The Role of Insulin-like Growth Factor-I (IGF-I) in Cerebral Cortex Development

by

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Abstract

The *in vivo* actions of insulin-like growth factor-I (IGF-I) on the embryonic and early postnatal development of the cerebral cortex were investigated in a line of transgenic mice that overexpress IGF-I in the brain under the control of regulatory sequences from the nestin gene. Transgene expression in these mice, designated nestin/IGF-I transgenics, begins by as early as embryonic day (E) 13 and continues into postnatal life, with the highest levels of expression detected in the cerebral cortex. To determine the effect of elevated IGF-I expression on cortex development, stereological analyses were conducted by light microscopy at postnatal day (P) 12. These analyses revealed a 31% increase in the total volume of the cerebral cortex and a corresponding 27% increase in the total number of cortical neurons in transgenic mice as compared to normal littermate controls. To investigate the mechanisms by which IGF-I overexpression promotes increased neuron number in nestin/IGF-I transgenic mice, an analysis of the cell cycle kinetics of neuron progenitors in the cerebral wall was conducted at E14, the approximate midpoint of cortical neurogenesis in normal mice (E11-E17). The lengths of the total cell cycle and all individual phases (G1, S, G2, and M) were measured in transgenic and control embryos. Total cell cycle length was decreased by 2.05 hr in transgenic embryos, due entirely to a reduction in G1 phase length. Analyses conducted to measure the proportion of cells exiting the cell cycle on E14-E15 revealed a 15% decrease in the exiting fraction of cells in transgenic embryos. A corresponding 27% increase in the proportion of Tbr2-positive intermediate progenitor cells was noted in the cerebral wall of transgenic embryos on E14. The anti-apoptotic actions of IGF-I in the cortex were assessed during early postnatal development, at P0 and P5. A significant decrease (31-39%) in the numerical density of apoptotic neurons in the cortex was documented in transgenic mice at these ages. Taken together, the results of the present set of experiments indicate that IGF-I acts both to promote neuron progenitor proliferation and to decrease neuron death by apoptosis in the developing cerebral cortex.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ALS</td>
<td>Acid labile subunit</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic protease-activating factor-1</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DAB</td>
<td>3, 3-diaminobenzidine</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain protein</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fibroblast growth factor-2</td>
</tr>
<tr>
<td>FL</td>
<td>Forelimb motor area</td>
</tr>
<tr>
<td>Fr</td>
<td>Frontal cortex</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GF</td>
<td>Growth fraction</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>Gu</td>
<td>Gustatory cortex</td>
</tr>
<tr>
<td>HL</td>
<td>Hindlimb motor area</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Insulin-like growth factor-I</td>
</tr>
<tr>
<td>IGF-I&lt;sup&gt;+&lt;/sup&gt;</td>
<td>IGF-I null mutant (knockout) mice</td>
</tr>
<tr>
<td>IGF-II</td>
<td>Insulin-like growth factor-II</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin-like growth factor binding protein</td>
</tr>
<tr>
<td>IGF1R</td>
<td>Type 1 insulin-like growth factor receptor</td>
</tr>
<tr>
<td>IGF1R&lt;sup&gt;−&lt;/sup&gt;</td>
<td>IGF1R null mutant (knockout mice)</td>
</tr>
<tr>
<td>IGF2R</td>
<td>IGF-II/mannose-6-phosphate receptor</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin-receptor substrate</td>
</tr>
<tr>
<td>ISEL</td>
<td><em>In situ</em> end labeling</td>
</tr>
<tr>
<td>ISHH</td>
<td><em>In situ</em> hybridization histochemistry</td>
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</tbody>
</table>
IZ  Intermediate zone
JNK  Jun N-terminal protein kinase
LI  Labeling index
MAPK  Mitogen activated protein kinase
MCx  Primary motor cortex
MI  Mitotic index
MZ  Marginal zone
Nv  Numerical density (number per mm$^3$)
P  Postnatal
Par1  Primary somatosensory cortex
Par2  Supplementary somatosensory cortex
PI3-K  Phosphatidylinositol 3-kinase
PIP$_3$  Phosphatidylinositol-3 phosphate
Rb  Retinoblastoma protein
RIA  Radioimmunoassay
RPA  Ribonuclease protection assay
Sos  Son of sevenless
SVZ  Subventricular zone
T$C$  Total cell cycle length
Tg  Transgenic
T$_{G1}$  G1 phase length
T$_{G2}$  G2 phase length
T$_{G2+M}$  The combined length of the G2 and M phases of the cell cycle
T$_S$  S phase length
TUNEL  Terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling
VZ  Ventricular zone
Acknowledgements

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Statement of Co-Authorship

Versions of this work have appeared in:


For the first publication listed above (Popken et al., 2004), Rebecca Hodge conducted the stereological analyses of the cerebral cortex at postnatal day 12. She was responsible for the tissue processing, data collection and analysis associated with these experiments, and contributed to the writing of the manuscript. Only these sections of the manuscript appear in the thesis as work done by the thesis author. For publications 2 and 3, Rebecca Hodge conducted all experiments presented in the paper and was the primary author of each paper.
Chapter I Introduction

1.1 General introduction to insulin-like growth factor-I (IGF-I) and related peptides

The insulin-like growth factor (IGF) system consists of structurally and functionally related peptides that function to promote growth during embryonic and postnatal development. The IGF system of peptides includes insulin-like growth factors -I (IGF-I) and -II (IGF-II), as well as the transmembrane receptors, the type I IGF receptor (IGF1R) and the mannose-6-phosphate/IGF-II receptor (IGF2R). Additionally, six soluble IGF binding proteins (IGFBP) have been identified, and appear to modulate the functions of IGF-I and IGF-II. IGF-I, a 70 amino acid basic peptide, shares structural homology with both IGF-II (a 67 amino acid peptide) and insulin. Human IGF-I and IGF-II share roughly 70% identity with respect to amino acid sequence, and both peptides share approximately 50% structural homology to pro-insulin (Daughaday and Rotwein, 1989). The IGF peptides appear to be highly conserved across species, with few structural differences noted between humans and rodents (Bell et al., 1986; Shimatsu and Rotwein, 1987; Daughaday and Rotwein, 1989). IGF-I and IGF-II are produced in a wide variety of tissues during development, with the liver being the main site of IGF-I synthesis.

The functions of IGF system peptides are controlled by interaction with a number of cell surface receptors. The actions of IGF-I are mediated in large part by binding to IGF1R. IGF-II is also capable of binding to IGF1R, although with lower affinity than IGF-I, and high concentrations of insulin can activate IGF1R signaling (reviewed in Ward et al., 2003). IGF-I, present at high concentrations (i.e. above normal physiological levels), may also activate the insulin receptor and IGF2R. As well, hybrids of the IGF1R and insulin receptors have been documented that appear to bind IGF-I with higher affinity than insulin. However, the biological significance of these hybrid receptors remains unclear (Jones and Clemons, 1995). Therefore, the majority of IGF-I’s actions during normal development appear to be dependent upon signaling through the IGF1R. The actions of IGF-II are dependent upon binding to a wider array of receptors including IGF1R, IGF2R, and an alternatively spliced variant of the insulin receptor (Butler et al., 1998; Ward et al., 2003). The IGF2R, which binds IGF-II with high affinity and does not bind insulin, appears to be involved in receptor-mediated degradation of IGF-II and may also have a role in controlling the mitogenic activity of IGF-II (reviewed in Spence and Nissley, 2003).
The actions of IGF system peptides are modulated in vivo by high affinity IGFBPs. To date, six structurally related peptides have been identified as members of the IGFBP family (for reviews see Jones and Clemmons, 1995; Baxter, 2000). IGFBP-1 through IGFBP-5 have similar affinities for IGF-I and IGF-II. IGFBP-6 differs from these other IGF binding proteins in that it has a much higher affinity for IGF-II (Baxter, 1997). IGFBPs have been shown to both inhibit the interactions of IGFs with their receptors and to enhance the functions of IGF peptides. The functions of IGFBPs appear to depend upon the cell type and developmental context under study (Baxter, 2000). For example, IGFBP-3 enhances IGF-I-mediated weight gain in hypophysectomized rats (Clark et al., 1993), while this same binding protein inhibits IGF-I-mediated DNA synthesis in human fibroblasts (DeMellow and Baxter, 1988). IGFBPs also function as carrier proteins of IGFs in circulation. In serum, the majority of IGF peptides (approximately 75%) are found in ternary complexes composed of IGFBP-3 and a non-IGF binding element called the acid labile subunit (ALS). IGFBP-5 also appears to form complexes with ALS and IGF peptides. The ALS is a glycoprotein composed of leucine rich domains that function in protein-protein interactions (Jones and Clemmons, 1995). Sequestration of IGFs in these ternary complexes appears to increase the half-life of IGFs in the serum (Guler et al., 1989).

1.2 Somatic functions of IGF-I and regulation by growth hormone

IGF-I is a critical factor involved in the regulation of embryonic and postnatal growth. Lines of mice carrying null mutations of the IGF-I gene (IGF-I-/- mice) exhibit abnormal growth during early embryonic development (Baker et al., 1993; Liu et al., 1993; Powell-Braxton et al., 1993). Intrauterine growth retardation is evident in IGF-I-/- mice by as early as embryonic day (E) 13 and body weights of IGF-I-/- mice are only approximately 60% of controls by birth (Baker et al., 1993). The survival of IGF-I-/- mice during the perinatal period is dependent upon the genetic background of the mice. Certain strains are not viable, while those that do survive reach only 25% of normal body weight by adulthood (Baker et al., 1993). Therefore, IGF-I appears to be necessary for both normal intrauterine and postnatal somatic growth in mice. Humans with IGF-I gene deletions also display intrauterine growth abnormalities and deficient postnatal growth (Woods et al., 1996), further supporting a role for IGF-I in the control of somatic growth. Transgenic (Tg) mice that overexpress IGF-I in somatic tissues during postnatal development also support a role for IGF-I in regulating somatic growth. In these mice, body weight is increased beginning at 3-4 weeks of age, and
body weights are approximately 30% greater in Tg mice by adulthood (Mathews et al., 1988; Ye et al., 1995a).

Mice carrying null mutations of the IGF1R gene (IGF1R^- mice) display a more severe reduction in intrauterine growth that begins earlier in development (Baker et al., 1993; Liu et al., 1993). In these mice, growth retardation is apparent by E10.5 and mice attain only 45% of normal body weight by birth. IGF1R^- mice die a short time after birth (Liu et al., 1993). Mice carrying mutations of both the IGF-I and IGF1R genes (IGF-I^- & IGF1R^-) have the same phenotype as IGF1R^- mice, as they reach only 45% of normal weight and do not survive during the perinatal period (Baker et al., 1993). The severity of the IGF1R^- phenotype likely results from loss of both IGF-I and IGF-II actions through this receptor. Humans lacking the IGF1R gene also display deficient in utero and postnatal growth, indicating that IGF1R function is necessary for normal human somatic development (Abuzzahab et al., 2003).

Interestingly, in mice IGF-II appears to be required only for normal embryonic growth, as opposed to postnatal growth. Mice lacking the IGF-II gene exhibit intrauterine growth retardation beginning at approximately E10.5. However, the mice are viable at birth and survive to adulthood, although their normal postnatal somatic growth does not compensate for deficiencies in growth during the prenatal period (DeChiara et al., 1990).

During postnatal development the liver appears to be the major source of IGF-I in circulation, and circulating IGF-I has traditionally been thought to act as a mediator of growth hormone (GH) action. GH is produced in the anterior pituitary, as well as in the brain, mammary gland, pineal gland and placenta. GH secretion from the pituitary is regulated by growth hormone releasing hormone and the inhibitory hormone somatostatin, both of which are produced in the hypothalamus, and also by IGF-I (Butler and LeRoith, 2001). The finding that IGF-I is a mediator of GH actions led to the development of the original somatomedin hypothesis, which states that IGF-I production and excretion from the liver is stimulated by GH, after which IGF-I is transported to target organs via circulation where it acts as an endocrine factor to promote growth. IGF-I in the circulation in turn acts as a negative regulator of GH secretion from the pituitary (reviewed in Butler and LeRoith, 2001). The somatomedin hypothesis was revised upon the discovery that IGF-I, as mentioned above, is produced by a wide variety of tissues, with peak expression varying between regions and developmental stages. The revised hypothesis is that the production of locally synthesized IGF-I is regulated, at least in part by GH, and appears to function in an autocrine and/or paracrine manner to
influence tissue growth (Butler and LeRoith, 2001). In fact, the functions of liver-derived IGF-I on somatic growth are uncertain, as evidence from studies of conditional knockout mouse models in which circulating IGF-I levels are reduced to 25% of normal levels indicate that somatic growth is normal in the absence of liver-derived IGF-I (reviewed in D'Ercole, 2003). Additionally, IGF-I appears to function independently of GH during embryonic development, as revealed by studies of mice lacking GH or GH receptor genes. In these mice, intrauterine growth is normal and body weights at birth are comparable to those of controls (Butler and LeRoith, 2001), whereas intrauterine growth is deficient in IGF-I knockout and IGF1R knockout mice, as discussed above.

1.3 Signaling through the type 1 IGF receptor

The actions of IGF-I are primarily mediated through binding to IGF1R, a transmembrane receptor tyrosine kinase. IGF1R is a heterotetramer consisting of two α and two β subunits (i.e. α₂β₂). The IGF1R gene is highly conserved between species with the human and rat cDNAs sharing 98% homology in the tyrosine kinase domain. As well, the IGF1R shares structural homology with the insulin receptor, another α₂β₂ transmembrane tyrosine kinase receptor. The IGF1R is expressed as a single precursor peptide which is cleaved into α and β subunits that are then linked by primary disulfide bonding. The mature α₂β₂ receptor is produced through secondary disulfide bonding of two αβ chains. The α subunits form the extracellular ligand binding domain of the receptor, and the β subunits form the transmembrane portion of the receptor. The tyrosine kinase domain is located within the cytoplasmic portions of the β subunits (reviewed in LeRoith et al., 1995; Ward et al., 2001).

Upon ligand binding to IGF1R, the receptor is autophosphorylated on tyrosine residues (tyr-1121, 1135, 1136), initiating a cascade of intracellular signaling events (Fig. 1-1). Signaling through IGF1R is complex, and a number of signaling pathways may be activated depending upon the situation and cell-type under study. IGF1R autophosphorylation results in the creation of binding sites for downstream signaling proteins. A number of these adapter proteins bind to and are phosphorylated by IGF1R, including insulin receptor substrates (IRS-1 to IRS-4), the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3-K), and Shc (reviewed in Folli et al., 1996; Butler et al., 1998). Activation of adapter proteins by phosphorylation provides a connection between IGF1R and downstream signaling pathways. In general, two major signaling pathways, the PI3-K pathway and the mitogen-activated protein kinase (MAPK) pathway, are known to be activated by IGF1R.
Fig. 1-1. Simplified schematic diagram of common signaling pathways activated by the binding of IGF-I to the type 1 IGF receptor (IGF1R). IGF1R is depicted as a heterotetrameric transmembrane protein. Upon binding of IGF-I to IGF1R, the receptor is autophosphorylated and activated. Several downstream targets may then be activated, including insulin receptor substrate-1 (IRS-1), which can, in turn, activate the phosphatidylinositol 3-kinase (PI3-K) signaling pathway. Initiation of PI3-K signaling leads to the activation of Akt, which can act on many downstream targets. PI3-K-mediated activation of Akt is often associated with the anti-apoptotic functions of IGF-I. Alternatively IGF1R may phosphorylate Shc, which then activates Ras. Activated Ras, in turn, activates members of the mitogen activated protein kinase (MAPK) signaling cascade. Initiation of MAPK signaling is often associated with the pro-mitotic functions of IGF-I.
The PI3-K pathway is activated by IGF1R signaling through binding of the p85 subunit of PI3-K directly to IGF1R or through binding of the p85 subunit to IRS-1 (Fig. 1-1). Binding of the p85 subunit to either of these proteins results in activation of the catalytic p110 subunit of PI3-K, leading to the formation of phosphatidylinositol-3 phosphate (PIP3) and phosphatidylinositol (3, 4) phosphate (Brunet et al., 2001; Sen et al., 2003). The serine/threonine kinase Akt (also known as protein kinase B) is activated by the PI3-K pathway, and has been shown to be an important mediator of IGF-I’s actions. Several mechanisms of Akt activation by PI3-K have been demonstrated. Akt may be activated by the kinases PDK1 and PDK2 (phosphoinositide-dependent protein kinase-1 and -2) or by binding of phosphatidylinositol (3, 4) phosphate to the pleckstrin homology domain of Akt (Butler et al., 1998; Brunet et al., 2001; Sen et al., 2003). Akt, in turn, regulates a number of important substrates. For example, Akt phosphorylates and inactivates glycogen synthase kinase 3β, which leads to glycogen synthesis (Butler et al., 1998; Cheng et al., 2000). Activated Akt also plays a role in regulating apoptosis through interactions with members of the Bcl-2 family of proteins. Akt has been shown to phosphorylate and inactivate BAD, which then forms a complex with 14-3-3, resulting in inhibition of the pro-apoptotic functions of BAD (Butler et al., 1998). Activation of the PI3-K pathway appears to also be responsible for the growth promoting and pro-mitotic effects of IGF-I in a number of tissues, including skeletal muscle (Chakravarthy et al., 2000) and fibroblasts (Y. Takahashi et al., 1997).

IGF1R activation also leads to initiation of the MAPK signaling pathway (Fig 1-1), which has been shown to regulate the proliferative effects of IGF-I on a variety of tissues. The initial step in MAPK pathway activation is the binding of Shc to IGF1R, which results in phosphorylation of Shc on tyrosine residues. These phosphorylated tyrosine residues serve as binding sites for the growth factor receptor-bound protein (Grb2). Grb2, in turn, binds Sos (son of sevenless), a guanine nucleotide exchange factor. Sos is responsible for catalyzing the exchange of GDP for GTP on Ras, a GTP binding protein. Ras is activated by the exchange of GDP for GTP, and activated Ras subsequently activates Raf. Activated Raf phosphorylates and activates MAP kinases (also known as extracellular signal related kinases or ERKs), which link the MAPK pathway with the nucleus (Jones and Clemmons, 1995). Activation of the MAPK pathway has been shown to promote proliferation of adipocytes, smooth muscle cells, and neuroblastoma cells, to name but a few cell types (reviewed in Dupont et al., 2003).
1.4 IGF-I expression in the rodent brain

In rodents, IGF-I expression is detectable in the brain by as early as E13 (Ayer-Le Lievre et al., 1991; Rotwein et al., 1988). Specifically, the olfactory bulb appears to be the first structure to express IGF-I (Ayer-Le Lievre et al., 1991). IGF-I mRNA expression is localized to neurons, with little expression detected in white matter, suggesting that glial cells express little IGF-I in the developing brain (Ayer-Le Lievre et al., 1991; Bartlett et al., 1991; Bondy, 1991). However, both embryonic neurons and glial cells have been shown to be capable of synthesizing IGF-I *in vitro* (Rotwein et al., 1988). IGF-I is expressed in numerous regions of the brain, with peak expression generally occurring during the first two weeks of postnatal life (Bach et al., 1991; Bartlett et al., 1991; Bondy, 1991). However, the time of peak IGF-I expression varies somewhat among different regions of the brain, as illustrated in Table 1-1. Regions of high postnatal IGF-I expression include the olfactory bulb, cerebellum, and subventricular zone (Rotwein et al., 1988; Bartlett et al., 1991, 1992; Bondy, 1991). Interestingly, these regions are associated with postnatal neurogenesis and gliogenesis, suggesting that IGF-I may be involved in these processes. IGF-I expression in other brain regions is also coordinated with periods of neuron production, process outgrowth and synapse formation (Bondy, 1991). For example, IGF-I expression has been noted in regions surrounding growing nerves (Bondy et al., 1990).

In most brain regions, IGF-I expression tapers off after early postnatal development (Rotwein et al., 1988; Bondy, 1991). However, IGF-I expression in the olfactory bulb, for example, persists into adulthood (Werther et al., 1990; Bondy et al., 1992). Within the olfactory bulb, IGF-I expression is localized to the internal granular and mitral cell layers (Werther et al., 1990). These sites of IGF-I expression are in close proximity to the glomerular layer, where IGF1R expression is present in the adult (Werther et al., 1990). Persistent expression of IGF-I into adulthood is also apparent in the Purkinje cells of the cerebellum (Rotwein et al., 1988; Torres-Aleman et al., 1994). Werther et al., (1990) noted IGF-I expression in the granule cell layer with IGF1R expression in the nearby molecular layer. IGF-I expression is also apparent in the adult hippocampus, where it is detectable in the dentate gyrus, the CA2 region, and the pyramidal cells in CA1 (Werther et al., 1990). As well, endogenously produced IGF-I has been implicated in the control of neural stem cell differentiation in adult mice (Brooker et al., 2000).
<table>
<thead>
<tr>
<th>Region</th>
<th>Approximate Peak Time of IGF-I mRNA Expression</th>
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<tbody>
<tr>
<td>Cerebral Neocortex</td>
<td>Postnatal day (P) 0-P28</td>
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<tr>
<td>Subventricular Zone</td>
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<tr>
<td>Olfactory Bulb</td>
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<td>- Mitral Cells</td>
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<td>- Tufted Cells</td>
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<td>Olfactory Bulb</td>
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<td>- Mitral Cells</td>
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<td>- Tufted Cells</td>
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<tr>
<td>Cerebellum</td>
<td></td>
</tr>
<tr>
<td>- Purkinje Cells</td>
<td>P4-P28</td>
</tr>
<tr>
<td>- Inferior Olive</td>
<td></td>
</tr>
<tr>
<td>- Deep Cerebellar Nuclei</td>
<td>E20-P21</td>
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<tr>
<td>- Red Nucleus</td>
<td>P4-P28</td>
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<tr>
<td>- Ventrolateral Nucleus</td>
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<td>Cerebellum</td>
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<td>- Purkinje Cells</td>
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<td>- Red Nucleus</td>
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<td>- Ventrolateral Nucleus</td>
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<tr>
<td>Hippocampus</td>
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<tr>
<td>- Dentate Gyrus</td>
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<tr>
<td>- Strata Oriens, Radiatum, Lacunosum-moleculare</td>
<td>E20-P28</td>
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<tr>
<td>Visual</td>
<td></td>
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<tr>
<td>- Retinal Ganglion Cells</td>
<td></td>
</tr>
<tr>
<td>- Superior Colliculus</td>
<td></td>
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<tr>
<td>- Lateral Geniculate Nucleus</td>
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<tr>
<td>- Lateral Posterior Nucleus</td>
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<td>- Anterior Pretectal Nucleus</td>
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<tr>
<td>Auditory-Vestibular</td>
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<tr>
<td>- Medial Vestibular Nucleus</td>
<td>E20-P21</td>
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<tr>
<td>- Superior Vestibular Nucleus</td>
<td>E20-P21</td>
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<tr>
<td>- Dorsal Cochlear Nucleus</td>
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<tr>
<td>- Ventral Cochlear Nucleus</td>
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<td>- Superior Olive</td>
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<td>- Lateral Lemniscal Nucleus</td>
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<td>- Medial Geniculate Nucleus</td>
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<td>- Inferior Colliculus</td>
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<td>- Interstitial Nucleus</td>
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<td>Somatosensory</td>
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<td>- Spinal Trigeminal Nucleus</td>
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<td>- Ventrobasilar Nucleus</td>
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<td>- Gelatinosa Nucleus</td>
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<td>- Intralaminar Nucleus</td>
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<td>- Anterior Pretectal Nucleus</td>
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<td>- Dorsal Column Nucleus</td>
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<tr>
<td>Medial Habenula</td>
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<tr>
<td>Lateral Septal Nucleus</td>
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<tr>
<td>Substantial Nigra, Pars Lateralis</td>
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</table>

This table was adapted from Table 1 in D’Ercole et al., 1996 and Table 1 in Bondy, 1991.

