Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author’s right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author’s permission before publishing any material from their thesis.

To request permissions please use the Feedback form on our webpage. http://researchspace.auckland.ac.nz/feedback

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the Library Thesis Consent Form and Deposit Licence.

Note : Masters Theses

The digital copy of a masters thesis is as submitted for examination and contains no corrections. The print copy, usually available in the University Library, may contain corrections made by hand, which have been requested by the supervisor.
A role for growth hormone in neurorestoration and neurogenic processes in the brain

Praneeti Pathipati

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Molecular Medicine, University of Auckland, 2010
ABSTRACT

The cerebral growth hormone (GH) axis plays an active role following ischemic injury to the brain. Studies have shown that both GH and its receptor are endogenously upregulated in response to ischemic injury and that GH administration post-injury confers significant neuroprotection. Furthermore, there is evidence that GH has trophic effects on neural stem cells (NSCs). However, whether GH can also aid long term recovery and/or have direct effects on neurogenic processes is unclear. Both in **vivo** and **in vitro** studies were carried out to address these issues.

*In vivo* studies using the endothelin-1 model of focal ischemic stroke in adult rats demonstrated that a long-term unilateral continuous intracerebroventricular (ICV) infusion of GH is capable of targeting specific areas of active remodelling and neurogenic processes. Immunohistochemistry analyses revealed that ipsilaterally infused GH localised specifically to neuronal and glial progenitor cells within the ipsilateral subventricular zone, white matter tract, lesion and penumbral regions. Treatment with GH commencing 4 days after stroke accelerated recovery in one out three tests of motor function and improved spatial memory on the morris water maze test with no effect on learning. *In vitro* studies were then carried out to further elucidate the role of GH in mediating neurogenic processes that could potentially contribute to long-term recovery. Studies were also conducted using the hormone prolactin (PRL) since it is closely related to GH and has similar trophic effects on NSCs. Using NSCs with properties of neurogenic radial glia derived from fetal human forebrains, it was determined that exogenously applied GH and PRL promote the proliferation of neural stem cells in the absence of epidermal growth factor or basic fibroblast growth factor. When applied to differentiating NSC’s, they both induce neuronal progenitor proliferation but only PRL has proliferative effects on glial progenitors. Both GH and PRL also promote NSC migration, particularly at higher concentrations. Interestingly, migration studies using receptor antagonists identified that both GH and PRL signal via the PRL receptor to promote migration.

In summary, these findings show that delayed treatment with GH may accelerate some aspects of functional recovery and improve spatial memory in the long-term. Furthermore, some of these beneficial effects may be mediated via its trophic effects on NSCs and thus is supportive of a role for GH in post-injury repair processes as well as developmental mechanisms in the brain.
ACKNOWLEDGEMENTS

Completing this thesis has certainly been an interesting and educational journey. Three primary supervisors, numerous funding issues and several technical issues later, I feel like I can face anything. All through this, I have been very fortunate to have the support of numerous people who helped make this thesis possible.

Dr. Mhoyna Fraser who helped me complete this journey – Your support and advice have helped me look forward and I am grateful to you for taking me on board. Dr. Arjan Scheepens who was not only my first supervisor but also a valuable mentor and friend: Thank you for initiating and seeing me through this journey, your guidance and mentorship have taught me a lot. Assoc. Prof. Christopher Williams for being an excellent director; constantly reminding me of the big picture and how to plan for the future. Dr. Thorsten Gorba for giving me a direction when I couldn’t find one. You gave me skills that helped me to not only develop as a researcher but also further my thesis work. I feel deeply indebted for your financial and intellectual support. Prof. Laura Bennett who has always been much more than just a scientific advisor; thank you for providing me with physical and emotional support when I needed it the most. I will always remain extremely grateful to you for always being there for me and giving me the strength to keep going forward.

I would also like to thank several people who have extended material and technical support. Mr. Andrzej Surus for his help with the IGF1 RIAs and GH RRA, Mrs Chris Keven for SEC analyses, Mr. Eric Thorstensen for CORT Mass Spec and Urea analysis, Mr. Wing Leong for help with the surgeries, Mr. Nagarajan Kannan, Mrs Prudence Grandison, Mr. Vijay Pandey, Mr. Nethaji Muniraj and Mr. Brahmanaspati Shastri for advice and guidance with molecular work. In addition, I’m extremely grateful to Prof. Peter Lobie and Dr. Jo Perry for giving me B2036, Prof. Vincent Goffin (InSerm, France) for his invaluable intellectual advice, critique and comment on my in vitro work along with providing me with the PRL and PRLR antagonist, Prof. Wayne Cutfield for the Genotrophin, Prof. Austin Smith and Dr. Yirui Sun (Wellcome Trust Centre for Stem Cell Research, Cambridge) for providing me with the very valuable human NSCs.

My heartfelt gratitude towards all members of what was the Baby Brain Injury group. Dr. Sumudu Ranasinghe, who was always there for any kind of support I needed and
gave me strength especially for the home run. Mrs. Larissa Christophidis who’s always been a constant source of inspiration and support both as a friend and as a fellow student. Dr. Tanja Moderscheim, the first out the door, for showing us the path and that it can certainly be done. Dr. Malin Gustavsson for being an awesome friend, supporter and well-wisher and Dr. Mariella Giovannangelo for being an excellent, efficient technician and a great friend.

Sincere thanks also to fellow residents of my Grad Room; Drs Sarah Hopkins, Severine Brunet-Dunand, Naeem Amiry and Nic Bougen. You guys certainly kept me sane in the most insane of periods and words can never express how much I appreciate all the fun times and fond memories. My precious friends Vinthiya, Swati, Pradeep, Megha, Anchal, Siva, Vishala, Ashwin, Akshat, and Wencke, thanks ever so much for all the fun times, hearing me out and putting up with me on strenuous days, giving me company on my late nights and for taking such good care of me when I couldn’t do it myself.

I would also like to acknowledge my in-laws Mr & Mrs Veeramachaneni for all their support and encouragement and the Param family for always being there, welcoming me with open arms every single time I needed them. My husband Ram, for putting up with my emotional last phase, patiently reminding me of my capabilities and constantly supporting me from the day I know him. Finishing would have been a hell of a lot rougher without him. And lastly but most importantly, my family. Mom, dad, sis and jiju – you have always believed in me a lot more than I did myself and to say that you’ve supported me always would be an understatement. This would have never even been possible without you. Thank you for everything. Finally, my lil bud Rayan; everything just got better since you stepped into this world. I hope one day you will understand what your smiles, hugs and ‘love yous’ did for me during my PhD. I love you to bits.
TABLE OF CONTENTS

ABSTRACT ....................................................................................................................................................... 1

ACKNOWLEDGEMENTS ................................................................................................................................. II

TABLE OF CONTENTS ...................................................................................................................................... IV

LIST OF FIGURES ............................................................................................................................................. VII

LIST OF TABLES ............................................................................................................................................... VIII

LIST OF ABBREVIATIONS ............................................................................................................................... IX

1 GENERAL INTRODUCTION ....................................................................................................................... 2

1.1 OVERVIEW .............................................................................................................................................. 2

1.2 STROKE .................................................................................................................................................. 3

1.2.1 Incidence and prognosis......................................................................................................................... 3

1.2.2 Risk Factors ....................................................................................................................................... 4

1.2.3 Etiology and subtypes .......................................................................................................................... 4

1.2.4 Animal models .................................................................................................................................... 6

1.2.5 Pathophysiology of ischemic stroke .................................................................................................... 11

1.2.6 Endogenous Response to Ischemic injury ........................................................................................... 18

1.2.7 Brain plasticity ................................................................................................................................... 20

1.3 CURRENT TREATMENTS FOR ISCHEMIC STROKE ............................................................................ 31

1.4 GROWTH HORMONE ............................................................................................................................. 35

1.5 PROLACTIN ............................................................................................................................................ 51

1.6 AIMS....................................................................................................................................................... 55

2 MATERIALS AND METHODS .................................................................................................................. 58

2.1 IN VIVO STUDIES .................................................................................................................................. 58

2.1.1 Animals ............................................................................................................................................ 58

2.1.2 Rat growth hormone buffer (rGH buffer) ............................................................................................. 58

2.1.3 Stereotactic Endothelin-1 (ET1) infusion surgery ............................................................................... 59

2.1.4 Overview of the in vivo studies ........................................................................................................... 61

2.1.5 ICV Cannula and pump placement ....................................................................................................... 62

2.1.6 Behavioural Testing ............................................................................................................................ 64

2.1.7 Blood and CSF sampling .................................................................................................................... 66

2.1.8 Catheter status at post-mortem ......................................................................................................... 66

2.1.9 Post-mortem, tissue harvestation and processing ............................................................................. 67

2.1.10 Radioimmunoassay (RIA) for IGFI .................................................................................................... 68

2.1.11 Corticosterone high performance liquid chromatography (HPLC) coupled with mass spectrometry 69

2.1.12 Urea measurements ......................................................................................................................... 70

2.1.13 Acid Fuschin-Thionin Staining and measurement of tissue survival ............................................... 71

2.1.14 Immunohistochemistry .................................................................................................................... 71

2.1.15 Quantification of GH and DCX labeling: dose response study ......................................................... 72

2.1.16 Statistics ......................................................................................................................................... 73

2.2 IN VITRO STUDIES ................................................................................................................................ 74

2.2.1 Cell culture ....................................................................................................................................... 74

2.2.2 Functional Assays ............................................................................................................................. 77

2.2.3 Molecular Biology ............................................................................................................................ 80

2.2.4 Statistics ......................................................................................................................................... 85

3 CENTRAL INFUSION OF GH POST-ISCHEMIA IN THE ADULT BRAIN: BEHAVIOURAL AND ENDOCRINE EFFECTS 86

3.1 INTRODUCTION .................................................................................................................................... 86

3.2 RESULTS ............................................................................................................................................... 87

3.2.1 Buffer formulation and testing .......................................................................................................... 87

3.2.2 Behavioural analysis .......................................................................................................................... 88

3.2.3 Plasma and CSF measurements ....................................................................................................... 91

3.2.4 Body and brain weights .................................................................................................................... 92

3.3 DISCUSSION ......................................................................................................................................... 94
4 CENTRAL INFUSION OF GH POST–ISCHEMIA IN THE ADULT BRAIN: REGION AND CELL-SPECIFIC TARGETING OF INFUSED GH

4.1 INTRODUCTION ................................................................. 98
4.2 RESULTS ............................................................................ 99
  4.2.1 GH infusion after stroke may alter the tissue survival .................. 99
  4.2.2 ICV GH following injury localises to neurogenic regions and the infarct penumbral area ......................................................... 100
  4.2.3 Quantification of GH immunoreactive cells ............................... 103
  4.2.4 GH immunopositive cells double-label with DCX and GFAP ....... 106
  4.2.5 Quantification DCX staining ............................................... 108
4.3 DISCUSSION ...................................................................... 111

5 DELAYED AND CHRONIC TREATMENT WITH GH AFTER STROKE MAY BE BENEFICIAL ................................................. 116
5.1 INTRODUCTION ............................................................... 116
5.2 RESULTS .......................................................................... 117
  5.2.1 Delayed onset of GH-treatment does not provide any neuroprotection ................................................................. 117
  5.2.2 Delayed and chronic treatment of GH after stroke may accelerate some aspects of functional recovery118
  5.2.3 Delayed and chronic GH treatment improved spatial memory ................................................................. 121
  5.2.4 Delivered GH was bioactive for the duration of infusion ................ 122
  5.2.5 GH treatment caused a transient increase in overall body weight but a decrease in spleen weight... 123
5.3 DISCUSSION .................................................................... 125

6 GH HAS PROLIFERATIVE AND CHEMOATTRACTIVE EFFECTS ON NSCS IN VITRO .......................................................... 130
6.1 INTRODUCTION ............................................................... 130
6.2 RESULTS .......................................................................... 130
  6.2.1 Characterisation of hNSCs .................................................. 130
  6.2.2 Physiological potency of Genotropin® .................................... 131
  6.2.3 Basal expression of GHR, IGF1R and IGF1 but no GH or IGF2 in hNSCs ......................................................... 132
  6.2.4 GH promotes the proliferation of hNSCs in the absence of EGF and bFGF ......................................................... 135
  6.2.5 GH promotes the proliferation of neuroblasts but not glial progenitors ......................................................... 136
  6.2.6 GH promotes the maturation of neurons but inhibits neurogenesis ......................................................... 137
  6.2.7 GH promotes the migration of hNSCs .................................... 140
6.3 DISCUSSION .................................................................... 142

7 PRL ALSO HAS PROLIFERATIVE AND CHEMOATTRACTIVE EFFECTS ON NSCS IN VITRO .................................................. 149
7.1 INTRODUCTION ............................................................... 149
7.2 RESULTS .......................................................................... 149
  7.2.1 hNSCs predominately express full length PRLR, with weak expression of the intermediate form ................................. 149
  7.2.2 rhPRL promotes the proliferation of hNSCs in the absence of EGF and bFGF ......................................................... 150
  7.2.3 rhPRL promotes the proliferation of neuroblasts and glial progenitors ......................................................... 151
  7.2.4 PRL can inhibit or promote migration of hNSCs .................... 152
7.3 DISCUSSION .................................................................... 154

8 GENERAL DISCUSSION .................................................................. 158
8.1 OVERVIEW ...................................................................... 158
8.2 MAJOR FINDINGS ............................................................ 159
  8.2.1 Central infusion of GH post ischemia in the adult brain; Behavioural and endocrine effects .................. 160
  8.2.2 Central infusion of GH post–ischemia in the adult brain; region and cell-specific targeting of infused GH .... 160
  8.2.3 Delayed and chronic treatment with GH after stroke may be beneficial ......................................................... 160
  8.2.4 GH has proliferative and chemotactic effects on NSCs in vitro ......................................................... 161
  8.2.5 PRL has proliferative and chemotactic effects on NSCs in vitro ......................................................... 161
8.3 IMPLICATIONS .................................................................... 162
  8.3.1 ET1 model of stroke and ICV infusion of GH ...................... 162
  8.3.2 Effects of GH on neurogenic processes .............................. 164
  8.3.3 Effects of GH on functional recovery following stroke .......... 166
  8.3.4 Effects of PRL on neurogenic processes ............................. 168
  8.3.5 Use of GH and PRL in the brain: Factors to consider ............ 171
8.4 LIMITATIONS .................................................................... 172
8.5 FUTURE DIRECTIONS.................................................................................................................. 173
8.6 SUMMARY .................................................................................................................................. 175
8.7 CONCLUSION ............................................................................................................................ 177

9 ADDENDUM.................................................................................................................................. 178

10 APPENDIX .................................................................................................................................. 179

10.1 MODIFIED BOUIN’S SOLUTION ............................................................................................. 179
10.2 0.1M PHOSPHATE BUFFERED SALINE (PBS) .......................................................................... 179
10.3 0.01M CITRATE BUFFER ......................................................................................................... 179
10.4 0.01M POTASSIUM PHOSPHATE BUFFERED SOLUTION (KPBS) ........................................... 180
10.5 2, 3-AMINOPROPYLsilane coating for slides ............................................................................ 180
10.6 BUFFERS FOR IGF1 RADIOIMMUNOASSAY ........................................................................... 180
  10.6.1 Buffer A: Acidic Dilution Buffer ...................................................................................... 180
  10.6.2 Buffer B: Antibody & Tracer dilution buffer ..................................................................... 181
  10.6.3 Buffer C: Acidic buffer for plasma .................................................................................. 181

11 LIST OF REFERENCES .................................................................................................................. 182
LIST OF TABLES

TABLE 1.1: A BRIEF DESCRIPTION OF THE IONIC AND METABOLIC CHANGES OCCURRING IN THE CORE AND THE PENUMBRA DURING ISCHEMIA 15
TABLE 2.1: PERCENTAGE OF IMPAIRMENT OF EACH MATCHED PAIR POST-STROKE ........................................................................................................ 64
TABLE 2.2: SEQUENTIAL PROCESS OF AUTOMATED TISSUE PROCESSING. ........................................................................................................... 68
TABLE 2.3: REHYDRATION OF SLIDE-MOUNTED SECTIONS PRIOR TO STAINING. ......................................................................................... 68
TABLE 2.4: FOUR-POINT SCALE USED FOR GH/DCX QUANTIFICATION ........................................................................................................... 72
TABLE 2.5: NANODROP RESULTS OF A REPRESENTATIVE RNA SAMPLE EXTRACTED USING THE RNEASY MINI KIT ...................... 81
TABLE 2.6: LIST OF PRIMERS. .................................................................................................................................................. 83
TABLE 2.7: LIST OF POSITIVE CONTROLS FOR EACH GENE EXAMINED USING PCR .................................................................................... 84
LIST OF ABBREVIATIONS

24h – 24 hours
AC – PKA – Adenylyl cyclise – protein kinase A
ACTH – Adenocorticotropic hormone
AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate
APCI – Atmospheric pressure chemical ionization
βIIIITubulin – Neuron-specific marker
DAB – 3,3′-Diaminobenzidine
DAPI - 4′,6-diamidino-2-phenylindole, DNA stain to label nuclei
BBB – Blood brain barrier
BDNF – Brain-derived neurotrophic factor
bFGF – Basic fibroblast growth factor
BrdU - Bromodeoxyuridine
BSA – Bovine serum albumin
BV – Blood Vessels
CBF – Cerebral blood flow
CM – Conditioned medium
CNS – Central nervous system
CSF – Cerebrospinal fluid
CXCL4 – Chemokine ligand 4
DCX – Doublecortin
DCX+ - Doublecortin positive
DG – Dentate gyrus
DPX – Dibutyl phthalate (mounting medium)
E# – Embryonic day #
EGF – Epidermal growth factor
ERK – Extracellular regulated kinase
EPO - Erythropoietin
ET1 – Endothelin-1
GABA – Gamma-amino butyric acid
GAP43 – Growth-associated protein 43
GCL – Granule cell layer
GF – Growth factor
GFAP – Glial fibrillary acid protein
GH – Growth hormone
GH+ - Growth hormone positive
GHBP – Growth hormone binding protein
GHD – Growth hormone deficiency
GHR – Growth hormone receptor
GHRA – Growth hormone receptor antagonist
GHRH – Growth hormone releasing hormone
GHRS – Growth hormone receptor substrate
GLDH – Glutamate dehydrogenase
GnRH – Gonadotrophin-releasing hormone
HCl – Hydrochloric acid
hGH/PRL/NSC – Human growth hormone/prolactin/neural stem cells
hpGH – Human pituitary growth hormone
HI – Hypoxia ischemia
HPLC – High-performance liquid chromatography
ICV - Intracerebroventricular
IGF1 – Insulin-like growth factor 1
IGFBP – Insulin-like growth factor binding protein
IRS – Insulin receptor substrate
JAK-STAT- Janus activated kinase - signal transducer and activator of transcription
KPBS – Potassium phosphate buffered saline
LV – Lateral ventricle
M1 – Primary motor cortex region
MAPK – Mitogen-activated protein kinase
min - Minutes
MCA – Middle cerebral artery
MCAO – Middle cerebral artery occlusion
MWM – Morris water maze
Na2B4O7 – Sodium tetraborate (borax)
NeuN – Neuronal nuclei
NGS – Normal goat serum
NMDA – N-Methyl-D-Aspartate
NSC – Neural stem cell
NZ – New Zealand
O/N – Overnight
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PFA</td>
<td>4% Paraformaldehyde</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol triphosphate</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PRL</td>
<td>Prolactin</td>
</tr>
<tr>
<td>PRLBP</td>
<td>Prolactin receptor binding protein</td>
</tr>
<tr>
<td>PRLR</td>
<td>Prolactin receptor</td>
</tr>
<tr>
<td>PRLRA</td>
<td>Prolactin receptor antagonist</td>
</tr>
<tr>
<td>RG</td>
<td>Radial glia</td>
</tr>
<tr>
<td>rGH/PRL/NSC</td>
<td>rat growth hormone/prolactin/neural stem cells</td>
</tr>
<tr>
<td>RG</td>
<td>Radial glia</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RRA</td>
<td>Radioreceptor assay</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>rtPA</td>
<td>recombinant tissue plasminogen activator</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SDS PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>S100β</td>
<td>S100 calcium binding protein B, marker for immature astrocyte</td>
</tr>
<tr>
<td>SEC</td>
<td>Size-exclusion chromatography</td>
</tr>
<tr>
<td>SGZ</td>
<td>Sub-granular zone</td>
</tr>
<tr>
<td>SHC</td>
<td>Src homology containing domain</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressors of cytokine signalling</td>
</tr>
<tr>
<td>SS</td>
<td>Somatostatin</td>
</tr>
<tr>
<td>STATs</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>SVZ</td>
<td>Sub-ventricular zone</td>
</tr>
<tr>
<td>WM</td>
<td>White matter</td>
</tr>
<tr>
<td>WMT</td>
<td>White matter tracts</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
1 General Introduction

1.1 OVERVIEW

Growth hormone (GH) is an anterior pituitary hormone that is best known for its effects on postnatal body growth. It stimulates body growth and cell reproduction and regeneration in humans and other animals [8]. The neural actions of GH were first documented in 1941 [9] and since then numerous studies have identified a significant role for the GH axis (including insulin-like growth factor 1 (IGF1)) in the brain during development and disease [10, 11, 12]. A significant role for the GH axis in neuroprotection and recovery from juvenile ischemic brain injury in particular has been elucidated by our group [13-19]. These studies, along with others [20-22] also provide firm evidence for the involvement of GH in aspects related to neurogenesis. However, while these studies have identified an important role for the GH axis in recovery processes in the injured juvenile brain (especially in the re-capturing of developmental processes related to recovery), the long-term effects of GH on recovery after ischemia, especially in the adult brain remain to be elucidated. Further, the direct role of GH in neurogenesis and thus promotion of recovery from injury needs to be clarified.

Stroke is an acute cerebrovascular incident characterized by a sudden disturbance in blood supply to the brain that can occur at any age. It results in severe cell damage that manifests as a loss of function in the affected area of the brain, and depending on its location, can lead to partial or complete paralysis or severe deficits in vision or speech or a combination of these. In children and infants, these can translate to long-term developmental disabilities. To date, despite extensive research, treatment options for stroke are very limited, especially if the patient is presented beyond 3-4.5 hours after stroke-onset [23]. Given that stroke is a major cause for death and disability worldwide [24], this presents an urgent need for successful treatment strategies that can be used outside of the limited time-frame.

Accordingly, the aims of this thesis were to a) study in vivo, the neuroprotective, neurorestorative, neuroendocrine and behavioural effects of central treatment with GH following ischemic stroke in the adult brain and b) examine in vitro, the direct effects of GH on the proliferation, differentiation and migration of fetal neural stem cells (NSCs). Further, since prolactin (PRL) is closely related to GH and therefore, like GH, has also
been elucidated to play a pivotal role in injury-related and neurogenic processes [16, 25-28] the direct proliferative and migratory effects of PRL on NSCs was also investigated.

The overall objective of this body of work is to further current knowledge of ischemic stroke and the role of GH axis in protection and recovery from such an insult. It provides evidence for GH being a promising therapeutic agent for long-term neurorestoration following stroke - as an augmenter of the natural recovery process following ischemic injury and as a potent neurogenic factor re-instating developmental growth processes to aid recovery. The following literature review provides the necessary background for these studies, starting with the pathophysiology, the endogenous responses to stroke and currently available therapeutic treatments. Shortcomings of currently available therapeutic modalities are discussed, followed by GH biology and current literature on its neuroprotective and possible neurorestorative effects. The biology of neurogenesis and PRL are also briefly described. Finally, the research aims of this thesis are detailed at the end of the review.

1.2 STROKE

1.2.1 Incidence and prognosis

Stroke is the third biggest killer and the greatest cause of long term adult disability in New Zealand (NZ). Around 21 NZers have a stroke every day, and approximately one-third of these people die within the first 12 months with another third becoming disabled [29]. Worldwide, in 2001 alone, an estimated 20.5 million people suffered from strokes with over 5.5 million of these cases resulting in fatalities [30]. It is thought that on average, 25% of stroke sufferers die from a stroke or its complications and at least 50% have health problems and long-term disabilities [30]. The incidence of perinatal stroke has been shown to range from 1 in 4000 live births [31] as high as 17% in autopsy studies of term newborns, with a mortality rate of about 10.1% in infants less than 30 days old [31]. Four out of five newborns that suffered a perinatal stroke develop neurologic disorders such as cerebral palsy, epilepsy, or language delay [32]. As a result of its severe impact, stroke critically impacts the functional independence and reduces the quality of life for both patients as well as their caregivers. Further, it imposes a great economical burden; about $138 million dollars in NZ [29] and about 2-4% of total health care costs worldwide [24] are spent per year for stroke-related hospital services alone. Given the rise
in the average life expectancy, it is expected that the incidence and prevalence of stroke will also increase, ultimately leading to a rise in disability [33].

### 1.2.2 Risk Factors

One of the major risk factors for stroke is age [30]. Incidence of stroke increases with advancing age, at least partly due to the parallel rise in medical conditions such as hypertension and diabetes. Prior occurrence [34] or family history [34, 35] is also associated with a greater risk of stroke. In addition to this, cerebrovascular risk factors such as atrial fibrillation, recent myocardial infarction and asymptomatic carotid stenosis, other modifiable risk factors such as cholesterol levels, smoking and stress all have well-confirmed roles in promoting stroke formation [36-38]. Strokes occurring during the perinatal and childhood periods have been associated with premature birth, maternal infections, maternal drug abuse, prior infertility treatments and other maternal conditions such as autoimmune disease and preeclampsia [39].

### 1.2.3 Etiology and subtypes

A stroke usually results from an interruption of or decrease in, cerebral blood flow (CBF) (also known as cerebral hypoperfusion) which, especially if prolonged, leads to a steady depletion in nutrients such as glucose and oxygen which are essential for physiological cellular function. This in turn causes a disruption in energy balance. Cell death involving various complex pathways (discussed in Section 1.2) ensues from this disruption, leading to loss of functional tissue [40, 41] which can ultimately lead to a loss of function.

Disruption of CBF can occur in one of two ways; spontaneous rupture of cerebral blood vessel(s) resulting in a hemorrhagic stroke or thrombolytic or embolic occlusion(s) leading to an ischemic stroke [24].

#### 1.2.3.1 Hemorrhagic Stroke

Hemorrhagic strokes ensue from the rupturing of a blood vessel, causing an internal bleed either into the brain tissue (intracranial haemorrhage) or onto the surface of the brain (subarachnoid haemorrhage). While 10% of all strokes are caused by intracranial haemorrhage, a minority of only 5% are of subarachnoid haemorrhage origin [30]. Haemorrhages result from a rupture of cerebral arteries (intracranial haemorrhage)
possibly due to an aneurysm (most common in subarachnoid haemorrhage) and are usually a consequence of hypertension or vascular malformations [42]. Rupturing of cerebral arteries invariably occurs in the basal ganglia, thalamus, pons and cerebellum with extravasation of blood into surrounding cerebral tissue and/or subarachnoid space. Such leakage of blood can lead to displacement and compression of surrounding tissue, and result in vasospasms of adjacent vessels, ultimately leading to ischemic injury of the tissue. Clot formation also occurs in this process that, although eventually resolves, results in the formation of edema and necrosis in surrounding tissue [36, 43]. The onset of spontaneous hemorrhagic strokes is usually abrupt and rapid, developing over 30 to 90 min although, if associated with anticoagulant therapy (discussed in section 1.3.2) can develop over 24-48 h [36]. In spite of their reduced occurrence, subarachanoid haemorrhage strokes are very dangerous and are associated with a mortality rate of about 50% [43].

1.2.3.2 Ischemic Stroke

About 80% of all strokes are ischemic [44]. Ischemic strokes result from obstructive blood clots called thrombi which form in the heart [39]. A thrombus usually develops from endothelial injury and inflammation which leads to a plaque formation. Loss of muscle cells leads to thickening of this plaque and consequent adherence of platelet cells which trigger the coagulation-clotting cascade [36]. This clot either stays in place or can break off as an embolus, travel and occlude a distal vessel [36], commonly the middle cerebral artery (MCA) in the brain. Since thrombic occlusion of blood vessel(s) is gradual, symptoms are often progressive and increase over several hours to days. An embolic stroke however, is often of sudden onset, with immediate, noticeable and maximal neurological deficits [36, 43]. Ischemia occurs when the blood flow to the brain tissue is less than 20 mL/100 g per minute [43]. If the total blood supply to the brain is reduced, such as after a cardiac arrest or severe hypotension, global ischemia occurs. Although easier to replicate in animal models, it is less relevant to humans as it is not a common feature of human stroke [45].

Focal ischemia, a more common kind of ischemic cell death in humans, results from a sudden disruption in CBF in a single, or group of vessels in the brain [2, 45]. Vessel occlusion eventually leads to a transient or permanent interruption in the blood flow to part(s) of the brain supplied by that artery leading to depletion of nutrients essential for
normal cellular function [40, 41]. This results in immediate necrotic and delayed apoptotic death. Although largely similar, some differences have been shown in the pathophysiology of global and focal ischemia [45].

At this point it is pertinent to mention that the primary focus of this review and ultimately, the thesis, is on unilateral focal ischemic stroke.

1.2.4 Animal models

Stroke studies usually employ animal models. Although epidemiological studies have contributed significantly to stroke research [46], detailed investigations into the mechanisms of ischemic damage and therapeutics are required, which cannot be extrapolated from these studies. Given the systemic risk factors of stroke (described in Section 1.2.2), it is also important to study the possible contributing effects of systemic diseases. In addition, stroke also has a large clinical variability especially in terms of duration, localization, severity of ischemic/damage, raising the need for very large patient group sizes which is not practically feasible [5]. As such, currently no \textit{in vitro} methods can satisfactorily simulate the complex interplay of vasculature, brain tissue, and blood flow during stroke [47]. Further, stroke produces behavioural and functional deficits by damaging neuronal circuits and clinical manipulation of these in response to treatment modalities under controlled conditions is not feasible. In order to design effective treatment strategies, it is necessary firstly, to model the complex pathophysiology occurring after stroke (described in section 1.3) and secondly, to develop and test various anti-ischemic treatment modalities focusing especially on behavioural and functional endpoints which are of most interest clinically.

Several experimental cerebral ischemia models have been developed to mimic ischemic stroke that now serve as indispensable tools (Figure 1.1). It is these models that have increased the understanding of the pathophysiology of ischemia enabling the development of treatment strategies [5], some of which have successfully been translated to the clinic [23, 39]
1.2.4.1 The advantages and limitations of using rodents

Rats have been used extensively as models for mimicking human stroke as their cerebrovascular anatomy and physiology (including the presence of a circle of Willis) closely resembles that of the human [48, 49]. Their moderate size allows for easy monitoring with low cost procedures and reproducible studies [50] while the relative homogeneity within strains (due to intensive inbreeding) minimizes the confounding effects of heterogeneity with the possibility of genomic modulation [51]. Further, the small brain volume of the rat facilitates various analytical procedures, enabling more extensive and comprehensive evaluation of the entire brain without excessive cost, time and labour [50]. More importantly however, rats allow for testing and studying functional outcomes in experimental stroke. The rat has been extensively studied in terms of its motor behaviour [52], and limb movement analyses have shown very similar motor components in human upper extremity and rat forelimb movement during certain behaviours [53]. A battery of sensorimotor tests are now readily available to measure various aspects of both motor impairment and recovery after ischemic insults in rats which can be used for stroke studies [52]. These tests, when combined with the extensive knowledge of the anatomical and neurophysiological organization of the rodent motor system [54], aid the development of novel adjuvant therapies that may enhance recovery.

Figure 1.1: Overview of animal models of global and focal ischemia. Given are brief descriptions of the procedures employed and the nature of ischemia elicited by each procedure (transient/permanent).
or limit impairment. Finally, without doubt, there is also greater acceptability for rats to be used in stroke research when compared to the use of dogs and nonhuman primates from both ecological and ethical perspectives [50].

Despite these advantages however, there has been considerable failure in translation of results from experimental studies to the clinic [24, 55-58]. Several limitations have led to this. Firstly, animal models of stroke in general, are unable to exactly replicate the complexity and heterogeneity of human stroke. Secondly, the degree of impairment between human stroke victims and rats is considered to be considerably different; while many human stroke victims have little or no upper extremity movements, rats rarely have a complete loss of forelimb movement in the current models. This also leads to a fundamental difference in rehabilitation studies as humans usually have hands-on assistance while current rat models are entirely ‘hands-off’ [52]. Lastly and most importantly, the size and distribution of infarction in rat models can be quite variable, resulting from inter-animal variations in collateral flow that can complicate the interpretation of results (especially if the n is small) [5].

These models nevertheless serve to provide information on fundamental neural and behavioural mechanisms involved in injury and subsequent recovery that can be used to guide the development of novel clinical therapies [52].

### 1.2.4.2 Rodent models of focal ischemic stroke

Based on the heterogeneity of human stroke in the clinic, several rodent models of ischemic stroke are now available. This has enabled the study of not only various drugs (for e.g. anti-thrombic agents for thrombic stroke) but also a variety of injury patterns and severities [59]. For example, transient ischemia models allow early reperfusion, enabling reperfusion injury studies whereas permanent models don’t [2] while global/focal ischemic models enable the study of reduced blood flow to the entire/selected parts of the brain respectively [59]. Various methods are used to induce cerebral ischemia, the most common one being the occlusion of the middle cerebral artery (MCAO) [60]. A brief overview of the various methods used to induce stroke in animals is shown in Fig 1.1. Complete global ischemia refers to a complete cessation of global blood flow while incomplete ischemia refers to a severe reduction (insufficient to maintain cerebral metabolism and function). In multifocal ischemia, the pattern of reduced CBF is patchy,
and several areas develop injury. In selective focal ischemia (the model employed in this thesis), the core of the ischemia has absolutely no blood flow but there is some flow that reaches the area via collateral circulation. As such, a gradient of blood flow develops, from the inner core reaching out to the boundaries of the ischemic area [2] (Fig 1.2, bottom panel).

In focal ischemic models, MCAO is commonly induced either by ligation, intraluminal occlusion using stiffened sutures, photothermogenesis using a laser-dye interaction to produce a platelet thrombus, or most commonly, vasoconstriction [61]. While MCAO generally causes a reduction in blood flow in the striatum and cortex, the degree and distribution of blood flow reduction depends on the duration and site of MCAO as well as the amount of collateral blood flow into the MCA territory. For example, proximal MCAO produces a big infarction involving cortical as well as subcortical areas while distal MCAO produces only cortical infarction [59]. Various factors such as the age, strain (including its origin), sex, brain/body temperature and physiology of the rat have also been implicated to affect lesion severity and size [5, 50] and so have experimental factors such as the anesthetic used and other model related factors (models requiring invasive surgery Vs non-invasive models) [5].

1.2.4.3  Endothelin-1 Model of focal ischemic stroke

Sharkey et al., [62] first characterized the Endothelin-1 (ET1) model of stroke in 1993. Since then, this model has been widely used to study the pathophysiology [63-66] and for exploring various therapeutic strategies for the treatment of stroke [67-70]. It has been successfully used in rats [54, 66, 71-73], mice [74] as well as nonhuman primates [75]. This model employs the perivascular microapplication of ET1, a potent vasoconstrictor peptide using calculated stereotaxic coordinates [62].

ET1 is a cerebral endothelium derived vasoconstrictor peptide which, although can dilate pial arterioles at low doses, potently constricts vessels at high doses [76]. Under normal physiological conditions, ET1 contributes to maintaining the endogenous vasomotor tone [77] and regulates cell proliferation via its receptors ETA and ETB [78]. Dysregulation of ET1 levels can have complex effects and lead to various hemodynamic disorders [78].
ET1 injections lead to a transient vasoconstriction of the local vasculature, causing a dose-dependent decrease in blood flow with dose-related lesions with large penumbral areas [79]. Previous studies in this model have showed that CBF decreases to less than 30-50% of control levels within 5min in the MCA territory [80] and remains reduced for about 16-22h after the insult [65]. Accordingly, although the lesion is small initially, its size increases markedly over 24h [81]. The damage pattern produced in the proximal MCA model is similar to that reported following the permanent surgical occlusion of the MCA but with considerably less invasive procedures [62] and elicits damage in the dorsal and lateral neocortex and striatum ipsilateral to the insult [82]. Infarction is seen in the lateral parts of frontal cortex extending through parietal and into insular cortex rostrally and through temporal and into occipital cortex caudally. Infarction is also seen in the dorsolateral portions of the caudate nucleus [62]. The ET1 model of focal stroke is widely accepted to be one of the more reliable and reproducible models of focal ischemic stroke and given its technical simplicity, confers the advantage of reduced side effects (such as on feeding) [62] and minimal morbidity [65] compared to other models. Additionally, the injury produced is unilateral (when injected unilaterally) [65] allowing a comparison to the contralateral hemisphere both in terms of histological as well as functional assessments [66]. Also, since the blood flow restriction is not immediate [83] and reperfusion occurs over several hours [71, 83], it is considered to be more representative of the human stroke than the intraluminal suture or clip models of MCAO [84]. Even the pattern of injury elicited (both cortical and subcortical structures are damaged) makes it a more clinically relevant model when compared to other pure cortical injury models [84, 85]. Finally, it is also possible with this model to alter the amount of injury and improve consistency by increasing the concentration of ET1 [83].

1.2.4.1 Endpoints of ischemic stroke studies

In animal ischemic stroke studies, histopathology (infarct volume) is usually one of the most studied outcome measure [5]. Infarct volumes are usually quantified at port mortem, using traditional histological staining techniques or non-invasive MRI measurements [5]. Traditional techniques commonly employ stains such as haematoxylin-eosin, triphenyltetrazolium chloride or acid fuschin-thionin and accurately and reliably distinguish infacted tissue from normal tissue at all stages of focal ischemia [86]. Although absolute measurements of infarct volumes is possible [87], given confounding factors such as edema (which can cause enlargement of infarct tissue) [87] and fixation
procedures (which can cause shrinkage of tissue) [5], relative measurements (comparing the infarcted hemisphere to non-infarcted area where possible) are commonly used [88].

Functional endpoints are quantified using sensorimotor tests that identify impairments produced by the insult. Tests can measure either acquired (skilled) or preexisting (unskilled) sensorimotor behaviours [52] and it is generally accepted that multiple testing/training sessions should carried out to enable proper elucidation of treatment effect both before and after stroke and/or treatment. Another important consideration for behavioural testing is that a battery of tests should be designed with sufficient sensitivity and precision to detect a range of motor impairments across a significant timespan [84]. Of equal importance is the elucidation of acute vs. chronic recovery. During the acute phase, various injury-related factors such as inflammation, edema and vasospasm can themselves confer impairments that spontaneously resolve in the long-term [52]. Alternatively, treatment effects can be delayed with most effects evident only after 6-8 weeks of treatment [89]. Hence the duration of the study and corresponding testing of behavioural outcomes is vital.

1.2.5 Pathophysiology of ischemic stroke

1.2.5.1 Pattern of cell death: the core and the penumbra

Ischemic stroke, particular of the focal type results in a characteristic pattern of damage. An ischemic ‘core’ or ‘infarct’ (Fig 1.2 bottom panel, blackened area) forms in the area with severely decreased blood flow (less than 15%). This is the main area supplied by the blocked artery and hence is rapidly and irreversibly injured [1, 90]. Lipolysis, proteolysis, disaggregation of microtubules, total bioenergetic failure and breakdown of ion homeostasis (described in the section 1.2.5.2) all lead to this acute, permanent damage [1]. It is almost impossible to prevent cell death in this region [91].

The ‘penumbra’ (Fig 1.2 bottom panel, gray area) on the other hand is a more distal area between the damaged core and the normal brain with less severely affected blood flow (less than 40%) [91]. Consequently, it becomes functionally impaired but remains structurally intact with partially preserved energy metabolism [90, 92, 93]. However, several deleterious cell death mechanisms are triggered here (elaborated in section 1.2.5.2), that result in ongoing cellular injury and infarct progression often for hours after stroke onset [1] (Fig 1.2). In human patients, functional impairment with structural
integrity is seen in up to 48h after stroke onset [40, 41] and MRI analyses show that the ischemic penumbral region is apparent in at least 80% of patients within 3h of stroke onset with the proportion diminishing with time [94, 95].

As a result of the extended timeframe to damage, this area is salvageable by improving blood flow and/or interfering with the continuing ischemic cascade (neurochemical processes triggered by ischemia, Fig 1.2) [1]. Indeed, several preclinical [96, 97] as well as some clinical studies have shown salvage of this tissue to be associated with neurological improvement and recovery [98].

![Graph](image)

Figure 1.2 Graph representing the temporal profile of the major pathophysiological events underlying acute focal cerebral ischemia. Their impact on the final ischemic damage is depicted underneath. With increasing time, cells in the ischemic penumbra (grey area) subside due to ongoing ischemic injury, resulting in expansion of the infarcted core (black area). Figure reproduced from [1, 2]

Interestingly, ionic and metabolic changes in the core and the penumbra are radically different (Table 1.1) and probably account for the great disparity in the efficacy of pharmacological interventions in protecting these two regions [90]. It is certain now
however, that the effects in the core contribute to damage in the penumbra; this is further discussed in the sections that follow.

