors that are necessary for SNS neurogenesis to be maintained at normal levels. Compared to other areas of the nervous system, the CNS for example, we have only very crude ways in which to measure proliferation and therefore only limited conclusions can be reached (discussed further in chapter 3, section 3.4.4). In addition to this, there is no study that has tried to evaluate proliferation over the course of sympathetic ganglion development in the mouse. There is not a single description that addresses proliferation in the precursors that have committed to a glial lineage and we do not know how the relative levels of proliferation for neuronal and glial lineages vary as development progresses. The chapters that follow attempt to address this. In the next chapter, I look at the development of the mouse sympathetic ganglion and attempt to monitor proliferation using a classical S-phase marker approach. I conclude that this approach yields insufficient information to understand how proliferation proceeds. In the subsequent chapter, I develop tools that provide the missing information which, for the first time, lead to a complete and quantitative picture of proliferation in the mouse sympathetic ganglion. In the final experimental chapter, I will use the tools to
investigate transgenic animals in order to demonstrate how this approach can yield new information about how two genes (*Ret* and *β-catenin*) influence final neuron number by regulating cell cycle parameters.

1.11 Aims of Thesis

**Chapter 3:** The aim of this chapter is to identify the changing phenotype and relative proportions of cells in the mouse stellate ganglion as it develops, using multiple label immunofluorescence to identify NCCs, neuroblasts and glial progenitors. Next, to measure the changes in a proliferation as development persists using single BrdU pulse labelling. This chapter aims to illustrate the significant limitations of using a single pulse of BrdU to measure proliferation.

**Chapter 4:** Using a combination of techniques, double s-phase labelling (using BrdU and EdU) combined with multiple labelling immunofluorescence, I aim to develop a new approach to measure the multiple parameters needed to properly characterise *in vivo* cell cycle dynamics. In doing so, I aim to fully characterise the proliferation in the major cell populations of the developing sympathetic (stellate) ganglion during the course of embryonic development.

**Chapter 5:** I aim to test the validity of my newly developed approach by applying these methods to situations where normal development has been perturbed. First, I examine the changes in proliferation that result from the removal of the glial family ligand co-receptor *Ret*. My aim is to see whether proliferation defects can account for the smaller sympathetic ganglia that are observed in *Ret* null mutant mice. Finally, I aim to identify how conditional over expression of stable β- catenin may affect proliferation and normal development of sympathetic ganglia at E16.5, a time when canonical Wnt signalling is thought to be important for maintaining neuroblast proliferation.
1.12 Chapter 1: Figures
Figure 1-1: Summarised anatomy of the ANS, and a functional schematic of the nervous system

A) Anatomical organisation of the autonomic nervous system: red) sympathetic or thoraco-lumbar outflows; blue) parasympathetic or cranio-sacral outflows; and green) enteric, neurons that lie in the alimentary canal

B) Functional organization of the nervous system. The motor system consisting of: the somatomotor (blue), the autonomic (pink) and the neuroendocrine (grey) components. All three efferent outflows act to influence behavior, and hence eventually the external environment. The afferent pathways return information from the internal (pink and blue) and external environments (black). Processing of this information occurs in the CNS and delivered to efferent motor neurons in three general types of input: c) from the cerebral hemispheres responsible for voluntary control and of the behavior; s) from the behavioral system controlling attention, arousal sleep/wakefulness and circadian timing; and r) from the sensory system monitoring processes in the body or the environment to all levels of the motor system generating reflex behavior. The three general input systems communicate bidirectionally with each other to generate behavior. This figure has been modified from Swanson (2000) and Jänig (2006)
Chapter 1: Literature Review
**Figure 1-2: Differentiation of NCCs to SNS neuronal or glial phenotype**

Showing the *in vivo* phenotypic differentiation program of NC stem cells into either a neuronal precursor, or glial progenitor (note this pathway is based largely on studies from the mouse). Large dotted arrow depicts the temporal progression and intervals are not scaled. For the gene interaction schematics; solid arrows indicate positive interaction, dotted arrows mean possible positive interaction and red connectors indicate repressive interaction. The figures are based on information from the text, section 1.5.3, and from the adjacent references (Callahan et al., 2008, Tsarovina et al., 2008, Rohrer, 2011).
NCCs migrate from the neural tube toward the dorsal aorta. BMPs released from the dorsal aorta result in the up regulation of transcription factors Ascl1 and Phox2b.

Unsure of the fate commitment of cells in the ganglion.

The combined actions of Ascl1 and Phox2b result in the up regulation of Insm1. Insm1 plays a role in regulating the expression of Ascl1.

Unsure of the fate commitment of cells in the ganglion.

Hand2, Gata2/3, and Phox2a are all now expressed and cross regulate each other.

Unsure of the fate commitment of cells in the ganglion.

Actions of the members of the transcriptional network result in the expression of TH and DBH.

Cells that express TH or DBH are likely to be committed to a neuronal noradrenergic phenotype.

Cells expressing TH and DBH have lost the expression of Sox10 and are now bona fide noradrenergic neuronal precursors.

The vast majority of Sox10 cells now express the glial marker BFABP and are considered glial precursors.
Figure 1-3: The cell cycle phases

Different stages of the cell cycle, including; the cyclin Cdk checkpoints that are necessary for progression through the cell cycle and to avoid cell death, and the Cip/Kip Cdk inhibitors that are associated with an increase in G1 and differentiation during CNS neurogenesis (Dehay and Kennedy, 2007).
Chapter 1: Literature Review

G1: Cells dividing

G0: Cells not dividing

M: Mitosis

S: Synthesis

G2: Gap 2

Cdk 1
Cyclin A/B

Cdk 4/6
Cyclin D

Cip/Kip
Cdk inhibitors

Cdk 2
Cyclin A

Cdk 2
Cyclin E
Figure 1-4: Progenitor pool differences in the chick compared to the mouse

Progenitor pool maintenance in the chick and mouse compared. On the left is Figure 3B from Tsarovina et al. (2008). Sox10+/Phox2b- are early progenitors, Sox10+/Phox2b+ cells are considered to be late progenitors and Sox10-/Phox2b+ (these cells are also positive for SCG10 and TH) are committed neuroblasts. On the right is an equivalent figure prepared from Table 2 of Callahan et al. (2008). Note that Sox10+/Phox2b- cells are only 5% of the total cells on E10.5 and are unlikely to contribute significantly to generation of neuroblasts after this time, whereas in the chick, these cells appear to differentiate into substantial numbers of neuroblasts at later times.
Chapter 1: Literature Review

Chick

- Sox10-/Phox2b+
- Sox10+/Phox2b+
- Sox10+/Phox2b-

Embryonic Day

Mouse

- sox10-/phox2b+
- sox10+/phox2b+
- sox10+/phox2b-

Embryonic Day
Chapter 2

2 Materials and Methods
This chapter summarises the methods used in this thesis and should be referred to for all of the following chapters.

2.1 Animals

The University of Melbourne Animal Experimentation Ethics committee approved all procedures relating to the following experiments. Mice were time plug-mated, and the morning of the detection of the plug was deemed to be embryonic day (E) 0.5. WT C57/Bl6 mice, $Ret^{TGM/TGM}$ mice (Enomoto et al., 2001) and $TH-Cre:β-Cat^{Ex3/+}$ (both transgenic lines on a C57/Bl6 background) were used. C57/Bl6 mice were from a colony maintained in our local animal house and all lines were maintained by inbreeding.

2.1.1 $Ret^{TGM/TGM}$ mice

$Ret^{TGM/TGM}$ mice have a fusion protein consisting of the N-terminal region of bovine tau, a full-length EGFP and three repeats of the human Myc tag inserted into the first exon of the $Ret$ gene by homologous recombination. Mice heterozygote for the $Ret$-TGM insertion ($Ret^{TGM/+}$) develop and survive normally, while homozygous animals die shortly after birth due to kidney and enteric nervous system deficits (Enomoto et al., 2001). $Ret^{TGM/TGM}$ animals were generated by mating $Ret^{TGM/+}$ males with $Ret^{TGM/+}$ females. $Ret^{TGM/TGM}$ embryos were genotyped by observation of GFP expression in the enteric nervous system of the stomach using a fluorescence microscope. WT mice have no GFP+ neurons in the stomach, $Ret^{TGM/+}$ animals have a dense plexus of GFP+ neurons throughout the stomach, while $Ret^{TGM/TGM}$ embryos have a sparse plexus of GFP+ neurons in the proximal stomach only (Stewart et al., 2007).

2.1.2 $TH-Cre:β-Cat^{Ex3/+}$

$TH-Cre:β-Cat^{Ex3/+}$ mice were generated by crossing mice that express Cre-recombinase under the TH promoter (Lindeberg et al., 2004), to mice in which exon 3 of the mouse $β$-catenin gene ($Catnb$) was surrounded by two $loxP$ sequences (Harada et al., 1999, Martínez et al., 2009). Cre-mediated
recombination at this locus removes the phosphorylation and ubiquitination sites in exon 3 and results in a stable form of catenin that constitutively activates Wnt/β-catenin signaling. TH-Cre:β-CatEx3/+ mice were genotyped by PCR of genomic tail DNA from tails removed at the time of harvesting, using primers described in Cain et al. (2008)

2.2 Injections

2.2.1 Single pulse of BrdU

Pregnant dams were injected intraperitoneally (i.p.) with 5-bromo-2’-deoxyuridine (BrdU) at 100µg g⁻¹ dissolved in 0.007N NaOH in 0.9% saline. 2 h after the injection, all embryos were dissected out and prepared for sectioning.

2.2.2 Double pulse of BrdU and EdU

Pregnant dams were injected i.p. with BrdU at 100µg g⁻¹ dissolved in 0.007N NaOH in 0.9% saline. 2 h later a second i.p. injection of 5-Ethyl-2’-deoxyuridine (EdU) at 50µg g⁻¹ dissolved and diluted to 2.5mg ml⁻¹ solution in dH₂O was administered. Thirty minutes after the injection of EdU, the pregnant dam was killed, embryos were dissected out, screened (if necessary) and prepared for sectioning.

2.2.3 Erythropoietin

Pregnant RetTGM/+ mice that were crossed to male RetTGM/+ mice were injected daily with 125 IU of erythropoietin i.p. daily on: E13.5, E14.5, and E15.5. On E16.5, the dam was killed and embryos were dissected and screened as above. Only RetTGM/TGM mice were further analysed.

2.3 Fixation/Tissue preparation

In all experiments, pregnant dams were killed by cervical dislocation. After a hysterectomy and isolation, embryos were decapitated and temporarily stored in chilled phosphate-buffered saline 0.01M PBS; containing in mM; NaCl
(145), \( \text{Na}_2\text{PO}_4 \) (7.5) and \( \text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O} \) (2.5). To screen for the \( \text{Ret}^{\text{TGM/TGM}} \) mice, the stomachs were removed and GFP expression examined on a Zeiss fluorescence microscope (Young 2004). Once screened, or immediately after removal (for the C57b6 mice), the anterior thorax, including the forelimbs, was removed and placed in fixative.

Fixation was in Zamboni’s fixative (2% formaldehyde plus 15% saturated picric acid in pH7.4 phosphate buffer). Embryos younger than E10.5 were immersion fixed immediately and the thoracic block dissected after fixation. All embryos were fixed at 4°C overnight (approx. 12-18 h). After fixation, embryos were washed 3 x 5 minutes, in 0.01M PBS. Prior to snap freezing, all tissue was cryoprotected overnight using 20% sucrose in distilled water. Tissue was embedded in OCT and snap-frozen in isopentane (\( \text{CH}_2\text{CH}_2\text{CH}(_3)\text{CH}_2 \)) cooled by liquid nitrogen. Tissue was stored at -70°C until used.

### 2.4 Immunofluorescence

Sections were cut on a cryostat at 5µm - 14µm thicknesses and a water repellent pen used to draw wells around individual sections. In all cases the primary antibody was applied for 24h, secondary and tertiary reagents were applied for 1 hour. In between each of these steps all tissue was washed in 0.01M PBS 3 x 5 minutes. Table 1 lists all of the primary antisera used, Table 2 list all secondary and tertiary reagents used.

#### 2.4.1 Immunofluorescence for Ki67 or Phox2b

When staining for Ki67 or Phox2B, all sections were put through antigen retrieval prior to the application of primary antibody to improve immunoreactivity. The sections were heated to 95°C in citrate buffer (tri-sodium citrate \( \text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \) 10mM at PH 6, using a standard microwave (1 minute on the highest power setting followed by 10 minutes on the lowest). Once sections had come to room temperature, they were washed in 0.01M PBS 3 x 5 minutes. Primary antibody was then applied.
2.4.2 Immuno-fluorescence when labelling with BrdU and, or EdU

BrdU was detected using immuno-fluorescence:

1) Identification of cells of interest, standard primary secondary and tertiary (if necessary) processing was carried out as described above

   a. To identify NCCs and glial progenitors Sox10-immunoreactivity (IR) was used as per above.

   b. To identify neuronal precursors and neurons TH-IR was used

2) Processing for BrdU (Primary and secondary processing)

   a. After both primary, secondary and tertiary staining, all tissue was washed in 2M HCl for 30 min at room temperature (22°C)

   b. Following the acid treatment all tissue was washed 2 x 5 minutes in 0.1M di-sodium tetraborate (Na₂B₄O₇.10H₂O)

   c. Tissue was transferred to and washed in 0.01M PBS 3 x 5 minutes

   d. Primary antibody (rat anti-BrdU) was applied followed by secondary (Donkey anti-rat Texas Red) with bisbenzimide (see table 1 and table 2).

3) Processing of EdU

   a. After labelling BrdU with the secondary antibody, all tissue was washed 3 x 5 minutes in 0.01M PBS and EdU reagents applied as per manufacturers instructions. The details of the procedure can be found at:
   https://commerce.invitrogen.com/index.cfm?fuseaction=viewCatalog.viewProductDetails&productDescription=38477

   b. Tissue was washed in 0.01M PBS 3 x 5 minutes and all slides were mounted using Dako mounting medium.

Sections were imaged on a Zeiss Meta 510 scanning confocal microscope and analysed using Zeiss Image Browser (v4.0.0.241, Carl Zeiss Microimaging) or ImageJ (v1.43u, NIH) software.
2.5 Calculation of S-phase, labelling index, growth fraction and cell cycle

2.5.1 Calculating the S-Phase length ($T_s$)

To calculate the S-phase length ($T_s$), single confocal images of transverse sections of embryos through the stellate ganglia were analysed. At E9.5, Sox10+ cells between the neural tube and the stellate ganlion were also analysed. All Sox10+/TH- or Sox10-/TH+ cells within the ganglion in an image were counted and scored as expressing either 1) BrdU, 2) both BrdU and EdU (BrdU/EdU) or 3) EdU alone. Cells labelled with the 1st tracer only, BrdU in this case, are cells that have left S-Phase and moved into G2 before administration of the 2nd tracer (EdU). Cells labelled with both BrdU/EdU were in S-phase for the entire duration of the injection interval; and cells positive for the EdU only were rare cells that have entered S-phase after administration of the EdU.

In previous studies (Hayes and Nowakowski, 2000b), the following mathematical relationship between the cells that have exited S-phase, versus the time of the injection interval has been described and is represented as:

$$ \frac{2h}{T_s} \propto \frac{\text{cells that exited S-phase}}{\text{cells that remained in S-phase}} $$

where; $T_s$ is the length of S-phase

The above relationship can be rearranged to solve for $T_s$ (Hayes and Nowakowski, 2000b). So, for all experiments in this study, the formula to calculate the $T_s$ is given by:

$$ T_s = 2 \times \left( \frac{\text{BrdU/EdU + EdU}}{\text{BrdU}} \right) $$

2.5.2 To calculate the Growth Fraction

The growth fraction (GF) was calculated from sections from the embryos treated with BrdU and EdU or, if all BrdU/EdU sections were required for calculating S-phase length, then from sections from additional littermates. In each case, sections were stained simultaneously for Ki67, Sox10 and either TH or Phox2b. The growth fraction was the proportion of total TH, Phox2b or Sox10 cells expressing Ki67.
2.5.3 To calculate the instantaneous labelling index

The instantaneous labelling index (LI₀) is the fraction of cells in S-phase at any instance. It takes approximately 15 minutes, post intraperitoneal injection, for thymidine analogues such as BrdU to become available at sufficient concentration for uptake by cells (Hayes and Nowakowski, 2000b). The LI₀ was calculated as the Y intercept of a regression fitted to three values (Nowakowski et al., 1989b, Hayes and Nowakowski, 2000b). Two of these values could be obtained from the double-labelling BrdU/EdU experiments. The first value is the fraction of cells labelled after 30 minutes or, cells that have incorporated the second tracer EdU (BrdU+/EdU+ cells plus BrdU-/EdU+ cells). The second value is the fraction of cells labelled after 2.5 h, which is all labelled cells in the double labelling experiment (BrdU+/EdU-, BrdU-/EdU+ and BrdU+/EdU+ cells). The third value comes from sections prepared from a second series of embryos from dams injected only with single doses of BrdU at E9.5, 10.5, 11.5, 12.5, 14.5, 16.5 or 18.5 and killed two hours later.

2.5.4 Calculation of the cell cycle length

With the S-phase (Tₛ), instantaneous labelling index (LI₀) and growth fraction GF determined, the cell cycle length (T_c) was calculated using the following relationship (Nowakowski et al., 1989b):

\[ T_c = T_s \times \left( \frac{GF}{LI_0} \right) \]

or cell cycle length = S-phase \( \times \left( \frac{Growth\ Fraction}{Inst.\ Labelling\ Index} \right) \)

Each parameter at each age was calculated from 3-6 embryos. Total cell numbers counted to calculate the growth fraction were 446 (E9.5), 413 (E10.5), 542 (E11.5), 952 (E12.5), 1688 (E14.5), 4446 (E16.5) and 7659 (E18.5). Cell counts for calculating S-phase were; 461 (E9.5), 591 (E10.5), 1320 (E11.5), 605 (E12.5), 2230 (E14.5) and 8174 (E16.5). No S-phase length for E18.5 TH+ cells could be calculated because of the low number of cycling cells.
2.6 Stereology

Three \( \text{Ret}^{\text{TGM/TGM}} \) and three WT (WT, \( \text{Ret}^{+/+} \) or \( \text{Ret}^{\text{WT}} \)) littermates were fixed by immersion in 4% formaldehyde and the thorax serially sectioned horizontally on a cryostat at 25\( \mu \)m. Sections were stained with Feulgen stain and counterstained with Light Green. All nucleated cells in the stellate ganglion were counted with the physical dissector method using Stereo Investigator software (v4, MBF Bioscience, Williston, USA). Final section thickness was measured separately for each section counted.

2.7 Density counts

Sections from either Ki67, or BrdU labeled tissues that were also processed for Sox10, TH and bisbenzimide immunofluorescence were examined to measure the relative density of either Sox10+/TH-, or Sox10-/TH+ cells (no of cells per mm\(^2\)). The area around the ganglion was traced using the polygon tool in Imagej 1.43u, all nuclei within the traced area that corresponded to either Sox10, or TH immunofluorescence were counted and expressed as a fraction of this area in mm\(^2\).

2.8 Modelling

The numbers of three cell types - neural crest cell-derived (NCC) progenitors, neuroblasts and glial progenitor cells - were modeled between E9.5 to E18.5. A system of differential equations accounted for the change in cell numbers in terms of rates determined from the experimental data for the growth fractions and cell cycle lengths.

NCC progenitors (\( p \)) proliferate as well as differentiate into neuroblasts (\( n \)) and glial precursors (\( g \)). The neuroblast and glial precursors also increase in number by proliferating themselves. The differential equation system describing the evolution of cell numbers in time \( t \) is given in Figure 2.1.

The proliferation rate functions \( \lambda_n \) and \( \lambda_g \) for neuroblasts and glial precursors were determined from the data (Eq. (5) in Figure 2.1). The growth
fraction value was divided by the cell cycle time (in days) and realistic nonlinear functional forms were fitted to the data (the adjusted R² values are 0.989 for both cell types; and also chapter 4 Fig. 4.5).

It was instructive to also predict the percentage of total cells from E12.5 assuming that the progenitor NCC population is insignificant from this time onwards (Callahan et al., 2008). In this case, we set \( p = 0 \) in the system and solved a system of two differential equations for \( n \) and \( g \) (Eqs (2)-(3) in Figure 2.1), where the initial data was chosen as \( n (12.5) = 89.29 \) (the measured proportion of neuroblasts).

From E9.5-E12.5, the properties of the progenitor NCCS are also needed. At early times, the experimental data are unable to distinguish adequately between NCC progenitors and glial precursors, as both express Sox10 (Kuhlbrodt et al., 1998; Callahan et al., 2008). The growth fraction of NCCS over E9.5 to E12.5 was chosen to be unity everywhere except at E10.5, where my data show that it decreased to 0.79. Cell cycle length was 10.6 h on E9.5 and 30.3 h thereafter.

There were no direct experimental data on the cell differentiation rates. These were estimated so that the results of the model approximate the percentage of cell data for the proportions of neurons and glia (\( \beta_n \) and \( \beta_g \) in Table 1). At E9.5, only NCC progenitors were present. NCC progenitors could differentiate into neuroblasts from E9.5 and glial progenitors only after E11.0, as the glial precursor marker, brain fatty acid-binding protein (BFABP), is first detected at E11.5 in the mouse stellate ganglion (Callahan et al., 2008). The “differentiation switch” for differentiation into glial progenitors was chosen as “off-on”, for simplicity (Eq. (6) in Figure 2.1). Nonetheless, if a smooth, but rapidly increasing function is chosen (eg a tanh type function), the results do not change appreciably.