Data contained in the table were compiled from Ayer-Le Lievre et al., 1991; Bach et al., 1991; Bartlett et al., 1991, 1992; Bondy 1991; and Lee et al., 1993.
IGF-I immunoreactivity is apparent during early embryonic development (E15) and IGF-I immunoreactive neurons are also evident during postnatal development (Noguchi et al., 1987; Garcia-Segura et al., 1991). IGF-I immunoreactivity is extensive in the embryonic brain where it is evident in regions such as the cortical plate, hippocampus, diencephalon, thalamus and brain stem. In general, a wider variety of cell types are immunoreactive for IGF-I than are positive for IGF-I mRNA (Andersson et al., 1988; Garcia-Segura et al., 1991). While glial cells do not appear to express IGF-I mRNA \textit{in vivo}, they are immunoreactive for IGF-I during early postnatal development (Garcia-Segura et al., 1991). As well, IGF-I immunoreactivity is apparent in capillaries, choroid plexus, and cell processes during this stage of development (Garcia-Segura et al., 1991). Similar to IGF-I mRNA expression, IGF-I immunoreactivity generally decreases in intensity during adulthood (Andersson et al., 1988; Garcia-Segura et al., 1991). For example, IGF-I immunoreactivity in the cerebellum was found to decrease after the second postnatal week (Andersson et al., 1988). However, the Purkinje cells of the cerebellum display IGF-I immunoreactivity into adulthood (Aguado et al., 1992), consistent with lasting IGF-I mRNA expression in these cells in the adult rodent brain. Within these Purkinje cells, IGF-I immunoreactivity is localized to the rough endoplasmic reticulum, which is in agreement with studies indicating that IGF-I is synthesized within the Purkinje cells themselves (Aguado et al., 1992). Using radioimmunoassay, Yamaguchi and coworkers (1990) were also able to demonstrate the presence of IGF-I in several regions of the adult rat brain including the olfactory bulb, brainstem, striatum, cerebral cortex, hippocampus and cerebellum. The highest level of IGF-I was documented in the pituitary gland, which may be associated with IGF-I's effects on somatostatin and GH levels (Yamaguchi et al., 1990).

\textbf{1.5 Expression of IGF1R in the rodent brain}

IGF1R, the principal receptor for IGF-I, is widely expressed in the rodent brain from early embryonic development (Bondy and Lee, 1993). In general, embryonic brain demonstrates higher IGF1R binding activity than adult brain (Ocran et al., 1988). Peak IGF1R expression appears to occur between E15 and E20 in rat embryos (Baron-Van Evercooren et al., 1991). In the embryo at E14-E15, IGF1R expression is particularly abundant in the ventral floorplate of the hindbrain, an area that is thought to be involved in axon guidance (Bondy et al., 1992). Within the developing brain, IGF1R expression is equally apparent in regions of active neuron progenitor proliferation and in regions containing newly generated cells, suggesting that IGF1R may be involved in multiple developmental processes (Bondy et al.,
1992). In the rat brain at E20, high levels of IGF1R expression are apparent in the olfactory bulb and cerebral cortex, and expression of IGF1R is evenly dispersed throughout these regions (Bondy et al., 1992). Other regions with notable IGF1R expression during embryonic development include the cerebellum, thalamic nuclei, and brainstem nuclei (Bondy et al., 1992).

During early postnatal development, IGF1R continues to be extensively expressed throughout the brain (Bondy et al., 1992) and IGF-I binding sites are present throughout most regions of the brain (Bohannon et al., 1988). In vivo, IGF1R is expressed by neurons and ependymal cells, with very low expression noted in the white matter (Marks et al., 1991; Bondy and Lee, 1993). However, IGF1R has been detected in primary cultures of both neuronal and glial cells taken from newborn rats (Shemer et al., 1987; Ocrant et al., 1988). High levels of IGF1R mRNA are present in the choroid plexus and circumventricular organs during postnatal development (Bondy et al., 1992). These regions also display an abundance of IGF-I binding sites (Bohannon et al., 1988). Other regions demonstrating high postnatal expression of IGF1R include the olfactory bulb, cerebellum, and hippocampal formation (Bondy et al., 1992). Interestingly IGF1R and IGF-I are differentially expressed during the development of some brain regions. For example, IGF1R expression is apparent in the pyramidal cells of Ammon's horn and the granule cells of the dentate gyrus in the hippocampal formation. However, IGF-I mRNA is not detected in these pyramidal and granule cells, but is found in cells occupying adjacent areas (Bondy et al., 1992). These results are consistent with a paracrine function of IGF-I during brain development. Similarly, IGF1R expression is seen to decrease in the Purkinje cells of the cerebellum during the first three postnatal weeks, while IGF-I mRNA is increasing in these cells during this developmental period (Bondy et al., 1992).

In general, IGF1R mRNA content decreases during early postnatal development of the brain (Ocrant et al., 1988; Werner et al., 1989; Bondy et al., 1992; Bondy and Lee, 1993). However, IGF1R mRNA and IGF-I binding sites remain present in many regions of the adult rodent brain (Lesniak et al., 1988; Marks et al., 1991). High levels of IGF1R mRNA are apparent in the olfactory bulb in adulthood (Marks et al., 1991; Bondy et al., 1992). As well, the choroid plexus continues to exhibit high IGF1R expression (Marks et al., 1991). Other regions with persistent IGF1R expression during adulthood include the granule cell layers of both the cerebellum and the dentate gyrus, as well as the pyramidal cells within the piriform cortex (Marks et al., 1991). Expression of IGF1R continues to be very low in white matter
regions of the adult rodent brain, with expression predominantly localized to axon tracts (Marks et al., 1991). The pattern of IGF-I binding sites generally correlates with the pattern of IGF1R mRNA in the adult brain, with an abundance of binding sites detected in the olfactory bulb, cerebellum and hippocampus (Marks et al., 1991).

1.6 Expression of IGF-I and IGF1R in the cerebral cortex

IGF-I and its cognate receptor the IGF1R are both expressed in the cerebral cortex of rodents during embryonic development (Ayer-Le Lievre et al., 1991; Bondy et al., 1992). IGF-I mRNA, as detected using in situ hybridization, is expressed diffusely throughout the embryonic cerebral cortex. However, expression of IGF-I in the embryonic cortex tends to be at lower levels than in other brain regions, such as the olfactory bulb (Ayer-Le Lievre et al., 1991). IGF-I immunoreactivity is apparent in the rodent cerebral cortex during embryonic development, where it is localized to neuronal somas (Garcia-Segura et al., 1991). IGF-I immunoreactivity is especially high in the cortical plate during the period of cortical neurogenesis in rats (Garcia-Segura et al., 1991). IGF1R is also widely expressed in the developing rodent cortex, where it is detectable in both neural progenitor cells in the ventricular zone (VZ) and in post-mitotic neurons at an apparently equal level (Bondy et al., 1992).

Peak expression of IGF-I in the cerebral cortex occurs during the early postnatal period, from postnatal day (P) 0 to P28, as is the case in many other brain regions (Table 1-1; Bondy, 1991; Bach et al., 1991). At this time, IGF-I mRNA is apparent in large interneurons which are present in all layers and cortical areas (Bondy, 1991; Lee et al., 1993). IGF-I immunoreactivity is evident in neuronal nuclei within all layers and areas of the cerebral cortex, and IGF-I immunoreactivity is detectable in pyramidal neuron dendrites within the cerebral cortex (Garcia-Segura et al., 1991). Expression of IGF1R is high in the neonatal cerebral cortex (P2) and appears to decrease during the early postnatal period, which may result, in part, from the increase in the volume of the cortex during postnatal development (Bondy et al., 1992). Interestingly, IGF-I and IGF1R display different expression patterns in the early postnatal cortex with IGF1R being particularly abundant in layer VI pyramidal neurons and IGF-I being dispersed throughout the neocortex, as mentioned above (Bondy et al., 1992; Lee et al., 1993). Binding sites for IGF-I, which may represent IGF1R and/or IGFBPs, are also present in the cerebral cortex, where they are abundant in layers II and VI and present to a lesser extent in layers III-V (Bohannon et al., 1988).
Expression of IGF-I in the adult rodent cerebral cortex is decreased compared to early postnatal development, although it is still present (Rotwein et al., 1988; Bach et al., 1991). IGF-I can also be detected in the adult rodent cerebral cortex using immunohistochemistry (Garcia-Segura et al., 1991) and radioimmunoassay (Yamaguichi et al., 1990). IGF1R expression is apparent in the adult rodent cortex where it is present in all cortical layers (Marks et al., 1991). However, IGF1R mRNA is particularly abundant in layers II, III, and V at this time (Marks et al., 1991). IGF-I binding sites are also abundant in layers II, III, and V of adult rat cerebral cortex, consistent with the presence high levels of IGF1R in these cortical layers (Marks et al., 1991).

1.7 Insulin-like growth factor binding proteins in the rodent brain

*IGFBP-1*

Expression of IGFBP-1 has not been documented in brain (D'Ercole et al., 1996; Naeve et al., 2000), but IGFBP-1 is expressed in the peripheral nervous system at neuromuscular junctions (Ma et al., 1994a, b). IGFBP-1 has been exploited to study the IGF system in brain growth using transgenic mouse models. In these models, IGFBP-1 expression has been shown to retard brain growth, reduce brain weight, and inhibit myelination, presumably by binding to and inactivating endogenous IGF-I (Ye et al., 1995a, b; Ni et al., 1997; Lee et al., 1999; Doublier et al., 2000). *In vitro* studies indicate that IGFBP-1 decreases oligodendrocyte precursor survival in the presence of IGF-I, indicating that IGFBP-1 may be capable of modulating the effects of IGF-I on these cells (Kuhl et al., 2002).

*IGFBP-2*

IGFBP-2, along with IGFBP-4 and IGFBP-5 are the most abundantly expressed IGFBPs in the rodent brain (D'Ercole et al., 1996). IGFBP-2 exhibits different expression patterns in the embryonic and postnatal rodent brain. During embryonic development, expression of IGFBP-2 mRNA becomes apparent very early, at approximately E7 in rats (Wood et al., 1992). Expression in the neural tube is visible by E10-E11 and by E14 IGFBP-2 mRNA is detectable in the choroid plexus, infundibulum and ventral floor plate (Wood et al., 1990, 1992). IGFBP-2 expression is also detectable in the embryonic spinal cord, and lower levels are present postnatally (Arnold et al., 2000).

IGFBP-2 expression is abundant in the perinatal rat brain, although it shows no discernible pattern of expression at this age (Lee et al., 1993). During early postnatal development, IGFBP-2 is expressed in astrocytes and colocalizes with GFAP expression in...
these cells (Lee et al., 1993). In some regions, the expression pattern of IGFBP-2 correlates with that of IGF-I (Lee et al., 1993). For example, IGFBP-2 and IGF-I expression are spatiotemporally associated in the developing cerebellum. In the cerebellum, IGFBP-2 is localized to Bergmann glial cells surrounding projection neurons (e.g. Purkinje cells) that express IGF-I (Lee et al., 1992b, 1993). Similar patterns are seen, for example, in the retina where IGFBP-2 is expressed in Muller cells and astrocytes, while IGF-I is localized to projection neurons (Lee et al., 1992b). These coordinated patterns of IGFBP-2 and IGF-I expression suggest that IGFBP-2 may have a role in modulating the actions of IGF-I in the brain, although the exact functions of IGFBP-2 during brain development have not been experimentally determined (D’Ercole et al., 1996). Current evidence indicates that IGFBP-2 is attached to cell membranes by way of proteoglycans in these brain regions (Russo et al., 1996).

While IGF-I and IGFBP-2 exhibit coordinated expression in regions such as the retina and cerebellum, the same does not hold true for IGFBP-2 expression patterns in other regions. For example, in the cerebral neocortex IGFBP-2 and IGF-I expression do not exhibit any discernible relationship with respect to their expression patterns. IGFBP-2 is widely expressed in the developing cerebral cortex of the newborn rat where it is found in both the VZ and the developing cortical plate (Lee et al., 1993). The types of cells expressing IGFBP-2 in the cortex were not identifiable at this stage of development (Lee et al., 1993). During later postnatal development (P20-P40), IGFBP-2 is detectable in the pyramidal neurons of layer V in the parietal region of neocortex. As well, IGFBP-2 is expressed by astrocytes in the neocortex between P12 and P40 (Lee et al., 1993). IGF-I mRNA is not expressed in cells anatomically associated with those expressing IGFBP-2 in the neocortex. Rather, IGF-I is expressed diffusely throughout the cortex during early postnatal development (Bondy 1991; Lee et al., 1993). Similar results were found for IGFBP-2 expression in the hippocampal formation where IGFBP-2 is expressed by astrocytes at approximately the same time as IGF-I is expressed by interneurons, but the cells expressing IGF-I and IGFBP-2 are not anatomically related (Lee et al., 1993).

In most regions of the developing rodent brain, IGFBP-2 expression decreases during late postnatal development (P20-P40) (Lee et al., 1993). However, IGFBP-2 expression can be induced in astrocytes as a response to CNS injury (Lee et al., 1993; Breese et al., 1996; Sandberg-Nordqvist et al., 1996). For example, Lee and collaborators noted increased IGFBP-
2 expression in reactive astrocytes in the retina after experimental optic nerve transection (Lee et al., 1993).

In addition to the documented expression of IGFBP-2 in the brain as discussed above, studies have also shown that IGFBP-2 is actively secreted by astrocytes in the brain (Ocrant et al., 1990; Olson et al., 1991). Specifically, IGFBP-2 was shown to be secreted by astrocytes in vitro that were derived from P21 rats (Olson et al., 1991). However, IGFBP-2 secretion was not detected using astrocytes derived from newborn (P1) rat brain, suggesting that IGFBP-2 secretion is developmentally regulated (Olson et al., 1991). Production of IGFBP-2 by astrocytes appears to increase during astrocyte proliferation (Chesik et al., 2004). However, the actions of IGFBP-2 proteases are also upregulated during astrocyte proliferation, resulting in degradation of IGFBP-2 (Chesik et al., 2004). These results suggest a role for IGFBP-2 in modulating the actions of IGF-I on developing astrocytes. IGFBP-2 may also have a role in regulating oligodendrocyte development, as it has been shown to reduce the survival of rat oligodendrocyte precursors in vitro (Kuhl et al., 2002).

While astrocytes appear to be a key source of IGFBP-2, this binding protein is also the major IGFBP found in the CSF of adult rats, although IGFBP-3 is also present at lower levels in the CSF (Ocrant et al., 1990). As well, IGFBP-2 is produced by the choroid plexus in culture (Ocrant et al., 1990).

**IGFBP-3**

The expression of IGFBP-3 in the brain has been less well studied. As mentioned above, IGFBP-3 is present in the CSF of adult rats. Ocrant et al., (1990) showed that IGFBP-3 was secreted by C6 cells, a glioma derived cell line, and by astrocytes in vitro. As well, IGFBP-3 may be expressed in the brain following injury (Gluckman et al., 1992).

**IGFBP-4**

IGFBP-4 expression is detectable in some regions of the rodent brain (Brar and Chernausek, 1993). During early embryonic development (E15), IGFBP-4 is expressed in the meninges and choroid plexus. In later embryonic development (E20 in rats) IGFBP-4 expression is apparent in neurons within the emerging basal ganglia. In the early postnatal period (P1-P5), IGFBP-4 is expressed in the hippocampal formation, caudate putamen, and piriform cortex. Expression of IGFBP-4 appears to be restricted to neurons within these brain regions. By adulthood, IGFBP-4 mRNA is highly expressed in the cerebral cortex where it is specifically localized to layers II and IV. High expression levels are also detectable in the
cingulate cortex. As well, IGFBP-4 is expressed by the olfactory bulb, limbic system (hypothalamus and amygdala), basal ganglia and thalamus in adult rodents. Expression of IGFBP-4 in the meninges and choroid plexus persists into adulthood (Brar and Chernausek, 1993). Low level expression of IGFBP-4 is also apparent in the developing spinal cord (Arnold et al., 2000).

**IGFBP-5**

IGFBP-5 expression is highly abundant during the development of the rodent brain. In general, peak IGFBP-5 expression occurs during the first and second weeks of postnatal life (Bondy and Lee et al., 1993). However, IGFBP-5 is expressed in the CNS from early embryonic development (Green et al., 1994). Similar to IGFBP-2, IGFBP-5 expression is coordinated with that of IGF-I in some brain regions. Such patterns are noticeable in the neurons of sensory relay systems including the olfactory bulb, and vestibular nuclei (Bondy and Lee et al., 1993). In the developing cerebellum, IGFBP-5 and IGF-I are expressed in anatomically associated cells where IGF-I mRNA is visible in the Purkinje cells and IGFBP-5 is localized to the external germinal zone (Bondy and Lee, 1993).

In the developing cerebral wall, IGFBP-5 expression is contained in the lateral VZ and subventricular zone (SVZ). Expression of IGFBP-5 decreases in the VZ in accordance with the disappearance of this zone. However, IGFBP-5 mRNA is detectable in persistent SVZ cells into adulthood (Bondy and Lee, 1993). During postnatal development, IGFBP-5 is expressed by pyramidal neurons located throughout layers III through V of the neocortex. Expression of IGFBP-5 is not developmentally or anatomically associated with that of IGF-I in the cerebral cortex. As well, while IGF-I expression tends to be low in white matter regions (Bartlett et al., 1991; Bondy 1991), expression of IGFBP-5 is evident in the subcortical white matter beginning at P12 (Bondy and Lee, 1993). Expression of IGFBP-5 is also visible in the developing spinal cord, where it is highest during embryonic development (Arnold et al., 2000).

Measurable expression of the IGFBP-5 gene is apparent in the adult rodent brain, where high expression is localized to the diencephalon and meninges (Stenvers et al., 1994). IGFBP-5 expression is evident in the adult rodent cerebral cortex; however, the level of expression is very low in the cortex. As well, IGFBP-5 is present in the thalamus and in mesenchymal structures throughout the adult brain (Stenvers et al., 1994).
In vivo studies of Tg mice that overexpress IGF-I in the brain during postnatal development indicate that IGF-I may regulate the expression of IGFBP-5 (Ye and D’Ercole, 1998). In MT-I/IGF-I Tg mice the cerebral cortex displays the highest level IGF-I transgene expression and IGFBP-5 expression is increased by 200% in this region. Conversely, in IGF-II/IGF-I Tg mice the highest level of transgene expression is in the cerebellum, and expression of IGFBP-5 is increased by 350% in this region. IGFBP-5 protein levels are also increased in the brains of both lines of Tg mice (Ye and D’Ercole, 1998). In vitro studies of vascular smooth muscle cells indicate that IGF-I-mediated IGFBP-5 expression requires activation of the PI3-kinase-Akt-p70 S6 kinase pathway (Duan et al., 1999).

IGFBP-6

Recent evidence indicates that IGFBP-6 is expressed in the rodent brain during normal development. Expression of IGFBP-6 is developmentally regulated with low levels of expression during embryonic and early postnatal development (Naeve et al., 2000). High levels of IGFBP-6 are found in the adult rat brain, particularly in the brain stem. The cerebral cortex is also a region of high IGFBP-6 expression in the adult rat, with abundant expression evident in the pyramidal cells of layers II/III and V and in the cingulate and retrosplenial cortices (Naeve et al., 2000). Interestingly, IGFBP-6 is expressed in all of the cranial nerves. Expression of IGFBP-6 is localized to γ-aminobutyric acid (GABA)ergic interneurons in the hippocampus and to the GABAergic basket cells of the cerebellum, although other types of interneurons do not express IGFBP-6 (Naeve et al., 2000).

The exact functions of IGFBP-6 in the rodent brain are unclear at this time. However, IGFBP-6 may have a role in modulating the functions of IGF-II in the brain, as it binds to IGF-II with high affinity (Naeve et al., 2000). In Tg mice in which human IGFBP-6 is expressed using a glial fibrillary acid protein (GFAP) promoter, the cerebellum is reduced in both weight and size and the number of GFAP positive astrocytes is decreased (Bienvenu et al., 2004). As well, IGFBP-6 has been shown to reduce both the survival and differentiation of oligodendrocyte precursor cells (Kuhl et al., 2003). IGFBP-6 has also been shown to arrest the growth and proliferation of neuroblastoma tumor cells (Babajko et al., 1997; Grelier et al., 1998; Seurin et al., 2002). Taken together, these results support a role for IGFBP-6 in brain growth and development.
1.8 IGF-II and IGF-II/mannose-6-phosphate receptor expression in the rodent brain

IGF-II is present in the rodent brain from early development. However, unlike IGF-I mRNA which is found in many regions of the brain, IGF-II is predominantly expressed in choroid plexus, meninges, and blood vessels (Hynes et al., 1988; Bondy et al., 1990, 1992; Ayer-Le Lievre et al., 1991; Lee et al., 1993; Logan et al., 1994; Couce et al., 1992). IGF-II mRNA is detectable in these structures from embryonic development through to maturity (Bondy et al., 1992). Within the choroid plexus, IGF-II is found in the epithelium where it may serve an autocrine role in promoting growth of the epithelial cells (Nilsson et al., 1996). Studies have also documented the expression of IGF-II by glial and granule cells in the cerebellum and by cells in the anterior pituitary (Gonzalez-Parra et al., 2001; Hetts et al., 1997). Interestingly, expression of the IGF-II gene in the cerebellum has been shown to be regulated by parental imprinting. In cerebellar granule cells only the paternal allele is expressed, whereas in the choroid plexus and meninges both alleles are expressed (Hetts et al., 1997).

Immunohistochemical studies have documented the presence of IGF-II in a wider range of brain regions than indicated by IGF-II gene expression studies. For example, IGF-II immunoreactivity is apparent in the medial basal hypothalamus, hippocampal glial cells, and brain stem neurons (Sullivan and Feldman, 1994). Studies indicate that IGF-II and IGFBP-2 are often colocalized in the developing brain within myelin sheaths of axons and in myelinated nerve tracts (Logan et al., 1994). In vitro studies indicate that both proteins are secreted by cells of the choroid plexus and by meningeal cells (Ishikawa et al., 1995; Nilsson et al., 1996). These findings indicate that IGFBP-2 may modulate the actions of IGF-II in the developing brain. Further evidence for an interaction between IGFBP-2 and IGF-II in the brain is derived from studies of Tg mice that overexpress IGF-II in the brain. In these mice, the expression of IGFBP-2 is upregulated 10-fold in the medulla oblongata (Reijnders et al., 2004).

Expression of the IGF-II/mannose-6-phosphate receptor (IGF2R) has been documented in the rodent brain during development (Gammeltoft et al., 1985). IGF2R mRNA is present in the hippocampus, and at lower levels in the choroid plexus and meninges (Couce et al., 1992). Expression of IGF2R is also extensive in the brain stem, where it has been documented in both motor and sensory nuclei (Nagano et al., 1995). IGF-II binding sites are more widespread in the brain and have been noted in regions such as the olfactory bulbs, cerebral cortex, anterior pituitary, hippocampus, and pineal gland (Smith et al., 1988; Dore et al., 1997). An abundance
of binding sites for IGF-II is obvious in the granule cell layers of the cerebellum (Mendelsohn et al., 1988; Kar et al., 1993). These binding sites likely represent IGF-II binding to IGF2R, but may also correspond to IGF-II binding to the IGF1R. Immunohistochemical studies confirm the presence of IGF2R in the rodent brain. High levels of IGF2R are apparent in the cortex and hypothalamus during early development. Expression in these regions tapers off at birth and further decreases during early postnatal development (Valentino et al., 1990). IGF2R immunoreactivity is also visible in the choroid plexus, pituitary, blood vessels, and retina from early embryonic development through to maturity (Valentino et al., 1990). Couce et al., (1992) documented IGF2R immunoreactivity in neurons of the forebrain, granule cells of the dentate gyrus, and pyramidal cells of the hippocampus. IGF2R immunoreactivity was not evident in glia (Couce et al., 1992).