1.2.5.2 Phases and mechanisms of cell death and injury

Ischemia-induced cell death manifests in two phases: An immediate or acute phase that occurs immediately after injury, and the delayed or chronic phase, which occurs after a time delay. The process of cell death occurring in these phases is extremely complex, involving extensive interplay between various factors (overviewed in Fig 1.3). It starts off with an early development of ionic and chemical changes (Table 1.1), a resulting activation of ‘executors’ of injury, and subsequently, changes in critical functions and structures that lead to cell death [90]. These changes manifest characteristically differently in the core and penumbral areas.
Figure 1.3: Overview of cell death processes that occur in response to ischemia. Processes involve the complex interplay between various factors which ultimately lead to immediate necrotic death or delayed, largely apoptotic cell death. Figure from: www.aeoluspharma.com/pre_other.php
Table 1.1: A brief description of the ionic and metabolic changes occurring in the core and the penumbra during ischemia

<table>
<thead>
<tr>
<th></th>
<th>Core</th>
<th>Penumbra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood flow alterations</td>
<td>&lt;15% of normal (during insult) [99, 100]</td>
<td>&lt;40%-50% of normal (both during and after insult) [100-104]</td>
</tr>
<tr>
<td>Ionic changes</td>
<td>• Rapid anoxic depolarisation within 1-2 min [105, 106]</td>
<td>• No permanent anoxic depolarisation due to severely reduced ATP levels (50-70% of normal) [105, 106]</td>
</tr>
<tr>
<td></td>
<td>• Concomitant rise in extracellular K+</td>
<td>• Sporadic transient depolarisations [102, 107] that may originate from glutamate release from core [107]</td>
</tr>
<tr>
<td></td>
<td>• May show some short, intermittent returns to baseline [107]</td>
<td></td>
</tr>
<tr>
<td>ATP levels</td>
<td>• Fall to ~25% of basal levels and remain there from 5min-4h of ischemia [108-111]</td>
<td>Fall to ~50-70% of basal levels during ischemia [109]</td>
</tr>
<tr>
<td></td>
<td>• After damaging durations of ischemia, return to ~two-thirds of basal levels at least for 4h [109]</td>
<td></td>
</tr>
<tr>
<td>Free radicals</td>
<td>No increase in core during 3h of ischemia, increase is seen after ~4h of reperfusion [112]</td>
<td>Early rise that elevated through 3h, further elevated with onset of perfusion [112]</td>
</tr>
<tr>
<td>Glucose utilisation</td>
<td>Drops rapidly to ~50% of normal and remains low [113]</td>
<td>• Elevated during insult [101, 113, 114]</td>
</tr>
<tr>
<td></td>
<td>• Drops to ~50% of normal by 3.5h [113]</td>
<td>•</td>
</tr>
</tbody>
</table>

The acute phase is characterized by primarily necrotic cell death and results from failure of cellular energy processes predominantly in the core of the infarct [103, 115] (Fig 1.2). Brain tissue has a relatively high consumption of oxygen and glucose and its main (and almost exclusive) source of cellular energy is ATP produced via aerobic metabolism. During ischemia, acute cerebral hypoperfusion causes a depletion of these essential nutrients, causing a switch to less-demanding anaerobic respiration and subsequent accumulation of lactate [116, 117]. Lactate accumulation rapidly leads to significant acidosis, consequently leading to failure of energy-dependent ion transport pumps and synaptic re-uptake mechanisms [40, 41]. Rapid depolarization of neurons and glia occurs, followed by influx of water into the cell leading to cell swelling and cytotoxic edema, and ultimately uncontrolled cell lysis. The rapid depolarization also causes somatodendritic as
well as presynaptic voltage-dependent calcium channels to become activated and release glutamate into the extracellular space [118]. This, coupled with impeded presynaptic reuptake of glutamate leads to NMDA (N-methyl-D-aspartate) receptor-mediated upregulation of intracellular calcium levels. Elevated intracellular calcium levels and inflammation are detrimental, upregulating various calcium-dependent destructive enzymes such as lipases, proteases, and endonucleases that attack the cytoskeleton [118-120]. This ultimately leads to blood-brain barrier (BBB) disruption, glial cell activation, white matter (WM) lesions [121] and extensive, free radical production that is very damaging [119] [122]. These WM lesions lead to a disruption in normal transmission and are considered to be a key predictor of outcome [123, 124]. BBB disruption can lead to cerebrovascular autoregulation [1] while activation of microglia and astrocytes leads to the activation of several inflammatory mediators such as matrix metalloproteinases, chemokines and cytokines, some cytotoxic (monocyte chemotactic protein-1, macrophage inflammatory protein 1α, fractalkine, interleukin-1 and tumour necrosis factor α amongst others) and others cytoprotective (protective effects discussed in section 1.4.1.1) [1]. Free-radical induced oxidative stress can also act to increase BBB permeability through activation of matrix metalloproteinases [122] and endothelial cell damage [119] causing vasogenic edema leading to secondary cell death in the delayed phase. In addition, the excitotoxic mechanisms also trigger expression of genes that promote apoptosis [1], making way for the delayed, largely apoptotic phase of cell death.

Neurons that survive/recover from the acute phase can die in this delayed phase and are largely located in the penumbral region of the stroke [125]. This stage occurs between 6 and 15 hours post-insult, can last for days and is triggered mainly by reperfusion of the ischemic tissue, which leads to the activation of various energy-dependent mediators of apoptotic death [126, 127]. This reperfusion-induced injury is key to the maturation of ischemic damage, especially after a transient ischemic event [81]. Free-radical production, triggered during the acute phase, is accentuated during this phase and oxidative stress-induced injury is another common feature of this delayed phase [119]. Free-radicals such as superoxide, hydrogen peroxide, the hydroxyl radical, nitric oxide (in neurons, glia and neutrophils [128]) and peroxynitrite are generated in the acute phase [129] that result in cellular effects during the delayed phase such as inactivation of enzymes, further release of Ca^{2+} ions, protein denaturation, lipid peroxidation, damage to cytoskeleton and DNA as well as chemotaxis [130]. BBB disruption caused by free radicals in the acute phase can also lead to secondary damage via intracranial
hypertension and hemorrhagic transformation [1]. Mitochondrial function is also impaired by free radicals which, along with disrupting ATP production triggers cytochrome c release which also initiates apoptosis [131].

Caspase-activation plays a central role in the execution of apoptotic cell death [131]. Binding of extrinsic signals/ligands to death receptors (Fas and TNFα1) lead to the activation of caspase-8. The activated caspase-8 activates the main ‘execution’ caspase 3 directly or indirectly (via Bid and cytochrome c release), which in-turn activates caspase–activated DNase leading to DNA fragmentation [131]. Alternatively or in sync with this, an internal mitochondrial pathway of caspase activation can also occur, where opening of the mitochondrial permeability transition pore plays a key role. Several pro-apoptotic proteins including Bax and Bak stimulate the opening of this pore, causing the release of several factors that initiate apoptosis, including cytochrome c, Smac/DIABLO, HtrA2/Omi, AIF and EndoG [132]. Multimeric Apaf-1/cytochrome c complexes can form as a result of this, which activate caspase-9. This activated caspase also subsequently cleaves and activates caspases such as caspases 3, 6, and 7, ultimately resulting in cell death [131]. High levels of Bax have been reported after an ischemic episode in neurons undergoing apoptosis [133, 134] and its downregulation is associated with significant neuroprotection [134], even of the new neurons produced by post-ischemic neurogenesis [135].

In addition to apoptosis, leukocyte-mediated endothelium damage and dysregulation of microcirculation further disrupt the BBB causing cytokine infiltration in the brain tissue propagating inflammation [136, 137]. Platelet and complement activation [136, 138] neutrophil infiltration, vascular plugging (occluded vessels) [139] and subsequent alterations in blood viscosity [140] all lead to dysregulation of the microcirculation while auxiliary extracellular and/or cellular edema, by compression of the the microvasculature, can further compromise reperfusion of the MCA territory [141] resulting in further cell death. As expected, CBF changes have been correlated with both the distribution and progression of neuronal damage with ischemia [142]. Recent studies have also identified lactate accumulation as another possible cause of secondary damage leading to infarct expansion and poor outcome [143].
1.2.6 Endogenous Response to Ischemic injury

The brain, like the rest of the body, is capable of defending and repairing itself during and after an injury. It produces a large number of neuroprotective and restorative factors which act, as their names suggest, to restrict the degree of injury and/or promote recovery respectively.

1.2.6.1 Inflammation

Vascular and tissue inflammation, mediated through altered expression of released and cell surface signalling molecules generally occurs following embolic stroke [144] and various studies have highlighted its importance in post-ischemic regenerative processes.

While a definitive role for some cytokines has been established in enhancing inflammation [137], others have been implicated in neuroprotection. Blocking interleukin 6 signalling leads to exacerbation of damage, and worsens neurological function [145] while administration of recombinant interleukin 6 and interleukin 10 following ischemia is neuroprotective [146, 147]. Erythropoietin (EPO) (a well known angiogenic and neurogenic cytokine [148]) is upregulated in response to ischemia [149] and preclinical treatment with EPO following stroke is beneficial (both structurally and functionally)[150]. Even matrix metalloproteinases, which promote brain damage by degrading the neurovascular matrix in the acute stage [121, 151] have been shown to have beneficial effects in the delayed phase [152, 153].

Focal ischemia-induced microglial activation, astrocytosis, and leukocyte infiltration into the ischemic areas [154] have all been shown to have beneficial effects as well. Microglia and astrocytes are immunoreactive cells of non-neural lineage resident in the CNS. Aarum et al., have shown previously that microglia, which are rapidly activated and upregulated following CNS injury, release various factors to promote differentiation and migration of neural stem cells [155]. Indeed, at least two other groups [154, 156] have identified some of these factors to be chemoattractant proteins which induce and direct neuroblast migration from neurogenic regions to the damaged regions after focal ischemia in adult rodents.

1.2.6.2 Regulators of Apoptosis

The apoptotic pathway is regulated at various steps. Proteins from the ‘inhibitors of apoptosis’ family (such as Bcl-2 and Bcl-xL) act to prevent cell death by preventing the
opening of the mitochondrial permeability transition pore (the deciding factor of intrinsically triggered apoptosis) [132]. In fact, pro-apoptotic proteins such as Bad and Bim elicit their damaging effects by absorbing Bcl-2 and Bcl-xL thereby enabling pore opening. As expected then, up-regulation of both Bcl-xL [157] as well as Bcl-2 [134] has been correlated with neuroprotection after ischemia. It is noteworthy that along with preventing apoptosis, Bcl-2 also promotes neurogenesis [158] and enhances the survival of newborn neurons [159] in adult stroke models, indicating a neurorestorative potential for this factor.

1.2.6.3 Growth Factors

Growth factors (GFs) are important regulators of cell growth, proliferation and differentiation in the brain and GF involvement in stroke injuries has been widely acknowledged [96, 160-167]. While most GF involvement has been studied in terms of treatment following injury (For e.g.[96, 162, 163, 167, 168]), there are a few reports documenting the increase in the endogenous levels of some GFs following ischemia.

There is evidence now that nerve growth factor, brain-derived neurotrophic factor (BDNF) and neurotrophin 3 are all rapidly expressed [169, 170] in the uninjured hemisphere immediately following juvenile hypoxia-ischemia (3-24h post-insult), presumably as a result of increased neuronal activity [170]. Increases in transforming growth factor β and activin have also been reported in the same model, especially within the injured hemisphere but only after 3-5 days post insult in regions undergoing neuronal death [171, 172] possibly to protect intact tissue from secondary damage. In the adult ischemic brain (focal, unilateral injury), a robust increase in the expression of basic fibroblast growth factor (bFGF) is seen from as early as 24h post-insult that lasts till at least 3 weeks post-insult [173-177]. As this increase occurs generally in parallel to an increase in bFGF receptor expression [178], localised to ‘reactive’ astroglia [173, 174, 176, 177] and some neurons [173] in areas within and surrounding the lesion [173, 174, 176], it is likely bFGF is involved in neuroprotective (at earlier stages) as well as restorative (in the longer-term) processes. Similar increases in levels of nerve growth factor [179], BDNF [180, 181], neurotrophin 3 [182], EPO [183], IGF1 [184, 185] and activinβa [186] as well as EPO [148, 149] and glial-derived neurotrophic factor receptors have also been reported in adult models of focal ischemic injury at either/or both early (at or within 2 days) and later (upto or at 30 days) stages.
Following injury to the brain, GFs are therefore released either within hours as an endogenous neuroprotective mechanism or after a delay as a rescue/restorative mechanism [170]. Additionally, they possibly act to pre-condition the brain, providing tolerance against future ischemic insults [187]. Although the mechanisms by which GFs influence cells are not completely understood, it has been recognized that transmembrane receptor proteins for GFs characteristically include a ligand-activated tyrosine kinase at the inner surface of the plasmalemma. Their effects are thus assumed to be mediated through receptor kinase-induced ‘phosphorylation cascades’ leading to changes in the activity of numerous proteins affecting transcription, translation, and structural cellular integrity [188]. Some individual GFs and their roles in neurogenesis are discussed further in Section 1.2.7.2.

1.2.7 Brain plasticity

Brain plasticity refers to the ability of the brain to anatomically and functionally reorganize its neuronal networks in order to compensate for an adverse event [189] or experience related [190, 191] loss or alteration(s) in function. It is the primary mode of recovery, especially of function, attempted by the injured brain. Reorganization of existing networks occurs via map and/or synaptic plasticity while new or ‘replacement’ networks can be formed by neurogenesis. Behavioural experience is the primary mediator of cortical structure and function (plasticity) during both developmental as well as pathological conditions [192].

1.2.7.1 Functional Remapping

White matter fiber pathways house an extensive anatomical network of connections between neurons within a functional area, enabling extensive communication [193]. Following a focal ischemic lesion, neuronal death resulting from the stroke can damage these pathways directly [194] effecting the performance of behaviours that rely on these pathways [195]. For example, insults affecting the cortical sensorimotor regions can disrupt connectivity with distant neurons (premotor areas and corticospinal outputs) providing/receiving afferent input to/from these areas, ultimately resulting in motor deficits [196]. Post stroke remapping of cortical sensorimotor areas has been widely acknowledged [197] and is the primary mediator of spontaneous (albeit slow and incomplete) functional recovery seen over time [198, 199]. This is one of the primary
endogenous, long-term compensatory/repair mechanisms of the brain, where the uninjured parts of the brain take over function of the injured parts enabling a ‘compensatory function’ mechanism.

Following unilateral ischemic stroke, altered connectivity can occur either a) within the injured cortex, b) from the injured cortex to the uninjured cortex and/or c) within the uninjured cortex. As such, axonal sprouting has been identified to occur in the peri-infarct areas following stroke [200] in different species, over long distances and between functionally different cortical areas [197] while cortical efferent fibers have been identified to be changeable after injury [201]. Axonal sprouting is triggered between 1 and 3 days after stroke and is initiated and maintained between 7 and 14 days post-stroke. It is completed, with new patterns of connections evident, approximately 28 days after stroke [197]. Accordingly, rehabilitation training is most efficient when started 5 days after stroke, less so at 14 days with no significant effects when started at 30 days [202]. Sprouting occurs in the favourable environment adjacent to and larger than, the glial scar [197].

The glial scar is the region where gliosis is seen, the region of the apoptotic cell death, which closely borders the infarct and extends several microns into the periinfarct cortex [203]. While the glial scar region has significant amounts of both growth-promoting and -inhibiting molecules, the periinfarct area outside of the glial scar largely expresses growth-promoting molecules [204]. GAP43 (growth-associated protein 43), a growth cone phosphoprotein highly linked to axonal sprouting for example, is upregulated following stroke in both animal [204, 205] as well as human [206] periinfarct cortexes for extended periods. It is accompanied by increased synaptogenesis [205, 206], dendritic arborisation [207] as well as neuronal sprouting [208, 209]. This re-organisation of the peri-infarct area probably underlies some of the alterations in projection fibers seen, such that they project to neighbouring (peri-infarct) regions after infarct to regain the somatosensory input lost due to damage [210]. Clinical studies in stroke patients have also shown that although sensory and motor stimulation of the affected limb leads to a bilateral cortical activation, over time with patient recovery, this cortical activation becomes more restricted to the side of the stroke [211]. This is seen with an expanded sensorimotor activation in the peri-infarct cortex and increased activation of any supplementary sensory and motor areas connected to it [197]. This finding is supported by functional mapping studies which have identified ‘ipsilateral’ (same side as injury)
cortical activation to correlate with good recovery while ‘contralateral’ (side opposite to the injury) activation was invariably correlated with reduced functional recovery [211]. Several other studies have also shown that ventral areas of the ipsilateral cortex undergo functional reorganization in patients with subcortical or cortical stroke [212-214]. Furthermore, a clear temporal relationship has been identified between recovery and task-related activation of the ipsilateral motor system after stroke [215].

While this represents remodeling within the injured cortex, there is also evidence for remodeling involving the uninjured cortex. Corticostriatal fibers, which are usually ipsilateral have been noted to sprout from infarct and uninjured cortex and terminate in injured striatum on the side of the lesion [201]. In the clinic, there is a bilateral increase in regional blood flow in premotor cortical areas when movements were made with the recovered hand [216] and increased activation of the contralateral sensorimotor cortex occurs in patients with good recovery [217]. In animal models, there is preferential use of the forelimb ipsilateral to the damage [207, 218, 219] [220] which is accompanied by a concurrent increase in dendritic arborisation in the contralateral cortex [66, 207, 218].

Thus in general, it appears that the pattern of neuronal reorganisation after ischemia is dependent on the size and distribution of injury [221] and is invariably predicted by dendritic outgrowth in remaining tissue [66, 71, 202, 222]. If the primary motor cortex is intact, recruitment of the undamaged hemisphere is transient. It is likely that the peri-infarct areas support/compensate in the long-term [210, 221]. However, when the primary motor cortex is extensively damaged, recruitment of the undamaged hemisphere is probably maintained long-term [221]. Ultimately, it appears that the lesion size is a major determinant of which cortex controls sensorimotor function; Small lesions likely lead to remodelling in adjacent regions while larger lesions cause a widespread remodelling (in adjacent as well as uninjured cortical areas) that is functionally less effective [211].

1.2.7.2 Neurogenesis and Neural Stem Cells

Neurogenesis or the birth of new neurons was thought to occur only developmentally. It was only relatively recently that adult (or even post-natal) neurogenesis has been confirmed [223-225] after much speculation. Since then, several studies have reported an increase in neurogenesis following injury in animal models [226-236] as well as humans [225, Jin, 2006 #295]. This suggests a possibility for endogenous neuronal renewal
following brain injury or degradation, highlighting an important therapeutic target. New neurons (or glia) are generated from neural stem cells (NSC), the most immature progenitor cells in the nervous system defined by their ability to self renew (via symmetric division) as well as produce more mature progenitors of all neural lineages (neurons, glia and oligodendrocytes, asymmetric division), termed multipotentiality [237]. Neurogenesis in the adult brain primarily occurs in two areas; The dentate gyrus (DG) of the hippocampus, in the subgranular zone (SGZ) at the border of the granule cell layer (GCL) [238] and the subventricular zone (SVZ) at the lateral walls of the lateral ventricles (LV) [239] (Fig 1.4). Although there have been suggestions that some neurogenesis occurs in other regions of the adult brain such as cortex, striatum, substantia nigra and amygdala, no consensus has yet been reached on this [240]. New neurons generated at the neurogenic sites proliferate and migrate toward their final destinations, where they differentiate into mature neurons to be integrated into the existing neuronal circuitry [241].

NSCs from the SGZ primarily express glial fibrillary acid protein (GFAP; a marker for mature astrocytes) and are morphologically and electrophysiologically similar to astrocytes [242]. They have prominent radial processes and also extend shorter tangentially oriented processes at the base of the SGZ and interact closely with their progeny [242, 243]. These NSCs continuously proliferate and generate new granule cells [243-245] that migrate out into the nearby GCL, differentiate into mature neuronal cells and extend axonal projections to the CA3 area [246] (Fig 1.4). Interestingly, although these hippocampal proliferative precursor cells (described above) are capable of proliferation and multipotential differentiation, they are unable to self-renew (and thus proliferate indefinitely), raising the possibility that these cells are neurogenic but are not pure stem cells [247].

The adult SVZ provides a specific microenvironment, the ‘stem cell/neurogenic niche’ where four types of cells are thought to exist (Fig1.4). These are astrocytes, transit-amplifying cells, newly generated neurons (neuroblasts) and ependymal cells [248, 249]. Similar to the DG, the astrocytes in the SVZ express not only express NSC markers such as nestin, vimentin and GLAST (glutamate aspartate transporter 1), but also GFAP despite being self-renewing NSCs [245, 250, 251]. These ‘B’ cells [252] are thought to be descendents of the embryonic ventricular radial glia (RG) that in turn, arise from the neuroepithelial cells of the neural tube [253]. In fact, they exhibit a unique glial
phenotype intermediate between RG and astrocytes [254]. In the embryonic brain, RG cells serve as both the parent as well as radial migratory guide for newborn neurons [253, 254] and are responsible for the generation of cortical neurons [255]. The ‘B’ cells are NSCs that are capable of repopulating the SVZ niche [250] and proliferate slowly and continuously to generate the rapidly proliferating transit-amplifying cells termed ‘C’ cells [252]. These cells are multipotent in early postnatal life or when cultured under specific conditions, but mostly differentiate into neuronal lineage-restricted migrating neuroblasts (type ‘A’ cells) in the adult brain. The migrating neuroblasts travel along the rostral migratory stream to the olfactory bulb where they further differentiate in olfactory interneurons under physiological conditions [251]. A small number of oligodendrocyte precursors are also produced, which migrate into the corpus callosum [256]. The ependymal cells are cuboidal and multiciliated cells that line the LV and play essential roles in the transport of cerebrospinal fluid (CSF) [257, 258] and in brain homeostasis [259]. These cells are also generated from RG, mostly between embryonic days 14 (E14) and E16 [260]. While some studies suggest they are post-mitotic and do not divide [260, 261] with no ability to generate multipotent stem cells in vitro [249, 262], others have suggested the contrary. Using BrdU labelling, Johansson and colleagues have identified that multi-ciliated ependymal cells could act as slowly proliferating NSCs in the adult mouse [263] as well as human [264] brain, and that these cells were truly multipotent when transplanted into irradiated mice [265].

In this thesis, NSCs are taken to be immature progenitor cells that are able to self-renew as well as produce mature progenitors of all neural lineages. The mature progenitors or precursor cells to a specific lineage of either neurons, glial or oligodendrocytes are referred to as neuronal, glial or oligodendroglial progenitor cells respectively. The specific cell typed used in this thesis is a NSC.
Figure 1.4: Sites of adult neurogenesis in the human and rodent brains. Neurogenesis primarily occurs along the lateral ventricle (LV) and the dentate gyrus (DG) of the hippocampus (a). Shown below the main images are coronal sections depicting the exact locations of the neurogenic regions in the brain. The architecture of each of these areas is shown in (b). In the SVZ, B cells (dark blue) are the GFAP-expressing astrocytes that are the self-renewing NSCs. Some of these B cells contact the ventricle lumen and have a single cilium (shown). C cells (green) are rapidly dividing, transit-amplifying cells derived from the B cells. C cells give rise to A cells (red), neuroblasts that migrate to the olfactory bulb, where they become local interneurons. A blood vessel (BV, pink) is shown with a perivascular macrophage (dotted fill); a basal lamina (BL, yellow) extends from the BV and interdigitates extensively with the SVZ cells. Ciliated ependymal cells (grey) line the ventricle walls. In the DG, astrocytes (As, dark blue) give rise to progenitors (D cells, orange), which mature into new granule cells (red G cells). These newly born granule cells integrate into the DG granule cell layer (brown G cells). Blood vessels (BV, pink) are found close to the SGZ layer, and a perivascular basal lamina (BL, yellow dotted line) possibly exists here similar to that found in the SVZ. Image and caption modified from: http://pubs.niaaa.nih.gov/publications/arh27-2/197-204.htm, [4-6].
### 1.2.7.2.1 Factors regulating neurogenesis

Age, environmental enrichment, exercise, growth and neurotrophic factors, transcription factors, neurotransmitters, certain inflammatory mediators, the neurogenic niche and injury have all been identified to be modulators of neurogenesis in the adult brain [240]. An age-related decline in neurogenesis has been extensively reviewed and studies suggest this to be a consequence of changes in the brain microenvironment rather than alterations in the numbers/properties of NSCs themselves[266, 267]. GFs in particular have been implicated as major regulators; bFGF, IGF1, vascular endothelial growth factor [268] levels as well as the level of epidermal growth factor (EGF) signalling [269] decline with age, and infusion of such factors into aged brains has lead to reversal of such age-related decreases [270, 271].

Given the importance of the hippocampus in functions such as learning and memory and in regulating mood and emotion, it is no surprise that there is significant involvement of hippocampal neurogenesis in these functions [272, 273]. Hippocampal-dependent learning tasks [272, 274], environmental enrichment [275] and exercise [276-278] increase the proliferation of NSCs in the SGZ and/or promote their survival, while stress [279-281] and psychiatric diseases [282] can have the opposite effect. Of late, ovarian steroids have also been extensively implicated in regulating hippocampal neurogenesis [283] along with several GFs, some of which are described below.

Various proteins such as (but not restricted to) bFGF, Notch1, noggin, BMPs, TGFα, Eph/ephrins, VEGF, sonic hedgehog, Stat3, Sox9 and ciliary neurotrophic factor are thought to play important roles in NSC maintenance and/or self renewal in the SVZ niche [4, 5, 284-288] and NSCs isolated from this niche can be extensively cultured and their multipotency maintained in vitro in the presence of GFs such as bFGF and EGF [289]. Several of these GFs are also implicated in regulating NSC proliferation; bFGF [238, 290, 291], EGF [292], ciliary neurotrophic factor [285], IGF1 [185, 293], neurotrophin-3 [294], transforming growth factor α [295], BDNF [296] and hematopoietic cytokines such as (but not limited to) granulocyte colony-stimulating factor, stem cell factor [166], EPO [148] and vascular endothelial growth factor [297] have all been shown to induce proliferation of NSCs, amongst others. Conversely, neurotransmitters glutamate and GABA (gamma amino-butryic acid) [298] act to down-regulate NSC proliferation. Cytokine withdrawal [299] or addition of various GFs results in NSC differentiation into one of three neural cell types: neurons, astrocytes and oligodendrocytes. Several cell
intrinsic and extrinsic signals control neuronal vs glial differentiation and various genes are differentially regulated for this. Basic helix-loop-helix transcription factors (such as neurogenins 1 and 2), suppressors of cytokine signalling 2 (SOCS2) and the EGF receptor are all pro-neuronal, eliciting pro-neuronal signals of GFs such as platelet derived growth factor, BDNF [300, 301] and EPO [183]. Meanwhile, leukemia inhibitory factor and its family members, ciliary neurotrophic factor and interleukin-6, through activation of the notch signalling pathway promote astroglial differentiation (via significant involvement of Smad1 and Janus-activated kinases – signal transducer and activator of transcription/JAK-STAT factors) [301]. Oligodendrogliogenesis also involves the basic helix-loop-helix transcription factor genes like Olig1 and 2, and relies on their interaction with Sox9 to prevent neurogenesis and gliogenesis, thus imposing an oligodendroglial fate on the newborn cells [302]. Transforming growth factor β [303] and IGF1 [304, 305] are examples of GFs involved in promotion of oligodendroglial differentiation. It is important to note however, that cell fate specification, including its modification by various factors is a very fluid and dynamic process that depends not only on the type of NSC but also on the age of the organism [301, 306]. Finally, neuroblast migration is regulated by various factors such as cytoskeletal modification [307], adhesion molecule expression [308, 309], extracellular matrix modelling [308, 310], all/some of which occur in response to guidance molecules that are either chemoattractive or repulsive [308, 311-313]. GFs are also implicated in NSC migration [314], at least partly by direct effects on chemokines such as chemokine ligand 4 (better known as CXCL4) and their receptors [135, 156]. Most of these molecular cues are produced by cellular substrates that can also serve as a physical scaffold for migrating neuroblasts [315] and both glia (gliophilic) and neurons (neurophilic) have been implicated [315]. For example, migration of neuroblasts along RG processes is gliophilic migration [316, 317] while the ‘chain’ migration of neuroblasts (in close association with each other) into the olfactory bulb via the rostral migratory stream is neurophilic migration [318]. In addition to neurons and glia, it was recently discovered that blood vessels could also mediate neuroblast migration [310, 314, 319] by providing migratory cues [314], a pathway, as well as metabolic substrates [320]. In fact, recent evidence suggests the existence of a ‘neurovascular niche’ where angiogenesis is associated with neurogenesis [230]. Evidence for this came from studies demonstrating the close association of blood vessels with NSCs in the SGZ and the SVZ (Fig 1.4b) [321, 322], the ability of endothelial factors (described earlier) to promote NSC proliferation and self-renewal [323] and that bursts of endothelial cell division are associated with
neural progenitor division [321]. Moreover, angiogenesis and neurogenesis share several cellular and molecular processes [324].

Interestingly, mature neurons arising from the hippocampal neurogenic niche are phenotypically different to those from the SVZ niche [325] and neither environmental enrichment nor exercise, both factors highly implicated in hippocampal neurogenesis [275], have significant effects on SVZ/olfactory bulb neurogenesis [326]. Instead selective gain or loss of function with olfactory stimuli promote or inhibit neurogenesis in the olfactory bulb [327-329] but with no effects in the SVZ [329]. These results suggest that neurogenesis in the SGZ and the SVZ are regulated independently and that the SVZ is not generally affected by normal behavioural paradigms.

Under physiological conditions, the rate of neurogenesis in the adult is significantly lower than that during development and most of the newly generated cells undergo programmed cell death rather than achieving maturity [330]. This is however extensively altered following injury; brain injury is one of the most important regulators of NSC activity/neurogenesis, predominantly via alterations in one or many of the factors listed above [331]. Injury-induced neurogenesis is thought to be beneficial in two ways; firstly, NSCs can be a potential source of new cells to replace those lost due to CNS injury and secondly, they can be a source of trophic molecules to minimize further damage and promote recovery [332]. Such properties render them therapeutic both in the acute as well as delayed phases.

1.2.7.2.2 Injury-induced neurogenesis

Extensive upregulation of neurogenesis occurs in pathological conditions [331] and upregulation in response to focal ischemic insults is reported both in the hippocampus [227, 229, 233, 333] as well as the SVZ [226, 229, 236] in animal models. Recently, post-mortem analyses revealed a similar increase in injury-induced neurogenesis in the adult human brain [334, 335].

Following a transient MCAO, a significant increase in the proliferation of hippocampal progenitor cells occurs around 7 days after stroke [229, 233, 336], which can last for at least 7 months [333]. Most of this upregulation occurs on the side ipsilateral to the insult [227, 229, 233, 333, 336, 337] and is thought to be mediated through glutamatergic
mechanisms acting via the NMDA receptors [227]. While a parallel increase in neurogenesis also occurs on the contralateral side [229], survival of these cells is significantly reduced compared to the ipsilateral side [233]. The newly generated neuronal cells co-express immature neuronal markers such as doublecortin (DCX) and proliferating cell nuclear antigen (PCNA) at earlier stages [229] and mature neuronal markers such as NeuN (neuronal nuclei) at later stages [226, 338]. However, the time course of maturation and the survival percentage of cells on the ipsilateral side adopting a neuronal fate in the hippocampus is similar to the normal brain [339]. This, together with observations that these newborn cells move only into the GCL (rather than to injured areas) [333, 338] leave uncertain roles for hippocampal neurogenesis in ischemic brain injury.

DCX immunoreactive cells (neuroblasts) generated in the SVZ (on the ipsilateral side) however have been shown to migrate to the ischemic penumbra of adjacent striatum and, via the rostral migratory stream and lateral cortical stream (through the corpus callosum) into the penumbra of the ischemic cortex [226, 338, 340]. Further, along with successfully integrating into the existing circuitry (albeit only fractionally), they also exhibit the phenotype of most neurons destroyed by the lesion [226, 234]. Although at least some of the neuroblasts seen in the peri-infarct cortex [226, 231, 236, 338] could be mature neurons that have been rendered mitotically active by ischemic injury [341], most of them (including and especially those in the striatum) certainly arise from neurogenesis in the SVZ. Focal ischemic stroke leads to a marked increase in the numbers of NSCs [235] and neuronal progenitors [338] in the ipsilateral SVZ and it is these cells that migrate out to the injured regions. In addition, at least a small proportion of the neuronal progenitors also arise from ependymal cells lining the lateral ventricle [342]. SVZ cell proliferation starts at 2 days after stroke and reaches a max 4 and 7 days post-stroke [235] but decreases up to 90% by 28 days thereafter [343]. These ‘activated’ NSCs divide more rapidly than non-stroke NSCs [235], with a greater proportion exiting the cell cycle phase to differentiate into neurons [344]. Striatal neuroblasts however, are generated without any decline at least 4 months [135] and possibly even a year [345] after stroke in adult rats suggesting a continuous, long-term role for endogenous NSCs in self-repair strategies following stroke. These neuroblasts generated in response to stroke even exhibit distinct migratory behaviours (to non-stroke derived cells) such as faster movement with a capacity for cell division during migration [346]. However, a vast majority of the newly generated neurons do not survive long-term [226, 231] and strategies to increase both
their generation and survival are critically needed. Development of such therapies requires an increased understanding of factors regulating post-injury neurogenic processes and those which specifically augment beneficial effects.

### 1.2.7.2.3 Regulation of post-injury neurogenesis

A major regulator of post-ischemic neurogenesis is GF signalling. Studies have identified a 3 to 102-fold increase in diffusible, mitogenic factors (such as bFGF, BDNF, IGF1, nerve growth factor and neurotrophin-3) commencing approximately 2 days after insult and reaching a maximum about a week later, when progenitor proliferation is maximal (Section 1.2.6.3) [154, 173-177, 179, 182, 347]. These factors have been shown previously to generate and maintain neurons from the adults SVZ [238, 348, 349], strengthening their role in post-ischemic neurogenesis. Also, several studies have shown significant benefits of GF infusions following stroke and that these benefits arise from GF-mediated improvement of neurogenesis [332, 350]. For example, bFGF has been shown by at least two separate groups to augment post-ischemic neurogenesis and promote recovery of sensorimotor function and cortical cell replacement [351, 352] and co-infusing it with EGF led to increased proliferation and survival of neural progenitor cells following MCAO [353, 354]. BDNF administration also induces neurogenesis and improves sensorimotor function [355] while ICV infusion of glial-derived neurotrophic factor increases neurogenesis, migration of the new neurons as well as their survival following MCAO [356]. Exogenous infusion of transforming growth factor α too has been beneficial, resulting in increased neurogenesis as well as angiogenesis [350].

Interestingly, increasing evidence now suggests that hematopoietic factors play important roles in regulating injury-induced neurogenesis. Granulocyte colony-stimulating factor is capable of mobilizing stem cells in ischemic stroke patients [357] while clinical studies are now underway using EPO in ischemic stroke patients [358, 359] owing to its success in providing neuroprotection (via increased neurogenesis and angiogenesis) in animal models of stroke [360-362]. Indeed, EPO is also involved in neuroblast migration to the site of injury [360]. ICV infusion of vascular endothelial growth factor also yields an increase in both neurogenesis and angiogenesis [363] and enhanced neuromigration [364] followed by neurite outgrowth [365] after cerebral ischemia in adult rats. The efficacy of hematopoietic factors is further evident in co-infusion studies in animals; a significantly delayed (starting 7 days after insult), combined infusion of EGF and EPO confers
functional recovery, cortical regeneration and mobilization of endogenous NSCs [96] while co-infusion of hematopoietic factors stem cell factor and granulocyte colony stimulating factor during the acute [166, 366] or chronic [367] post stroke periods enhances neurogenesis and improves functional recovery where EGF alone does not. Experimental evidence indicates a close link between neurogenesis and angiogenesis in the adult brain; angiogenesis occurs extensively in the ischemic region [323, 368, 369], even preceding neurogenesis and vascular endothelial cells release soluble factors [369] that promote NSC proliferation and neurogenesis in the SVZ [323]. Further, neuroblasts migrate in association with blood vessels in the striatum [234] and new neuroblasts are recruited to the peri-infarct area exhibiting endothelial cell proliferation [230] following stroke. It is likely that vascular endothelial cells produce factors such as stroma-cell derived factor 1 and angiopoietin 1 which, act via the chemokine receptor 4 and Tie2 receptors to control neuroblast migration [156] [135, 230]. In fact, the vasculature in the SVZ has been shown to be an important component of stem cell niches [370, 371] (Figs 1.2 and 1.4). In addition, several genes associated with angiogenesis and neurogenesis are upregulated in the peri-infarct [369] as well as the SVZ post-stroke [372], all contributing to the importance of angiogenic factors and events in neuroregeneration following stroke.

It is noteworthy, that not all GFs are stimulatory in function; some GFs (such as transforming growth factor β [373]) block the self-renewal cells by inhibiting cell division while others (such as monocyte chemotactic protein-1, expressed by activated microglia and astrocytes in the damaged area [154, 374]) do so by promoting migration away from the mitogenic niche by cell cycle exit. While most GF (and their receptor) expression following stroke arises in ischemic tissue [375] there is also significant evidence for expression in the SVZ [183, 185]. GF secretion is also thought to be one of the mediators of the beneficial effects of stem cell transplantation therapies, to the extent that even their entry into the brain may not be required [376].

1.3 CURRENT TREATMENTS FOR ISCHEMIC STROKE

There are three stages for the management of cell death; (i) reduction of death via recanalisation therapies (remove blockage), (ii) neuroprotection or preventing damage via interrupting cell death processes and finally, (iii) neurorestoration or repair of occurred damage via drug therapy and/or replacement of lost tissue. The primary focus of treatment immediately after a stroke is to restrict existing damage, repair immediate
damage as soon as possible and reduce the possibility of further damage occurring (i.e. a combination of neuroprotection and restoration). A secondary and more long-term focus is on promoting reorganization and compensatory improvement in the physical condition of the patient, i.e. long term neurorestoration either via cell therapy or physical therapy. Thus, ultimately, the efficacy of stroke therapies depends on the time point at which treatment is applied.

1.3.1.1 Recanalisation (reperfusion) therapies

Recanalisation therapies aim to restore blood flow to all affected parts of the brain before irreversible damage occurs. Reperfusion is achieved by thrombolysis, either with a thrombolytic agent (administered intravenously or intra-arterially) or by mechanical disruption and removal of the occluding thrombus, usually done in conjunction with arterial thrombolysis [23].

Pharmacological thrombolytic agents work by converting plasminogen into plasmin, an active protease that dissolves clot-forming fibrin as well as its degradation products [377], thus removing the obstructing thrombus. Of all the thrombolytic agents clinically trialled to date (Streptokinase, Ancrod and recombinant tissue plasminogen activator/rtPA) [23], rtPA is the only most successful food and drug administration-approved treatment for acute cerebral ischemia which has consistently demonstrated improvement in long-term outcomes following ischemic stroke [378]. However, clinical studies have demonstrated a 300 minute time window (to treatment initiation) to achieve early and complete recovery from stroke with rtPA treatment [379] and studies have identified this time limit to be the result of more than just the thrombolytic actions of rtPA [380]. Further, there exists important exclusion criteria such as uncontrollable hypertension, anticoagulation, recent surgical interventions, presence of signs of haemorrhage [381] etc., that render rtPA (and thrombolytic therapy in general) administrable to only about 5-10% of all presented patients [23]. rtPA/thrombolytic therapy has also been associated with the risk of haemorrhage and possible amplification of excitotoxic currents, promoting matrix metalloproteinase dysregulation, BBB leakage and edema [382, 383] making it a possible cause of secondary derangement of tissue integrity affecting hemodynamics as well as neuronal survival [383-385] mainly due to its extravascular effect on them [386]. Indeed, most of these deleterious effects are results of administration beyond the efficacy window [381] and some add-on treatments may be able to prevent these injurious effects [387].
Even the success of endovascular therapies (involving mechanical methods to reopen blocked arteries) is determined by time to treatment initiation, with an overall threshold of around 8h [388, 389]. Currently, attempts are being made at broadening the timeframe for rtPA [167, 385] as well as developing more selective plasminogen activators without these deleterious side effects [23].

Reperfusion can also be promoted by antiplatelet/anticoagulant agents which impair the formation of clots and several clinical trials have now tested their efficacy using (mostly) aspirin and heparin in acute ischemic stroke patients [390, 391]. Overall, it was found that neither anticoagulants nor antiplatelet agents had significant clinical outcome that was comparable to rtPA. Furthermore, early anticoagulant therapy (less than 14 days), although significantly decreased the incidence of deep venous thrombosis, pulmonary embolism and recurrent stroke, increased the incidence of symptomatic haemorrhage [390]. Antiplatelet therapy on the other hand, if given within 48h of ischemic stroke onset, reduced the risk of recurrent stroke and improved long-term outcome without a major risk of early haemorrhage [391]. Consequently, antiplatelet/anticoagulant therapy is recommended only for thromboembolic prophylaxis in acute ischemic stroke [391].

1.3.1.2 Neuroprotectants

Reduction in damage requires the restriction of the ischemic cascade in order to prevent ischemic brain tissue from evolving into infarcted tissue [1]. As discussed earlier in section 1.2.5, secondary damage evolves largely over 72h after stroke onset, providing an attractive target for therapeutic intervention. However, since the amount of viable tissue rapidly decreases in the first few hours [392], it is vital for treatments to be initiated as soon as possible. Given neuroprotective agents are generally not associated with the high adverse risks of thrombolysis and they can be applied over a longer time span (up to days) after stroke [23], they serve as one of the primary interests for stroke therapy.

Owing to extensive success in preclinical models [58], clinical trials have now examined the efficacy of various factors that block the progression of the ischemic injury cascade. Calcium channel antagonists, free radical scavengers, anti-inflammatory agents, trophic peptides, membrane stabilizers, glutamate antagonists, $\alpha$-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) antagonists, indirect glutamate modulators, opioid antagonists, GABA agonists as well as other neurotransmitter modulators have all been
used for acute stroke [47]. However, none of these have proven to provide significant clinical benefits in a Phase III trial, raising questions regarding the matching of preclinical and clinical trials [58]. Even agents with several mechanisms of actions such as EPO that have shown significant promise in clinical pilot studies [150] appeared to fail in larger, Phase III clinical trials, particularly in patients receiving systemic thrombolysis [359]. In preclinical models, EPO has been shown to increase both neurogenesis and angiogenesis [148], be antiapoptotic [393-395] as well as restore local blood flow to the ischemic area [396], presenting a true multimodal therapeutic option. Despite doubts about the viability of pharmacological neuroprotection as a treatment, preclinical and clinical research in this field continues, particularly with putative agents, such as EPO, showcasing several mechanisms of action [397]. It is now generally accepted that the timing and dosage of drug administration, window of opportunity, length of ischemia, gender and age differences, medical history and in particular, clinical trial design (i.e. selection criteria and outcome measures appropriate for heterogeneous stroke patients) all need to be better matched in order to translate success in the laboratory to the clinic [47, 55, 58, 61]. As a result, a Stroke Therapy Academic Industry Roundtable criteria has now been established that delineates specific recommendations for rigorous preclinical evaluation of putative neuroprotectants [398].

1.3.1.3 Neurorestoration

The role of CNS re-organization in promoting recovery from injury has been extensively reviewed [210, 215, 399]. It is now obvious that CNS reorganization, especially in the peri-infarct tissue occurs after injury (elaborated in section 1.2.7.1) and is aided by the production of endogenous GFs partly through re-instating developmental processes (discussed in section 1.2.7.2). Preclinically, restoration of neurological function after ischemic insults has been achieved by the administration of molecules that directly [96, 148, 205, 400] or indirectly (by inhibiting outgrowth-inhibitor molecules) [89, 401] enhance neurite outgrowth and/or promote neurogenesis and angiogenesis. Moreover, even the success of cell-based therapies such as the administration of mesenchymal stem cells has been attributed to their ability to regulate GFs that promote remodelling of the brain [402]. This highlights the importance of post-injury re-organisation of neural circuits in promoting recovery and emphasizes the importance of factors that are able to regulate this re-organization.
Promotion of reorganization of brain tissue and improving compensatory mechanisms in the stroke patient is enabled by the fact that the brain continuously modifies its neural circuitry in a functionally appropriate manner [403, 404]. Accordingly, constraint therapy remains the most effective, long-term treatment for stroke patients [405]. Furthermore, it also means that the window of opportunity for this treatment is far longer than recanalisation or neuroprotective therapies.