The model assumes that there is no cell death braced on the fact that prior to E18.5 cell death is minimal and therefor unlikely to significantly contribute to the overall numbers of cells predicted (Fagan et al., 1996).
2.9 Statistical analysis and mathematical modelling

Growth fraction and labelling index were calculated on pooled values from all embryos at each age and treatment group. Cell cycle length and S-phase length are presented as ±SEM for each embryonic age. Growth fractions are presented as proportions ± 95% confidence intervals. Means were compared using either unpaired t tests or one way ANOVA with Tukey’s post hoc test, as appropriate, using GraphPad Prism v5.04 (GraphPad Software Inc. La Jolla, USA). All modelling was done using Mathematica 8 (Wolfram Research, Champaign, USA).
2.10 Chapter 2: Tables and Figures
Figure 2-1: Equations used for modeling

Equations describing the time and evolution of the three cell types present in the mouse stellate ganglion

\[
\begin{align*}
\text{Precursor NCC:} \quad & \frac{dp}{dt} = \frac{\lambda_p p}{\text{cell division}} - K_n p - K_g(t) p \\
\text{Neuroblasts:} \quad & \frac{dn}{dt} = \frac{\lambda_n(n)}{\text{cell division}} + \frac{K_n p}{\text{from differentiation}} \\
\text{Glial:} \quad & \frac{dg}{dt} = \frac{\lambda_g(g)}{\text{cell division}} + \frac{K_g(t) p}{\text{from differentiation}} \\
p(9.5) = p_0, \quad & n(9.5) = 0, \quad g(9.5) = 0
\end{align*}
\]

\[
\begin{align*}
\lambda_p(t) &= \frac{\log_2 G_p(t)}{T_p(t)}, \quad & \lambda_n(t) &= \frac{\log_2 G_n(t)}{T_n(t)}, \quad & \lambda_g(t) &= \frac{\log_2 G_g(t)}{T_g(t)} \\
G(t) & \text{ : growth fraction, subscript represents cell type} \\
T(t) & \text{ : cell cycle length, subscript represents cell type}
\end{align*}
\]

\[
\begin{align*}
K_n &= \log_2 \beta_n, \quad & K_g(t) &= \begin{cases} 
0, & t < 11.1 \\
\log_2 \beta_g, & t \geq 11.1
\end{cases}
\end{align*}
\]
Table 2-1: Primary antisera

<table>
<thead>
<tr>
<th>Antisera</th>
<th>Host</th>
<th>Dilution</th>
<th>Supplier</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TH</td>
<td>Chicken</td>
<td>1:100</td>
<td>Chemicon</td>
<td>Chemicon lot# PS01410197</td>
</tr>
<tr>
<td>Sox10</td>
<td>Goat</td>
<td>1:200</td>
<td>Santa Cruz</td>
<td></td>
</tr>
<tr>
<td>Phox2B</td>
<td>Guinea</td>
<td>1:500</td>
<td>H. Enomoto (gift to H. Young)</td>
<td></td>
</tr>
<tr>
<td>Ki67</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Thermo Scientific</td>
<td>Thermo Scientific lot#9106s 1002c</td>
</tr>
<tr>
<td>BrdU</td>
<td>Rat</td>
<td>1:40</td>
<td>Abcam</td>
<td>Abcam #lot 570168</td>
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</tbody>
</table>

Table 2-2: Secondary antisera and tertiary reagents

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<th>Antisera</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dk × Rabbit Texas Red</td>
<td>1:200</td>
<td>Jackson lot# 77917</td>
</tr>
<tr>
<td>Dk × Rabbit Ig biotin</td>
<td>1:400</td>
<td>Jackson lot# 98327</td>
</tr>
<tr>
<td>Dk × Chicken FITC</td>
<td>1:100</td>
<td>Jackson lot# 88953</td>
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<tr>
<td>Dk × Sheep Ig biotin</td>
<td>1:200</td>
<td>Jackson lot# 76225</td>
</tr>
<tr>
<td>Dk × Sheep 647</td>
<td>1:500</td>
<td>Invitrogen Molecular Probes lot# 404236</td>
</tr>
<tr>
<td>Dk × Guinea Pig FITC</td>
<td>1:500</td>
<td>Chemicon</td>
</tr>
<tr>
<td>DK × Guinea Pig DyLite 649</td>
<td>1:500</td>
<td>Jackson lot#92605</td>
</tr>
<tr>
<td>Strept Avidin Cy5</td>
<td>1:40</td>
<td>Abcam lot# 570168</td>
</tr>
<tr>
<td>Strept Avidin Alexa 405</td>
<td>1:100</td>
<td>Jackson lot# 95129</td>
</tr>
<tr>
<td>Bisbenzamide</td>
<td>100,000µg ml⁻¹</td>
<td>Sigma St Louis MO</td>
</tr>
</tbody>
</table>

Dk = Donkey
### Table 2-3: Parameter values for model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Units</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_p$</td>
<td>$\frac{\lambda_p(t)}{\log_e 2} = \frac{G_p(t)}{T_p}$</td>
<td></td>
<td>experimental data</td>
</tr>
<tr>
<td></td>
<td>$10.6/24$</td>
<td>day</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\frac{\lambda_n(t)}{\log_e 2} = \frac{G_n(t)}{T_n(t)}$</td>
<td></td>
<td>experimental data</td>
</tr>
<tr>
<td></td>
<td>$1.95 (t - 9.5)^3 e^{-1.25(t-9.5)}$</td>
<td>day$^{-1}$</td>
<td>experimental data</td>
</tr>
<tr>
<td></td>
<td>$\frac{\lambda_g(t)}{\log_e 2} = \frac{G_g(t)}{T_g(t)}$</td>
<td></td>
<td>experimental data</td>
</tr>
<tr>
<td></td>
<td>$4.04 (t - 9.5)^4 \left( e^{-1.79(t-9.5)} + 0.0017 e^{-0.58(t-9.5)} \right)$</td>
<td>day$^{-1}$</td>
<td>experimental data</td>
</tr>
<tr>
<td>$\beta_n$</td>
<td>$\frac{1}{(10.6/24)}$</td>
<td>day$^{-1}$</td>
<td>estimate</td>
</tr>
<tr>
<td>$\beta_g$</td>
<td>$\frac{1}{(21.2/24)}$</td>
<td>day$^{-1}$</td>
<td>estimate</td>
</tr>
</tbody>
</table>
Chapter 3

3 Proliferation in the developing SNS: The limitations of using BrdU
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3.1 Introduction

The SNS is produced by NCCs that differentiate into either neuroblasts or glial progenitors, and it is the proliferation of these two cell populations that ultimately generates the mature sympathetic ganglia. The mechanisms that govern proliferation in the developing SNS are not well understood. Previously, many loss of function experiments in genetically modified animals, have shown that the removal of any of a wide range of extrinsic signalling molecules, cell surface receptors or transcription factors often results in a reduction in proliferation, seen as smaller sympathetic ganglia containing fewer cells (see section 1.8). However, how the reduction in proliferation comes about in each case is unknown. In particular, we lack even the most basic knowledge of how cell division changes to bring about alterations in the number of cells present in the growing sympathetic ganglion.

In this chapter my aims are two fold: 1) to identify the relative changes in the proportions of neuronal precursors and glial progenitors during the course of development; and 2) identify the changes in proliferation of these cells as development progresses. In order to do this, I need to differentiate NC progenitors from the differentiated neuronal precursors and glial progenitors. In addition, I need to identify how their relative proliferation changes as development progresses.

3.1.1 Identifying cell types in the developing sympathetic ganglion

Sympathetic ganglia are founded by NCCs that migrate from the neural tube early in embryonic life. Within the ganglion, they differentiate into either neuroblasts or into glial precursors, both of which continue to proliferate (Rothman et al., 1978, Rohrer and Thoenen, 1987, Rohrer, 2011). NCCs are characterised by the expression of the transcription factor Sox10. Sox10 is essential to the survival of undifferentiated NCCs and has been shown to maintain multipotency in NC-derived sympathetic progenitor cells (Southard-Smith et al., 1998, Paratore et al., 2001, Kim et al., 2003, Callahan et al., 2008).
Sox10 is expressed by all migrating NCCs but is not by sympathetic neuroblasts (Kim et al., 2003, Callahan et al., 2008, Tsarovina et al., 2008).

Sox10 continues to be expressed once NCCs differentiate into glial precursors. However, the glial marker, BFABP is detectable by E11.5 in all Sox10-positive glial precursors cells (Callahan et al., 2008). Hence, from E11.5, either Sox10 or BFABP expression can be used to identify glial progenitors.

Neuroblasts express a wide range of pan-neuronal markers, such as SCG10, neurofilament, Tuj1 and PGP9.5, as well as markers specific for noradrenergic neurons, such as tyrosine hydroxylase (TH) and dopamine β hydroxylase ((DβH) – see Fig.2A in Rohrer, 2011). A small fraction of sympathetic neurons are cholinergic and so express the pan-neuronal markers but not noradrenergic markers. Many cholinergic sympathetic neurons are believed to be generated postnatally by transdifferentiation of noradrenergic neurons, controlled by signals provided by their target tissues (Glebova and Ginty, 2005, Rohrer, 2011). A second, small population of sympathetic neuroblasts appears to be cholinergic from embryonic times and is not likely to be identified by immunofluorescence against noradrenergic markers. Other than these cells, immunofluorescence to a noradrenergic marker such as TH should identify all neuroblasts in a sympathetic ganglion.

Thus, a combination of immunofluorescence to TH and Sox10, should identify nearly all neuroblasts in the ganglion and depending on the age, NCCS or glial precursors in developing sympathetic ganglia. This will allow their relative change in proportion to be tracked as development proceeds.

3.1.2 Measuring proliferation in the developing sympathetic ganglion

By far the most common way to examine proliferation in the development of the SNS has been to take advantage of labeling with thymidine analogues such as H³thymidine or BrdU (Rothman et al., 1978, Rohrer and Thoenen, 1987, Andres et al., 2001, Coppola et al., 2010, Reiff et al., 2011). There are other ways to look at proliferation (see the Discussion in this chapter), however, I wish to provide an overall description of proliferation in both neuronal and glial
precursors that will help interpret the results from gain of function and loss of function experiments. The vast majority of similar studies have used BrdU to examine proliferation, which is the approach I will adopt in this chapter.

3.2 Materials and methods

For all materials and methods relating to this chapter refer to chapter 2 sections 2.1 – 2.4. A list of antibodies used can be found in table 2.1 and 2.2

3.3 Results

I analysed transverse sections through the embryo at the level of the forelimbs, which is the level at which the ganglion primordium for the stellate, or first thoracic ganglion develops. The stellate ganglion is the target of all of the studies that follow in this thesis.

3.3.1 Sox10 and TH immunoreactive cells in the StG – E11.5 to E18.5

NCCs identified by their expression of Sox10 migrate to the site of the ganglion primordia, lateral to the dorsal aorta, around E9.5 (Durbec et al., 1996). At E10.5, transverse sections at the level of the forelimb reveal that the cells in the developing stellate ganglion have clustered but are not yet tightly packed together (Fig. 3.1A-E). The same transverse sections through the embryo also show that, in addition to the sympathetic ganglia, NCCs positive for Sox10-immunoreactivity (IR) are present in the primitive DRG, the developing nerve trunks and developing gut (Fig. 3.1A, B & D). At E10.5, cells positive for TH-IR are present only in the developing sympathetic ganglion and, rarely, in the developing gut (Fig. 3.1A, C & D). Double-labelling with antibodies against TH and Sox10 reveals that there are 3 populations of cells in the sympathetic ganglia at E10.5; cells that express Sox10-IR only (Sox10+/TH-), cells that express both Sox10-IR and TH-IR (Sox10+/TH+); and cells that express TH-IR only (Sox10-/TH+). The intensity of Sox10-IR in the Sox10+/TH+ population was always faint, when compared with the intensity of staining in the Sox10+/TH- population (Fig. 3.1 A-E).
At E11.5 the ganglia have a circular profile and the cells more tightly packed together (Fig. 3.2A). All three types of cell (Sox10+/TH-, Sox10+/TH+, Sox10-/TH+) are still present, although Sox10+/TH- and Sox10+/TH+ cells are rarer and Sox10-/TH+ cells more common (Fig. 3.2). Consistent with previous reports (Callahan et al., 2008), from E11.5 to E12.5, transverse sections through the developing stellate ganglion showed that Sox10+/TH- cells are more often around the periphery of the developing ganglia, with occasional Sox10+/TH- cells found in more central parts (Fig. 3.2A-B). From E11.5 to E18.5 the ganglion rapidly increases in size and the stereotypical, peripheral location of the Sox10 cells disappears, so that after E13.5, there is no apparent bias to the distribution of the Sox10+ cells (Fig. 3.2A-F). From E10.5 to E18.5, the average size of Sox10-/TH+ cells very obviously increases (compare the size of cells in Fig. 3.2E-F to 3.2A-B).

### 3.3.2 BFABP and Sox10 expression

Callahan et al. (2008) reported that BFABP is first expressed in the developing stellate ganglion at E11.5 in 80% of Sox10+/TH- cells and in 97% on E12.5. BFABP expression is absent from cells lacking Sox10 (Callahan et al., 2008). In the study by Callahan et al. (2008), overlap of BFABP and Sox10 after E12.5 was not examined. To show that Sox10 can be used as a marker for glial progenitors, after E12.5, double-labelling immunofluorescence for both BFABP and Sox10 was performed on embryos at E12.5 to E18.5. I found only a handful of Sox10+ cells that lacked BFABP at these times, with nearly all Sox10 cells being double labelled (Fig. 3.3A-I). After E11.5, it appears that Sox10 identifies glial progenitors almost exclusively (Fig. 3.3A-I).

### 3.3.3 Changes in the proportions of TH and Sox10 cells from E10.5 to E18.5

I next measured the change in proportions between Sox10+/TH- cells, Sox10+/TH+ cells and Sox10-/TH+ cells. From E10.5 to E18.5, there is a dramatic increase in cell number in the ganglion and I was interested in how the relative proportions of the three cell types changed over this time. I counted all cells in transverse sections through the stellate ganglia and scored them for the presence
of Sox10 or TH. Over the period examined, there were dramatic changes in the proportions of the three cell types in the stellate ganglion (Fig 3.4).

On E10.5, 52% of the total cells in the stellate ganglion were Sox10+/TH-, 47% Sox10+/TH+ and only 1% of all cells were Sox10-/TH+ (Fig. 3.4). The proportion of Sox10-/TH+ cells increased rapidly from E10.5 until E13.5 (to 89% of total cells) and then deceased slowly to 67% on E18.5 (Fig. 3.4). Sox10+/TH+ cells decreased in proportion from E10.5 and were only 6% of the total on E11.5 and virtually absent on E12.5 (0.9% - Fig. 3.4). However, note that there was always a small population (~1%) of Sox10+/TH+ cells up until at least E18.5 (Fig. 3.4). After E10.5, the Sox10+ /TH- population also decreased exponentially, reaching their lowest levels on E13.5 (10%) and then slowly increasing at the expense of Sox10-/TH+ cells until they were 32% of the total on E18.5 (Fig. 3.4).

3.3.4 Proliferation in the developing stellate ganglion

In conjunction with the double-labelling protocol described earlier, I also injected BrdU into pregnant dams on E10.5-14.5, 16.5 and 18.5, took the embryos 2 h later and processed sections from the embryos for BrdU-IR, TH-IR, Sox10-IR and bisbenzimide labelling (Fig. 3.5). This enabled me to track both the overall and relative changes in proliferation in all three populations of cells (Sox10+/TH-, Sox10+/TH+ and Sox10-/TH+).

I examined cell proliferation in the developing stellate ganglion using BrdU, which is incorporated into the genome of proliferating cells during the synthesis phase (S-Phase) of the cell cycle (Eidinoff et al., 1959, Stockdale et al., 1964, Gratzner, 1982, Nowakowski et al., 1989b, Takahashi et al., 1993, Enomoto et al., 2001). BrdU is substituted for thymidine when cells synthesise new DNA, therefore, cells that incorporate BrdU represent cells that were in S-phase during the injection interval.

BrdU labelling was only observed in the nucleus of labelled cells and was found in both Sox10 and TH populations (Fig. 3.5). When I examine proliferation in all cells (Fig 3.6A), ignoring the individual subpopulations of cells identified by immunofluorescence to Sox10 and TH, at E10.5 14% of all cells are positive for
BrdU labelling. This increases to a peak of BrdU labelling at E12.5, with 50% of all cells positive for BrdU (Fig. 3.6A). From E12.5, there is a decline in labelling so that, by E18.5, the proportion of BrdU-positive cells is down to 8% (Fig. 3.6A). The fact that I combined BrdU labelling with immunofluorescence for TH and Sox10, means that I can identify the proportion of BrdU-labelled cells in each of the three phenotypically distinct populations of cells identified earlier (Fig 3.6 A - i, ii & iii).

The pattern of labelling with BrdU for each type of cell was calculated separately. These results are shown in Figure 3.6A - i, ii & iii, as the percentage of BrdU cells as a proportion of all cells in the ganglion. I was interested in identifying relative changes in proliferation between the Sox10-/TH+ neuronal precursors and Sox10+/TH- glial progenitors. However, presenting these data as a percentage of all cells does not reveal any information about the relative efficacy with which both cells incorporate the tracer and makes a comparison difficult. So, Figure 3.6B shows the same data replotted as the proportion of BrdU+ cells of each type, relative to the total number of cells of that type. Viewed in this way, it is clear that both Sox10+/TH- and Sox10-/TH+ cells have similar patterns of BrdU uptake, rising to a peak at E12.5 and falling away to E18.5 (Fig. 2.5). Interestingly, during the period from E11.5-14.5 there appears to be no significant difference in the proliferative behaviour of either population of cells (Fig. 3.6B). However at E10.5, it is clear that the differentiating Sox10+/TH+ cells only very rarely incorporate BrdU, in fact, they are the least proliferative cells found throughout development (Fig. 3.6B). From E16.5 there is a statistical difference between the Sox10+/TH- glial progenitors and Sox10-/TH+ neuroblasts, and this difference increases at E18.5, indicating that the glial cells are relatively more proliferative later in development (Fig. 3.6B).
3.4 Discussion

I have examined the localisation of the transcription factor Sox10 and the noradrenaline synthesising enzyme, TH, from E10.5 to E18.5 in the developing murine stellate ganglion. In doing so, I have identified NCCs and glial progenitors (Sox10+/TH- cells) and neuronal precursors (Sox10-/TH+ cells), as well as a third class of cells (Sox10+/TH+ cells). In addition to this, I have tracked the temporal changes in the relative proportions of these three populations of cells and shown that Sox10-/TH+ neuronal precursors are the dominant cell after E10.5, while the proportion of Sox10+/TH- glial progenitors increases steadily up to E18.5. The Sox10+/TH+ population is a transient population that is virtually absent from the ganglion after E11.5. These changes in proportions are matched by similar changes in the proportions of cells taking up BrdU. Early in development, the vast majority of BrdU-positive cells are also positive for TH, while by E18.5, the majority of BrdU positive cells in the ganglion are Sox10+. Sox10+/TH+ cells show only very low levels of BrdU labelling.

3.4.1 Sox10+/TH+ cells are adopting a noradrenergic phenotype

This is the first report showing that cells in the murine SNS at E10.5 co-express both Sox10 and TH. On E10.5, it has previously been shown that many Sox10+ cells also express Phox2b (Callahan et al., 2008). Phox2b is a transcription factor necessary for proper noradrenergic differentiation, including the expression of TH (Goridis and Rohrer, 2002, Rohrer, 2011). It seems likely that the Sox10+/TH+ cells, observed in this study, are a subset of the Sox10+/Phox2b+ population observed in Callahan et al. (2008) that have gone on to express TH. As such, the Sox10+/TH+ cells are likely to be in the process of down regulating the expression of Sox10 and up regulating the expression of TH as they differentiate into neuroblasts. This is likely the reason for the low expression of Sox10-IR when Sox10 is co-localised with TH (figure 2.0 B-E). As Sox10 has been shown to maintain multipotency in NC progenitors (Kim et al., 2003), it is not clear if the Sox10+/TH+ cells are restricted to differentiating into neuronal phenotype. It may be that some of them will lose TH and give rise to glial progenitors. However, nothing in my data sheds light on this issue.
In the absence of evidence to the contrary, it is likely that the dramatic reduction in the proportion of Sox10+/TH+ cells, from E10.5 to E11.5 (47% to 5% of the total), is the result of differentiation of NCCs into neuroblasts. Over the same period, from E10.5 to E11.5, the Sox10-/TH+ population moves from 1% to 76% of the total cells. I speculate that this rapid reduction in the Sox10+/TH+ cells is due to these cells losing the expression of Sox10 and contributing to the Sox10-/TH+ population. The massive increase in proportion of Sox10-/TH+ cells, observed from E10.5 to E11.5, cannot be accounted for solely by proliferation of the 1% of Sox10-/TH+ cells observed at E10.5.

### 3.4.2 Differentiation of Sox10+/TH- cells

At E11.5, the glial marker, BFABP, is expressed by 80% of Sox10+/TH-cells (Callahan et al., 2008) and not at all by cells that express TH (Jessen and Mirsky, 2005, Taylor et al., 2007, Nishino et al., 2010). Hence, the majority of Sox10+/TH- cells on E11.5 are already glial progenitors and, thereafter, Sox10 expression identifies the glial lineage (Fig. 3.3). Thus, over E10.5 to E11.5, Sox10+/TH- NCCs give rise to two sorts of cell, Sox10+/BFABP+/TH- glial progenitors and Sox10-/BFABP-/TH+ neuroblasts, both of which continue to proliferate. A third group of cells, Sox10+/TH+, are transiently present and likely to represent cells differentiating from NCCs into neuroblasts. By E12.5, Sox10+/BFABP-/TH- NCCs are only 0.3% of total cells (Callahan et al., 2008).

Currently, there are no mechanisms identified to explain how sympathetic NC precursors can almost simultaneously give rise to two cell types. In chick sympathetic ganglia, Notch and Delta signalling has been shown to regulate the maintenance, or depletion, of the NC progenitor pool (Tsarovina et al., 2008). I speculate that the 52% of Sox10 cells that I observed at E10.5, are analogous to the NCC progenitors maintained by Notch signalling reported in Tsarovina (2008). In the chick, Delta1 (the ligand for Notch receptor signalling) is expressed on cells that have already adopted a neuronal phenotype and lost the expression of Sox10 and so Notch/Delta signalling may maintain a pool of Sox10+/TH- NCCs in murine tissue. Tsarovina et al (2008) have shown that, in the chicken, cells with a phenotype consistent with being NCCs (Sox10+/SCG10-)
are present in significant numbers for much longer, in comparison to what I have observed in this study. In the chicken, NCCs continue to differentiate into neuroblasts for more than three days (Tsarovina et al., 2008), while my data suggests that this happens for barely two days in the mouse. Note that the chicken does not appear to have cells expressing both Sox10 and a neuronal marker (SCG10 in this case).