1.9 Interaction of IGF-I with the blood brain barrier

Numerous studies, as discussed in the above sections, have shown that IGF-I is expressed and synthesized in the rodent brain. However, IGF-I is also abundant in circulation, raising the possibility that IGF-I may be taken up from circulation and cross the blood brain barrier to reach the brain. Early in vitro studies showed that IGF-I could both bind to and be endocytosed by brain microvessels (Frank et al., 1986; Rosenfeld et al., 1987; Duffy et al., 1988). As well, these studies demonstrated that IGF1R is present on brain microvessels (Rosenfeld et al., 1987; Duffy et al., 1988). More recently, radiolabeled IGF-I has been shown to cross the blood brain barrier following carotid artery infusion of the ligand (Reinhardt and Bondy, 1994). In these studies, radiolabeled IGF-I was detectable in the choroid plexus, cerebral vasculature, median eminence, and within the brain parenchyma (Reinhardt and Bondy, 1994). IGF-I also crosses the blood brain barrier following intravenous administration of radiolabeled ligand. Radiolabeled IGF-I was detectable in the brain parenchyma as early as 20 min post-injection (Pan and Kastin, 2000). Evidence indicates that IGF-I crosses the blood brain barrier into the CSF by way of a carrier-mediated uptake system (Pulford and Ishii, 2001). This carrier system appears to function without influence from IGFBPs, IGF1R, and IGF2R, as IGF-I analogs that have reduced affinity for either the IGFBPs or the IGF receptors cross into the CSF (Pulford and Ishii, 2001). The ability of IGF-I to cross the blood brain barrier is consistent with studies showing that peripheral administration of IGF-I promotes neurogenesis in the dentate gyrus (Aberg et al., 2000), ameliorates cognitive decline in diabetic
rats (Lupien et al., 2003), and improves neurological functioning after brain injury (Hatton et al., 1997; Saatman et al., 1997; Fernandez et al., 1988; Pulford et al., 1999).

1.10 Regulation of IGF-I gene expression in the brain

Relatively few studies have addressed the factors responsible for regulating IGF-I gene expression in the brain. GH, a major regulator of IGF-I expression in the liver (Daughaday and Rotwein, 1989), appears to be involved in regulation of IGF-I in the brain. Early evidence for GH-mediated regulation of IGF-I gene expression came from studies of hypophysectomized rats, in which the pituitary is surgically removed. In these rats, the quantity of IGF-I mRNA is reduced in the brain (Hynes et al., 1987). Intracerebral GH administration ameliorates the reduction in IGF-I mRNA, increasing levels to 80% of normal (Hynes et al., 1987). Similarly, IGF-I mRNA is reduced in the hypothalamus of hypophysectomized rats by approximately 60% and peripheral infusion of GH results in increased IGF-I mRNA abundance (Wood et al., 1991). Studies of Tg mice that express an IGF-I-luciferase fusion gene provide further evidence for GH-mediated IGF-I gene expression in the brain. In these mice, luciferase is expressed under the control of regulatory sequences from the rat IGF-I gene in neurons and astrocytes during early postnatal development (Ye et al., 1997). Administration of GH to Tg mice via intraperitoneal injection results in increased transgene expression in the olfactory bulb, cerebral cortex, hippocampus, and brain stem (Ye et al., 1997). These results suggest that GH upregulates IGF-I expression in several brain regions. GH receptors are expressed in a number of brain regions coincident with peak IGF-I gene expression in the brain (Garcia-Aragon et al., 1992; Lobie et al., 1993), further supporting a role for this hormone in regulating IGF-I expression during brain development.

The effects of glucocorticoids on IGF-I expression have also been analysed in the developing brain. Addition of dexamethasone to neurons and glia in vitro appears to decrease IGF-I mRNA levels (Adamo et al., 1988). In Tg mice expressing an IGF-I-luciferase fusion gene, as described above, dexamethasone administration by i.p. injection significantly decreases transgene luciferase activity in the cerebral cortex, brainstem, cerebellum, and hippocampus. As well, transgene mRNA abundance is significantly reduced in the cerebral cortex in these mice following dexamethasone administration (Ye et al., 1997), suggesting that dexamethasone is a negative regulator of IGF-I expression in the brain.

Nutritional status appears to have regulatory effects on IGF-I expression in the brain. For example, fasting for a 48 hr period has been shown to reduce IGF-I mRNA by 35% in the
brains of adult rats (Lowe et al., 1989), and fasting for 72 hr decreases IGF-I mRNA in the rat hypothalamus by 65% (Olchovsky et al., 1993). Expression of IGF1R appears to be unaffected in these experiments (Lowe et al., 1989; Olchovsky et al., 1993). Conversely, undernutrition results in increases in IGF-I mRNA levels in several brain regions of mice during early postnatal development. At P7, IGF-I mRNA abundance is increased in the cerebral cortices of insufficiently nourished pups, but decreased in the diencephalon (Calikoglu et al., 2001). By P14, IGF-I mRNA is upregulated in the diencephalon in undernourished mice, suggesting that the effects of nutritional status on IGF-I expression depend on the stage of development and brain region under study (Calikoglu et al., 2001). Studies of cultured C₄ glioma cells indicate that glucose is capable of upregulating IGF-I expression, further supporting a role for nutrition in regulating IGF-I expression in the brain (Straus and Burke, 1995). However, several studies have shown that altered nutritional status has minimal or no effect on IGF-I expression in the brain (Philipps et al., 1989; Shambaugh et al., 1995). For example, protein restriction during early postnatal development appears to have only minimal effects on reducing IGF-I mRNA in the cerebellum (Shambaugh et al., 1995).

IGF-I gene expression in the brain also appears to be influenced by other growth regulatory factors. Cultured neural crest cells (Drago et al., 1991) and hypothalamic neurons (Pons and Torres-Aleman, 1992) release IGF-I in response to stimulation by basic fibroblast growth factor (bFGF). As well, primary rat astrocytes in culture express increased levels of IGF-I when stimulated with epidermal growth factor (EGF). Estrogens also influence IGF-I expression in the brain. In monkeys, treatment with estrogen results in upregulation of IGF-I mRNA expression in the brain with no apparent effect on IGF1R expression (Cheng et al., 2001).

Numerous studies indicate that IGF-I expression is upregulated in the brain in response to injury. In many cases, astrocytes appear to be the site of IGF-I expression following injury (Yamaguchi et al., 1991; Garcia-Estrada et al., 1992; Komoly et al., 1992; Lee et al., 1992a; Yao et al., 1995). Various experimental injury models produce upregulated IGF-I expression in astrocytes including models of hypoxia/ischemia (Lee et al., 1992a), and demyelination (Komoly et al., 1992). IGF-I is also upregulated in animal models of multiple sclerosis (Liu et al., 1994, 1995). These studies suggest that injury is an important mediator of IGF-I gene expression. The anti-apoptotic functions of IGF-I following injury are discussed in Chapter 6.
Taken together, the above findings suggest that numerous factors are involved in the regulation of IGF-I expression in the brain. These factors include GH, nutritional status, developmental stage, and injury. Further studies are required to precisely determine the relative roles of each of these factors in controlling IGF-I gene expression in the brain.

1.11 General effects of IGF-I on the brain

Current evidence indicates that IGF-I has an important role in brain growth and development. IGF-I has been reported to serve a number of functions during brain development. Various *in vitro* studies indicate that IGF-I promotes both neuron (Lenoir and Honegger, 1983; DiCicco-Bloom and Black, 1989; Drago et al., 1991; Arsenijevic et al., 2001) and oligodendrocyte precursor proliferation (McMorris and Dubois-Dalcq, 1988; Mozell and McMorris, 1991). IGF-I also appears to promote both neuronal and oligodendroglial differentiation *in vitro* (Mozell and McMorris, 1991; Nataf and Monier, 1992; Arsenijevic and Weiss, 1998). IGF-I has been shown to increase neuron and glia survival *in vitro* by inhibiting apoptosis (Aizeman and DeVellis, 1987; Torres-Aleman et al., 1990a, 1990b, 1992; Pons et al., 1991; Ang et al., 1992, 1993; Barres et al., 1992; D’Mello et al., 1993; Hughes et al., 1993; Neff et al., 1993; Sortino and Canonico, 1996). As well, IGF-I has been shown to enhance neurite outgrowth from several types of neurons including motor (Caroni and Grandes, 1990), hypothalamic (Torres-Aleman et al., 1990b), cortical (Aizeman and DeVellis, 1987), and sympathetic neurons (Zackenfels et al., 1995). IGF-I stimulates the growth of dendrites in the rat somatosensory cortex, as illustrated using organotypical slice cultures (Niblock et al., 2000). In these studies, IGF-I was shown to increase both the total length of dendritic segments and the number of dendritic branches (Niblock et al., 2000). Studies suggest that IGF-I also promotes neuronal cell body hypertrophy (Aizeman and DeVellis, 1987; Liu and Lauder, 1992). Taken together, these results indicate that IGF-I is an important factor influencing various aspects of brain development.

1.12 Evidence from mouse models of IGF-I overexpression in the brain

Mouse models of IGF-I function strongly support a role for IGF-I in regulating the development of the CNS. Various lines of mice carrying genomic alterations of IGF-I and related proteins have been generated, and many of these mice exhibit specific brain phenotypes. Analyses of Tg mice in which IGF-I is overexpressed in the brain indicate that IGF-I acts to increase brain weight and volume (Gutierrez-Ospina et al., 1996; Ye et al., 1996; Dentremont et al., 1999; O’Kusky et al., 2000). In IGF-II/IGF-I Tg mice, IGF-I is
overexpressed in the brain during postnatal development under control of regulatory sequences from the mouse IGF-II gene, with the highest level of transgene expression apparent in the cerebellum (Ye et al., 1996). Brain weight is increased by 35% in adult IGF-II/IGF-I Tg mice, and cerebellar weight is increased by 90% at the same age (Ye et al., 1996). Additionally, the weights of the hippocampus (34%), diencephalon (28%), brainstem (28%), and cerebral cortex (9%) are significantly increased in IGF-II/IGF-I mice (Ye et al., 1996). Body weight does not differ between Tg and normal littermate control mice, as transgene expression is not detectable outside of the brain (Ye et al., 1996). Consistent with high transgene expression in the cerebellum, increases in the volume of the internal granule cell layer (92%), total number of granule neurons (82%), and total number of Purkinje cells (20%) are also apparent in these mice (Ye et al., 1996). Increased neuron number in the cerebella of IGF-II/IGF-I mice has been shown to result from a combination of IGF-I-mediated increased proliferation and decreased apoptosis (Ye et al., 1996; Chrysis et al., 2001).

In addition to increases in cerebellar volume and neuron number, increases in the volumes of brainstem structures including the medulla (27%), nucleus of the solitary tract (59%), dorsal motor nucleus of the vagus (84%), hypoglossal nucleus (29%), and facial nucleus (21%) are also apparent in IGF-II/IGF-I Tg mice (Dentremont et al., 1999). Interestingly, total neuron number is increased only in the nucleus of the solitary tract (50%) and in the nucleus of the vagus (53%) in Tg mice. Given that neuron production in these nuclei occurs during early embryonic development well before transgene expression is initiated, these increases in neuron number likely result from IGF-I-mediated inhibition of neuron death by apoptosis (Dentremont et al., 1999; D’Ercole et al., 2002). While total neuron number does not differ from controls in the hypoglossal nucleus or in the facial nucleus, the numerical density of neurons is decreased in both regions and neuronal profile area is increased. These results suggest that increased volume in the facial and hypoglossal nuclei of IGF-II/IGF-I mice results from increases in neuropil volume and cell size, consistent with in vitro evidence indicating that IGF-I promotes neurite outgrowth and cell body hypertrophy. The finding that IGF-I overexpression does not increase total neuron number in these brainstem nuclei is likely due to the fact that in these regions both neuron production and neuron elimination by apoptosis occur during embryonic development, well before the initiation of transgene expression in IGF-II/IGF-I Tg mice (Dentremont et al., 1999; D’Ercole et al., 2002).
Studies of the hippocampal formation of IGF-II/IGF-I Tg mice indicate that IGF-I influences the growth and development of this brain region. In the dentate gyrus, the volume of both the granule cell layer and molecular layer are increased during postnatal development by 27-69% (O'Kusky et al., 2000). As well, total neuron number in the granule cell layer is increased by 29-61% from P14 to P130, with no apparent change in the numerical density of neurons at any age. Transgene expression in IGF-II/IGF-I mice coincides with the periods of both neuron production and neuron elimination by apoptosis in the developing dentate gyrus. Thus, the observed increase in neuron number in the granule cell layer of these mice likely results from a combination of enhanced neuronal proliferation and inhibited apoptosis (O’Kusky et al., 2000).

Analyses of other lines of IGF-I Tg mice provide further evidence that IGF-I augments brain growth and development. In MT-I/IGF-I Tg mice, IGF-I expression is elevated in the brain during postnatal development under the control of a metallothionein-I promoter. The highest level of transgene expression is localized to the cerebral cortex in these mice (Ye et al., 1995a; Gutierrez-Ospina et al., 1996). Various lines of MT-I/IGF-I Tg mice exhibit increases in brain weight ranging from 22-91%, depending on the level of transgene expression in particular lines of these mice (Mathews et al., 1988; Behringer et al., 1990; Ye et al., 1995a; Gutierrez-Ospina et al., 1996). The area of the cortex is increased by 22-91% in lines of adult (P90) MT-I/IGF-I Tg mice, consistent with high transgene expression in this region. Within the S1 barrel field of the primary somatosensory cortex, the average number of neurons is increased in individual barrels in Tg mice by 24% and neuronal cell area is increased by 33%. The density of neurons in these structures is decreased by 39%, indicating that IGF-I may increase neuropil volume within the cortex (Gutierrez-Ospina et al., 1996). The production of neurons destined for the cerebral cortex occurs during embryonic development in mice (Takahashi et al., 1995a), well before expression of the IGF-I transgene in MT-I/IGF-I mice. Therefore, the increase in cortical neuron number documented in MT-I/IGF-I mice most likely results from reduced neuronal apoptosis in response to elevated IGF-I (Gutierrez-Ospina et al., 1996).

IGF-I Tg mice in which IGF-I is overexpressed specifically in astrocytes (IGF-IAst/Tet-Off) exhibit similar increases in brain weight (34%) during postnatal development when compared to the above discussed lines of Tg mice (Ye et al., 2004). As well, both the content of DNA (21%) and protein (37%) are increased in whole brain samples. In the dentate gyrus the
volume of the granule cell layer is increased by 55% and the total number of neurons within this layer is increased by 49% in Tg mice as compared to normal littermate controls (Ye et al., 2004). These studies suggest that elevated IGF-I expression restricted to astrocytes promotes neurogenesis in the dentate gyrus, indicating that IGF-I may act in a paracrine manner to influence neuron development.

The effects of IGF-I on synaptogenesis have been studied in mouse models with elevated expression of IGF-I. IGF-I increases synaptogenesis within the developing hippocampal dentate gyrus of IGF-II/IGF-I Tg mice (O’Kusky et al., 2000). As noted previously, transgene expression occurs during postnatal development in these mice (Ye et al., 1996). In Tg mice, the total number of synapses in the molecular layer is increased after the second postnatal week and peaks at P28 (105% increase in Tg mice). The $N_v$ of synapses is 36% greater in Tg mice at P28 and 21% greater at P35, and there is a corresponding increase in synapse-to-neuron ratio at these ages in Tg mice (60% increase at P28, 24% increase at P35). However, while the total number of synapses is still greater in Tg mice, the synapse-to-neuron ratio does not differ between Tg and control animals by adulthood (P130). In adult Tg animals, the increase in synapse number corresponds to the increase in neuron number documented in the granule cell layer of these mice (O’Kusky et al., 2000).

IGF-I has also been shown to augment synaptogenesis in the hypoglossal nucleus of IGF-II/IGF-I Tg mice (O’Kusky et al., 2003). As discussed earlier, the volume of this nucleus is increased in Tg mice due to increased neuropil volume without a corresponding change in neuron number (Dentremont et al., 1999). In Tg animals, the $N_v$ of synapses in the hypoglossal nucleus does not differ from controls at any time during postnatal development. However, total synapse numbers are increased by 42-52% after P14 in Tg mice. Synapse numbers are seen to decrease in both groups by adulthood, suggesting that IGF-I augments only the progressive phase of synaptogenesis and has no effect on synapse elimination (O’Kusky et al., 2003). An effect of IGF-I on synaptogenesis is also apparent in MT-I/IGF-I Tg mice. In these mice, the number of synapses is increased in cortical columns within the primary somatosensory cortex without a corresponding change in synapse density (Gutierrez-Ospina et al., 2004).

In addition to promoting increased neuron numbers in Tg mice, elevated levels of IGF-I also appear to stimulate increases in the number of oligodendrocyte progenitors and mature oligodendrocytes. Initial studies of MT-I/IGF-I Tg mice did not find any increase in the
number of carbonic anhydrase II-positive oligodendrocytes (Carson et al., 1993). However, subsequent studies using different markers for oligodendrocytes (NG2, proteolipid protein) found increases in the numbers of oligodendrocytes and precursors in the cerebral cortex (18%) and corpus callosum (37%) of MT-I/IGF-I Tg mice (Ye et al., 1995a; Mason et al., 2000). In IGF-I Tg mice where elevated IGF-I expression is restricted to astrocytes (IGF-I<sup>Ast/Tet-Off</sup>), the number of oligodendrocytes is increased in the corpus callosum by 12% (Ye et al., 2004). This study indicates that elevated IGF-I expression augments oligodendrocyte number even when the IGF-I transgene is not expressed in oligodendrocytes themselves, suggesting that IGF-I can function in a paracrine manner to influence oligodendrocyte numbers.

1.13 Evidence from mouse models of deficient IGF-I expression in the brain

Deficient expression of IGF-I in the developing mouse brain results in brain growth retardation and measurable decreases in neuron number. In IGF-I<sup>−/−</sup> mice, brain weight is decreased by 38% in 2 month old animals. Body weight is also decreased at this age, but by an even greater magnitude (74%) in comparison to wild type mice (Beck et al., 1995). Several brain regions exhibit decreased volume including the granule cell layer of the dentate gyrus (59%), hippocampus (38%), and striatum (28%). In general, cell density is increased in IGF-I<sup>−/−</sup> mice likely as a result of reduced neuropil volume. In a number of brain regions, neuron numbers appear to be unaffected by the lack of IGF-I expression, such as in the striatum and basal forebrain where the numbers of cholinergic neurons do not differ from controls. The number of dopaminergic neurons in the mesencephalon also appears to be normal in IGF-I<sup>−/−</sup> mice. However, decreased neuronal cell numbers are apparent in some brain regions. For example, the number of parvalbumin immunoreactive neurons is decreased in the dentate gyrus (59%), striatum (52%), and hippocampus (32%) of null mutant mice (Beck et al., 1995). IGF-I<sup>−/−</sup> mice demonstrate abnormal inner ear development, as evidenced by decreases in cochlear (34%) and cochlear ganglion (27%) volumes (Camarero et al., 2001). Neuron number in the cochlear ganglion of IGF-I<sup>−/−</sup> mice is reduced (22%), as is the mean volume of individual neuronal cell bodies within this structure (Camarero et al., 2001).

Further support for IGF-I's role in regulating brain development comes from studies of IGFBP-1 Tg mice. These mice express elevated IGFBP-1 in the brain, which results in apparent inhibition of IGF-I function in the brain (D'Ercole et al., 1994). Brain weights are decreased by 8-16% in IGFBP-1 Tg mice during early postnatal development (D'Ercole et al., 1994). In lines of adult IGFBP-1 Tg mice brain weights are reduced by 22-24%. Regional
decreases in weight are also apparent in IGFBP-1 mice, with the hippocampus (20%), cerebral cortex (18%), and diencephalon (12%) all exhibiting reductions in weight (Ye et al., 1995a). The area of the cerebral cortex has been shown to be reduced by 22-24% in these mice, and a corresponding 10% decrease in neuronal profile area and 39% decrease in neuronal density have also been demonstrated within the barrel field of the primary somatosensory cortex (Gutierrez-Ospina et al., 1996). As well, the average number of neurons within individual barrels is decreased by 15% in IGFBP-1 Tg mice when compared to normal littermate controls (Gutierrez-Ospina et al., 1996). In general, these results are similar to those noted for IGF-I-/- mice. Therefore, current evidence indicates that brain growth and development are hindered by the absence of IGF-I, suggesting that this growth factor is an important regulator of normal brain development.

Mice with deficiencies in IGF-I expression exhibit altered synaptogenesis, further supporting a role for IGF-I in this process. Abnormal synaptophysin immunostaining patterns have been demonstrated in the cochlear ganglion and organ of Corti in IGF-I-/- mice during early postnatal development, suggesting that IGF-I may be necessary for synapse refinement in these structures (Camarero et al., 2001). As well, IGFBP-1 Tg mice exhibit decreased synapse numbers within cortical columns of the primary somatosensory cortex (Gutierrez-Ospina et al., 2004). Therefore, evidence from both mouse models of deficient IGF-I expression and models of IGF-I overexpression indicates that IGF-I promotes synaptogenesis during normal development of the brain.

Studies of mice with deficient IGF-I expression support a role for IGF-I in oligodendrocyte development. Certain studies indicate that the number of oligodendrocytes in the brains of IGF-I-/- mice is reduced (Beck et al., 1995). However, Cheng and collaborators did not find any decreases in oligodendrocyte number in IGF-I-/- mice; rather they reported that the number of oligodendrocytes in these mice is proportional to brain weight (Cheng et al., 1998). Discrepancies in the results of studies of IGF-I-/- mice may reflect compensatory actions of IGF-II on oligodendrocyte development in the absence of IGF-I (D’Ercole et al., 2002). In IGFBP-1 mice, there is a slight reduction in the number of oligodendrocytes in the corpus callosum (5%), suggesting that oligodendrocyte development is impaired in the absence of functional IGF-I activity. Taken together, the above results indicate that IGF-I influences oligodendrocyte development in vivo.
Abnormal development of the brain is also apparent in mice with null mutations of the IGF1R gene (IGF1R<sup>-/-</sup>). IGF1R<sup>-/-</sup> mice exhibit increased cellular density in the mantle zone of the spinal cord and in the brainstem during embryonic development, which appears to result from a decrease in neuropil volume (Liu et al., 1993). Thus, mice with ablated expression of IGF-I and its cognate receptor IGF1R generally exhibit opposite effects on brain growth, volume, and neuron number when compared to mice with elevated IGF-I levels, consistent with an important role for IGF-I in regulating brain development.

1.14 Overview of cerebral cortical development

The adult mammalian cerebral neocortex is a laminar structure composed of six individual layers. Each layer in the cortex possesses a characteristic neuronal density and is composed of pyramidal neurons and nonpyramidal or granular neurons (Jacobson 1991; McConnell, 1995). In certain layers, such as layer V, pyramidal neurons are the dominant neuronal population, whereas in other layers (e.g. layer IV) nonpyramidal neurons dominate. The characteristic morphological appearances of each layer derive from these differences in neuronal composition. As well, the cerebral cortex is divided into different cytoarchitectonic areas, each of which serves distinct functions.