1.4 GROWTH HORMONE

GH, previously known as somatotrophin is a key mediator of the complex process of growth. Following isolation and characterization in the late 1940s [406], numerous studies have established its biological functions and importance in the growth process. Of all of its functions, GH is known mostly for its involvement in physiological events that regulate growth, body composition, energy and bone metabolism and cardiac functions [407]. For the sake of conciseness, most of this review will focus on aspects of GH related to the brain.

1.4.1.1 Protein, receptor and binding protein structures

GH is a non-glycosylated polypeptide hormone that is encoded for by a single GH gene in rats and mice (located on chromosomes 10 and 11 respectively) but a five-gene cluster (located on chromosome 17) in humans [408]. Each of the 5 highly similar genes in humans is comprised of five exons and each can utilize one or more alternative splicing pathways during RNA processing [409]. While the mice/rats GH gene is expressed only in the pituitary, the human GH (hGH) gene cluster, which is comprised of hGH-N, hCS-L, hCS-A, hGH-V and hCS-B is expressed in a tissue specific manner [410]. Four of the genes are expressed in the placenta (hCS-L, A & B, hGH-V) and only one is expressed in the pituitary (hGH-N) and it is this pituitary gene that generates the predominant form of GH protein seen in the pituitary and circulation [410]. hGH-N is expressed as two distinct GH isoforms; the 20kDa and the 22kDa isoform [409]. The 22kDa isoform accounts for 90% of GH seen in the pituitary and circulation and is thought to be the only one with anabolic growth-promoting actions [411]. It consists of a single amino acid chain of 191 amino acids (aa), stabilized by two sulfide bridges [412].

Cellular effects of GH are mediated via the growth hormone receptor (GHR), a class 1 cytokine receptor [413]. Members of this cytokine receptor family include PRL, EPO, leptin and interleukin receptors and share the common features of a single transmembrane
domain and no intrinsic enzyme activity [414]. The GHR is a protein of 620 aa, consisting of 10 exons that has a molecular mass of 70kDa [415] and is encoded by a gene located on the short arm of chromosome 5 in humans [416]. Exons 2-7 (246 residues) encode the extracellular domain, Exon 8 (23 residues) the single transmembrane domain and exons 9 and 10 (351 residues) the intracellular (cytoplasmatic) domain [415]. The intracellular domain consists of two proline-rich boxes which bind the tyrosine kinase JAK2 (Janus Kinase 2), and several tyrosine residues that are substrates for phosphorylation by JAK2 [417]. The extracellular domain consists of limited aa homology and a single arginine at residue 43 that determines specificity for the receptor [418]. Variants of the human GHR are generated by alternative splicing and include one with a deletion encoding 22 aa in the extracellular domain (exon 3) that is predominantly found in the placenta [419, 420] and short isoforms with truncated cytoplasmic domains (exon 9) that are present in the liver [421, 422]. While the expression of either of these isoforms can modulate either or both GH binding as well as the function of the full length GHR [419-421], it is the short isoforms that act as sources for GH binding protein or GHBP [422].

The GHBP is similar in structure to the extracellular domain of the GHR, given that it is essentially generated from the GHR transcript either by alternative splicing (such as in mice and rats), [423]) or specific proteolysis (as in most other species [424]). It is a 55kDa protein that binds to GH with somewhat lower affinity than the GHR [425] that is not implicated in any direct biological effects on cells or GH signaling [426]. Although its biological functions remain to be clarified, it is likely there is a reservoir of GHBP in the circulation, which reduces the degradation as well as metabolic clearance of GH [427]. Alternatively, it could serve to block GH actions, preventing further binding to membrane receptors [426].

1.4.1.2 Signal transduction and modulators

GHR activation occurs via receptor homodimerization. One GH molecule binds to two molecules of GHR in two steps – the initial ‘high affinity binding’ at site 1 and the subsequent ‘lower affinity binding’ at site 2 [428]. This dimerization of the GHR brings two JAK2 molecules within close proximity together allowing auto- and cross-phosphorylation of their activating tyrosine-residues on the cytoplasmic domain, locking JAK2 in an active confirmation [429] (Fig 1.5). Following this docking, various
molecules are recruited to the GHR including signal transducers and activators of transcription (STATs), Src homology 2/α collagen-related (SHC), Insulin Receptor Substrate proteins (IRS), members of the mitogen activated protein kinase (MAPK) family and phospholipase C-γ [430].

The STAT cytoplasmic proteins are the major mediators of GH signalling that, upon activation by JAK2, form hetero- or homo- dimers, translocate to the nucleus, bind to DNA and stimulate transcription [429]. STAT5 is the predominant STAT utilized by the GH and it is the JAK-STAT pathway that is necessary for the transcriptional regulation of IGF1 [430]. MAPK phosphorylation can also occur directly via JAK2 and results in the activation of various molecules such as STATs, phospholipase A2 and proteins which
induce gene transcription (such as c-fos, egr-1, jun B and IGF1) [430]. GHR mediated SHC signaling can occur independent of JAK signaling, ultimately leading to activation of the extracellular regulated kinases (ERKs) 1 and 2 (p44/42 MAPK) [430, 431]. Signalling via the IRS proteins involves the activation of the major effector molecule phosphatidylinositol 3 kinase (PI3K), which, ultimately activates downstream signalling pathways involved in cell proliferation and survival, cytoskeletal reorganization and cellular metabolism [430]. PI3K signaling results in protein kinase C recruitment to the plasma membrane, which also mediates several GH functions including lipogenesis, an increase in intracellular Ca^{2+} concentration and transcriptional events [430]. Protein kinase C activation also occurs via the phospholipase C pathway, which can also be activated by GH. The phospholipase C pathway involves the hydrolysis of phosphatidylinositol-4,5-bisphosphate to produce the second messengers 1,4,5-trisphosphate and diacylglycerol, which increase cytosolic Ca^{2+} and activate protein kinase C [430].

GHR signaling can be negatively regulated by feedback inhibitors such as SOCS which are induced by cytokines (such as interleukins and interferons) and cytokine hormones (such as GH, PRL and leptin) [432]. The SOCS proteins prevent the binding of effector molecules to phosphorylated tyrosines on the GHR or JAK2 [430, 433] thus leading to inhibition of GH-induced transcription. SOCS1 and SOCS3 inhibitory effects are mediated via repression of JAK2/STAT5 activity, while SOCS2, the most important of all, elicits inhibition via competitive binding of STAT5 and SH2 [433]. This competitive binding is thought to be the reason behind the dual effect of SOCS2 on GH signaling – lower levels inhibit approximately half of STAT5 activity whereas higher levels result in enhancement of signaling [434].

In addition to SOCS molecules, negative regulation of GH signaling can also be mediated by receptor-related events. Removal of the GHR/JAK2 complex from the cell surface by internalization or reduced translocation of newly synthesized GHR to the cell membrane can both lead to an inhibition of signaling [435]. The dephosphorylation of the phosphorylated tyrosine residues on the cytoplasmic domain of GHR by phosphatases such as SH2-domain containing protein tyrosine phosphatases (SHPs) can also inhibit GH signalling [430, 433]. Additionally, GHBPs have also been implicated in the negative regulation of GH signaling; absence of GHBP leads to express clearance of GH within seconds [436]. They do this either via direct sequestration of GH away from the cell
membrane-bound GHR or by heterodimerization with the full length, wild-type receptor on cell surface [426].

1.4.1.3 GH and GHR expression in the brain

While the main source of GH is the anterior pituitary gland, several studies have now established evidence for extra pituitary, neural expression of GH. In fact, GH gene expression in the brain is seen prior to its ontogenic appearance in the pituitary gland, suggesting that GH may have evolved phylogenetically as a neuropeptide rather than an endocrine entity [437]{Costa, 1993 #1554, 438, 439}. GH immunoreactivity is measurable in samples of whole brain, hypothalamus, thalamus, caudate, cortex and hippocampus from day 10 of gestation (as opposed to day 12 in the pituitary) in the fetal rat [437]. Similarly, GH mRNA is detectable in the chick neural retina within the first trimester days [438], prior to its expression in the pituitary somatotrophs [439]. In humans, GH is present in brain extracts from embryos (at 8 weeks of development; E8) prior to its appearance in the pituitary at the end of the first trimester [440]. GH immunoreactivity has also been reported in the embryonic choroid plexus [441] and the ventricular zone [433, 441]. In fact, in chicks, intense GH immunoreactivity is seen in the divisions of the brain (the telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon) as early as E3 [441]. Interestingly, GH immunoreactivity in the late fetal-early neonatal stage of the rat brain appears to be transient, as levels drop off (to pituitary levels) within 24h after birth but subsequently rise to adult levels around puberty [437]. The pattern of GH expression is largely conserved in the adult brain too, with abundant GH immunoreactivity evident in the midbrain, cortex, hippocampus, striatum, olfactory bulb, hypothalamus, thalamus amygdaloid nucleus and cerebellum of adult rodent [442-445], primate [446] as well as chicken [447] brains. In adult humans, although the presence of GH mRNA is uncertain [448] GH is measurable in the CSF [449].

Evidence that this GH is indeed of neural origin comes from several observations; Firstly, levels of GH immunoreactivity in most CNS areas do not change after hypophysectomy [446]. Moreover, levels in the amygdaloid nucleus and hypothalamus, although initially fall, actually rise above control levels several weeks following hypophysectomy [446, 450]. Furthermore, GH gene transcription in the lateral hypothalamus has been shown to occur independently of pituitary GH expression in the rat [451]. Secondly, dispersed cells from both intact as well as hypophysectomised rat CNS [446] and amygdaloid nucleus
continuously release somatostatin (SS)-responsive GH in culture. SS is a hypothalamic hormone primarily responsible for suppressing GH release [452] (discussed in the following section). Thirdly, SS receptors are found in CNS areas where GH mRNA is present [453, 454]. Finally, not only has the presence of CNS GH been confirmed by various methods (in situ hybridization [444], radioimmunoassay [446] and immunohistochemistry [441]), it has been demonstrated in the presence of a BBB intact to circulating pituitary GH [437, 446]. Furthermore, the fact that GH levels in the CSF are usually much lower than in the plasma is thought to be a function of the BBB being generally exclusive to the movement of GH from the cerebrovasculature [449, 455].

Specific binding of GH has been demonstrated in several CNS areas in a variety of species during all ages and using a variety of techniques. In neural tissues of chick embryos, the widespread distribution of GH immunoreactivity was mirrored by the distribution of GHR immunoreactivity [441]. Immunoreactive GHRs are also present in neural ganglia in fetal rats and in non-neural cells, including the ependymal cells lining the ventricles, choroid plexus, and pia mater [456, 457]. In humans, GHRs are present in the brain at the beginning of the second trimester [458]. In both rodents as well as humans, GHR immunoreactivity in the CNS declines with postnatal age, in contrast to its ontogeny in the liver [457, 459]. Overall, the choroid plexus has the highest density of GHRs followed by the pituitary [459-462]. Other areas with significant GHR immunoreactivity include the parietal cortex, striatum, hippocampus (DG in particular), hypothalamus, the thalamus septal region, putamen, amygdala as well as the cerebellum, midbrain and spinal cord [17, 459, 461, 463-465]. mRNA for GHRs is seen in glial, neuronal, as well as endothelial cells [17, 457, 464]. Although the sequence of the GHR mRNA in the spinal cord, hippocampus [466] as well as choroid plexus [467] is identical to that in the liver, there is some evidence for unique ligand specificity for the neuronal GHR [17]. Furthermore, GHR gene transcription and splicing, at least in the rat brain, are acutely autoregulated in a tissue specific way; hypophysectomy or GH treatment only affect GHR/BP in the brain tissues and not in the liver [468].

1.4.1.4 Regulation

The major site of GH expression is the pituitary. While most of the GH produced in extrapituitary sites is thought to play an autocrine/paracrine role, pituitary GH is considered to be the primary mediator of the endocrine actions of GH [469]. The pulsatile
nature of GH release from the pituitary is thought to be the result of the interaction and feedback primarily from two separate hypothalamic hormones, GHRH (growth-hormone releasing hormone) and SS; refer to Figure 1.6.

GHRH is synthesized in the arcuate nucleus and the ventromedial nucleus of the hypothalamus [470] and acts on the pituitary somatotrophs as a potent regulator of GH release. It acts via the AC-PKA (adenyl cyclase, protein kinase A) pathway to regulate cAMP levels to promote cell depolarization leading to GH release [469]. A strong GHRH regulated GH-release is seen in humans [471] as well as rats [472] and administration of an anti-GHRH serum completely abolishes GH release [473].

SS is a brain gut peptide secreted from the hypothalamus [474] which acts to suppress GH release. In contrast to the stimulatory actions of GHRH on the AC pathway, SS inhibits this pathway, preventing depolarization of the cell, thus inhibiting GH release [475]. Part of this inhibition is also mediated via indirect suppression of GHRH secretion from the arcuate nucleus which, interestingly, is thought to be mediated by GH itself (which acts on the GHRs on SS neurons in the periventricular nucleus) [476, 477].

In addition to GHRH and SS, other molecules such as the GHR secretagogues (GHRS, primarily ghrelin) and peptide hormones such as leptin (secreted by adipocytes) have also been identified to have important roles in GH secretion and regulation [469]. These agents link the body metabolic status to GH secretion, with obese people exhibiting higher levels of leptin and inversely related serum GH concentration [478]. Leptin has a stimulatory action on GH secretion both from direct actions on the pituitary as well as from regulating GHRH, SS and Ghrelin levels [479, 480]. Ghrelin is a potent stimulator of GH secretion, both via the PLC (phospholipase C)/PKC as well as AC/PKA pathways and belongs to the family of GHRS, which are all thought to be potent GH-releasing peptides. Other members of this family include GHRP-6 (growth hormone releasing peptide 6) and hexarelin [469].

GH itself is another significant regulator of its secretion [481]. Exogenous administration of GH decreases hypothalamic synthesis and release of GHRH along with an increase in SS tone, subsequently blunting/inhibiting endogenous GH secretion GH (See Fig 1.6) [482-484]. Additionally, GH also reduces stomach ghrelin mRNA and plasma ghrelin levels [485]. Considering IGF1 is the primary executor of GH mediated actions on cells,
understandably, IGF1 is also a mediator of GH biosynthesis and release, with IGF1 addition suppressing basal GH secretion, GH mRNA level and GHRH-stimulated GH simulation in rat pituitary cells *in vitro* [486, 487]. These inhibitory effects of IGF1 on GH synthesis and release are thought to be mediated via inhibition of the AC- and PKC-mediated GH secretion [488]. Interestingly, the autoregulatory effects of GH appear to be highly species specific; exposure to hGH significantly reduces GH secretion in the bovine pituitary but not from the rat pituitary [489] while ICV administration of hGH inhibits GH secretion in rats [490] but not in sheep [491].
1.4.1.5 Transport

The existence of a BBB to GH is controversial [446, 492, 493]. In mice, it was demonstrated that the BBB is permeable to GH in the neonatal period but effectively impermeable in later postnatal life and consequently, CNS requirements for GH are supplied by local synthesis [494]. However, Hojvat and colleagues have shown the BBB to be intact to circulating GH during late fetal as well as early neonatal and adult life [437, 446]. In humans, it was demonstrated that prolonged peripheral hGH treatment leads to an dose-dependent increase in CSF levels of hGH [495] and elevated levels of hGH have been reported in patients with acromegaly [449]. It appears then that at least in humans, hGH crosses the BBB. The very high presence of GHRs on the choroid plexus [459-462] suggests that GH can gain access to the CSF by means of a saturable, carrier-
mediated transport system [496]. A ligand-dependent, endoysytic mechanism-mediated receptor internalization has been suggested for the GHR [497, 498] followed by nuclear translocation of the complex resulting in transcriptional regulation [499, 500]. In spite of the concept of receptor/ligand complex formation and internalization, the function of the internalized hormone/receptor complex is yet to be fully understood.

1.4.1.6 Function and importance in brain function

The presence of GH and GHR in various regions of the brain (see section 1.4.1.3) itself indicates that GH has autocrine-paracrine functions in the CNS. Evidence suggests that IGF1 is, like in the peripheral tissues, the primary mediator of GH actions in the brain [469]. It is thought that GH acts on target cells to stimulate IGF1 release, which acts in an autocrine or paracrine manner to elicit specific differentiation effects. Central and systemic GH treatment of hypophysectomised rats increases brain IGF-I mRNA levels [501] and GH increases expression of IGF-I promoter-controlled luciferase in cultures of fetal mouse brain [494]. Increased levels of plasma and CSF IGF1 are also seen in GH-deficient (GHD) patients undergoing GH replacement therapy [495]. Further, the ontogenic regulation of IGF1 in the CNS is parallel to that of the GHR [502]. However, contrary to earlier beliefs that all of GH effects were IGF1 mediated i.e. the ‘Somatomedian Hypothesis’ [503], it is now evident that GH can indeed act independent of IGF1 via direct signaling through the GHR as well to elicit distinct but overlapping roles, giving way to the ‘Dual Effector Hypothesis’ [504]. Additionally, GH is also capable of undergoing enzymatic degradation in the circulation to yield bioactive fragments that enter and exert effects in the CNS [505]. This has been shown to be true for GH fragments generated by plasma enzymes that are structurally similar to the opioid peptides and subsequently interact with opioid receptors [505].

1.4.1.7 Developmental

The GH/IGF axis is active in the developing brain (See section 1.4.1.3) [506]. Both GH and GHRs have been identified in the developing brain [446, 460] and neuronal development deficits are seen in dwarf mice models lacking GH [446, 507]. Conversely, overexpression of GH increases brain and spinal cord weights and coordinately increases the size (nuclear and cell body) of lumbar spinal motor neurons [508]. Additionally, hypophysectomy studies demonstrated that GH increased the thickness of diencephalic structures such as anterior and posterior commissure in the growing postnatal brain [509].
Human children deficient in GH exhibit a smaller than normal head circumference (used as a measure of brain growth [510]) that demonstrates rapid catch-up growth once hGH treatment is instituted [511, 512]. The findings of Noguchi et al [507] show that GH is capable of influencing the growth and maturation of neuronal networks and synapses and GH replacement partially normalizes some of these deficits when given at critical stages of fetal development [513]. Hypomyelination seen in these mouse models was found to be due to arrested glial cell proliferation, suggesting a role for GH in the proliferation and maturation of both glial and neuronal cells [507]. In addition to this, in a recent study, GH has been shown to promote oligodendrocyte development and myelination in the CNS of fetal rats [514]. The Ajo group have further confirmed a role for GH in the regulation of fetal NSCs as well as neuronal and glial growth and differentiation in vitro [515]. GH may also be involved in gap junction formation in the brain [516]. While most of the above-mentioned effects (such as myelination, proliferation and differentiation of neuronal and glial cells) have been shown to be IGF1 mediated [517] [515], others (such as that on motor neuron size) are not [508]. There is further evidence for GH-mediated regulation of neurogenesis and angiogenesis (processes highly important in brain growth and development) which is discussed in sections 1.4.1.12 and 1.4.1.9.

### 1.4.1.8 Learning, memory and cognition

Both GH [445, 446] and GHR [460] are expressed in the hippocampus and the age related decline in the GH axis [459] has been implicated in the decreased survival of hippocampal DG neurons [518]. The hippocampus is highly involved in memory acquisition and GH, by altering NMDA receptor gene expression, plays a role in enhancing learning, memory and cognitive capabilities [519, 520]. GH induces long-term memory improvement in young rats [521] and GH replacement therapy in GH-deficient (GHD) patients results in an improvement in both long-term and working memory [522] as well as cognitive function [523]. Further, the age-related decline in circulating GH axis has been attributed to a reduction in cognitive function with age [445]. GH mRNA gene upregulation occurs following an intense hippocampal-dependent learning paradigm [445], chronic GH increases hippocampal receptor expression, along with altering the expression of NMDA receptor subunits in the hippocampus [519], and attenuates hippocampal short-term plasticity and spatial learning [524]. Furthermore, Mahmoud and Grover [525] have recently shown a direct and powerful short-term effect of GH on excitatory synaptic activity and hippocampal function. Coincidentally, ghrelin, an
endogenous ligand for the GHS receptor and stimulator of GH release [526] is also implicated in dendritic spine synapse formation and LTP generation along with enhanced spatial learning and memory [527]. These effects of GH on hippocampal function are thought to be mediated largely by IGF1 [528].

1.4.1.9 Vascular Effects

Angiogenesis or the outgrowth of new blood vessels from pre-existing vasculature is essential for tissue growth during development and normally stops during adulthood. Although constitutive in some organs such as the female reproductive organs, angiogenesis in most other adult tissues only occurs as a process of repair after tissue injury by wounding and inflammation. Pro- and anti-angiogenic molecules determine the level of angiogenesis, and most of their effects are mediated via modulation of the endothelial cells to digest the basement membrane, proliferate, differentiate and migrate to form new capillary networks. Increasing numbers of GFs are now implicated in regulating angiogenesis [529].

A significant role for GH in angiogenesis was evident in clinical observations of diabetic retinopathy, where retinal neovascularization is associated with circulating GH levels [529]. Also, GHRs are present in certain blood vessels of humans [530] and rats [531] and in vitro analyses have confirmed the stimulatory effects of GH on proliferation of human retinal microvascular endothelium [532]. GH can also stimulate ischemia-induced neovascularization in mice [533]. Given that cerebrocortical angiogenesis is associated with improved clinical outcome after embolic stroke in aged rats [235] and increased blood vessel density in the penumbra correlates with longer survival after stroke in human patients [534] it is apparent that promotion of cortical angiogenesis presents a valuable target for stroke treatment. GH effects on cerebral cortical vasculature has been explored to some extent, and Sonntag et al., [535] have shown that long-term (28 days) injection of GH stimulates neovascularization in the cerebral cortex of aging rats. There is also a positive correlation between plasma levels of GH/IGF1 and cerebral cortical vasculature [535]. Although the exact mechanism of action of GH on angiogenesis is not clear, GH is thought to promote endothelin-induced endothelial cell migration [536]. Indeed, some of the pro-angiogenic effects may be IGF1 mediated and various studies have shown IGF1 to stimulate endothelial cell proliferation, migration, and angiogenesis in vivo [529]. Intriguingly, it is only the ‘parent’ GH protein (GH-N) that is pro-angiogenic. Enzymatic
processing of GH produces a 16K N-terminal GH fragment which has been shown to inhibit endothelial cell proliferation [537].

1.4.1.10 Other effects

GH also regulates various functions such as appetite, sleep, energy metabolism and mood [436]. GHD patients demonstrate increased food intake when subjected to GH replacement therapy [538] and increases in CNS levels of GH leads to hyperphagia in mice [539]. GH-mediated effects on appetite in mice are likely regulated by increased hypothalamic expression of appetite regulating proteins [539, 540]. GH has also been shown to cause an increase in rapid eye-movement (REM) sleep with a concomitant decrease in slow-wave sleep in humans [541]. Administration of GH antiserum or inhibition of GH secretion in the rat suppresses the duration of both REM and non-REM sleep [542, 543]. Clinically, GHD patients have been noted to suffer from suboptimal well-being and impaired psychological functions and GH therapy in these patients has beneficial effects on alertness, vitality and mood [544, 545] as well as the overall quality of life [546]. It is possible that a GH-induced increase in plasma levels of β-endorphin is involved in this [495, 504] since an increase in this peptide may reflect activity in the neural pathways leading to activation of the brain reward system and subsequent euphoric effects [504]. In addition to all these effects, GH is also significantly involved in the autoregulation of its secretion as described in section 1.4.1.4.

Interestingly, GHD has been identified as a risk factor for cerebrovascular stroke while hypertension (which is also a risk factor for stroke) is common in acromegaly, suggesting a possible link between GH, stature and arterial function [547].

1.4.1.11 Implications in neuroprotection

Several lines of evidence suggest GH to be neuroprotective. GH expression in the developing chick neural retina elicits neuroprotective effects on the retinal ganglion cells, contributing to the regulation of developmental cell death [548]. GH levels also determine the survival of newborn neurons and apoptosis in the hippocampus [270, 518, 549] as well as oligodendrocyte turnover in the corpus callosum [550]. In vitro, GH protects fetal hippocampal cells [551], mature neurons [17, 552-554] and primary neurospheres [555] from death. Further, age related changes in GHR expression on endogenous precursors
are thought to induce cell death or differentiation [556], possibly mediating the age-related decrease in neurogenesis that has been attributed to a decrease in GH/IGF1 signalling [268, 270, 278, 518, 557].

GH is also implicated in neuroprotection following injury. Both GH [19] and GHR [18] are upregulated in response to focal hypoxic-ischemic injury and ICV administration of rGH (rat GH) close to the time of injury elicits neuroprotection both via as well as independent of the IGF1-mediated pathway [19] in the 21day old rat. A similar neuroprotective effect has been reported in the hypoxic-ischemic (HI) 7day old rat following GH treatment [558, 559]. Furthermore, even GH-releasing peptides such as GHRP6, hexarelin and ghrelin are neuroprotective in both in vivo and in vitro models of ischemic and excitotoxic neural injury. GH is also neuroprotective in rodent models of spinal cord injury [436] and pediatric neuroAIDS [555].

A mechanism of action for these neuroprotective effects of GH was suggested by studies reporting that anti-apoptotic effects of GH were coincident with downregulation of the pro-apoptotic proteins Bax and Bad as well as iNOS and eNOS [559], increase in the anti-apoptotic protein Bcl-2 [560] and reduction in caspase 3 [551]. In vitro studies suggest this to be a result of GH signalling via both the JAK/STAT and PI3K signalling pathways [552] with some possible involvement of the MAPK pathway [561]. It is noteworthy the neuroprotective effects are ligand specific; bovine GH (bGH) is not neuroprotective in the rat in vivo [17, 18] or in cerebrocortical neurons in vitro [17] while rGH is. This is likely due to the existence of a unique ligand specificity for the rat GHR [17], highlighting the importance of using species-specific molecules especially for neuroprotective strategies.

To what extent IGF1 is involved in the mediation of GH-induced neuroprotection is unclear. The role of IGF1 in brain injury has been studied extensively [13, 562-564]. Astrocyte-produced IGF1 and IGFBP3 (IGF binding protein 3) mRNA levels increase after HI injury in infant rats [558] [565] and ICV IGF1 treatment reduces neuronal loss in adult rats [565, 566]. Further, IGF1 treatment provides neuroprotection [567] that is largely mediated via anti-apoptotic mechanisms similar to that described for GH [560, 568]. While some studies have shown GH-mediated neuroprotection to be a factor of IGF1 actions [18, 560], the finding that GH elicits neuroprotective effects which, in a spatiotemporal pattern are distinct from IGF1-induced neuroprotective effects [19], suggests GH can have IGF1-independent effects.
1.4.1.12 Implications in neurogenesis

GH has an important role in brain development (section 1.4.1.7) [446, 506, 509], presumably due to its significant effects not only on neuroprotection (section 1.4.1.11) but also neurogenesis and neuronal plasticity. Apart from the fact that the GH axis is active during early development (section 1.4.1.7), various studies have shown that GH significantly regulates the activity of neural cells. Evidently, neural progenitor cells, neurons as well as glial cells all express GH and its receptor [14, 17, 433, 515] (See section 1.4.1.3).

Proliferative effects of GH have been observed mostly in vitro, where exogenous GH induces the proliferation of cerebrocortical [515] and neuronal hybrid [569] cells from the embryonic rat, NSCs from the embryonic mouse [14], hippocampal progenitor cells from the adult rat [22] and SVZ neurospheres from the adult mouse [20]. In vivo, GH has been shown to be the primary mediator of exercise-induced proliferation of NSCs in the adult mouse [278] while in the goldfish retina, GH administration results in a dose-dependent increase in the number of NSCs and progenitors [570]. In addition to this, models of GHD exhibit reduced brain weight and glial proliferation 1 week after birth [571] while GH administration to adult hypophysectomised rats increases the number of newborn cells in both neurogenic regions [22]. Similarly, treatment with GH antiserum causes a decrease in proliferation and myelination in growing rats [572].

In addition to promoting proliferation, GH may play a role in neural differentiation. Addition of GH to embryonic neural cultures enhanced gliogenesis [515, 573] as well as neurogenesis [515]. This was supportive of findings in GHD mice where exogenous GH was thought to accelerate glial cell division and myelinogenesis, along with increased content of gangliosides (marker of neuronal maturation) in neurons [507]. However, evidence suggests that the effects of GH on differentiation maybe age-dependent. GH inhibits neurogenesis in cerebrocortical cells from neonatal mice [433], and SOCS2 knockout mice at a young adult age (which would be hyper-responsive to GH) have fewer neurons and more astrocytes in several areas of the brain, but not in the hypothalamus [574]. Conversely, GHR knockout mice exhibit a higher proportion of neurons and fewer astrocytes in several brain regions [574]. However, addition of GH to cerebrocortical cells from the fetal rat brain increases the number of neuronal progenitor cells [515],
suggesting an age-dependent action of GH on NSCs. In addition to increased neuron numbers, GHR knockout mice show decreased dendritic branching and neuronal somas in some regions [574]. This suggests GH may also have a role in the maturation of neuronal cells, and is supported by other studies reporting enhanced neurite outgrowth and dendritic branching of neuronal progenitors with GH treatment [515]. GAP43 is a growth/plasticity protein that is expressed at high levels in neuronal growth cones during development and axonal regeneration [575]. GH administration increased expression of this protein in the brain [516], suggesting that this could be a possible mechanism for GH-induced neurite outgrowth. Recent evidence suggests GH to be involved in post-injury neurogenic processes. GHR is upregulated in various regions following focal hypoxic-ischemic brain injury [18, 19] including the SVZ [14], coinciding spatially and temporally with injury-induced neurogenesis.

It is possible that GH acts via local IGF1 synthesis to elicit its actions. IGF1 is extensively involved in neurogenic processes and several studies have elucidated specific roles for IGF1 in these processes. During development, IGF1 overexpression leads to increased brain size and myelin content [576] and IGF1 knockout animals exhibit abnormal formations of the olfactory bulb and altered RG morphology [577] along with decreased cell numbers in the hippocampus [578, 579]. Postnatally, increases in IGF1 are associated with increased cell proliferation, neurogenesis and synaptogenesis in the hippocampus [580, 581] as well as other regions [582]. This is reflected in vitro, where IGF1 has robust proliferative effects on hippocampal progenitor cells [583]. In terms of differentiation, IGF1 promotes neurogenesis [580, 584] and oligodendrocyte differentiation [305]. It is also extensively involved in post-injury processes [565, 568, 585-587] including neurogenesis [184]. However, similar to its effects on neuroprotection, it is possible that GH may have effects independent of IGF1. Postnatally, while IGF1 primarily promotes neurogenesis and oligodendrocyte differentiation [580, 584] [305], GH tends to promote a glial fate [21, 433]. Also, GH-induced increase in astrocyte numbers (paralleled with a decrease in neuron number) can be overcome by SOCS2 overexpression in vitro [433], suggesting the involvement of predominantly, the JAK-STAT pathway. Since IGF1 signals predominantly via cellular pathways other than the JAK-STAT pathway, this observation also supports an IGF1-independent role for GH. Further, the motor neuron size is larger in postnatal GH transgenic animals than in IGF1 transgenic animals [508]. These observations heighten the importance of studying both IGF1-mediated and independent effects of GH.
1.5 **Prolactin**

PRL is an anterior pituitary hormone that, based on structural homology and overlapping biological properties, belongs to the same family of proteins as GH [408]. It however, has a much broader spectrum of biological activities compared to GH, with significant involvement in lactation, reproduction, metabolism, osmoregulation, immunoregulation and behaviour [588]. Given the high structural similarities between the PRL and GH genes, their expression, as well as their receptor signalling mechanisms [589], it is evident that like GH, PRL too can elicit important actions in the brain.

### 1.5.1.1 Structure, signaling and regulation

PRL is a single chain polypeptide of 23kDa with three intramolecular disulfide bridges [590] coded for by a gene present on chromosomes 6 and 17 in humans and rats respectively [591]. While rodents also express many other PRL-related genes that are specific to the uterus and placenta, rodents as well as humans mainly express a single PRL gene (hPRL) that is synthesised and secreted by lactotroph cells of the anterior pituitary gland [592]. In humans, the same gene is also expressed in the placenta, amnion, decidua and uterus, the brain, mammary gland and immune system [593]. hPRL mRNA is composed of 6 exons and is more than 15kb long [591] with known variants (>100kDa, 40-60kDa and 16kDa), all arising from alternative splicing, proteolytic cleavage and posttranslational modifications or aggregations [591]. PRL secretion at the cellular level in the pituitary is sexually dimorphic; females exhibit continuous release [594] while males exhibit intermittent release [594]. At the level of the whole organism however, PRL release is governed by physiological conditions, such as reproductive status, maternal behaviour, stress, circadian rhythms etc., [592].

Regulation of PRL secretion predominantly occurs at the hypothalamic level. PRL release from the pituitary is governed by the hypothalamic dopaminergic system, which, via the release of dopamine (which in turn controls the release of prolactin release factor) tonically inhibits PRL release [595, 596]. PRL also affects its own secretion by stimulating dopamine synthesis and release, thus forming a negative short-loop feedback mechanism [597]. Many other hormones, neurotransmitters and GFs are also involved in the regulation of PRL gene expression; ligands that bind to G-protein linked receptors
(like thyrotropin releasing hormone and vasoactive intestinal peptide), oxytocin, vasopressin, neurotensin, insulin, leptin, EGF and bFGF simulate while glucocorticoids and TGFβ suppresses PRL gene expression [592, 598]. Estradiol is also implicated, and regulates PRL secretion at the level of the pituitary lactotroph, by controlling its gene expression and sensitivity to stimulators and inhibitors of PRL secretion [592].

Like GH, PRL elicits its cellular effects via its receptor (PRLR). The PRLR encodes a 291 aa protein that in humans (hPRLR), is encoded by a single gene containing 11 exons and is located in close proximity to the GHR gene (on chromosome 5) [593]. In addition to the long form of the hPRLR, intermediate [599, 600] and short [599, 601] isoforms have also been identified, which vary primarily in the length of the cytoplasmic domains as a result of alternative splicing. One such variant, reflecting alternate splicing from exons 7-11 encodes a secreted soluble PRL-binding protein (PRLBP) [601]. The PRLR gene is expressed in virtually all tissues of vertebrates [602], although short isoforms containing exon 11 have only been detected in samples of human breast, placenta, kidney, liver and pancreas [601]. In addition to PRL, the PRLR (even in rodents) is also able to bind hGH with high affinity [603, 604], and can also bind placental lactogen (PL) [605]. Incidentally, the serum PRLBP also binds hGH, and thus may play a significant role in the biological activity of GH as well as PRL [606]. The PRLR is also a member of the cytokine receptor superfamily and consequently, the signalling pathways utilised by PRL are essentially similar to that of GH [593] (See Fig 1.5) [605]. Receptor dimerisation by one molecule of PRL initiates signalling molecules such as STATs, SHC proteins and Fyn, as discussed in section 1.4.1.2.

1.5.1.2 PRL and PRLR expression in the brain

Studies demonstrating sustained PRL levels after hypophysectomy [607] provided the first evidence for PRL expression in the brain. In general, PRL-like immunoreactivity is differentially distributed and is more concentrated in the brain of female rats than male rats [608]. PRL-containing cell bodies are primarily located in the hypothalamus [609], with PRL-positive neurons evident in the arcuate, supraoptic, paraventricular, dorsomedial and ventromedial nuclei [610]. PRL expression is also seen in several other brain areas including the choroid plexus, cerebral cortex, hippocampus, amygdala, septum, caudate/putamen, thalamus, midbrain, pons-medulla and even in the cerebellum and spinal cord [591, 610]. PRL is also present in the CSF, possibly derived from both local and external sources [611] since PRL can be transported to the brain from the
pituitary by retrograde blood flow [612] and can be taken up from the circulation by the choroid plexus [496]. Evidence for this comes from studies showing immunoreactive PRL in ependymal cells located at the ventricular lining of almost all circumventricular organs [613]. On the other hand, given there is no tight correlation between PRL levels in the blood and CSF during hyperprolactinemia [614] and PRL levels in the CSF are sustained following hypophysectomy [608], it is likely this PRL originates in the brain.

The choroid plexus highly expresses both the short and long forms of the PRLR [615-619] and is the site with the highest density of PRLRs of all brain regions [496, 617, 620]. Moreover, elevations in circulating PRL levels markedly increase PRLR expression and binding capacity in this organ. In addition to the choroid plexus, high affinity and specific binding sites for PRL are evident on neurons in the various nuclei of the hypothalamus, limbic structure, amygdala complex, hippocampus, striatum (including the substantia nigra), the piriform cortex, neocortex thalamus and the periaqueductal gray [591, 621]. Like expression of the PRL gene in the brain, PRLR mRNA is increased in female rats, especially during proestrous [622], and is also higher during pregnancy and lactation [618]. Recent information on their ontogeny indicates that the mRNA for both long and short forms of the PRLR is detectable in the choroid plexus, olfactory system, ependymal cells, trigeminal and dorsal root ganglia, neuroepithelial cells of the cerebral ventricles as well as the anterior and intermediate lobes of the pituitary [623]. In fact PRLR expression within the fetal olfactory system is significantly higher than in adults [624].

1.5.1.3 Function and importance in the brain

PRL is involved in the regulation of diverse functions within the brain. To begin with, PRL is a major regulator of the hypothalamic dopaminergic system. An increase in either endogenous or exogenous PRL results in higher activity of the dopaminergic tuberoinfundibular neurons (TIDA) in the hypothalamus as well as activation and induction of tyrosine hydroxylase, both of which result in increased dopamine synthesis and secretion into the hypophysial portal blood [591]. Excess PRL, by acting on the brain, pituitary and/or gonads is thought to have antigonadal effects, presumably through decreasing gonadotrophin-releasing hormone (GnRH) mRNA expression [625] and reduced pulsatility and frequency of luteinizing hormone release [626]. As such, hyperprolactinemia results in reproductive dysfunction and anovulation in humans and other species [591]. Normal PRL levels however may stimulate GnRH release [627] thus
playing an important role in normal reproductive function. In fact, PRL may also play a role in the migration or differentiation of GnRH neurons during fetal development [591]. The presence of high affinity PRLRs in many regions of the brain involved in behaviour, along with its stimulatory effects on catecholamine, acetylcholine and GABA levels all suggest its action on several neural circuits [591]. It is highly implicated in sexual and parental behaviour; hyperprolactinemia suppresses male copulatory behaviour [628, 629] and acute and chronic ICV injections of PRL have stimulatory and inhibitory effects on male sexual behaviour respectively [628]. Moreover, twice-daily PRL surges triggered by mating are responsible for the maintenance of luteal function during the first half of pregnancy in the rat [630]. Further, together with ovarian steroids, PRL is important for the induction and maintenance of maternal/parental behaviour in rodents [631] as well as birds [632]. Notably, PRL can stimulate maternal behaviour even in juvenile male rats ‘exposed’ to pups [629]. In addition to this, increased levels of PRL when regulated centrally (but not peripherally) lead to increased food intake and body weight [591]. Finally, PRL is also implicated in regulating sleep-wake cycles in experimental animals [633] with a rise in plasma PRL levels evident in humans (which is disrupted in patients with sleeping sickness) [634].

1.5.1.4 Implications in Neuroprotection and Neurogenesis

Several lines of evidence now suggest that PRL, like GH, has significant neurotrophic as well as neuroprotective effects.

PRL mediates the development and maturation of TIDA neurons in the hypothalamo-pituitary system [635-638]. The number and activity of these neurons decreases significantly in both the Ames and Snell dwarf mice which have undetectable serum or pituitary PRL [639, 640] and PRL treatment restores the level of TIDA neurons if started within the first two postnatal weeks [641]. This finding highlights the importance of PRL signalling during a critical period in postnatal brain development. However, since targeted disruption of the PRL gene alone does not elicit developmental deficits in TIDA neurons (although these neurons have reduced levels of dopamine and tyrosine hydroxylase) [639], it is possible that GH or thyroid stimulating hormone compensate for the lack of PRL signalling in these mice. PRL is also a known mitogen for embryonic [642, 643] as well as post-mitotic astrocytes [25, 643], as well as a differentiation factor for embryonic astrocytes [643]. In addition to this, recent evidence shows that PRL is able
to induce proliferation of cells in the SVZ [27], the olfactory epithelium [26] as well as oligodendrocyte precursor cells [644] as seen in pregnant mice which have high levels of PRL. In fact, PRL is the mediator of pregnancy-induced neurogenesis, and administration of PRL to male mice has the same effect [27]. Furthermore, exogenous administration of PRL [27] or male pheromone-induced PRL release in females [645, 646] leads to an increase in SVZ neurogenesis in the adult. NSCs in the adult SVZ express receptors for PRL, and PRL, in the presence of EGF increases the proliferation and differentiation of these cells [27] with a reduction in PRLR being associated with significantly less proliferating cells in this region [27]. While the proliferative effects are likely mediated by PKC/phospholipase D [643, 647], its effects of promoting astrocyte differentiation likely result from prolonged stimulation of Stat 1 [643].

PRL is also involved in injury-related processes. In humans, there is an increase in the level of circulating PRL in the acute phase of traumatic brain injury [648, 649], while PRL upregulation is seen following HI in the cerebral cortex [25] and hypothalamic injury [650] in rats. Upregulation occurs most robustly on astrocytes, reactive microglia [25, 650] and some neurons [25] in the vicinity of the injury, as well as on neuroblasts in the SVZ, DG and striatum [16]. This, together with the fact that PRLR is also upregulated in parallel to PRL in most of these cells [25], strongly suggests a role for PRL in injury-related neurogenic as well as recovery processes. In support of this view, PRL has been shown to induce extensive neurogenesis and functional recovery after focal ischemic injury in adult rats [651]. Interestingly however, while PRL failed to elicit neuroprotection after HI in juvenile rats [25], it has been shown to be able to prevent the chronic stress-induced loss of proliferating cells in the DG of adult mice [28].