### 3.4.3 Proliferation in the developing mouse stellate ganglion

Examining changes in the proportions of different cell populations as a fraction of the total cells does not provide much insight into the proliferative behaviour of the cells concerned. In contrast, BrdU labelling marks cells actively in S-phase and hence committed to divide. For this reason, the proportion of BrdU cells in a population is normally considered to be a direct measure of proliferation.

The first significant observation from the BrdU data is that there is a very low level of proliferation in the Sox10+/TH+ cells compared with proliferation in the Sox10+/TH- population on E10.5. Only 4% of Sox10+/TH+ cells were BrdU positive on E10.5, vs 24% positive for Sox10+/TH-. In fact, based on BrdU uptake, the Sox10+/TH+ cells are the least proliferative cells observed in the study (Fig. 3.6B).

Sox10+/TH+ cells were identified as differentiating NCCs. In the CNS, upon differentiation from neuronal stem or precursor cells, committed neurons withdraw from the cell cycle and are no longer proliferative (Dehay and Kennedy, 2007). In contrast, in the SNS, cells continue to proliferate after they have differentiated and up-regulated both neuronal and noradrenergic markers (Rohrer and Thoenen, 1987, Rohrer, 2011). However, it may be that differentiation is inherently incompatible with continued proliferation and as a result, Sox10+/TH+ cells have very low BrdU labelling. In the chick, cumulative labelling studies with $^3$Hthymidine showed that, around E2-E3, some NCCs might start withdrawing from the cell cycle (Rothman et al., 1978). The equivalent time in the mouse is E10-E11 when Sox10+/TH+ cells have low levels of BrdU labelling. It seems unlikely that 52% percent of differentiating NCCs are
permanently withdrawing from the cell cycle at this time, as it would make the rapid expansion of neuroblast numbers reliant on too few cells. An alternative is that the cell cycle length is increasing, so that a smaller proportion of cells are in S-phase at any time. However, like Rothman et al. (1978) in the chick, I cannot determine whether the low number of S-phase cells is due to fewer cells dividing (cell cycle withdrawal), or lengthening of the cell cycle, or both.

A second observation is that, from E11.5 to E16.5, both the Sox10+/TH-cells and the Sox10-/TH+ cells incorporate BrdU in very similar relative proportions (Fig. 3.6B). This raises the possibility that both of these cell types are dividing at similar rates. However, based solely on a single pulse of BrdU, it is impossible to determine whether this is the case. It is possible that one population of cells are all dividing, but at a slow rate, while the second population has most cells withdrawn from the cell cycle, with the remainder cycling rapidly. It is conceivable that, in these circumstances, both populations will show identical patterns of uptake of BrdU, but quite different underlying proliferation patterns. Hence I would next like to consider exactly what does S-phase labelling with a pulse of BrdU actually tell us about cell proliferation overall?

3.4.4 Measuring proliferation with BrdU: limitations

Cell division is one of the most important processes in biology. The rate of cell division is the driving force behind the production of tissues and organs during embryogenesis; it is also the driving force behind other important processes such as wound repair and cancer. The rate of growth, for a population of cells is governed by the fraction of dividing cells and how fast they divide. A single pulse of BrdU labelling provides only indirect information about these two parameters.

3.4.4.1 What conclusions can be reached from BrdU labeling alone

BrdU labelling reveals the probability that any cell in a population will be in S-phase over the injection period. The tracer can only be incorporated during S-phase of the cell cycle, so by injecting a single dose of BrdU and killing the animal 2h later, I have effectively measured an S-phase labelling index (LI) for a 2-hour
interval (LI₂₀) (Nowakowski et al., 1989b). It is clear that, during the period from E11.5-E16.5 the LI₂₀ for both TH-IR and Sox10-IR cells are almost the same (Fig. 3.6B), giving the impression that proliferation is very similar for both of these cell populations. However, before I make any assumptions about the proliferation observed in both cell populations from E11.5-E16.5, I need to ask what factors can have an effect on the LI₂₀, or in other words, what factors could alter the probability that a cell will incorporate BrdU over the 2-hour period? Four factors turn out to be important and are considered below:

1) The growth fraction (GF), which is the fraction of proliferating cells in the population, or the proportion of cells that are proliferating and not withdrawn from the cell cycle;
2) The time it takes for a cell to move completely through the cell cycle, or the cell cycle length (Tc);
3) The time it takes for a cell to move through the DNA synthesis phase, the S-phase the cell cycle (Ts); and
4) The instantaneous labelling index (LI₀), that is the fraction of cells that are in the S-phase of the cell cycle at any given instant.

The way in which these factors impact on the LI is best explained by the equation:

\[ LI_{0+} = GF \times \left( \frac{T_{s2.0}}{T_c} \right) \]

In the above equation n is the time (in hours) of the BrdU labelling period (Nowakowski et al., 1989a). So in my case, the formula would be:

\[ LI_{0+2.0} = GF \times \left( \frac{T_{s2.0}}{T_c} \right) \]

My data confirms that both Sox10+/TH- cells and Sox10-/TH+ cells have very similar LI₂₀’s from E11.5-E16.5. However, I may not assume anything else beyond the fact that the LI₂₀ is similar in each case, as BrdU labelling alone does not estimate any of the three parameters that contribute to the LI (GF, Ts or Tc). In fact, LI’s for different populations can be similar, but the factors underlying the LI, the GF, Tc and Ts, may vary significantly. While differences in the labelling
index do indicate that there are differences in proliferation, the LI does not identify what parameter has changed to bring about a change in the LI. To fully understand proliferation, I need to identify the factors that govern proliferation and underlie the LI (the GF, $T_c$ and $T_s$), and until I do so my description of proliferation in sympathetic ganglia will remain incomplete.

**3.4.5 Methods to measure the GF, $T_s$ & $T_c$**

In this section I will discuss different methods that can be used to identify the GF, $T_c$, $T_s$ and LI and, in doing so, I will establish the most appropriate way to measure proliferation in the mouse sympathetic ganglion. These techniques will form the basis for the next chapter.

**3.4.5.1 Estimating the GF**

In order to understand and compare changes in proliferation, the GF of the cell populations of interest must be determined. The GF represents the proliferating proportion of cells out of the total cell population. In other words, the GF is the percentage of cells in a population that are not in G0 (withdrawn from the cell cycle). There are many ways to estimate the GF and choosing the most appropriate method is essential. However, the most appropriate method depends on the experimental paradigm, the tissue that is being examined and the overall questions being asked. There are 4 different methods that can be used to calculate the GF. These will all be considered before I determine the best method for use in the developing sympathetic ganglia.

**3.4.5.1.1 1) Using an antibody directed against a proliferation specific antigen**

There are a number of proteins that occur only in proliferating cells. To calculate the GF I can use an antibody directed against one of these antigens, but it must satisfy two criteria; 1) the antigen must be expressed during all proliferative stages of the cell cycle (G1, S, G2 and M), and 2) the antigen must not be expressed when the cells are withdrawn from the cell cycle in G0, when the cell is quiescent and not proliferating. Two antigens that satisfy these criteria are proliferating cell nuclear antigen (PCNA) and Ki67 (Eisch and Mandyam, 2007). In my experimental paradigm, I want to compare and identify the GF's during development at consecutive stages that are only 24 h apart. PCNA has a very long
half-life $\sim 24h$, in contrast to Ki67, which has a very short half-life of only about an hour (Eisch and Mandyam, 2007). Given that Ki67 has such a short half-life an antibody directed against Ki67 would be better for my application. The long half-life of PCNA means that a cell could withdraw from the cell cycle but still be positive for PCNA 24 h or more later. In addition, PCNA has been identified in quiescent (non-cycling) cells that are repairing DNA (Eisch and Mandyam, 2007). This is unlikely to be of much significance during early development, however, it potentially could introduce some experimental error (Eisch and Mandyam, 2007).

In contrast, the short half life of Ki67 makes it ideal to use when the time points to be sampled are 24h apart. Ki67 is also suitable for my application because it has been combined successfully with immunofluorescence (Hendershot et al., 2008, Armstrong et al., 2011), and in this study with immunofluorescence against Sox10 and TH, enabling the GF of both cell populations to be determined simultaneously. Finally, using Ki67 gives a measure of the instantaneous GF at the instance the embryo was fixed.

3.4.5.1.2 2) Calculate the GF using a cumulative labelling strategy

Another way to determine the GF is to identify the absolute loading fraction of a population of cells. This can be done through a cumulative labelling paradigm. If a single injection of a tracer such as BrdU is administered at time 0 and the cells harvested one hour later, then $X_1$ number of cells will be labelled. If two injections of BrdU are administered; the first at time 0, the second 1 hour later and the cells are harvested 1 hour after the second injection, the number of cells labelled will be $= X_1 + X_2$. This can be repeated so that the number of cells labelled is $= X_1 + X_2 \ldots + X_n$ (where $n =$ the total number of hourly injections administered). Each consecutive injection will label any cells that enter S-phase in that hour, until all dividing cells in the population have been labelled. At this point, when all cycling cells are labelled, no new cells will be identified by another injection. The proportion of BrdU labelled cells is then the GF for that population. This method has been effectively utilised to identify the GF’s of neural progenitors in the CNS (Nowakowski et al., 1989b). However, this protocol would not be practical in my system as acquiring the growth fraction via
a cumulative labelling protocol as it is likely that the total cumulative labelling period will exceed 12h. My objective is to compare and contrast the instantaneous growth fractions on consecutive 24 h intervals over development. A cumulative protocol would yield a GF over a time when the GF itself could changing rapidly, so that the resultant number is difficult to relate to any point in that period, a significant problem when the sampling interval is only 24 h.

3.4.5.1.3 3) **Using a flow cytometer and FACS to determine the GF**

Fluorescent-Activated Cell-Sorting (FACS) using a flow cytometer, can sort cells that have had their DNA fluorescently marked based the intensity of fluorescent nuclear staining. The fluorescence intensity correlates with the various stages of the cell cycle and so various cell cycle parameters determined. However, there are problems with the potential use of this protocol for my studies. One problem is the fact that there needs to be a large number of cells for the FACS process to work accurately. In the early stages of SNS development there are but a handful of cells that are very fragile, so, extracting enough cells to perform FACS sorting would be extremely difficult. The other reason why the FACS process would not be practical for my use is that it would require the cells to be sorted twice. First, for the cell type using some kind of cell surface marker, and second by the intensity of nuclear staining, to determine the stage of the cell cycle. At this point I do not have access to reliable cell surface markers that can separate the neuronal population from the glial population in my samples.

3.4.5.1.4 4) **Using genetically modified mice**

Finally, it is possible to identify the GF of mice that have been genetically engineered so that they produce fluorescent proteins that are differentially expressed in cells as they pass through different stages of the cell cycle (Sakaue-Sawano et al., 2008). In these mice, cells that are in G1 express red fluorescent protein, cells in S/G2/M express green fluorescent protein and, consequently, upon the transition from G1 to S, they express a mixture of the two and are visualised as yellow. It would be possible to determine a GF by identifying all of the fluorescent cells as a fraction of the total cells for any given population. This would be an excellent way to get an instantaneous growth fraction and would be
a good tool if I were dealing with a homogenous population of cells. However, I am not. In the developing stellate ganglion, there are multiple cell populations that are not anatomically segregated. Therefore, I would need to use these genetically modified animals in conjunction with antibodies directed against cell specific antigens, such as TH or Sox10, to identify the GF’s for neuroblasts and glioblasts respectively, as well as a nuclear marker. Given that the genetically engineered mice already use 2 of the possible fluorescent channels, combining any additional fluorescent markers would be difficult. In addition, these animals are not yet widely available. Although these mice are likely to be a valuable tool for identifying cell cycle parameters, they are not ideal for use in my study.

3.4.5.2 Estimating the $T_c$ requires an estimate of the $T_s$ in vivo

There are only a limited number of ways to measure the cell cycle length ($T_c$) in vivo. One way is to image living cells using time-lapse photography. This requires fluorescent tagging of the cells (using a GFP reporter for instance) and some form of organotypic culture to image a mouse stellate ganglion. The other way to estimate the cell cycle length in vivo is by using the relationship $L_{10} = GF \times \left(\frac{T_s}{T_c}\right)$. As long as I can determine the LI, the GF and $T_s$, I can re-arrange the formula so that $T_c = T_s \times \left(\frac{GF}{L_{10}}\right)$ and solve for $T_c$ (Nowakowski et al., 1989a).

BrdU labelling measures the LI, I have discussed several methods for measuring GF and so that leaves only the measurement of $T_s$. A method for calculating the $T_s$ in vivo, using a double labelling protocol with two S-phase markers already exists (Hayes and Nowakowski, 2000b). The method used in this study been described in the materials and methods section 2.5.1.

My situation has an additional level of complication that is not a problem for studies in the CNS. In the CNS, the proliferating neural progenitors have been identified solely by their anatomical position in relation to the ventricular surface (Nowakowski et al., 1989a, Hayes and Nowakowski, 2000b). The fact that these proliferating precursors are assumed to be a homogenous population of cells that are confined to a specific anatomical region means that the double-labelling protocol alone is sufficient to determine the $T_s$. I do not have the same conditions in the developing SNS. Although the cells of the developing sympathetic ganglion
are anatomically defined, they are not a homogenous population of cells. I need to identify the different homogenous populations within the ganglion as well as measure the $T_s$ of these individual populations. To do this, I will need to combine the double-labelling protocol (described in section 2.5.1) with immunofluorescence against cell the specific markers Sox10 or TH, to identify the $T_s$ in specific cell populations.

Recently, an alternative thymidine analogue, 5-ethyl-2’-deoxyuridine (EdU), has been developed and is easily detectable through fluorophore labelling using a copper-mediated covalent reaction (Salic and Mitchison, 2008). EdU is detected with the same efficiency as BrdU and is becoming the preferred technique to identify cells that are in S-phase. Both EdU and BrdU, can be independently detected and also be combined with immunofluorescence (see methods section 2.5.1). This allows me to calculate the $T_s$ of the different populations of cells, identified by specific immunofluorescence, simultaneously and in vivo.

**3.4.5.3 Estimating and instantaneous LI ($LI_{0}$)**

In this study I have already estimated the $LI_{2.0}$ for both the Sox10+/TH- (glial progenitor) and Sox10-/TH+ (neuronal precursor) populations of cells. However, there is some inherent error in the measured $LI_{2.0}$. BrdU is injected into the pregnant dam at time 0 ($T_0$). It takes time ($T_x$) before the BrdU is available at sufficient concentration for incorporation by cells in the embryos (Hayes and Nowakowski, 2000b). During the period $T_x$ (the time necessary for sufficient concentration of the tracer to become available to S-phase cells in the embryos), some cells that were in S-phase, when the pregnant dam was injected at $T_0$ may already have progressed through S-phase to G2, before the tracer is available for uptake. These cells will have been in S-phase during the BrdU-pulse but will not incorporate the tracer. So, the proportion of cells labelled in the estimation of $LI_{2.0}$’s will always be less than the true proportion of cells that should be labelled in a $LI_{2.0}$. I will refer to this error as the $T_x$ error. This means if I use my $LI_{2.0}$ (in conjunction with my estimates of GF, and $T_s$) in the relationship $LI_n = GF \times (T_{s+n}/T_c)$, I will always underestimate the $T_c$. To resolve this issue I need to be
able to calculate the true instantaneous labelling index ($LI_0$) at $T_0$, when the pregnant dam was injected. This can be achieved in either one of 2 ways:

3.4.5.3.1 Using a flow cytometer and FACS to determine the $LI_0$

FACS of cells labelled with a fluorescent DNA marker can be used to identify the fraction of cells that are in the S-phase of the cell cycle in a particular sample. However the problem still exist of getting enough cells from embryonic ganglia and finding reliable cell cycle markers so this is not an option if I wish to obtain these measurements for SNS development.

3.4.5.3.2 Using a cumulative labelling protocol

I have used a 2-hour pulse of BrdU to identify the $LI_{2.0}$ for separate populations of dividing cells. If I had used a 1-hour pulse of BrdU then I would have observed fewer labelled cells and the $LI$’s would be smaller compared to the 2-hour experiment. It is also true that if I had used a 3-hour pulse the $LI$ would be larger and that more cells would be labelled with the tracer. This seems obvious, but it is important to note that the $T_x$ error can only have its impact once. So, if I compare the proportion of cells labelled in a 1-hour versus, a 2-hour or a 3-hour labelling interval, the incremental increase in the number of cells for each extra hour will be higher than the first hour but no different for every hour after the first. If I plot the proportion of cells labelled against time, for different timed pulses of BrdU injections, and fit a least of squares best fit, I can extrapolate the measured $LI$ to time 0 (Nowakowski et al., 1989b). This would give the true instantaneous labelling index ($LI_0$ see methods section 2.5.3). The advantage of using this cumulative labelling approach is that I can get two time point measurements using the same double-labelled mice that are used to calculate the S-phase lengths (at 0.5 h and 2.5 h). In order to accurately estimate the instantaneous $LI$, I will need at least a third point which will need to be measured on a separate group of animals (see methods section 2.5.3).
3.5 Chapter 3 figures
Figure 3-1: NCCs differentiation in the ganglion primordia at E10.5

A) Tile scanned transverse plane section of an E10.5 mouse embryo at the level of the forelimbs, showing Sox10 immunofluorescence in green, TH in red and Bisbenzimide (Bisb) in green (bar 100µm); B-E) Single plane confocal image showing the developing stellate ganglion. The asterisk (*) indicates cells that are Sox10+/TH+, arrow heads for cells that are Sox10+/TH- and the º showing cells that are positive for Sox10-/TH+. (NT) neural tube, (Ntc) notochord, (Sn) spinal nerve, (DA) dorsal aorta, (Spg) sympathetic ganglion and (ENS) enteric nervous system (bar 10µm)
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E10.5 Sox10 (green) TH (red)

A

B

C

D

E

E10.5

Sox10

TH

Sox10 & TH

Bisb
Chapter 3: Proliferation in the developing SNS: The limitations of using BrdU
Chapter 3: Proliferation in the developing SNS: The limitations of using BrdU
Figure 3-2: Change in morphology of the developing StG

Single optical plane confocal images of transverse sections, all at the same scale. Sections were processed for Sox10-IR (green), to identify glial progenitors, and TH-IR (red), to identify neuronal precursors. All images are of the developing stellate ganglion at the level of the forelimbs; A) E11.5, B) E12.5, C) E13.5, D) E14.5, E) E16.5 and F) E18.5 bar 20µm
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E11.5 to E18.5 Sox10 (green) TH (red)
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Figure 3-3: BFABP is expressed in Sox10+/TH- glial progenitors after E12.5

Z-projections of transverse sections through developing stellate ganglia; immunofluorescent for Sox10 (green) and BFABP (red). A-F) Stellate ganglia from E12.5 and E16.5 embryos, the BFABP staining becomes more prevalent in the processes of glial progenitors as development goes on. At E12.5 cells are located around the periphery of the developing ganglion. This is lost by E16.5 (bar 20µm). G-I) E18.5 high power image showing that BFABP staining persists late in development and is co-localised with Sox10 (bar 10µm)
Chapter 3: Proliferation in the developing SNS: The limitations of using BrdU

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**Sox10**

**BFABP**

**Sox10 & BFABP**
Chapter 3: Proliferation in the developing SNS: The limitations of using BrdU
Figure 3-4: Changes in proportions of cell populations in the developing StG

Percentage changes in the proportions of Sox10+/TH- NCCs and glial progenitors, Sox10-/TH+ neuronal precursors and Sox10+/TH+ differentiating NCCs, during the course of development in the developing stellate ganglion.

Figure 3-5: Example of the 4 channel labeling protocol

A-C) Transverse section through an E13.5 stellate ganglion at the level of the forelimbs. A) Showing IR for both Sox10 (purple) and TH (red). B) Sox10, TH and BrdU-IR (Green). C) Sox10, TH, BrdU & bisbenzimide (blue). Bisbenzimide was used to identify nuclei and was used as a counting aid. Scale bar for A-C 20µm
Figure 3.4:

% of cell populations / total cells

Sox10-/TH+
Sox10+/TH-
Sox10+/TH+

Developmental Stage

Figure 3.5:

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<thead>
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E13.5
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Chapter 3: Proliferation in the developing SNS: The limitations of using BrdU
Figure 3-6: The proportions of dividing cells

A) Proportions of dividing cells as a percentage of total cells. A) BrdU positive cells (black), BrdU negative cells (white) both expressed as a proportion of total cells.

- **i)** Sox10-/TH+/BrdU+ cells (red) and Sox10-/TH+/BrdU- (grey red outline) expressed as a proportion of total cells in the ganglion;
- **ii)** Sox10+/TH-/BrdU+ cells (purple) and Sox10+/TH-/BrdU- cells (grey purple outline) expressed as a percentage of total cells in the ganglion; and
- **iii)** Sox10/TH/BrdU positive cells (blue) and Sox10/TH/BrdU negative cells (grey blue outline) expressed as a proportion of total cells in the ganglion.

Note that the percentages in i), ii) and iii) when added together are equal to the percentages given in A.

B) The relative proportions of dividing cells. Cells expressed as relative proportions to compare the relative changes in BrdU uptake.

- Sox10-/TH+/BrdU+ cells (solid red bars) and Sox10-/TH+/BrdU- (grey bar outlined by red line).
- Sox10+/TH-/BrdU+ (Solid purple bars) and Sox10+/TH-/BrdU- (grey bar outlined by purple line).
- Sox10+/TH+/BrdU+ (solid blue bar) and Sox10+/TH+/BrdU- (grey bar outlined by blue line).