The development of the neocortex begins with production of the preplate, which contains the earliest generated neurons that have migrated away from the proliferative zones of the cerebral wall (Mountcastle 1997). Subsequently generated neurons migrate through the intermediate zone (IZ), a region distinguished by the presence of migrating newly born neurons and their developing processes, to form the cortical plate (Jacobson, 1991). The cortical plate is a transient structure that ultimately gives rise to the layers II-VI of the cortex (Jacobson 1991; Mountcastle 1997). The cortical plate inserts into the cortical preplate, which results in the splitting of the preplate into the marginal zone (MZ) and the subplate (Mountcastle 1997). The MZ is located at the farthest edge of the developing cerebral wall, above the cortical plate and adjacent to the pial surface. The MZ contains some of the earliest generated cells of the cortex, the cortical pioneer neurons and Cajal-Retzius cells, and will ultimately develop into layer I of the neocortex (Bayer and Altman, 1990; Derer and Derer, 1990; Hestrin and Armstrong, 1996; Meyer et al., 1998).

The subplate is located below the cortical plate, adjacent to the IZ. The subplate is a transient structure populated by neurons that, in mice, are generally fated to die (Price et al., 1997; Wood et al., 1992; Gilles and Price, 1993). However, prior to its elimination by cell
death, the subplate seems to be important for guiding thalamocortical projections to their target
cells (Ghosh et al., 1990; Ghosh and Shatz, 1992a; Allendorfer and Shatz, 1994) and for the
refinement of cortical afferents (Ghosh and Shatz, 1992b). In rodents, the subplate is small,
while in more phylogenetically advanced species, the subplate is a great deal larger (Wood et
al., 1992; Allendorfer and Shatz, 1994; Price et al., 1997). Neurons generated after the
splitting of the preplate migrate into the cortical plate. In general, the neurons of the deepest
cortical layers (e.g. layers VI and V) are formed first, followed by the generation of neurons
that reside in upper cortical layers (IV, III, and II). Thus, the cortical layers are generated in an

The cerebral hemispheres are derived from the dorsal telencephalon of the neural tube
(Jacobson 1991). Projection neurons of the cerebral cortex are produced by progenitor cells
located in the morphologically defined VZ and SVZ zones of the developing cerebral wall
(reviewed in Parnavelas, 2000). Neurogenesis in the VZ is initiated in rostral lateral regions of
the cerebral wall and subsequently proceeds rostrocaudally and lateromedially as development
progresses (Hicks and D’Amato, 1968; Caviness and Sidman, 1973; Miyama et al., 1997). The
VZ consists of radially aligned neuron progenitor cells and lies adjacent to the lateral ventricle
(Jacobson 1991; McConnell 1995). These cells undergo characteristic interkinetic nuclear
movements during different phases of the cell cycle, such that cell somata move away from the
ventricular surface during S phase, and ultimately descend back to the ventricle to undergo M
phase (Jacobson, 1991). Most VZ cells have the morphological appearance of radial glial cells
(Noctor et al., 2002), which were initially thought to serve only as guides for migrating
neurons (Rakic, 1972). However, in recent years radial glial cells have been identified as the
predominant neuron progenitor cells in the VZ, responsible for producing a large proportion of
cortical projection neurons (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001,
2002; Heins et al., 2002).

The SVZ, which is composed of randomly oriented cells, overlies and is derived from
the VZ. This zone first appears several days after the initial development of the VZ (Takahashi
et al., 1995b). Early studies of the SVZ revealed its ability to produce glial cells (Levinson
and Goldman, 1993) and olfactory bulb neurons (Luskin, 1993). However, more recent studies
indicate that the SVZ is a source of cortical projection neurons (Tarabykin et al., 2001;
Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Englund et al., 2005). The
neuron progenitors in the SVZ characteristically divide away from the ventricular surface (i.e.
at non-surface or basal positions) and have been termed intermediate progenitor cells. The exact contribution of these progenitor cells to the overall complement of cortical projection neurons is the subject of many current studies. These studies indicate that intermediate progenitor cells may produce more than half of the projection neurons in the cortex (Tarabykin et al., 2001; Haubensak et al., 2004; Miyata et al., 2004).

Recent evidence indicates that cortical GABA-producing interneurons are produced in the ventral telencephalon (subpallium) and migrate into the cerebral cortex during embryonic development (Anderson et al., 1997, 1999, 2002; Lavdas et al., 1999; Witchterle et al., 2001). Few of these interneurons appear to be produced by progenitors in the VZ and SVZ of the dorsal telencephalic wall (Anderson et al., 2002). GABAergic interneurons have been shown to migrate tangentially from the ventral telencephalon through the MZ and descend into the cortical plate where they reach their appropriate positions and integrate with projection neurons (Ang et al., 2003).

1.15 The kinetics of neuron production in the cerebral wall

The total number of projection neurons produced during the period of cortical neurogenesis is influenced by a number of factors, including cell cycle kinetics and the fates of cells following mitosis (reviewed in Caviness et al., 1995, 1999, 2003). Neurogenesis in the cortex of the mouse occurs from E11 to E17. Essentially all of the progenitor cells within the VZ are actively dividing between E11 and E16 (Takahashi et al., 1993, 1995a). Individual phase lengths for each component of the cell cycle have been determined at each of ages E11 through E16. On E11 the total cell cycle is approximately 8 hr (Takahashi et al., 1995a). As neurogenesis progresses, the length of the cell cycle increases, reaching a maximum of approximately 18 hr on E16. The increase in cell cycle length during neurogenesis results from a four-fold increase in G1 phase length, with no significant alterations in the lengths of the S, G2, and M phases (Takahashi et al., 1995a). During the 6 day period of neurogenesis, cells in the VZ complete a total of 11 cell cycles. As the rate of cell division is higher during early neurogenesis, the majority of these 11 cell cycles are completed by the midpoint of neurogenesis (Takahashi et al., 1995a). Consistent with the rostral to caudal gradient of neurogenesis, rostral regions of the cerebral wall are developmentally advanced in comparison to caudal regions, and this developmental difference is reflected in the cell cycle parameters of the two regions. On a given embryonic day, the cell cycle is longer in rostral regions than it is in caudal regions, owing to the earlier initiation of
neurogenesis rostrolaterally. However, cells in all regions ultimately undergo 11 rounds of cell division (Miyama et al., 1997).

Neuron output from the cerebral proliferative zones is also dependent upon the fate decisions of cells following a given round of mitosis. Progenitor cell divisions produce two daughter cells. One or both daughter cells may either exit the cell cycle as a post-mitotic neuron and migrate away from the proliferative zones to the cortical plate. The fraction of cells that exits the cell cycle is termed the leaving fraction. Alternatively, the daughter cells may retain their proliferative potential and undergo further rounds of division. As discussed above, these progenitor cells may either remain as radial glia in the VZ, or the radial glial progenitors may produce intermediate progenitor cells which migrate into the SVZ (Noctor et al., 2004; Englund et al., 2005). The fraction of cells that re-enters the cell cycle is termed the proliferative fraction.

During early neurogenesis, the majority of cells produced by progenitor divisions remain in the cell cycle (Takahashi et al., 1994, 1996). The large proliferative fraction leads to expansion of the progenitor population. As neurogenesis progresses more cells exit the cell cycle as post-mitotic neurons, and the leaving fraction increases correspondingly (Takahashi et al., 1994, 1996). The switch from progenitor expansion to neuron output occurs when the proliferative fraction decreases below 0.5 and the leaving fraction increases above 0.5. In mice, this switch normally occurs on E14 (Takahashi et al., 1996). At the end of neurogenesis (E17 in mice) the majority of the progenitor cells in the VZ exit the cell cycle, and the VZ is depleted as a result.

1.16 Apoptosis during normal development of the rodent cerebral cortex

During normal development of the cerebral cortex as many as 50% of the neurons produced during cortical neurogenesis are eliminated by a process known as naturally occurring programmed cell death (Oppenheim, 1991). The majority of neurons eliminated by programmed cell death in the cortex have been identified as undergoing cell death by apoptosis (Kerr et al., 1972). Apoptosis is characterized morphologically by a number of features including condensation of chromatin, cytoplasmic shrinkage, nuclear pyknosis, DNA fragmentation, membrane blebbing, and the development of membrane bound apoptotic bodies (Kerr et al., 1972; reviewed in Yuan et al., 2003). In addition to occurring during normal development, apoptotic cell death is also a feature of many neurodegenerative diseases.
Apoptosis in the normal cerebral cortex occurs from early embryonic development through to early postnatal development, although differing estimates of the magnitude of apoptosis during the embryonic period are found in the literature (reviewed in Chun and Shatz, 1999; Gilmore et al., 2000). Many studies have shown that the majority of apoptotic neuronal death in the rodent cortex occurs during the first two postnatal weeks of life, after the bulk of neurons destined for the cortex have been generated (Ferrer et al., 1992; Finlay, 1992; Spreafico et al., 1995; Verney et al., 2000). At the time of birth, apoptotic neurons are present at low density in the cortex (Verney et al., 2000). Increasing numbers of apoptotic cells are evident by P4 in mice (Verney et al., 2000). In rats, peak neuronal death by apoptosis occurs between P5 and P8 (Spreafico et al., 1995). After P8, the number of apoptotic cells in the cortex decreases in both rats and mice, such that few apoptotic cells are present by P14 and essentially no apoptotic cells are present in the normal adult rodent cortex (Ferrer et al., 1992; Spreafico et al., 1995; Verney et al., 2000). In a given cortical layer, approximately 10-15% of cells are eliminated by apoptosis during postnatal development (Verney et al., 2000).

Varying estimates of apoptotic death have been reported during embryonic development of the cortex. For example, estimates by Thomaidou et al. (1997) using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method and electron microscopy indicate that apoptotic cells are present in the rat cortex from E14, although in fewer number than during postnatal development. At E14 approximately 2% of cells in the VZ and SVZ are undergoing apoptosis. By E16, approximately 9% of cells in these zones are apoptotic, and by E18 this value rises to 18% (Thomaidou et al., 1997). The number of apoptotic cells in the SVZ rises significantly at birth (P0) to approximately 37%. Of these TUNEL-labeled cells, approximately 71% incorporate BrdU and are assumed to be proliferating. The death of proliferating cells appears to occur during G1 phase, indicating that commitment to an apoptotic fate may occur following mitosis (Thomaidou et al., 1997). Therefore, apoptosis appears to occur in both proliferative and post-mitotic cell populations during normal cortical development.

While the extent of apoptosis in the VZ and SVZ documented by Thomaidou and colleagues (1997) was significant, the estimated amount of embryonic cortical apoptosis they documented is modest in comparison to studies of apoptosis conducted using the in situ end labeling (ISEL) method. Using the ISEL method, which is proposed to be a more sensitive detection technique than TUNEL, Blaschke et al., (1996, 1998) documented extensive
apoptosis in the cortex during embryonic development. Specifically, on E14 approximately 70% of cells in the cerebral wall were labeled with ISEL and on E18 approximately 50% of cells in the cerebral wall were estimated to be undergoing apoptosis. The majority of these labeled cells were noted in the proliferative zones (VZ and SVZ), and a significant number co-labeled with BrdU, indicating that the dying cells were actively dividing (Blaschke et al., 1996). The implications of such a high level of apoptosis in embryonic cortical progenitor populations have been the subject of much discussion in the literature. It has been noted, for example, that death of greater than 50% of the progenitor population would preclude expansion of the cortical neuron population (Gilmore et al., 2000). Clearly, a consensus on the extent of apoptosis during embryonic development of the cortex has yet to be reached.

1.17 Construction of the nestin/IGF-I transgene

The present research was designed to determine the effects of IGF-I on the growth and development of the cerebral cortex. IGF-I and the type 1 IGF receptor (IGF1R) are normally expressed in the brain from embryonic development. However, relatively few studies have addressed the in vivo role of IGF-I in the development of the cerebral cortex. To address this question, experiments were conducted on nestin/IGF-I Tg mice and normal littermate controls to determine the effect of elevated IGF-I expression in the brain on different aspects of cortical development in vivo.

Nestin/IGF-I mice carry a transgene composed of regulatory elements of the human nestin gene, which included the second intron of this gene in combination with the minimal promoter of the ICP4 herpes simplex immediate early gene, human IGF-IA cDNA, a signal sequence from rat somatostatin, and polyadenylation sites taken from the human growth hormone gene (Popken et al., 2004). Nestin is an intermediate filament protein highly expressed in neuron progenitor cells during early development of the CNS (Hockfield and McKay, 1985). Nestin expression is abundant during periods of active neuron production, and decreases during later development after cells have committed to neuronal or glial fates (Zimmerman et al., 1994; Dahlstrand et al., 1995; Matsuda et al., 1996). Previous studies indicate that the above described nestin promoter directs transgene expression to proliferating neuron progenitor cells in the CNS (Yaworsky and Kappen, 1999).

Methods used in the construction of the nestin/IGF-I transgene have been described in detail in a previous publication (Popken et al., 2004). Briefly, the transgene was constructed using two plasmids: 1) The pNesIE4 plasmid (Dr. Claudia Kappen, Mayo Clinic, Scottsdale,
AZ) which contained the second intron of the human nestin gene and a minimal promoter of the ICP4 gene. 2) The plasmid pMIG which contained a mouse metallothionein promoter I (MT-I) fused to the rat somatostatin sequence and human IGF-I cDNA (Mathews et al., 1998). The MT-I promoter was deleted from the pMIG plasmid and a 1.8 kb fragment of the nestin 5' regulatory sequence was inserted. The whole transgene was excised and purified for the purposes of microinjection (Popken et al., 2004).

1.18 Generation of nestin/IGF-I transgenic mice

Nestin/IGF-I Tg mice were produced using classical microinjection techniques as described previously (Popken et al., 2004). Eggs derived from C57BL/6 mice were used as recipients of transgene DNA. Homozygous Tg mice did not survive to birth, but heterozygous mice were viable and developed normally. Therefore, only heterozygous nestin/IGF-I Tg mice were studied. Birth weights of Tg mice were normal and body weights remained similar to controls well into adult life. Both male and female Tg mice were fertile, and produced litters of pups similar in number to those produced by the mating of normal C57BL/6 mice. As well, Tg mice did not exhibit any obvious variations in behaviour when compared to normal control animals (Popken et al., 2004).

1.19 Analyses of IGF-I expression in nestin/IGF-I Tg mice

Expression of the nestin/IGF-I transgene was assessed in mice of various ages using Northern blot, in situ hybridization histochemistry (ISHH), and radioimmunoassay techniques. Transgene expression was compared to that of endogenous nestin using a ribonuclease protection assay (RPA) and immunohistochemistry (Popken et al., 2004). During embryonic development the abundance of IGF-I transgene mRNA in the brain, assessed using Northern blots, was substantially increased in Tg mice as compared to endogenous IGF-I mRNA. Expression of the transgene increases up until P5, after which time it begins to decrease gradually until expression levels reach an apparent steady-state level at P20 (Popken et al., 2004). Even after reaching this steady-state level, expression remains elevated above control levels. This pattern of transgene expression is generally similar to previously documented patterns of nestin expression in the brain (Dahlstrand et al., 1995). The cerebral cortex exhibited the greatest level of transgene expression, and high levels of expression were also detected in the hippocampus, cerebellum, brainstem and diencephalon (Popken et al., 2004).

Increased expression of transgene IGF-I mRNA, assessed using ISHH, was apparent by as early as E13, and at all other ages examined (P0, P6). The transgene was expressed
primarily in neuronal cells, with little expression apparent in white matter or in glial cells (Popken et al., 2004). These results are consistent with previously documented patterns of the expression of a reporter gene under control of the same nestin gene regulatory sequence used in nestin/IGF-I Tg mice, which demonstrate that this sequence directs gene expression to neuronal cells (Yaworsky and Kappen, 1999). At E13, transgene expression was apparent in the neuroepithelium, VZ, cortical plate, developing cerebellum, and primordial hippocampus. At P0, expression increased in the cerebral cortex and hippocampus, and became visible in the habenular complex. Transgene expression in the hippocampus continued to increase on P6, and high expression was also noted in the habenular complex (medial and lateral habenular nuclei). As well, high levels of transgene expression were detected in the medial nucleus of the amygdala and in the Purkinje cells of the cerebellum. In the cerebral cortex, transgene expression remained homogeneous, except for higher levels of expression noted in the piriform and retrosplenial cortices (Popken et al., 2004), which may result from the high cell densities typical of these cortical areas (particularly in layer II/III).

Augmented IGF-I expression in nestin/IGF-I Tg mice was confirmed using a radioimmunoassay at P0 to quantify relative amounts of IGF-I protein in Tg and control mice. At this age, IGF-I protein was increased in Tg mice by approximately 60%, as compared to normal controls. These results indicate that increased expression of transgene mRNA correlates with increased IGF-I protein levels in Tg mice (Popken et al., 2004).

To determine if nestin expression was altered in Tg mice, expression of the native nestin gene was measured in Tg and control mice using RPA. At P0, levels of endogenous nestin expression did not differ significantly between Tg and control mice, indicating that expression of the IGF-I transgene did not alter nestin expression nor affect the number of cells expressing nestin (Popken et al., 2004). Patterns of nestin expression were further examined in Tg and control mice using immunostaining techniques. These experiments illustrated that nestin staining patterns were similar in Tg mice to those previously documented in normal mice (Dahlstrand et al., 1995). For example, the intensity of nestin staining in Tg mice began to decrease after P6, consistent with previous reports of normal nestin expression (Dahlstrand et al., 1995). On P6 nestin was detected in the choroid plexus, cerebellum, and ventricular zone of Tg mice, consistent with normal patterns of nestin expression (Dahlstrand et al., 1995; Popken et al., 2004). By P45, nestin staining was detected only in the choroid plexus and
ventricular zone of Tg mice (Popken et al., 2004). These results indicate that expression of endogenous nestin is not altered by expression of the nestin/IGF-I transgene in Tg mice.

1.20 Nestin/IGF-I Tg mice exhibit increased brain weight and volume during embryonic and postnatal development

Nestin/IGF-I Tg mice exhibited augmented brain growth during early embryonic development of the brain, and measurable changes in the volumes of many brain regions were apparent at different stages of development. Tg mice did not exhibit signs of morphological abnormalities, neuronal ectopias, gliosis, or gross malformations at any of the ages examined (Popken et al., 2004). Significant increases in the brain weights of nestin/IGF-I Tg mice were evident by as early as E18 (6.5% increase). By P5, brain weights of Tg mice were significantly increased by 23% as compared to normal controls. Similar increases in brain weights were documented in Tg mice through to P45 (Popken et al., 2004).

Stereological analyses performed at E16 showed that regional volumes and cell numbers were significantly increased in Tg embryos during embryonic development of the nervous system. Significant increases in the volumes of the dorsolateral telencephalic wall (25%), cortical plate (52%), intermediate zone (12%) and the combined regions of the VZ and SVZ (26%) were documented in Tg embryos at E16. The volume of the marginal zone (MZ) of the dorsolateral telencephalic wall did not differ significantly between groups. A significant increase of 20% in pial surface area overlying the cortical plate contributed to the increased volume of the dorsolateral telencephalic wall. However, the total surface area of the lateral ventricle adjacent to the VZ did not differ between Tg and control embryos at E16. Analyses of the numerical density (Nv, number of cells per unit volume of tissue) and total number of cells in the cortical plate revealed no significant difference in the Nv, but the number of cells in the cortical plate was increased by 54% in Tg embryos due to the significant increase in the volume of the cortical plate in these mice. Unlike the dorsolateral telencephalic wall, the total volume of the ventrolateral telencephalic wall did not differ between Tg and control embryos. Similarly, the volumes of the combined regions of the VZ and SVZ and the primordial caudate-putamen were not affected, and the area of the ventricular surface adjacent to the VZ also did not differ between groups (Popken et al., 2004).

The above results indicate that the volumes of structures involved in the generation of the cerebral cortex are significantly augmented at E16 in Tg embryos. As well, cell number is already increased in the cortical plate of Tg embryos at E16. This increase in cortical plate cell
number precedes the major phase of neuron elimination in the cerebral cortex, suggesting that the increase in cell number results, at least in part, from augmented mitosis during the period of cortical neurogenesis (Popken et al., 2004). The observed increase in the volume of the VZ and SVZ regions, which contain progenitor cells responsible for the production of a large proportion of the neurons of the cortex, further supports a role for IGF-I in neuronal proliferation.

To determine if augmented IGF-I expression in nestin/IGF-I Tg mice results in measurable changes in regional volumes and neuron numbers during postnatal brain development, stereological analyses were performed on P12. Volume, $N_v$, of neurons, and total number of neurons were measured in various brain regions. The volumes of numerous brain regions were augmented in Tg mice, including the total forebrain (26%), caudate putamen (37%), hippocampus (49%), dentate gyrus (71%), and habenular complex (48%). Interestingly, the volume of the olfactory bulb did not differ between groups. The $N_v$ of neurons did not differ between Tg and control animals in any of the regions examined. However, due to the significant increases in regional volumes, the total number of neurons was increased in the caudate putamen (27%), dentate gyrus (69%), medial habenula (61%), and lateral habenula (36%) in Tg mice as compared to controls. These results show that augmented expression of IGF-I during prenatal and postnatal development results in increased tissue volume and neuron number in many brain regions of nestin/IGF-I Tg mice.

1.2.1 Thesis theme and statement of hypotheses

The present set of experiments was designed to determine the in vivo effects of IGF-I on several aspects of cerebral cortex development. Evidence indicating that IGF-I acts as both a pro-mitotic and anti-apoptotic factor during neural development led to the development of a general working hypothesis for the effects of IGF-I on cerebral cortex development. Working hypothesis #1: Elevated IGF-I expression in Tg mice stimulates cortical growth by increasing neuron progenitor proliferation and/or by decreasing neuron death by apoptosis, either of which would ultimately result in an increase in the final number of neurons in the cerebral cortex. In the event that IGF-I both augmented progenitor proliferation and inhibited neuron death, the goal was to determine the relative magnitude of each effect. Initial studies were conducted at P12 to determine the overall magnitude of the effects of augmented IGF-I expression on growth of the cerebral cortex. Stereological analyses were conducted to determine if IGF-I overexpression produced increases in cortical volume and neuron number in
Tg mice. Experiments were conducted on P12 because both the phase of cortical neurogenesis and the phase of neuron death by apoptosis are essentially completed by this time.

Studies of IGF-1<sup>−/−</sup> mice indicated that the development of several types of cortical neurons did not seem to be affected by a lack of IGF-I during development. Specifically, the number of projection neurons in layers III and V of the parietal and temporal regions of the cortex was normal in IGF-1<sup>−/−</sup> mice (Beck et al., 1995). These studies suggested that IGF-I may differentially effect the growth of specific neuron types and/or cytoarchitectonic regions of the cerebral cortex. Working hypothesis #2: IGF-I overexpression in nestin/IGF-I Tg mice may differentially influence the development of specific cortical regions and layers. To examine this hypothesis, stereological analyses were conducted on P12 to determine the volume and total number of neurons within individual layers of the primary motor and primary somatosensory regions of the cortex in Tg mice and littermate controls.

Subsequent experiments were designed to determine the mechanisms by which IGF-I overexpression produces increases in total neuron number in the cerebral cortex of Tg mice. Evidence from some in vitro studies showed that IGF-I is capable of promoting neuron progenitor proliferation (Lenoir and Honegger, 1983; DiCicco-Bloom and Black, 1989; Drago et al., 1991; Arsenijevic et al., 2001). However, many of the IGF-I Tg lines of mice developed to date have used promoters to drive transgene expression that have restricted expression to postnatal development. In many brain regions, neuron production occurs during embryonic development, and, as a result, the majority of in vivo studies of IGF-I’s actions during brain development have focused on the anti-apoptotic effects of IGF-I.