1.6 AIMS

Most neuroprotective compounds identified using animal models need to be administered before or immediately after, the incidence of an ischemic event [146, 147, 652]. In the clinical scenario however, most stroke patients are identified only after a considerable time delay, when significant damage has already occurred. Long-term neurorestoration therefore, is a more functional target for stroke therapy, enabling treatment of a larger number of presented stroke patients. Since there are endogenous mechanisms by which the brain can attempt to protect itself from ischemia and long-term recovery from stroke is possible even in untreated patients, it is logical that all attempts at augmenting this
natural recovery process are pursued. Thus the main focus of the majority of current stroke research is that the plasticity of the brain itself be targeted to enhance recovery from stroke. Recent discoveries that injury is an evolving process that can be interrupted up to days later, that neurogenesis persists in the adult brain [227], can occur after injury [226, 229] and is augmented by various neurotrophic factors [96, 297, 351] provides useful information for research in the development of neurorestorative therapeutic strategies. Research in our group in particular, has established important roles for the GH (and PRL) axis in these processes in the juvenile HI brain [14, 16-19, 25], serving as vital evidence for the endorsement of GH (and PRL) as a therapeutic option for long-term neurorestoration.

Taking this into consideration, the focus of the research presented in this thesis is aimed towards elucidating a role for GH in neurorestoration following adult stroke. Starting with a dose reponse study to determine the ideal treatment dose, studies are extended towards studying the long-term neurorestorative properties of GH administered following a unilateral focal ischemic stroke. These studies are designed to allow elucidation of a) a dependable method of long-term GH delivery, one that targets primary regions of interest (penumbral and neurogenic regions), b) establishment of reliable surrogate measures that can confirm the bioactivity of the infused GH for the duration of such a long term study and c) the ability of GH to promote motor recovery following a delayed, long-term infusion after a stroke. Complementing these, in vitro studies utilizing NSCs have been designed to clarify the role of GH in mediating neurogenic processes in the brain; to study the effects of exogenously applied GH on the proliferation, differentiation and migration of NSCs. The in vitro analyses are extended to include PRL as well, considering this closely-related hormone has also been implicated to mediate several neurogenic processes in the brain. Further, given the similarity of the signalling mechanisms, it was of interest to identify the similarities and differences between GH and PRL in mediating such processes.

These studies would allow for the elucidation of the role of GH in mediating neurorestorative processes in the brain; both in terms of motor recovery as well as in terms of mediating neurogenic processes which, now have been widely acknowledged to play important roles in long-term recovery from injury processes. These findings would clarify not only the potential of GH as a long-term neurorestorative factor in the brain but also the importance of GH in mediating developmental neurogenic processes. Taken
together with findings from the PRL studies, they will highlight the importance of careful consideration of closely-related hormones in terms of their similar and distinct functions and the possibility of adjunct therapies with such factors.
2 Materials and Methods

2.1 In Vivo Studies

2.1.1 Animals

All experiments were approved by the Animal Ethics Committee of the University of Auckland. Adult male Sprague Dawley rats weighing between 280 and 350g were habituated to handling for several weeks prior to surgery. Animals were housed in groups of 4 or 5 at 22 ± 2°C with a 12 hourly light/dark cycle and ad libitum access to rat chow and water.

2.1.2 Rat growth hormone buffer (rGH buffer)

Based on the developments in the formulation of liquid human GH [653], and taking into account the isoelectric point of rGH [654] a special buffer was designed that would retain rGH intact and bioactive for 2 weeks at mammalian body temperature for use in minipumps. The final buffer was designed to consist of: 8.5mg/ml NaCl (for isotonicity with mammalian body compositions), 3.0 mg/ml Phenol (as a preservative/bacteriocide to protect the formulation from microbial degradation or contamination [653]), 2.0 mg/ml Tween-20 (as a non-ionic surfactant and to prevent aggregation, permitting the formulation to be exposed to shear and surface stresses without causing denaturation of the GH protein [653, 655]) and 31.5mM NaHCO3 with 11mM Na2CO3 (as buffers in order to maintain the pH of the solution within a narrow, predetermined range [653]). The ideal final pH of the solution was determined to be around 9.4 as a) a pH greater than 6.2 was found to be necessary to inhibit or reduce GH crystallization during storage at various temperature [655] and b) this pH has been safely used for similar rGH preparations [656]. The efficacy of the rGH buffer was tested via size exclusion chromatography (SEC) and radioreceptor assays (RRA) (described below), examining rGH bioactivity after a 2-week incubation at 37°C in this buffer as compared to a freshly prepared rGH solution.

2.1.2.1 Size exclusion chromatography

Fresh and 2 week old rGH solutions were size-fractionated under neutral conditions (using phosphate buffered saline or PBS, at a pH of 7.4) using SEC. For this, about
0.24mL of the rGH buffer (1mg rGH in 9mL of buffer) were filtered through a 0.22 µm Millex filter and applied to a Superose 12 size-exclusion column (Pharmacia Akta Explorer). The Superose 12 was chosen for its high resolution separation of proteins between the molecular weights $1 \times 10^3$ to $3 \times 10^5$ daltons – GH has a molecular weight of $2.2 \times 10^3$ daltons. Filtered and de-gassed 10mM PBS (phosphate-buffered saline, at pH of 7.4, recipe given in appendix) containing 0.02% sodium azide (as a preservative) was used as a buffer to help reduce non-specific interactions between the protein and the column. The column was washed with a flow rate of 0.5 ml/min and the total column volume was 24mLs. 170 fractions of 0.46 ml were collected and monitored at 254 and 280 nm using the NanoDrop® ND-1000 (Biosciences, New Zealand). Nearly all proteins absorb at 280nm due to their content of aromatic amino acids tryptophan and tryrosine [657].

### 2.1.2.2 GH RRA

Fractions corresponding to the monomeric GH ligand separated in the SEC were subjected to a RRA to determine their bioactivity. rbGH (rabbit GH) preparation was radiolabelled to a specific activity of 70-90 µCi/µg [658]. All assays were performed within 3 days of iodination and purification of the radioligands on a Sephadex G-100 (Pharmacia, Uuosala, Sweden) column (90 x 2.0 cm). There was no significant degradation of the radioligands during incubations of the assays. The assay buffer consisted of 0.025 M Tris, 0.01 M CaCl$_2$, 0.2% (wt/vol) BSA, 0.02% (wt/vol) sodium azide, and aprotinin (10$^6$ KIU/liter). 100µL membrane preparations were incubated with a range of concentrations of unlabelled hormone (fresh or 2 week old rGH, 0-500 ng/tube) and approximately 40,000 cpm of [$^{125}$I] rbGH, in an incubation volume of 0.5 ml for 20 h at 4ºC. Nonspecific binding was determined by the addition of an excess of the appropriate unlabelled ligand (1000 ng/tube). Incubation was terminated by the addition of 3ml cold (4ºC) 0.025 M Tris-0.01 M CaCl$_2$ buffer (pH 7.6). Bound and free hormones were separated by centrifugation at 3000 x g for 30 min at 4ºC. Separate experiments showed that equilibrium was achieved under the incubation conditions described and that the binding of [$^{125}$I] rbGH was fully reversible. Specific binding was corrected for protein content.

### 2.1.3 Stereotactic Endothelin-1 (ET1) infusion surgery
The stroke model employed in our study is the ET1-induced MCAO model that has been widely characterized [62]. Rats were anaesthetized with 5% halothane/oxygen and placed in stereotactic frame supported by a heating pad to maintain their body temperature at 37°C. For the duration of the surgery, anaesthesia was maintained with a mixture of 3% halothane/oxygen delivered to via a nose cone attached to the frame. Before surgical manipulation, animals were checked for their state of unconsciousness by a toe pinch as toe withdrawal would be expected if the animal is still conscious. Fur on the surface of the head was shaved off using an electric shaver (Panasonic, Japan), the skin sponged with iodine solution (for sterility) and wiped dry. A midline skin incision was made through the scalp to expose the coronal suture line (bregma) of the skull. A small opening was drilled in the cranium bone using the following stereotaxic coordinates: 0.2mm anterior to Bregma, 5.9 mm right of the midline and 7.5mm below the surface of the skull (an illustration of this area is shown in Fig 2.1). These co-ordinates lead to the piriform cortex, approximately 0.5 mm dorsal to the MCA [62, 82]. A 26 gauge hypodermic needle was attached to a 5μL Hamilton microsyringe and vertically lowered 7.5mm ventral to the surface of the skull. 100 pM of porcine ET1 (Sigma Aldrich, New Zealand) in a 3 µl volume of saline containing 3% acetic acid was infused over 3 minutes (i.e. 1µl/min) [82]. Following the infusion, the needle was left in place for a further 3min before being withdrawn in order to prevent any backflow.

Figure 2.1: An illustration of the rat skull showing the target sites for Endothelin-1 infusion and ICV cannula placement. Figure outline derived from [3].
2.1.4 Overview of the *in vivo* studies

Two *in vivo* studies were conducted; the overall design for each of these studies is shown in Fig 2.2:

A **dose response** pilot study was first carried out to examine the endocrine dose response to infusion of rGH, verify the placement of the cannula for the delivery of GH into the brain and the neuroanatomical distribution of the chronically infused GH by immunohistochemistry. This study also allowed investigation of any possible technical difficulties arising from stereotaxic surgeries and ET1 infusion enabling easier manipulation in future studies. There were four treatment groups in this 6-week long study; Vehicle, and three different rGH doses (5, 20 and 80µg/day). GH infusion was started immediately after induction of stroke. Endocrine measures were plasma and CSF IGF1 and plasma corticosterone and urea. Behavioural assessment (bilateral asymmetry, postural reflex, forepaw asymmetry and forepaw inhibition tests) was performed to evaluate if there were any beneficial effects of a central GH infusion starting immediately after induction of stroke.

A **delayed treatment** study was then carried out at a dose of 20µg/day, based on previous studies [19] and the neuroanatomical distribution seen in the pilot study, to examine the effect of a central, long term infusion of rGH on the rate of recovery of motor function and memory. There were two treatment groups in this study; Vehicle and 20µg/day rGH. GH infusion was started 4 days after the induction of stroke and continued for 6 weeks followed by a further 6 weeks of recovery to allow for all neurorestorative processes to take complete effect [89]. Endocrine measures in this study were plasma IGF1 and corticosterone. Behavioural assessment was performed using the forepaw asymmetry, forepaw inhibition, the postural reflex and the Morris water maze (MWM) tests.
2.1.5 ICV Cannula and pump placement

A second burr hole was made in the skull while animals were under halothane/oxygen anesthesia during the ET1 surgery. An ICV cannula (Alzet brain infusion kit II, Alzet Corp, USA) was stereotaxically placed onto the skull and fixed with dental cement at the coordinates of 0.8mm posterior to Bregma and 1.5mm right of the midline to a depth of 4mm below the surface of the skull (See Fig 2.1) [659]. The size of the holes for both ET1 and ICV cannula infusions was between 1-1.5mm. The ICV cannula was attached to a primed mini osmotic pump (model 2002, Alzet Corp. USA) via a catheter preloaded with infusate (either vehicle or GH solution). The pump works based on the principle of osmosis and was ‘primed’ (placed in sterile 0.9% saline at 37°C) in advance for atleast 6 hours based on the manufacturer’s recommendations. It accurately delivers 0.5µl/hour for 14 days and was replaced twice giving a total infusion of 6 weeks. The pump was then implanted in a small subcutaneous pocket between the shoulder blades leaving the catheter loose to allow for free head movement. The skin incision was sutured with silk and 2% Lignocaine jelly (National Veterinary Supplies, NZ) applied to the wound site to minimize any discomfort. The rat was then moved to a warmed incubator until fully awake after which it was transferred to its cage. Sutures were removed approximately 7-9 days later depending on how well the wound had healed. Enrofloxacin (Bayer) was given by daily i.p. injection for three days at 7.5mg/kg as prophylactic antibiotic starting 2 hours prior to surgery. The pumps were replaced every 14 days whilst the animals were

Figure 2.2: Timeline and schematics of the dose response and delayed treatment studies. The open arrows represent behavioural assessments which include bilateral asymmetry, postural reflex, forepaw asymmetry and forepaw inhibition tests, the numbers refer to weeks and MWM refers to the morris water maze test.
under 3% halothane/oxygen inhalation anaesthesia. For this, the subcutaneous pocket of skin was re-opened, the pump replaced and the skin sutured close as before followed by application of Lignocaine gel. Once again, sutures were removed after 7 to 9 days depending on how well the wound had healed. A blood sample was taken from the tail (see section 2.1.7) whilst the animal was still under anesthesia and the solution remaining in the pump examined for volume and clarity.

For the dose response pilot study, the ICV cannula and minipump were fitted at the same time as the endothelin infusion surgery, as outlined above and shown in Fig 2.2. A randomized block design was used and animals were allocated to 0, 5, 20 or 80µg/day GH treatment groups (n=5/group). This study was concluded following a 6-week infusion.

Based on information from previous studies [19, 490, 660, 661] as well as utilising GH localisation observations from the dose response study, a delayed treatment study was carried out using the 20µg/day dose. rGH infusions were commenced 4 days after the injury. This allowed for: 1) matching of the animals on the severity of their injury and thus overcoming the inherent variability previously observed in experimental stroke models and 2) exclusion of any potential neuroprotective effects of GH in this treatment paradigm such that any beneficial effects seen would be the result of GH facilitating neurorestoration. For this study (n=10/group), the animals were allowed to recover from the ET1 infusion surgery for 3 days and at day 3, tested with the postural reflex and forepaw asymmetry tests (described in sections 2.1.6.1 and 2.1.6.2). The animals were then matched into 10 pairs according to the severity of their behavioural deficits and one of each pair randomly assigned to a treatment group. Table 2.1 shows the percentage of impairment of each animal and its corresponding matching pair. All the asymmetry scores aligned suitably enough to have two animals within a maximum of 5% score of each other and both final treatment groups had an equal distribution of severity of injury (Table 2.1). Four days after the endothelin infusion, the primed minipumps and ICV cannulae were fitted, starting infusion of rGH at the dose of 20µg/day or vehicle (buffer) only. Once again, pumps were replaced every 2 weeks with fresh infusate. Following 6 weeks of infusion, the animals had their minipumps removed and ICV cannula sealed with dental cement for a further 6 week assessment. All through the 14 weeks of study (2 weeks baseline, 6 weeks treatment and 6 weeks post-treatment), animals were assessed weekly for motor function using the postural reflex, forepaw asymmetry and forepaw
inhibition tests (described in section 2.1.6). In the last week (prior to the end) of the study
the animals were assessed for cognitive function using the MWM (Section 2.1.6.5). The
motor function tests were analyzed in three phases; 1) pre-treatment phase including the
baseline measures before injury and the testing between the injury and the start of
treatment, 2) treatment phase during the 6 week rGH infusion and 3) the post-treatment
phase from removal of the pumps to post mortem.

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>GH</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>92</td>
</tr>
<tr>
<td>89</td>
<td>90</td>
</tr>
<tr>
<td>83</td>
<td>82</td>
</tr>
<tr>
<td>81</td>
<td>73</td>
</tr>
<tr>
<td>73</td>
<td>71</td>
</tr>
<tr>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td>61</td>
<td>61</td>
</tr>
<tr>
<td>60</td>
<td>59</td>
</tr>
<tr>
<td>42</td>
<td>55</td>
</tr>
<tr>
<td>-</td>
<td>54</td>
</tr>
</tbody>
</table>

Table 2.1: Percentage of impairment of each matched pair post-stroke. The level of impairment
was calculated from the forepaw asymmetry test by assessing the percentage of time the animal
used the ipsilateral paw as opposed to the contralateral paw. *this animal had infection following
surgery and as a result was unable to perform in the motor function tests at week 1. It was
eventually euthanased.

2.1.6 Behavioural Testing
All animals were handled for 10-15 minutes per day for a week prior to commencement
of behavioural testing to reduce the effects of stress on task performance [662]. All
behavioural analyses were taped and scored offline by an observer blinded to the
treatment groups.

2.1.6.1 Bilateral Asymmetry test:
Round sticky labels one-half inch in diameter (Avery, Brea, CA, USA) were applied to
the animal’s forelimbs bilaterally in a random order [663]. Animals were placed in an
observation cage and timed for latency to contact and remove the tape from each
forelimb. Testing sessions consisted of three trials per animal for each testing day (once a
week). The difference between latency to remove the tape from the contralateral (injured)
and ipsilateral (uninjured) sides was calculated and expressed as an average ratio per session.

### 2.1.6.2 Postural reflex test:
To evaluate sensorimotor function, the postural reflex test developed by Bederson et al [664] was employed. The degree of abnormal posture was estimated by suspending rats with their tails 20 cm above the floor. Intact rats extended both forelimbs toward the floor (score = 0). Unilaterally brain-injured rats reaching only with the forelimb contralateral to the side of the injury were given a score of 2, while those rotating their contralateral shoulder towards the tail were given a maximum score of 4. This test was repeated three times in one day, once a week. The scores were then averaged per animal per behaviour session and compared to the pre-injury score [664].

### 2.1.6.3 Forepaw asymmetry test:
Rats were placed in a clear Perspex cylinder and videotaped. The number of times, out of a total of 10 rears that the rat placed either, both or one paw on the side of the cylinder was counted. Results were expressed as a ratio of ipsilateral (injured limb) use, calculated as follows: (No. of ipsilateral placements/ipsilateral + contralateral placements). This test was performed once per week.

### 2.1.6.4 Forepaw inhibition test:
Rats were placed in a 120cm long water tank containing 22°C water and a visible platform at the end. The rats were videotaped completing three straight swims to the platform per session [665]. The number of times each front paw was used to swim was counted and totaled (ipsilateral strokes - contralateral strokes) over three swims. This test was employed once per week, one session per animal.

### 2.1.6.5 Morris Water Maze (MWM):
A round black pool 1.5m in diameter was filled with clean 22°C water. A black platform 12cm in diameter was placed in the middle of the north east quadrant, 1.5cm under the level of the water. Animals were started off at random points (chosen using a die) of the pool, four times daily (at half an hour intervals) during the 5 day acquisition (trial) phase [534]. If the animals were not able to locate the platform within 60 seconds, they were placed on the platform for 10 seconds before being removed from the pool. The animals
were tracked using ANY-maze software (v4.15b, Stoelting Co, USA). On day 6, the platform was removed and the animals were tracked for 60 seconds starting opposite the site where the platform used to be. The platform zone was defined as where the top of the platform used to be.

2.1.7 Blood and CSF sampling

Blood samples were generally collected whilst the animal was under 3% halothane/oxygen anaesthesia for pump replacement and at post-mortem. For blood sampling, an area of the tail vein was swabbed with an alcohol swab and the blood (around 500µL) drawn using a 26 gauge needle attached to a 3ml syringe with the rat lying on its back whilst under 3% halothane inhalational anaesthesia. The size of the needle was chosen to eliminate any possible hemolysis. The blood sample was loaded into a 500µL eppendorf tube, transferred onto ice intermittently before being centrifuged at 3000g for 15 minutes at 4°C. Plasma was carefully isolated into fresh 500µL eppendorf tubes and stored at -20 ºC till further analysis. The needle entry point was then held closed with cotton wool until any possible bleeding had stopped. The rats were then allowed to recover in their home cage whilst being monitored.

CSF samples were collected from the dose response study animals at post-mortem to determine IGF1 levels. Rats were placed in a stereotaxic head frame with the head flexed downward while under 3% halothane/oxygen anesthesia. The skin over the depression caudal to the occipital prominence was incised and a blunt dissection was made over the cistern magna until the dura was exposed. CSF was then collected, using a fine 30 gauge insulin needle (BD biosciences, Australia), into eppendorf tubes, chilled on ice and finally stored at -80 ºC. CSF samples that were contaminated with blood were excluded from the study. Following CSF collection, animals were euthanised by i.p. overdose of pentobarbitone (150mg/kg).

2.1.8 Catheter status at post-mortem

At post-mortem in the dose response study, 20µL of methylene blue dye (1.4% w/v in 95% ethanol; Sigma Aldrich, NZ) was injected into the catheters in order to assess whether there was any blockage of the cannulas. If the dye was easily injectable into the brain, it was assumed that catheter was not blocked. If the dye exited at the surface of the
brain, it was assumed that the catheter was at least partially blocked. A fully blocked catheter provided significant resistance to dye injection.

2.1.9 Post-mortem, tissue harvestation and processing

Following euthanasia with an i.p. overdose of pentobarbitone (150mg/kg), animals were dissected to open the ribcage to allow access to the heart. They were then transtracheally perfused (using 26 gauge needles) with sterile 0.9% saline solution to flush out blood. Saline was then replaced with freshly prepared and cold modified Bouin’s solution (recipe provided in the Appendix) for approximately 5-10 minutes until the tip of the nose appeared yellow. The animals were further dissected and organs (brain, spleen, liver and heart) removed and weighed. The brains were stored in the same Bouin’s fixative for at least 24 hours (h) afterwards to ensure thorough fixation. The fixative was then replaced with 50% ethanol daily for 3-4 days until processing. Using an adult rat brain matrix (Pelco international, USA), 7 2mm coronal slices (labelled a-g) were cut and placed into plastic histology cassettes (Technoplas, Australia) for processing in an automated machine (Model ASP300, Leica, USA) for dehydration, delipidation and paraffin wax embedding (outlined in Table 2.2). The processed brain slices were then blocked in wax using an embedding machine (Model EG1140C, Leica, USA) to allow for sectioning. Using a manual rotary microtome (Leica, USA), 8μm coronal sections at every 2mm were cut onto 2,3-aminopropylsilane (Sigma Aldrich, NZ; coating protocol listed in Appendix) coated slides and baked overnight (O/N) at 65°C. Slides were deparaffinized in xylene and subsequently re-hydrated in a descending alcohol series as shown in table 2.3 prior to implementation of any staining protocols.
Table 2.2: Sequential process of automated tissue processing. Steps for dehydration, delipidation and paraffin wax embedding as carried out in the automated Leica processing machine.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% Ethanol</td>
<td>Holding Period (can be altered)</td>
</tr>
<tr>
<td>70% Ethanol</td>
<td>30 minutes</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>20 minutes</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>20 minutes</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>30 minutes</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Chloroform</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Chloroform</td>
<td>30 minutes</td>
</tr>
<tr>
<td>WAX 1</td>
<td>10 minutes</td>
</tr>
<tr>
<td>WAX 2</td>
<td>10 minutes</td>
</tr>
<tr>
<td>WAX 3</td>
<td>10 minutes</td>
</tr>
</tbody>
</table>

Table 2.3: Rehydration of slide-mounted sections prior to staining.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Xylene</td>
<td>8 minutes</td>
</tr>
<tr>
<td>100% Xylene</td>
<td>8 minutes</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>70% Ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>50% Ethanol</td>
<td>2 minutes</td>
</tr>
<tr>
<td>Milli Q water</td>
<td>5 minutes</td>
</tr>
<tr>
<td>0.01M KPBS</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>

2.1.10 Radioimmunoassay (RIA) for IGFI

Plasma and CSF IGFI concentrations were measured using a previously established IGF binding protein (BP)-blocked IGFI RIA protocol [666] described below.
2.1.10.1 Standards, Samples and reagents

The IGF1 antiserum was raised in rabbits [667] and diluted in 200mM phosphate buffer containing sodium azide, bovine serum albumin (BSA) and Triton (Buffer B, See Appendix) and used at a final dilution of 1:150000. IGF2 (Eli Lilly) was added in excess (83ng/mL) to displace IGF1 from IGFBPs, thus eliminating the need for IGF1 extraction [668]. IGFBP interference was eliminated by diluting the standard (recombinant human IGF1 (Genentech, CA) as well as samples (plasma or CSF) in two acidic buffers; a 20mM phosphate buffer and a 0.5M Ortho-phosphoric acid buffer also containing protease inhibitors (Buffers A and C, See Appendix) [668]. The standard was used at final concentrations ranging from 0.08-10ng/mL, while samples were diluted 300X. Samples were stored at -20ºC prior to execution of the assay. $^{125}$I-IGF diluted in Buffer B served as tracer and was used to a specific activity of 10-15000cpm.

2.1.10.2 RIA procedure

100µL of samples, controls or standards were combined with 100µL of acidic buffers (Buffers A + C, See Appendix) and 100µL of IGF1 antiserum for 1hr at RT (room temperature). IGF1 antiserum was replaced by 100µL of Buffer B (See Appendix) for NSB (non-specific binding buffer) tubes. Standards and NSB were run in triplicate while samples and controls were run in duplicate. After the 1hr incubation, 100µL tracer was added into all tubes, tubes vortexed and incubated for a further 48hrs at 4ºC.

After this period, bound and free ligands were separated by addition of 1% sheep anti rabbit IgG and 0.01% normal rabbit serum and 1h incubation at 4ºC. Immunoprecipitation was supported by addition of 8% m/v polyethylene glycol (PEG) in 0.01MPBS to each vial before 45min centrifugation at 3000G. The supernatant was discarded off carefully and the pellet quantified on a gamma counter. Recovery of unlabelled IGF-I in CSF was 89.6% (n=2). The ED-50 was 0.1ng/tube and the intra- and inter assay coefficients of variation were 5% and 9% respectively.

2.1.11 Corticosterone high performance liquid chromatography (HPLC) coupled with mass spectrometry

Corticosterone was extracted from plasma using ethyl acetate. First, 100µL of 100ng/mL solution of corticosterone D8 was added as an internal standard to 50µL of the plasma sample. To this, 1mL of ethyl acetate (Merck KGaA Darnstadt, Germany) was added and the solution vortex mixed for 30s followed by centrifugation to separate the organic and
aqueous layers. The organic supernatant was vacuum dried, resuspended in 100µL of 60% methanol (Merck KGaA Darmstadt, Germany) by vortex mixing and 25µL was then separated by HPLC (Thermo Finnigan Surveyor; Thermo Electron Corporation San Jose, CA) in HPLC injector vials. The HPLC used an isocratic mobile phase consisting of 80% methanol and 20% water flowing at 600µl/min through a Phenomenex Luna 3u C18(2) 100A, 50 x 3mm column held at 35 °C giving a retention time of 2.1 min. The resulting eluent was then analyzed using a Thermo Finnigan TSQ Quantum Ultra AM mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA) with the following settings: APCI (atmospheric pressure chemical ionization) source in positive mode, discharge current 3A, vapor temperature 400°C, sheath gas pressure 35 psi, aux gas pressure 5 psi and the transfer capillary set at 300°C. Then, the selected ion, corticosterone (parent ion; mass/charge (m/z) – 347; peak width Q1 – 0.3 mass units) was fragmented using Argon (collision pressure – 1.2mTorr; collision energy – 28V) to produce a product ion (daughter ion; m/z 121; Q3 – 0.7 mass units). Corticosterone-d8 was analysed simultaneously as internal control (parent – m/z 355.30; daughter – m/z 125.2) with the same parameters.

2.1.12 Urea measurements

Urea concentrations in the plasma samples were measured on a Hitachi 902 Autoanalyser (Hitachi High Technologies Corporation, Tokyo, Japan) by kinetic UV assay (Roche, Mannheim, Germany).

The kinetic UV assay works on the enzymatic reaction principle of the hydrolysis of urea; urea is hydrolysed in the presence of urease, and one of the products, ammonia, helps to turn NADH to NAD+ with the catalysis of GLDH (glutamate dehydrogenase). The absorbency decrease (at 340nm) is directly proportional to the concentration of urea. This reaction principle is given below [669]:

\[
\text{Urea} + 2\text{H}_2\text{O} \xrightarrow{\text{Urease}} 2\text{NH}_4^+ + \text{CO}_3^{2-}
\]

\[
\alpha - \text{Oxyglutarate} + \text{NH}_4^+ + \text{NADH} \xrightarrow{\text{GLDH}} \text{L - Glutamate} + \text{NAD} + \text{H}_2\text{O}
\]

The calibrators and controls used were the Roche Calibrator for automated systems (C.f.a.s, Roche, Mannheim, Germany) and PreciNorm U and Precipath U (Roche, Mannheim, Germany).
2.1.13 Acid Fuschin-Thionin Staining and measurement of tissue survival

Following re-hydration in alcohol, slides were transferred to a coplin jar with thionin staining solution for 10 min. Following a quick wash in distilled water, they were transferred to acid fuchsin for 30s (seconds), immediately followed by another wash in distilled water. This was then followed by sequential dips (6 each) in 95% and 100% ethanol, followed by 10 dips each in 50/50 mix of 100% ethanol and xylene and 100% xylene. The final step was to allow a 5min incubation of the slides in fresh 100% xylene prior to mounting. Slides were mounted in dibutyl-phthalate-xylene (DPX) medium (BDH laboratory supplies, England) and left to air dry in the fume hood for atleast 4h. Slides were visualized under light using the Axioskop 2 plus microscope (Carl Zeiss Inc., Germany) and images were obtained with the 1.25x objective using the Axiovision software (v3.1, Carl Zeiss Inc., Germany) for measurement. Damaged cells appeared pink while intact cells had a blue nissl colour due to acid fuchsin and thionin respectively.

The area of intact cortical tissue was measured over 5 coronal levels through anterior and mid-caudate and the anterior hippocampus (levels b-f) of each animal while blinded to the treatment groups. The extent of tissue loss was estimated by demarcating the area of intact tissue in each hemisphere for each section and expressing the intact area on the ipsilateral side as a percentage of the corresponding contralateral intact area ((ipsilateral intact area/contralateral intact area) *100). The mean percentage value for each group (vehicle and treatment) was then calculated and statistically analysed. Expressing the area of intact tissue as a percentage of that in the contralateral hemisphere enabled to reduce the errors associated with the processing of tissue for histological analysis.

2.1.14 Immunohistochemistry:

All sections were subjected to antigen retrieval using the citrate buffer method before processed for immunohistochemical labelling. Following dewaxing and re-hydration, sections were washed for 20min at RT in fresh citrate buffer (See Appendix) containing 0.05% tween20 (Sigma Aldrich, NZ) as surfactant. Sections were washed out in PBS and then quenched in 1%H2O2/methanol for 30min at RT. After further washes in PBS (5min each), all sections were blocked for 2h at RT in 0.01M KPBS containing 5mg/mL of BSA (Sigma) and 10% animal serum specific to the species that the secondary antibody was made in. All primaries were diluted in dilution buffer consisting of 5mg/mL BSA and
2% animal serum in 0.01M KPBS (Recipe listed in Appendix). Washes were done in 0.01M KPBS with 0.05% tween-20 as surfactant.

2.1.14.1 GH, DCX, GFAP

To detect GH immunoreactivity, a polyclonal rabbit anti-rat GH antibody (NHPP, USA) was used at 1/600 with a biotinylated goat anti-rabbit IgG secondary (Sigma) at 1/500 and detected using the Vectastain Elite ABC kit (Vector), following the manufacturers' instructions with diamino benzidine (DAB)(Sigma) as chromagen. Positive controls included staining of pituitary sections and negative controls included using non-immune normal rat serum, antibody diluting buffer or the GH antibody pre-absorbed with affinity-purified rGH or rGH diluting buffer at a ratio of 1:10 (antibody:protein) instead of the primary antibody for 24h at 4°C. DCX immunoreactivity was detected using the same protocol (goat anti-human DCX, 1/150, Santa Cruz). A Cy3-conjugated glial fibrillary acidic protein (GFAP) primary was used at 1/200 (mouse anti porcine GFAP, Chemicon). For GHR staining, a highly specific anti rat GH-R antibody was used at a 1/700 (a kind gift of Dr Thordarson, UC, Santa Cruz, USA).

Double labeling to identify cell types was performed with the same protocols as above using the fluorescent markers Alexa 488, 568 and Cy3 instead of DAB. For DCX staining, a conjugated secondary (donkey anti-goat Alexa 488, Sigma) was used. Following visualization of the first antibody, sections were washed, blocked and incubated O/N at 4°C with the second antibody followed by the secondary (or conjugated secondary) antibody and fluorophore.

<table>
<thead>
<tr>
<th>Approximate number of positive cells</th>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Absent</td>
<td>0</td>
</tr>
<tr>
<td>1 - 20</td>
<td>Occasional</td>
<td>1</td>
</tr>
<tr>
<td>21 - 70</td>
<td>Low levels</td>
<td>2</td>
</tr>
<tr>
<td>71+</td>
<td>Present</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 2.4: Four-point scale used for GH/DCX quantification

2.1.15 Quantification of GH and DCX labeling: dose response study

Sections were stained for GH or DCX (individually; single labeling as described in the previous section) and number of positively stained cells assessed in various areas. Positive immunostaining was then compared between each (ipsi- and contralateral)
hemisphere and between each of the four treatment groups. Quantification of GH and DCX labeling in the dose response study was carried out in two ways:

Absolute quantification - The number of GH/DCX positive cells lining and within close proximity to the LV, SVZ and the infarct area (core + peri-infarct) were counted in mid-striatal sections (levels c & d). Only these two levels were chosen as these sections exhibited the maximum extent of infarct, peri-infarct, dorsolateral SVZ and LV areas.

Semi-quantification – The GH/DCX positive staining in the cortex (all area outside of the infarct region, cells lining the 3rd ventricle, cells lining and within close proximity to the lateral lining of the LV, infarct area (core + penumbral region), white matter tracts (WMTs), cortical blood vessels, dorsolateral SVZ, hippocampus (dentate gyrus + CA1,2,3) and the meninges/pia mater was semi-quantified based on the four-point scale shown in Table 2.4. The score was collated over all levels (a-g) and the mean score for each group graphed.

2.1.16 Statistics

Statistical analyses were carried out using Sigmastat™ v3.1 (Jandel Scientific, San Rafael, CA, USA). Two way ANOVA with time as a repeated measure was used to analyse data from the animal studies. If data was not normally distributed, or did not have equal variance, the two-way ANOVA was carried out on rank transformed data. Post hoc statistics were performed using bonferroni t-tests or non parametric equivalent where applicable. Correlation of IGF1 levels with the rGH dose was done using Pearson’s correlation. All results are expressed as mean ± SEM and significance was assumed to exist at P<0.05.
2.2 **IN VITRO STUDIES**

2.2.1 **Cell culture**

2.2.1.1 **Source**

Human neural stem cells (hNSCs) were sourced from Prof Austin Smith and Yirui Sun, Wellcome Trust Centre for Stem Cell Research at the University of Cambridge, UK. These cells were isolated from human fetal neural tissue from fetal cortices at embryonic 50-55 days, equivalent to Carnegie stage 19-22 [670].

2.2.1.2 **Expansion**

Cells were expanded continuously in monolayer culture on flasks coated with 10mg/mL laminin (mouse, Invitrogen) in PBS, O/N at 37°C/5%CO₂. The expansion medium comprised of Euromed-N medium (Euroclone, Italy), 2mM L-Glutamine (Invitrogen, New Zealand), 1x N2 supplement (Invitrogen, New Zealand), 20ml/L B27 (Invitrogen, New Zealand), 10mL/L Penicillin-Streptomycin (Invitrogen, New Zealand) and 10ng/mL each of recombinant human EGF and bFGF (Sigma Aldrich, New Zealand) [670]. The doubling time of these hNSCs is 3-4 days [670, 671] and once confluent, cells were harvested using Accutase (Sigma Aldrich, New Zealand) and passaged at 1:2 to 1:3 ratios every 5-7 days. Proliferating hNSC cultures contain both small bipolar cells and more flattened apolar cells. All assays were carried out on hNSCs that were between passages 14-19. It has been shown already that these cells can be successfully expanded for over a year for around 50 passages without any obvious cell senescence, crisis or spontaneous differentiation [670].

2.2.1.3 **Freezing and thawing cells**

To freeze cells, cultures were detached, centrifuged, and re-suspended in expansion medium with 10% DMSO. A 60-90% confluent T25 flask was usually frozen down into 1 vial, and one T75 flask into 3 vials. Cells were frozen at 1ml per cryotube (Nunc, Germany) at -80°C O/N in a slow-freeze container, then transferred to liquid nitrogen for long term storage.
Cells were thawed by rapidly bringing the vial to 37°C in a water bath and then re-suspended in the appropriate volume (7mLs for a T25 flask, 12mLs for a T75) of pre-warmed expansion medium. This suspension was then transferred to a laminin coated flask. Flasks were coated with 10mg/mL laminin (mouse, Sigma Aldrich, New Zealand) for approximately 12h. After 6h following resuspension, all medium in the flask was replaced with fresh prewarmed expansion medium with the appropriate concentration of GFs (see below). Medium was prewarmed in a 37°C water bath.

2.2.1.4 Hormones and Antagonists

This study exclusively employed recombinant proteins.

Both rhPRL (recombinant human PRL) and the hPRLR antagonist were a generous gift from Prof. Vincent Goffin (InSerm, France). Recombinant wild type (WT) PRL and Del1-9-G129R-hPRL (WT PRL with a deletion of the 9 N-terminal residues and single substitution of Arg for Gly 129, resulting in a pure PRLR antagonist (PRLRA)) were produced in Escherichia coli and purified, and have been extensively characterized [672-678]. Lyophilised proteins were resuspended in 50mM Tris-HCl at 1mg/mL stock concentration and stored at -20°C until use.

Genotropin® (Pfizer, New York, USA; a kind gift from Prof. Wayne Cutfield, CRU, Liggins Institute) was resuspended in sterile mQH2O (to a final concentration of 1mg/mL) and used as the source of GH. Genotropin® contains recombinant hGH (rhGH) produced in Escherichia coli. This form of rhGH has been shown to be structurally identical to pituitary GH [679] and has been widely used as an exogenous, recombinant source for hGH [551, 680] [681]. The hGH antagonist B2036 (Pfizer, stock solution at 10.4mg/mL)(GHRA) was kindly provided by Prof. Peter Lobie (Liggins Institute). B2036 is a recombinant analogue of the hGH that has been genetically engineered to function as a GHR antagonist [682].

Since the freeze-dried formulation of Genotropin® contains inactive preservative and stabilizing agents, it was necessary to determine the concentration (w/v) and integrity of the recombinant hGH in solution prior to use.
2.2.1.4.1 Protein Assay

Protein content of the Genotropin® solution (re-suspension was in sterile milli-Q water) was measured using the BioRad DC colourimetric Assay. This assay is a modified version of the Lowry assay [683] and is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent leading to colour development. The assay was carried out in triplicate, using 1µg/mL BSA (Sigma Aldrich, NZ) solution as standard. 5µL of Genotropin® solution (1µg/µL) was loaded. It was determined that 5µL of solution contained 2.2µg of rhGH.

2.2.1.4.2 SDS-PAGE Gel Electrophoresis

In order to confirm the integrity of the Genotropin®, Genotropin® solution (1µg/µL) was run on an SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis). It was expected that a single band would be seen at 22kDa (molecular weight of GH) with no other bands at lower molecular weights (which would indicate degradation).

NuPAGE Bis-Tris 4-12%, 1.5mm pre-cast gel was used with the Bio-Rad electrophoresis apparatus (Bio-Rad Laboratories Inc., USA). Genotropin® solution (4µg of protein) was incubated 10min at 70°C with reducing sample buffer (with DTT added; NuPage MES buffer, Bio-Rad Laboratories Inc., USA) to break disulfide bonds and linearize the protein.10µg of protein was loaded per well alongside a protein standard (SeeBlue Plus2 pre-stained standard, Invitrogen) for 40min at 200V, 400mA. 4µg of pituitary extracted hGH 200ug/mL in CBS (a kind gift from Prof.Richard Faull, FMHS, University of Auckland) served as positive control. The gel was then stained with Comassie Blue for 1h and then transferred to de-staining solution (50% MetOH, 10% Acetic Acid and 40% mQH₂O) for a further 1h before imaging. Fig 2.3 shows validation of the SDS-PAGE gel electrophoresis for Genotropin® solution integrity.
2.2.2 Functional Assays

2.2.2.1 Proliferation Assay

Proliferative effects of GH and PRL were assessed on hNSCs using the BrdU labeling method. 48well culture plates (Nunc, Denmark) were coated with 10mg/mL Laminin in PBS O/N. The following day, the laminin was removed and wells washed out with PBS. Cells were harvested from confluent flasks using accutase, spun down and resuspended in seeding medium. 20000 cells suspended in 500µL of seeding medium (without EGF or bFGF) were added to each well of the 48 well plates. 24h later, 250µL of medium was replaced with an equal amount of seeding medium containing 2µM BrdU and either rhGH or rhPRL (no EGF/bFGF) of the required concentration. Concentrations were doubled in the replaced medium since only half volumes were added. Following a further 48h incubation, cells were washed, fixed in cold 4%PFA and processed for BrdU immunostaining. Wells with medium only (no EGF/bFGF) served as negative controls while wells with medium containing EGF and bFGF served as positive controls. Each assay was carried out in quadruplicate.

2.2.2.1.1 BrdU Immunostaining

Fixed cells were incubated with 2N HCl (hydrochloric acid) for 30 minutes at 37°C, followed by neutralisation with 0.1M Na₂B₄O₇ (sodium tetraborate – Borax (Sigma Aldrich, New Zealand) – 2g/100ml Millipore water) 2x5min, washed 3x with PBS and blocked with 5% normal goat serum (NGS) in PBS for 30min before immunostaining with 1:50 mouse anti-BrdU antibody (Oncogene, USA) at 4°C O/N (PBS+2% NGS+1%
BSA + 0.1% triton). The next day, cells were washed and further incubated with 1:200 goat anti-mouse Cy3-coupled secondary antibody (Sigma Aldrich, New Zealand) for 2h at RT. Following 3x5min washes, cells were incubated in DAPI solution (1µg/mL, Roche, New Zealand) for 15min at RT. Cells were then washed again and mounted using Vectashield (Vector laboratories, USA).

2.2.2.2 Neurotrophic Assay

The aim of this assay was to identify the proliferative effects, if any, that GH and PRL would have on neuroblasts and glial progenitor cells (i.e. differentiating hNSCs). Once confluent, hNSCs were detached with accutase, spun and plated at a density of 25,000 cells per well, in polyornithine/laminin-doublecoated 48 well plates (Nunc, Denmark). The plating medium used was a 1:1 mixture of EuromedN® supplemented with N2, and Neurobasal® medium supplemented with B27, 2mmol/L glutamine and 2ng/ml bFGF. After 24h half of the medium was replaced with bFGF-free medium (1:1 mixture, to trigger differentiation) supplemented with 0.5, 5, 50, 150 and 500 ng/ml rhGH or rhPRL. Medium only wells served as negative controls whereas 10ng/mL BDNF (RnD Systems, NZ) or 3% fetal calf serum (Invitrogen, NZ) was used as positive controls for neuroblast and glial differentiation respectively. Cells were incubated for a further 72h and subsequently fixed for immunocytochemical analysis (described later on). Cells were double-labelled with antibodies directed against PCNA (marker for proliferating cells) and either β-tubulin (marker for neuroblasts) or S100β (marker for glial progenitors) followed by DAPI as a nuclear stain. Since RG are known to express low levels of GFAP [670, 684, 685], we chose to use S100β, a glial marker expressed by immature cells of the astrocytic lineage [686] to identify glial progenitors.

The number of cells positive for both PCNA and either β-tubulin or S100β in each field of view (at 20x) were counted and expressed as a proportion of the total number of cells (DAPI stained) in that view. At least 5 fields of view were counted per well.