Significance calculated with students T-tests * indicating significance; * when p<0.05, ** when p<0.01 and *** when p<0.001
Chapter 3: Proliferation in the developing SNS: The limitations of using BrdU

A. BrdU+ / Total cells

B. Proportional data for dividing cells

**Table:**

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Chapter 3: Proliferation in the developing SNS: The limitations of using BrdU
Chapter 4

4 Proliferation and cell cycle dynamics in the developing SNS
Chapter 4: Proliferation and cell cycle dynamics in the developing SNS
The following chapter, Chapter 4, Proliferation and cell cycle dynamics in the developing mouse stellate ganglion, (including limited parts of Chapter 5) has been submitted as a full paper to the Journal of Neuroscience. The author line is DG Gonsalvez, KN Cane, KA Landman, H Enomoto, HM Young and CR Anderson. I am claiming 80% of the experimental work, the remainder shared by my supervisors, Kylie Cane and Colin Anderson. Professor Kerry Landman has carried out 100% of the mathematical modelling, Professor Hideki Enomoto and Dr Heather Young helped interpret the results. All authors contributed to the writing of the manuscript, of which I am claiming 80%.

Adjustments have been made to the submitted manuscript to adapt it for inclusion in this thesis
4.1 Introduction

Although cell division underlies the growth of the developing nervous system, the dynamics of cell proliferation have only been extensively studied in the mouse cerebral cortex (Takahashi et al., 1997, Caviness et al., 2003, Mitsuhashi and Takahashi, 2009). In the cortex, neural stem cells divide for up to eleven cell cycles and generate waves of neurons and, later, glial cells progressively populate the forming cortical layers (Takahashi et al., 1999). Stem cell cycle length increases with developmental age, due to a lengthening of G1 (Takahashi et al., 1995). Neural stem cells are progressively lost as they exit the cell cycle (Mitsuhashi and Takahashi, 2009), which is thought to be the trigger for differentiation of neurons and glia (Cremisi et al., 2003).

In sympathetic ganglia, a well studied and accessible system for understanding neuronal development, commitment to the neuronal lineage does not immediately lead to withdrawal from the cell cycle (Rothman et al., 1978, Rothman et al., 1980, Rohrer and Thoenen, 1987). Instead, neuronal precursors differentiate from NCCs and continue to divide, while expressing a variety of neuronal markers. Although the differentiation program leading to a noradrenergic neuronal phenotype has been extensively studied, what happens to the cell cycle dynamics of these NCCs as they undergo differentiation has not been studied at all. After initial differentiation of NCCs in sympathetic ganglia of embryonic mice, division of existing neuronal precursors, rather than division of stem cells, drives the increase in the numbers of sympathetic neurons (Rohrer, 2011). However, we do not have any information about the cell cycle dynamics of these cells either. Our only understanding about the cell cycle kinetics in the developing SNS comes indirectly from studies in the chick, where it was shown that neuronal precursors, identified by formaldehyde-induced fluorescence, were capable of dividing in excess of three times over a three day interval (Rothman et al., 1978). No equivalent information exists for murine SNS development. There is no information about the proliferation of NCCs prior to the acquisition of
neuronal phenotype, and there is no information about cell cycle dynamics of the glial progenitor cells.

Understanding how proliferation proceeds and is regulated is important because sympathetic ganglia give rise to the embryonic cancer, neuroblastoma, where the control of proliferation is lost (Maris et al., 2007, Janoueix-Lerosey et al., 2010, Jiang et al., 2011). Neuroblastoma is a developmental pathology and understanding the genesis of this tumor requires understanding the normal progress and regulation of sympathetic ganglion growth in the embryo. Genes that regulate sympathetic ganglion development, such as Phox2b (Reiff et al., 2010) and the anaplastic lymphoma kinase receptor (Reiff et al., 2011), are mutated in familial forms of the disease. A number of molecules, including Ret (Enomoto et al., 2001), GDNF and artemin (Nishino et al., 1999), frizzled-3 and β-catenin (Armstrong et al., 2011) and Sox11 (Potzner et al., 2010), are all thought to regulate proliferation in the developing sympathetic ganglion. However, without a detailed understanding of how changes in cell cycle length and cell cycle withdrawal contribute to normal growth, it is impossible to determine precisely how these signalling pathways regulate the number of cells in sympathetic ganglia or contribute to the genesis and progress of neuroblastoma.

In the previous chapter I discussed experimental techniques to identify the necessary parameters to determine: the instantaneous labelling index (LI₀), S-phase lengths (Tₛ), growth fractions (GF) and cell cycle lengths (Tc), of TH+ neuronal precursors and of Sox10+ NCCs or glial progenitors. I have applied these techniques here and, for the first time, have systematically measured the in vivo LI₀, Tₛ, GF and Tc for the cell types mentioned above. These data have subsequently been used to develop a mathematical model of normal ganglionic growth, which can be used in future experiments to understand how growth will be affected when normal development is perturbed.
4.2 Materials and methods

For materials and methods refer to Chapter 2, in particular sections 2.5, 2.6 and 2.7.

4.3 Results

4.3.1 Changes in the GF

To identify the GF of NCCs or cells in the neuronal or glial lineage, I used quadruple fluorescence labelling, for combinations of either Ki67, Sox10 and Phox2b, or Ki67, Sox10 and TH (see section 2.5.2) and the fluorescent nuclear marker, bisbenzimide. Ki67 is present in the nucleus of cycling cells during all stages of the cell cycle, but is absent from cells in G0 (Ross and Hall, 1995, Scholzen and Gerdes, 2000). I have investigated Phox2b in addition to TH as it is a transcription factor that is expressed early in developing neuroblasts, before TH, and so may mark the first stage of differentiation of NCC.

4.3.1.1 Change in proportions and GF’s from E9.5 to E12.5

We first observed how the GF changes as NCCs give rise to neuronal precursors or glial progenitors. E9.5-E10.5 is the developmental period when murine NCCs coalesce into the ganglion primordia and first express markers of neuronal noradrenergic differentiation (see section 1.5 and Goridis and Rohrer, 2002, Rohrer, 2011). At E9.5 when Sox10+ NCCs have coalesced into the ganglion primordia, 23% are positive for Phox2b, indicating that some NCCs have started to differentiate (Figures 4.1B and 4.2A-B). At E9.5, all Sox10+ cells are Ki67+, so the initial expression of Phox2b does not appear to affect the GF, which remains at 100% (Fig. 4.2A-D and Fig. 4.4). No TH-IR was detected at this age. Sox10+ migrating NCCs cells between the dorsal root ganglia and stellate ganglion did not show detectable levels of Phox2b-IR, but were all positive for Ki67 staining.

By E10.5, most of the NC progenitors had up-regulated the expression of Phox2b and were Sox10+/Phox2b+, with only ~2% of the total cells in the ganglion Sox10+/Phox2b - (Fig. 4.1B, Fig. 4.2E-H). Phox2b is needed for the
expression of TH and expression remained on in TH+ cells (Coppola et al., 2010). Double-labelling for Sox10 and TH showed that 34% of the cells in the ganglion had up-regulated the expression of TH, 19% were Sox10+/TH+, while the remaining 15% had lost the expression of Sox10 and were Sox10-/TH+ (Fig. 4.2I-L and Table 4.1). In the previous chapter, 47% of the cells at E10.5 were TH+, the differences are largely due to the subtle differences in the age of embryos that can vary by ~0.25-0.5 of a day. At E10.5, Ki67 labelling revealed that virtually all of the TH+ cells have withdrawn from the cell cycle and were Ki67- (Fig. 4.2I-L, Table 4.1, and 4.4). However, the Sox10+/TH- cells are cycling with a GF of 79% (Fig. 4.4, Table 4.1 and Fig. 4.2J-L). So, upon acquisition of a neuronal phenotype, marked by the expression of TH, neuronal precursors lack the expression of Ki67, indicating they have withdrawn from the cell cycle (Fig. 4.2I-L and Table 4.1).

At E11.5 almost all of the cells in the ganglion have re-entered the cell cycle with the GF ≥ 90% in both populations (Figure 4.4). The GF remain ≥ 90% at E12.5, again with no apparent difference in the GFs of either Sox10-/TH+ or Sox10+/TH- (Figure 4.4). It is important to note that for TH+ cells, there is a dramatic increase in the growth fraction from E10.5, where virtually all the cells are withdrawn from the cell cycle and the GF is ~0%, to E11.5, where over 90% of the cells are now cycling (Figure 4.4).

From E9.5 to E10.5, in the developing murine SNS, there is no evidence of the appearance of a glial phenotype in any cells. However, BFABP, a glial marker, is expressed by most of the Sox10+/TH- cells at E11.5 (Callahan et al., 2008) and virtually all of the Sox10+/TH- cells by E12.5 (see chapter 3 Fig. 3.3). Thus, from E12.5 and, for the most part on E11.5, a Sox10+/TH- cell is almost certainly a glial progenitor. There is no evidence for a temporary withdrawal from the cell cycle on E11.5, when these cells start to express the glial marker, BFABP. It is possible that they, like the Sox10+/TH+ cells, also withdraw from the cell cycle on E10.5, as the growth fraction of the Sox10+/TH- cells drops to 79%, but this may reflect cells differentiating down the TH+ neuroblast lineage, which have withdrawn from the cell cycle before they have detectable levels of TH.
4.3.1.2 Change in proportions and GF’s from E12.5 to E18.5

At E12.5, over 85% of cells were TH+ neuronal precursors and virtually all of these cells are again positive for Ki67 (Fig. 4.1A, 4.2M-O and 4.4). From E12.5 onward, TH+ cells make up a decreasing proportion of the cells in the ganglion and the GF decreases rapidly, so that by E18.5, only 2% of these cells are cycling and positive for Ki67 (Fig. 4.4). The proportion of Sox10+/TH- cells decreased between E11.5 and E12.5 (to 10% of the total – Fig. 4.1A), but thereafter increased (Fig. 4.1A). The GF for the Sox10+/TH- glial progenitors decreased from 90% at E12.5 and to 26% at E18.5 (Fig. 4.4).

Sox10+/TH+ cells were very rare after E12.5 (Fig. 4.1A), as are Sox10+/Phox2b+ (Callahan et al., 2008), but persistently made up around 1% of cells on each embryonic day examined. However, there were not enough of these cells to estimate a GF for the Sox10+/TH+ after E10.5.

4.3.2 Changes in cell cycle kinetics

Proliferation was measured by adapting methods from previous studies on proliferation in the cortex (see chapter 2 and - Nowakowski et al., 1989a, Hayes and Nowakowski, 2000b). Quadruple-labelling involving two S-phase markers (BrdU and EdU) combined with nuclear staining with bisbenzimide and immunofluorescence for Sox10, or TH was performed (Figure 4.3A-H). The use of two S-phase markers injected with a two hour interval allowed the calculation of the length of S phase, and combined with the single 2 h pulse data from the previous chapter, enabled the calculation of the LI₀ (see section 2.5.3 in chapter 2). This was done for Sox10+ cells at all ages and TH+ cells, from E10.5 onwards. After E11.5, there is a small population ~1% of cells that are Sox10+/TH+, these cells would have been included into the Sox10+ cell counts when identifying the GF, but are so few in number that they do not significantly affect any counts. Prior to E10.5 we cannot detect any TH-IR. In addition to this, at E10.5, we do not detect BrdU/EdU labelling as Sox10+/TH+ and Sox10-/TH+ cells are withdrawn from the cell cycle, so, our first data point for the TH+ population is E11.5, when the TH+ cells have re-entered the cell cycle and are highly proliferative. Together, the LI₀ and T₅ could be combined with the GF to calculate the overall Tᵥ for all developmental ages examined (see chapter 2). These data are summarised in Fig
4.4. We were unable to examine cell cycle length of Phox2b+ cells, as the Phox2b immunostaining did not survive the acid treatment required to reveal BrdU-IR. However, after E11.5 Phox2b protein is only co-expressed in cells positive for TH and is therefore equivalent to the Sox10-/TH+ population.

4.3.2.1 Change in cell cycle dynamics from E9.5 to E10.5

At E9.5, all Sox10+ cells in the migratory stream between the neural tube and dorsal aorta and within the ganglion primordia itself, were highly proliferative and had a cell cycle length of only 10.6 h, which was the shortest cell cycle length of any cell type examined in this study (Fig 4.4).

At E10.5, a cell cycle length was calculated for Sox10+ cells, which included both Sox10+/TH- and Sox10+/TH+ cells. As 95% of Sox10+/TH+ cells have withdrawn from the cell cycle but would have contributed to calculating the cell cycle length, we adjusted the number of Sox10 cells recorded as not being in S-phase by subtracting the proportion of Sox10 cells that co-express TH at E10.5 so that the calculated cell cycle length is representative only of Sox10+/TH- cells. The adjusted cell cycle length of Sox10+/TH- cells had increased to 30.0 ± 4.3 h, significantly different from that at E9.5 (one way ANOVA and Tukey’s post hoc test, F7,30=5.5, p=0.001). Between E9.5 and E10.5, S-phase length of Sox10+/TH- cells decreased significantly from 7.2 h to 4.1 ± 0.7h (one way ANOVA and Tukey’s post hoc test, F7,30=8.2, p=0.0001). The change in the proportion of the cell cycle occupied by S phase between E9.5 and E10.5 was striking and significantly decreased between E9.5 and E10.5 from 65% (±5.6%) to 14% (±2.2%, unpaired t test, t= 11.10, df= 9, p < 0.0001).

4.3.2.2 Change in cell cycle dynamics from E11.5 to E18.5

At E11.5 virtually all of the cells in the ganglion had re-entered the cell cycle with the GF over 90% (Fig. 4.4) for all cell types. For Sox10-/TH+ neuroblasts, the S-phase lengths of around 7 h, and total cell cycle lengths of around 20.7 h, did not significantly change (one way ANOVA and Tukey’s post hoc test, F7,30=5.5, p=0.001) from E11.5 to E16.5. The number of dividing cells at E18.5 is too few to accurately measure their cell cycle kinetics.
The cell cycle dynamics for the Sox10+/TH- population was quite different to that of the Sox10-/TH+ cells. At E11.5, the $T_c$ for Sox10+/TH- cells was 16 h, this decreased slightly (but was not significantly different) at E12.5 to 14 h, and from E12.5 increased so that by E18.5 the cell cycle lengths were 37 h. Overall, between E9.5 and E18.5, Sox10+/TH- cells showed a significant lengthening of S-phase length from 7.2 h at E9.5 to 16.7 h at E18.5 (one way ANOVA and Tukey's post hoc test, $F_{7,30}=8.2$, $p=0.0001$) and total cell cycle length (one way ANOVA and Tukey's post hoc test, $F_{7,30}=5.5$, $p=0.001$) from 10.6 h at E9.5 to 37.0 h at E18.5 (Fig. 4.4).

As a control, we examined the effect of decreasing the period of BrdU incubation as if the BrdU incubation period (2.5 h) is too long relative to the S-phase length, then a cell that incorporates BrdU in S-phase early in the period of exposure to BrdU could progress through metaphase within that period and so appear as two cells (see section 6.6.1 and Fig. 6.1). At E12.5, the age with the shortest S-phase, shortening the BrdU incubation to 1.5 h gave similar S-phase lengths for Sox10+ cells to the 2.5 h BrdU incubation (1.5 h, S-phase 3.6 h vs 2.5 h, 2.9 h, unpaired t-test, two tailed, $t=1.41$, $df=4$, $p=0.23$, $n=3$). S-phase length in the TH+ cells was similarly unaffected (1 h, 6.2 h vs 2h, 6.8, unpaired t-test, two tailed, $t=1.94$, $df=6$, $p=0.66$, $n=3$).

### 4.3.3 Modelling

We examined whether a mathematical model based on the cell cycle parameters measured could accurately predict the changes in proportions of cell types shown in Figure 4.1. Initially, we modelled ganglionic growth from E12.5 to E18.5. During this period, 98% of the Sox10+/TH- cells in the mouse stellate ganglion also express the glial precursor marker, BFABP (Callahan et al., 2008), and therefore there are essentially only two populations of cells; Sox10+/TH- cells (glial progenitors) and Sox10-/TH+ cells (neuronal precursors). As Sox10+ cells lacking BFABP expression (presumed stem/progenitors) comprise a maximum of 2% of total cells between E12.5-E18.5 (Callahan et al., 2008), TH+ cells were assumed to arise only from the division of pre-existing TH+ cells in the model. Using the E12.5-E18.5 cell cycle length and growth fraction data, and
starting the model from the observed proportions at E12.5, led to a very close fit for predicted cell proportions to observed proportions over the three succeeding time points (Figure 4.5B). It is important to note that 3 key assumptions are built into the model: 1) we assume a differentiation rate that results in a fit through the proportions observed at E11.5; 2) that the model accounts for all of the cells in the ganglion during the developmental period modeled; and 3) that cell death is insignificant over the period from E10.5-E18.5 (Fagan et al., 1996).

The sensitivity of the model to potential systematic errors in our measured parameters was then explored. Doubling the cell cycle length or halving the growth fraction lead to a large mismatch between observed and modelled values (Figure 4.5B, dashed lines). Halving the cell cycle length or doubling the growth fraction also perturbed the match, but to a lesser extent (Figure 4.5B, dotted lines). While the fit of calculated to observed values could be improved very slightly by arbitrary adjustments in parameters, for instance increasing the starting proportion of neuroblasts at E12.5 by 1%, the adjustment needed was always very small, suggesting the initial model was quite accurate.

To explore ages earlier than E12.5, a significant additional population of cells, NCC progenitors, had to be included in the model. Sox10+/TH- cells include both NCC progenitor cells and glial precursors, which can be identified by the expression of BFABP. The model assumes that at E9.5, all cells were NCC progenitors, which, with time, both proliferated and differentiated. Proliferation rates were based on the cell cycle lengths measured for Sox10+ cells at E9.5 and E10.5. We modelled the differentiation of NCC progenitors (“p”) by including fixed probabilities that the progenitors would differentiate into neuroblasts from E9.5 and glial progenitors only after E11.0, as BFABP is first detected at E11.5 in the mouse stellate ganglion (see methods section 2.7 - Callahan et al., 2008). The differentiation probabilities are the only values used in the model that were not derived directly from observation and were selected on the basis that they gave rise to the observed proportions of the two main cell types at E12.5. The model started from E9.5 and generated very similar changes in the relative proportions
of cell types to those we observed in developing mouse stellate ganglia from E12.5-E18.5 (Figure 4.5A).

The selected differentiation rate for NCC progenitors was 10 h. That is, a NCC progenitor would differentiate into a neuroblast (TH+ cell) on average every 10 h. After E11.0, NCC progenitors could also give rise to glial progenitors, on average every 22 h. These values were determined by trial and error and gave the closest fit to the proportions from E12.5 onwards. For neuroblasts, as the differentiation rate is close to the measured cell cycle length (10.6 h) there is an important consequence. In the model, NCC progenitors increase in number up until the time they start to differentiate into neuroblasts (E9.5 in our model). At this point, because of the similarity between cell cycle rate and differentiation rate, their overall numbers remain static because, on average, one of the daughter cells differentiates after each division. Therefore, from E9.5 up to E11, for each 10 hour period the behaviour of a NCC progenitor approximates the typical asymmetric division of a neural stem cell, with a probability of 0.5 in turning into either another neural crest progenitor or into a neuroblast. After E11.0, Sox10+/TH- NCC progenitors were also permitted to differentiate into a glial progenitor as well as a neuroblast. Hence, over a 10 h period (approximately), our model predicts a NCC has the probability of 0.4 of turning into a NCC, 0.4 into a neuroblast and 0.2 into a glial progenitor. This results in a decrease in the proportion of NCC progenitors in the ganglion to very low levels around E12.5, which matches the observation of very few Sox10+/BFABP- cells (0.3%) present at E12.5 (Callahan et al., 2008).

The number of neuroblasts and glial progenitors present at any time were proportional to the initial number of NCC progenitors \((p_0)\) present at E9.5. The model results determined the total number of cells \((p + n + g)\) at E18.5 to be equal to \(81.47p_0\). Therefore, if the total count is 41,906 cells in E18.5 WT ganglia (from stereology), the model predicted that the number of NCC progenitors present at E9.5 is \(p_0 = 41,906/81.47 = 514\). Given that there are only progenitors present at E9-E9.5, their number was \(p_0e^{-\lambda p_0/2} = 235\) cells at E9.0. This number is plausible and independently suggests that the model is realistic.
4.4 Discussion

In most parts of the nervous system, differentiation of neural stem cells into neurons is accompanied by permanent withdrawal from the cell cycle. This is the first study to examine in vivo cell cycle dynamics in murine sympathetic ganglia where proliferation continues following expression of neuronal markers (Rothman et al., 1978, Rohrer and Thoenen, 1987). We show that NCCs undergo dramatic changes to their cell cycle dynamics as they differentiate into Sox10-/TH+ neuronal precursors, or Sox10+/TH-glial progenitors (Fig. 4.6). In addition to this, we have developed a model that validates our cell cycle estimations, allows us to make predictions about the starting cell number, the numbers of neuroblasts and glioblasts at any given developmental stage and will allow us to make predictions about the development of the SNS when development has been perturbed (see chapter 5).

4.4.1 Cell cycle dynamics of neuroblasts from E9.5 to E10.5

At E9.5 Sox10+/TH- NCCs have a short 10.6 h $T_c$ and these highly proliferative cells spend 63% of their time in S-phase. These values are within the range previously reported for ES cells and early embryonic cells (Mac Auley et al., 1993, White and Dalton, 2005). A day later, nearly all of the Sox10+/TH-cells have up-regulated Phox2b and many of these have gone on to express TH. At E10.5, Sox10+/TH- NCCs take 30 h to divide, spend only 14% of their time in S-phase and 80% of them are in the cell cycle. Virtually all TH+ cells (whether or not they co-express Sox10) have withdrawn from the cell cycle and are no longer Ki67+.

Previous studies have suggested that differentiation of pluripotent stem cells may be triggered by an increase in $G_1$ in an otherwise fast cycling cell (Orford and Scadden, 2008, Singh and Dalton, 2009, Salomoni and Calegari, 2010). The increase in $G_1$ is postulated to allow a signal to accumulate to levels that can change cell behaviour. The techniques needed to identify the length of $G_1$ are outside the scope of this study. However, we speculate that the three-fold
increase in cell cycle length at E10.5, is likely to be the result of an extended G1, in part at the expense of S-phase.