Transgene expression in nestin/IGF-I Tg mice begins by as early as E13 (Popken et al., 2004), which coincides with the normal period of neurogenesis in the mouse (E11-E17; Takahashi et al., 1995a). Given that transgene expression in nestin/IGF-I Tg mice begins during embryonic development, these mice represent a unique opportunity to determine the effects of IGF-I on neuron progenitor proliferation in vivo. A number of studies conducted on non-neural cell types have shown that IGF-I is capable of promoting proliferation by increasing the rate at which cells progress through the cell cycle (Leof et al., 1982; Chen and Rabinovitch 1989, 1990; Lu and Campisi, 1992; Adesanya et al., 1999; Chakravarthy et al., 2000; Dupont et al., 2000; Stull et al., 2002). Cell cycle progression rate is one of several factors known to be involved in the regulation of neuron output during cortical neurogenesis (Takahashi et al., 1993, 1995a, 1996; Caviness et al., 1995). Working hypothesis #3: As IGF-I has been shown
to increase cell cycle progression rate in some non-neural cells, elevated IGF-I expression in Tg mice may augment neuron progenitor proliferation by increasing cell cycle progression rate during the period of neurogenesis in the cortex. This hypothesis was tested on E14, the approximate midpoint of cortical neurogenesis, in Tg and littermate control embryos using a cumulative S-phase labeling protocol, which allowed for the determination of individual phase lengths for all phases of the cell cycle.

While the rate of neuron progenitor cell progression through the cell cycle is an important determinant of neuron output during cortical neurogenesis, it is not the only factor involved in the regulation of neuron production. The fraction of daughter cells produced after a given round of division that subsequently exit the cell cycle as post-mitotic neurons is a key factor involved in the determination of neuron output. For example, if more daughter cells remain in the cell cycle as progenitors following a given round of cell division, the potential for generating greater numbers of neurons is increased as a result of expansion of the progenitor cell population (Takahashi et al., 1996; Caviness et al., 1995, 2003). There was little evidence in the literature to suggest that IGF-I would influence the re-entry of progenitors into the cell cycle following a given round of division. However, IGF-I is known to promote the entry of several types of quiescent cells into the cell cycle in vitro (Stiles et al., 1979; Clemmons et al., 1980; DiCicco-Bloom and Black, 1988; Lorenzo et al., 1993; Reiss et al., 1997). Working hypothesis #4: IGF-I may be capable of regulating cell cycle re-entry in the cerebral wall in vivo. As this is a critical factor in determining neuron output, the effect of elevated IGF-I expression on the fraction of cells returning to the cell cycle was examined in Tg mice and normal littermate controls on E14-E15.

During normal neurogenesis in the cerebral cortex, several different types of neuron progenitor cells exist within proliferative zones of the cerebral wall and produce the majority of projection neurons destined for the cerebral cortex. These progenitors include radial glial and intermediate progenitor cells (Miyata et al., 2001, 2004; Noctor et al., 2001, 2002, 2004; Englund et al., 2005). The effects of IGF-I on the development of these different types of progenitor cells has not been examined previously. Working hypothesis #5: IGF-I may differentially influence the development of specific progenitor populations during cortical neurogenesis. In order to determine how IGF-I influences neuron production during cortical neurogenesis, the effect of augmented IGF-I expression on the proportion of radial glia and
intermediate progenitor cells within the cerebral wall was examined in Tg and control embryos on E14.

The experiments discussed to this point were designed to examine the effects of IGF-I on neuron progenitor proliferation during embryonic development of the cerebral cortex, with the intent of assessing the mechanisms by which IGF-I overexpression produces increased neuron number in the cerebral cortex of Tg mice. However, a number of studies indicate that IGF-I promotes neuron survival in vitro (Aizeman and DeVellis, 1987; Torres-Aleman et al., 1990a, b; Pons et al., 1991; Bozyczko-Coyne et al., 1993; Sortino and Canonico, 1996; Russell and Feldman, 1999; Yamada et al., 2001). As well, IGF-I-mediated inhibition of apoptosis has been suggested as a mechanistic explanation for the increases in neuron number documented in numerous brain regions of mice with elevated expression of IGF-I during postnatal development (Gutierrez-Ospina et al., 1996; Dentremont et al., 1999; O’Kusky et al., 2000; reviewed in D’Ercole et al., 2002). Working hypothesis #6: Augmented IGF-I expression in the cortex may reduce the number of neurons eliminated by apoptosis in Tg mice, which would result in an increase in the final number of neurons in the cerebral cortex. To ascertain the contribution of inhibited neuron apoptosis to the determination of final neuron number in the cortex, the density of apoptotic cells present in the cortex was assessed in Tg and control mice during embryonic development at E16 and at several ages during early postnatal development (P0, P5).
1.22 Bibliography


Chapter II  Stereological analyses of the cerebral cortex in nestin/IGF-I transgenic and control mice at postnatal day 12

2.1 Introduction

Evidence from recent studies indicates that IGF-I has a role in CNS development (for review see D’Ercole et al., 1996, 2002; M.F. Anderson et al., 2002). However, relatively few studies, either in vitro or in vivo, have specifically addressed the effects of IGF-I on cerebral cortex development. Both IGF-I and IGF1R are expressed in the primitive cerebral cortex of rodents during embryonic development (Ayer-Le Lievre et al., 1991; Bondy et al., 1992). IGF1R, the predominant receptor for IGF-I, is expressed by neural progenitor cells in the VZ of the developing cerebral cortex and post-mitotic cortical neurons (Bondy et al., 1992). In situ hybridization studies indicate that IGF-I mRNA is expressed in a relatively uniform pattern throughout the embryonic cortex (Ayer-Le Lievre et al., 1991). High levels of IGF-I and IGF1R expression are apparent in the rodent cerebral cortex during the first three weeks of postnatal life (Bartlett et al., 1991; Bondy, 1991; Bondy et al., 1992).

Mutant mouse models of IGF-I function support a role for IGF-I in cerebral cortex growth and development. Tg mice that overexpress IGF-I in the brain during postnatal development exhibit increased cortical surface area, as well as increased total barrel area and neuron number within individual barrels in the posterior medial barrel subfield of the somatosensory cortex (Gutierrez-Ospina et al., 1996). Conversely, IGF-I knockout mice (IGF-1/−) exhibit decreased cortical thickness and increased cell density in the cerebral cortex in comparison to normal control mice (Beck et al., 1995). However, the apparent number of projection neurons in layers III and V in the parietal and temporal regions of the cerebral cortex of IGF-1−/− mice does not differ from normal mice (Beck et al., 1995). These studies indicate that IGF-I influences the development of the cerebral cortex and suggest a differential effect of IGF-I on specific neuronal populations and/or functional areas of the cerebral cortex.

The present study was designed to determine the in vivo effects of IGF-I on the prenatal and early postnatal development of the cerebral cortex in nestin/IGF-I Tg mice. Nestin/IGF-I mice carry a transgene which directs expression of IGF-I to progenitor cells within the CNS.

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Transgene expression begins during prenatal life (E13 or earlier) and continues into postnatal development, with regional expression being highest in the cerebral cortex (Popken et al., 2004). Given that IGF-I is well documented *in vitro* to promote neuron proliferation (DiCicco-Bloom and Black, 1988; Torres-Aleman et al., 1990; Drago et al., 1991; Werther et al., 1993; Zackenfels et al., 1995; Arsenijevic et al., 2001) and to inhibit neuron death by apoptosis (D’Mello et al., 1993; Werther et al., 1993; Yamada et al., 2001), augmented IGF-I expression in nestin/IGF-I Tg mice may result in increased volume and neuron number in the cerebral cortex. As well, IGF-I overexpression may differentially affect the development of distinct cytoarchitectonic regions and layers of the cerebral cortex. To test these hypotheses, stereological analyses were conducted to determine the effects of IGF-I on the total volume and mean thickness of the neocortex, and on the \( N_v \) and total number of neurons in the cortex. Separate stereological analyses were conducted to measure all morphometric variables for individual cortical laminae in each of two different cytoarchitectonic regions, the primary motor and primary somatosensory cortices.

### 2.2 Materials and Methods

**Nestin/IGF-I transgenic mice**

Heterozygous male nestin/IGF-I Tg mice were mated with normal non-Tg littermate control females to generate litters consisting of approximately half Tg mice and half normal non-Tg controls. Tg mice were routinely identified by PCR of tail genomic DNA.

Nestin/IGF-I Tg mice were generated at the mouse facility of the University of North Carolina at Chapel Hill and housed at 22°C with alternating 12-hour light and dark cycles. Institutional review committees of the University of British Columbia and University of North Carolina at Chapel Hill approved all procedures involving the use of animals.

Expression of the nestin/IGF-I transgene is evident by as early as E13 and gradually increases to peak values at P5-10. Expression levels then gradually decline to steady state values by P20 (Popken et al., 2004). The cerebral cortex has been shown to exhibit the highest level of IGF-I transgene expression, which at E13 occurs predominantly in the neuroepithelium of the VZ (Popken et al., 2004).

**Tissue collection and processing**

On P12, Tg and control mice were deeply anaesthetized with an intraperitoneal (i.p.) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Individual mice were perfused through the left ventricle for 45 minutes at a perfusion pressure of 90-100 mm Hg using a
fixative solution containing 4% paraformaldehyde and 1% glutaraldehyde in 0.1M phosphate buffer (pH 7.4). Brains were removed, weighed and stored in additional fixative solution at 4°C for 48-72 hours. Tissue blocks were cryoprotected prior to sectioning by immersion in a solution of 15% sucrose in 0.1M phosphate buffer at 4°C overnight. Serial frozen sections (30 μm) were cut in the coronal plane through the rostrocaudal extent of the cerebral hemispheres, mounted on glass slides and air-dried overnight. All sections in the series were stained with 0.1% thionine in acetate buffer (pH 3.7) for the detection of Nissl substance, dehydrated in ascending grades of ethanol, cleared in xylene and mounted with Permount. All stereological analyses were conducted on coded histological sections to prevent observer bias in the measurements.

Stereological analyses of the cerebral cortex and subcortical white matter

Stereological analyses were performed to measure the total volume of the cerebral neocortex, pial surface area, mean cortical thickness, anteroposterior cortical length, and mean cortical width. The numerical density ($N_v$, cells per mm$^3$) and total number of both neurons and glia were determined for the neocortex. Additionally, analyses were conducted to measure the total volume of the subcortical white matter, and the $N_v$ and total number of glia in the subcortical white matter.

The cerebral neocortex was defined as all six-layered regions of the cerebral cortex according to established boundary criteria (Zilles, 1985), which exclude the piriform and entorhinal regions. Measurements of total cortical volume, pial surface area, mean cortical thickness, anteroposterior length and mean cortical width were performed on 6 Tg and 6 control animals. Total volume measurements were performed using Cavalieri's direct estimator (Gundersen et al., 1988a). Sections were examined with an Olympus BH-2 compound microscope (4X planapochromatic objective) interfaced to a Bioquant TCW98 image analysis system (R&M Biometrics, Nashville, TN) and visualized on a monitor at a final magnification of 75X. The outline of the cerebral neocortex was traced and its area was measured in μm$^2$. The total volume of the neocortex was calculated from (1) $V = \Sigma A \times T \times 4$, where $\Sigma A$ is the sum of the area measurements, $T$ is the section thickness, and 4 is the periodicity of the section sample. The length of the pial surface overlying the neocortex was measured in μm. The total pial surface area was calculated from (2) $S = \Sigma L \times T \times 5$, where $\Sigma L$ is the sum of the length measurements, $T$ is the section thickness, and 5 is the periodicity of the section sample. Mean cortical thickness was determined for each animal by dividing the total
cortical volume by the pial surface area. The anteroposterior length of the cortex was calculated from (3) \( L = \Sigma N \times T \times 4 \), where \( \Sigma N \) is the total number of sections in the sample, \( T \) is the section thickness, and 4 is the periodicity of the section sample. The mean width of the cerebral cortex was determined for each animal by dividing the pial surface area by the anteroposterior length.

The \( N_v \) of neurons in the cerebral cortex (\( N = 6 \) per group) was determined using the optical disector method (Gundersen et al., 1988b). Seven sections that were equally spaced along the caudal-to-rostral extent of the cerebral hemispheres were selected from the serial frozen sections for analysis. A random set of sampling points was generated over the neocortex on each section using the Bioquant TCW98 Stereology Toolkit (R&M Biometrics). Approximately 25-45 points were sampled on each section. Sections were examined at each sampling point using a 100X oil-immersion planapochromatic objective at a final magnification of 1,880X. For a given sampling point, a square counting frame measuring 30 \( \mu \text{m} \) on a side was positioned approximately 3 \( \mu \text{m} \) from the section surface and moved through 10 \( \mu \text{m} \) of section thickness in the Z-axis plane. Nuclei of neurons that came into focus entirely within the counting frame or were intersected by its inclusion edges (the top and right edges of the frame and the final plane of focus) were counted. Approximately 400-500 neurons were counted per animal. Neurons were typically larger than glia with visible, defined nucleoli and dark staining cytoplasm. The \( N_v \) of neurons was calculated using the equation (4) \( N_v = \Sigma Q' / \Sigma V_{\text{Dis}} \), where \( \Sigma Q' \) is the sum of the neurons counted and \( \Sigma V_{\text{Dis}} \) is the sum of the disector volumes. The disector volume was calculated from (5) \( V_{\text{Dis}} = a_{\text{Dis}} \times h \), where \( a_{\text{Dis}} \) is the area of the counting frame and \( h \) is the disector height, which is equal to the distance traveled in the Z-axis plane (10 \( \mu \text{m} \)). Estimates of neuronal \( N_v \) and total cortical volume were used to calculate the total number of neurons in the cerebral cortex of each animal. The \( N_v \) of glia in the neocortex (\( N = 4 \) per group) was determined using the optical disector method as described for the \( N_v \) of neurons (equations 4 and 5), and the total number of glia in the neocortex was calculated from estimates of glial \( N_v \) and total cortical volume.

The volume of the subcortical white matter (\( N = 4 \) per group) was determined using Cavalieri's direct estimator as described above (equation 1), with measurements taken on every eighth section in the series (\( P = 8 \)). Boundaries for the subcortical white matter were determined using established criteria (Franklin and Paxinos, 1997), and included the corpus callosum. For volume measurements, individual sections were visualized using a 10X...
planapochromatic at a final magnification of 188X. The \( N_v \) and total number of glia in the subcortical white matter (\( N = 4 \) per group) was determined using the optical disector method as described above for the neocortex (equations 4 and 5).

**Stereological analyses of the primary motor and primary somatosensory cortices**

Stereological analyses of the primary motor cortex and the primary somatosensory cortex were conducted using the regional boundary criteria of Zilles (1985) to define cytoarchitectonic regions and cortical layers. On coronal sections the primary motor cortex (Fig. 2-1, MCx) was characterized by a prominent layer V, containing a relatively low packing density of large pyramidal neurons, and a narrow layer IV, sparsely populated by granular neurons. This region corresponds to the combined forelimb (FL) and hindlimb (HL) motor areas described by Zilles (1985). The motor region is bounded dorsomedially by the frontal cortex (area Fr) and ventrolaterally by the primary somatosensory cortex (area Pari) along its entire rostrocaudal extent (Zilles, 1985), as illustrated in Figure 2-1. The primary somatosensory cortex (Fig. 2-1, Par1) was distinguished from surrounding cytoarchitectonic regions by a prominent layer IV containing densely packed granular neurons. At levels where the plane of section was roughly perpendicular to the pial surface, somatosensory barrels could be identified in layer IV (Fig. 2-1 C-D). The primary somatosensory region is bounded dorsomedially by the frontal cortex most rostrally, by the motor cortex more caudally, and by the visual/occipital cortex most caudally. Ventrolaterally, it is bounded by the gustatory cortex (Gu) most rostrally, by the supplementary somatosensory cortex (Par2) more caudally, and by the auditory/temporal cortex most caudally. Individual cortical layers were delineated in each cytoarchitectonic area according to the boundary criteria of Zilles (1985). Given the lack of distinct and reliable boundaries between layer II and layer III in the motor and somatosensory cortices, they were combined and measured as a single layer (layer II/III) in each cytoarchitectonic area.

Stereological analyses were conducted to measure the total volume (equation 1), the pial surface area (equation 2), and the mean cortical thickness of both the primary motor and somatosensory cortices as described above for the neocortex, using the serial frozen sections stained for Nissl substance and every third section in the series (\( P = 3 \)). In addition, tissue volume and both the \( N_v \) and total number of cortical neurons were determined separately in individual cortical layers within each cytoarchitectonic region. Tissue volumes for each layer were measured as described for the neocortex. Individual estimates of neuronal \( N_v \) for each
Fig. 2-1. Representative sections (30 μm thick) stained with thionine for Nissl substance, through the cerebral hemisphere of a non-Tg littermate control mouse at P12 to illustrate the boundary criteria used to identify cytoarchitectonic areas. Panels A through D illustrate progressively more caudal sections through the primary motor cortex (MCx) and the primary somatosensory cortex (Par1). Adjacent areas include the frontal cortex (Fr), the gustatory cortex (Gu), and the supplemental somatosensory cortex (Par2). Boundary criteria for these cytoarchitectonic areas were identical in Tg mice. Scale bar, 500 μm.
layer were determined using the optical disector method as described previously (Gundersen et al., 1988b). For each individual estimate of neuronal $N_v$, at least 200-250 neurons were counted in each layer.

**Statistical analysis**

For all variables pertaining to the total cerebral neocortex, the statistical significance of differences between means was determined using Student's $t$-test. Measurements of pial surface area and mean cortical thickness for the primary motor and primary somatosensory areas were compared between Tg and control mice using Student's $t$-test. For estimates of tissue volume, neuronal $N_v$ and total neuron number in individual cortical layers of the primary motor and primary somatosensory cortices, the statistical significance of differences among means was assessed using a $2 \times 5$ (groups $\times$ layers) analysis of variance (ANOVA). For the $N_v$ and total number of neurons in individual layers, the data failed to demonstrate homogeneity of error variances in both the motor and somatosensory cortices, due to extremely low values for neuron density and total number in layer I. For these variables, log transformed data were used in the $2 \times 5$ ANOVA (Winer, 1971). For the direct comparison of the effects of IGF-I on motor versus somatosensory cortex, a $2 \times 5$ (areas $\times$ layers) ANOVA was employed for percentage increases. Briefly, for each variable in each Tg mouse, the percent increase relative to the corresponding control mean was calculated. The statistical significance of differences in percentage increases between motor and somatosensory cortices was determined using the $2 \times 5$ ANOVA.

For all ANOVA tests, *post hoc* comparisons of the statistical significance of differences between individual pairs of means were analyzed using estimated marginal means with a least significant differences adjustment for multiple comparisons. For all statistical analyses, values of $P < 0.05$ were considered statistically significant.

**Reelin immunostaining on E14**

Nestin/IGF-I Tg and normal littermate control embryos were collected from timed-pregnant dams on E14. Timed-pregnant dams were generated by breeding male nestin/IGF-I Tg mice with control C57BL/6 females from 8:00 A.M. to 10:00 A.M. Conception was determined by the presence of a vaginal plug, and the day of conception was considered E0. Embryos were collected by hysterotomy on E14 and fixed in 70% ethanol overnight at 4°C. Following fixation, embryos were embedded in paraffin and sectioned at 4 $\mu$m in the coronal plane. For each animal ($N = 3$ per group), four non-adjacent sections (i.e. every second section
in a series of seven consecutive sections) were processed for the immunohistochemical detection of reelin. Briefly, sections were deparaffinized in xylene and rehydrated in ethanols to distilled water. Slides were boiled in antigen unmasking solution (Vector Laboratories, Burlingame, CA) for 10 min, after which they were incubated in blocking solution (5% normal horse serum, 0.1% Tween-20 in phosphate buffered saline) for 1 hr at room temperature. Sections were incubated in a mouse monoclonal anti-reelin antibody (G10 clone; 1:400 dilution; Calbiochem, San Diego, CA) overnight at 4°C. Further processing was carried out using a Vector Elite ABC Kit for mouse IgG (Vector Laboratories) according to the manufacturer's protocol.

Counts of reelin-immunoreactive cells were conducted within the marginal zone (primordial layer I) of the developing cerebral wall on the four sections per animal, as described above. On each section, the area of the marginal zone was measured over the entire cerebral wall using a 20X planapochromatic objective and Bioquant TCW98 software. Reelin-immunoreactive cells within the outlined area of the marginal zone were counted using a 100X oil-immersion planapochromatic objective. The number of reelin-immunoreactive cells per mm² was determined by dividing the sum of all reelin-immunoreactive cells counted by the total marginal zone area. The statistical significance of differences among mean values was determined using Student’s t-test. As well, profile areas of 100 reelin-immunoreactive cells were measured per animal to determine if neuronal profile areas differed between Tg and control embryos. Mean profile areas were generated for each animal and compared between groups using Student’s t-test.

2.3 Results

At P12, brain weights of Tg mice were significantly increased by 23% as compared to littermate controls (t = 6.312; P< 0.001), indicating that brain growth of Tg mice was significantly increased by IGF-I overexpression at this age (Table 2-1). As transgene expression is restricted to the brain, body weights of Tg mice did not differ significantly from those of normal littermate controls (Table 2-1). Upon inspection of the serial frozen sections stained for Nissl substance we noted an obvious increase in overall brain volume in Tg mice (Fig. 2-2). There were no signs of morphological abnormalities, gross malformations, neuronal ectopias, neuronal degeneration, or gliosis in the brains of Tg mice. However, there was a slight reduction in the volume of the lateral ventricles because of the increased brain growth in Tg animals (Fig. 2-2).
Fig. 2-2. Representative sections (30 μm thick) stained with thionine for Nissl substance, through the cerebral hemispheres of (A) nestin/IGF-I Tg and (B) non-Tg littermate control mice at P12. Scale bar, 1 mm.
Growth and development of the cerebral cortex

Histological examination of the serial sections revealed that the overall pattern of lamination and neuronal packing density appeared normal in the cerebral cortex of nestin/IGF-I Tg mice. However, the cerebral cortex was noticeably larger than that of controls, both in thickness and pial surface area (Fig. 2-2). The results of stereological measurements for the cerebral cortex in both hemispheres are presented in Table 2-1. The volume of the neocortex was significantly increased by 31% in Tg mice (t = 5.934; P< 0.001) compared to controls. Significant increases in both the pial surface area of the cortex (17%; t = 6.234; P < 0.001) and the mean cortical thickness (12%; t = 4.669; P = 0.001) contributed to the increase in cortical volume in Tg mice. The increased pial surface area in Tg mice resulted from significant increases of 8% in both the anteroposterior length (t = 4.485; P = 0.001) and the mean mediolateral width (t = 4.262; P = 0.002) of the cerebral hemispheres. The overall Nv of neurons, from pia to white matter (including all cytoarchitectonic areas), did not differ between Tg and control mice (t = 0.566; P > 0.05). However, the total number of neurons in both hemispheres was greater in Tg mice (27%; t = 6.886; P < 0.001) as compared to controls, due to the significantly greater cortical volume in Tg mice. Similarly, the Nv of glia did not differ significantly between Tg and control animals at P12 (t = 1.313; P = 0.237), while the total number of glia was significantly increased by 37% in Tg mice (t = 4.601; P = 0.004).

A disproportionate increase of 52% in the volume of the subcortical white matter was detected in Tg mice at P12 (t = 6.447; P = 0.001; Table 2-2). The Nv of glia in the subcortical white matter did not differ significantly between Tg and normal littermate controls (t = 1.150; P = 0.294). A significant increase of 42% was detected in the total number of glia in the subcortical white matter in Tg mice (t = 8.359; P < 0.001), due to the significant increase in the volume of the white matter.