2.2.2.3 Differentiation Assay

In order to determine if GH had any effects on the differentiation of hNSCs, a differentiation assay was carried out. For the differentiation assay, 24-well plates were double coated with 5mg/mL poly-ornithine (Sigma) and 10mg/mL laminin for 2h each. Following this, 2x10⁵ hNSCs (well volume = 500µL) were plated into each well in
expansion medium (with EGF and bFGF) for 48h. Differentiation was triggered by removing of EGF from expansion medium. After 10 days, the culture medium was changed to Euromed-N® mixed with Neurobasal® medium in a ratio of 1:1 supplemented with N2 (0.5X), B27 (1X), and bFGF (10ng/ml). 4 days later, bFGF was withdrawn from medium, and after a further 4 days, medium was switched to Neurobasal® medium, supplemented with B27 (1X) and the specific concentration of mitogen (rhGH/rhPRL, BDNF (10ng/ml, R&D systems NZ as positive control)) without N2 or bFGF for 10-20 days. The mitogen concentrations were calculated such that only 1µL of solution containing the required amount of mitogen for 500µL was added to each well during all replacements to ensure minimal dilution/contamination. During the whole progress of neuronal differentiation, 1/2 volumes of media were replaced every alternate day.

**2.2.2.4 Migration Assay**

Cell migration was measured using 12-well transwell culture plates and inserts with 12-µm-pore polycarbonate membranes (Corning Costar). Plates and inserts were pre-coated with 10mg/mL laminin/PBS for 1h and 50µg/mL poly-l-lysine/PBS (Sigma Aldrich, NZ) for 30min and rinsed twice in sterile PBS and milliQ water respectively. Control wells of the transwell plates were then coated in 1.5mL of seeding medium containing 10ng/mL BSA (Sigma Aldrich, NZ) as vehicle while the treatment wells were coated in seeding medium containing various concentrations (0.5, 5, 50, 150 and 500ng/mL) of rhGH/rhPRL. Where receptor antagonists were used, they were added along with BSA or rhGH/rhPRL (no pre-treatment). 75000 cells were seeded into each insert in a total volume of 500µL. The plates were then incubated at 37°C 5%CO2 for 24h. The following day, wells and inserts were rinsed out with PBS and fixed in 4% PFA for 30min at RT. Cells were subsequently stained with Gill's haematoxylin (Sigma Aldrich, NZ) for 1h, followed by two rinses in PBS. All cells that displayed neurite outgrowth and had travelled to the bottom chamber were counted as migrating cells.

**2.2.2.4.1 JAK2 Inhibitor – AG490**

AG490 (Calbiochem, USA) was kindly provided by Prof. Peter Lobie (Liggins Institute) and was used at a final concentration of 10µM. AG490 has been shown to completely prevent hGH-stimulated tyrosine phosphorylation of JAK2 [431]. AG490 was added along with GH to each well at either of the 3 concentrations tested (1, 5 or 10µM) (i.e. there was no pre-treatment with AG490).
2.2.3 Molecular Biology

2.2.3.1 Immunocytochemistry for stem cell markers and receptors

For immunostaining, hNSCs were plated in expansion medium, at a density of $2 \times 10^3$ cells per well into a 24well plate precoated O/N with 10mg/mL Laminin (Invitrogen, NZ). The following day, cells were rinsed out with PBS and fixed in ice-cold 4%PFA for 15min at RT. Reactive sites were blocked out by incubating the cells in blocking solution containing 8% NGS in a 1% BSA/PBS solution with 0.1% triton added as a surfactant. After 2h at RT in blocking solution, cells were incubated in primary antibodies; rabbit anti musashi (1/500, Chemicon, USA), mouse anti nestin (1/500, Chemicon, USA), rabbit anti Sox2 (1/1000, Chemicon, USA), mouse anti rat PRLR (1/100, Affinity bioreagents, USA) and mouse anti rat GHR (1/200, Chemicon, USA). Rabbit and mouse IgG only (1/200, Chemicon, USA) wells served as negative controls. Primaries were diluted in modified blocking solution (with 2% NGS instead of 8%) were added for 1h at RT, followed by O/N at 4°C. Following 3 gentle washes in PBS, conjugated secondary antibodies were used at the following dilutions for 2h at RT: Goat anti mouse Cy3 (1/200, Amersham Biosciences, UK) or biotinylated goat anti rabbit (1/200, AbCam, USA). Where biotinylated goat anti rabbit was used, following 3 x 5min washes, cells were further incubated with 1/200 Streptavidin Alexa 488 (Invitrogen, NZ) for 1h at RT. All wells were rinsed out once again in PBS and finally incubated with the nuclear stain DAPI (1µg/mL, diluted in methanol, Roche, NZ) for 15min at RT. Following a final wash in PBS, the wells were coverslipped using vectashield (Vector labs, USA) as the mountant. Wells were viewed under UV light and imaged at 10x (musashi, nestin and sox2) or 20x (GHR/PRLR).

2.2.3.2 RNA Extraction

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen). Cells grown to confluence were detached using accutase and centrifuged to a pellet. Cells were disrupted using 350µL of lysis buffer containing β-mercaptoethanol to lyse cells and deactivate endogenous DNase and RNase enzymes. The lysate was homogenized using QIAshredder spin column, treated with 70% ethanol (to promote selective binding of RNA to the RNeasy membrane) by pipetting and then centrifuged for 15s at 10,000RPM to enable total RNA to bind to the membrane. The 3D structure of RNA is disrupted and
subsequent contaminants are efficiently washed away using buffers containing guanidine thiocyanate. Finally, RNA was eluted in RNase-free water and the quantity and purity derived by measuring the absorbance of each RNA sample at 260 and 280nm. Absorbance (quality) and concentration of the extracted RNA were measured using the NanoDrop® ND-1000 (Biosciences, NZ). Table 2.5 shows Nanodrop® results for a representative sample of RNA extracted from hNSCs. The absorbance at 260nm provides an indication of nucleic acids, that at 280nm of proteins and at 230nm, of other contaminants such as residual solvents. A 260/280 and 260/230 ratio greater than 1.8 is considered to be an indicator of good quality RNA [687]. All RNA samples were stored at –80 °C until use.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>263.9</td>
<td>6.597</td>
<td>3.198</td>
<td>2.06</td>
<td>1.71</td>
</tr>
</tbody>
</table>

Table 2.5: Nanodrop results of a representative RNA sample extracted using the RNeasy mini kit

### 2.2.3.3 Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

Reverse transcription of cDNA was carried out using the Superscript III Reverse Transcriptase System® (Invitrogen, NZ). For synthesis of the first-strand cDNA, 1µg of RNA was incubated at 65°C for 5min in a total volume of 13 µL containing 1 µL oligo(dt)20 primer and 1 µL 10 mmol/L dNTPs (Invitrogen Inc., Carlsbad, CA, USA). Following this, 4 µL 5x first-strand buffer, 1 µL 0.1M DTT, 1 µL of RNaseOUT recombinant RNase inhibitor, and 200U of reverse transcriptase (Superscript III; Invitrogen) were added to the mixture and incubated at 50°C for 60 min. The cDNAs were kept at -20°C until PCR amplification.

For RT-PCR, 1uL of the reverse transcription product was combined with 100pmol each of forward and reverse primers, 25µL of PCR GoTaq Green MasterMix (Promega) and nuclease free water to make up a final reaction volume of 50µL. The PCR reaction was as follows: Denaturation at 94°C for 2min, followed by 33-42 cycles of sequential incubations at 94°C for 30s, annealing at the primer-specific annealing temperature for 30s, elongation at 65°C for 1min followed by a final elongation step at 65°C. PCR products were held at 4°C until further analysis. β-actin was run as the housekeeping gene. A list of all primers used for identifying specific genes are listed in Table 2.6. The positive controls used for each of these genes are
listed in Table 2.7. To determine if the anticipated DNA fragment size was generated, PCR products were run on 2% agarose gel (Invitrogen, NZ, made up in tris-EDTA buffer) for 65min at 65V. A 100bp DNA ladder (Invitrogen, NZ) was run on each gel.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer direction</th>
<th>Primer sequence</th>
<th>Expected product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH</td>
<td>Sense</td>
<td>CAGGGAGGAAACACAAACAGAAA</td>
<td>155 bp</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TTAGGAGGTCATAGACGTTGCT</td>
<td></td>
</tr>
<tr>
<td>hGHR</td>
<td>Sense</td>
<td>GCTAACTAGCAATGGTGTTACAG</td>
<td>103 bp</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GACGTTCAGTAAAGTCCAGTTGA</td>
<td></td>
</tr>
<tr>
<td>hGHR (variants;</td>
<td>Sense</td>
<td>GGATAAGGAATATGAAGTGCA</td>
<td>GHR (full length): 453bp</td>
</tr>
<tr>
<td>shorter isoforms)</td>
<td></td>
<td></td>
<td>GHR1-279: 427bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GHR1-277: 383bp</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>GATTTCCTCATGGTCACCTGC</td>
<td></td>
</tr>
<tr>
<td>hGHR (variants;</td>
<td>Sense</td>
<td>GTCCTACAGGTATGGATC</td>
<td>GHR3+ = 383 bp</td>
</tr>
<tr>
<td>exon 3 deletion)</td>
<td></td>
<td></td>
<td>GHR3- = 317 bp</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>AGGTATCCAGATGGAGTG</td>
<td></td>
</tr>
<tr>
<td>hIGF1</td>
<td>Sense</td>
<td>ATCCCTTTGCTCTGCACGAGT</td>
<td>108 bp</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TTACTTGTGTATTTTCATTGAGGG</td>
<td></td>
</tr>
<tr>
<td>hIGF2</td>
<td>Sense</td>
<td>CCTCCAGTCTGGGTCCTGTC</td>
<td>163bp</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CAGGTCCCTCTGGACTTGTG</td>
<td></td>
</tr>
<tr>
<td>hIGF1R</td>
<td>Sense</td>
<td>ATTGAGGAGGTCACAGAGAAC</td>
<td>755bp</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TTCATATCCTGGGTCCCTGTC</td>
<td></td>
</tr>
<tr>
<td>hPRL</td>
<td>Sense</td>
<td>TTCTGGCCAGATATGTCTT</td>
<td>423 bp</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CTGAGGAGGAGTATGGA</td>
<td></td>
</tr>
<tr>
<td>hPRLR</td>
<td>Sense</td>
<td>GTGGCATCTGCAACCGTTCCTGCTGTA</td>
<td>275bp</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CCACATGGAGGCTGTTGACCTGCAAGTG</td>
<td></td>
</tr>
<tr>
<td>hPRLR (variants;</td>
<td>Sense</td>
<td>AGTGAGGCTTGGAAGGGCTAT</td>
<td>PRLR (long) = 856bp</td>
</tr>
<tr>
<td>long &amp; intermediate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>forms)</td>
<td></td>
<td></td>
<td>PRLR (intermediate) = 284bp</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>AGGAGTCCCGGCGCTT</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Sense</td>
<td>GATCAGCAACGAGGAGTAAGTG</td>
<td>579bp</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TCATAAGATATCTGTTGAGG</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.6: List of primers. The annealing temperature was 60 °C
<table>
<thead>
<tr>
<th>Gene</th>
<th>Positive control (cDNA)</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGH</td>
<td>MDA-hGH (forced expression of GH in MDA-MB-231)</td>
<td>MDA-MB-231 (Human breast cancer cell line) forced to express GH</td>
<td>[688]</td>
</tr>
<tr>
<td>hGHR</td>
<td>Human astrocytes</td>
<td>Primary astrocytes (glial cells) isolated from the normal adult human brain</td>
<td>[448]</td>
</tr>
<tr>
<td>hIGF1</td>
<td>Primary cells from human placenta</td>
<td>Primary cells isolated from the adult human placenta collected at birth</td>
<td>[689]</td>
</tr>
<tr>
<td>hIGF2</td>
<td>Primary cells from human placenta</td>
<td>Primary cells isolated from the adult human placenta collected at birth</td>
<td>[689]</td>
</tr>
<tr>
<td>hIGF1R</td>
<td>MCF7</td>
<td>Human breast adenocarcinoma cell line</td>
<td>[690]</td>
</tr>
<tr>
<td>hPRL</td>
<td>Placental decidual cells</td>
<td>Human Endometrial Stromal cells (HESC cell-line) that have been treated with medroxy progesterone acetate (MPA), &amp; estradiol/cAMP, to become decidual cells</td>
<td>[691]</td>
</tr>
<tr>
<td>hPRLR</td>
<td>RL95</td>
<td>Human endometrial carcinoma cell line</td>
<td>[692]</td>
</tr>
</tbody>
</table>

Table 2.7: List of positive controls for each gene examined using PCR
2.2.3.4 IGF1 Enzyme-linked immunosorbent assay

Quantification of the endogenous levels of IGF1 produced by hNSCs was performed using an enzyme-linked immunosorbent assay (ELISA) to detect human IGF1. ELISA analysis of IGF1 was preferred over RIA as preliminary analyses utilising RIA demonstrated insufficient sensitivity to measure the quantities produced by hNSCs. Measurements were carried out using the Quantikine human IGF1 ELISA kit (R&D Systems, NZ) as per the manufacturer’s directions. This is a 3.5 hour solid-phase ELISA with high specificity and sensitivity (the minimum detectable dose ranges from 0.007-0.056ng/mL). The assay employs a quantitative sandwich enzyme immunoassay technique where an IGF1-specific monoclonal antibody is pre-coated onto the microplate. Samples (and standards) are pipetted onto the microplate and any present IGF1 is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IGF1 is added, followed by a substrate solution that, according to the levels of enzyme (and thus IGF1) present, develops colour in proportion. The intensity of colour is then measured in a spectrophotometer, and using data from the known concentration standards, the concentration of IGF1 in the samples calculated.

Cell culture medium from GH-treated, BDNF-treated (as negative control) and untreated cells (conditioned medium) was run in triplicate while standards (0-60ng/mL) were run in duplicate. Medium was collected from cells treated with 0.5, 5, 50, 150 and 500ng/mL of rhGH for 3 and 6 days. Absorbances at 450 and 540nm were recorded and the optical density calculated (abs at 450nm – abs at 540nm).

2.2.4 Statistics

All statistical analyses were carried out using SigmaPlot v12 for Windows (Systat Software Inc, San Jose, CA, USA). Two way analysis of variance (ANOVA) and Holm-Sidak post-hoc tests were applied with replicate number and treatment as factors. Where data was found not normally distributed or not equally variant, the same tests were carried out on rank-transformed data. All results are expressed as mean ± SEM and significance was assumed to exist at P<0.05. All graphing was done using GraphPad PRISM v5 (GraphPad Software, CA, USA).
3 Central infusion of GH post-ischemia in the adult brain: behavioural and endocrine effects

3.1 INTRODUCTION

Previous research has identified a significant role for GH in mediating post-ischemic processes in the brain. Evidence for this came from observations that both GH as well as its receptor are upregulated ipsilaterally following a unilateral ischemic insult in the juvenile brain [18, 19] while GH administration following such an insult was neuroprotective [559, 693], some of it independent of IGF1 [19]. These neuroprotective properties of GH have also been confirmed *in vitro* [17, 694, 695]. Its role in the adult ischemic brain however, remains to be elucidated.

The primary aim of this study was thus to establish a dose-response to a long-term ICV infusion of GH, starting immediately after a focal ischemic stroke in adult rats. Prior to this however, a buffer was designed specifically for this long-term (6 week) study, one that would successfully maintain the bioactivity of the infusate for the duration of the study. Endpoints of this study were behavioural and endocrine effects of such treatment, which would enable 1) determination of whether GH is neuroprotective in the ischemic adult brain and 2) characterisation of an endocrine response to such a treatment paradigm. Based on previous studies, it was hypothesized that a dose of 20µg/day would be ideal, given that this dose is sufficient to express bioactivity [660, 661] and is sufficient to elicit neuroprotection in the juvenile rat model of HI [19]. As such, GH treatment is expected to offer neuroprotection and consequently, reduce injury-induced sensorimotor deficits. It was anticipated that ICV delivery of GH would result in an increase in CSF concentrations of IGF1 accompanied by a decrease in plasma IGF1 concentrations.
3.2 Results

3.2.1 Buffer formulation and testing

The primary methodology used in this study was a chronic infusion of GH and one of the concerns of chronic infusions is the maintenance of bioactivity of the infusate. Based on suggestions for clinical liquid human GH infusates [653], a GH buffer was designed in order to keep rGH intact for 2 weeks at mammalian body temperature. In order to determine whether rGH (dissolved in this buffer) would remain bioactive when placed in a minipump in vivo, its bioactivity was tested after a two week incubation at 37°C, and compared to a freshly prepared rGH solution using the same buffer.

SEC enables the separation of solute molecules of a solution based on their molecular hydrodynamic volume or size [696]. Results from SEC analysis indicate that the rGH was primarily dimerised following this incubation (larger dotted peak in Fig 3.1a). A monomeric GH fraction was also eluted although this was very minor (smaller dotted peak at 15mL of buffer in Fig 3.1a). In order to determine if this dimerisation affected the bioactivity, or more specifically, receptor binding of rGH, a GH RRA was employed. The monomeric fraction of the 2 week old rGH was tested along with fresh rGH and rbGH (rabbit GH) solution for its binding affinity to GHR on liver membrane. Binding studies revealed that the dimerisation did not have a significant effect on the 2 week old rGH in solution in its ability to bind to, or be displaced from, the rat hepatic GHR in a RRA (Fig 3.1b). The ED50 for the fresh solution was 4000ng/mL while that for the 2 week old GH was 16000ng/mL.
3.2.2 Behavioural analysis

rGH dissolved in buffer was loaded into osmotic minipumps and implanted into rats subjected to ET1 stroke. A chronic (6-week) ICV infusion of GH at 3 different doses; 5, 20 and 80µg/day was given as treatment (vehicle group were infused with buffer only), commencing immediately after stroke. As both GH [17, 19] and its major downstream effector IGF1 [566, 567] have been shown to be neuroprotective and IGF1 to improve somatosensory function after stroke [697], it was of interest to evaluate the effects of GH treatment on stroke-induced sensorimotor deficits.

Figure 3.1: Specific activity of rGH incubated for 2 weeks in the designed rGH buffer. Size exclusion chromatographic analysis of rGH following a 2-week incubation at 37 °C showing nearly complete dimerisation (a). Panel b shows results from a GH radioreceptor assay demonstrating that a 2-week incubation at 37 °C had little effect on receptor binding activity of rGH. Rabbit GH (rbGH) was used as the radiolabelled hormone and binding studies were carried out on rat hepatic GHRs.
A total of five animals (out of 20) were lost in the study; three in the immediate postoperative phase, one due to intracerebral haemorrhage, one due to infection and one due to idiopathic reasons. Two more animals were lost in weeks 4 and 5 of the study, one from anaesthesia-induced complications and one due to intraabdominal hemorrhage from injections. The behavioural results from the first three animals were excluded from all analyses. The final n for each of the groups for behavioural analyses was as follows: vehicle = 5, 5 µg/day GH = 3, 20 µg/day GH = 3, 80 µg/day GH = 4.

Behavioural testing results are shown in Fig 3.2. Overall, there was no significant difference between any of the treatment groups for any of the tests carried out. Behaviour is expected to worsen after injury.
Figure 3.2: ICV administration of GH commencing immediately after stroke has no significant effects on sensorimotor function at any of the doses studied. Endothelin was infused adjacent to the middle cerebral artery to cause a transient vasoconstriction. GH was then infused at various doses via ICV osmotic pumps for 6 weeks. An array of behavioural endpoints was investigated. Shown are results for a) Bilateral asymmetry, b) Postural reflex, c) Forepaw asymmetry and d) Forepaw inhibition tests. Although all groups exhibited impairment in behaviour following stroke induction, there was no significant difference between the vehicle and any of the treatment groups over the period of observation. The dotted lines represent the value expected for normal function. Colours are as follows: vehicle = white, green = 5µg/day, red = 20µg/day and blue = 80µg/day. Shown are mean ± SEM. The x-axis denotes weeks from stroke/GH infusion; -2 and -1 denote 2 and 1 week before induction of stroke/GH infusion. GH infusion was continued for 6 weeks. The final n for the groups was: vehicle = 5, 5µg/day GH = 3, 20µg/day GH = 3, 80µg/day GH = 4.
3.2.3 Plasma and CSF measurements

As GH was delivered ICV for a prolonged period, it was necessary to confirm that the delivered GH remained bioactive during the entire treatment period. GH is a key regulator of IGF1 concentrations both systemically [698, 699] as well as centrally [501, 699, 700]. Theoretically, it is expected that central treatment with GH will increase CSF IGF1 and reduce circulating IGF1 concentrations via activation of the hypothalamic negative feedback system [701], giving a relatively simple surrogate measure to determine that the infusate is bioactive at any time during treatment. In addition to IGF1, plasma urea and corticosterone concentrations were also measured to ensure the wellbeing of the animals. IGF1 concentrations were measured using an IGFBP blocked RIA, urea concentrations using a kinetic UV assay on the Autoanalyser while corticosterone concentrations were determined using HPLC coupled with mass spectrometry (detailed in Chapter 2).

Predictably, plasma IGF1 concentrations in all 3 GH groups were significantly lower compared to those in the vehicle group (p<0.01, Fig 3.3a) while CSF IGF1 exhibited a dose-dependent increase with GH treatment (r=0.7, p<0.01; Fig 3.3b). Plasma urea concentrations in the GH-treated animals were also significantly higher than those in the vehicle group (p<0.05, Fig 3.3c), with corticosterone concentrations also exhibiting an increasing trend with GH treatment at the higher doses (Fig 3.3d). Whilst plasma corticosterone concentrations at week 3 were lower in all animals compared to that at week 2 or 6, concentrations in animals which received high GH doses were greater than in controls or 5µg/day groups. The difference between the vehicle and 20µg/day group and the 5 and 20µg/day groups at week 6 (end of pump 3/post-mortem) was significant (p<0.01 and 0.001 respectively). For plasma IGF1: n=5, 4, 3, 4; CSF IGF1: 4,3,2,4; Urea levels: 5,4,3,5; Corticosterone: 5,4,3,5 for vehicle, 5, 20 and 80µg/day groups respectively.
Animals from all groups were weighed once a week in order to ensure their wellbeing and to study any possible treatment effects during the study. Major organs such as the brain, spleen, liver and heart were removed and weighed at post-mortem to assess if there were any systemic effects of central GH infusion.

Although there was a gradual increase in body weights over time as expected, chronic 6-week ICV GH infusion caused a significant increase in the body weight (p<0.001, Fig 3.4a). The mean body weights of the GH-treated groups (combined) were significantly higher than those of the vehicle group. Post-hoc analysis revealed that there was a significant difference between the vehicle and 5µg/day group (p≤ 0.01), vehicle and 20 µg/day group (p≤ 0.01), 5 and 80µg/day groups (p< 0.01) and 20 and 80µg/day groups (p≤ 0.01). In terms of organ weights however, GH-treatment did not have any significant effects (Fig 3.4b).
Figure 3.4: GH treatment resulted in a significant rise in body weight but no significant change in any of the organ weights. The mean body weights of the treatment groups (combined) were significantly greater than those of the vehicle group (a). Individually, the mean body weights of both the 5 and 20µg/day groups were significantly greater than that of the vehicle group during the treatment period. This significant increase in body weight was not reflected in the weights of any of the organs as measured at post-mortem (b). Colours are as follows: vehicle = white, green = 5µg/day, red = 20µg/day and blue = 80µg/day *p<0.05, **p<0.01, ***p<0.001. Values represent Mean±SEM. The final n for the groups was: vehicle = 5, 5µg/day GH = 3, 20µg/day GH = 3, 80µg/day GH = 4
3.3 DISCUSSION

Results from this study demonstrated for the first time, the behavioural and endocrine effects of a chronic (6-week) ICV infusion of GH following an ET1 induced stroke in adult rats. GH treatment commencing immediately after stroke did not induce any significant amelioration in sensorimotor deficits as assessed by bilateral asymmetry, postural reflex, forepaw asymmetry and forepaw inhibition tests. The rGH buffer specially formulated for this long-term infusion study was successful in maintaining the bioactivity of the GH for the duration of the study, as shown by suppressed plasma IGF1 and elevated CSF IGF1 concentrations in response to a central delivery of GH at all doses. GH treatment caused a significant increase in body weight while having no significant effects on brain, spleen, liver or heart weights at post-mortem. Together, these results provide evidence for a successful formulation of a buffer for the long-term central delivery of GH as well as highlight specific surrogate measures that could be used to ensure the bioactivity of GH for the duration of such a chronic infusion. However, given the observed variability in severity of injury amongst the treatment groups, the effects of GH treatment on stroke-induced sensorimotor deficits remains to be further elucidated.

The first challenge of long term central treatment studies utilizing indwelling mini osmotic pumps is to ensure the bioactivity of the infusate. Taking into account suggestions for and recent developments into the formulation of liquid human GH for clinical use [653], a special buffer was designed to maintain the integrity and bioactivity of rGH intact over a 2 week period at mammalian body temperature. Interestingly, assessment of this buffer revealed that almost 100% of the rGH was dimerised after a 2 week period of incubation at 37°C (Fig 3.1a) but this dimerisation did not appear to significantly affect its ability to bind to or be displaced from the GHR (Fig 3.1b). This is noteworthy since hGH dimers have been suggested to be less potent than hGH monomers [702-704]. It is possible that the dimerized rGH acts as a reservoir of stabilized rGH which is able to monomerize when exposed to a low GH containing environment, such as in the binding assay or when it is in contact with CSF in vivo. Indeed, this has been shown to be true for an isoform of the human GH [705]. It is worth mentioning here that the RRA, while is a potential measure of bioactivity, is not a measure of bioactivity per se. However, the strong reduction in plasma IGF1 levels (Fig 3.3a), as measured in blood samples taken throughout the study and dose-dependent
increase in CSF IGF1 levels (Fig 3.3b) at the end of the 6-week infusion clearly demonstrate that the rGH preparation was bioactive.

Behavioural assessment of animals during this study revealed no specific effects of GH on the amelioration of these injury-induced sensorimotor deficits. This was unexpected, considering both GH [19, 558-560] as well its primary downstream effector IGF1 [706-708], have been shown to be neuroprotective in various models of HI injuries. All the tests used in this study have been extensively characterised and used for testing sensorimotor deficits elicited by stroke in various animal models [52]. However, the behavioural response of an animal to a given therapy can be influenced by a number of factors, including the size and location of the damage, the nature/efficacy of the treatment as well as the timing of the treatment after insult [52]. Unfortunately, as is apparent from the behavioural results (Fig 3.2a-d), the injury elicited in this study appeared extremely variable; visual examination showed infarct sizes to vary greatly between animals. This was most obvious in the forepaw asymmetry and inhibition tests (Fig 3.2c, d). There are several possible explanations for this. First and foremost, due to unexpected post-surgical and anaesthesia complications, the final n for all treatment groups was reduced. A higher n would certainly have provided more reliable results especially considering the expected variability involved in all experimental models of stroke [709]. Secondly, despite careful application of the stereotaxic coordinates (see Chapter 2), it is possible that the ET1 infusion was somewhat inaccurate in the precision of localisation due to the infusion setup used; a sharp needle was used and reference points for bregma were taken from the end of this needle. However, as the infusate comes out about 1mm above the end of the needle, ET1 infusions could have been inconsistent. Thirdly, in addition, there were several unexpected difficulties during the ET1 infusion with the infusion needle blocking due to a narrow lumen. Fourthly, when calculating the stereotaxic co-ordinates for the ET1 infusion sites, it appeared that there might be discrepancies in these from animal to animal resulting from possible discrepancies in animal strain. Since wistar rats have a slightly shorter head than sprague-dawleys, the infusion point for the ET1 which should have been on the lateral ridge of the skull, was not. This was confirmed by Mr.Wing Leong, who has extensive surgical expertise, especially involving stereotaxic surgeries. This could contribute significantly to the variability of the treatment outcome as histopathological outcome after stroke has been shown to be variable in different rat strains [710, 711], probably as a result of variability of the collateral circulation present in each rat strain [710].
However, since staff of the ARU have confirmed that this discrepancy is impossible and that all animals were definitely Sprague-Dawley rats, there is no way to prove this discrepancy. Accordingly, this reasoning although can be considered, can be under-valued and accordingly wasn't highlighted in the paper published from this work. And lastly, since increased corticosterone levels are known to detrimentally effect behaviour [712, 713], it is possible that any beneficial effect may be masked.

Given the various experimental difficulties experienced (as described above), it would have been ideal to carry out further studies using a larger n. While this would have enabled a definitive elucidation of a neuroprotective role for GH, funding constraints, coupled with supervisory changes did not allow for this study to be repeated with more animals. In addition to this, the primary aim of this study was to elucidate if and how a dose-response to a GH treatment would exist and where exactly such ICV-infused GH would localise to. Given that these aims were addressed despite the small n, the study was considered satisfactory.

Despite the inaccuracy of stereotaxic placement, GH infusion into the lateral ventricles appeared successful as evident from measurements of plasma and CSF concentrations. Visual assessment of the tissue slices at the cannula level also confirmed this. Brain IGF1 mRNA levels have been shown to increase in response to ICV GH treatment [501, 699] and to our knowledge, this study shows for the first time, a dose-dependent increase in CSF IGF1 levels too (r = 0.69, p<0.01; Fig 3.3b). Indeed, GH has an independent paracrine activity on brain cells, promoting IGF1 transcription via the JAK-STAT pathway [714]. Plasma IGF1 concentrations however, declined significantly with ICV infusion of GH, presumably through activation of the hypothalamic GH negative feedback loop [701]. Treating animals with GH for extended periods of time (greater than 24h) can block endogenous GH release from the pituitary due to feedback inhibition via SS release from the hypothalamus when administered systemically [715] and decreased expression of GHRH when administered centrally [716]. Consequently, this would lead to decreased production of IGF1 from the liver, thereby reducing plasma levels of IGF1. These data illustrate the exquisite sensitivity of the hypothalamic feedback system as plasma IGF1 appears to be maximally inhibited by ICV GH even at the lowest dose of 5µg/day (Fig 3.3a). The moderately increased levels of plasma urea may reflect this decrease in circulating IGF1 and indicate the induction of a mildly catabolic state, as IGF1 is a major anabolic hormone postnatally [717]. Conversely, the increased urea levels may be due to an enhanced nitrogen turnover caused by an
increase in food intake by central GH [718]. Alternatively, it could also be a result of the increased corticosterone levels (Fig 3.3d). Glucocorticoids are widely associated with a decrease in protein synthesis and increase in protein catabolism [719, 720], an effect which is mediated by decreased GH secretion [721]. Interestingly, the strong catabolic action of glucocorticoid analogues was successfully counteracted by the administration of recombinant human IGF1 and its analogues [722]. Additionally, some glucocorticoids have been identified to act directly at the tissue level [723], interfering with local IGF1 production [724] and antagonising insulin action [725]. The increase in plasma corticosterone levels seen with central GH infusion has been reported previously in transgenic animals over-expressing GH either throughout the body [726, 727] or in the brain [728] and is thought to be the result of chronic rises in plasma ACTH (adrenocorticotropic hormone) concentrations [729]. Indeed, corticosterone concentrations are generally taken to be an indicator of stress [730] but given the levels in GH-treated animals are higher compared to the vehicle group, it can be assumed that the rise in corticosterone is a result of GH action.

The increase in body weight of GH-treated animals is possibly related to enhanced food and water intake in these animals (Fig 3.4a) [718]. Both systemic and ICV GH [718] and ICV infusion of GH releasing peptides [731] have been shown to increase food intake and a consecutive increase in body weight in rats. Similar increases in food intake have been reported with GH treatment in humans [732]. Accordingly, no significant changes in brain, spleen, liver or heart weight were observed at post-mortem (Fig 3.4b).

In conclusion, this study provides several important standpoints for chronic ICV infusion of GH. Firstly, it provides a successful buffer formulation for maintaining bioactivity of rGH infusions for at least a 2 week interval in rats. Secondly, it demonstrates that plasma and CSF concentrations of IGF1 and plasma concentrations of corticosterone and possibly urea are useful endpoint measures for determining bioactivity of infused GH and confirmation of functional and accurate placement of cannulae. Finally, given the observed discrepancies and marked variability in behavioural results, this study highlights several important surgical and post-operative issues that require being addressed in future studies so that an in-depth investigation of the effects of GH on sensorimotor deficits induced by stroke can be successfully carried out.
4 Central infusion of GH post–ischemia in the adult brain: region and cell-specific targeting of infused GH

4.1 INTRODUCTION

There is now growing evidence to suggest a trophic and neurogenic role for GH in the brain [14, 16, 17, 20-22, 515, 518, 550]. These studies have demonstrated that GH is capable of inducing progenitor proliferation and differentiation while other studies have shown that post-injury upregulation of GH and GHR seen in the juvenile brain occurs largely on neuronal and glial cells within regions known to undergo active remodelling [18, 19]. From these studies, it is apparent that GH may have direct actions on stem/progenitor cells and subsequent beneficial therapeutic effects, especially when appropriately regionally/cellularly targeted within the brain.

This histological study was designed to identify areas that a long-term GH infusion would localise to and thus subsequently, its cellular modes of action. Since the accurate delivery of GFs to regions of injury (where re-modelling and/or replacement is needed) and neurogenesis (where new neurons that enable remodelling/replacement are generated) is a key determinant of efficacy, another objective of this study was to establish whether unilateral infusion of GH ipsilateral to the injury alone is sufficient. The primary aims of this histological study thus, were to characterise 1) the effect of unilateral ICV GH treatment on tissue survival, 2) the areas to which the delivered GH localised and finally 3) the particular cell types that the delivered GH associates with. The hypothesis for this study was that such a unilateral, ICV delivery of GH would elicit significant neuroprotection, by localising to regions of injury (cortical and subcortical penumbral regions of the infarct) as well as neurogenesis.
4.2 RESULTS

4.2.1 GH infusion after stroke may alter the tissue survival

Previous work has identified GH to be neuroprotective when administered following HI injury in the juvenile brain [19]. Here, the neuroprotective effects of GH when administered after an ischemic insult in the adult brain were examined via gross histology. GH was administered ICV commencing immediately after a unilateral focal ischemic insult, for 6 weeks in adult rats. At the end of this period, coronal sections of the brain tissue were stained with acid fuchsin-thionin and imaged for measurements of tissue survival. The area of intact tissue on the ipsilateral (injured and ET1 infused) side was measured and expressed as a percentage of that on the contralateral side (uninjured/no ET1 infused).

Fig 4.1a shows a representative coronal acid-fuchsin stained section depicting the infarct and surrounding areas at different levels in the brain (levels b-f). The alphabet on the bottom right hand corner of each image denotes the level of the section, which was relatively consistent for all the animals. Overall, there was a significant difference in tissue survival (p<0.01) and post-hoc tests revealed that this difference arises from the significantly lower mean of the 5µg/day GH group. There was no significant difference in tissue survival between the 20 and 80 µg/day groups and the vehicle group (Fig 4.1b, c). The mean±SEM for the vehicle, 5, 20 and 80µg/day groups were 88.6±2.22, 77.1±2.86, 87.9±3, 82.1±2.56, respectively.
4.2.2 ICV GH following injury localises to neurogenic regions and the infarct penumbral area

8µm sections from the vehicle and GH-treated animal brains were labelled with antibodies against rGH to identify areas of localisation of infused GH. The specificity of the GH staining was confirmed by pre-absorption of the antibody and pituitary sections were used as positive and negative controls for staining. The number of GH positive (GH+) cells lining and near the lateral side of the LV, dorsolateral corner of the SVZ and infarct area (core + penumbra) were counted at the two levels that these areas were.
most prominent at (levels c & d). Cells were counted on both ipsi- and contralateral sides of the brain section.

Labelling with an anti-GH antibody revealed that GH+ cells were located specifically in the ependymal cells lining the lateral and ventral sides of the LV in the ipsilateral hemisphere (Fig 4.2a,d, e closed arrows), cells of the dorsolateral SVZ on the same side (Fig 4.2a, d open arrows), blood vessels (BV, Fig 4.2 g, h open arrows) and neuron-like cells within the ischemic penumbra (Fig 4.2g, h, closed arrows) and ipsilateral WMTs between the dorsolateral SVZ and ischemic penumbra (WMT, Fig 4.2 i, j). Panel j shows the distribution of GH+ cells in the ipsilateral dorsolateral SVZ and a column of these cells in the WMTs between the dorsolateral SVZ and the ischemic penumbra. Little staining was observed on the contralateral side of the GH treated brain (b) and little to no GH staining was found in vehicle only treated brains (c). Pre-absorption of the primary antibody completely abolished all GH+ staining seen (f). GH+ cells were also evident lining the 3rd ventricle (Fig 4.2k). Once again, vehicle infused brains did not contain GH+ cells lining the 3rd ventricle (open arrows) but did have GH+ cells in the hypothalamus on both ipsi- and contralateral sides (closed arrows) (l).

Panels m, n and o of Fig 4.2 show positive and negative controls of GH staining run on serial adult rat pituitary sections. Replacement of the primary antibody with pre-immune serum (normal rat serum) (m) was used as a negative control for the primary antibody while the primary antibody on a normal adult rat pituitary section was used as positive control (n). GH+ staining is evident in the anterior pituitary but is absent in the posterior section (n) as expected [733]. Preabsorption of the primary antibody for 24h eliminated GH+ staining in the pituitary sections too (o), proving that the observed GH staining was specific.
Figure 4.2: GH delivered via implanted minipumps localises to cells in known neurogenic regions as well as those surrounding the infarct penumbra. Immunohistochemistry revealed that the infused GH bound to ependymal cells lining the lateral side of LV and dorsolateral SVZ (closed arrows in panel a,d and e). Little staining was observed on the contralateral side of the GH treated brain (b) and almost no GH staining was observed in vehicle-treated brains (c). GH staining was completely abolished with pre-absorption of the primary antibody (f). Neuron-like cells (closed arrows) and blood vessels (open arrow) within the infarct penumbra were GH positive (g and h). GH positive cells were also evident within the ipsilateral WMTs between the dorsolateral SVZ and infarct penumbra (i, j) as well as lining the 3rd ventricle (k). Vehicle infused brains did not contain GH positive cells lining the 3rd ventricle (open arrows) but did have positive cells within the hypothalamus on both ipsi- and contralateral sides (closed arrows) (l). Positive and negative controls for GH staining were run on pituitary sections to confirm specificity of GH staining; Normal rat serum (m) was used as a negative control for the primary antibody while a normal adult rat pituitary section was used as a positive control (n). GH positive staining is evident in the anterior pituitary but is absent in the posterior section (o). Preabsorption of the primary antibody for 24h eliminated GH positive staining. LV = lateral ventricle, SVZ = sub-ventricular zone, 3rd V = 3rd ventricle, WMT = white matter tracts, Ant = anterior pituitary, Post = posterior pituitary. Scale bars j= 160µm, a,b,c,f,g, i = 80µm, k,l,m,n,o = 40µm, d,e,h = 20µm. Images are representative of 20 and 80µg/day GH-treated brains. The final n for the groups was: vehicle = 5, 5µg/day GH = 3, 20µg/day GH = 3, 80µg/day GH = 4

4.2.3 Quantification of GH immunoreactive cells

Since there was considerable localisation of GH to cells lining the lateral side of the LV, within the dorsolateral SVZ and within and surrounding the infarct penumbra, the number of GH+ cells were quantified in these regions.

Quantification of GH+ cells at the two levels (mid-striatal; levels c & d) most prominently exhibiting the majority of infarct penumbra, lateral lining of the LV and dorsolateral SVZ revealed that GH infusion considerably increased the number of GH+ cells in these areas (Fig 4.3). There were somewhat higher numbers of GH+ cells in the lateral side of LV (Fig 4.3a), dorsolateral SVZ (Fig 4.3b) and infarct penumbra (Fig 4.3c) of the 20 and 80µg/day (Groups C and D respectively) GH-treated animal brains, when compared to those in vehicle-infused (Group A) and 5µg/day (Group B) brains. At the lowest dose of GH (5µg/day), there was very little GH+ staining visible in the ipsilateral lateral lining of the LV and dorsolateral SVZ (Fig 4.3a, b), that in the dorsolateral SVZ being at levels similar to the vehicle infused brain (Fig 4.3b). Interestingly, GH+ cells were also visible in the contralateral dorsolateral SVZ of most groups (Fig 4.3b). In the infarct penumbra on the ipsilateral side, there appeared to be a dose-dependent increase in the number of GH+ cells with GH treatment; the increase with 80 µg/day GH being significant when compared to the mean number of the vehicle group (Fig4.3c, p<0.01).
GH+ cells in other brain regions were also semi-quantified in order to determine the distribution of the delivered GH. 8µm sections were taken every 2mm, stained for GH and the number of GH+ cells in each of the following areas semi-quantified on both ipsi- and contralateral sides: Cortex (all area including the infarct region), cells lining the 3rd ventricle, cells lining and within close proximity to the lateral side of LV, infarct penumbra, WMTs, cortical blood vessels, dorsolateral SVZ, hippocampus (dentate gyrus + CA1,2,3) and the meninges/pia mater. Each area was assigned a score based on the approximate number of positive cells present: (0) absent, (1) occasional, (2) low levels and (3) present, as described in Table 2.4, pg75 and the mean score for that area calculated for each treatment group.

Semi-quantification over all areas revealed the presence of GH+ cells in the ipsi and contralateral cortical and predominantly ipsilateral infarct areas of the GH- treated groups with little visible staining in the vehicle group (Fig 4.3d, g). In general, the GH groups had a higher mean score than the vehicle group (not significant in the cortex). There was a significant difference in the mean score for GH+ cells around the lateral side of LV between the treatment groups (Fig 4.3e, p=0.013), and post-hoc testing showed that the mean score for GH+ cells was significantly higher for the ipsilateral side of the 20µg/day GH group when compared to the same side of the vehicle group (Fig 4.3e, p<0.05). There was also a significant difference in the number of GH+ cells in the infarct penumbra between the treatment groups (Fig 4.3g, p=0.006) with the GH groups exhibiting higher ipsilateral mean scores compared to that of the vehicle group. The 20µg/day exhibited a significant difference in the ipsilateral mean score when compared to the vehicle group (p<0.05, Fig 4.3g). The mean score for the WMTs and dorsolateral SVZ had a slightly similar pattern, with higher doses of GH (20 and 80µg/day) showing elevated mean scores (not significant, Fig4.3 h, i). Interestingly, there was a considerable presence in GH+ cells in the contralateral dorsolateral SVZ of both of these groups – an effect absent in the vehicle and 5µg/day GH groups (Fig 4.3i). The vehicle and 5µg/day groups also lacked the GH+ staining around ipsilateral cortical BVs that was seen at the higher doses of GH (not significant, Fig 4.3j). No GH+ cells were evident in the hippocampal regions (DG and CA 1,2, 3) for any of the treatment groups (results not shown).
A - Ipsilateral
A - Contralateral
B - Ipsilateral
B - Contralateral
C - Ipsilateral
C - Contralateral
D - Ipsilateral
D - Contralateral

**Number of GH+ cells**

LV

Dorsolateral SVZ

Peri-infarct area

Cortex

Third Ventricle

**Mean score**

Overall p=0.006*

Overall p=0.013

Overall p=0.006
4.2.4 GH immunopositive cells double-label with DCX and GFAP

Given the significant numbers of GH+ cells visible in neurogenic and infarct penumbral regions, double-labeling studies were carried out to identify the phenotypes of these cells. Double-labeling was carried out using antibodies against GH, doublecortin (DCX) and glial fibrillary acid protein (GFAP).