4.4.2 Cell cycle withdrawal and re-entry

I have two independent (and consistent) measures that point to a withdrawal, or an extreme slowing of the cell cycle at E10.5 in the Sox10-/TH+ cells: 1) The lack of Ki67 labeling; and 2) virtually non existent labeling index obtained from the BrdU experiments in Ch3. This is the first report showing that sympathetic neuroblasts undergo cell cycle arrest as they differentiate and then re-enter the cell cycle. At E10.5, withdrawal from the cell cycle is by cells that have just adopted a neuronal phenotype, marked by the expression of TH. Hence, like the CNS, cell cycle withdrawal coincides with the expression of neuronal markers, however unlike the CNS, in the SNS virtually all of the Sox10-/TH+ neuronal precursors re-enter the cell cycle and then continue to proliferate. The mechanisms that control this cell cycle exit and re-entry are unknown, but obviously are of great interest.

The only alternative explanation consistent with this data, is that there are two populations of Sox10-/TH+ progenitor cells, one of which withdraws on E10.5 and another that appears and starts to divide on E11.5. If two such groups exist, then the group of cells that differentiates early and then withdraws from the cell cycle must die, because there are too few non-cycling cells present at E11.5 and E12.5 to account for the 30% of cells that have differentiated and withdrawn on E10.5. A calculation based on Fig. 4.5D suggests that 30% of the 1333 cells present on E10.5 would be 400 cells, equivalent to 12% of the neuroblasts present on E12.5. Instead, we observe that only 1.5% of cells are not cycling at E12.5. Since a previous study has shown that there is no detectable cell death at E12.5 in the mouse, our study does not provide any evidence for multiple populations of sympathetic neuron progenitors and points directly to cell cycle withdrawal.

In both the CNS and PNS, Notch signalling has been shown to maintain progenitor status, prevent cell cycle withdrawal and prevent differentiation (Wakamatsu et al., 2000, Cornell and Eisen, 2005, Mizutani et al., 2007, Shimizu
et al., 2008, Woodhoo et al., 2009). In the developing chick SNS, Notch is expressed by migrating NCCs and Sox10+ NC progenitors in the ganglion primordia. Notch is not expressed on cells that have acquired a neuronal phenotype, marked by the expression of the neuronal marker, SCG10 (Wakamatsu et al., 2000, Tsarovina et al., 2008). In DRGs and sympathetic ganglia, Notch/Delta signalling has been shown to regulate the epistasis between the progenitor pool and neuronal precursors (Wakamatsu et al., 2000, Tsarovina et al., 2008). In the chick, the Notch+ NCC progenitors are Sox10+/SCG10-. These cells are analogous to the Sox10+/TH- cells we observe in the mouse. The Notch ligand Delta1 is expressed on chick Sox10-/SCG10+ neuronal precursors (Tsarovina et al., 2008). These are phenotypically equivalent to the Sox10-/TH+ neuronal precursors in the mouse. Delta1 binds to Notch1 that is expressed only by Sox10+/SCG10- progenitors cells and maintains their undifferentiated phenotype (Tsarovina et al., 2008). In vivo experiments in the chick have shown that the loss of Notch signalling is accompanied by an increase in initial differentiation toward a neuronal phenotype. We may speculate that, in addition to keeping Sox10+ NCC progenitors in an undifferentiated state, Notch signalling may also prevent the cell cycle withdrawal that we observed at E10.5.

Other candidates that may bring about cell cycle withdrawal are Phox2b and the zinc finger transcription factor Insm1. Ectopic, or over-expression of Phox2b in the spinal cord has been shown to induce premature cell cycle withdrawal of neuronal precursors (Dubreuil et al., 2000). However, Phox2b is expressed by more than 20% of NCCs on E9.5 but all stay in the cell cycle. It must also be expressed by almost all of the Sox10+/TH- cells on E10.5, but again most of these stay in the cell cycle. However, neural precursors begin to adopt a neuronal phenotype as a consequence of Phox2b transcription activity, and these do eventually withdraw from the cell cycle temporarily. While Phox2b may not be directly responsible for cell cycle withdrawal, it may be required to initiate events that are.

Insm1 is expressed downstream of Phox2b and is present by E10.5 in all cells that will give rise to neuronal precursors (Wildner et al., 2008). Insm1 is
part of the cascade of transcription factors that induce a noradrenergic phenotype in sympathetic neuroblasts (Wildner et al., 2008, Rohrer, 2011). It has been shown that Insm1 can bind to cyclin D1 and act as a transcriptional repressor of BHLH genes that possess E-box binding domains (Liu et al., 2006). Cyclin D1 is accumulated in G1 and, bound to CDK4 (Cyclin dependant kinase 4), is a key regulator of the G1/S transition (Liu et al., 2006). Insm1 can compete for Cdk4 binding resulting in cell cycle arrest in pancreatic islet cells and it has been shown that inhibition of Cdk’ s can cause a lengthening of G1, resulting in neuronal differentiation (Calegari and Huttner, 2003, Zhang et al., 2009). Insm1 acting via cyclinD1 is a candidate that may be involved in both the lengthening of the cell cycle and the initial cell cycle arrest of sympathetic neuronal precursors.

BMP signalling is necessary for both proliferation and differentiation of NCCs into noradrenergic neuronal precursors (Rohrer, 2011). In the primitive node, signalling via the BMP receptor Acvr1 (Alk-2) results in cell cycle withdrawal that is identified by the loss of Ki67 staining (Komatsu et al., 2011). This withdrawal in proliferation is necessary for the proper differentiation of the primary cilium (Komatsu et al., 2011). In mice, where Acvr1 has been conditionally removed, cells do not properly withdraw from the cell cycle resulting in incomplete differentiation of the primary cilia in the nodal cells (Komatsu et al., 2011). The Acvr1 (Alk-2) receptor is expressed by NCCs (Kaartinen et al., 2004), so, components of the BMP signalling pathway(s) may also be candidates capable of mediating the cell cycle withdrawal observed in the TH+ cells at E10.5. It is known that the dorsal aorta releases BMP2 in vivo (Shah et al., 1996), also that BMP2 has a spacio-temporal expression pattern that may identify it as a potential signal, generated in the local environment that may bring about the temporary hiatus (Danesh et al., 2009). However currently this is just speculation, other than BMP2, there are no obvious external signals that have the spacio-temporal expression pattern to suggest that they may be responsible for the triggering cell cycle withdrawal, although one may exist.

There is nothing in the existing literature that points to potential mechanisms that would induce the re-entry to the cell cycle. I pointed out, in
section 1.8.4.2, that there was a large reduction in proliferation observed in the 
Sox11\textsuperscript{-/-} mice (Potzner et al., 2010). Interestingly, tissue specific deletion of Sox11 
from the NCCs in the Wnt1-Cre:Sox11\textsuperscript{flox/flox} mice, did not result in any major 
proliferation defects (Potzner et al., 2010). It is known that cells of the dorsal 
aorta and the tissue surrounding the ganglion primordial express Sox11. In the 
Sox11\textsuperscript{-/-} mice, these cells are likely to be affected suggesting that the proximal 
environment may be critical to the re-entry into the cell cycle, given that NC 
tissue specific deletion of Sox11 does not appear to affect proliferation.

4.4.3 Development of CNS compared to the SNS

It is of interest to compare proliferation in the mouse cortex to that in the 
mouse sympathetic ganglion. In the cortex, T\textsubscript{C} increases from around 8 h to 18 h 
(Takahashi et al., 1997), due to a steady increase in G1, but not S-phase 
(Mitsuhashi and Takahashi, 2009). In the developing SNS, the T\textsubscript{C} is 10.6 h at E9.5 
in NCC, increases to 30.3 h on E10.5 and drops to 20.7 h in Sox10-/-/TH+ 
neuroblasts at E11.5 and remains steady thereafter.

The neuronogenic interval (NI) is the period between when the first 
cortical cell withdraws from the cell cycle to the time when the last progenitor 
differentiates (Takahashi et al., 1997). The mouse cortex develops over a NI of six 
days that equates to eleven cell cycles (Takahashi et al., 1997). In the sympathetic 
ganglion, the first cell withdraws around E11.5-E12.5 and most neuroblasts have 
withdrawn by E18.5 (7 days). We estimate this as being around eight cell cycles 
of 20.7 h. Because of the higher number of cell cycles and the shorter mean cell 
cycle time in the cortex, expansion in the cortex represents an increase of 140 
fold over the starting number of cells (Takahashi et al., 1997) while our model 
calculates that from E9.5 to E18.5, there is an 84 fold increase in sympathetic 
cells. The proportion of cycling cells (the GF) in both tissues decreases at a 
similar rate and both tissues reach 50\% on E14.0 (Takahashi et al., 1997) but 
note that cell division of sympathetic neuroblasts is reported even after birth in 
rodents (Hendry and Campbell, 1976, Shi et al., 2008). However, postnatal 
proliferation is likely to be rare for neuroblasts. We can use our model to predict 
that only \textasciitilde650 cells are dividing at E18.5. This equates to a growth fraction of 
around 2\%.
4.4.3.1 Notch signalling may regulate progenitors in both the CNS and SNS

In both the CNS and SNS, Notch signalling has been shown to maintain progenitor status, prevent differentiation and ultimately cell cycle withdrawal (Wakamatsu et al., 2000, Tsarovina et al., 2008). In the CNS, differential intracellular notch signalling is what governs the balance between apical and basal progenitors (Mizutani et al., 2007). In both types of CNS progenitors, ligand activated Notch signalling can be identified by the presence of the cleaved intracellular signalling domain of the Notch receptor ((NICD) - Tokunaga et al., 2004). For the apical progenitors, NICD interacts with the canonical Notch effector C-promoter binding factor 1 (CBF1) but in the basal progenitors this signalling is attenuated (Mizutani et al., 2007). In addition to this, pharmacological inhibition of the CBF1 signalling pathway results in a conversion from apical to basal progenitors, illustrating that Notch-CBF1 maintains an apical phenotype and ultimately the ratio of apical to basal progenitors (Mizutani et al., 2007).

In the SNS, the signalling pathways that regulate the differentiation between the Sox10+/Phox2b- cells and the Sox10+/Phox2b+ cells are unknown. However, we may speculate that like the CNS, differential Notch signaling may be a potential mechanism. Canonical NICD-CBF1 signalling targets the Hes genes (Hes1 and Hes5), which are potent suppressors of BHLH transcription factors (Kageyama et al., 2009, Kameda et al., 2012, Shi et al., 2012). Recent studies on the dorsal rhombomere 1, the area from which the locus coeruleus develops, showed that conditional inhibition of NICD-CBF1 (also known as Notch-Rbpj) signalling, resulted in the conversion of undifferentiated progenitors to differentiated LC progenitors that expressed the BHLH genes Ascl1, Phox2a and Phox2b (Shi et al., 2012). Initial differentiation from a Sox10+/Phox2b- cell into a Sox10+/Phox2b+ cell, requires the rapid up regulation of BHLH genes (Rohrer, 2011). So, it is conceivable that NICD-CBF1 signaling could also be a way to limit the up regulation of these BHLH genes and maintain the Sox10+/Phox2b- phenotype.
4.4.4 Proliferation of satellite glial progenitors

The cell cycle length of glial progenitors, unlike that of neuroblasts, increases significantly with time. While this resembles the situation for neural stem cells in the developing mouse cortex, the underlying mechanism is likely to be quite different. In the cortex, cell cycle lengthens due to an increase in G1, not S-phase (Mitsuhashi and Takahashi, 2009). In contrast, sympathetic glial progenitors show a significant increase in the length of S-phase, so that, from E16.5 to E18.5, S-phase length increases by 14 h and cell cycle length by 20h. At this stage we can only speculate as to the reasons why s-phase gets longer in the Sox10+ population, however, the change in cell cycle kinetics appears to coincide with the timing of further differentiation. From E16.5, Sox10+/Bfab-P+ cells begin expressing more mature glial markers such as S100b (our preliminary observations - Callahan et al., 2008). So, the differences in cell cycle dynamics may reflect a further differentiated state, however this is yet to be confirmed. Interestingly the majority of glial progenitors have withdrawn from the cell cycle by E18.5, while a significant minority (26%) remains cycling. Shi et al. (2008) have identified Ki67–IR satellite glia postnatally in the mouse and the same cells take up BrdU. In normal DRG, satellite glia continue to divide postnatally (Cecchini et al., 1999, Elson et al., 2004), but the estimated half life of a satellite cell (>1 year in Elson et al., 2004) may be an overestimate if the majority of DRG satellite cells drop out of the cell cycle as they do in sympathetic ganglia (overestimating the growth fraction may result in a longer estimated Tc).

4.4.4.1 Notch (NICD-CBF1) signaling may be important for the acquisition of a glial phenotype

In the chick the Sox10+/SCG10- cells (equivalent to the Sox10+/TH- cells in the mouse) are maintained as NC progenitors by Notch signaling (Tsarovina et al., 2008). Based on our data and the study by Tsarovina et al. (2008), it is clear that in both mouse and chick, Sox10+/TH- cells (either Phox2b positive or negative) give rise to both glial progenitors and to neuroblasts. In the DRG, transient activation of Notch signalling in NC stem cells is sufficient to cause rapid and irreversible loss of neurogenic capacity accompanied by accelerated glial differentiation (Morrison et al., 2000). Interestingly, in the developing SNS,
removal of the NICD-CBF1 signaling pathway leads to a decrease in the number of BFABP glial cells observed at both E14.5 and E18.5 (Taylor et al., 2007) without affecting levels of BrdU uptake by BFABP cells (Taylor et al., 2007). It may be that the NICD-CBF1 signalling pathway may play a role in the acquisition of a glial phenotype in sympathetic ganglia. However, in the absence of further analysis of proliferation we cannot confirm whether a proliferation defect exists that may account for the reduction in the number of BFABP cells observed at both E14.5 and E18.5.

4.4.5 Modelling

The model generated from the measured cell cycle lengths and growth fractions can generate a number of realistic predictions. It predicts almost exactly the changes in observed relative proportions of Sox10+ and TH+ cells from E12.5 to E18.5. The model can be used to estimate the absolute number of total cells, total number of Sox10+ or TH+ cells, and the total number of neuroblasts or glioblasts at any given developmental stage. With the addition of an estimated rate at which NCCS differentiate into neuroblasts and glial progenitors, it provides testable estimates of the rate of appearance of these cells and still maintains the fit to later time points. More accurate estimates of when and the rate at which the first neuroblast and glial progenitors appear, as well as better markers of uncommitted NCC, will allow this part of the model to be refined.

Manipulating development via mutagenesis has become the most popular way to try and elucidate the in vivo signalling that regulates the development of the SNS. Neuroblastoma, the most common solid childhood cancer, results when the control of this developmental process is lost, therefore, understanding the signalling pathways controlling the development of the SNS is of paramount importance. Our model provides an exciting tool that can be used to make predictions about SNS development when the system has been perturbed, leading to a greater understanding of the developmental process involved in the generation of the SNS. This aspect will be explored in the next chapter.
4.5 Chapter 4: Figures and Tables
Figure 4-1: Changes in the proportions of different phenotypic classes of cells in the developing mouse stellate ganglion

Percentages are expressed as proportions of total cells counted. A) The proportions of cells expressing Sox10 and/or TH. Note that at E10.5, some cells expressed both Sox10 and TH. B) The proportions of cells expressing Sox10 and/or Phox2b at E9.5 and 10.5. Note the large proportion of cells expressing both Sox10 and Phox2b at E10.5.

Table 4-1: Ki67 in the mouse stellate ganglion at E10.5

<table>
<thead>
<tr>
<th></th>
<th>Percentage of total</th>
<th>Percentage that is Ki67+</th>
<th>Number of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sox10+/TH-</td>
<td>66.1</td>
<td>79.4</td>
<td>199 (n=9)</td>
</tr>
<tr>
<td>Sox10+/TH+</td>
<td>18.6</td>
<td>5.4</td>
<td>56 (n=9)</td>
</tr>
<tr>
<td>Sox10-/TH+</td>
<td>15.0</td>
<td>2.2</td>
<td>46 (n=9)</td>
</tr>
<tr>
<td>Sox10+/Phox2b-</td>
<td>2.2</td>
<td>40</td>
<td>15 (n=6)</td>
</tr>
<tr>
<td>Sox10-/Phox2b+</td>
<td>8.4</td>
<td>34.5</td>
<td>55 (n=6)</td>
</tr>
<tr>
<td>Sox10+/Phox2b+</td>
<td>90</td>
<td>32</td>
<td>604 (n=6)</td>
</tr>
</tbody>
</table>
**Figure 4-2: Ki67 expression in different classes of cells during SNS development**

Transverse cryostat sections of mouse embryos through the forelimbs showing distribution of cell specific markers relative to the cell cycle indicator, Ki67 in the stellate ganglion at various ages. Each row of images shows triple label immunofluorescence for either; Phox2b, Sox10, Ki67 and a merged image (A-H) or, TH Sox10, Ki67 and a merged image (I-P). Note that at E9.5 (A-D) the majority of cells are Sox10+, with a few also showing colocalised Phox2b. All Sox10+ cells at this age also express Ki67. At E10.5 (E-H), nearly all cells are Sox10+/Phox2b+, but many cells lack Ki67. (I-L) Showing that TH+ cells lack the expression of Ki67. By E12.5 (M-P) TH and Sox10 are expressed by non-overlapping populations of cells. Nearly all cells are now immunoreactive for Ki67. Arrows in A-D and E-F shows examples of cells that are immunoreactive for Ki67, Sox10 and Phox2b. Arrows in I-L showing an example of a cell immunoreactive for TH, Sox10, but lacking Ki67.
Chapter 4: Proliferation and cell cycle dynamics in the developing SNS

A Sox10
B Phox2b
C Ki67
D Merge

E Sox10
F Phox2b
G Ki67
H Merge

I TH
J Sox10
K Ki67
L Merge

M TH
N Sox10
O Ki67
P Merge
Figure 4-3: Double S-phase labelling combined with IR for TH or Sox10

Transverse single optical plane confocal images of sections through the forelimbs of E12.5 mouse embryos showing BrdU and EdU. **A-D:** Incorporation of BrdU and EdU into Sox10-/TH+ cells. **E-H:** Incorporation of BrdU and EdU into Sox10+/TH- cells. Arrows show (from left to right in each image) a Sox10+ cell without BrdU or EdU, a Sox10+ cell with both BrdU and EdU and a Sox10+ cell showing only BrdU. Scale bar in **A** applies to all images.
Chapter 4: Proliferation and cell cycle dynamics in the developing SNS
Figure 4-4: Changes in the GF, $T_C$ and $T_S$ during SNS development

Summarised data for proportion of cycling cells (growth fraction), overall cell cycle length and S-phase length for Sox10-/TH+ cells (left) and for Sox10+/TH+ cells (right). At E9.5, only Sox10+ cells were present, at E10.5 only Sox10+ cells were in sufficient numbers to calculate an S-phase and a cell cycle length and on E18.5 there were too few cycling Sox10-/TH+ cells to calculate an S-phase or cell cycle length.
Chapter 4: Proliferation and cell cycle dynamics in the developing SNS

**Sox10+/TH- Growth Fraction**

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>E9.5</th>
<th>E10.5</th>
<th>E11.5</th>
<th>E12.5</th>
<th>E14.5</th>
<th>E16.5</th>
<th>E18.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion of total cells</td>
<td>1.5</td>
<td>1.0</td>
<td>0.5</td>
<td>0.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Sox10-/TH+ Growth Fraction**

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>E9.5</th>
<th>E10.5</th>
<th>E11.5</th>
<th>E12.5</th>
<th>E14.5</th>
<th>E16.5</th>
<th>E18.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion of total cells</td>
<td>1.5</td>
<td>1.0</td>
<td>0.5</td>
<td>0.0</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

**Sox10+/TH- Cell Cycle Length**

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>E9.5</th>
<th>E10.5</th>
<th>E11.5</th>
<th>E12.5</th>
<th>E14.5</th>
<th>E16.5</th>
<th>E18.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle length (h)</td>
<td>50</td>
<td>40</td>
<td>30</td>
<td>20</td>
<td></td>
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</tbody>
</table>

**Sox10-/TH+ Cell Cycle Length**

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>E9.5</th>
<th>E10.5</th>
<th>E11.5</th>
<th>E12.5</th>
<th>E14.5</th>
<th>E16.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle length (h)</td>
<td>40</td>
<td>30</td>
<td>20</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Sox10+/TH- S-phase Length**

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>E9.5</th>
<th>E10.5</th>
<th>E11.5</th>
<th>E12.5</th>
<th>E14.5</th>
<th>E16.5</th>
<th>E18.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-phase length (h)</td>
<td>25</td>
<td>20</td>
<td>15</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Sox10-/TH+ S-phase Length**

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>E9.5</th>
<th>E10.5</th>
<th>E11.5</th>
<th>E12.5</th>
<th>E14.5</th>
<th>E16.5</th>
<th>E18.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-phase length (h)</td>
<td>15</td>
<td>10</td>
<td>5</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4-5: Modeling SNS cell cycle dynamics

A) Curve fits to observed proliferation rates, (calculated by dividing growth fraction by cell cycle time/24h) for Sox10-/TH+ cells (blue points) and Sox10+ cells (red points). Black points are for NCCS present on 9.5 and 10.5. For NCCS after E10.5, proliferation rate was assumed to return to that seen on E9.5 (dotted line) Note that for any age, the proliferation rate of Sox10+ cells is always larger than for Sox10-/TH+ cells.

B) Comparison of the predictions of the model (solid lines) compared to the observed proportions of each cell type (Sox10-/TH+ cells = n, blue solid line and Sox10+ cells = g, red solid line), from E12.5 to E18.5. The model accurately predicts the observed proportions of Sox10-/TH+ cells (blue squares) and Sox10+/TH cells (red squares). Arbitrarily doubling (dashed lines) or halving (dotted lines) proliferation rates to mimic systematic errors in the data results in poorer fits.