Growth of the primary motor cortex is augmented in nestin/IGF-I transgenic mice

Stereological analyses of the primary motor cortex revealed that the total volume of this cytoarchitectonic area, measured from the pia to the white matter and including all cortical layers, was 42% greater in Tg mice (5.310 ± 0.108 mm³) as compared to controls (3.731± 0.126 mm³; P < 0.001). Individual laminar volumes were significantly increased in all layers in Tg mice (35% to 50%; Fig. 2-3), with the greatest increase observed in layer I (50%; Table 2-3). The increase in total motor cortex volume was primarily due to a significant increase in
### Table 2-1

Morphometric variables of the cerebral neocortex measured in both hemispheres for nestin/IGF-I transgenic and control mice on postnatal day 12\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Nestin/IGF-I Transgenic</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>6.93 ± 0.50</td>
<td>7.10 ± 0.19</td>
</tr>
<tr>
<td>Brain weight (mg)</td>
<td>428.7 ± 10.5**</td>
<td>347.2 ± 7.5</td>
</tr>
<tr>
<td>Volume (mm(^3))</td>
<td>103.776 ± 3.480**</td>
<td>79.467 ± 2.161</td>
</tr>
<tr>
<td>Pial surface area (mm(^2))</td>
<td>137.602 ± 2.762**</td>
<td>117.931 ± 1.526</td>
</tr>
<tr>
<td>Anteroposterior length (mm)</td>
<td>7.900 ± 0.109*</td>
<td>7.320 ± 0.069</td>
</tr>
<tr>
<td>Mean cortical width (mm)</td>
<td>17.413 ± 0.172*</td>
<td>16.117 ± 0.251</td>
</tr>
<tr>
<td>Mean cortical thickness (mm)</td>
<td>0.753 ± 0.012*</td>
<td>0.674 ± 0.012</td>
</tr>
<tr>
<td>(N_V) of neurons (neurons/mm(^3))</td>
<td>124,869 ± 4,557</td>
<td>128,609 ± 4,780</td>
</tr>
<tr>
<td>Total number neurons</td>
<td>12,896,530 ± 303,440**</td>
<td>10,179,481 ± 252,252</td>
</tr>
<tr>
<td>(N_V) of glia (glia/mm(^3))</td>
<td>130,382 ± 6712</td>
<td>123,927 ± 7156</td>
</tr>
<tr>
<td>Total number of glia</td>
<td>13,775,215 ± 706,558*</td>
<td>10,082,012 ± 380,937</td>
</tr>
</tbody>
</table>

*All values are presented as the mean ± standard error of the mean (SEM).  
*\(P < 0.01\), **\(P < 0.001\) compared to control mice using Student’s t-test.

### Table 2-2

Morphometric variables of the subcortical white matter measured bilaterally in nestin/IGF-I transgenic and control mice at postnatal day 12\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Nestin/IGF-I Transgenic</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total volume (mm(^3))</td>
<td>6.546 ± 0.291*</td>
<td>4.302 ± 0.190</td>
</tr>
<tr>
<td>(N_V) of glia (glia/mm(^3))</td>
<td>235,437 ± 6712</td>
<td>253,793 ± 14,476</td>
</tr>
<tr>
<td>Total number of glia</td>
<td>1,536,592 ± 42,929*</td>
<td>1,085,046 ± 32,786</td>
</tr>
</tbody>
</table>

*All values are presented as the mean ± SEM  
*\(P < 0.001\) compared to control mice using Student’s t-test
pial surface area (32%; \( t = 5.301; \ P < 0.001; \) Table 2-4), as mean cortical thickness was not significantly different between Tg and control mice in this region (\( t = 2.099; \ P > 0.05 \)).

The \( N_v \) of neurons did not differ significantly from controls in layers II through VI. However, there was a significant increase of 24% in the \( N_v \) of neurons in layer I of the primary motor cortex in Tg mice (\( P < 0.001; \) Table 2-5). The total number of neurons in the motor cortex was significantly increased by 44% in Tg mice (Tg = 794,269 ± 17,663 neurons; control = 551,865 ± 20,153 neurons), with increases of 34% to 53% in layers II-VI (Fig. 2-3; Table 2-6). Total neuron number was increased by 93% in layer I of Tg mice, due to significant increases in both the \( N_v \) of neurons and laminar volume.

**Stereological analyses of the primary somatosensory cortex**

Stereological analyses of the primary somatosensory cortex indicated a significant increase of 35% in the total volume (including all cortical layers) of this area in Tg mice (11.562 ± 0.439 mm\(^3\)) as compared to controls (8.587 ± 0.246 mm\(^3\); Fig. 2-4). Significant increases in volume were observed in all layers (29% to 38%; Table 2-3; Fig. 2-4). Significant increases in both pial surface area (22%; \( t = 3.883; \ P = 0.003 \)) and mean cortical thickness (10%; \( t = 4.254; \ P < 0.01; \) Table 2-4) contributed to the increased total volume for the primary somatosensory cortex documented in Tg mice.

The \( N_v \) of neurons did not differ significantly between Tg and control mice in layers II through VI of the somatosensory cortex (Table 2-5). However, the \( N_v \) of neurons in layer I was 29% greater in Tg mice as compared to controls (\( P < 0.001 \)). The total number of neurons in the somatosensory cortex was increased by 28% in Tg mice (1,742,335 ± 65,525 neurons) as compared to controls (1,364,697 ± 42,309 neurons). Significant increases in individual laminar neuron numbers were observed in layers II to VI of Tg mice (22%-36%; Table 2-6). The largest increase in neuron number in the primary somatosensory cortex of Tg mice was observed in layer I (76%; Fig. 2-5), due to significant increases in both the \( N_v \) of neurons and laminar volume.

**IGF-I differentially augments the growth of the motor and somatosensory cortices**

IGF-I overexpression produced relatively greater overgrowth of the primary motor cortex in nestin/IGF-I Tg mice compared to the primary somatosensory cortex in these mice. The percentage increases (relative to corresponding control means) in the regional volumes of the motor and somatosensory cortices in Tg mice were compared using a 2 × 5 ANOVA (areas × layers).
Fig. 2-3. Photomicrographs of the primary motor cortex in (A) nestin/IGF-I Tg and (B) normal control mice at P12. The limits of the individual cortical layers are indicated at the left side of each panel. Note that the overall pattern of cortical lamination and the neuronal packing density in Tg mice appear normal. Scale bar, 200 μm.

Fig. 2-4. Photomicrographs of the primary somatosensory cortex in (A) nestin/IGF-I Tg and (B) normal control mice at P12. The limits of the individual cortical layers are indicated at the left side of each panel. Note that the overall pattern of cortical lamination and the neuronal packing density in Tg mice appear normal. Scale bar, 200 μm.
Table 2-3

Volumes (mm$^3$) of individual cortical layers in the primary motor and somatosensory regions of the cerebral cortex for nestin/IGF-I Tg and control mice on postnatal day 12$^a$

<table>
<thead>
<tr>
<th></th>
<th>Motor Cortex</th>
<th>Somatosensory Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tg (N = 6)</td>
<td>Control (N = 6)</td>
</tr>
<tr>
<td>Layer I</td>
<td>0.536 ± 0.031$^{***}$</td>
<td>0.358 ± 0.031</td>
</tr>
<tr>
<td></td>
<td>(50%)$^b$</td>
<td>(37%)</td>
</tr>
<tr>
<td>Layer II/III</td>
<td>1.327 ± 0.023$^{***}$</td>
<td>0.952 ± 0.027</td>
</tr>
<tr>
<td></td>
<td>(39%)</td>
<td>(30%)</td>
</tr>
<tr>
<td>Layer IV</td>
<td>0.452 ± 0.011$^{**}$</td>
<td>0.334 ± 0.017</td>
</tr>
<tr>
<td></td>
<td>(35%)</td>
<td>(29%)</td>
</tr>
<tr>
<td>Layer V</td>
<td>1.571 ± 0.049$^{***}$</td>
<td>1.086 ± 0.041</td>
</tr>
<tr>
<td></td>
<td>(45%)</td>
<td>(38%)</td>
</tr>
<tr>
<td>Layer VI</td>
<td>1.403 ± 0.026$^{***}$</td>
<td>1.003 ± 0.027</td>
</tr>
<tr>
<td></td>
<td>(40%)</td>
<td>(38%)</td>
</tr>
<tr>
<td>Total</td>
<td>5.310 ± 0.108$^{***}$</td>
<td>3.731 ± 0.126</td>
</tr>
<tr>
<td></td>
<td>(42%)</td>
<td>(35%)</td>
</tr>
</tbody>
</table>

$^a$Volume was measured bilaterally with values presented as the mean ± SEM.

$^b$Values represent the percent increase in Tg mice relative to the control mean in each cytoarchitectonic area.

$^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ for individual paired comparisons with control mice.
### Table 2-4

Pial surface area and mean cortical thickness in the primary motor and somatosensory regions of the cerebral cortex for nestin/IGF-I Tg and control mice on postnatal day 12\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Motor Cortex</th>
<th>Somatosensory Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tg (N = 6)</td>
<td>Control (N = 6)</td>
</tr>
<tr>
<td>Pial surface area (mm(^2))</td>
<td>4.791 ± 0.140(^*)</td>
<td>3.635 ± 0.167</td>
</tr>
<tr>
<td>Mean cortical thickness (mm)</td>
<td>1.111 ± 0.071</td>
<td>1.031 ± 0.062</td>
</tr>
</tbody>
</table>

\(^a\)Variables were measured bilaterally with values presented as the mean ± SEM.

\(^*\)P<0.01, \(^*\)P<0.001 compared to control mice using Student’s t-test.

### Table 2-5

Estimates of the numerical density of neurons (N\(_V\), neurons/mm\(^3\)) in individual layers of the primary motor and somatosensory regions of the cerebral cortex for nestin/IGF-I Tg and control mice on postnatal day 12\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Motor Cortex</th>
<th>Somatosensory Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tg (N = 6)</td>
<td>Control (N = 6)</td>
</tr>
<tr>
<td>Layer I</td>
<td>23,365 ± 934(^*)</td>
<td>18,779 ± 1,723</td>
</tr>
<tr>
<td>Layer II/III</td>
<td>194,262 ± 6,129</td>
<td>183,675 ± 4,939</td>
</tr>
<tr>
<td>Layer IV</td>
<td>206,195 ± 6,517</td>
<td>209,730 ± 5,961</td>
</tr>
<tr>
<td>Layer V</td>
<td>118,902 ± 3,995</td>
<td>111,926 ± 4,434</td>
</tr>
<tr>
<td>Layer VI</td>
<td>174,967 ± 5,539</td>
<td>178,596 ± 6,131</td>
</tr>
</tbody>
</table>

\(^a\)N\(_V\) was measured bilaterally with values presented as the mean ± SEM.

\(^*\)P<0.001 for individual paired comparisons with control mice using 2 × 5 ANOVA.
Fig. 2-5. Representative photomicrographs of layer I in the primary motor cortex of (A) nestin/IGF-I Tg and (B) normal control mice. Relatively few neurons were observed in layer I of control mice (B, arrows), while a substantial increase in the $N_v$ of neurons was observed in layer I of Tg mice. Scale bar, 25 μm.
Table 2-6
Total number of neurons in individual layers of the primary motor and somatosensory regions of the cerebral cortex in nestin/IGF-I Tg and control mice on postnatal day 12

<table>
<thead>
<tr>
<th></th>
<th>Motor Cortex</th>
<th>Somatosensory Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tg (N = 6)</td>
<td>Control (N = 6)</td>
</tr>
<tr>
<td>Layer I</td>
<td>12,474 ± 714**</td>
<td>6459 ± 194</td>
</tr>
<tr>
<td></td>
<td>(93%) b</td>
<td>(76%)</td>
</tr>
<tr>
<td>Layer II/III</td>
<td>257,440 ± 7,414**</td>
<td>175,038 ± 7,940</td>
</tr>
<tr>
<td></td>
<td>(47%)</td>
<td>(26%)</td>
</tr>
<tr>
<td>Layer IV</td>
<td>93,196 ± 3,644**</td>
<td>69,603 ± 2,665</td>
</tr>
<tr>
<td></td>
<td>(34%)</td>
<td>(22%)</td>
</tr>
<tr>
<td>Layer V</td>
<td>186,035 ± 4,066**</td>
<td>121,547 ± 7,151</td>
</tr>
<tr>
<td></td>
<td>(53%)</td>
<td>(36%)</td>
</tr>
<tr>
<td>Layer VI</td>
<td>245,124 ± 6,238**</td>
<td>179,218 ± 8,479</td>
</tr>
<tr>
<td></td>
<td>(37%)</td>
<td>(26%)</td>
</tr>
<tr>
<td>Total</td>
<td>794,269 ± 17,663**</td>
<td>551,865 ± 20,153</td>
</tr>
<tr>
<td></td>
<td>(44%)</td>
<td>(28%)</td>
</tr>
</tbody>
</table>

*aNeuron number was measured bilaterally with values presented as the mean ± SEM.
bValues represent the percent increase in Tg mice relative to the control mean in each cytoarchitectonic area.

*P<0.01, **P<0.001 for individual paired comparisons with control mice.
A significant main effect of regions \((F = 6.03, P = 0.017)\) was observed, indicating that the increase in motor cortex volume was disproportionately greater by 22% than that in the somatosensory cortex (Table 2-3). Evaluation for an interaction among regions and layers was not significant \((F = 1.29, P > 0.05)\); thus, a direct comparison between individual layers of the motor and somatosensory cortices was not warranted. The percentage increase in total neuron number was also influenced by cytoarchitectonic region \((F = 21.95, P < 0.001)\), indicating a disproportionately greater increase in total neuron number in the motor cortex (44%) compared to that in the somatosensory cortex (28%). Again, no interaction among regions and layers was detected \((F = 0.22, P > 0.05)\). In both the motor and somatosensory cortices the proportional increases in total neuron number among laminae exhibited the following descending order of increase: layer I > V > II/III > VI > IV.

*Indices of within group variability*

An index of within group variability can be obtained by calculating the standard error of the mean as a proportion of the mean. For estimates of total cortical volume, this index was found to be 3.4% for Tg mice and 2.7% for controls. For the volumes of individual cytoarchitectonic areas, it was found to be 2.0-3.8% for Tg mice and 2.9-3.8% for controls. Estimates of within group variability for the volumes of individual cortical laminae across both regions were 1.7-5.8% for Tg mice and 1.6-8.7% for controls. For estimates of total neuron number, this index was found to be 2.4% for Tg mice and 2.5% for controls. Within group variability indices for total neuron number estimates in the individual cytoarchitectonic areas were found to be 2.2-3.8% for Tg mice and 3.1-3.7% for controls. Similarly, variability indices for total neuron estimates in individual cortical laminae of both regions were 2.2-5.7% for Tg mice and 1.3-6.9% for controls.

*Reelin-immunoreactive cells on E14*

The results of the stereological analyses of layer I in the primary motor and primary somatosensory regions of the cortex at P12 indicated that total neuron number was greatly augmented in this layer in both cortical regions of Tg mice. To determine if this increase in neuron number was due to enhanced production of layer I neurons during embryonic development counts of reelin-immunoreactive cells were conducted in Tg and control embryos on E14, shortly after the normal time of layer I neuron production. Reelin is secreted by many layer I neurons, including Cajal-Retzius cells, and has been shown to play a critical role in directing proper cortical lamination (reviewed in Tissir and Goffinet, 2003). On E14, reelin-
immunoreactive cells were localized to the marginal zone of the developing cerebral wall (Fig. 2-6). As well, reelin staining was apparent in regions of the ventral telencephalon, particularly in the mantle zone of this region (Fig. 2-6). Cell counts conducted in the marginal zone revealed a significant increase of 28% (t = 2.883; P < 0.05) in the number of reelin-immunoreactive cells/mm$^2$ in nestin/IGF-I Tg embryos (2861 ± 72.6 cells/mm$^2$) as compared to normal littermate controls (2238 ± 203.6 cells/mm$^2$). Profile areas of reelin-immunoreactive cells did not differ significantly between Tg (28.30 ± 1.16 μm$^2$) and control embryos (27.08 ± 1.32 μm$^2$).

2.4 Discussion

The results of this study demonstrate that increased expression of IGF-I in the brain during embryonic and early postnatal development promotes growth of the cerebral neocortex, as evidenced by the significant increases in total neocortical volume (31%) and neuron number (27%) documented in nestin/IGF-I Tg mice at P12. Additionally, the volume of the subcortical white matter was significantly augmented by IGF-I overexpression in Tg mice. However, the increase in subcortical white matter volume in Tg mice was slightly greater than that observed in the neocortex (52% versus 31%). This disproportionate increase in white matter volume may result from an increase in axon diameter or in the number of axons in the white matter of Tg mice.

The N$_v$ of neurons in the neocortex of Tg mice did not differ significantly from controls. In fact, the only significant change in N$_v$ noted in Tg mice was the increased neuronal N$_v$ in layer I of both cytoarchitectonic regions examined. Several studies have shown that IGF-I overexpression during later stages of development results in reduced neuronal N$_v$ and increased dendritic outgrowth in a number of brain regions (Gutierrez-Ospina et al., 1996; Dentremont et al., 1999; O'Kusky et al., 2000). The present results suggest that increased IGF-I expression during prenatal and early postnatal development did not augment dendritic outgrowth sufficiently to reduce neuronal packing density by the time of examination on P12. However, the current findings are in general agreement with previous studies of Tg mice, which have demonstrated that augmented expression of IGF-I in the brain produces increased volume and neuron number in various regions of the CNS (Gutierrez-Ospina et al., 1996; Ye et al., 1996; Dentremont et al., 1999; O'Kusky et al., 2000; Popken et al., 2004).
Fig. 2-6. Representative photomicrographs of reelin immunostaining in the developing telencephalon of a control embryo at E14. Reelin immunostaining was localized to the marginal zone of the dorsal telencephalic wall (A, arrow), and was also present in the mantle zone of the ventral telencephalic wall (A, double arrowhead). At higher magnification individual reelin-positive cell bodies were seen in the marginal zone, immediately adjacent to the pial surface (B, arrows). Immunostaining patterns were similar in Tg embryos at E14. Scale bar in A = 100μm. Scale bar in B = 30μm.
Projection neurons of the cerebral cortex are produced largely from progenitor cells in the dorsal telencephalic wall between E11 and E17 (Takahashi et al., 1995). Following this period of neurogenesis, a phase of naturally occurring neuron death by apoptosis occurs in the cortex during the first two postnatal weeks (Spreafico et al., 1995; Verney et al., 2000). Augmented expression of IGF-I in nestin/IGF-I Tg mice coincides with both of these phases of cortical development. Previously, we have documented increases in cortical plate volume (52%) and total cell number (54%) in nestin/IGF-I embryos at E16 (Popken et al., 2004), indicating that increased cortical neuron number in Tg mice precedes the major period of apoptotic neuron death. Therefore, the observed increase in cortical neuron number in Tg mice at P12 likely results, at least in part, from an IGF-I-mediated enhancement of neural proliferation during the period of cortical neurogenesis.

IGF-I, besides its ability to promote neuron proliferation, is a well-documented inhibitor of neuron apoptosis in vitro (Bozyczko-Coyne et al., 1993; Hughes et al., 1993; Neff et al., 1993; Matthews and Feldman, 1996; Dudek et al., 1998; Blair et al., 1999; Yamada et al., 2001) and postnatally in vivo (Baker et al., 1999; Chrysis et al., 2001). Thus, IGF-I-mediated inhibition of neuron death by apoptosis likely contributes to the increased number of cortical neurons in nestin/IGF-I Tg mice. Experiments designed to determine the relative contributions of IGF-I-mediated enhancement of neuronal proliferation and inhibition of neuronal apoptosis to the observed increase in total neocortical neuron number in nestin/IGF-I Tg mice will be discussed in later chapters.

Cortical surface area was increased in nestin/IGF-I Tg mice in approximately equal proportions in both the anteroposterior and mediolateral dimensions. Sections stained for the presence of Nissl substance showed that the topographical distribution of cytoarchitectonic areas through the rostrocaudal extent of the cerebral cortex was essentially normal in Tg mice. These findings suggest that IGF-I may influence areal growth, but likely does not influence the regional patterning of the cortex. Current evidence indicates that a number of growth factors exert their effects from specific signaling centers. For example, fibroblast growth factor-8 (FGF8) is expressed in the anterior telencephalon where it imparts anteroposterior position in the cortical field (Fukuchi-Shimogori and Grove, 2001). The present data indicate that the growth promoting effects of IGF-I may differ from those of growth factors acting at specific signaling centers, which in turn influence brain patterning.
In addition to the documented increase in total neuron number in the neocortex of nestin/IGF-I Tg mice, the results of this study also revealed increases in the total number of glia in the neocortex and subcortical white matter of these mice. Similar to the \( N_v \) of neurons, the \( N_v \) of glia was not significantly altered in Tg mice at P12. These data are consistent with previous reports of Tg mice which showed that augmented expression of IGF-I during postnatal development produces increased numbers of glia (Ye et al., 1995a, b). However, given that IGF-I expression begins prenatally in nestin/IGF-I Tg mice, the observed increase in glial cell number on P12 may reflect an initial overproduction of glia during embryonic development of the cortex. While the mechanisms responsible for the increased number of glia in Tg mice have not been examined directly, the finding that IGF-I influences the cell cycle kinetics of progenitors in the VZ of embryonic mice (see Chapter 3) suggests that augmented prenatal production of glia is possible. Conversely, the increase in glia may be due, in part, to an enhancement of postnatal proliferation or an inhibition of glia cell death by apoptosis, as IGF-I has been shown both to promote oligodendrocyte proliferation (McMorris and Dubois-Dalcq, 1988; Mozell and McMorris, 1991; Jiang et al., 2001) and to inhibit oligodendrocyte apoptosis (Barres et al., 1992; Ye and D’Ercole, 1999) \textit{in vitro}. Further study is required to specifically address the mechanisms that contribute to the increased numbers of glia in nestin/IGF-I Tg mice. Regardless of the mechanism responsible for the increase in glial cell numbers in the neocortex and subcortical white matter of Tg mice, the present observations indicate that IGF-I is an important factor in glial cell development.

\textit{IGF-I overexpression differentially augments the growth of the primary motor and primary somatosensory cortices in nestin/IGF-I Tg mice}

In the present study, the boundary criteria of Zilles (1985) were used to define the limits of the primary motor and primary somatosensory cortices and to delineate individual cortical laminae within these cytoarchitectonic regions. Indices of within-group variability demonstrate that taking measurements from successively smaller compartments of the cerebral cortex did not result in substantial increases in within-group variability. For example, this index was estimated to be 2.2-3.8% for Tg mice and 3.1-3.7% for controls with respect to measurements of total neuron number in the individual cytoarchitectonic areas, which are similar values to those calculated for measurements of total neocortical neuron number for control (2.5%) and Tg (2.4%) mice. Thus, these boundary criteria resulted in relatively precise...
and reliable estimates of tissue volume and neuron number for both cortical regions and laminae.

The effects of augmented IGF-I expression on volume and neuron number were proportionately greater on the motor cortex than on the somatosensory cortex of nestin/IGF-I Tg mice. In mice, neocortical neurogenesis is initiated in rostrolateral regions of the telencephalic VZ and progresses caudomedially from E12 to E17 (Miyama et al., 1997). These spatial gradients in neuronal proliferation do not appear to have contributed to the disproportionately greater growth of the motor cortex relative to the somatosensory cortex in nestin/IGF-I Tg mice, as the motor and somatosensory cortices occupy adjacent positions along this axis. Alternatively, differences in the relative rates of layer production in adjacent cytoarchitectonic areas have been documented (Polleux et al., 1997a) and may contribute to the disproportionate effect of IGF-I on motor cortex growth. Differences in layer production rates result from regional variations in both laminar cell cycle duration and the proportion of cells exiting the cell cycle as post-mitotic neurons (Polleux et al., 1997a, b). The effects of IGF-I on cell cycle duration and cell cycle exit will be discussed in a later section.