Double labeling showed that with chronic treatment, GH (red) strongly associated with endothelial cells and a closely associated population of DCX positive (DCX+, green) cells within the striatal (Fig 4.4 a, b) and cortical (Fig 4.4c) penumbral regions. Several GH-DCX double labelled cells were also present in the infarct region in the penumbra close to the core (Fig 4.4d), as well as near to (Fig 4.4e) and within the WMTs (Fig...
4.4f) on the ipsilateral side. There was also occasional colocalisation of GH-DCX in cells within the striatum (Fig 4.4g) and the lateral lining of the LV and dorsolateral SVZ (Fig 4.4h).

Figure 4.4: Infused GH localised to DCX positive cells lining blood vessels, in the infarct penumbral region as well as in and surrounding the WMTs on the ipsilateral side. Within the striatal (a and b) and cortical (c) penumbral region, the endothelial cells of blood vessels and a closely associated adjacent population of DCX (Green) positive cells were strongly immunopositive for GH (Red). Some GH-DCX double-labelled cells were also evident in the infarct region (d) as well as the penumbral region near (e) and within (f) ipsilateral WMTs. Occasional colocalisation of GH-DCX in cells within the striatum (g) and those lining the lateral side of the LV and dorsolateral SVZ (h) were also observed. Scale: a, b, c=8µm, d= 16µm, e, f, g= 20µm and h=80µm. Exploded bodes show the selected cells at a 40x magnification. All these images are representative of the 20 and 80µg/day GH groups.

Several GH+ cells (green) were also immuoreactive for GFAP (red) (Fig 4.5). This colocalisation was particularly evident in the penumbral region of the infarct (Fig 4.5a)
as well as WMTs (Fig 4.5b) on the ipsilateral side. There was also some co-localisation of GH-GFAP evident in cells bordering the dorsolateral SVZ (Fig 4.5c) as well as ependymal cells lining the lateral side of the LV (Fig 4.5d i(GFAP), ii(GH), iii(Merge)).

![Image](image_url)

### 4.2.5 Quantification DCX staining

DCX is a marker for differentiating and migrating neurons and DCX+ cells have been shown to appear after stroke in the ischemic penumbra, most of which originate in the SVZ [360, 734]. As there was considerable colocalisation of DCX and GH in cells lining the lateral side of the LV and dorsolateral SVZ, WMTs and within the ischemic penumbra, the number of DCX+ cells in these areas was counted.

Fig 4.6 shows the mean number of DCX+ cells per group. There was no significant difference between the groups in any of the areas quantified.
As with GH staining, semi-quantification of DCX+ staining was carried out in various regions and scores assigned on the four-point scale described in section 2.1.15. Figure 4.6 (panels d-i) show results from this analysis. Although, the mean score for DCX+ cells was similar in all treatment groups, there appeared to be a slight trend towards higher levels for the contralateral cortex compared to the ipsilateral cortex (Fig 4.6d, not significant). There was a significant difference between the mean score of treatment groups for the infarct area (p=0.03, Fig 4.6e). However, due to interference from the methylene blue dye injected at post-mortem, quantification was hindered for 1 animal from each of the vehicle and 20µg/day groups, resulting in a smaller n value. There was no significance difference in the mean score for any of the LV, WMT or the dorsolateral corner of the SVZ areas (Fig 4.6 f-h). Within the hippocampus however, there was a significant effect of GH treatment (p=0.002, Fig 4.6i). Post-hoc testing revealed a significant decrease in the mean DCX score on the contralateral side in the 80µg/day group compared to the vehicle group (p<0.01).
DISCUSSION

Together, these results provide the first evidence of the distribution of GH when delivered ICV into the injured brain. The aim of this study was to evaluate the specific areas which were targeted by a unilateral continuous infusion of GH following stroke. Although there were no significant effects on tissue survival with 20 and 80µg/day GH treatment, it was largely at these doses that there was widespread distribution of GH immunopositive cells in brain. GH+ cells were visible lining and within the immediate proximity of the lateral side of the LV, within and lining the dorsolateral SVZ, within the ipsilateral WMTs extending from the ipsilateral SVZ to the infarct area, within and surrounding the ischemic penumbra and lining the third ventricle. There was significant co-localisation of GH and DCX within ipsilateral cortical and striatal BVs, cells within and lining the ischemic penumbra (including WMTs), as well as in some cells lining the LV near the dorsolateral SVZ. GH also colocalised with GFAP expressing cells within the ischemic penumbra, ipsilateral WMTs and cells lining the ipsilateral LV. These results indicate that ICV delivery of GFs ipsilateral to the side of the injury is an effective way to target regions of interest following focal ischemia.

**Figure 4.6:** Quantification of DCX immunoreactive cells. Shown are results from the lateral ventricle, SVZ and the infarct area of ipsi- (clear) and contra-lateral (shaded) sides. The number of DCX immunopositive cells were counted and collated from 2 levels closest to the infarct. (levels c and d). GH treatment did not have any significant effect on the number of DCX immunoreactive cells lining and near the lateral ventricle (A), dorsolateral SVZ (sub-ventricular zone, B) and the infarct penumbral area (C) on either the ipsi- or contra- lateral sides. DCX+ cells were evident in the contralateral LV & SVZ in all animals, but not in the infarct area. Inset photomicrographs are representative of all animals and depict the areas of analysis as shown in the histograms corresponding to the labels. A=vehicle, n=5 (clear bars); B=5µg/day, n=3; C=20µg/day, n=3; D=80µg/day, n=4. Bar denotes the mean±SEM for each group. The final n for the groups was: vehicle = 5, 5µg/day GH = 3, 20µg/day GH = 3, 80µg/day GH = 4
Stereotaxic injection of ET1 into the brain near the MCA causes a reversible decrease in CBF leading to the formation of a characteristic focal ischemic lesion with a highly injured ischemic core and corresponding penumbral region [62, 65]. This model of stroke has been extensively used and characterised [62, 63, 82, 735]. The damage elicited by ET1 infusion seen in this study is in agreement to that previously reported [62, 82], with damaged tissue readily discernible in acid fuschin-thionin stained sections collected at the end of the study (6 weeks later) (Fig 4.1a). The distribution of brain damage in general was similar to that reported previously [70, 82], with infarction in the lateral parts of the frontal cortex extending through temporal and into occipital cortex caudally and through the parietal and into the insular cortex rostrally. Although some striatal infarction was clearly visible in all animals (Fig 4.1a, figures c,d), some animals exhibited extended striatal damage, with one animal (5µg/day) having a clear infarct extending almost till the LV (not shown).

As mentioned in Chapter 3, a few difficulties were encountered during ET1 infusion with the needle blocking and potential discrepancies in the strain of the rats supplied for this study. It has been established that experimental stroke can vary between individual rats [709] and in this model of focal ischemic stroke, a minimum n of 7-8 was required to allow significant detection of a 50% reduction in the volume of ischemic brain damage [82]. Since the final n for this study was considerably smaller, it is very likely that the inherent technical difficulties and induced variability of injury together with a small n all significantly contributed to the overall variation in damage observed across animals. Given this, the accuracy of the results from the histological analysis, especially that suggesting 5µg/day GH significantly decreases tissue survival, is questionable. Previous work in this lab has elucidated a significant neuroprotective role for GH when administered following HI injury in the developing brain [19, 693] while several others have confirmed GH to be neuroprotective after ischemic brain injury [10, 558, 560]. Given this, and the fact that there was no similar significant effects at the other two doses of GH tested (Fig 4.1 b,c) it is unlikely that GH causes a decrease in tissue survival. Indeed, when examined individually, the animal with extensive striatal damage in the 5µg/day group had a much lower mean percentage of surviving tissue (across all levels) compared to all other animals in the study (62.69% compared to means ranging from 75.76- 99.09%). This discrepancy obviously would have led to considerable skewing of the data.Regardless, the small n of this study makes it difficult to definitively assign a role for GH in ameliorating tissue survival following adult stroke.
One of the primary aims of this study was to verify the placement of the cannula for the delivery of GH into the brain. To this effect, the study design allowed for determination of the neuroanatomical distribution of the chronically infused GH as the pumps were active at the time of euthanasia. This was an important endpoint considering recent clinical trials have highlighted targeting problems as a key issue determining the efficacy of CNS delivery of growth factors [736]. Immunohistochemical analysis revealed most of the infused GH appeared to localise to the side ipsilateral to the injury/infusion, clearly associating with cells within the dorsolateral SVZ and ependymal cells lining the ipsilateral side of the LV near the site of infusion (Fig 4.2 a, d, e). This binding of the infused GH to ependymal cells primarily on the injured side of the brain is likely due to this being the side of the GH infusion and relates to the known flow of CSF forward through the LVs and down the 3rd ventricle (Fig 4.2a, d and k). Accordingly, there is dense staining in the lining of the 3rd ventricle of the GH-treated groups that is absent in the vehicle group (Fig 4.2k, l and Fig 4.3f). GH+ staining lining the LV including the dorsolateral SVZ regions was most prominent with 20 and 80µg/day doses although some staining was visible at the lowest dose of GH (Fig 4.3a, b, e, i). This probably reflects the minimum dose of GH needed for binding to the ventricular ependymal cells. Along with the lateral lining of the LV and dorsolateral SVZ, GH binding appeared to be maximal in the penumbral regions of the infarct, particularly in the areas corresponding to the MCA territory where most of the damage is elicited in this model (Fig 4.3). These results suggest that the binding density of GH is enhanced in the injured as well as neurogenic regions in the injured brain. It is possible that the high level of GH binding in these regions could be due to the upregulation of GHR. Although not examined in the adult brain to date, GHR upregulation following ischemic injury has been reported in the juvenile brain, both in injured areas [18, 19] as well as the SVZ [14]. Although this upregulation in the juvenile brain is significant only at 5 days after injury [14], it is possible that the continuous infusion and circulation in the CSF of exogenous GH in this study is able to maintain constitutive GHR expression in these regions. While a majority of the CSF is generated in the choroid plexus and circulated around the ventricles and subarachnoid spaces [737], a small amount of it is also transported through the parenchyma along perivascular spaces and subcortical WMTs, comprising extra-cerebral fluid [738, 739]. The ipsilateral WMT and vascular staining likely reflects this convective flow of CSF.
from the ipsilateral LV and perivascular spaces (Fig 4.3h, Fig 4.4 e and f).[586, 740, 741].

The appearance GH+ cells within the SVZ, and co-localisation with lesion and penumbral vascular associated DCX+ cells after stroke in our model likely relates to its specific action on NSCs, which are known to be activated as a consequence of stroke [235, 236, 240, 342]. SVZ neurogenesis plays a key role in post-injury processes with neuroblasts [346] as well as GFAP-expressing cells [234] originating in the SVZ migrating out towards the ischemic boundary regions of the striatum and the cortex where they differentiate into mature neurons. Since GH+ staining is visible in cells lining the LV and within the dorsolateral SVZ (Fig 4.2a,d,e) and co-localises with DCX (Fig 4.4h) and GFAP (Fig 4.5c, d) in these and other (WMT) regions, it is probable that GH has specific actions on these cells. Indeed, recent studies have identified GHR to be expressed by a subset of NSCs within normal [278] as well as ischemic [14] rodent SVZ’s and that GH induces symmetrical cell divisions in these cells [278]. Furthermore, GH has also been shown to promote proliferation of NSCs in both adult [20] and fetal [14, 515] rodent brains. Further evidence for the direct actions of GH on NSCs is outlined in Chapter 6. It is pertinent to note that GH+ doesn't necessarily mean GH acts on these cells. Ideally, GHR staining would have enabled clarification of this. However, when examined closely (Fig 4.4) most of the GH+ staining looks cytoplasmic, possibly relating to exogenous GH that has been internalised post-receptor binding. This likely represents the infused GH rather than endogenously produced GH (Ref. Chapter 1) as, if endogenously produced, a significant amount of staining would have been visible in the vehicle brains (not seen here).

The strong association between GH, BVs and closely coupled populations of DCX+ cells likely reflects GH localising to a population of NSCs within the ‘vascular niche’. Several studies have now confirmed that stroke induces both angiogenesis and neurogenesis and that these processes are coupled [167, 230, 297, 400, 742-748]. Angiogenesis occurs in the ischemic hemisphere and peri-infarct area [368]{Chopp, 2007 #8031, 749, 750] and neuroblasts generated in the SVZ migrate to this ischemic boundary by closely associating with cerebral vessels [230, 748, 751]. This phenomena of newly generated cells associating closely with BVs has also been reported in the ischemic human brain [334, 335, 752]. Interestingly, the pericytes of capillaries and vascular adventitial cells of arterioles have also been shown to be a novel potential
source of neuronal progenitor cells in the postischemic primate hippocampus [753]. While the origin of the DCX+ cells lining striatal and cortical blood vessels in this study is not clear, it is evident from the GH-DCX co-localisation that GH has specific effects on these cells at higher doses (Fig 4.4j). It is plausible that the GH may have reached these vessels via the peri-vascular CSF flow paths described earlier [586, 741]. Together, this outlines GH following ipsilateral ventricular, WM and vascular CSF flow paths and localizing to the SVZ, lesion and vascular associated stem cells.

Given this localization of GH to DCX+ cells in various regions, it was somewhat disappointing to see no specific significant effect of GH-treatment on the number of DCX+ cells (Fig4.6). Increased neurogenesis and DCX expression in the brain following stroke has been widely reported in experimental animals [226, 229, 231, 236, 338] as well as the human brain [334, 335, 752]. Consistent with these reports, there was significant DCX expression both in the infarct (penumbral) regions as well as in known neurogenic regions (SVZ and hippocampus) (Fig4.6), even at 6 weeks after injury [748].

In summary, this study shows for the first time, that chronic GH treatment via ICV infusion localizes to the subventricular neurogenic region known to play an important role in stroke-induced neurogenesis (SVZ) as well as to the penumbral regions of the ischemia-induced infarct and to the neurovascular niche. Here, it co-localises with DCX expressing neuroblasts, and some GFAP-expressing cells, suggesting possible effects of GH on these cells. While a definitive role for GH in ameliorating tissue survival in this model of ischemic injury in the adult remains to be elucidated, the results suggest that a unilateral infusion of GH is sufficient to target it to regions of interest, namely the ischemic penumbral region, the neurovascular niche and the neurogenic LV/SVZ.
5 Delayed and chronic treatment with GH after stroke may be beneficial

5.1 INTRODUCTION

In the juvenile brain, post-injury GH upregulation is seen especially on neuronal and glial cells within injured regions as well as blood vessels and ventricular ependymal cells [19]. When infused into the injured adult ischemic brain, GH appeared to localise to these same cells both within and around the region of injury and neurogenesis (Chapter 4). Although not examined in the study conducted in Chapter 4, GHR upregulation (as reported in the juvenile model) coincides with this pattern of GH upregulation/binding; particularly with injury-induced neurogenesis [465] [14]. Considering there is strong association of GH/GHR with areas where neurogenic processes are known to take place [230, 324, 342, 343], combined with the fact that GH has been shown to mediate neurite outgrowth and maturation [21, 515, 552, 569], it is evident that GH may mediate long-term neurorestorative processes within the brain; Neurogenesis has been identified to mediate long-term recovery from injury (refer to section 1.2.7) while neurite outgrowth and dendritic arborisation have been long accepted to be mechanisms mediating brain plasticity such as that needed for recovery [10, 71, 207].

Seeing that GH appears to have effects on long-term neurorestorative processes, it was the aim of this study to examine the functional effects of long-term unilateral ICV GH treatment initiated 4 days after stroke. Information from previous studies conducted by this group [19] coupled with results from Chapter 4 showing GH targeting, it was determined that 20µg/day rGH might be an ideal dose. While all 3 doses exhibited similar endocrine responses, it was at this dose that considerable GH labelling was seen on the ipsilateral side without significant precipitation. The time delay was selected based on previous reports utilising the ET1 model of stroke, which have shown that the volume of cortical and striatal damage in this model is maximally developed by 3 days after ET1 induced occlusion [65, 82]. As such, this delay would ensure the elimination of any possible neuroprotection that could be elicited by the infused GH and the results would identify purely, the neurorestorative function of GH (if any). Functional benefits were measured using a battery of three sensorimotor tests throughout the study and one
cognitive function test performed at the end of the study. Endocrine measurements were also made along with histological measurements of the surviving brain tissue. It was hypothesized that delayed long-term GH treatment would produce significant improvement in sensorimotor as well as cognitive function in the absence of any obvious neuroprotective effects.

5.2 RESULTS

5.2.1 Delayed onset of GH-treatment does not provide any neuroprotection

One of the main aims of this study was to investigate the effects of GH on long-term neurorestoration. Since the majority of cell death processes continue to occur well after the primary insult but mostly resolve within days [91], GH treatment was commenced at 4 days after ET1 infusion/stroke induction. It was anticipated that this delayed treatment would eliminate any neuroprotective actions of GH [17, 19, 558, 560] and all effects seen would thus be the result of the neurorestorative actions of GH. To confirm this, acid fuchsin-thionin stained coronal sections at every 2mm were photomicrographed and the percentage of non-infarcted tissue on the ipsilateral side quantified and expressed as a percentage of that on the contralateral side.

The final n for the vehicle group was 7 and GH group was 10. As expected, delayed treatment with 20µg/day of GH did not alter the percentage of non-infarcted tissue on the ipsilateral side of the brain (Fig 5.1). Fig 5.1a shows a representative coronal section series from a GH-treated brain showing the extent of the infarct at different levels (b-f) as observed at post-mortem (12 weeks after stroke). As depicted in these images, the majority of the infarct area is visible at level d; accordingly, the percentage of non-infarcted tissue at this level is considerably lower at this level for both groups in agreement with the location of the ET1 infusion (Section 2.1.3) (Fig 5.1b). Similar to the dose response study, ET1-induced MCA occlusion caused ischemic injury to areas in the frontoparietal cortex and the dorsolateral striatum. Panel c shows the overall percentage of non-infarcted tissue for both treatment groups. There was no significant difference between the groups (86±2 for vehicle Vs. 87±2.1 for GH group).
5.2.2 Delayed and chronic treatment of GH after stroke may accelerate some aspects of functional recovery

Since neurorestoration was the primary focus of this study and functional recovery is one of the most important benchmarks and clinical endpoints of interest for neurorestoration [754], animals were tested for sensorimotor deficits elicited by stroke and the effect of GH treatment on these deficits. Baseline measures were established before stroke was induced. After ET1 surgery, sensorimotor deficits elicited by stroke were assessed by way of the forepaw asymmetry, forepaw inhibition and postural reflex tests and the animals were pair-matched based on their performance in the forepaw
asymmetry test (please refer to table 2.1, page 66). One of each pair was allocated to each treatment group (vehicle or 20µg/day GH) and all animals subsequently fitted with the appropriate pump. Behaviour was then assessed weekly till the end of the study.

Animals were lost from each of the groups due to adverse events (infection (1), severe stroke (1) and death during anaesthesia (1)) and results from these animals and their matched pair was excluded from all analyses. The motor function tests were analyzed in three phases; 1) pre-treatment phase including baseline measures (timepoints -2 and -1) prior to injury and testing between injury and commencement of treatment (timepoint 1), 2) treatment phase during the 6 week rGH infusion (timepoints 2-7) and 3) the post-treatment phase from removal of the pumps to post-mortem (timepoints 8-12).

There were no statistically significant differences between the groups in any of the pre-treatment measures in the sensorimotor tests (Fig 5.2). Within each group however, animals exhibited significant p<0.01) motor deficits after stroke induction in the pre-treatment phase. The forepaw asymmetry test (Fig. 5.2a) did not reveal any significant differences between the groups during any of the three phases. In the postural reflex (Fig 5.2b) test, although there was a statistically significant difference (p<0.05) between the two groups at 7 weeks post injury (timepoint labelled post 7 on the graph; the last behavioural measure taken before treatment was stopped), this was only transient. In these two tests, the animals did not appear to recover function over time. In the forepaw inhibition test however, recovery of the GH treated group was more immediate than the vehicle treated group (Fig 5.2c). Overall, GH treatment improved performance during the treatment phase (p = 0.006); post hoc analysis revealed a specific improvement during the early stage of this phase (p<0.001) for this test. No significant differences were observed between groups during the post-treatment phase.
Figure 5.2: Delayed and long term ICV GH treatment accelerates motor function recovery as measured by the forepaw inhibition test. Following baseline assessment of motor function (weeks -2 and -1), endothelin stroke was induced. All groups exhibited significant impairment in behaviour (p<0.01) following stroke induction, as seen at week 1 (where behavioural testing was carried out before commencement of GH-treatment). Animals were then pair-matched based on their function in the forepaw asymmetry test at week 1 and one of each pair assigned to either of the treatment groups. GH was administered at a dose of 20µg/day and effects of treatment on motor deficits assessed by various motor tests. While GH treatment had no significant effects in the forepaw asymmetry (a) and postural reflex (b) tests, GH-treated animals exhibited an accelerated recovery of forepaw inhibition (c) elicited by stroke. GH was infused for 6 weeks (treatment) and the animals assessed for a further 6 weeks (post-treatment). -1 and -2 represent baseline (pre-experimental) behavioural assessments. The dotted lines represent a normal (uninjured) response. *p<0.05, ***p<0.001. Shown are mean ± SEM. The final n for vehicle group = 7 and GH group = 10.
5.2.3 Delayed and chronic GH treatment improved spatial memory

In addition to effects on sensorimotor deficits, this study was designed to assess effects of GH-treatment on the cognitive function of rats subjected to stroke. Accordingly, all rats were subjected to a 4 day acquisition training period followed by a probe trial in the MWM test during the last week of the study. Previous studies suggest a strong influence of GH on hippocampal memory processes in both the rat [755] as well as human [522].

Behavioural analysis with the MWM showed an improved performance in the probe trial with the GH animals having a significantly higher number of entries into the platform zone (p<0.05, Fig 5.3a) as well as spending more time in the platform zone (p<0.05, Fig 5.3b). Although there was no significant difference in the latency to find the platform during the acquisition trials (Fig 5.3c), there was a considerable decrease in the latency to find the actual platform in GH-treated animals during the probe trial (80% of the GH group found the platform within 30s compared to 43% of the vehicle group) (not significant, Fig 5.3d). There was no difference in the total distance swum between groups during any part of the MWM (data not shown).
5.2.4 Delivered GH was bioactive for the duration of infusion

As mentioned in chapter 3, ensuring bioactivity of the infusate is an integral part of chronic treatments involving continuous infusions. Accordingly, blood samples acquired at various timepoints during the study were analysed and IGF1 and corticosterone concentrations assessed via RIA and HPLC-coupled mass spectrometry respectively. Both factors have been confirmed to be specific surrogate measures for GH activity in the dose response study (Chapter 3).

There were no significant differences between plasma IGF1 and corticosterone concentrations of the two treatment groups prior to treatment (baseline) (Fig 5.4). Following commencement of treatment, plasma IGF1 levels were significantly reduced (by 54%) during the GH infusion (486.69ng/mL in the vehicle group versus 264.68ng/mL in the GH group, p<0.05) and normalized once the pumps were removed (Fig 5.4a). Conversely, plasma corticosterone concentrations were increased during the GH infusion period (p<0.001) and normalised thereafter (Fig 5.4b).

Figure 5.3: Delayed and long-term ICV GH treatment improved spatial memory as assessed by the Morris water maze. Following 4 days of acquisition training with the platform in the pool, animals were tested in a probe trial where the platform was removed. In the probe trial, the number of entries into (a) and the mean time spent in (b) the platform zone were both significantly higher in the GH group compared to the vehicle group. Although there was no significant difference in the latency to find the platform between the two groups during the acquisition trials (c), there was an apparent decrease in the latency to find the platform in the GH group once the platform was removed during the probe trial but this was not significant. *p<0.05, **p<0.01. Shown are mean ± SEM. The final n for vehicle group = 7 and GH group = 10
5.2.5 GH treatment caused a transient increase in overall body weight but a decrease in spleen weight

Animals from both treatment groups were weighed once a week in order to ensure their well-being and to assess any possible treatment effects during the study. Major organs such as the brain, spleen, liver and heart were also removed and weighed at post-mortem to determine whether there were any systemic effects of central GH infusion.
As observed previously with the dose response study, GH treatment caused a significant increase in body weight during the treatment phase (p=0.002, Fig 5.5a) which normalized 2 weeks following the completion of infusion. At post-mortem, the mean spleen weight of the GH-treated animals was significantly lower (p<0.05, Fig 5.5b) whereas no significant changes were observed in regards to post-mortem liver, heart or brain weight (Fig 5.5b) or in the brain % body weight ratio (not shown).

Figure 5.5: GH treatment leads to a transient increase in body weight for the duration of infusion. Body weights were measured weekly throughout the study, while organ weights were measured at post-mortem to ascertain both the wellbeing as well as any possible treatment effects on the animals. GH-treated animals exhibited a significant but reversible increase in body weight during the treatment phase (a). In terms of organ weights however, the GH group exhibited a decrease in the mean spleen weight compared to the vehicle group, but no changes in the brain, liver or heart weights (b). All data represent Mean± SEM values, *p<0.05. The final n for vehicle group = 7 and GH group = 10
5.3 DISCUSSION

The present studies show that chronic and delayed central treatment of unilateral stroke with GH conveys some neurorestorative properties in the adult rat. Accelerated recovery in a specific subset of motor function parameters and improved spatial memory performance were seen in the long term, but with no effects on learning. Once again, a reduction in plasma IGF1 and an increase in corticosterone concentration were used as specific surrogate measures to confirm bioactivity of the infusate. An increase in body weight was also observed during the treatment phase which eventually reversed after cessation of GH infusion.

GH has been shown to be neuroprotective in various neuropathological paradigms [19, 558, 560, 756, 757] [552, 555, 695]. There is however, limited data on effects of GH on long-term neurorestoration, particularly after ischemic insults. As growing evidence now highlights significant roles for GH in modulating neurorestorative processes such as neurogenesis [14, 20, 22, 515, 552, 555], neurite/axon outgrowth (synaptic plasticity) [20, 21, 515, 552, 569, 758] and angiogenesis [529, 680] it was hypothesized that GH would have significant effects on neurorestoration following unilateral stroke. Accordingly, GH treatment was started 4 days after stroke in order to exclude neuroprotection. Since the volume of cortical and striatal damage in this model is maximally developed by 3 days after ET1 induced occlusion [65, 82], it is unlikely that there was any remodeling of the infarct area (either spontaneously or GH-induced) at or after 4 days. This was confirmed by histological analysis of the brain tissue, which showed no significant differences in the infarct volume between the two groups (Fig 5.1). In order to eliminate any possible discrepancies due to injury variation as seen in the dose response study (Chapter 3 and 4), animals were behaviourally tested after stroke but before GH treatment was commenced (Fig 5.2, week 1). This enabled the animals to be divided into pairs based on their deficits as assessed by their performance in the bilateral asymmetry test.

Examination of the animal deficits at week 1 revealed that the performance deficit elicited by stroke, at least in the bilateral asymmetry test ranged from 42-92%; i.e., the percentage of time injured animals used their contralateral limb (affected limb) ranged from 42-92, suggesting that the degree of injury remained considerably variable. Several aspects of surgery and post-surgical procedures were modified based on observations from the previous study; specially made blunt atraumatic needles were
used for ET1 infusion (to prevent discrepancies in the infusion site), thicker walled catheters were used to prevent elasticity (and thus catheter blockage) and all possible steps were taken to ensure that all rats were of the same strain. The n for each group was also larger. All these modifications considerably reduced the variability of the histological injury as observed after post-mortem. Thus, the behavioural deficit variability seen at week 1 most likely is attributable to the inherent variability of the model [82, 709].

Despite the delayed-onset of treatment, GH led to an acceleration of functional recovery in the forepaw inhibition test (p<0.001, Fig 5.2c). The forepaw inhibition test is widely used to assess unilateral stroke deficits and has been shown to be very sensitive to brain injury and resistant to recovery [665, 759]. Recovery of normal function in the vehicle group was seen at 4 weeks after stroke, the time at which post-lesion neuromorphological changes corresponding to functional recovery have been reported [71, 207]. In the GH group however, functional recovery was seen at just 2 weeks after stroke (one week after treatment) (Fig 5.2c). As GH has significant effects on neurite outgrowth and dendritic arborisation [20, 21, 515, 552, 569, 758] and functional recovery after stroke has been primarily attributed to morphological changes within both the ipsi as well as contralateral corticies [66, 72, 207, 218] (particularly in forelimb representation area [71]), this finding is of considerable significance. However, due to problems encountered with tissue processing, analysis of GH on the histological changes associated (if any) with treatment could not be undertaken. Given the effect observed in the forepaw inhibition test, it was surprising however, that no improvement was evident in the forepaw placement and postural reflex tests (Fig 5.2a, b). Interestingly, other groups, using the same model of unilateral stroke similarly failed to detect a functional recovery in the forepaw asymmetry test despite observing significant and complete recovery in other tests (skilled reaching and/or beam traversing) [71, 760] that corresponded to the neurological changes they observed [71]. The authors attributed this to differences in the nature of the test, with the other test(s) being ‘learned’ tasks with positive re-inforcement. It is possible a similar difference exists in this study; the chance to come out of water can be treated as a ‘reward’ for the forelimb inhibition test and the accelerated recovery effect seen with GH treatment is a result of a combination of factors. In this case, the ‘reward’ would provide motivation and the swimming would provide a form of training (such as that during rehabilitation) and this would ultimately lead to a natural recovery (accelerated recovery in the GH group) for
this particular task that is not obvious in either of the other two tests which examine spontaneous, unskilled limb use/movements (Fig 5.2). Alternatively, it is plausible that a process of suppressed movement or “learned nonuse” [761] is reason for part of the preferential limb use during postural support. That is, when an animal uses a single forelimb for extended periods (such as due to injury to the other forelimb), it may ‘learn’ to not use the other limb thereafter. Given that forced use of the affected forelimb is highly beneficial for functional recovery [405], it is likely that the ‘learned nonuse’ of the affected forelimb could restrict functional recovery. Ultimately, it may be that restoration of sensorimotor function such as that associated with the forelimb inhibition test [759] is much quicker compared to pure motor functions such as forepaw asymmetry given the extent of the neural networks involved [54]. It is likely that this also explains the results obtained with the postural reflex test [762]. In this study, the treatment effects seen with other tests were undetectable in the postural reflex test. It is also conceivable that since we observed behavioural improvements in light of the increased corticosterone concentrations, which is known to detrimentally affect behaviour [712, 713], the treatment effect of GH may be somewhat underrepresented.

It is interesting that despite a majority of the lateral frontal cortex was spared in this model of injury (Fig 5.1a) [760], significant motor deficits were seen in the forepaw asymmetry test (Fig 5.2a). The forepaw asymmetry test is considered a pure motor deficit sensitive primarily to M1 (the primary motor cortex) and secondarily to subcortical structure injury [54]. Thus, even if the M1 is largely intact, ischemia in subcortical territories does not retain the neurophysiological properties of the M1 due to damage to descending fibers which would essentially disrupt neural circuits linking it with the basal ganglia, brainstem, and spinal cord [54, 760].

The MWM test on the other hand, is considered to be a prototype for cognitive tests that is dependent almost entirely on hippocampal function [763]. In this study, GH-treated animals performed better in the MWM in terms of the number of entries into, the mean time spent in and the latency to the platform zone (Fig 5.3a, b, d). This finding is in agreement with reports showing that the GHR is widely expressed in the cerebrum and specifically in regions involved with learning and memory, including the DG of the hippocampus [19, 574]. Further, several lines of evidence now suggest a complex interplay between different regions of the brain to be responsible for spatial learning and in particular, MWM performance [764]. Both striatal [765] as well as cortical lesions
have previously been shown to elicit deficits in MWM performance and since GHR is expressed in both these regions [19, 457] it is possible that GH acts multimodally. That this effect was observed even 6 weeks after treatment was discontinued, suggests this effect was permanent.

Bioactivity of the infused GH was assessed, once more by measuring plasma IGF1 and corticosterone concentrations (Fig 5.4a and b respectively). As observed previously in the dose-response study, plasma concentrations of IGF1 fell significantly whereas those for corticosterone increased during the treatment phase, highlighting the exquisite sensitivity of the hypothalamic feedback system (as discussed earlier in Chapter 3), even in the event of an ischemic injury. The considerably higher corticosterone concentrations observed at the end of pump-3 in the GH-treated group could be an additive effect of GH infusion as a similar rise is seen in the dose-response study (Chapter 3).

As with the dose response study, GH treatment caused a significant increase in the body weights of animals during the treatment phase (Fig 5.5a). However, since this study was carried out for a further 6 weeks after completion of treatment, it appears that this GH-mediated increase in body weight is reversible. A similar increase in body weight post ICV GH administration was reported previously [767-769] and is suggested to be the result of an alteration in hypothalamic systems controlling satiety and orexic behaviour [768].

In addition to altering body weights, long term GH infusion also induced a significant decrease in spleen weight as measured at post-mortem (Fig 5.5). This is also seen in transgenic mice expressing a GH-R antagonist [770] and further confirms that central GH infusion significantly decreased circulating concentrations of GH as well as IGF1 as this effect is not seen when IGF1 signalling alone is reduced [771].

Overall, these findings support a significant role for GH in neurorestorative processes (including long-term cognition), and the general concept that chronic treatment with selected growth factors may have potential to accelerate functional recovery from stroke even after a delayed onset of treatment. Importantly, they also highlight the selective nature of the apparent functional benefits and the various behavioural tests, along with the importance of assessing the systemic impact of central treatment.
Acknowledgements for Chapters 3, 4 and 5

Mr Wing Leong and Dr. Arjan Scheepens were responsible for carrying out the skillful stereotaxic surgeries. Dr. Arjan Scheepens was also responsible for planning out the overall logistics of these studies, buffer formulation as well as providing support, advice and training with the scientific writing. Mr. Andrzej Surus was instrumental in executing the IGF1RIAs and GH RRA, Mrs Chris Keven in the SEC analyses and Mr. Eric Thorstensen for CORT Mass Spec and Urea analysis.

A paper on part of the results presented in Chapters 3, 4 and 5 was published in Behavioural Brain Research.

6 GH has proliferative and chemoattractive effects on NSCs in vitro

6.1 INTRODUCTION

Findings from Chapter 4 of this thesis support a role for GH in neurogenic processes. There is significant localisation of ICV infused GH to neuronal and glial progenitor cells in regions of active remodelling as well as neurogenesis (Chapter 4). This finding is supported by several other studies [14, 17, 22, 515, 569], which have shown GH to have significant trophic and neurogenic effects in vitro. However, other studies have largely employed human GH on rodent cells, making it difficult to separate the effects of GHR/PRLR signalling (hGH is lactogenic in the rodent [772]). Moreover, GH as well as GHR localise specifically to migrating neuroblasts (Chapter 4, [14]) and there are no studies which have studied the role of GH in regulating NSC migration.

The aim of this study was to test in vitro, the direct effects of exogenous GH on NSC activity. For this purpose, NSCs harvested from the human fetal forebrain were used [670], and exposed to rhGH (Genotropin®; [679]). The primary aim of this study was to elucidate the dose-dependent effects of exogenous GH on regulating NSC proliferation, differentiation and migration as well as its effects on the proliferation of neuronal and glial progenitor cells. The hypothesis for this study was that GH would promote proliferation and migration of NSCs, through signalling via GHR. A further hypothesis was that GH would also promote the proliferation of neuroblast and glial progenitors but, would decrease neuronal differentiation in accordance with previous findings [433].

6.2 RESULTS

6.2.1 Characterisation of hNSCs

The hNSCs used in this study were sourced from fetuses at Carnegie stages 19-22 and have been established to exhibit similarities to RG cells [670]. The doubling time for hNSCs cultured in the conditions listed in this thesis is longer than that for mouse NSCs [671, 773, 774] and as a result, all experiments with these cells require much longer time frames.
Proliferating hNSC cultures contain both small bipolar cells and more flattened apolar cells as shown in Fig 6.1a. It has previously been established that hNSCs isolated and cultured in conditions similar to those detailed here homogenously express markers for immature NSCs as well as RG [670]. Here, immunocytochemistry was used to confirm the undifferentiated NSC phenotype of these cells using the markers musashi (Fig 6.1b), nestin (Fig 6.1c) and Sox2 (Fig 6.1d).

Figure 6.1: hNSCs are grown in an adherent monolayer and propagated in EGF and bFGF. Expansion cultures contain both small bipolar cells and more flattened apolar cells (a). They express radial glial markers such as Musashi (b), Nestin (c) and Sox2 (d). Cells were counterstained with DAPI. Scale: 80µm.

6.2.2 Physiological potency of Genotropin®

Genotropin® is a recombinant form of hGH, expressed by *E. coli, strain K12* and widely used for GH replacement therapies. It consists of a single polypeptide chain containing 191 amino acids and two disulfide bonds (C_{53}-C_{165}; C_{182}-C_{189}) [679] with a molecular mass of 22,124 Da – representing the most abundant form of GH in humans [409]. Genotropin® for this study was kindly donated by Prof.Wayne Cutfield of the CRU, Liggins Institute, University of Auckland. Since this freeze-dried formulation also
contains various excipients, it was deemed necessary to determine the concentration (w/v) and integrity of rhGH in the resuspended solution prior to use.

The concentration of GH protein was noted to be approximately 2.2µg in 5µL of Genotropin® solution, as measured by a protein assay. Fig 6.2 shows results from an SDS-page gel-electrophoresis to evaluate solution integrity. The protein band for Genotropin® was seen at approximately 22kDa (Fig 6.2, Lane G), corresponding with that of the human pituitary-extracted GH (Fig 6.2, Lane hpGH). There was no obvious denaturation visible in Lane G (i.e. no bands of lower molecular weight such as those seen with the older hpGH), confirming the stability and integrity of Genotropin® solution.

![SDS-page gel-electrophoresis](image)

**Figure 6.2:** The physiological potency of Genotropin is equivalent to pituitary-derived hGH. 4µg each of Genotropin (G) and human pituitary-extracted GH (hpGH) were loaded per well. Proteins were separated by gel-electrophoresis. The standard shows the approximate molecular weights in kDa.

6.2.3 Basal expression of GHR, IGF1R and IGF1 but no GH or IGF2 in hNSCs

To establish whether cultured hNSCs were a valid system to evaluate the actions of GH, it was first confirmed that these cells expressed receptors for hGH. Fig 6.3 shows the identification of human GHR protein (panel a) and mRNA (b, lower image) by immunocytochemistry and RT-PCR respectively. No endogenous expression of hGH
mRNA was observed (panel b, upper image). β-actin was run as the house-keeping gene for all mRNA (including positive controls) used.

Short isoforms of hGHR resulting from alternative splicing in exons 3 and 9 have been reported [422, 775]. The expression of these isoforms can modulate the function of the full length GHR as well as regulate GHBP generation, subsequently altering GH signalling [422, 775]. Hence the expression of hGHR isoforms by hNSCs was verified. Using primers in exons 7 and 10 [422] and exons 2 and 5 [775] of the hGHR, RT-PCR revealed that only the full length isoform of the hGHR is expressed. The expected bands for exon 9 deletions in the GHR transcript were at 427bp and 383bp (lane 1 of Fig 6.3c), whereas that for the exon 3 deletion was at 317bp (lane 2 of Fig 6.3c). The bands seen in lane 1 (453bp) and lane 2 (383bp) were the bands expected for the full length isoforms of GHR. The ladder shown is a 100bp ladder.

Figure 6.3: hNSCs express hGHR but not hGH. Immunostaining and RT-PCR were used to confirm hGHR protein (a) and mRNA (b, lower image) expression respectively. Specific analysis using primers for various isoforms of the hGHR showed that hNSCs express only the full length form of hGHR (c) (lane 1, 453bp). They do not express any of the known shorter isoforms (Exon 9 deletion bands expected at 427 & 383bp in lane 1 and Exon 3 deletion band expected at 317bp in lane 2). Positive controls were: MDA-hGH (for description, see table 2.7) for GH and human astrocytes for GHR. β-actin bands for the GH and GHR +ve controls as well as hNS are also shown.
IGF1 is a major gene target of GH and mediates many of the actions of GH on growth and development [407] and there are several reports of significant levels of IGF1/IGF1R expression in neural progenitor cells [178]. IGF2, another member of the IGF family is also expressed in the brain where it likely modulates several IGF1 functions [776]. The expression human of IGF1, IGF2 and IGF1R by hNSCs was evaluated using RT-PCR, immunocytochemistry and ELISA (Fig 6.4). Immunocytochemistry was used to confirm IGF1R protein expression (Fig 6.4a) while RT-PCR was employed to confirm expression of IGF1 and IGF1R mRNA (Fig 6.4b). No IGF2 expression was noted (Fig 6.4b). The ELISA (enzyme-linked immunosorbent assay) method was used to quantify the levels of IGF1 expressed by hNSCs (Fig 6.4c). IGF1 protein content was measured in a) conditioned medium (CM) and b) medium from cells treated with BDNF (as negative control) or varying concentrations of GH. The average concentration of IGF1 in the CM was 0.12 ± 0.01 ng/mL. Interestingly, the mean concentration of IGF1 in both BDNF and GH treated cells was significantly lower than that in the CM (p<0.05, p<0.01, Fig 6.4c).
6.2.4 GH promotes the proliferation of hNSCs in the absence of EGF and bFGF

As GHR is expressed by NSCs (Fig 6.3, section 6.1.3) [433], and GH promotes the proliferation of embryonic [14, 515] and adult [20, 22] rat NSCs, we hypothesized that GH would promote the proliferation of NSCs from the human fetal CNS. To test this, hNSCs were incubated with GH at varying concentrations for 48h with BrdU and the proportion of positively labelled cells per well quantified (Fig 6.5a).