C) A complete model of the change in proportions of Sox10-/TH+ cells (n, blue line) and Sox10+ cells (g, red line) in the stellate ganglion from E9.5. The model includes differentiation of neural crest progenitors (p, black line) into Sox10-/TH+ cells from E9.5 and Sox10+ cells from E10.5. The model accurately predicts the observed proportions of Sox10-/TH+ cells (blue squares) and Sox10+ cells (red squares). The green line (g + p) indicates the period where significant numbers of NCCS overlap with glial progenitors. Both cell types will contribute to Sox10+ cells.

D) Predictions of changes in absolute cell numbers in the ganglion based on the model, starting number adjusted to match stereological counts at E18.5. The increase in number in the WT ganglion of Sox10-/TH+ cells (assumed to be neuroblasts, n) and Sox10+ cells (assumed to be glial progenitors, g) are shown in the solid lines.
Chapter 4: Proliferation and cell cycle dynamics in the developing SNS

A

B

C

D

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Figure 4-6: Changes in the Tc and phenotypic differentiation of NCCs

Diagram showing how the Tc changes with respect to differentiation as development progresses. Arrows indicating the possible daughter cells and the broken lines indicating at this stage there is no data on cell cycle dynamics. Brown nucleus Sox10-IR only, brown/pink nucleus depicting Sox10-IR & Phox2b-IR, bright pink nucleus depicting Phox2b-IR only, red cytoplasm indicating TH-IR and yellow cytoplasm indicating BFABP-IR. On the left hand side is a time line (not drawn to scale) indicating developmental age.
Chapter 4: Proliferation and cell cycle dynamics in the developing SNS

- **E9.0**
  - Sox10+ NCC undifferentiated stem cell
  - 10.6h

- **E10.0**
  - Sox10+/Phox2b+/differentiating NCC progenitor
  - 30.3h
  - Sox10+/Phox2b+/TH+ differentiating neuronal precursor
  - Withdrawn in G0

- **E11.0**
  - Phox2b+/TH+ neuronal precursor
  - 17.8h
  - Sox10+/BFABP+ glial precursor
  - 12.8h

- Sox10+ differentiating neuronal or glial progenitor
  - Withdrawn in G0 or dividing?
Chapter 5

5 Insights into the function of Ret, Epo and β-catenin
Chapter 5: Insights into the function of Ret, Epo and β-catenin

5.1 Introduction

In Chapter 3, I showed that using a single dose of BrdU allows only limited conclusions about proliferative behaviour to be drawn. In contrast, Chapter 4 shows that using techniques to separately determine $L_{I0}$, $T_s$, $T_c$ and GF, combined with quadruple-label immunofluorescence yields a wealth of insights into proliferation in the developing sympathetic ganglia. Using the latter approach, I completed a detailed description of cell cycle kinetics during normal embryonic development of the mouse stellate ganglion (see chapter 4). We have also generated a mathematical model that successfully describes the growth, observed in SNS ganglion development. Now I will focus on using the techniques and the model developed in the previous chapter to investigate the development of the stellate ganglion when development has been perturbed in genetically manipulated animals.

These experiments were done towards the end of my PhD candidature and are unfinished at the time of writing. However, they are included here because they demonstrate the power of the approach described in the previous chapter to provide new insights into the processes controlling cell signalling in sympathetic ganglia.

GOF or LOF experiments have provided us with virtually all of the existing information about the actions of transcription factors and signaling pathways that influence proliferation in the developing SNS (see sections 1.5 and 1.8). However, no previous study has combined a GOF or LOF approach with a detailed analysis of cell cycle dynamics. The questions I aim to address here are: 1) can I apply my methods to reveal changes in cell cycle dynamics when normal control systems have been altered; and 2) does applying these methods give us any extra insight into the how proliferation is regulated? To answer these questions, I have decided to perform the analysis of proliferation on strains of mice that have LOF of the Ret gene, and GOF of the Ctnnb1 gene, encoding β-catenin.
5.1.1 Ret signaling in the SNS

Members of the glial-line derived neurotrophic family of ligands (GFL’s) have been shown to play key roles in the development of the SNS (see section 1.8.1.3). GFL’s signal through a receptor complex that is composed of a signaling subunit, the tyrosine kinase receptor Ret, and a binding subunit consisting of one of the GFRα family of glycosylphosphatidylinositol (GPI)-linked cell surface proteins (Enomoto et al., 2001). LOF experiments have shown that Ret is important for the development of motor neurons and many NC-derived structures such as the enteric, parasympathetic and sympathetic nervous systems (Enomoto et al., 2001, Airaksinen and Saarma, 2002). The loss of Ret results in a reduction in the size of sympathetic ganglia, particularly the SCG, which is also found caudally displaced in the Ret knock out (RetTGM/TGM) mice (Enomoto et al., 2001, Airaksinen and Saarma, 2002). In addition to the migration defects and the reductions in ganglion size, axonal projection defects of developing SNS neuronal precursors have also been reported when Ret is removed (Enomoto et al., 2001).

It is interesting to note that LOF experiments for the GDNF family ligand artemin, or its cognate GFRα receptor GFRα3 (that forms the receptor signaling complex with Ret), almost identically phenocopy the defects observed in the RetTGM/TGM mice (Nishino et al., 1999, Honma et al., 2002). Also, in all three knockouts (Ret, Artemin, or GFRα3), elevated cell death is observed and is suggested to be a cause for reduction in the size of the ganglia (Nishino et al., 1999, Enomoto et al., 2001, Honma et al., 2002). The increase in cell death, observed in the RetTGM/TGM mice, is likely to be a consequence of the axonal projection defects leading to a loss of trophic support, however, it is not known to what extent this accounts for the overall loss of cells during development.

Mice carrying the RetMEN2B mutation (caused by a point mutation (M918T) in the tyrosine kinase-coding domain of RET) have constitutively active Ret signaling (Gestblom et al., 1999, Rajan et al., 2001). Transgenic mice that express this mutation, under the control of the DβH promoter (DβH:Cre-RetMEN2B), have profound sympathetic ganglion hyperplasia (Gestblom et al., 1999, Rajan et al., 2001).
While it is clear that Ret signaling can influence proliferation in the developing SNS, the mechanism is unknown. In the Ret\textsuperscript{TGM/TGM} mice, 2 h pulse labelling with BrdU did not show any difference in proliferation compared to WT animals, suggesting that the removal of Ret does not have any significant effects on proliferation (Enomoto et al., 2001). However, I have shown that single pulse BrdU labelling alone may not reveal changes in cell cycle dynamics that can alter proliferation (see chapter 3). Hence, the Ret\textsuperscript{TGM/TGM} mice provide a good model for us to test the validity of my technique, to see if there are changes to proliferation that may not be detectable by single pulse BrdU labeling alone.

### 5.1.2 Beta-catenin signaling in the SNS

Frizzled-mediated Wnt signaling has recently been reported to play a role in SNS development (Armstrong et al., 2011). Removal of the seven-pass transmembrane receptor, Frizzled 3, results in both proliferation and final target innervation defects in mice (Armstrong et al., 2011). β-catenin, the key downstream target of canonical frizzled-mediated Wnt signaling, does not appear to play a role in the innervation defect, however, it does account for the proliferation defects observed in the Frizzled 3 knockout mice (Armstrong et al., 2011). Conditional removal of β-catenin under the TH promoter (TH:Cre-\textsuperscript{βcat\textsuperscript{flox/flox}}) mimicked proliferation defects observed in the Frizzled 3-knockout mice, however, it did not affect final target innervation (Armstrong et al., 2011).

Using innovative techniques that combined EdU-labelling with subsequent Ki67 staining, Armstrong et al. (2011) showed that there was premature cell cycle withdrawal of sympathetic precursors in embryos that lack Frizzled 3/β-catenin. Here I aim to use my methods for detecting changes in cell cycle dynamics to try and understand how proliferation in the stellate ganglion is altered when β-catenin signaling is constitutively activated.

### 5.2 Materials and methods

For materials and methods see Chapter 2
5.3 Results

5.3.1 Ret null mutants have fewer cells at E18.5 by stereology

In Ret\textsuperscript{TGM/TGM} mice at birth, neuronal number in the SCG is reduced by 35% (Enomoto et al., 2001, Airaksinen and Saarma, 2002) and thoracic ganglion size reduced (Enomoto et al., 2001). However, there is no quantitative analysis of the reduction in cell number in thoracic ganglia in the Ret\textsuperscript{TGM/TGM} mice. By stereology (see methods section 2.6), Ret\textsuperscript{TGM/TGM} mice have a total of 23,760 cells in the stellate ganglion compared to 41,906 cells in WT controls (Figure 5.1). This difference is significant (unpaired t test, t= 4.33, df=4, P=0.012) confirming that previously reported changes in the size of Ret\textsuperscript{TGM/TGM} sympathetic ganglia are associate with reductions in total cell number. It has also been reported that neuronal precursors appear smaller in the SCG's of Ret\textsuperscript{TGM/TGM} mice (Enomoto et al., 2001). Analysis of transverse sections though WT and Ret\textsuperscript{TGM/TGM} stellate ganglia in fact show that there is no significant difference in neuroblast or glial progenitor density at E16.5 (Figure 5.1).

5.3.2 Changes in the cell cycle dynamics of Ret\textsuperscript{TGM/TGM} mice

5.3.2.1 GF changes in the Ret\textsuperscript{TGM/TGM} mice

The growth fraction in Ret\textsuperscript{TGM/TGM} mice was measured using Ki67-labelling in conjunction with Sox10 or TH-immunofluorescence. The GF of both Sox10- /TH+ and Sox10+ /TH- cells are summarised in Figure 5.2. At E12.5, the growth fraction of TH+ neuronal precursors in Ret\textsuperscript{TGM/TGM} mice is 92.8%, this is significantly lower than the 98.2% GF that is observed for WT mice at the same developmental age (paired t test, t=4.10, df=6, p=0.014, Figure 5.2). At E14.5 to E16.5 there is no statistical difference between the GF of TH+ neuronal precursors in the Ret\textsuperscript{TGM/TGM} mice compared to the WT. The GF of the Sox10+ glial precursors in the Ret\textsuperscript{TGM/TGM} embryos is not statistically different from WT embryos in the period from E12.5-E14.5. However, at E16.5 in the Ret\textsuperscript{TGM/TGM} mice there is a significant increase in the GF of Sox10+ glial cells (54.3%), compared to 41.3% in the WT (paired t test, t=4.23, df=4, p=0.013, Figure 5.2).
5.3.2.2 $T_S$ changes in the Ret$^{TGM/TGM}$ mice

The $T_S$ was calculated using the double-labelling BrdU/EdU protocol, previously described and the data is summarised in Figure 5.2. Compared to WT animals, there were no significant differences in the $T_S$ of Sox10-/TH+ neuroblasts in Ret$^{TGM/TGM}$ mice, at E12.5 or E14.5. However at E16.5, the $T_S$ of TH+ neuronal precursors, in the Ret$^{TGM/TGM}$ mice, was almost double that of the WT, 14.4 h vs 7.7 h respectively (paired t test, t=5.18, df=4, p=0.007, Figure 5.2). For the Sox10+/TH- glial progenitors, there was a significant difference in the $T_S$ of Ret$^{TGM/TGM}$ mice at E12.5, 4.5 h compared to the 2.8 h in the WT (paired t test, t=2.76, df=5, p=0.040, Figure 5.2). However, at either E14.5 or 16.5, there was no significant difference in the $T_S$ of Sox10+ cells between the Ret$^{TGM/TGM}$ and WT embryos (Figure 5.2).

5.3.2.3 $T_C$ changes in the Ret$^{TGM/TGM}$ mice

The $T_C$ was determined for both TH+ and Sox10+ cells in Ret$^{TGM/TGM}$ mice from E12.5 to E16.5. These data are summarised in Figure 5.2. For the Sox10+ glial progenitors, there was no statistical difference between the $T_C$ of Ret$^{TGM/TGM}$ and Wt mice at any developmental stage examined (Figure 5.2). This was the same for TH+ neuroblasts at both E12.5 and E14.5. However, at E16.5, the $T_C$ of the Ret$^{TGM/TGM}$ mice increased dramatically to 100 h (paired t test, t=8.40, df=4, p=0.007), Figure 5.2). This was a five-fold increase in the $T_C$ compared to WT mice, which have a cell cycle length of about 20 h.

5.3.2.4 Modeling proliferation in the Ret$^{TGM/TGM}$ mice

The parameters for the Ret$^{TGM/TGM}$ mutant mice at E12.5, 14.5 and 16.5 were substituted into the mathematical model. I used a starting number of 520 cells at E9.5, as calculated by the model for the WT cells (see Chapter 4). Using the Ret$^{TGM/TGM}$ data, the model predicted 35,628 total cells at E18.5, compared to the 23,760 cells actually counted by stereology in sections from Ret$^{TGM/TGM}$ mice. Thus, the changes to cell cycle length measured after deletion of Ret appears to account for only some of the loss of cells actually observed in Ret$^{TGM/TGM}$ mice. The mathematical model also predicted that, while there should be fewer neuroblasts, the number of glial progenitors in Ret null mutants should remain
unchanged (Figure 5.3) and, as a consequence, their proportion relative to neuroblasts should increase. In contrast, my count of cells in sections from RetTGM/TGM mice showed that the proportions of neuroblasts and glial precursors matched those seen in WT animals. That is, in RetTGM/TGM mice, the numbers of glial cells must have decreased by the same proportion as did the neuroblasts.

5.3.3 The effect of Epo on the cell cycle dynamics of RetTGM/TGM mice

The timing of the effects of deletion of Ret on neuroblast cell cycle length is puzzling. While all neuroblasts in the ganglion express Ret prior to about E13.5, from E14.5 onwards, only a small proportion of TH+ cells in the ganglion express Ret (Enomoto et al., 2001, Callahan et al., 2008). Therefore, the five-fold increase in the Tc of RetTGM/TGM TH+ neuronal precursors occurs when Ret is not expressed on these cells. This means that the increase in Tc observed in the RetTGM/TGM mice at E16.5 must be due either to some persistent effect of the lack of Ret signaling earlier in development or to the absence of some external signal that is normally induced by Ret on E16.5 and which drives proliferation.

One obvious deficit in RetTGM/TGM animals is the absence of normal kidney organogenesis that is due to defects in the branching of the uretic bud (Moore et al., 1996, Enomoto et al., 2001, Airaksinen and Saarma, 2002). By E16.5, RetTGM/TGM animals either lack kidneys, or have only a small rudiment in the vicinity of where the kidneys would normally be (Enomoto et al., 2001, Airaksinen and Saarma, 2002). RetTGM/+ heterozygous mice appear to have normal kidneys and do not have any defects in SNS development.

The developing kidneys are a major source of the hormone erythropoietin (Epo) during late mammalian embryonic development (Clemons et al., 1986, Wintour et al., 1996, David et al., 2002, Noguchi, 2008). In the embryo, the yolk sac first produces Epo, then the liver and finally production of Epo is by the kidney, which is the primary source of Epo throughout adult life (Clemons et al., 1986, Wintour et al., 1996, Lee et al., 2001, Noguchi, 2008). The kidney-derived production of Epo would be lost in RetTGM/TGM mice. Epo is a mitogen that signals via the erythropoietin receptor (EpoR). It drives proliferation during erythropoiesis and has also shown to be important for CNS neurogenesis (Tsai et
al., 2006). Epo and EpoR are strongly expressed in sympathetic ganglia at E11.5 and the EpoR remains expressed by postnatal sympathetic neurons (Knabe et al., 2005).

To investigate the idea that kidney agenesis in the Ret<sup>TGM/TGM</sup> mice may affect the proliferation of cells in the developing SNS due to the loss of Epo production, I injected pregnant Ret<sup>TGM/+</sup> dams crossed to male Ret<sup>TGM/+</sup> mice, with 125IU of Epo daily for 3 days (at E13.5, E14.5 and E15.5 – see methods section 2.2.3). In most species, Epo does not cross the placenta (Schneider and Malek, 1995, Widness et al., 1995) but in rodents, levels in the embryo of 7-10% of the maternal systemic levels can be achieved (Widness et al., 1995). Cell cycle parameters on E16.5 were measured using the methods described in Chapter 4. I have completed Epo treatment experiments on five Ret<sup>TGM/TGM</sup> (Epo-Ret<sup>TGM/TGM</sup>) animals from two litters at the time of writing.

### 5.3.3.1 Cell cycle dynamics in the Epo-Ret<sup>TGM/TGM</sup> mice

Epo treatment for three days largely reversed the effect of loss of Ret, taking T<sub>c</sub> from around 100 h in Ret<sup>TGM/TGM</sup> mice to around 40 h in the Epo-Ret<sup>TGM/TGM</sup> mice (Figure 5.4D). The T<sub>s</sub> is also reduced in the Epo-Ret<sup>TGM/TGM</sup> embryos, but this is not statistically different from the T<sub>s</sub> observed in the Ret<sup>TGM/TGM</sup> mice.

The GF of Epo-Ret<sup>TGM/TGM</sup> mice is not statistically different from that of the C57b6 mice but is significantly lower than the GF of the Ret<sup>TGM/TGM</sup> mice (Figure 5.4C). All data were analyzed using an ANOVA (one way) with Tukey’s post hoc tests for a significance of p<0.05.

### 5.3.4 Effects of expressing a stable form of beta-catenin in the developing SNS

I also had the opportunity to apply my method for analyzing proliferation to a single litter of E16.5 transgenic animals that constitutively expressed beta catenin. β-catenin is a key down-stream element of the canonical Wnt signaling pathway and has been shown to play an important role in proliferation in the SNS after E13.5 (Armstrong et al., 2011). Embryos that over-express β-catenin
specifically in TH+ cells were generated by crossing mice that express Cre recombinase driven by the TH promoter to homozygote mice that have the exon3 component of the β catenin gene flanked by loxP sites. This produces a heterozygous mouse with one copy of the constitutively active β-catenin gene (TH-Cre:βcatEx3/+).

5.3.4.1 Cell cycle dynamics in the TH-Cre:β-catEx3/+ mice

At E16.5, the GF of TH+ stellate ganglion neuronal precursors is significantly reduced to 4.9% in the TH-Cre:βcatEx3/+ mice, compared to 17.3% in WT mice (Figure 5.5A). This reduction in growth fraction means that very few cells were cycling and therefore hardly any TH+ cells were labelled using the double-labelling protocol of BrdU/EdU. Due to the lack of BrdU/EdU labeling, it was not possible to determine any other cell cycle parameters for the TH+ cells in TH-Cre:βcatEx3/+ mice.

Sox10+/TH- glial progenitors do not express TH, so these cells will not express the stable form of β-catenin. To see if there were any indirect effects on the proliferation for the Sox10+/TH- glial progenitors in the TH-Cre:βcatEx3/+ mice, I conducted a cell cycle analysis on this population as well. The GF for Sox10+/TH- glial progenitors in the TH-Cre:βcatEx3/+ mice was significantly different (t=5.895 , df=5, P<0.05) compared to WT mice (Fig. 5.5B-C). In addition, the Ts was longer (Fig 5.5C) in the TH-Cre:βcatEx3/+ mice, but just failed to reach statistical significance (p=0.09 ). However, the L0 and the Tc of TH-Cre:βcatEx3/+ mice, was almost identical to what was observed for the WT population (Fig. 5.5D-E). The Sox10+/TH- glial progenitors made up 17.0% of all cells in the stellate ganglion of TH-Cre:βcatEx3/+ mice versus 15.1% in WT animals. The density of Sox10-/TH+ neuronal precursors was slightly decreased and that of Sox10+/TH- glial progenitors slightly increased (Figure 5.1).
Chapter 5: Insights into the function of Ret, Epo and β-catenin

5.4 Discussion

5.4.1 Proliferation in the stellate ganglia of Ret\textsuperscript{TGM/TGM} mice

Enomoto et al. (2001) suggested that the effects of loss of Ret seen at E16.5 and P0 must be indirect and likely due to a dysfunction in earlier artemin signalling via GFRα3 and Ret. Artemin signalling normally guides sympathetic axons to peripheral target tissues (Honma et al., 2002) which then supply trophic support to the developing neuroblasts (Glebova and Ginty, 2005). Failure to project axons successfully to a suitable target will then presumably result in loss of cells by apoptosis. However, while this accounts for some of the loss of sympathetic neurons in the Ret\textsuperscript{TGM/TGM}, it cannot account for the change in cell cycle length, as this is affecting neurons that are still in the cell cycle and which presumably are yet to extend an axon to a target tissue. The loss of cells seen in Ret\textsuperscript{TGM/TGM} animals is therefore likely to be due to both the change in cell cycle length and the loss of cells due to failed axon projection. Our mathematical model includes only the effect on cell cycle, which may explain why the modelling of the effects of deleting Ret predicts a smaller change in cell number than measured with stereology.

My results showed only minor changes in proliferation parameters in Sox10+/TH- glial progenitors and these values did not result in any change in the predicted number of these cells when the new values were substituted into the mathematical model. However, the relative proportion of neuronal precursors to glial progenitors is unchanged in Ret\textsuperscript{TGM/TGM} ganglia. That is, the final number of glial progenitors is also reduced. As glial progenitors never express Ret (Callahan et al., 2008), the effect of Ret deletion must be either on the NCC, which all express Ret at E9.5 (Pachnis et al., 1993), prior to differentiation, or be indirect, perhaps via the effect on neuroblasts, which are likely to signal to glial progenitors using Lgi4 (Nishino et al., 2010)

5.4.2 The effect of Epo on the Ret\textsuperscript{TGM/TGM} mice

The lengthening in Tc may be due to the lack of an extrinsic mitogenic signal that has been lost due to the Ret\textsuperscript{TGM/TGM} phenotype. Epo has been shown to
act as a mitogen in CNS development and drives proliferation in erythropoiesis (Tsai et al., 2006, Chen et al., 2007). Ret<sup>TGM/TGM</sup> mice lack kidneys, a major source of Epo late in mammalian development (Clemons et al., 1986, Wintour et al., 1996, Lee et al., 2001, Noguchi, 2008), so I tested the idea that the loss of Epo might account for the cell cycle phenotype observed in the Ret<sup>TGM/TGM</sup> animals at E16.5.