The increases in neuron number documented in the motor and somatosensory cortices of nestin/IGF-I Tg mice differed between individual cortical laminae in both cortical regions. However, the magnitude of increases in neuron number in both the motor and somatosensory regions followed similar patterns, such that layer I > V > II/III > VI > IV. Relatively greater increases in the number of neurons in a given layer may reflect a differential effect of IGF-I on the distinct cell types which predominate in that layer.

Few studies have addressed the effects of IGF-I on specific types of neurons. In IGF-I knockout mice, significant decreases in the number of parvalbumin-immunoreactive interneurons (30%) have been reported in the cerebral cortex (Beck et al., 1995). However, the numbers of cholinergic and dopaminergic neurons were unaffected in these mice, as were the numbers of large projection neurons in layers III and V of the parietal and temporal cortices of the IGF-I knockout mice (Beck et al., 1995). These results suggest that distinct neurotransmitter-specific neuron populations may be differentially affected by IGF-I and that cortical interneurons may depend more on IGF-I for their survival than projection neurons. During the normal period of neuron elimination by apoptosis in the cerebral cortex, the survival rate for GABAergic interneurons has been shown to be only half that of projection
neurons (Miller, 1995). Therefore, the anti-apoptotic effects of IGF-I might be expected to differentially augment the survival of cortical interneurons.

The cell counting method employed in this study involved the use of a thionine stain for the detection of Nissl substance, which did not allow for precise differentiation between projection neurons and interneurons. However, the increased neuron numbers documented in nestin/IGF-I Tg mice were relatively greater in layers V and II/III, where projection neurons predominate, than in layer IV, whose population consists largely of stellate neurons. These findings indicate that projection neurons may be amplified by IGF-I overexpression to a greater degree than stellate neurons.

Projection neurons, as noted earlier, are derived from the VZ and SVZ of the dorsal telencephalon (pallium) corresponding to the primordial cerebral cortex. Cortical interneurons, on the other hand, arise from progenitor cells in the proliferative zones of the ventral telencephalon (subpallium) and migrate to their final positions within the cortical plate (Anderson et al., 1997, 1999, 2002). It has been shown previously that the volume of the dorsolateral telencephalic wall is significantly increased by 25% in nestin/IGF-I Tg embryos at E16 (Popken et al., 2004). This augmented volume was due to significant increases in the volumes of the VZ/SVZ (26%), intermediate zone (12%) and cortical plate (52%). In contrast, the total volume of the ventrolateral telencephalic wall did not differ significantly between Tg and control embryos. Similarly, VZ and SVZ volume in this region of the telencephalon, which corresponds to the proliferative regions responsible for the production of a number of cortical interneurons, did not differ between groups (Popken et al., 2004). Therefore, IGF-I appears to differentially affect the development of the dorsolateral and ventrolateral regions of the embryonic telencephalon. Given the increased volume of the dorsolateral telencephalic wall, the pro-mitotic effects of IGF-I may be expected to differentially augment the production of cortical projection neurons.

In nestin/IGF-I Tg mice the greatest increases in neuron number were observed in layer I, with significant increases in both the motor (93%) and somatosensory (76%) regions. The neurons of layer I are some of the first post-mitotic cells to be produced during cortical neurogenesis (E10-E12), and are typically transient cells that undergo cell death during late embryonic and early postnatal development (Bayer and Altman, 1990; Derer and Derer, 1990; Hestrin and Armstrong, 1996; Meyer et al., 1998, 1999; Hevner et al., 2003). The increase in layer I neurons was noticeably greater than that observed for any other layer in Tg mice,
suggesting that one or more of the types of neurons populating layer I might be exceptionally responsive to IGF-I. Several types of neurons that reside in layer I, such as Cajal-Retzius cells, cortical pioneer neurons, subpial pyriform cells and neurogliaform cells, perform critical functions in the determination of cortical structure (Bayer and Altman, 1990; Derer and Derer, 1990; Hestrin and Armstrong, 1996; Marin-Padilla, 1998; Meyer et al., 1998, 1999).

The present study shows that the number of reelin-immunoreactive cells per unit area of marginal zone is increased by 28% in Tg animals during early embryonic development (E14). The documented increase in the density of reelin-immunoreactive cells is not influenced by the profile areas of these cells as no significant change in profile area was apparent between groups. Reelin is a large secreted extracellular matrix protein produced by many layer I neurons that has been shown to play a critical role in directing proper cortical lamination (see Rice and Curran, 2001 for review). Reelin is expressed by several types of layer I neurons, including Cajal-Retzius cells (Derer and Derer, 1990; Meyer et al., 1998, 1999; Hevner et al., 2003). The increased density of reelin-immunoreactive cells documented in Tg animals is apparent during embryonic development, prior to the phase of normal apoptotic elimination of many of these cells, which occurs during early postnatal development. Therefore, these data indicate that the increase in layer I neurons documented during postnatal development in Tg mice likely results, at least in part, from an IGF-I-mediated enhancement of the production of reelin-immunoreactive cells during embryonic development. However, the 28% increase in reelin-immunoreactive cells on E14 does not appear to account entirely for the very large increases in layer I neurons in Tg mice on P12. Therefore, this large increase in layer I neurons in Tg mice may result from both enhanced neuronal proliferation and decreased elimination of layer I neurons by apoptosis. Further studies would be required to determine if layer I neurons persist beyond the end of the second postnatal week in Tg mice. As well, more detailed studies using different markers for layer I neurons would be necessary to determine the relative effects of IGF-I on specific subpopulations of cells within this layer.

In conclusion, the present findings demonstrate that augmented IGF-I expression promotes growth of the cerebral cortex and associated subcortical white matter by increasing the numbers of neurons and glia in these structures. Distinct cytoarchitectonic regions of the cortex are differentially affected by IGF-I, with the growth of the primary motor cortex being augmented to a greater degree than that of the primary somatosensory cortex in nestin/IGF-I
Tg mice. Additionally, the neurons of layer I, many of which are involved in cortical layer formation, appear to be particularly sensitive to the effects of IGF-I.
2.5 Bibliography


Chapter III The effects of IGF-I on the cell cycle kinetics of neuron progenitors and cell cycle exit during neurogenesis in the cerebral cortex

3.1 Introduction

In the previous chapter, elevated IGF-I expression in nestin/IGF-I Tg mice was shown to produce increases in cortical volume and neuron number, with differential effects on specific cortical layers. However, it was not possible to determine the mechanisms responsible for the increase in neuron number using the methods described in the previous study. The increase in cortical neuron number documented in nestin/IGF-I Tg mice may result from enhanced neuron output, inhibited apoptosis, or a combination of these effects. Previous studies have shown that IGF-I overexpression in nestin/IGF-I Tg mice begins by as early as E13, during the normal period of neurogenesis (E11-E17) in the mouse cerebral cortex (Popken et al., 2004). As well, increases in the number of cells in the cortical plate and in the combined volume of the VZ and SVZ have been documented in Tg mice at E16, well before the major period of neuron apoptosis in the cortex (Popken et al., 2004). Based on this evidence, the hypothesis that increased neuron number in the cerebral cortex of nestin/IGF-I Tg mice results, at least in part, from an IGF-I-mediated increase in neuron production during embryonic development was generated. The present study was designed to investigate the in vivo effects of IGF-I on several factors involved in controlling neuron output during embryonic development.

Neuron progenitor cells in the dorsal telencephalon produce the vast majority of projection neurons destined for the cerebral cortex (for review see Parnavelas, 2000). Recent evidence indicates that progenitors in VZ produce neurons, intermediate progenitor cells and some glial cells (Takahashi et al., 1995a; Miyata et al., 2001; Noctor et al., 2001, 2002, 2004; Englund et al., 2005). Progenitors in the morphologically defined SVZ produce neurons during cortical neurogenesis, and give rise to glial cells later in embryonic development, (Takahashi et al., 1995b; Tarabykin et al., 2001; Noctor et al., 2004; Englund et al., 2005). In rodents, cortical interneurons arise predominantly from the ventral telencephalon (Anderson et al., 1997, 1999, 2002). Neocortical neurogenesis in the VZ of the normal mouse extends from E11 through E17 (Takahashi et al., 1995a). During this time the mean length of the cell cycle (\(T_c\)) increases approximately 2-fold, due entirely to an increase in the length of the G1 phase (Takahashi et al., 1995a). For progenitor cells in the VZ, the proliferative fraction (proportion

\[ \text{proliferative fraction} = \frac{\text{proliferating cells}}{\text{total cells}} \]

of daughter cells that re-enter the cell cycle) gradually decreases, while the exiting fraction (proportion of daughter cells that exit the cell cycle) gradually increases, indicating that initially neurons are generated slowly, while the VZ expands rapidly (Takahashi et al., 1994, 1996).

The number of neurons produced by progenitors during cortical neurogenesis is influenced by several factors, including the cell cycle kinetics of proliferating progenitor cells in the VZ of the dorsal telencephalon (Takahashi et al., 1993, 1995a). Using cumulative S-phase labeling with 5-bromo-2'-deoxyuridine (BrdU) several cell cycle parameters, including the growth fraction (GF, proportion of proliferating cells), total cell cycle duration ($T_c$, mean length of the cell cycle), and individual phase lengths ($T_{G1}$, $T_s$, $T_{G2}$, and $T_M$; lengths of the G1, S, G2, and M phases, respectively) were measured in nestin/IGF-I Tg and control mice on E14. Neuron output is also dependent upon the fate decisions made by progenitor cells following a given round of division. Cells may either exit the cell cycle as post-mitotic neurons or re-enter the cell cycle and undergo further rounds of division. If more cells re-enter the cell cycle as progenitors, the potential for enhanced neuron output is increased (Takahashi et al., 1996; Chenn and Walsh, 2002). To determine if IGF-I overexpression influences progenitor fate determination, single-injection BrdU labeling was combined with double-labeling immunohistochemistry to measure both the proliferative fraction and the exiting fraction on E14-E15. The developmental length of neurogenesis may also influence the number of neurons produced during this period. For example, a prolonged period of neurogenesis beyond E17 may result in increased neuron output. Using BrdU labeling at E11, E17, E18, the time-span of neurogenesis was determined in nestin/IGF-I Tg and control embryos.

3.2 Materials and Methods

Embryo collection and tissue processing

In total, 90 embryos derived from 20 separate litters were used in the present study. To generate timed-pregnant dams, male nestin/IGF-I Tg mice were placed with normal C57BL/6 female mice (Charles River Laboratories, Wilmington, MA) for a period of 2 hr (8:00 A.M. to 10:00 A.M.). Vaginal plug checks were conducted at the end of the 2 hr period. The presence of a vaginal plug indicated conception had occurred, and the day of conception was taken as E0. Litters generated by breeding heterozygous Tg males with normal C57BL/6 females typically consisted of half Tg and half normal non-Tg littermate control embryos. Embryo genotype was assessed by PCR analysis of genomic DNA derived from somatic tissues.
Embryos were collected from timed-pregnant dams at various gestational ages, including E11, E14, E15, E17 and E18. Individual dams were deeply anesthetized with i.p. injections of ketamine (100 mg/kg) and xylazine (10 mg/kg). A hysterotomy was performed and the uterus was removed to ice cold 0.1 M PBS (pH 7.4). In the case of embryos at E11- E17, individual fetuses were dissected from the uterine tissue, decapitated, and the whole heads were placed in 70% ethanol overnight at 4°C. E18 animals were decapitated and the brain was dissected free of surrounding tissue and fixed by immersion as described above. Following fixation, embryos were embedded in paraffin and sectioned at 4 μm in the coronal plane. Sections were collected on Colorfrost Plus glass slides (Fisher Scientific, Ottawa, ON) and air-dried.

Cumulative BrdU labeling at E14

The mean lengths of the total cell cycle (T_c) and S phase (T_s) were determined on E14 in Tg and control embryos using a cumulative BrdU labeling protocol. The growth fraction (GF), defined as the proportion of VZ cells actively proliferating on E14 (Takahashi et al., 1993), was also determined using this method. Details of the BrdU labeling protocol have been published previously (Takahashi et al., 1992, 1993, 1995a). Pregnant mice were injected i.p. with BrdU (50 μg/g body weight) beginning at 9:00 A.M. on E14. Subsequent injections were administered at maximum intervals of 3 hr, with the last BrdU injection administered 0.5 hr before sacrifice of the dam (Takahashi et al., 1993). For example, animals from the 8.0 hr survival time received BrdU injections at 9:00 A.M., 12:00 P.M., 3:00 P.M., and 4:30 P.M., with the hysterotomy performed at 5:00 P.M. Embryos were collected at survival time points 0.5, 2.0, 3.5, 8.0, 11.0, 12.5, and 14.0 hr after the initial BrdU injection. Four animals per group were collected at each time point, except for the 14.0 hr survival time where 3 Tg and 3 control embryos were sampled. In total, 54 embryos from 12 litters were used in this analysis.

Embryos were fixed and processed for paraffin embedding as described above. Coronal sections (4 μm) were stained immunohistochemically for the detection of BrdU. Briefly, sections were deparaffinized, rehydrated in descending grades of ethanol, and incubated in 2 N HCl for 1 hr. Sections were incubated with a mouse monoclonal anti-BrdU antibody (1:75; BD Biosciences, San Diego, CA) for 1 hr at room temperature. Control slides were processed without incubation in the primary antibody. Following incubation with the primary antibody, sections were processed using a Vectastain ABC Elite Kit for mouse IgG (Vector Laboratories), according to the manufacturer’s protocol. Slides were reacted for 3 min
with 0.05% diaminobenzidine (DAB), 0.025% cobalt chloride, 0.02% nickel ammonium sulfate and 0.01% hydrogen peroxide. Sections were counterstained with 0.1% aqueous basic fuchsin.

Cell counts were conducted on four non-adjacent coded histological sections selected for each embryo at each survival time point. Details of the counting methods employed have been published elsewhere (Takahashi et al., 1993, 1995a). Counts were conducted in a sector of the dorsomedial cerebral wall corresponding to the primordial primary somatosensory cortex. The sector measured 100 μm in its mediolateral dimension and 4 μm in its anteroposterior dimension and was positioned over a constant region of the dorsomedial cerebral wall in each embryo. It was divided into bins, 10 μm in height, that were oriented parallel to the ventricular surface. Within each bin, BrdU-labeled and unlabeled nuclei (Fig. 3-1) were counted according to criteria established by Takahashi and collaborators (1993). After examining the distribution of labeled cells relative to the ventricular surface, Bins 1-7 were identified as best representing the VZ in both Tg and control embryos. A labeling index (LI), defined as the proportion of BrdU-labeled cells to total cells in Bins 1-7, was determined for the VZ.

Graphs of LI versus post-injection survival time were generated for Tg and control embryos. For each group, a least-squares fit to all of the data points was generated using Microsoft Excel (Nowakowski et al., 1989). The GF was determined as the maximum LI value attained for each group during the experimental period. $T_c$ and $T_s$ were determined from the following equations: (1) $y$-intercept = $GF \times T_s / T_c$, and (2) time to reach maximum LI = $T_c - T_s$ (Takahashi et al., 1993).

The statistical significance of differences among mean LI values was assessed using a 2 × 7 (groups × survival times) ANOVA of data from the 0.5 – 14.0 hr survival times. Post hoc analysis of the statistical significance of differences between individual pairs of means was conducted using estimated marginal means with a least significant difference adjustment for multiple comparisons.

**Measurement of $T_{G2+M}$ on E14**

To determine the lengths of the G2 and M phases of the cell cycle in Tg and control embryos on E14, a single injection BrdU labeling protocol was used (Takahashi et al., 1993). Single i.p. injections of BrdU were administered to timed-pregnant dams at 9:00 A.M. on E14. Dams were allowed to survive 1.0, 1.5, and 2.0 hr post-injection. At the specified survival
times, hysterotomies were performed and whole embryo heads were removed and fixed by immersion in 70% ethanol overnight (N = 3 per group for each survival time). Embryos were embedded in paraffin and sectioned at 4 μm in the coronal plane. Sections were processed for the detection of BrdU immunoreactivity as described in the previous section. Cell counts were conducted on 4 non-adjacent coded histological sections within a dorsomedial sector of the cerebral wall equivalent to that used for the cumulative BrdU labeling study. Within each sector the percentage of BrdU-labeled VZ mitotic figures was determined. Mitotic figures were considered to belong to the VZ only if they occurred within Bin 1 of the sector (i.e. adjacent to the ventricular surface). The combined length of the G2 and M phases of the cell cycle (T_{G2-M}) was determined as the time required to label all ventricular mitotic figures with BrdU (Takahashi et al., 1993). The statistical significance among means was assessed using a 2 x 3 (groups x survival times) ANOVA.

**Mitotic Index (MI) estimates at E14**

Estimates of MI, defined as the proportion of cells in mitosis compared to the total number of cells in the VZ, were determined for Tg and control embryos at E14. Counts of mitotic figures were conducted on mice from the 0.5, 2.0, and 3.5 hr survival times (N = 4 embryos per group for each time point) according to the method of Takahashi et al. (1993). Five sections adjacent to those used for estimating T_c and T_s were selected from each brain. Sections were stained with 0.1% aqueous thionine in acetate buffer (pH 3.7) for 15 min, dehydrated in alcohols, cleared in xylene, and coverslipped with Permount.

Mitotic figures were counted in the same dorsomedial sector of the cerebral wall described in previous sections. Counts were conducted in both hemispheres of each brain. Mitotic figures falling within Bin 1 were taken to represent VZ mitoses. Abventricular mitotic figures located in other bins (i.e. those mitotic cells not immediately adjacent to the ventricular surface) were considered separately. The total number of cells in the VZ (Bins 1-7) was determined for each animal. MI was determined from the equation (3) \[ \text{MI} = \frac{N_m}{N_{tot}} \], where N_m is the number of VZ mitotic figures and N_{tot} is the total number of cells in the VZ (Takahashi et al, 1993). Estimates of T_M were determined from the equation (4) \[ T_M = \text{MI} \times T_C \]. The statistical significance among mean MI values, numbers of ventricular mitoses per sector, numbers of abventricular mitoses per sector, and numbers of cells in the VZ were assessed using 2 x 3 (groups x survival times) ANOVA.

**Calculation of GI phase length (T_{GI})**
\( T_{G1} \) was calculated from the equation (5) \( T_{G1} = T_C - (T_S + T_{G2+M}) \) (Takahashi et al., 1993).

**Cell cycle exit at E14-E15**

The proportion of cells in the cerebral wall that exit the cell cycle (exiting fraction) and the complementary proportion of cells that re-enter the cell cycle (proliferative fraction) were assessed in Tg and control embryos on E14-E15. A cohort of proliferating cells was labeled with a single i.p injection of BrdU (50 \( \mu \text{g/g body weight} \)) administered at 9:00 A.M. on E14. At 9:00 A.M. on E15, 24 hr later, embryos were collected by hysterotomy (Chenn and Walsh, 2002). For each group, 3 embryos were fixed by immersion in 70% ethanol, embedded in paraffin, and sectioned at 4 \( \mu \text{m} \) in the coronal plane.

Five non-adjacent sections (every second section in a series of 10 serial sections) were selected from each embryo for cell counts. Slides were processed for the immunohistochemical detection of BrdU and Ki67, an endogenous marker of proliferating cells (Scholzen et al., 2000; Kee et al., 2002). Sections were deparaffinized, rehydrated and heated in Antigen Unmasking Solution (Vector Laboratories) for 5 min, until the solution came to a boil. The temperature was then reduced, and sections were heated for a further 20 min. Slides were incubated in blocking solution (0.3% triton X-100 and 5% normal goat serum in PBS) for 1 hr at room temperature. Primary antibodies were diluted in blocking solution and slides were incubated overnight at 4 °C. The primary antibodies used were mouse monoclonal anti-BrdU (1:200; BD Biosciences) and rabbit polyclonal anti-Ki67 (1:500; Novocastra Laboratories, Newcastle upon Tyne, UK). Primary antibody binding was detected using the fluorochrome-conjugated secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG (1:500; Molecular Probes, Eugene, OR) and Alexa Fluor 633 goat anti-mouse IgG (1:250; Molecular Probes), which were applied to the slides for 1 hr at room temperature in the dark. Sections were mounted in ProLong Gold antifade reagent (Molecular Probes) and dried overnight.

Immunofluorescence was detected using an Olympus Fluoview 500 confocal laser scanning microscope. Z-axis optical sections (1 \( \mu \text{m} \) in thickness) were collected over the dorsomedial cerebral wall. Specimens were scanned sequentially with an argon laser and a red helium neon laser to prevent cross-talk between fluorescence channels. Differential interference contrast (DIC) images of the specimens were collected with each scan. Scans were collected in sectors of both the right and left hemispheres on each section (i.e. 10 scans per brain). Images were viewed using Olympus Fluoview software, and cells were counted by
hand. Within the 100 μm x 4 μm sector of the dorsomedial cerebral wall, BrdU-labeled cells were counted and scored for the presence or absence of Ki67 immunofluorescence. The proportions of BrdU-positive/Ki67-positive (BrdU+/Ki67+; proliferative fraction) and BrdU-positive/Ki67-negative (BrdU+/Ki67-; exiting fraction) cells were determined for each animal (Chenn and Walsh, 2002). The statistical significance of differences among means was assessed using a 2 x 2 (groups x cell types) ANOVA. Post hoc analysis of the statistical significance of differences between individual pairs of means was conducted using estimated marginal means with a least significant difference adjustment for multiple comparisons.

**BrdU labeling on E11, E17, E18**

Single injections of BrdU were administered to timed-pregnant dams on E11, E17, and E18 to determine the duration of neurogenesis during embryonic development in Tg and control embryos. All BrdU injections (50 μg/g body weight) were administered at 9:00 A.M. and the dam was sacrificed 1 hr post-injection. Embryos were removed by hysterotomy and processed as described above. Representative sections were selected and stained immunohistochemically for the detection of BrdU with basic fuchsin as a counterstain as described in previous sections.

### 3.3 Results

**Cell cycle kinetics in nestin/IGF-I Tg and control embryos on E14**

Detailed analyses of cell cycle kinetics were performed in nestin/IGF-I Tg and control embryos on E14, which is the approximate midpoint of neurogenesis in normal mice. No signs of gross malformation or morphological abnormalities were apparent in Tg embryos upon histological examination. At this age, the size and lamination pattern of the telencephalic wall was similar in Tg and control embryos, and the two groups were visually indistinguishable (Fig. 3-1). In both groups the thickness of the VZ was approximately 70 μm over the dorsomedial sector analyzed, and VZ thickness remained constant during the 14.0 hr period of cumulative BrdU labeling.