Figure 6.4: hNSC endogenously express IGF1. Immunocytochemistry (a) and RT-PCR (b) confirmed expression of IGF1 receptor protein and mRNA respectively. ELISA analysis (c) confirmed the presence of IGF1 protein in the conditioned medium (CM) from these cells. However, these levels dropped following exposure to BDNF or GH. β-actin bands for the IGF1 and IGF1R +ve controls as well as hNS are also shown. Values are mean ± SD. All significances are shown against CM except where indicated. Positive controls for the PCR were: primary cells from the human placenta for IGF1 and MCF7 for IGF1R. Values are mean ±SEM * p<0.05 **p<0.01
Treatment with rhGH increased the proportion of cells immunolabelled for BrdU by 1.2 – 1.4 fold (Fig 6.5a, p<0.001) over all the concentrations of GH tested as compared to medium alone with no growth factors added. Control mean ± SEM = 100 ± 4.3, and that for GH was 135 ± 2.9, 121 ± 2.5, 120 ± 3.3, 129 ± 4.6 and 130 ± 5.8 % for 0.5, 5, 50, 150 and 500ng/mL respectively.

6.2.5 GH promotes the proliferation of neuroblasts but not glial progenitors

NSCs as well as post-proliferative migratory neuroblasts (DCX+ cells) express GHRs [15] during neurogenic responses following injury. Consequently, it is possible that GH regulates NSCs at intermediate stages of differentiation (progenitors). Accordingly, the effects of GH on neural and glial progenitor proliferation were studied. First, hNSCs were induced to differentiate by plating them in a mixed medium for 24h. They were then cultured in varying concentrations of rhGH for 72h, and subsequently labelled with PCNA (to label proliferating cells) and S100β (for glial progenitors) or βIIIITubulin (for neuronal progenitors).

rhGH treatment of hNSCs lead to a robust increase in the proportion of PCNA+/βIIIItubulin+ cells relative to the total number of cells, especially at the lowest concentration (0.5ng/mL, p< 0.001; mean ± SEM: 674± 83) tested (Fig 6.5b, p<0.001) compared to the negative control (mixed medium only; mean ± SEM: 100±24.2). Even at 5, 50, 150 and 500ng/mL concentrations, GH exhibited significant induction of neuroblast proliferation (Fig 6.5b, p< 0.001 for 5, 50 and 150ng/mL and p ≤ 0.01 for 500ng/mL; mean ± SEM: 370±71, 366±42, 399±29, 360±62 respectively). The mean±SEM for positive control (BDNF) was 300±55 (p<0.05); n=15 & 16 for negative and positive controls respectively and n=15 for all GH concentrations.

In contrast, GH had no significant effect on the proportion of PCNA+/S100β+ cells relative to the total number of cells at any of the concentrations tested (Fig 6.5c) There was a slight trend towards reduced glial progenitor proliferation at the lowest concentration of GH (0.5ng/mL) although this was not significant (Fig 6.5c). The mean±SEM for the negative control was 100±12.5 and for the positive control (fetal calf serum, FCS) was 169±15.3 (p<0.001); n=13 & 12 for negative and positive controls respectively and n=15 for all GH concentrations.
6.2.6 GH promotes the maturation of neurons but inhibits neurogenesis.

It has been previously reported that GH enhances neurogenesis and gliogenesis in embryonic NSCs but inhibits neurogenesis in NSCs from neonatal [515] and adult [21] mice. There are also several reports of the effects of GH on neuronal morphology, as an indication of maturation [515, 552, 569]. Here, a preliminary investigation was carried out to delineate the effects of GH on the differentiation of human fetal NSCs. hNSCs were cultured for over 3 weeks in the presence or absence of rhGH, at the end of which time, they were labelled with anti-βIII tubulin antibodies. Brain BDNF served as a positive control for neuronal differentiation. In replicate #1, βIII tubulin+ (positive) neurites were counted per field of view at 20x magnification. In replicate #2, the total
number of βIIITubulin\(^+\) cells with neuronal processes per well were counted at 10x magnification.

The differentiation assay was a very lengthy assay, with the minimum time required for completion being over 6-8 weeks. There were several complications encountered with regard to staining and long-term adhesion that prevented successful replication of the assay within existing time constraints. Given this, the assay was successfully carried out only twice, and since different parameters were measured both times, statistical analysis on this data is not possible. Preliminary results from both these replicates are depicted in Fig 6.6.

Fig 6.6a shows the effects of GH on the number of βIIITubulin\(^+\) neurites. A trend towards an increase in the number of neurites was seen at 5ng/mL GH (intra assay mean±SEM: 0.74±0.07). As the concentration of GH increased however, there was a trend towards a decrease in the number of positive neurites GH (Fig 6.6a, 6 wells for negative control, 5ng/mL, 100ng/mL; 5 wells for 50ng/mL; 4 wells for BDNF (positive control), 10ng/mL, 500ng/mL; 2 wells for 1000ng/mL. Intra assay mean ± SEM for negative and positive controls were 0.33±0.03 and 0.40±0.02 respectively.
There was a considerable increase in the number of βIII tubulin⁺ cells at 1 ng/mL GH (Intra assay mean ± SEM: 10.66±0.72; Fig 6.6b, 3 wells for all concentrations). At all other concentrations tested however, there was a trend for GH treatment to suppress the proportion of βIII tubulin⁺ cells. Intra assay mean ± SEM for negative and positive controls were 8.82±1.05 and 13.45±1.64 respectively.

Figure 6.6: Preliminary results show rhGH has considerable effects on the neuronal differentiation of hNSCs. There is a trend towards an increase in the number of βIII tubulin neurites at 5 ng/mL rhGH (a) (6 wells for negative control, 5 ng/mL, 100 ng/mL; 5 wells for 50 ng/mL; 4 wells for positive control, 10 ng/mL, 500 ng/mL; 2 wells for 1000 ng/mL). In terms of the number of βIII tubulin positive cells, a slight increase in the number of positive cells is evident at 1 ng/mL (b). (3 wells for all concentrations) Negative control = differentiation medium without growth factors; Positive control = Brain derived neurotrophic factor (10 ng/mL). Each assay was only carried out once and hence, statistical analysis was not possible.
6.2.7 GH promotes the migration of hNSCs

In vivo studies showed that GH delivered ICV to rats subjected to ischemia localises to migratory neuroblasts (Chapter 4) [777] while other studies report significant post-injury upregulation of GHR specifically on migratory neuroblasts [14]. These observations postulate a role for GH in the migration of NSCs. To test this, hNSCs were plated in inserts placed in transwell plates coated with either protein control (BSA at 10ng/mL) or rhGH (0.5, 5, 50, 150 and 500ng/mL). The total number of cells that haptotactically migrated into the bottom chamber were stained with haematoxylin and counted. All results are representative of at least 2 independent assays run in duplicate or triplicate.

GH increased the migration of hNSCs at various concentrations compared to control as shown in Fig 6.7a (p<0.001, 2 replicates). There was a significant increase in the number of migrated cells at the 0.5, 50 and 150ng/mL concentrations (p<0.001, p<0.05 and p<0.01 respectively), with the 0.5ng/mL hGH showing the maximum increase in migration. Intriguingly, there was no significant effect on migration at 5 and 500ng/mL (Fig 6.7a).

In order to test the specificity of the effects, a specific GHR antagonist, B2036 (GHRA) was used to antagonize the GHR (Fig 6.7b). B2036 is generated by introducing a single point mutation within the region of GH that binds site 2 of GHR (which confers antagonism) along with 8 other amino acid substitutions in site 1 (which increase GHR binding while abolishing PRLR binding) [778] [779]. Surprisingly, co-treatment with GH (300ng/mL) and GHRA (50, 500, 1500ng/mL, 10 & 20µg/mL, 2 replicates) did not reduce GH-induced migration at any of the concentrations of antagonist tested (Fig 6.7b). There was however, a significant increase in migration when GH and GHRA were administered simultaneously (p<0.01 and p<0.001 respectively, for 500 and 1500ng/mL GHRA).

hGH also binds to the PRLR [780, 781]. Hence, it was postulated that hGH might be signaling via the PRLR to induce hNSC migration. To investigate this possibility, Del1-9-G129R-hPRL, a pure PRLR antagonist (PRLRA) [677] was employed. As for the GHRA, the PRLRA was devoid of any significant effect when used alone (Fig 6.7c). In
contrast, migration of hNSCs induced by 300ng/mL GH (p<0.001) was significantly inhibited by adding 10-fold molar excess (3000 ng/mL) of the PRLRA (p<0.001, 2 replicates). Interestingly, adding both GHR and PRLR antagonists together prevented this inhibition of GH-induced migration (Fig 6.7c, p<0.001). GH-induced migration of hNSCs was also inhibited by the addition of 5µM AG490, a potent inhibitor of JAK2; a GHR/PRLR signaling pathway effector (Fig 6.7d, p<0.001; 2 replicates). There was significant reduction in the number of migrated cells in both the control and GH-treated wells when AG490 was added at 10 µM (p<0.001), suggesting it was cytotoxic at this dose.
DISCUSSION

The GH axis has been identified to have significant roles in post-injury processes [14, 18, 19, 25, 777], where part or most of it can be attributed to its ability to regulate neurogenic processes in the brain [10]. Using NSCs derived from human fetal
forebrains, it was confirmed that undifferentiated hNSCs express receptors for both hGH as well as hIGF1 protein but not rhGH protein. Exogenously added GH promoted the proliferation of undifferentiated hNSCs as well as neuronal progenitors, along with increasing neurite outgrowth in differentiating neurons. In contrast to several previous findings, it was noted that GH potentially has the capability to promote the differentiation of hNSCs into neurons, at least at physiological doses, although this is subject to further verification. Finally, a significant chemoattractive role for GH was also distinguished, and that this results largely from signaling via the PRLR. These studies contribute to, as well as further knowledge gathered from previous findings of the role of the GH/IGF1 axis in regulating neurogenesis in the CNS.

The clonal cell line of NSCs used in this study was derived from human fetal forebrains and can be propagated for prolonged periods (at least a year, up to around 50 passages) in vitro without any genetic manipulation [670]. When grown as an adherent monolayer in vitro, cultures contain both small bipolar cells as well as more flattened apolar cells (Fig 6.1a). These cells exhibit many characteristics of RG cells [670] and provide a good model system for pharmaceutical screening. Along with fundamental NSC markers such as Nestin, Sox2 ([670], Fig 6.1 c,d) and Musashi (Fig 6.1b) they express genes for GHR, IGF1R and IGF1 (Figs 6.3 & 6.4). This is consistent with previous findings that both GH and IGF1 have significant roles during brain development [10, 458, 574, 782] and their receptors are expressed in neurogenic regions as well as in progenitors isolated from neurogenic regions in the brain [14, 20, 433, 460, 783-785].

IGF1 plays fundamental role in growth & development and is expressed in a highly regulated manner during brain development [782]. IGF1 expression is seen as early as the embryonic stage [515, 786] and increases greatly during fetal development, peaking during the first 2 postnatal weeks, especially in neurons and glial progenitors [786-789]. Along with lineage-restricted progenitors, IGF1 is also expressed in NSCs [577, 790] and this expression increases with GH treatment [515]. In this study, surprisingly, there was a decrease in the amount of IGF1 protein secreted into the medium (as measured by ELISA, Fig 6.4c) with GH treatment. Although IGFBP3 was not measured in this study, Ajo et al [515] identified a dose-dependent increase in IGFBP3 protein in medium from cell cultures grown in presence of GH for 2 days. In human circulation, IGFBP3 carries almost 80% of the circulating IGF1 [791], is regulated mainly by GH [792], and is mostly saturated [793-795] thus presenting itself as an important modulator of IGF1
actions. Since the ELISA system used in this study discourages pre-treatment to remove binding of protein-bound hormone in culture medium, it is possible that ELISA quantification did not include the entire amount of IGF1 produced in response to GH. Alternatively, since IGF1 in the CM was measured only at 3 or 6 days after GH addition, it is possible that negative regulators of GH/IGF1 signalling such as the suppressors of cytokine signaling (SOCS2) might have come into play, preventing IGF1 induction (See Section 1.4.1.2). Consequently, a more acute GH stimulation (24-48h) might lead to increased IGF1 secretion [515].

IGF2 is a close relative of IGF1 and is thought to have some of the same growth-promoting actions of IGF1 during fetal life [796, 797]. In rats, IGF2 mRNA is expressed in various regions of the brain (See Chapter 1) from E13-E18 and levels gradually decline with maturation [798, 799]. However, evidence pertaining to its expression in cells of neuroepithelial origin, particularly in the human fetal brain is controversial [800-802]. RG arise early in development from neuroepithelial cells [803], and current analysis suggests IGF2 is not expressed by hNSCs (Fig 4b).

Interestingly, no endogenous expression of GH was observed (Fig 6.3). Previous in vitro studies by the Turnley group identified neurospheres generated from both newborn [433] as well as adult [20] rodent brains to endogenously produce GH. Moreover, these studies also observed positive GH immunostaining in the cells of the ventricular zone (VZ) of the E14 mouse cortex [433]. In addition, immunoassayable GH has also been reported in fetal rat [437] as well as human [440] brain extracts. The VZ of the developing fetal brain has been shown to house both RG as well as some intermediate progenitor cells [306, 804], while neurospheres have been determined to contain GF-responsive stem as well as progenitor cells [805]. Given that hNSCs used in this study were specifically selected to exclude any progenitor cells [670], it is possible that progenitor cells, rather than RG express GH. Also, since in vivo studies reporting GH immunoreactivity in the brain report on findings from whole brain extracts, it is difficult to ascertain the exact origin of this GH (See Chapter 1, section 1.4.1.3).

GH, acting both directly and indirectly (via IGF1) has been widely recognized as a pro-proliferative factor for stem cells from various organs [806-808] including the CNS [14, 20, 22, 278, 515, 569, 809]. Consistent with these reports, the present study shows significant effects of GH on the proliferation of fetal NSCs as well as neuronal
progenitor cells (Fig 6.5a, b; P<0.001). Treatment with rhGH (at all concentrations) for 48h significantly increased the number of BrdU immunopositive cells relative to the total cell number by at least 1.2 fold (p<0.001) with no obvious dose-dependent (bell-shaped) effect as reported previously with rat (r) NSCs [515] (Fig 6.5a). Given that hNSCs have a significantly longer doubling time compared to rodent NSCs [670], it is possible a similar effect would be evident with a longer assay (i.e. 72h of treatment). Indeed, the aforementioned study does report a similar lack of a dose-dependent effect on cell proliferation at 24h [515]. Alternatively, an inhibitory effect of GH might have been obvious at much higher concentrations of rhGH [569]. The difference in doubling time of NSCs between the two species also explains the difference in the percentage of proliferation induced by GH [14, 515]. Both investigations noted a minimum 2.4 fold increase in the number of BrdU labelled cells as opposed to the minimum 1.2 fold increase observed in this study. A longer treatment with subsequent BrdU labeling might render similar effects. Although the effect of GH on the survival of NSCs and progenitors was not assessed here, previous reports suggest significant neuroprotective effects of GH on NSCs [518, 549, 555] at lower concentrations [17]. If this were true here, a similar increase in numbers would be expected for glial progenitor cell proliferation, at least at the lower concentrations of 0.5 and 5ng/mL but this was not observed in this study (Fig 6.5c). Furthermore, GH treatment has been shown to increase the fraction of cells in the G1 phase of cell cycle [569] and neurospheres from GHR knockout mice appeared to proliferate less [20, 278]. These findings further support a direct role for GH in proliferation of NSCs. The maximal effect was observed at the concentration of 0.5ng/mL of GH, which corresponds to the reported cerebrospinal fluid (CSF) concentrations of GH in adult human subjects [449, 810]. Notably this concentration also corresponded to a slight decrease in the number of proliferating glial (PCNA+/S100β+) progenitors (Fig 5c, not significant). Since the maximal effect of GH on neuronal (PCNA+/βIIIITubulin+) progenitors was at 0.5ng/mL, it is possible that there is some degree of active inhibition of glial differentiation at this concentration.

Given the the proliferative effects of GH on neuronal progenitors, it was postulated that GH would influence long-term neuronal differentiation/maturation of NSCs. To investigate this, neuronal differentiation was first induced in hNSCs by EGF withdrawal [670] and they were then cultured in the presence of GH for over 3 weeks. Preliminary results support previous findings of GH inducing morphological changes in neuron-
committed progenitors [515] as well as neuronal hybrid cells [569]. At doses close to physiological concentrations reported in CSF [449, 810], there was an increase in both βIIIItubulin+ neurites (Fig 6a, P<0.001) as well as βIIIItubulin+ cells (Fig 6.6b, not significant) as compared to negative controls. This is consistent with the findings of Byts et al, who have shown an increase in neurite initiation and arborization of embryonic hippocampal neurons with GH treatment at physiological concentrations [552]. In this study, at higher concentrations however, GH appeared to reduce both neurite outgrowth as well as neuronal differentiation (Fig 6, not significant). While an inhibitory effect of GH on neuronal differentiation has been previously reported [433, 515], its effects on neurite outgrowth as seen here, particularly at higher concentrations remain to be further validated as they do not reflect previous findings [515, 552]. The small number of wells examined in this study, particularly at higher concentrations (2 wells at 1000ng/L), probably accounts for this discrepancy. Alternatively, the decrease in neuronal differentiation has been reported earlier and could be a result of increased glial differentiation [433, 515] or the result of apoptosis in the hNSCs. The potential increase in neuronal differentiation at a more physiological concentration of GH (Fig 6.6b) however, could be a novel finding that further supports previous findings that GH has differential effects on differentiation based on dose as well as cell-type [515, 569].

When applied to hNSCs in a Boyden chamber assay, GH exhibited significant chemoattractive properties, at almost all the concentrations tested (Fig 6.7), although the highest effect was seen once again, at the concentration closest to physiological concentrations of GH in the CSF. A direct chemoattractive role GH has been reported previously in precursor cells from the thymus [811] as well as carcinoma cells [812], although not in cells of neural origin. There is also a significant GH/GHR colocalisation with migrating neuronal precursors in the recovering ischemic brain (Chapter 4), [777] [14] as well as an increase in migration of GH-induced NSC progeny [809]. Results from this in vitro study complement these findings and demonstrate for the first time, a direct role for GH in regulating NSC migration. Interestingly, this GH-induced migration appeared to be mediated by signaling via the PRLR [780, 781], as indicated by reduced migration following antagonism of the PRLR (Fig 7c) but not with GHR at the concentrations tested (Fig 6.7d). Significant antagonism by B2036 (the GHRA used in this study) of GH-mediated effects has already been reported at 10-fold higher concentrations of B2036 [813], and effective competition between exogenously added B2036 and hGH is seen at a 1:1 ratio [814]; phenomena that were not evident in our
study (Fig 6.7b). It is known that hGH has lactogenic properties [780, 781] and given that GH-induced migration is reduced with the addition of the PRLRA (Fig 6.7c), it appears that at least in hNSCs, PRLR signalling is involved in mediating the chemoattractive properties of GH. However, since only a higher concentration of GH (300ng/mL, Fig 6.7c) was tested in this study, it is unclear if the involvement of PRLR is GH concentration-dependent. This PRLR-mediated migration of NSCs appears to be a result of signaling via the JAK-STAT pathway (Fig 6.7d) and other studies have shown involvement of Ras-induced PI3K signaling downstream of this hNSC migration [815]. True inhibition of the JAK/STAT pathway (specifically Stat5b, which is the main downstream effector of GHR/PRLR signaling [408, 602] is seen at 5µM of AG490 (Fig 6.7d), consistent with previous findings in studies utilising NSCs [288, 816]. At 10µM, it is likely that AG490 was cytotoxic (Fig 6.7d) [288] as there was a significant reduction in migration in both control as well as treatment wells.

An increase in migration was observed when GH and GHRA were added simultaneously (Fig 6.7b, c). It is possible that when GH and GHRA are added together, B2036 antagonizes the receptor, preventing GH from binding it. This could result in binding of GH to the PRLR, leading to an additive effect of GH and GHRA on migration, since addition of GH with PRLRA prevents GH-induced migration. Furthermore, the addition of GH with GHRA and PRLRA might cause GH to have access only to the PRLR, thus increasing the ‘effective’ concentration of GH that the PRLRA would have to compete against and potentially contribute to reduced antagonism of PRLRA (Fig 6.7c). Another possibility is that the increase in migration with the addition of GH and GHRA together is caused by the relatively ‘low’ concentrations of GHRA ‘aiding’ GH signaling since the GHRA a) has a lower affinity for the receptor surface, and b) can also downregulate the negative effect of GHBP by partially displacing GH from GHBP [813]. However, as GHBP was not measured in this study, the likelihood of this remains purely speculative. PRL is capable of inducing NSC migration [27] (Chapter 7) and these results, together with those in Chapter 7, indicate this to be a result of signaling via the PRLR.

Given the design and results from this study, it was not possible to elucidate the extent of IGF1 involvement in mediating GH-induced effects. The study by Ajo et al [515] showed complete reversal of GH-induced proliferation and differentiation by incubation with IGF1 antibodies and several groups have highlighted its importance in
proliferation, maintenance and differentiation of NSCs [580, 582, 784, 785, 817-820] while others have identified IGF1 to increase neuronal progenitor proliferation [776, 817, 821, 822] and promote neurite outgrowth [823, 824]. While it is tempting to assume a role for IGF1, several lines of evidence suggest some IGF1-independent effects for GH. Firstly, as the proliferation assay was carried out in the presence of insulin, N2 and B27 as supplements, the insulin concentration in the medium was in excess to that required to activate IGFRs [825-827]. Secondly, at least two reports have shown neurosphere proliferation to be dependent on GHR signaling as proliferation is significantly decreased in neurospheres from GHR knockout mice [20, 278]. Thirdly, in terms of differentiation, it is interesting to note that IGF1 has been shown to promote neuronal [557, 580, 784, 828] and oligodendroglial differentiation [304, 305, 829] with no effect on astrocyte numbers [580] whereas GH has been shown to increase astrocyte numbers [433]. This would suggest that the effects of GH on NSC differentiation are at least partly independent of IGF1 actions (Fig 6.5). And lastly, since GH signals via the PRLR to promote NSC migration (Fig 6.7), the involvement of IGF1 in mediating the chemoattractive properties of GH at least in hNSCs is unlikely.

In conclusion, this study demonstrates for the first time, the effects of rhGH on NSCs isolated from the human fetal brain and in turn supports previous findings from in vitro work on NSCs derived from rodents. These results further highlight the importance of GH in regulating neurogenic events during brain development and subsequently in developmental processes recapitulated post-injury at other ages. GH-mediated effects in the hNSCs at this developmental age are in accordance with the sequential occurrence of neurogenic events during development, as shown by its pro-proliferative effects on undifferentiated NSCs and neuronal progenitors but a possible slightly suppressive effect on glial progenitor proliferation at concentrations closest to physiological ranges. A role for GH in regulating NSC migration is also implicated, and that it occurs as a result of GH signaling via the PRLR thus highlighting the importance of cross-signalling mechanisms.
7 PRL also has proliferative and chemoattractive effects on NSCs in vitro

7.1 INTRODUCTION

PRL is an anterior pituitary hormone belonging to the same protein family as GH [408]. As such, GH and PRL are closely related in terms of structure and signalling mechanisms (See section 1.5.1.1) and previous work in this lab has identified the PRL axis to have notable presence following HI injury in the juvenile brain [16, 25]. Most importantly, post-injury responses of the PRL axis are related to that of the GH axis, in that upregulation of the PRL axis occurs in the neurogenic regions, and also on proliferating and migrating neuroblasts [16, 25, 589]. These findings, combined with those from Chapters 4 and 6 defining a role for GH in mediating neurogenic processes, potentiate a role for PRL in regulating NSC activity. Although its role in promoting NSC proliferation has been investigated to some extent [27], there is a paucity of data demonstrating a) its effects on neuronal and glial progenitor cells and b) the direct chemotactic properties of PRL. Given GH appears to regulate the proliferation of neuronal and glial progenitors differently, it is possible PRL does the same. Its effects on NSC migration on the hand, are of particular importance considering GH appears to signal via the PRLR to promote NSC migration (Chapter 6, Fig 6.7).

Thus, the primary aim of this study was to examine in vitro, a dose-dependent effect of rhPRL in regulating human fetal NSC and neuronal and glial progenitor proliferation and hNSC migration. It was hypothesized that PRL, in a manner similar to that of GH, would have significant positive effects on NSC proliferation and migration.

7.2 RESULTS

7.2.1 hNSCs predominately express full length PRLR, with weak expression of the intermediate form

The expression of both PRL as well as PRLR protein has been reported in the brain, in both pituitary and non-pituitary sites [591]. Although there is some data on PRLR expression in neurogenic regions [25, 27], few studies have examined expression of PRL protein. Here, the expression of hPRLR and hPRL by hNSCs was examined. It
was found that hNSCs express the full length hPRLR. Fig 7.1 shows identification of PRLR protein (panel a) and mRNA (b) by immunocytochemistry and RT-PCR respectively. No endogenous expression of hPRL mRNA was observed (panel b, upper image).

Based on the variation in the intracellular domain, several splice variants of the PRLR have been reported that can differentially modulate PRL signaling [7, 601, 830]. Using primers from these studies, it was determined that hNSCs predominantly express only the full length hPRLR (Fig 7.1c, band at 856bp). There was also weak expression of the intermediate form (band at 284bp) but no expression of any of the shorter isoforms generated from alternate splicing of Exon 11 as shown by Trott et al [601].

![Figure 7.1: hNSCs express hPRLR but not hPRL. Shown are protein (a) and mRNA (b) expression of hPRLR. No endogenous expression of hPRL mRNA was observed (b). While hNSCs strongly express the full length form of hPRLR, they also weakly express the intermediate (int) form of hPRLR (expected band is at 284bp) (c).](image)

### 7.2.2 rhPRL promotes the proliferation of hNSCs in the absence of EGF and bFGF

Studies by Shingo et al have identified a) strong expression of PRLR in the SVZ and b) PRL to be responsible for pregnancy-induced neurogenesis [27]. Given this, it was hypothesized that rhPRL would promote the proliferation of hNSCs. To test this hypothesis, hNSCs were incubated with varying concentrations of rhPRL and BrdU for 72h. The number of BrdU^+ cells were counted and expressed as a proportion of the total number of cells.
rhPRL treatment promoted the proliferation of hNSCs as determined by BrdU immunolabelling by at least 25% in the absence of EGF or bFGF (Fig 7.2a, p<0.001 for 0.5, 5, 500ng/mL and p<0.01 for 50 and 150ng/mL, 2 replicates). This effect was most robust at the lowest concentration of PRL tested in this study (0.5ng/mL). The mean±SEM for the control was 100 ± 18.72 while that for PRL at 0.5, 5, 50, 150, 500ng/mL was 143 ± 3.22, 135 ± 6.3, 122 ± 4.45, 122 ± 3.88, 132 ± 6.022 respectively.

7.2.3 rhPRL promotes the proliferation of neuroblasts and glial progenitors

Recent work has identified a trophic and pro-proliferative role for PRL on glia following injury [25]. Also, given that GH had significant effects on neuroblast and glial progenitor proliferation, it was hypothesized that closely-related PRL would also have similar effects. Differentiating hNSCs were incubated with varying concentrations of rhPRL for 72h, labelled with PCNA (marker for proliferating cells) and counter-labelled with markers for neuroblast (DCX) or glial (S100β) progenitors.

Figure 7.2b shows the strong inductive role PRL has on neuroblast proliferation at all concentrations (p<0.001). This effect is highest at the lowest concentration of PRL used in this study (0.5mg/mL, mean%±SEM: 698±84 vs 100±26.69 of control). Thereafter, PRL exhibits a dose-dependent increase in promoting neuroblast proliferation. The mean±SEM for 5, 50, 150 and 500ng/mL of PRL respectively are as follows: 416±31.30, 483±49.68, 563±53.42, 727±106.5 %; 2 replicates were carried out for all concentrations except at 500ng/mL.

In contrast to neuroblast proliferation, PRL exhibits a classic cytokine response (bell-shaped curve) for glial progenitor proliferation (Fig 7.2c), with no positive effect at the lower and higher concentrations but a significant pro-proliferative effect at 5 and 50ng/mL (p<0.05 and 0.01 respectively). Mean±SEM for control, 5 and 50ng/mL PRL respectively are as follows: 100±16.68, 187±20.25 and 259±38.09 %.
7.2.4 PRL can inhibit or promote migration of hNSCs

Since GH promotes haplotactic migration of hNSCs, and it appears to do so by signalling via the PRLR (Chapter 6), it is highly likely that closely-related PRL would also have significant chemoattractive properties. To evaluate this, boyden chamber migration assays was carried out using rhPRL at 0.5, 5, 50, 150 and 500ng/mL concentrations.

Interestingly, rhPRL produced a J-shaped dose response curve for hNSC migration (Fig 7.3a, 3 replicates). At lower concentrations (0.5 and 5ng/mL; p<0.05 and 0.01 respectively) rhPRL significantly inhibited, while at higher concentrations (150 and 500ng/mL, p<0.05 and 0.001 respectively) rhPRL significantly promoted, the migration of hNSCs. There was no significant effect at 50ng/mL. Fig 7.3b shows the results of
antagonism of the hPRLR using various concentrations of the highly specific PRLRA [677]. There was complete inhibition of PRL-induced migration at a 10-fold higher concentration of the PRLRA as compared to the PRL ligand (Fig 7.3b, p<0.001 and 0.001 for 10-fold and 15-fold PRLRA respectively, 3 replicates). To assess the specificity of the PRLRA, cells were exposed to denatured (boiled) antagonist. Denaturing the antagonist restored PRL-induced migration (Fig 7.3c, p<0.05 when comparing the denatured antagonist to intact antagonist, 2 replicates).

Thus, rhPRL, acting via its own receptor, can both inhibit or promote the migration of hNSCs at lower or higher concentrations respectively.

Figure 7.3: PRL can suppress or induce migration of hNSCs. At lower, closer to the physiological range of concentrations found in CSF, PRL inhibits the migration of hNSCs whereas at higher concentrations, it induces migration (a). This PRL-induced migration of hNSCs at higher concentration is diminished by the addition of a PRLR specific antagonist PRLRA at a 10-fold excess concentration. Denaturing the antagonist significantly reduces the inhibition of migration (c). Clear bars represent control wells and grey bars represent PRL-treated wells. Shading represents wells with antagonist. Control = 10ng/mL BSA, PRLRA = PRLR antagonist, hPRL = recombinant human prolactin, boiled = denatured PRLRA. In (c), PRLRA concentration was 1500ng/mL. All significances are shown against control except where indicated. Values are mean ± SEM. * p<0.05 **p<0.01 ***p<0.001. Data shown is from 2-3 replicates run in duplicate or triplicate analysed using a two-way ANOVA.
7.3 DISCUSSION

Prolactin is a 23kDa member of the cytokine receptor superfamily that is closely related to GH based on structural homology, overlapping biological properties [593]. Recently, a role for PRL in regulating cell genesis in the adult [27, 644, 645] and juvenile [16, 25] CNS has been elucidated. Results from our present study contribute to and extend these findings, and specify a direct role for PRL in regulating important developmental neurogenic processes. Like rhGH (See Chapter 6), rhPRL also promotes the proliferation of hNSCs. It also has significant proliferative effects on neuronal and glial progenitors, the latter effect being in contrast to that seen with GH. Of note is the observation that PRL elicits a bell-shaped dose response curve for glial progenitor proliferation but not for hNSC or neuronal progenitor proliferation. Another significant finding was PRL induced a J-shaped dose response curve for hNSC migration, by inhibiting migration at lower, more physiological concentrations while promoting it at higher concentrations. PRL-induced migration of hNSCs appears to be a PRL-receptor mediated mechanism, as suggested by the complete inhibition of migration seen with PRLR antagonism.

Although PRL is mainly synthesized in the pituitary [594, 831] several reports have now established PRL-like immunoreactivity in several extra-pituitary sites in the adult brain including the hypothalamus, cerebellum, thalamus, brain stem, hippocampus, cerebral cortex and caudate [592, 608, 610, 832-835]. There is however, very little and somewhat conflicting information regarding PRL expression in the fetal and neonatal brain. In the hypothalamus, PRL mRNA was detected only on postnatal days 1 through 8 but not prior to birth [836] but PRL imunoreactive cells were detected in hypothalamic cultures prepared from embryonic rats [837] and both PRL protein and mRNA expression is seen in the hypothalamus and amygdala of fetal sheep [838]. Results from the present study suggest that PRL is not expressed in NSCs isolated from the 12-14 week old human fetus (Fig7.1). There is however, significant expression of both the full-length as well as weak expression of the intermediate forms of PRLR, as detected by RT-PCR. This is consistent with previous findings that PRLR mRNA is expressed in the fetal rat brain [623, 839] and also in the fetal human brain as early as D52 of gestation (~13 GW) [840]. The intermediate form of PRLR differs from the full-length PRLR in that it lacks part of the intracytoplasmic domain, rendering it slightly variable in physiological functions compared to the full length isoform [600]. Although this isoform has a similar affinity for PRL, it can be deficient in its abilities to stimulate
cellular proliferation in the presence of PRL and also exhibits some signaling disparities compared to the longer isoform [600].

The presence of PRL and its receptor in the brain suggests that the PRL axis is of considerable importance in the brain. Recently, PRL has been identified as a regulator of brain cell proliferation, with significant proliferative effects on a variety of CNS cells [26, 27, 643, 644, 841, 842]. Here, exogenous PRL administration for 48h elicited a significant increase in hNSC proliferation as quantified by BrdU labelling (Fig 7.2a, p<0.001). At least three independent groups have reported an increase in SVZ neurogenesis in response to PRL [27, 645, 646] in vivo, but in vitro studies suggest PRL alone is not sufficient to induce proliferation of adult NSCs [27]. NSCs exhibit age-related differences in their response to growth factors [20, 433, 515, 843] and it is possible that as the hNSCs employed in this study were derived from fetal brains as opposed to adult brains, they exhibit different responses. Recent results by T.Gorba in our laboratory identified that PRL (without any additional growth factor) increases the proliferation of E15 mouse-derived NSCs in a dose-dependent manner [16], while it was demonstrated earlier in Chapter 6 that closely related GH was also sufficient to enhance the proliferation of hNSCs. Furthermore, since both GH as well as PRL employ a similar signaling pathway of JAK/STAT activation [605], and activation of this pathway is associated with increased proliferation [844], it is possible that PRL is indeed self-sufficient in inducing hNSC proliferation, at least at or around the gestational age they were derived in this study. Alternatively, it is possible that PRL functions as a co-activator; it has been shown to temporally increase the expression of various growth-associated cytokines such as tumor necrosis factor (TNF-α), interleukin1α, and tumor growth factor [845] which in turn, could synergise with PRL in promoting proliferation of cultured astrocytes [841]. As previously observed with GH (Chapter 6), 48h of treatment with PRL was not sufficient to induce a bell-shaped dose-response in NSCs – this effect might have been more obvious with 72h of treatment [16] (Fig 2a).

PRL levels in the human CSF range from 0.6-4.7ng/mL, with a mean of about 1.3ng/mL [846, 847]. Maximal proliferative effects of PRL on both NSCs as well as neuronal progenitors were evident at a concentration closest to this (0.5ng/mL), although similar effects were also seen at the highest concentration of 500ng/mL (Fig 7.2a,b). Interestingly, it was at these concentrations that PRL exhibited no significant
effects on glial progenitor proliferation (Fig 7.2c). From 5ng/mL, PRL elicited a dose-dependent increase in neuronal progenitor proliferation till the maximal concentration it was tested at; 500ng/mL (Fig 7.2b). The gestational age at which these hNSCs were derived correlates with the period of corticogenesis when neurogenesis is occurring rapidly [306, 803, 848] with cell intrinsic factors [849] as well as extrinsic signals from the changing environment [255, 850] mediating it. The fact that PRL is detected in the human fetal pituitary gland by as early as 10 weeks of gestation and that its concentration rises throughout gestation into the post-partum period [851], combined with its obvious effects on NSC, neuronal and glial progenitor proliferation suggest a role for endogenous PRL in regulating neuro- and glio-genesis during cortical development.

Given the important regulatory role PRL has on NSC, neuronal and glial progenitor proliferations, it was not surprising to observe that it was able to regulate NSC migration as well (Fig 7.3). Interestingly however, PRL appeared to inhibit migration at lower, more physiological concentrations (0.5 and 5 ng/mL) but promote migration at higher concentrations (150 and 500 ng/mL) (Fig 7.3). NSC migration is a complex process tightly regulated by a number of factors, especially during development, to ultimately generate the complex cytoarchitecture that is generated. Temporal and spatial regulation of these factors is necessary for normal development. Accordingly, inhibition of cellular migration when inappropriate is vital and at lower concentrations PRL is possibly one such factor. For example, GABA (the primary inhibitory neurotransmitter in the adult CNS) inhibits neuroblast migration to regulate the number of neurons produced [852] while FILIP (Filamin A interacting protein), is expressed in the neuroepithelium adjacent to the ventricles to contribute to the neuroepithelial layer structural integrity by preventing premature migration of cortical neurons [853]. Even reelin, a glycoprotein secreted by the Cajal-Retzius cells of the marginal zone of the developing cortex, inhibits neuronal migration, acting as a stop signal for radially migrating neurons to regulate the laminar patterning of the cortex [313, 854, 855]. Interestingly however, in the cerebellum it has been suggested to act as a chemoattractant for migrating Purkinje cells [856]. At higher concentrations, possibly such as those seen after injury [16, 25] or with increasing developmental age [851], PRL promotes migration. This positive effect of PRL on mediating NSC migration (at a higher concentration, 400ng/mL) has been demonstrated earlier using fetal mouse NSCs in vitro [16]. As hNSCs do not endogenously produce PRL (Fig 7.1), but detectable
levels of PRL are evident in the hypothalamus and pituitary [837, 838, 851] as well as fetal serum [851], it seems likely that some of this PRL is transported via the CSF to the ventricular/ependymal zone containing NSCs. Several findings support this; 1) in the growing brain, the properties of the choroid plexus (CP) epithelial barrier are largely similar to those of the typical adult blood-CSF barrier [857] and PRL can be transported via receptor uptake across the CP in adults [496], 2) functional PRLRs are expressed by the fetal CP [623, 858] and 3) in the fetal brain, CP-CSF fluid prominently provides nutrients/GFs to fetal neural tissue [859, 860].

It is apparent from results presented here that PRL, like GH, acts via the PRLR to elicit its effects on migration (Fig 7.3b). Antagonising the PRLR at a 10-fold molar excess of PRL in a competition assay demonstrated significant reduction in the PRL-induced effect with a dose-dependent decrease in migration (although not-significant) evident with increasing concentrations of the PRLR antagonist (Fig 7.3b). This has been confirmed in earlier characterisation studies of this particular antagonist, Del1-9-G129R-hPRL [677]. Denaturing the antagonist restored PRL-induced migration of NSCs (Fig 7.3c), confirming the significance of PRLR signaling in NSC migration. This PRLR-mediated migration of NSCs appears to be a result of signaling via the JAK-STAT pathway as observed in Chapter 6 and other studies have shown involvement of Ras-induced PI3K signaling downstream of this [815].

Taken together, these results support an important regulatory role for PRL in mediating crucial developmental (and thus, subsequent post-injury recovery) processes in the brain. Although a role for PRL in NSC regulation has been suggested earlier, results from this study, for the first time, show the direct effects of PRL on hNSCs. To what extent PRL is involved in the differentiation of hNSCs remains to be elucidated.

Acknowledgements for Chapters 6 and 7
Work in this chapter was carried out with extensive intellectual assistance, practical training, financial and materialistic support from Dr.Thorsten Gorba. Drs Arjan Scheepens and Dr.Mhoyra Fraser provided intellectual assistance while Dr. Mhoyra Fraser also provided advice on scientific writing and editing along with supervisory support. Dr.Chris Triggs (Dept of Statistics) kindly provided statistical advice. Finally, Dr.Vincent Goffin also provided invaluable suggestions towards the work and its final presentation.
8 General Discussion

8.1 Overview

Ischemic brain injury is one of the leading causes of death and disability worldwide. It can occur at all ages and when not detrimental, can lead to a significant reduction in the quality of life for patients and caregivers alike. Currently, there is an urgent need for treatment modalities that can be implemented well after the onset of the primary insult. Scientifically this is possible, as a significant amount of the injury occurs/worsens over at least a few days after the onset of the insult which can be prevented or attenuated. It is during this time that there is a considerable but insufficient endogenous repair response to brain injury which, if augmented, may promote recovery, at least in the functional sense (the primary interest of all patients). These endogenous repair mechanisms initiated in response to injury act to promote recovery of the injured regions by enhancing the function of existing neuronal circuitry and/or enhancing birth of new neurons that can compensate for neuronal loss, thus restoring function. Since GFs are agents that can affect both these mechanisms of recovery, the use of GFs to treat brain injuries has received considerable interest over the years.

GH is one such factor that has reputed important roles both during development as well as in post-injury processes in the brain [10]. Previous research by our group had identified a key role for neural GH in post-injury processes, some of which were protective [18, 19] while others promoted recovery [14, 17, 19] after HI injury in the juvenile rat brain. It appeared from these studies that the GH axis is not only activated rapidly after injury but also co-localises with stem and progenitor cells which are part of the post-injury neurogenic mechanisms [14, 18, 19]. Several other lines of research also supported this by illustrating GH to have some role in neuro- and gliogenic processes [21, 22, 433, 515, 571] including neurite outgrowth (necessary for neuronal plasticity mechanisms) [20, 21, 433, 515, 516], as well as angiogenesis [18, 19, 535, 861]. Furthermore, IGF1, the downstream mediator of most GH effects has also been extensively characterised and promoted as a neuroprotective as well as neurotrophic agent in both juvenile as well as adult rats [565, 567, 862, 863]. Given all of these processes augment post-ischemic recovery in the brain, these findings strongly support a role for GH in long-term neurorestoration. To date however, there are no reports on
the long-term neurorestorative effects of GH treatment following ischemia in the adult brain. Further, data regarding the direct effects of exogenous GH application on NSC proliferation and differentiation is limited while that on their migration is non-existent.

Along with GH, closely related PRL has been implicated in similar post-injury mechanisms [16, 25, 589], including neurogenesis [16, 25, 27]. In the brain, there is significant interaction of these two hormones as they have highly similar signalling pathways and hGH can also signal via the hPRLR [589]. However unlike GH, PRL is not neuroprotective [25] and is involved more in glial rather than neuronal responses [16, 25] implying effects distinctly different to that of GH. Presently however, there is a distinct paucity of evidence on the role of PRL in regulating NSC activity. This potentiated interest in PRL and its neurogenic effects and whether it was possible to delineate the effects of PRL from those of GH in neurogenic processes considering their convergence in signalling pathways.