Epo injections into pregnant dams rescued 60% of the cell cycle defect observed in the Ret<sup>TGM/TGM</sup> mice, reducing the T<sub>c</sub> from ~100 h to ~40 h. For Epo treated Ret<sup>TGM/TGM</sup> embryos (Epo-Ret<sup>TGM/TGM</sup>), the T<sub>c</sub> for TH+ neuroblasts was not significantly different from Ret<sup>+/+</sup> or C57b6 mice, indicating that the T<sub>c</sub> had been restored to a value within the range observed in normal development.

It is important to note that although Epo treatment appears to ‘rescue’ the T<sub>c</sub> defect observed in the Ret<sup>TGM/TGM</sup> mice, it does not completely restore the WT pattern of proliferation parameters. Another interesting point to note is that the LI remains unchanged between the Ret<sup>TGM/TGM</sup> mice and Epo-Ret<sup>TGM/TGM</sup>. The substantial reduction in cell cycle length comes about because the cell cycle estimation is the product of the GF times the S-phase, which are both reduced (but not to statistical significance). This observation shows one of the benefits of using the approach developed in this thesis. Because I measure the GF, LI<sub>0</sub> and the T<sub>S</sub> separately in order to allow to calculate the formula T<sub>c</sub> = T<sub>S</sub> x (GF/LI<sub>0</sub>), I can evaluate the effect of Epo separately on each of these. If the study had relied on a single 2 h pulse of BrdU (estimating the LI<sub>0+2h</sub> only), the effect of Epo would have been missed, as the LI<sub>0</sub> did not change.

Knabe et al. (2005) only examined the distribution of Epo and EpoR at a single time point in the mouse embryo (E11.5). The response of sympathetic ganglia to injected Epo suggests that the receptor is expressed from E13.5 to E15.5, consistent with its continuing presence on postnatal sympathetic ganglia (Knabe et al., 2005). Epo is produced first by the liver and then the kidney throughout embryonic life so one can speculate that Epo may act as an extrinsic mitogen regulating proliferation throughout sympathetic ganglion development.
5.4.3 Proliferation in the $\text{TH-Cre}:\beta$-cat$^{\text{floxflox}}$ mice

The results for the $\text{TH-Cre}:\beta$-cat$^{\text{Ex3/+}}$ mice are preliminary. However, the results support the usefulness of my approach to studying proliferation in sympathetic ganglia. Again, it was possible to identify a specific aspect of proliferation that was affected by the constitutive expression of $\beta$-catenin. The conditional over-expression of $\beta$-catenin in TH+ neuroblasts results in a significantly reduced GF at E16.5 (Figure 5.5A), which is most readily interpreted as premature cell cycle withdrawal. Recall that the number of cells generated depends primarily on the GF and the $T_C$, so a severe reduction in the GF is likely to have a significant impact on the total number of cells produced. This result was surprising, given the fact that conditional removal of $\beta$-catenin also results in a reduction in the GF of TH+ neuroblasts in the developing SNS (Armstrong et al., 2011). Taken together, it would appear that either overexpression or removal of $\beta$-catenin results in a premature cell cycle withdrawal. $\beta$-catenin has multiple intracellular functions and at this stage I do not have an explanation of the mechanism.

In addition to the effects seen on the neuroblasts, there are significant changes to the cell cycle dynamics, in particular the GF, of the Sox10+/TH- glial progenitors in the $\text{TH-Cre}:\beta$-cat$^{\text{Ex3/+}}$ mice (Fig. 5.5B-C). There was no significant difference in the $L_0$, $T_C$ or the $T_S$ of the Sox10+/TH- cells in the $\text{TH-Cre}:\beta$-cat$^{\text{Ex3/+}}$ mice compared to the WT (Fig. 5.5D-E). The lack of effect on the $T_C$, for the Sox10+/TH- cells in the $\text{TH-Cre}:\beta$-cat$^{\text{Ex3/+}}$ mice, is due to the fact that the changes in the GF is countered by a (non-significant) change in $T_S$ ($T_C = T_S \times \text{GF}/L_0$). As the stable $\beta$-catenin is not expressed in the Sox10+/TH- cells, this demonstrates that the Sox10+/TH- glial progenitors are responding to altered Sox10-/TH+ neuroblast behaviour. The major identified signalling mechanisms that are believed to regulate sympathetic gliogenesis, Lgi4/ADAM22 (Nishino et al., 2010) and Notch/Delta (Taylor et al., 2007), both require cell-to-cell mediated interactions between the neuronal and glial populations. The conditional expression of stable $\beta$-catenin in neuroblasts is leading to indirect effects on the cell cycle dynamics of the Sox10+/TH- glial progenitor population.
5.4.4 Conclusion

I set out to address two main questions: 1) can I apply our method to reveal previously unidentified changes in cell cycle dynamics when normal development has been altered; and 2) does applying these methods give us any extra insight into the how proliferation is controlled when the system is perturbed? It is clear that the application of my method of analysis of proliferation has revealed new insights about the Ret\textsuperscript{Ftm/Tgm} phenotype. I have identified that the cell cycle length increases by 5 fold at E16.5 in Ret\textsuperscript{Ftm/Tgm} mice and my modelling indicates that this partly accounts for loss of cells identified by stereology. Previous investigations, using BrdU-labelling alone, came to the conclusion that there was no significant difference in proliferation between Ret\textsuperscript{Ftm/Tgm} and WT mice. I have also been able to show that Epo has an effect on neuroblast cell cycle kinetics and so identified a novel mitogen regulating sympathetic ganglion growth. In addition, I have shown that there are significant changes to the cell cycle dynamics of both Sox10−/TH+ neuroblasts and Sox10+/TH- glioblasts, in the TH-Cre:βcat\textsuperscript{Ex3/+} mice. Of note was the fact that the Sox10+/TH- glial progenitors had identical LI\textsubscript{0} in both the WT and TH-Cre:βcat\textsuperscript{Ex3/+} mice. This means that a single pulse-label with BrdU would not have identified the changes in proliferation that I have observed, further validating this approach.
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Figure 5-1: Density changes and stereology counts for the mouse stellate ganglion

Left: Changes in density of cells per mm² over the course of development. Transverse sections through the stellate ganglia were analysed for ganglion cross sectional area and the number of TH+ and Sox10+ cells in WT animals (red and purple lines). Ret<sup>TGM/TGM</sup> (black triangles) and TH-Cre:βcat<sup>Ex3/+</sup> (blue diamond and circle) are displayed only for E16.5.

Right: At E18.5, cell counts using stereology show that there is a significant reduction in the total number of cells in the stellate ganglia of Ret<sup>TGM/TGM</sup> (23,760 cells) mice compared to the Ret<sup>WT</sup> (41,906 cells).
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Figure 5-2: Cell cycle dynamics: Ret\textsuperscript{TGM/TGM} vs. WT

Summarised data for overall cell cycle lengths, proportions of cycling cells (growth fraction) and S-phase lengths for Sox10-\textasciitilde TH+ cells (left) and for Sox10+/TH- cells (right). Cell cycle lengths, Growth fraction and S-phase lengths for WT animals are shown in white bars, Ret\textsuperscript{TGM/TGM} animals in black. WT values, from the previous chapter, were compared to Ret\textsuperscript{TGM/TGM} values with unpaired t-tests and significant differences (p<0.05) are indicated with asterisks.
Chapter 5: Insights into the function of Ret, Epo and β-catenin

**Sox10-/TH+ Cell Cycle Length**

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**Sox10+/TH- Cell Cycle Length**

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**Sox10-/TH+ Growth Fraction**

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**Sox10+/TH- Growth Fraction**

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**Sox10-/TH+ S-phase Length**

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**Sox10+/TH- S-phase Length**

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Figure 5-3: Changes in cell number in WT vs. Ret\textsuperscript{TGM/TGM} mice, as predicted by the model

Predictions of changes in absolute cell numbers in the ganglion based on the mathematical model, starting number adjusted to match stereological counts at E18.5 for WT animals. The increase in number in the WT ganglion of Sox10-/TH+ cells neuroblasts (n) and Sox10+/TH- cells glial progenitors (g) are shown in the solid lines. The dotted lines show the result when parameters from Ret\textsuperscript{TGM/TGM} animals are used.
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Figure 5-4: Cell cycle dynamics: Epo-Ret\textsuperscript{TGM/TGM} vs. Ret\textsuperscript{TGM/TGM}, Ret\textsuperscript{+/+} and C57

Differences between E16.5 animals that are C57, Ret\textsuperscript{+/+}, Ret\textsuperscript{TGM/TGM} and Epo-Ret\textsuperscript{TGM/TGM}. All values are for Sox10-/TH+ neuroblasts:

In each case the four lines of animals are compared for: \textbf{A)} growth fraction; \textbf{B)} labelling index; \textbf{C)} S-phase length; and \textbf{D)} cell cycle length. In each case, WT values were compared to Ret knockout values using a one way ANOVA and Tukey's \textit{post hoc} tests, significant differences (p<0.05). ns indicating that the difference between uncapped bars are not significant. The * indicates that there is a significant difference between capped lines.
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**A** Effect of Epo on GF

**B** Effect of Epo on LI

**C** Effect of Epo on Ts

**D** Effect of Epo on Tc

**Effect of Epo on GF**

**Effect of Epo on LI**

**Effect of Epo on Ts**

**Effect of Epo on Tc**
Chapter 5: Insights into the function of Ret, Epo and β-catenin
Chapter 5: Insights into the function of Ret, Epo and β-catenin

Figure 5-5: Cell cycle dynamics of TH-Cre:β-cat<sup>Ex3/+</sup> vs. WT

GF data for E16.5 embryos: black bars represent TH-Cre:βcat<sup>Ex3/+</sup> compared to WT values represented by the white bars: A) GF for the TH-Cre:βcat<sup>Ex3/+</sup> embryos is significantly lower than WT animals at the same developmental stage; B) GF of Sox10+/TH- glial progenitors in the TH-Cre:βcat<sup>Ex3/+</sup> is embryos significantly lower than what is observed for the WT. C) T<sub>S</sub> of the Sox10+/TH- glial progenitors is longer in the TH-Cre:βcat<sup>Ex3/+</sup> compared to the WT embryos, however this was just below a significant difference; D) there is no difference between the LI₀ in both TH-Cre:βcat<sup>Ex3/+</sup> or WT embryos; and E) the T<sub>C</sub> of Sox10+/TH- glial progenitors, at E16.5, is virtually the same for TH-Cre:βcat<sup>Ex3/+</sup> and WT embryos (WT = C57b6 embryos)
Chapter 5: Insights into the function of Ret, Epo and β-catenin

**Panel A: Sox10-/TH+ Growth fraction**

**Panel B: Sox10+/TH- Growth fraction**

**Panel C: Sox10+/TH- S-phase Length**

**Panel D: Sox10+/TH- Labelling index**

**Panel E: Sox10+/TH- Cell cycle length**
Chapter 6

6 Final discussion
### 6.1 Introduction

In the previous three results chapters (3, 4 & 5) I have considered some aspects of the measurement of cell cycle parameters. In this section, I will consider some additional aspects, critically evaluate the technique overall and look ahead to what will grow out of this approach.

### 6.2 Are there similarities between proliferation in the SNS, CNS and other parts of the PNS?

Development of the cortex starts with a pseudostratified neuroepithelium (NE). Neuroepithelial cells initially undergo symmetrical divisions to increase in number (Kriegstein and Alvarez-Buylla, 2009). Neuroepithelial cells then differentiate into radial glial cells (collectively NECs and radial glial called apical progenitors), expressing both stem cell and glial markers which start to give rise to cortical neurons via asymmetric divisions, or to intermediate precursor cells (IPC) that go on to divide symmetrically in the SVZ and give rise to neurons (Kriegstein and Alvarez-Buylla, 2009). When a neuron arises from division of a radial glial cell or IPC it differentiates and withdraws from the cell cycle (Salomoni and Calegari, 2010). Canonical Notch/Delta signaling has been identified as an important regulator of this process, and is thought to maintain progenitor status, preventing cell cycle withdrawal (Kageyama et al., 2009).

Dorsal root ganglia form from neural crest cells, but their subsequent differentiation and proliferation is more like the CNS than sympathetic ganglia. In the chicken DRG, neural crest progenitors undergo asymmetric divisions that renew the progenitor and give a daughter cell that differentiates as a neuron (Rohrer, 2011). The latter cell withdraws from the cell cycle and expresses neuronal markers (Rohrer, 2011). Numb distributes asymmetrically between the two daughter cells and the cell inheriting Numb becomes a neuron that expresses high levels of Delta (Wakamatsu et al., 2000). Delta signalling from the newborn neurons acts on Notch on the progenitors to maintain them in that form.
In the DRG, neurons aggregate centrally and progenitor cells peripherally. Glial cells appear to arise from progenitors later in development under the influence of Notch and neurogenin (Morrison et al., 2000, Wakamatsu et al., 2000). Hence, the pattern of proliferation and differentiation in DRG resembles that seen in the CNS. Increase in cell number is predominantly due to asymmetric division of a neural stem cell, differentiation into a neuron is accompanied by cell cycle withdrawal and glial cells are generated somewhat later than neurons.

The question then arises whether the pattern of proliferation and differentiation in sympathetic ganglia is fundamentally different from these examples. In one sense, it is possible to find functional equivalence in the cells involved between proliferation in the cortex and in sympathetic ganglia. For instance, CNS neuroepithelial cells are the equivalent of NCCs as they both act as founder cells. In addition, NCCs arise from the ventricular zone of the neural tube and undergo an EMT and delaminate from the NE. Radial glia, which can give rise to both neurons and glia, are likely the equivalent of the Sox10+/Phox2b+/TH-cell that arises from the NCCs on E10.5. IPCs are the equivalent of sympathetic neuroblasts, and perhaps glial progenitors, as each give rise only to a single type of cell that ultimately withdraws from the cell cycle.

The DRG share other similarities with sympathetic ganglia. The starting cell is obviously very similar (NCCs in each case). Both show the same pattern of development, with neuroblasts/neurons tending to appear centrally in the ganglion with NCCs and glia appearing peripherally. In each case, Notch/Delta signalling plays a role in regulating the balance between progenitor, neuronal and possibly glial phenotype (Morrison et al., 2000, Wakamatsu et al., 2000, Taylor et al., 2007).

While one can find cells with apparently analogous roles in cortex and sympathetic ganglion, there is a dramatic difference in the contribution each cell makes. In the cortex, asymmetric division of neural stem cells (radial glia) provides a constant stream of neurons and IPCs over many days to fuel growth of the cortex. In the sympathetic ganglia, the neural stem cells (NCCs and
Sox10+/Phox2b+/TH- cells) are consumed quickly over no more than two days, presumably by a high rate of symmetric divisions into neuroblasts or glial progenitors. It is the neuroblast (likely equivalent to the IPC) that is responsible for most of the increase in neuron number in the ganglion. Neuroblasts are presumably dividing symmetrically on E11.5 and E12.5, to give rise to more neuroblasts, while after that time they must have a rapidly increasing probability of dividing asymmetrically to give one neuroblast and a cell that withdraws from the cell cycle to become a mature neuron. In the cortex, IPCs divide symmetrically to produce daughter IPCs, or two neurons that differentiate and withdraw from the cell cycle (Kriegstein and Alvarez-Buylla, 2009).

While the comparison of sympathetic ganglion to cortex or DRG can find parallels between the cell types involved, it does not lead to a deeper understanding. While it is clear that the differences are likely due to the regulation of the rate of differentiation and cell cycle withdrawal, other than Notch/Delta, few common signalling pathways link proliferation in each part of the nervous system. However, this may reflect the absence of truly comparative studies rather than an absolute difference. Further studies are needed to resolve this.

Finally, we are left with the timing of differentiation into a neuron like cell as the major, indisputable difference between cortical and DRG development on the one hand and sympathetic development on the other. In the cortex/DRG, differentiation coincides with withdrawal from the cell cycle, i.e. it is the final act during proliferation. In sympathetic ganglia it occurs much earlier, while the cell is still differentiating. SNS neuronal precursors then re-enters the cell cycle as phenotypically neuronal cells, proliferating to produce bulk growth. The mechanism for cell cycle withdrawal from this point is unknown.
6.3 CNS cell cycle measurements: What cells are being measured?

The measurement of the $T_c$ using the methods described by Nowakowski et al. (1989a) and others makes the assumption that the cells for which the $T_c$ is calculated are homogenous. That is, they are a single type of cell sharing common cell cycle parameters. If cells with different cell cycle parameters are present but not distinguished, then the cell cycle parameters calculated are averages that represent no one cell type. This may in fact be the situation in the cortex, where early studies may have inadvertently pooled different cell types in the calculations. It is now known that the apical progenitors found in the VZ of the developing CNS are heterogeneous and are comprised of both neuroepithelial cells and radial glial cells (Hartfuss et al., 2001). In addition to this, there exist sub-populations of radial glial cells including those that give rise to neurons and those that give rise to glial precursors (Noctor et al., 2008). Further complicating the issue is the fact that apical progenitors can divide symmetrically to replace themselves, or asymmetrically to produce neurons (Wang et al., 2009).

Recent studies have started to address these problems, using a similar approach to that used in the present study. In the CNS, neuroepithelial cells and radial glia can either self renew via symmetrical divisions or divide asymmetrically to produce neurons (Dehay and Kennedy, 2007, Salomoni and Calegari, 2010). Using $Tis27GFP$ mice (Calegari et al., 2005, Arai et al., 2011), it is possible to distinguish between symmetrically dividing and asymmetrically dividing cells. Only the asymmetrically dividing cells that produce neurons express GFP under the control of $Tis27$ (Haubensak et al., 2004). At E15.5 there is a 50:50 ratio between the symmetrically and asymmetrically dividing cells and they have $T_c$’s at E15.5 of 14.8 h and 19.1 h respectively (Calegari et al., 2005, Arai et al., 2011). If all cells were pooled, the average cell cycle length is 17.4 h, consistent with what has been previously reported (Nowakowski et al., 1989a, Calegari et al., 2005). It must still be noted that both the symmetrically and asymmetrically dividing cells are themselves a mix of neuroepithelial cells and radial glial cells, suggesting that the measurements of $T_c$ in the cortex may not even now represent that of homogenous cell populations.
The strength of my study is that it has shown that immunofluorescence can be combined with cell cycle measurements to allow subpopulations of dividing cells to be identified (chapter 4). Immunofluorescence is simpler and more flexible than using transgenic animals. For instance multiple probes can be used on sections from the one animal. The challenge is to use more than one marker for identifying cell populations. In my four-channel fluorescence, two channels are taken for the S-phase markers, BrdU and EdU and one for the nuclear marker, bisbenzimide, leaving only one as a cell marker. One could forgo the nuclear marker and use that channel for another cell marker or a fifth channel could be added. With current fluorophores, the latter approach may require use of spectral unmixing techniques, which have not yet been widely adopted. Alternatively, fluorophores that excite and emit on much narrower wavelengths must be developed.

6.4 Are the populations of cells in developing sympathetic ganglia homogenous?

In my studies I have relied on simple dichotomous use of immunofluorescent markers to distinguish cells in sympathetic ganglia. From E12.5 onwards, there are only two significant populations of dividing cells, Sox10+/TH- glial precursors and Sox10-/TH+ neuroblasts. A tiny proportion (<1%) of the cells are Sox10+/TH+ are present throughout development (see chapter 3). Their low numbers are unlikely to affect the measurements. Prior to E12.5, the situation is more complex. On E9.5, Sox10 cells exist both with and without colocalised Phox2b. The cell cycle parameters measured, at E9.5, are dominated by Sox10+/Phox2b- cells (77% of the total), but I cannot rule out that the Sox10+/Phox2b+ cells have a different cell cycle length to Sox10+/Phox2b-. Similarly, on and after E10.5, many cells exhibit mixed phenotypes that combine Sox10, Phox2b and TH in various combinations. However, examination of Table 4.1 suggests that 98% of these cells contain Phox2b, suggesting that using three markers (Sox10, Phox2b and TH) instead of two (Sox10 and TH) is unlikely to significantly alter the cell cycle parameters reported.
6.5 The control of cell cycle dynamics: Insights into the downstream effectors of Epo and β-catenin signalling

At the most basic level, progression from one phase in the cell cycle to the next, is governed by the activity of cyclins and cyclin dependent kinases (cdk). The catalytic subunit, a cdk, is activated when bound by its regulatory subunit, a cyclin (Israels and Israels, 2000). Specific cyclin/cdk complexes regulate the progression through different phases of the cell cycle (Israels and Israels, 2000): G1 (cyclin D with cdk4/6), G1-S-phase transition (cyclin E with cdk2), S-phase (cyclin A with cdk2) and G2-M (cyclin A/B with cdk1 – see Fig. 1.3). The levels of each cyclin independently increase or decrease with specific phases of the cell cycle and it is this tightly regulated periodicity that controls the transition between the cell cycle phases. Changing the timing of accumulation and degradation of specific cyclin/cdk complexes will ultimately bring about changes in the duration of the discrete cell cycle phases, and possibly the overall Tc.

In order to have an influence on cell cycle dynamics, transcription factors, mitogens and receptor-mediated signalling pathways must have a way of impacting the timing and accumulation of specific cyclin/cdk complexes, or, have an impact on the factors that regulate their production. At the moment, this seems to be uncharted territory regarding SNS development. We are yet to identify the links between the cell cycle control mechanisms and the transcription factors, mitogens and receptor signalling pathways involved in SNS development. This is in some way to be expected, given the lack of information about how cell cycle dynamics are affected in GOF and LOF experiments. It follows that to identify candidates that may affect cell cycle dynamics one needs to be able to measure how cell cycle dynamics are altered when development has been perturbed.

6.5.1 Epo/EpoR signalling has the potential to regulate SNS cell cycle dynamics during embryonic development

I showed that Epo, has the ability to rescue the lengthened Tc observed in the RetTGM/TGM mice. This is primarily by restoring the GF and T3 back to values that are more like those observed in WT mice (chapter 5). However, the question
remains; what Epo-mediated signalling mechanisms could facilitate these changes in cell cycle dynamics?

Epo signals via the Erythropoietin receptor (EpoR), which can activate down-stream signalling pathways that have been shown to impact on cell cycle control mechanisms (Komatsu et al., 1997, Matsumura et al., 1999, Bouscary et al., 2003, Fang et al., 2007). In bone marrow erythroblasts, Epo/EpoR signalling has been shown to up-regulate at least five cell cycle progression factors: nuclear protein 1 (Nurp1), G1 to S phase transition 1 (Gsp1), Early growth response 1 (Egr1), Ngf-A binding protein 2 (Nab2), and cyclin D2 (Fang et al., 2007). In addition to this, Epo/EpoR signalling can dramatically down regulate the cell cycle inhibitory factors, cyclin G2, p27/Cdk1b and B-cell leukemia lymphoma 6 ((Bcl6) - Fang et al., 2007). These factors are thought to play important roles in controlling the progression through G1 and S-phases of the cell cycle (Fang et al., 2007). In addition, bone marrow erythroblasts that carry null mutations in the EpoR are abnormally distributed in G1/G0 and also accumulate aberrantly in S-phase (Fang et al., 2007). The bone marrow erythroblast defects observed when Epo signalling is perturbed correlates with the cell cycle defects that I observe in the Sox10-/TH+ neuronal precursors of the Ret^TGM/TM mice at E16.5. Cell cycle and S-phase are both lengthened, consistent with a lack of drive through EpoR and a consequent failure to push on through G1. EpoR is expressed early in SNS development (Knabe et al., 2005), so Epo/EpoR signalling has the potential to drive proliferation over a considerable time.

6.5.2 Overexpression of β-catenin results in altered cell cycle dynamics

It is well known that Wnt signalling is important for both proliferation and differentiation during embryonic development. Wnt signalling typically promotes progression through the cell cycle by inducing myc (either c-myc or n-myc), which is up regulated in response to β-catenin signalling in the nucleus (He et al., 1998, Mill et al., 2005). Myc has been shown to up regulate cyclin D and repress the cdk inhibitors p21 and p27, and consequently pushes the cell through the cell cycle faster while maintaining a proliferative state (Daksis et al., 1994, Gartel et al., 2001). In SNS development, removal of the seven pass trans-membrane Wnt receptor, Fz3, or conditional removal of β-catenin under the TH promoter (TH-
Cre:β-catenin\textsuperscript{lox/lox} mice), results in a reduction in the GF due to premature exit from the cell cycle and depletion of the proliferating pool (Armstrong et al., 2011). In chapter 5, I showed that conditional expression of a stable form of β-catenin in the heterozygote \textit{TH-Cre:β-catenin}\textsuperscript{lox/+} mice, resulted in a reduced growth fraction at E16.5 (see chapter 5). This was surprising given the fact that conventional Wnt signalling would predict that stable β-catenin should promote an increase in proliferation. This raises an interesting question: how can the removal of Wnt signalling via deletion of Fz3, have similar outcomes to increasing down stream Wnt signalling by the expression of stable β-catenin?

There is a body of literature that has been focused at understanding the role of Wnt/β-catenin signalling in the mitotic program (Salinas, 2007, Niehrs and Acebron, 2012). This includes the role of Wnt/β-catenin signalling in microtubule (MT) dynamics, spindle formation and centrosome division (Huang et al., 2007, Salinas, 2007). Members of the canonical Wnt signalling pathway, β-catenin, Axin2 and GSK3, accumulate at the centrosomes and can regulate microtubule growth (Huang et al., 2007). Disruption to any of these Wnt signalling components can lead to premature splitting of the centrosome ((or multiple centrosomes) - Huang et al., 2007, Bahmanyar et al., 2008), disruption of radial microtubule growth (Huang et al., 2007) and also the phenomenon where proteins destined for proteasomal degradation localize preferentially to only one of the two centrosomes (Fuentealba et al., 2008). The last feature, may lead to asymmetric protein distribution, even in otherwise ‘non-polarized’ cells (Fuentealba et al., 2008). Furthermore, in the CNS, disruption of the centrosome can lead to asymmetric division that results in premature exit of the proliferating pool, depleting the number of proliferating progenitors in favor of neurons (Wang et al., 2009).

It is possible that the perturbed canonical Wnt signalling in both \textit{TH-Cre:β-catenin}\textsuperscript{lox/+} and \textit{TH-Cre:β-catenin}\textsuperscript{lox/lox} mice, may lead to improper formation of the centrosome and mitotic machinery. I speculate that this may lead to premature asymmetric division that depletes the proliferating pool of neuroblasts in both cases (\textit{TH-Cre:β-catenin}\textsuperscript{lox/+} and \textit{TH-Cre:β-catenin}\textsuperscript{lox/lox} mice). This would provide a
Reason for the similarities between the results that I observed in chapter 5, and those observed by Armstrong et al. (2011).

### 6.6 Measuring cell cycle dynamics: Problems and solutions

Other than visually identifying dividing cells, the only way to study in vivo cell cycle dynamics is to apply experimental techniques that allow calculation of the T_c by measuring the GF, T_S, and LI. All of these parameters are needed to understand proliferation. However, there are some inherent technical limitations or caveats to this approach that must be discussed. These are considered below.

#### 6.6.1 Limitations in calculating the T_s

To calculate the T_S we used BrdU and EdU in a double-labelling protocol, modified from Hayes et al. (2000b). To ensure that the T_S is calculated accurately, there needs to be a sufficient number of cells that will incorporate both tracers during the injection interval and a significant number of them must leave S-phase (the S-phase leaving fraction) during the injection interval. This means that T_S is hard to estimate when you have very few cycling cells and/or S-phase is very long. An example of this was my failure to calculate the T_c for WT TH+ neuroblasts at E18.5 and for TH-Cre;β-cat^Ex3/+ embryos at E16.5. The problem of too few cycling cells can be overcome by increasing the number of cells examined, but at a major cost in effort.

When the T_S of a particular population of cells is extremely long (Figure 6.1B), this results in too few cells being measured in the S-phase leaving fraction. To overcome this, it is possible to lengthen the interval between the injections of the two S-phase markers. However, during this interval, the availability of the first tracer may drop to levels that fail to label S-phase cells if the interval is too long. It would be possible to make a second injection of the first S-phase marker, but care would need to be taken that there was always a level of the first marker present that would label all S-phase cells.

The opposite problem is the situation where S-phase is very short relative to the injection interval. If the S-phase is very short relative to the injection interval, cells may leave S-phase and progress through G2 and M before the
animal is killed. Division of labelled cells will overestimate the S-phase leaving fraction and underestimate the true value. The appropriate control is to shorten the injection interval, as I did in Chapter 4 to see if the $T_S$ changes.

6.6.2 Problems with immunofluorescence

Immunofluorescence allows identification of proliferating cells that may otherwise be intermingled, but this must be combined with treatments that open DNA to the antibodies needed for detection of BrdU. I used a 30 minute 2M HCl wash (see methods). Acid exposure can make the immunofluorescent detection of certain antigens impossible. All immunofluorescence for TH or Sox10 had to be completed before acid treatment. The secondary antibody labelling of the two antigens survived the acid treatment for both TH and Sox10. However, I found that Phox2b labelling did not survive acid treatment, even when completed before the acid wash. It may be possible to get around the problems caused by acid treatment by using DNAase treatment to expose the BrdU epitopes necessary for detection, but this was not investigated in this study.

Another important consideration is that while this technique purports to measure proliferation, it does not count actual cell divisions. Instead, the method calculates parameters that together predict the rate of cell division at that time, if the parameters remain unchanged. Thus, all parameters are estimates of the instantaneous growth fraction or instantaneous $T_C$, and give no indication of how rapidly that parameter is changing. Often the parameters are changing rapidly. When the $T_C$ of Sox10+/TH- cells is calculated on E10.5 as 30.3 h and E11.5 as 12.9 h it is clear that at least one or perhaps all three parameters ($GF$, $LI_0$, and $T_S$) are changing rapidly between the two time points.

6.6.3 Calculated $LI_0$ vs. individual $LI_0$

The $LI_0$ was calculated as the Y intercept from a least of squares linear regression through 3 data points from a cumulative S-phase labelling protocol (see methods section 2.5). Two of these data points come from the double-labelling (BrdU/EdU) protocol (0.5 h and 2.5 h) and the third, from a separate set of experiments where 2 h pulse of BrdU was administered. A line was fitted to the three time points (0.5, 2.0, 2.5 h) to estimate the Y intercept. This means that the labelling index is calculated from pooled data rather than individually for each
animal. Simple mathematical caution demands that the Y-intercept representing the LI is calculated by fitting to three rather than two points. However, if the LI could be calculated for each individual, it may provide insights not possible at the moment. For instance, from E9.5-E11.5, embryos can vary in developmental stage significantly, even within litters, so, calculating an individual labeling LI may be more beneficial for assessing individual variation. It would also simplify the experiments and eliminate the need for a second cohort of animals. Alternatively, one could estimate the Y intercept only from the 2 values derived from the double labelling protocol (LI0.5 and LI2.5). The relationship between time and S-phase marker uptake is known to be linear over this time (Nowakowski et al., 1989a, Hayes and Nowakowski, 2000b), so the intercept estimated from only two values will be only slightly less accurate than for three. In practice, when the difference between a LI0 calculated from 2 vs 3 values was calculated, it was never very great. Ultimately, if a third S-phase label was available, it would be possible to get the three values from a single animal and this should be adopted as soon as the technology becomes available.

6.6.4 BrdU toxicity

BrdU is a thymidine analogue that can be incorporated into the genome of cells that are synthesizing DNA (Bardos et al., 1955). The fact that BrdU is a thymidine substitute means that it can have toxic effects if incorporated into the genome at high dosage. Single injections of at 200-500mg/kg by bodyweight, and cumulative injections over a period of several days at lower dosages around 25-100 mg/kg by body weight (b.b.w), can cause side effects depending on the developmental age of the animal (Taupin, 2007). It has been reported that in the embryonic murine CNS (at E10), single injections of BrdU at low doses (>25mg/kg b.b.w) can result in an increase of pyknotic nuclei in some regions of the developing cortex 10h after the injection of BrdU (Nagao et al., 1998). However, it is generally accepted that single injections of BrdU, at 50-100mg/kg b.b.w over the short term (2.5 h), are safe for use when studying the cell cycle dynamics in the developing murine cortex (Miller and Nowakowski, 1988, Takahashi et al., 1995).
Anatomical location and the method by which BrdU is administered, will also affect the relative concentration and toxicity that cells are exposed to (Taupin, 2007). Injections of BrdU directly into target tissues (intracerebroventricular for example) will mean that cells are exposed to an effectively higher concentration of the tracer. I injected no more than 100mg/kg b.b.w in a single injection and all injections are delivered i.p into the pregnant dam. Using my techniques, pregnant dams are sacrificed no longer than 2.5 h after the initial injection of BrdU (see methods sections 2.5 & 2.5.1). Therefore, it is highly unlikely that we will see any effects on proliferation or development. This is also confirmed by the fact that the modeling data that uses our calculated values for $T_{c}$, almost perfectly predicts the changes proportions, of Sox10+ and TH+ cells, which we observe for mice that do not receive any BrdU injections at all. This confirms that my experimental program using BrdU labelling does not appear to alter cellular behavior in any significant way. Similarly, in all of my studies, the GF is not calculated via a cumulative labelling protocol, which requires repeated measurements of BrdU over many hours. Instead we use Ki67 labelling that eliminates the chance of BrdU toxicity. Note that EdU and BrdU are both thymidine analogues and in terms of toxicity can be considered equivalent, so the discussion above presumably applies to EdU as well.

### 6.6.5 Other methods to analyse cell cycle dynamics

The process I have developed to study proliferation is relatively time consuming and requires significant effort. If one is looking at 7 stages of embryonic development, multiple litters and embryos for each developmental stage, the time/cost benefit becomes a real consideration. However, there are several ways to study in vivo cell cycle dynamics that will not give you the full range of detail discussed in this discourse, but will yield more detail than using a single injection of a thymidine analogue.

A single Ki67 measurement tells you how many cells are in the cell cycle at an instant but does not tell you how many cells will continue to divide. At least two time points need to be measured to calculate the rate of change of GF. However the leaving fraction of a cell population can be calculated for a single time point by injecting BrdU (or EdU), and then sometime later, labelling for Ki67
Chapter 6: Final discussion

6.7 Future direction

6.7.1 Investigating the role of Ret in the development of the SNS

I have identified that the removal of Ret results in a 5 fold increase in the cell cycle length of neuroblasts at E16.5, when compared to WT mice. However, our model predicts that this is not sufficient to account for the difference in cell numbers that I observed in the stereological counts for E18.5 Ret<sup>TGM/TGM</sup> and WT embryos (see chapter 5). Our modelling does not account for the elevated cell death that has been reported in Ret<sup>TGM/TGM</sup> mice due to axon projection deficits (Enomoto et al., 2001), and this likely explains the discrepancy with my counted numbers. However, before we accept elevated cell death as the reason for the discrepancy between our modeled and counted numbers, I must investigate whether the removal of Ret also effects early development (prior to E12.5). So, to complete this study, I need to apply my techniques for measuring proliferation at E9.5-11.5 to see if Ret plays a role early in development.

6.7.2 The role of Epo and the EpoR in the development of the SNS

One of the most exciting results was the discovery that Epo can significantly rescue the cell cycle phenotype observed in the Ret<sup>TGM/TGM</sup> mice. However, I am yet to confirm that the cell cycle defect in the Ret<sup>TGM/TGM</sup> mice is actually caused by a loss of Epo. The organs that produce Epo in the embryo are the liver and kidney (Clemons et al., 1986, Wintour et al., 1996, Lee et al., 2001, Noguchi, 2008). I will have to measure the levels of Epo produced by both of these organs late in

(Armstrong et al., 2011). The cells that are BrdU (or EdU) positive that lack the Ki67-IR are cells that have withdrawn from the cell cycle.

It is also possible to detect other cell proliferation markers alongside BrdU (or EdU). One such marker is phosphorylated histone H3 (Ph3). Ph3 is expressed during M phase only. Staining for Ph3 after a pulse of BrdU (or EdU) will give information about how quickly cells progress from S phase to M phase. In fact using a double S-phase labelling protocol, followed by staining of Ph3 will allow for the estimation of the length of G2.
development, for both WT and Ret^{TGM/TGM} animals, to confirm whether or not Epo levels are actually reduced in the Ret^{TGM/TGM}.

In some respects, the fact that Epo appears to affect the T_c in the Ret^{TGM/TGM} mice is far more exciting. It is known that many NB tumors are positive for EpoR (Sartelet et al., 2007) expression, however, it has been concluded that Epo has no proliferative effects on NB cells in vitro (Sartelet et al., 2007). Based on this one study, the authors recommend that Epo is safe for use in the treatment of NB (Sartelet et al., 2007). Recently it has been shown that EpoR signaling plays a role in maintaining proliferation in glioblastoma cells, and that targeted reduction in EpoR signaling reduces their proliferation (Peres et al., 2011). However, the way that cells behave in culture can be quite different to the way that they behave in vivo, and the role that Epo plays in normal SNS development is yet to be examined. There is at least one study that reports the EpoR is strongly expressed in SNS precursors at E11.5 (Knabe et al., 2005). First, I will need to determine whether the EpoR is expressed on SNS precursors throughout the course of development. Second, I will have to measure the cell cycle kinetics in WT Epo-treated animals to see if Epo has any effect on normal development. Finally, I would like to use EpoR conditional knockout mice, using Epo^{floxed/floxed} animals crossed to either TH-Cre or Phox2b-Cre mice. This will produce conditional Epo LOF embryos, and may shed insight into its normal function.

The EpoR, like ALK, is a member of the cytokine receptor family and can induce proliferation and support cell survival (Chen et al., 2007). Canonical EpoR signaling is via the Jak/Stat pathway, and this is yet to be examined in the development of the SNS. Non-canonical EpoR signaling overlaps with pathways also shared by ALK, and both can activate ERK that is known to be important for proliferation (Chiarle et al., 2008, Peres et al., 2011). As described above, Epo is widely used in treatment of NB, and understanding its mechanism of action and its roles in vivo may be of paramount importance.

6.7.3 The role of Wnt/β-catenin signalling in the development of the SNS

Earlier, I described a possible mechanism for stable β-catenin playing a role in the organisation of the mitotic machinery, suggesting that the reduction in GF
observed in chapter 5, could be due to premature asymmetric division of neuroblasts leading to the production of neurons. One way of initially testing this idea, is to identify if the GF early in development in \(TH-Cre:\beta\text{-cat}\text{Ex}3/+\) mice is altered. From E11.5-E12.5, the GF is nearly 100% meaning that TH+ neuroblasts do not appear to be asymmetrically dividing to produce neurons. If, during this period, we observe a decrease in the GF, we may be able to conclude that cells are prematurely exiting and that β-catenin plays a role in this decision. In addition, it would be of interest to know whether the cell cycle dynamics of TH+ neuroblasts in the early stages of development is altered in the \(TH-Cre:\beta\text{-cat}\text{Ex}3/+\) mice. Given the fact that β-catenin is involved in the up regulation of factors that can reduce the time that a cell spends in the G1 phase of the cell cycle, I would hypothesise that TH+ neuroblasts would be dividing faster in \(TH-Cre:\beta\text{-cat}\text{Ex}3/+\) mice.

### 6.7.4 The role of Notch/Delta in the developing SNS

Earlier I discussed the idea that Notch/Delta signaling may simultaneously maintain proliferation and inhibit differentiation of Sox10+/TH- NC progenitors. Little is known about the importance of Notch/delta signalling in mouse sympathetic development. Activated Notch signalling can be detected using a commercially-available antibody that recognizes NICD (Tokunaga et al., 2004). I could use it to investigate Notch signalling in the Sox10+/TH- population. I could also investigate the role of differential downstream NICD signaling pathways, (CBF1 dependent and CBF1 independent) in the differentiation and proliferation of NCCS sub populations (Sox10+/Phox2b- and Sox10+/Phox2b-) using the transgenic \(\text{Notch}_{\text{CBF1active-GFP}}\) mice generated by Mizutani et al. (2007). The \(\text{Notch}_{\text{CBF1active-GFP}}\) mice express GFP when the CBF1 pathway is active (Mizutani et al., 2007). The hypothesis would be that undifferentiated Sox10+/Phox2b- would express high levels of GFP and the initially differentiated Sox10+/Phox2b+ cells, would express low or no levels of GFP.

It is also possible to test the function of NICD signaling through GOF experiments where, floxed NICD-overexpressing mice (Dong et al., 2010) are crossed to mice that express Cre-recombinase, driven by either the TH or Phox2b promoters. I could use my methods to investigate how proliferation is altered in mice that conditionally over-express notch
6.8 Neuroblastoma and cell proliferation

Neuroblastoma is the most common solid extra cranial tumor in children, it accounts for 7% of all malignancies and 15% of all pediatric oncological deaths in patients younger than 15 years (Maris et al., 2007). Neuroblastoma arises during the development of the sympathetic nervous system (Pahlman et al., 2004) and is thought to occur when control mechanisms for normal SNS development are deregulated (Axelson, 2004, Pahlman et al., 2004, Maris et al., 2007). The worst forms of neuroblastoma are associated with a lack of cellular differentiation (Axelson, 2004, Pahlman et al., 2004, Maris et al., 2007) and there is evidence that suggests that neuroblastoma cells are halted in an early embryonic state of development in which their proliferative capacity is maintained (Stockhausen et al., 2005).

While neuroblastoma has previously not been considered in this thesis, the results of my study are directly relevant to this disease as neuroblastoma appears to arise during development from disorders in the processes studied here. It is no coincidence that a growing body of evidence highlights the fact that genes commonly mutated in the more aggressive forms of neuroblastoma, either directly or indirectly, converge on to cell cycle mechanisms that result in faster progression through G1, leading to the maintenance of a stem cell phenotype and suppression of the cyclin/cdk inhibitors that act as the G1 restriction point. Examples of these genes are; N-MYC (Alam et al., 2009), ALK (Reiff et al., 2011), Notch (Axelson, 2004, Pahlman et al., 2004) and Phox2b (Reiff et al., 2010), for a good general review see Jiang et al. (2011). However, until the techniques outlined in this thesis are more widely used, their contribution to cell cycle dynamics during normal SNS development in vivo is unknown.

One of the most relevant observations in this thesis may be that differentiating SNS neuroblasts temporally withdraw from the cell cycle and then re-enter as proliferating neuronal precursors. This unique behaviour means that SNS neuroblasts must demonstrate the capacity to override the G1 restriction point imposed on them as they acquire a neuronal phenotype. The mechanisms that permit the overriding of the G1 restriction point, thus facilitating re-entry
into the cell cycle, are unknown, but this peculiar developmental behavior may be linked to the origin of neuroblastoma.
Chapter 6: Figures
Figure 6-1: Limitations of the double-labelling protocol

Illustration of the double labelling BrdU/EdU protocol: BrdU is injected first, cells in S-phase incorporate BrdU, EdU is injected 2 h later and the cells are harvested 0.5 h after the EdU injection. At the end of the protocol, BrdU+/EdU- cells (red nucleus) are those that left S-phase prior to the EdU injection. The BrdU+/EdU+ cells are those that remained in S-phase for the duration of the injection interval, and on the rare occasion, it is possible to see BrdU-/EdU+ cells (not depicted in this diagram). BrdU-/EdU+ cells represent cells that have just managed to enter the S-phase close to the end of the injection interval. The S-phase length is calculated using:

\[ T_s = \text{inj}_{\text{interval}} \times \left( \frac{\text{all cells positive for 2nd tracer}}{\text{cells positive for 1st tracer only}} \right) \quad \text{or} \quad T_s = 2 \times \left( \frac{\text{EdU}^* + \text{BrdU}^*}{\text{EdU}^*} \right) \]

A) Indicating the ‘ideal situation’, where cells have left S-phase, over the injection interval, but have not gone on to divide. B) This situation is not ideal as cells have a very long S-phase, and therefore no cells leave S-phase over the injection interval (no BrdU+/EdU- cells). This is the equivalent of the injection interval being too short. C) This situation is not ideal, here the injection interval is too long and BrdU+/EdU- cells that have left S-phase have gone on to divide, this will make the calculation of the leaving S-phase leaving fraction incorrect.
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7 List of references


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