Consequently, in vivo studies were carried out to a) develop, characterise and employ an ICV, long-term, continuous GH infusion starting immediately after stroke in adult rats, b) execute a dose-response to such a treatment, followed by elucidation of the cell types the infused GH would localise to, c) to test the neurorestorative potential of long-term GH treatment of a focal ischemic insult in adult rats, especially when treatment was initiated after a delay. Furthermore, studies to characterise the direct effects of GH on NSC proliferation, differentiation and migration were conducted in vitro to elucidate a possible mechanism of GH-induced neuroprotective and neurorestorative effects. These in vitro analyses were also extended towards PRL, to examine the effects of PRL on NSC proliferation and migration in order to identify similarities and differences between the two hormones in these processes.

8.2 MAJOR FINDINGS

The major findings from each of the studies have been analysed and discussed in detail in each respective chapter. Key findings are reviewed below. Ultimately, the focus of this chapter is to provide an overall description of the thesis and discuss how the findings complement or conflict each other. Limitations of these studies will also be deliberated upon and finally, future directions arising from these findings will be offered.
8.2.1 Central infusion of GH post ischemia in the adult brain; Behavioural and endocrine effects

Treatment with GH starting immediately after stroke, at any of the doses tested, did not provide any significant amelioration in sensorimotor deficits. However, considering the small n and discrepancies in severity of injury amongst the treatment groups, the effect of GH treatment on functional recovery and neuroprotection in this particular treatment paradigm remains to be clarified. Nevertheless, this study addressed several important issues associated with long-term GF infusions in the brain. Firstly, it presents a successful buffer formulation that can be used for future long-term infusion studies employing rGH in particular. Secondly, it identifies reliable, extremely specific surrogate measures that can be employed for ICV GH studies; plasma and CSF concentrations of IGF1 and plasma corticosterone. Thirdly, it highlights several important surgical and post-operative issues that require to be addressed in future studies for successful elucidation of treatment effects.

8.2.2 Central infusion of GH post–ischemia in the adult brain; region and cell-specific targeting of infused GH

One of the primary issues of long-term GF infusions in the brain is targeted delivery to the site of injury. This study identified the histological outcomes of this treatment paradigm. Although there was no significant effect of GH treatment on augmenting tissue survival at any dose, immunohistochemistry revealed that the unilaterally infused GH localized to penumbral areas of injury as well as to the SVZ, where neurogenesis is known to occur following stroke. Moreover, GH co-localised with markers for neural progenitor and glial cells, suggesting involvement in neurogenic (and possibly angiogenic) processes occurring as an endogenous response to brain injury. While a definitive role for GH in ameliorating tissue survival in this model of ischemic injury in the adult remains to be elucidated, it is apparent from these results that a unilateral infusion of GH is sufficient to target it to regions of interest, namely, the ischemic penumbral region, the neurovascular niche and the neurogenic SVZ.

8.2.3 Delayed and chronic treatment with GH after stroke may be beneficial

This study addressed the question of whether long-term treatment with GH is beneficial if treatment is initiated after a delay. That is, whether GH has the capacity to promote
neurorestoration without any obvious neuroprotection. The lack of neuroprotection was confirmed via histological measurements. GH treatment significantly accelerated recovery in one out of the three sensorimotor tests carried out, and improved spatial memory as assessed using the MWM test. GH infusion also caused a significant increase in body weight during the treatment phase, which eventually reversed after cessation of treatment. Findings from this study support a considerable beneficial role for GH in the acceleration of functional recovery and long-term cognition following focal ischemia. Further, they highlight the selective nature of the apparent functional benefits and the various behavioural tests, along with the importance of assessing the systemic impact of central treatment.

8.2.4 GH has proliferative and chemoattractive effects on NSCs in vitro

Using NSCs derived from human fetal forebrains, in vitro analyses were carried out to study the direct effect of GH on NSC activity. These hNSCs express various markers of RG cells as well as hIGF1 protein. Exogenously added rhGH promoted the proliferation of these hNSCs as well as of neuronal progenitors, along with (possibly) increasing neurite outgrowth in differentiating neurons. Preliminary differentiation analyses suggested that contrary to previous reports, GH promotes neuronal differentiation at least at physiological doses. It is also demonstrated for the first time, that GH can promote NSC migration, and that this effect is elicited by GH signalling via the PRLR. Consequently, this study highlights the importance of GH in regulating neurogenic events during brain development and subsequently in developmental processes recapitulated post-injury at other ages.

8.2.5 PRL has proliferative and chemoattractive effects on NSCs in vitro

PRL belongs to the same family of hormones as GH and it has been shown previously that the PRL axis is also responsive following HI. It was the aim of this study to identify the specific role of PRL in regulating NSC activity. This in vitro study identified that like GH, PRL also promotes the proliferation of NSCs and neuronal progenitor cells. However, unlike GH, PRL also promoted glial progenitor proliferation, at least at some concentrations. Interestingly, the chemoattractive properties of PRL appeared to be two-fold. While at lower, more physiological concentrations PRL inhibited migration, at higher concentrations, it significantly augmented it. rhPRL-induced migration of hNSCs
was blocked by a PRLR antagonist, thus confirming it to be a PRLR-mediated mechanism. These results support an important regulatory role for PRL too in mediating crucial developmental (and thus, subsequent post-injury recovery) processes in the brain. Although a role for PRL in NSC regulation has been suggested earlier, results from this study, for the first time, show the direct effects of rhPRL on hNSCs.

8.3 IMPLICATIONS

This thesis aimed to further knowledge about GH, its potential use for the treatment of ischemic brain injury and its direct role in the neurogenic processes that are important for recovery from brain injury. Since most of these processes are a recapitulation of developmental processes, these findings also have significance in elucidation of GH’s role in brain development.

8.3.1 ET1 model of stroke and ICV infusion of GH

In order to study the effects of a continuous, long-term infusion of GH on outcome after focal ischemic stroke, adult rats were subjected to ischemic stroke. Here, the ET1 model was chosen since it offers various advantages over several other focal ischemic stroke models (see Chapter 1), especially in providing an excellent representation of embolic strokes reported in the clinic [864] [65] [84]. The sustained reductions in CBF, combined with the gradual development of the ischemic lesion are not only highly representative of human clinical stroke but also make this an ideal model to study delayed treatment strategies [65]. Much of the large inherent variability of this model is a consequence of the variability in vessel location and size; the MCA can vary slightly from animal to animal and hence accurate placement of ET1 adjacent to the MCA can be difficult [84]. This presumably explains the continued variability of injury that was observed in the delayed treatment study, despite significantly rectifying the issues identified in the dose response study. Further, it is possible that this variability contributed to the variation in functional recovery seen in the sensorimotor tests (Chapter 5). In fact, studies by Windle and colleagues [84] comparing the various methods of ET1 induced stroke, suggest that the stereotactic infusion of ET1 proximal to the MCA is the least successful in terms of eliciting functional deficits. Although results from the dose response study were variable and relatively inconclusive due to the small final n (chapter 3), the damage and functional deficit patterns seen in the delayed
treatment study (larger n, chapter 5) suggest that there was a reasonable consistency of damage across all groups in this study compared to the dose response study. Further, since animals were behaviourally tested and pair-matched based on their functional deficits, the variability was reasonably accounted for.

As mentioned in chapter one, targeted delivery of GFs are fundamental to treatment especially in the brain, since several GFs have largely local effects especially when administered in the brain. From the in vivo study described in chapters 4 and 5, it was apparent that a unilateral, ICV delivery of GH ipsilateral to the injury is beneficial in terms of targeted GF delivery following a focal ischemic stroke. Using osmotic pumps was an important factor in these studies. GFs have been shown to be rapidly cleared following intraventricular infusions, in as little as 12 min [865] and using a continuous infusion paradigm in this study eliminated the possibility of rapid clearance before significant uptake/distribution. The endocrine responses in terms of plasma and CSF IGF1 concentrations as well as plasma corticosterone and urea concentrations all confirm the continuous bioactivity of the infused GH (chapters 3 and 4), while immunohistochemical analyses confirmed GH was well-distributed within the brain tissue. GH+ cells were visible in various regions outside of, but in addition to, the known convective CSF flow paths (Chapter 4) [741] such as around the penumbra of the infarct and other cortical regions in the GH-treated animals (Figs 4.2 and 4.3, chapter 4). Since these regions have been established to be regions that can be potentially restored after stroke [866] and are primary areas of cortical remodelling [203, 204, 867] and the target of new neurons generated in response to stroke [236, 324, 346], this finding suggests that the unilateral, continuous GH infusion paradigm was successful in terms of targeting specific areas of interest in this model.

In summary, despite the large inherent variability, the ET1 model provides a valuable model of focal ischemic stroke. A long-term continuous infusion paradigm using osmotic pumps is also a very valuable tool for studying the longer-term effects of GF treatment. Importantly however, consideration must be given to ensure variables are carefully accounted for, surrogate measures are established to confirm the bioactivity of the infusate and the ultimate targets of the infused GF elucidated.
8.3.2 Effects of GH on neurogenic processes

The considerable co-localisation of GH and DCX especially in the penumbral region of the infarct (Fig 4.4, Chapter 4) suggested GH might have regulatory effects on these cells. Consequently, in vitro studies were carried out using NSCs from the human fetal brain (Chapter 6). Findings from the in vitro investigations support in vivo findings; GH has significant pro-proliferative effects on neuronal progenitors (Fig 6.5b). It was somewhat surprising given this, that there were no significant effects of GH treatment on DCX expressing cells in vivo (Fig 4.6, Chapter 4). There are several possible explanations for this. Firstly, it could be due to the difference in the antigens labelled; while DCX (a marker for late mitotic neuronal precursors and early post mitotic migrating neurons [868, 869]) expression was analysed for the in vivo studies, βIII-tubulin (marker for new immature postmitotic neurons [870]) was used to label neuronal progenitors in vitro. Although there is some overlap between the expression of these two markers, they can also label distinct stages of neuronal maturation [871]. As GH has differential effects on neural stem and progenitor cells based on dose and cell-type [515, 569], it is possible that the proliferative capacity of GH is restricted to a certain stage of development of the neuronal progenitor cell. Alternatively, it could be due to discrepancies in neuronal progenitor marker expression at different stages of CNS maturation [872]. As the in vitro assays were carried out using NSCs and subsequent progenitors from the fetal brain (Chapter 6) while the in vivo studies were in adults, the differential effects of GH observed could be a result of either differential marker expression [872, 873] and/or a difference in the effects of GH [20, 515] based on developmental age. For example, while exogenous GH appears to significantly increase the number of βIIIItubulin expressing neuronal progenitors from the fetal brain (Fig 6.5b), it has no effects on the same from the adult brain [20]. Further, at least in cells from the adult brain, GH appears to increase NSC numbers at the expense of their differentiation [20]. It is possible that this is a mechanism for stem cell quiescence and therefore confers protection of the stem cell niche [874]. Additionally, given that GH appears to promote neurite outgrowth and maturation (Chapter 6) [507, 515, 516, 574], and DCX expression in neuronal progenitors decreases with neuronal maturation [869], another possibility is that GH treatment causes an increase in neuronal maturation rather than an increase in neurogenesis [433, 574]. If so, we would expect an increase in the expression of mature neuronal markers (such as NeuN, MAP2, etc., rather than DCX).
with such a GH treatment. However, as this was not assessed in this study, this possibility remains purely speculative.

Colocalisation of GH with GFAP-expressing cells lining the LV, WMTs and the penumbral region of the infarct in our study provides further evidence for GH involvement in post-ischemic neurogenic processes (Fig 4.5, Chapter 4). As mentioned in Chapter 1, type B cells of the SVZ are astrocyte-like NSCs [250], and previous studies indicate that the most immature neurons in peri-infarct tissue are derived from GFAP-expressing NSC in the SVZ [230, 234]. After cortical ischemia these cells migrate out along the uninjured WMTs to reach areas of damage (the penumbral regions) [230]. In this study, immunohistochemical analyses revealed considerable co-localisation of GH and GFAP in all of these regions (Fig 4.5, Chapter 4) while *in vitro* findings reveal that exogenous GH has robust pro-proliferative and migratory effects on NSCs (Fig 6.5a, 6.7a; chapter 6); both findings supporting a role for GH in promoting stroke-induced proliferation and migration of NSCs. Moreover, recent reports from rodent studies have also indicated that GH is involved in promoting SVZ NSC proliferation [14, 278] and that there is a rapid upregulation of GHR in the SVZ after hypoxic-ischemic injury [14].

Additional evidence for a role for GH in post-ischemic neurogenesis comes from the presence of GH/DCX double-labelled cells along the lining of the blood vessels (Fig 4.4a,b,c). Several studies have now established that after stroke, neuroblasts migrate out to the peri-infarct areas in close association with blood vessels [230, 748]. Importantly, these neuroblasts have been noted to localise specifically to BVs in areas of active vascular remodelling in and along the border of the infarct [230]. Findings from this study are in agreement with these reports since GH/DCX colocalisation was evident in BVs of the striatum and the cortical areas close to the infarct (Fig 4.4 a, b, c). That the delivered GH is visible in these neuroblasts, once again, probably relates to the convective flow path of the CSF which is known to be aided by the pulsation of the cerebral arteries [739]. It is possible that GH binds to these neuroblasts and promotes their migration and/or maturation especially in the peri-infarct areas (Chapter 6).

Peri-infarct areas exhibiting active vascular remodelling consist of BVs with newborn endothelial cells [230]. These cells not only secrete several GFs or chemokines (discussed in chapter 1) that possibly mediate the localisation of neuroblasts in the peri-
infarct cortex [230, 360, 747, 875, 876] but also increase NSC proliferation and promote neuronal differentiation [747]. Since there was significant co-localisation of GH/DCX in the peri-infarct areas and both GH and IGF1 have been reported to stimulate endothelial cell proliferation, tube formation and angiogenesis in many tissues [680, 877] and the age-related decline in GH and IGF1 levels has been linked to a decrease in cerebral microvascular density [535], it is indeed possible that ICV delivered GH may additionally play a role in promoting post-stroke angiogenesis [748]. Previous findings that GHRs are rapidly up-regulated in the endothelia of BVs within a few hours after ischemic injury [18] support this. Although BV numbers were not examined in this study, previous studies have shown long-term (28 days) GH treatment to increase microvascular density [535]. Since increased angiogenesis has been shown to be beneficial for functional recovery following stroke [324, 878], this could be yet another beneficial effect of GH treatment following stroke. Since we observed accelerated recovery of function in one of the motor tests in the delayed treatment study (Chapter 5), it is possible that GH-mediated angiogenesis may potentially be involved. Of course, GH may also aid in the proliferation of these BV-associated neuroblasts (Chapter 6), thus augmenting neurogenesis that occurs following stroke [324].

In summary, effects of GH on neurogenic processes appear to be largely mitogenic and chemoattractive. When given ICV following ischemia, GH localises to differentiating and migrating neuronal progenitors in areas established to contain NSCs as well as those with active remodelling. Exogenous GH application in vitro promotes the proliferation and migration of NSCs. These results strongly support a role for GH in promoting NSC and neuronal progenitor cell migration post-injury. Its effects on neuronal and glial progenitor cells however, may be age-dependent; while it promotes the proliferation of neuronal progenitors from the fetal brain, it does not appear to have a significant effect on those from the adult ischemic brain. GH however, does appear to promote the maturation of neuronal progenitor cells, as is evident from its effects on neurite outgrowth. Finally, a possible role for GH in injury-related angiogenesis also cannot be discounted.

### 8.3.3 Effects of GH on functional recovery following stroke

Given GH has a considerable effect on neurogenic processes, it is reasonable to assume it would be beneficial for long-term recovery. As hypothesised, GH treatment appeared to accelerate recovery of some sensorimotor function (Chapter 5; Fig 5.2c) even when
started 4 days after stroke. Commencing treatment after such a delay ensured no apparent neuroprotection as reported earlier with GH [19] and subsequently, any effects observed presumably are the result of GH effects on long-term neurorestoration. Interestingly however, there was a difference in the recovery profile observed in forepaw inhibition test from that in the forepaw asymmetry and postural reflex tests, (Chapter 5, Fig 5.2) with GH treatment accelerating recovery only in the former test. As discussed in Chapter 5, several studies have reported similar discrepancies in functional outcome when measured using a battery of tests and have been attributed to the difference in the nature of the test [71, 760]. As the forelimb inhibition test involves a form of motivation (to come out of the water) along with learning (swimming towards the platform), it might be comparable, at least partly to other ‘learned’ tasks such as skilled reaching and beam traversing [71] that have been shown to be highly sensitive to treatment interventions, more than the forepaw asymmetry test [71, 760]. Alternatively, spontaneous, ‘non-skilled’ use tests such as forelimb asymmetry and postural reflex could reflect motor recovery resulting from adaptive compensatory strategies that are not necessarily comprised of the original posture or forelimb movement patterns [879]. While these factors still applied to the dose response study (Chapter 3), the small final n and variability in severity of injury encountered confounded identification of possible neuroprotective effects of GH [19, 548, 552, 558, 560, 756] and consequently, its effects on sensorimotor function in this treatment paradigm. Equally possible was that the use of ET1 may have resulted in under-representation of GH-induced neuroprotection. ET1 has been shown to be expressed by astrocytes in response to injury, is strongly associated with injury-induced gliosis [880] and ET1 receptor antagonists are neuroprotective [88, 880]. Given this, the use of ET1 to create ischemia may have exacerbated the injury and hence led to an under-representation of possible neuroprotection by GH in this treatment paradigm.

WM injury plays a significant role in behavioural recovery/performance. It is now known that myelin damage begins in the core at 1 day and progressively increases for at least 7 days [881, 882]. WM has been shown to be specifically susceptible to inflammatory mediators and inflammation after stroke can cause WM damage at times and in locations with no obvious loss of neurons [881, 883]. Given that WM fiber pathways form the brain's communication network and its physical condition influences how effectively information is transmitted and behaviour is performed [193], [195] at least part if not most of the behavioural deficits that manifest are a result of WM damage.
In fact, measures of WM microstructure have been suggested to be useful predictors of responses to therapeutic interventions [885, 886]. As such, it is possible that WM damage caused in this model has contributed to at least some of the behavioural deficits. Recently, a clinical study reported that remodeling of WM may occur in regions distant to the lesion after stroke and correlates with improved recovery [887]. Relating this to animal studies, a re-routing of premotor cortex axons was seen, going around the lesioned area to innervate the somatosensory cortex which projects directly to the spinal cord possibly playing a role in functional recovery [210]. It is possible that some of these mechanisms came into play in these studies, enabling recovery in the forepaw inhibition test.

In terms of spatial memory function, GH treatment elicited a long-lasting improvement that was detectable even 6 weeks after treatment was discontinued (Chapter 5). Several previous studies have highlighted the important role of the GH axis in regulating cognitive function (See Chapter 1), showing that GH-deficiency confers memory and learning impairments [888] [889] that can be significantly improved with long-term GH treatment [890, 891]. In rats of 3 months of age, exogenous administration of GH or GH releasing hormone has been reported to improve long-term memory [521]. Since GH increases neurogenesis (Chapter 6, [14, 20, 22, 515] and increased neurogenesis has been associated with improved learning and memory [276], GH may mediate this improvement via its effects on neurogenesis. Moreover, GH may also enhance formation and maintenance of connections critical for learning and memory functions; an fMRI study of patients treated with GH for 6 months identified a significant and more efficient activation of the neural networks involved in both long-term and working memory functions [522]. Additionally, especially in young adult rats, GH increases hippocampal NMDA receptor subunit expression [519] and consequently could increase learning and memory via receptor-mediated actions on glutamatergic neurotransmission [519] and increased synaptic plasticity [892, 893]. Observations that GH increases neurite outgrowth and neuronal maturation (see Chapter 6 and Section 1.4.1.2) support this view.

8.3.4 Effects of PRL on neurogenic processes

As members of the PRL/GH/placental lactogen family, GH and PRL are closely related hormones (See Chapter 1). In fact, h GH not only binds to its cognate receptor (GHR)
but also to the PRLR and mimics some of PRL actions [780]. Given the similarities in their signal transduction pathways, distribution of receptors, production of binding proteins and extra pituitary sources especially in the brain (Chapter 1, [589]), it is not surprising that PRL too like GH, has effects in the CNS. Several lines of evidence from rodent studies have now established that PRL is also involved in post-injury recovery mechanisms such as glial responses [16, 25] and neurogenesis [16, 26, 27, 645]. One of the main aims of this study was to extend these previous findings from largely rodent *in vitro* and *in vivo* studies to studies on cells of human origin. This is important especially since PRL has considerably more isoforms of both protein and receptor in humans compared to rodents and as a result, can have variable effects in humans [593]. The main focus in particular, was on investigating the direct effects of rhPRL on hNSCs. Results from these studies demonstrate that PRL, although having similar proliferative effects to GH on undifferentiated fetal NSCs (Chapter 7, Fig 2a) and neuronal progenitors (Chapter 7, Fig 2b), has its own distinctive effects on glial progenitors (Chapter 7, Fig 2c). This novel finding that PRL increases the proliferation of glial progenitors is in line with previous studies identifying a gliatrophic role for PRL [25, 643, 841] likely highlights the difference in function between these two related, yet distinct hormones. Given the robust proliferative effects of PRL, it could similarly to GH, have an important role in post-injury recovery mechanisms. In support, it has been previously reported that both PRL and its receptor are upregulated locally in close proximity to both immature neurons as well as glia [16, 25] following unilateral ischemic injury. There is rapid upregulation of both PRL and its receptor as early as 1 day after injury, with a peak at 5 days [25] when most neurorestorative mechanisms start taking effect (See Chapter 1). The increased levels of PRL following injury [25, 648, 649] could be part of an endogenous mechanism to increase the proliferation as well as migration of NSCs (Chapter 7) and subsequent proliferation of neuronal and glial progenitors (Chapter 7). It is possible that *in vivo*, such effects are mediated in a spatio-temporal fashion; PRL promotes the proliferation and migration of undifferentiated NSCs (probably ‘B’ cells) [27] and neuroblasts (probably ‘A’ cells) [16, 27] out to the peri-infarct areas where remodelling occurs [16, 226, 231, 340]. Simultaneously, PRL probably elicits similar proliferative effects on glial progenitor cells both in the SVZ [234, 324] as well as peri-infarct areas [25], enabling multiple neurorestorative processes to take effect.
Although the effects of PRL on differentiation were not investigated in this study, Shingo et al., have shown PRL to be responsible for the pregnancy-associated increase in neurogenesis and that neurospheres generated in the presence of PRL and EGF produce twice as many neurons as those generated in the presence of EGF alone [27]. Prior to this, studies had already established a pivotal role for PRL in the differentiation of the tuberoinfundibular dopaminergic (TIDA) neurons of the hypothalamus [636, 894, 895]. These findings suggest that along with the proliferation and migration (Chapter 7), PRL may also be involved in the differentiation of NSCs. It is important to note however, that since the post-injury upregulation in PRL/PRLR is sustained in glial cells rather than neuronal cells [25], it is possible that PRL may exhibit more gliotrophic than neurogenic properties at least in the postnatal brain. Indeed, its trophic effects on astrocytes [25, 643, 647], combined with its involvement in the formation of the glial scar [25, 650] further strengthen this theory. Alternatively, PRL too, like GH, may have age-specific effects on neurogenic events in the brain since proliferative effects of PRL on NSCs appear to be restricted to those from the embryonic/fetal brain (Chapter 7, [16]) and similar in vitro studies using NSCs from the adult brain have failed to detect a significant proliferative effect when PRL was administered in the absence of EGF [27, 874]. It is likely that PRL especially in the adult brain, may require the presence of a ‘supplementary’ GF such as EGF [27] or estrogen [896] since lack of either of these hormones appears to ameliorate PRL-mediated proliferation. A somewhat similar age-related effect is seen on neuronal differentiation; PRL-induced differentiation of TIDA neurons only occurs when PRL is administrated in the early postnatal period [636, 641] [897].

Overall, results from this current in vitro study highlight several important effects that PRL can have on NSC function in the brain and contribute significantly to the limited information currently available regarding the role of PRL in mediating neurogenic events in the brain both during development and subsequently following injury. They provide evidence, for the first time, of the effects of an exogenous application of hPRL in mediating hNSC activity; that it regulates the proliferation of NSCs as well as neuronal and glial progenitor cells along with concentration-dependent actions on regulating NSC migration. Taken together, findings from this study suggest that similar to GH, PRL has substantial effects on NSC function and thus support a supplementary role for PRL in regulation of neurorestorative processes.
8.3.5 Use of GH and PRL in the brain: Factors to consider

As discussed in Section 8.3.1, one of the most important factors to consider while employing GFs/hormones for the treatment of brain injuries is the method of delivery. Targeted and continuous delivery of GFs in a medium that can maintain bioactivity for the duration of infusion is crucial for both experimental and clinical trials, as is the elucidation of useful surrogate measures that can confirm such bioactivity. In reviewing experimental literature on the therapeutic potential of GH and PRL careful consideration must also be given to species-specific effects of these hormones since both hormones can have variable effects based on their origin and target species. The most important species-specific effect of GH is that hGH is highly lactogenic [772, 898] and can significantly influence PRL-sensitive circuits in the brain [589] including that of maternal behaviour [899]. This is evident also in the in vitro studies presented in this thesis, where GH-induced migration of NSCs is mediated (at least largely) by signalling via the PRLR (Chapter 6, Fig 6.5). Given that hGH can also bind to PRLR, the biological availability of GH can be influenced by serum PRLBPs that are essentially the extracellular portion of the PRLR [606, 8310]. Further, since signalling pathways for both GHR and PRLR are similar, in cells such as NSCs expressing both receptors, the intracellular response might be expected to be comparable; overall similar effects for both hormones were observed with respect to proliferation of NSCs (at higher doses) and neuronal progenitors as well as the migration of NSCs (Chapters 6 and 7). Moreover, there is a similarity in their receptor distribution in the brain, possibly reflecting the fact that GH and PRL have related functions [589] and might actually be mutually compensatory for loss of action [750]. This is of considerable importance especially for studies employing recombinant hGH due to its cost effectiveness in experimental animals. Other evidence includes findings related to neuroprotection; rGH but not bovine GH is neuroprotective following ischemia [17, 19] and has trophic effects on primary neuron-enriched cultures from the fetal rat cortex [17]. A similar species-specific effect appears to exist for PRL as well, since recombinant mouse PRL but not ovine PRL increases SVZ neurogenesis [27] and cell proliferation in the periventricular region surrounding the third ventricle and median eminence [897] in mice. Interestingly however, ovine PRL is more potent than recombinant mouse PRL in inducing TIDA neuron differentiation [900] in mice. Moreover, ovine PRL has been shown to have somatogenic properties in mice [897, 900] that homologous PRL lacks [900]. All of these observations highlight the significance of using homologous proteins.
for experimental studies and consequently, further substantiate findings reported in this thesis as the hormones used were of the same origin as the recipient (rat or human fetal NSCs).

Despite these similarities, it is important to note that both hormones have several distinct and specific functions especially in the brain, most of which are evident from differences in the distribution of their receptors in the brain (Chapter 1). For example, regulation of hippocampal function is specifically mediated by GH but not PRL as receptors are not expressed for the latter in this region [457, 459, 461, 615, 901-903]. From studies conducted in this thesis it is apparent that effects of GH and PRL on glial progenitor proliferation are distinct (Chapters 6 and 7), while other studies from our laboratory have reported neuroprotective actions to be restricted to GH [19, 25]. Ultimately, it is of utmost importance to keep in mind the convergence and divergence of the functions of these hormones.

### 8.4 Limitations

The primary limitation for the dose-response study was the limited number of animals that were successfully investigated within each group. As discussed in Chapters 3 and 4, the final \( n \) at post-mortem for each group in the dose response study was relatively small owing to mortality as a consequence of technical issues. This made it difficult to confidently ascertain a neuroprotective role for GH in the ischemic adult brain. It also imparted significant restriction on the extent of immunohistochemical analyses that could be carried out especially at the level of the cannula placement. Although the problems identified in the dose response study were rectified (see Chapter 5) for the delayed treatment study, there were issues relating to histological processing of tissue in this study. As a result, detailed immunohistochemical analysis of these tissues was not possible and the mechanism of action which GH-conferrered behavioural recovery and memory improvement in such a delayed, long-term treatment paradigm could not be examined (although this was outside the scope of this study which was aimed at studying the effects on longterm motor recovery). The possible mechanism of action of GH can only be suggested quoting findings from other studies (See section 1.4.1.12) as well as those from the \textit{in vitro} studies presented in this thesis (Chapter 6).
Another aspect that cannot be ascertained from the results presented in this thesis is the extent of IGF1 involvement in mediating the effects of GH. As IGF1 is the principal mediator of the peripheral actions of GH and IGF1 has been shown to have neuroprotective as well as neurotrophic actions following injury in the brain [585, 862] it is possible that IGF1 may mediate at least some of the aforementioned effects. However, IGF1-independent effects of GH have been observed in earlier studies (See Chapter 1), especially in terms of regulating neuroprotection [19]. Further possible discrepancies between GH and IGF1-mediated effects have been discussed in detail in Chapter 6.

Given the length of differentiation assays and the expense involved in using human hNSCs, there were limitations to the number of replicates that could be carried out for this assay. Accordingly, the effects of GH and PRL on the differentiation of NSCs could not be explored beyond preliminary analyses. High expenses also did not allow for exploration into the mechanism of receptor signaling for proliferation assays and the concentration-dependent migratory effects for both hormones.

### 8.5 Future Directions

Work presented in this thesis suggests an important role for GH both following injury as well as developmentally in regulating neurogenic activity in the brain. In doing so, it also provides several important avenues for future research in this field.

Although the limitation of small $n$ for the dose response study did not hinder addressing the primary aims of the study, it precluded accurate and reliable assessment of neuroprotection by GH in this treatment paradigm. As such, whether GH confers protection following a focal ischemic injury in the adult brain and subsequent benefits in terms of motor function remains to be evaluated. An in vivo study could be carried out utilising the ET1 model and continuous infusion treatment paradigm, starting a 20µg/day (dose based on results from Chapters 3 and 4) GH infusion immediately after stroke induction. A battery of sensorimotor tests are recommended, including those that would test specific ‘learned’ behaviours such as the beam-traversing and skilled reaching tasks [66, 71]. BrdU labelling could be employed to specifically quantitate GH-induced neurogenesis, while double-immunohistochemical analyses of the fate of these progeny could be assessed using a variety of markers for neuronal (βIIIITubulin,
DCX) and glial progenitor (S100β, GFAP) cells. It would also be interesting to study the expression of NSC markers such as nestin, especially in the SVZ. Since GH has a considerable role in promoting neuronal maturation (Chapter 6, [515, 552]), assessment of changes in levels of GAP43 (marker for a brain specific cell membrane glycoprotein associated with neurite outgrowth [904]) and neuN (a marker for mature neurons [905]) would provide further insight into mechanisms of GH-induced neurorestorative processes, along with clarification of whether GH promotes neuronal maturation following stroke. Simultaneously, molecular analyses using techniques such as laser capture microdissection of the SVZ and infarct penumbra followed by microarrays for genes regulating NSC activity (such as transcription factors and growth factors), angiogenesis (such as vascular endothelial growth factor, EPO, angiopoietin, ephrin) [372] and GH effects (such as IGF1 and SOCS2; see section 1.4.1.4) would elucidate the mechanism of action of GH treatment following stroke. Finally, an investigation of the pattern of GHR expression would collectively strengthen current findings and further clarify the role of GH post-ischemic injury in the adult brain. Such an in vivo study could be carried out in different groups of animals (young adults Vs aged, SOCS2 -/- Vs normal) in order to specify the age-related and SOCS2-mediated effects of such a GH treatment.

Since GH primarily promotes NSC proliferation, at the expense of their differentiation, in the adult brain [20] and NSC proliferation post-stroke is initiated within 7 days [235, 236, 906], it would be ideal to administer GH relatively soon following stroke. However, since GH in the adult brain does not have any significant proliferative effects on neuronal progenitors [20] but PRL does [27], an extended co-treatment with GH and PRL might prove to be highly beneficial.

Preliminary findings presented in Chapter 6 suggest that GH has significant effects on NSC and progenitor cell differentiation and further studies are required to clearly delineate these specific effects. The effects of GH on neurite outgrowth observed in Chapter 6, particularly at higher concentrations remain to be further validated; whether this is a result of reduced neuronal and increased glial differentiation [433] or the result of increased apoptosis needs to be clarified. An in vitro GH dose-response study would be appropriate as GH appears to have differential effects on differentiation based on dose as well as cell-type [515, 569]. Double-immunolabelling of the differentiated cells using markers for both progenitor (βIIIITubulin and S100β) as well as mature (neuN and...
GFAP) neurons and glia, combined with markers of apoptosis such as caspase 3 and TUNEL would provide all this information.

GH-mediated migration of NSCs appeared to be the result of GH signalling via the PRLR rather than GHR (Chapter 6). However, in this study, only one (300ng/mL) concentration of GH was tested when the antagonists were employed. It was evident from the in vitro analyses that the responses of cells to more physiological levels of hormones (0.5ng/mL or 5ng/mL) were quite distinct to those at higher doses (50, 150 and 500ng/mL; Chapters 6 and 7). Thus, it would be interesting to determine whether PRLR signalling is involved even at smaller, more physiological concentrations of GH (such as 0.5ng/mL). A similar analysis could be carried out using PRL as well, since PRL appears to have differential effects on NSC migration depending on the concentration it is used at (Chapter 7). Additionally, since GH utilises the PRLR to regulate NSC migration (Chapter 6), it is plausible that it interacts with the PRLR to regulate other NSC activity such as proliferation and differentiation. This could be clarified using GHR and PRLR receptor antagonists in proliferation and differentiation assays such as those performed in Chapters 6 and 7.

8.6 SUMMARY

Research presented in this thesis has addressed several important points pertaining to the use of GH to promote neurorestoration following brain injury. Firstly, it highlights the numerous technical issues that require to be addressed when utilising rGH infusions in the brain. Namely, a buffer formulation that maintains bioactivity of the rGH for extended periods in vivo, surrogate measures (plasma and CSF IGF1 and plasma corticosterone and urea concentrations) that can confirm bioactivity for the duration of the study, the careful implementation of the ET1 model of unilateral focal ischemic stroke, accurate cannula and pump placements for rGH infusions and the establishment of a dose-response for the rGH infusion to identify optimal dosage for treatment. Secondly, although a definitive role for GH in mediating neuroprotection after focal stroke in the adult brain could not be elucidated, it was evident that GH infused ICV into the ischemia-injured brain is capable of targeting all major regions of interest. These regions were the neurogenic region SVZ, the WMTs that NSCs utilise to ‘travel’, the infarct penumbra where active remodelling occurs as well as to the ependymal cells of blood vessels in parts of the injured striatum and cortex on the side ipsilateral to the
injury. Further, there is significant co-localisation with markers of differentiating and migrating neurons as well as possible glial progenitor cells suggesting GH has significant effects on these cells even at 6 weeks following injury. Thirdly, it presents GH as a potential neurorestorative factor following focal ischemic stroke in the adult brain as a long-term GH treatment initiated even 4 days after stroke confers some advantage in terms of accelerating motor recovery and improving spatial memory in the long-term without any apparent neuroprotection. Fourthly, it provides in vitro evidence to confirm a direct role for GH in mediating NSC activity; exogenously administered GH promotes the proliferation of NSCs in the absence of EGF or bFGF, promotes neuronal progenitor proliferation and maturation with no effects on glial progenitor proliferation and promotes NSC migration. Finally, it identifies similarities and differences between the GH and PRL circuits in the brain as an exogenous PRL treatment, although like GH has proliferative effects on NSCs and neuronal progenitors, unlike GH also has proliferative effects on glial progenitors. Evidence suggests that both GH and PRL act via the PRLR to induce migration (at least at the concentration tested in the study), although PRL can also inhibit migration at physiological levels. This highlights the importance of not only delineating species, target and dose-specific effects of these hormones but also the importance of considering simultaneous treatment with such closely-related factors.
8.7 Conclusion

The present studies show that GH has significant effects on the brain both developmentally as well as following injury. Given its significant involvement in regulation of neurorestorative and neurogenic processes, GH potentially represents a promising therapeutic option for the treatment of injuries in the brain.
There are some inadequacies in this thesis that have resulted from various setbacks that were out of the student's control. Frequent change in supervision caused various funding and practical difficulties that did not allow for some of the studies to be completed and/or carried forward, especially for Chapters 3, 4 and 5. Furthermore, these studies were also complicated by other issues such as possible incorrect provision of animal strains (Chapters 3 and 4) and loss of histological specimens due to a mixup of solutions in the tissue processor by the person responsible for maintaining it (Chapter 5). As such, some of the obvious inadequacies were unfortunate but inevitable.
10 Appendix

10.1 MODIFIED BOUIN’S SOLUTION

Recipe provides 1L of solution containing 0.1M PBS, 4% paraformaldehyde, 0.08% gluteraldehyde, 15% picric acid at pH 7.4

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraformaldehyde</td>
<td>40g</td>
</tr>
<tr>
<td>Picric acid</td>
<td>150mLs</td>
</tr>
<tr>
<td>Gluteraldehyde</td>
<td>800μL</td>
</tr>
<tr>
<td>0.1M PBS</td>
<td>To final volume of 1L</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
</tr>
</tbody>
</table>

10.2 0.1M PHOSPHATE BUFFERED SALINE (PBS)

Recipe provides 1L of buffer at pH 7.4

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2M NaH2PO4·2H2O</td>
<td>100mLs</td>
</tr>
<tr>
<td>0.2M Na2HPO4</td>
<td>400mLs</td>
</tr>
<tr>
<td>NaCl</td>
<td>9g</td>
</tr>
<tr>
<td>mQH2O</td>
<td>To final volume of 1L</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
</tr>
</tbody>
</table>

10.3 0.01M CITRATE BUFFER

Recipe provides 1L of buffer at pH 6

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M Citric Acid (19.24g citric acid in 1L distilled H2O)</td>
<td>16mLs</td>
</tr>
<tr>
<td>0.1M Sodium Citrate (29.41g sodium citrate in 1L distilled H2O)</td>
<td>84mLs</td>
</tr>
<tr>
<td>mQH2O</td>
<td>900mLs</td>
</tr>
<tr>
<td>pH</td>
<td>6</td>
</tr>
</tbody>
</table>
10.4 0.01M POTASSIUM PHOSPHATE BUFFERED SOLUTION (KPBS)

Recipe provides 1L of solution at pH 7.4

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1.15g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.2g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2g</td>
</tr>
<tr>
<td>mQH₂O</td>
<td>1L</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
</tr>
</tbody>
</table>

10.5 2, 3- AMINOPROPYL SILANE COATING FOR SLIDES

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rocking wash in detergent H₂O</td>
<td>30min</td>
<td>RT</td>
</tr>
<tr>
<td>Rinse in distilled H₂O</td>
<td>30min</td>
<td>RT</td>
</tr>
<tr>
<td>Dip in distilled H₂O with a dash of HCl</td>
<td>10s</td>
<td>RT</td>
</tr>
<tr>
<td>Dry in oven</td>
<td>O/N</td>
<td>50°C</td>
</tr>
<tr>
<td>Dip in 2% 2,3 aminopropylsilane in acetone</td>
<td>5s</td>
<td>RT</td>
</tr>
<tr>
<td>Dip in 100% acetone</td>
<td>5s</td>
<td>RT</td>
</tr>
<tr>
<td>Dip in distilled H₂O</td>
<td>5s</td>
<td>RT</td>
</tr>
<tr>
<td>Dry in oven</td>
<td>O/N</td>
<td>42°C</td>
</tr>
</tbody>
</table>

10.6 BUFFERS FOR IGF1 RADIOIMMUNOASSAY

10.6.1 Buffer A: Acidic Dilution Buffer

Recipe provides 1L of 20mM Phosphate buffer with pH 2.8

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5926 mM ortho-phosphoric acid</td>
<td>205µL</td>
</tr>
<tr>
<td>16.409 mM NaH₂PO₄H₂O</td>
<td>2.265g</td>
</tr>
<tr>
<td>5mM EDTA (Titriplex)</td>
<td>1.86g</td>
</tr>
<tr>
<td>120mM NaCl</td>
<td>7.01g</td>
</tr>
<tr>
<td>0.02% NaN₃</td>
<td>0.20g</td>
</tr>
<tr>
<td>Reagent</td>
<td>Amount</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>--------</td>
</tr>
<tr>
<td>0.2% BSA (fat-free)</td>
<td>2.00g</td>
</tr>
<tr>
<td>0.1% Triton X-100</td>
<td>1mL</td>
</tr>
<tr>
<td>pH</td>
<td>2.8</td>
</tr>
</tbody>
</table>

10.6.2 Buffer B: Antibody & Tracer dilution buffer

Recipe provides 1L of 200mM phosphate buffer at pH 7.8

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>151.1mM NaH₂PO₄·H₂O</td>
<td>26.89g</td>
</tr>
<tr>
<td>48.9mM NaH₂HPO₄ (anhydrous)</td>
<td>5.87g</td>
</tr>
<tr>
<td>40mM NaCl</td>
<td>2.34g</td>
</tr>
<tr>
<td>0.02% NaN₃</td>
<td>0.20g</td>
</tr>
<tr>
<td>0.2% BSA (fat-free)</td>
<td>2g</td>
</tr>
<tr>
<td>0.1% Triton X-100</td>
<td>1mL</td>
</tr>
<tr>
<td>pH</td>
<td>7.8</td>
</tr>
</tbody>
</table>

10.6.3 Buffer C: Acidic buffer for plasma

Recipe provides 1L of buffer at pH 1.25.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5M ortho-phosphoric acid</td>
<td>34.1mL</td>
</tr>
<tr>
<td>1% BSA</td>
<td>10g</td>
</tr>
<tr>
<td>1% Triton-X100</td>
<td>10mL</td>
</tr>
<tr>
<td>0.10% NaN₃</td>
<td>1g</td>
</tr>
<tr>
<td>EDTA</td>
<td>3g</td>
</tr>
<tr>
<td>Antipain</td>
<td>3g</td>
</tr>
<tr>
<td>Benzamidine</td>
<td>3g</td>
</tr>
<tr>
<td>Trasylol</td>
<td>30g</td>
</tr>
<tr>
<td>pH</td>
<td>1.25</td>
</tr>
</tbody>
</table>
11 List of References


29. StrokeFoundationNZ, 10 Key facts about stroke in New Zealand. 2008, New Zealand Stroke Foundation Inc.: Wellington, NZ.


Le Greves, M., et al., Growth hormone replacement in hypophysectomized rats affects spatial performance and hippocampal levels of NMDA receptor subunit and PSD-95 gene transcript levels. Experimental Brain Research, 2006. Apr 22 Epub (ahead of print).


Gluckman, P.D., et al., The role of IGF-1 in the response to organ injury - studies in the central nervous system., in Insulin-like growth factors and their regulatory