**$T_c$ is reduced in Tg embryos**

To determine if IGF-I influences overall cell cycle length during cortical neurogenesis, a cumulative BrdU labeling protocol was used to generate LI values for the VZ on E14. Plots of LI versus survival time were generated for both Tg and control embryos. These plots illustrated that LI increased linearly to a maximum value at a given survival time and then leveled off in both groups (Fig. 3-2). The GF (equivalent to maximum LI) was 0.98 in both Tg
Fig. 3-1. Photomicrographs illustrating BrdU labeling in E14 control (A, C) and nestin/IGF-I transgenic (Tg) (B, D) embryos 0.5 hr after injection of a timed-pregnant dam. Counts of BrdU labeled and unlabeled cells were conducted over the dorsomedial cerebral wall (A, black rectangle). BrdU labeled cells were identified by the accumulation of brown reaction product in cell bodies (D, arrows). Unlabeled cells appear pink due to the use of a basic fuchsin counterstain. As illustrated in the low power photomicrographs, Tg embryos (B) were not visually distinguishable from controls (A) on E14. Figures C and D illustrate the structure of the cerebral wall from the ventricular surface to the pia at 9:30 A.M. on E14. The cerebral wall consisted mainly of the ventricular (VZ) and subventricular zones (SVZ) in both control (C) and Tg (D) embryos at this time, and a morphologically distinguishable cortical plate was not evident in either group. The height of the VZ and the total cerebral wall were similar in Tg and control embryos. Scale bar in A = 100 μm. Scale bar in C = 30 μm. Reprinted with permission from: Hodge RD, D’Ercole AJ, O’Kusky JR (2004) Insulin-like growth factor-I accelerates the cell cycle by decreasing G1 phase length and increases cell cycle reentry in the embryonic cerebral cortex. J Neurosci 24:10201-10210. Copyright 2004 by the Society for Neuroscience.
Fig. 3-2. Plots of BrdU labeling indices (LI) determined by cumulative labeling with BrdU in nestin/IGF-I Tg and control embryos on E14. The cumulative injection experiments were initiated at 9:00 A.M., with the final survival time point collected 14.0 hr later. LI was determined for the VZ, which was 7 bins in height for both Tg and control animals. For each group, a least squares curve was fit to all data points from each survival time in the analysis. The plots illustrate the linear advance of LI towards maximum levels in Tg (filled squares, solid line) and control (open squares, dashed line) embryos as the number of BrdU injections administered increases over the experimental period. As maximum labeling is reached LI values level-off accordingly in each group. However, LI values plateau at an earlier time in Tg embryos indicating that LI progresses to a maximum at a faster rate in Tg embryos and that $T_c-T_s$ is reduced in Tg mice. *Reprinted with permission from: Hodge RD, D’Ercole AJ, O’Kusky JR (2004) Insulin-like growth factor-I accelerates the cell cycle by decreasing G1 phase length and increases cell cycle reentry in the embryonic cerebral cortex. J Neurosci 24:10201-10210. Copyright 2004 by the Society for Neuroscience.*
and control embryos, indicating that effectively all cells in the VZ were proliferating over the experimental period.

The relationship between LI and survival time was compared between Tg and control embryos using a 2 × 7 ANOVA (groups × survival times). A significant main effect of groups was observed (F = 66.568, P < 0.001), indicating that LI was significantly greater in Tg mice. The interaction between groups and survival times was also significant (F = 6.686, P < 0.001), allowing for direct comparisons between individual mean LI values. LI did not differ significantly between Tg and control embryos at the 0.5 hr, 12.5 hr, or 14.0 hr post-injection survival times. However, LI was significantly greater in Tg embryos at the 2.0 hr (16%, P < 0.001), 3.5 hr (12%, P < 0.001), 8.0 hr (13%, P<0.001), and 11.0 hr (7%, P < 0.001) survival times. These data suggest that the proportion of cells in S-phase at 0.5 hr after the initial BrdU injection was approximately the same in Tg and control embryos, as LI values did not differ between groups at this survival time. However, the rate of labeling increased in Tg embryos at subsequent time points, as illustrated by the significantly higher LI values at the 2.0 hr – 11.0 hr survival times. After reaching the inflection point in the LI versus survival time graph, LI values leveled off in Tg and control embryos. Consequently, the LI values for the 12.5 and 14.0 hr survival times did not differ between groups. The time required to reach maximum LI (i.e. T<sub>C</sub>-T<sub>S</sub>, according to equation 2) was calculated to be 10.81 hr in Tg embryos and 12.66 hr in controls, indicating that the time required to reach the maximum LI was significantly decreased by 1.85 hr in Tg embryos.

Individual phase lengths for T<sub>C</sub> and T<sub>S</sub> were derived from the graphs of LI versus survival time using equations 1 and 2, cited in the methods section above. T<sub>S</sub> was slightly shorter (12 min) in Tg embryos, but the difference in T<sub>S</sub> between groups was insubstantial (Table 3-1). However, T<sub>C</sub> was 2.05 hr shorter in Tg mice (Table 3-1), indicating that the length of one or more phases of the cell cycle must be reduced in Tg embryos.

T<sub>G2-M</sub> is normal in Tg embryos

The combined length of the G2 and M phases of the cell cycle was determined from single injection BrdU experiments conducted on E14. At post-injection survival times of 1.0, 1.5, and 2.0 hr, the proportion of VZ mitotic figures labeled with BrdU was determined in Tg and control embryos (Fig. 3-3). A 2 × 3 ANOVA comparison of the data showed that the proportion of labeled mitotic figures did not differ significantly between groups (F = 0.065, P > 0.05). The percentage of labeled figures was similar in Tg and control embryos at each
Table 3-1
Total cell cycle and individual phase lengths measured in nestin/IGF-I transgenic and normal littermate control embryos on embryonic day 14<sup>a</sup>

<table>
<thead>
<tr>
<th>Cell Cycle Parameter</th>
<th>Nestin/IGF-I Transgenic</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time required to reach maximum LI</td>
<td>10.81</td>
<td>12.66</td>
</tr>
<tr>
<td>T&lt;sub&gt;S&lt;/sub&gt;</td>
<td>5.06</td>
<td>5.26</td>
</tr>
<tr>
<td>T&lt;sub&gt;G2+M&lt;/sub&gt;</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>T&lt;sub&gt;M&lt;/sub&gt;</td>
<td>0.61</td>
<td>0.56</td>
</tr>
<tr>
<td>T&lt;sub&gt;G2&lt;/sub&gt;</td>
<td>1.39</td>
<td>1.45</td>
</tr>
<tr>
<td>T&lt;sub&gt;G1&lt;/sub&gt;</td>
<td>8.81</td>
<td>10.66</td>
</tr>
<tr>
<td>T&lt;sub&gt;C&lt;/sub&gt;</td>
<td>15.87</td>
<td>17.92</td>
</tr>
</tbody>
</table>

Cell cycle phase lengths were measured using cumulative and single injection BrdU labeling protocols. T<sub>C</sub> represents total cell cycle length. T<sub>G1</sub>, T<sub>S</sub>, T<sub>G2</sub>, and T<sub>M</sub> denote the lengths of the G1, S, G2 and M phases, respectively. T<sub>G2+M</sub> denotes the combined length of the G2 and M phases. <sup>a</sup>All values are expressed in hours.
survival time, with approximately 25% of figures labeled at 1.0 hr post-injection and 55% of figures labeled at 1.5 hr post-injection (Fig. 3-3). By 2.0 hr post-injection, virtually 100% of mitotic figures were BrdU-labeled in both Tg and control embryos (Fig. 3-3). \( T_{G2+M} \) was estimated as the time required to label all mitotic figures with BrdU, which was 2.0 hr in both groups. Therefore, \( T_{G2+M} \) was invariant at 2.0 hr in both Tg and control embryos.

**Increased mitotic index in Tg embryos**

MI, the proportion of mitotic figures relative to the total number of cells in the VZ, was determined on sections stained for Nissl substance from the 0.5, 2.0, and 3.5 hr cumulative BrdU labeling survival times (Equation 3). The mean number of cells in the VZ did not differ significantly between Tg (139.42 ± 4.98; mean ± SEM) and control (151.50 ± 6.89) embryos on E14, indicating that cellular density in the VZ was similar in both groups. The mean number of ventricular mitotic figures per sector did not differ significantly between groups (Tg = 5.208 ± 0.242; control = 4.667 ± 0.319; \( P > 0.05 \)). Similarly, the mean number of abventricular mitotic figures per sector was not significantly different in Tg and control embryos (Tg = 1.125 ± 0.118; control = 1.117 ± 0.108; \( P > 0.05 \)). However, analysis of the MI data using a 2 x 3 ANOVA (groups x survival times) showed a significant main effect for groups (\( F = 7.287, P = 0.015 \)), indicating that MI was significantly increased by 21% in Tg embryos (0.382 ± 0.003) as compared to controls (0.314 ± 0.003). The interaction (groups x survival times) was not significant, so individual paired comparisons of mean values were not warranted.

MI data were used to calculate an estimate of \( T_M \) for each group (Equation 4). \( T_M \) values were similar in Tg and control embryos (36.60 min and 32.87 min, respectively), indicating that \( T_M \) did not differ considerably between groups. Individual estimates of \( T_{G2} \), determined by subtracting \( T_M \) values from 2.0 hr (\( T_{G2+M} \)), also did not differ greatly between groups (Table 3-1). Individual estimates of \( T_M \) and \( T_{G2} \) were not appreciably different in Tg and controls, verifying the estimate of a 2.0 hr \( T_{G2+M} \) in both groups.

**Reduced G1 phase length accounts for decreased cell cycle length in Tg embryos**

Data from the cumulative and single injection BrdU experiments demonstrated that \( T_C \) was reduced in Tg embryos on E14, while \( T_S \) and \( T_{G2+M} \) were invariant. \( T_{G1} \) was calculated in control and Tg embryos using equation 5. \( T_{G1} \) was 1.85 hr shorter in Tg embryos as compared to controls (Table 3-1). The reduced G1 phase length accounts for the majority of the observed decrease in \( T_C \) in Tg embryos, with the remainder of the difference in \( T_C \) between groups.
Fig. 3-3. Graph of the percentage of labeled mitotic figures over the dorsomedial sector of the cerebral wall at 1.0 hr, 1.5 hr, and 2.0 hr after a single injection of BrdU was administered to a timed pregnant dam on E14 (Tg, open bars; Control, solid bars). Error bars represent the standard error of the mean. The graph shows that the percentage of labeled mitotic figures does not differ significantly between groups at any of the times examined. By 2.0 hr post-injection, essentially all mitotic figures are labeled in Tg and control embryos. Therefore, the combined length of the G2 and M phases is 2.0 hr in both groups. Reprinted with permission from: Hodge RD, D'Ercole AJ, O'Kusky JR (2004) Insulin-like growth factor-I accelerates the cell cycle by decreasing G1 phase length and increases cell cycle reentry in the embryonic cerebral cortex. J Neurosci 24:10201-10210. Copyright 2004 by the Society for Neuroscience.
accounted for by the slight decrease in T₅ in Tg embryos. Therefore, cell cycle length is reduced in Tg embryos as a result of shortened G1 phase length.

*IGF-I promotes cell cycle re-entry on E14-E15*

During cortical neurogenesis, daughter cells produced by divisions of progenitor cells within the proliferative zones of the cerebral wall (VZ and SVZ) may either exit the cell cycle and migrate away from the proliferative zones to assume their final positions in the cortical plate or re-enter the cycle and undergo further rounds of cell division. One of the key factors influencing total neuron output during cortical neurogenesis is the fraction of cells that exit the cell cycle following a given round of cell division. To better elucidate the role of IGF-I in the decision to proliferate versus exit the cell cycle, the proportion of cells that exit the cell cycle and the complimentary proportion that re-enter the cell cycle were determined on E14-E15. Cells that had returned to the cell cycle following division on E14-E15 were immunostained with both BrdU and Ki67, an endogenous marker of proliferating cells. Cells that had exited the cell cycle between E14 and E15 were BrdU-positive, but did not express Ki67, indicating that they were no longer proliferating.

Examination of single channel images from histological sections double stained for BrdU and Ki67 (Fig. 3-4) showed that BrdU+ cells (red fluorescence; Fig. 3-4C) were located in several different zones within the dorsomedial cerebral wall in both Tg and control embryos. BrdU+ cells were found within the VZ, SVZ, and IZ (Fig. 3-4C). Additionally, some BrdU+ cells were present in the cortical plate. Ki67+ cells (green fluorescence; Fig. 3-4B) were located predominantly within the proliferative VZ and SVZ; as well, a number of Ki67+ cells were found scattered throughout the IZ. Within the VZ, Ki67 immunostaining was detected in the vast majority of cells (Fig. 3-4B). A small number of Ki67- cells was observed in the VZ on some sections; these cells likely represented newly post-mitotic neurons that had not yet completed their migrations out of the VZ. DIC images were examined in combination with fluorescence images to ensure that immunostaining was localized within distinct cell bodies. Individual cell bodies were readily distinguishable on the DIC images (Fig. 3-4A). Double-labeled cells were identified on merged images of the red, green, and DIC channels. These cells were characterized by the presence of yellow punctate staining within the cell bodies (Fig. 3-4C). Similar BrdU and Ki67 staining patterns were observed in Tg and control embryos, and the two groups could not be easily distinguished by visual examination alone. On each
Fig. 3-4. Confocal microscope immunofluorescence scans of the dorsomedial cerebral wall in a nestin/IGF-I Tg embryo at E15. Embryos were labeled with BrdU on E14 and examined 24 hr later on E15 for the presence of BrdU (red; C-E) and Ki67 (green; B, D, E) immunostaining. Differential interference contrast (DIC) images were collected with each scan to ensure that immunostaining was localized to cell bodies (A). All images are presented as fluorescence channels merged with corresponding DIC images. Ki67 stained proliferating cells localized within the VZ and SVZ, as well as occasional cells in the IZ (B). Nearly all VZ cells were immunoreactive for Ki67. BrdU labeled cells were found in the VZ, SVZ/IZ and cortical plate (C). Counts of BrdU+/Ki67- cells (red; D, E, white arrow) and BrdU+/Ki67+ cells (yellow; D, E, white asterisk) were conducted on merged images of the green, red and DIC channels. Scale bar in A = 50 μm, and applies to images A-D. Scale bar in E = 20 μm. Reprinted with permission from: Hodge RD, D’Ercole AJ, O’Kusky JR (2004) Insulin-like growth factor-I accelerates the cell cycle by decreasing G1 phase length and increases cell cycle reentry in the embryonic cerebral cortex. J Neurosci 24:10201-10210. Copyright 2004 by the Society for Neuroscience.
scanned image, the proportions of BrdU+/Ki67- (exiting fraction) and BrdU+/Ki67+ (proliferative fraction) cells were determined.

The mean proportions of BrdU+/Ki67- and BrdU+/Ki67+ cells within the dorsomedial cerebral wall were compared in Tg and control animals using a 2 x 2 (groups x cell types) ANOVA. The analysis revealed a significant main effect for cell types (\( F = 395.137, P < 0.001 \)). The interaction term (groups x cell types) was also significant (\( F = 273.600, P < 0.001 \)), which warranted comparisons of individual mean values. Individual paired comparisons showed that the mean proportion of BrdU+/Ki67- cells (exiting fraction) was 15% lower (\( P < 0.001 \)) in Tg embryos (0.416 ± 0.004) as compared to controls (0.492 ± 0.005). Correspondingly, the mean proportion of BrdU+/Ki67+ cells (proliferative fraction) was 15% greater (\( P < 0.001 \)) in Tg animals (0.584 ± 0.004) than in controls (0.508 ± 0.004). These data indicate that the proportion of cells returning to the cell cycle on E14-E15 was significantly greater in Tg embryos. Conversely, the proportion of cells that exited the cell cycle as post-mitotic neurons was significantly reduced in Tg embryos. Therefore, these data show that IGF-I promotes cell cycle re-entry during cortical neurogenesis on E14-E15.

The duration of neurogenesis is normal in Tg embryos

Neurogenesis in the VZ of the developing cerebral wall normally begins on E11 and is essentially completed by E17 in normal mice (Takahashi et al., 1995a). Embryos were collected on E11, E17, and E18 to determine if the duration of neurogenesis differed between nestin/IGF-I Tg mice and controls.

On E11, the thickness of the dorsomedial cerebral wall measured from the ventricular surface to the pia was, on average, 70 µm in both groups. The VZ was the most prominent feature of the cerebral wall at this age, and the beginning of a primitive plexiform zone was also apparent. The VZ was approximately 60 µm thick in both Tg and control embryos (Fig. 3-5A, B). Cellular density in the VZ, measured as the number of cells within a 100 µm wide sector of the dorsomedial cerebral wall, did not differ significantly between Tg embryos (119.5 ± 9.5) and controls (125.5 ± 6.5). Similarly, the number of cells in the primitive plexiform zone did not differ noticeably between groups (Fig. 3-5A, B). Cells in the primitive plexiform zone were not immunoreactive for Ki67 and, therefore, were assumed to be post-mitotic. BrdU labeling revealed a distinct S-phase zone of cells in the VZ, which did not appear to differ between Tg and control embryos (Fig. 3-5A, B). The presence of post-mitotic cells in the
Fig. 3-5. Representative photomicrographs of the dorsomedial cerebral wall at E11, E14, E17, and E18 in control (A, C, E, G) and nestin/IGF-I Tg (B, D, F, H) embryos. Embryos were labeled with BrdU and sacrificed at 1 hr post injection. At E11 the cerebral wall consisted of the VZ and an adjacent zone of post-mitotic cells (A, arrow). By E14, the SVZ was apparent adjacent to the VZ (B, C). The height of the VZ did not differ between Tg (D) and control (C) embryos. At E17, the VZ was reduced in height in comparison to E14 embryos in both control (E) and Tg (F) embryos, but a zone of BrdU labeled cells in S-phase was still evident. Therefore, neurogenesis was still occurring at E17, but the reduced height of the VZ suggested that this process was nearing completion. Increased neuron output from the VZ was apparent in control (G, K) and Tg (J, L) embryos at E17, as the IZ and cortical plate (CP) were increased in size. By E18, a distinct VZ was not apparent in either control (G) or Tg (H) embryos. BrdU labeling showed random staining in the cells adjacent to the ventricular surface, which were likely ependymal cells. Nestin/IGF-I Tg embryos were visibly larger than control embryos by E17 (I, Control; J, Tg). Closer examination of histological sections showed that the CP was much larger in Tg embryos (L) as compared to controls (K). Scale bars in A, C, E, G = 20 μm. Scale bar in I = 100 μm. Scale bar in K = 70 μm. Reprinted with permission from: Hodge RD, D’Ercole AJ, O’Kusky JR (2004) Insulin-like growth factor-I accelerates the cell cycle by decreasing G1 phase length and increases cell cycle reentry in the embryonic cerebral cortex. J Neurosci 24:10201-10210. Copyright 2004 by the Society for Neuroscience.
primitive plexiform zone and a zone of S-phase cells in the VZ indicated that neurogenesis had begun in both groups on E11.

At 9:30 A.M. on E14, the mean radial thickness of the dorsomedial cerebral wall, measured from ventricular surface to pia, was approximately 130 μm in both Tg and control embryos. The cerebral wall consisted of a prominent VZ, which did not differ noticeably between groups with respect to thickness or cellular density (Fig. 3-5C, D). A cortical plate was not evident over the dorsomedial sector of the cerebral wall at this time in either Tg or control embryos (Fig. 3-1A, B). By 11:00 P.M. on E14 (i.e. 13.5 hr later), the average thickness of the dorsomedial cerebral wall had increased to 230 μm in Tg embryos and to 220 μm in controls. A cortical plate over the dorsomedial cerebral wall was evident in both groups. The cortical plate did not appear to differ with respect to thickness or cellular density between Tg and control embryos.

By E17, the area and thickness of the VZ were substantially reduced in both Tg and control embryos, indicating that the VZ progenitor population was becoming depleted and cortical neurogenesis was nearing its endpoint (Fig. 3-5E, F). The height of the VZ in the dorsomedial cerebral wall had decreased to approximately 50 μm in both groups. BrdU labeling showed a small band of S-phase cells at the superficial margin of the VZ in both groups, as well as a very thin band of unlabeled cells deep to this S-phase band and adjacent to the ventricular surface. These unlabeled cells had the characteristic radially aligned appearance of VZ cells, indicating that some neuron production was still occurring at this age (Fig. 3-5E, F). The cortical plate was visibly larger in Tg embryos on E17 (Fig. 3-5I, J). However, there were no apparent differences in the thicknesses of the VZ, SVZ or IZ between Tg and control embryos on E17 (Fig. 3-5K, L).

By E18, a morphologically distinct VZ was not readily apparent in Tg and control embryos (Fig. 3-5G, H). BrdU-immunoreactive cells were observed immediately adjacent to the ventricular surface, but a definite S-phase band of cells was not distinguishable in either group (Fig. 3-5G, H). The VZ appeared to be largely replaced by randomly oriented neuroepithelial cells, characteristic of the SVZ, and by cuboidal ependymal cells, adjacent to the ventricular surface. These observations indicate that neuron production from the VZ was essentially completed some time between E17 and E18 in both groups. Therefore, the overall duration of neurogenesis in the VZ during embryonic development did not change appreciably in Tg embryos.
3.4 Discussion

The present study was designed to elucidate the *in vivo* role of IGF-I in governing the dynamics of mitosis during cortical neurogenesis. Previously, it has been shown that increased expression of IGF-I in the dorsal telencephalon during embryonic development produced increases in cortical plate volume (52%) and total cell number (54%) in Tg embryos at E16 (Popken et al., 2004). As well, augmented IGF-I expression during early postnatal development produced increases in total cortical volume (31%) and neuron number (27%) in Tg mice at P12 (Popken et al., 2004; Hodge et al., 2005). However, given that IGF-I is a well documented inhibitor of apoptosis *in vitro* (Bozyczko-Coyne et al., 1993; Hughes et al., 1993; Neff et al., 1993; Matthews and Feldman, 1996; Dudek et al., 1998; Blair et al., 1999; Yamada et al., 2001) and postnatally *in vivo* (Baker et al., 1999; Chrysis et al., 2001), it was not clear to what extent, if any, this increased cell number resulted from an IGF-I-mediated enhancement of cellular proliferation. Parameters that regulate neurogenesis and influence neuron output include cell cycle kinetics, progenitor cell number, and the proportion of cells exiting the cell cycle following any given division (Caviness et al., 1995; Takahashi et al., 1996, 1997; Haydar et al., 2000; Chenn and Walsh, 2002). The results of the present study clearly demonstrate that increased expression of IGF-I reduces cell cycle length and augments progenitor cell re-entry into the cell cycle during an otherwise normal length of cortical neurogenesis.

The present data indicate that IGF-I influences proliferation in the VZ of the dorsomedial cerebral wall, which contains progenitor cells responsible for the generation of cerebral cortical projection neurons, by decreasing total cell cycle length. In general, these findings are in agreement with *in vitro* studies showing that IGF-I promotes neuron proliferation (DiCicco-Bloom and Black, 1988; Drago et al., 1991; Ye et al., 1996; Arsenijevic et al., 2001). As well, the current results indicate that IGF-I acts specifically to reduce the length of the G1 phase of the cell cycle, without affecting the lengths of the G2, M, and S phases. The G1 phase of the cell cycle has been identified as a key regulatory point during neurogenesis in the mouse cortex (Takahashi et al., 1995a; Caviness et al., 1999, 2003). During the period of normal cortical neurogenesis, the length of G1 increases significantly from E11 to E17 as progenitors undergo successive cell cycles, while the lengths of other phases remain relatively constant. Consequently, $T_c$ increases as neurogenesis progresses (Takahashi et al., 1995a).
The results of the present study are in agreement with several studies of non-neural cell types which showed that IGF-I has a role in regulating G1 phase progression. Early studies of the mitogenic properties of IGF-I, performed with cultured fibroblasts (BALB/c-3T3), indicated that IGF-I acts to stimulate progression through G1 or the G0/G1 transition (Stiles et al., 1979; Clemmons et al., 1980; Wharton et al., 1981; Russel et al., 1984; Olashaw et al., 1987). More recent studies in both myocytes (Reiss et al., 1997; Chakravarthy et al., 2000) and mammary epithelial cells (Stull et al., 2002) similarly showed that IGF-I promotes progression through the G1 phase of the cell cycle. However, studies of estradiol-induced mitosis in the endometrium suggest that IGF-I regulates progression through G2 phase (Adesanya et al., 1999). Therefore, it is possible that IGF-I may differentially affect the cell cycle in specific tissues or when its actions are coordinated with those of other mitogens, such as estrogens.

The control values for $T_c$, $T_s$, and $T_{G1}$ documented in this study vary slightly from previous analyses using the cumulative BrdU labeling method (Takahashi et al., 1993, 1995a). The combined length of the G2 and M phases of the cell cycle determined in the present study is consistent with previously published reports of $T_{G2+M}$ (Takahashi et al., 1993, 1995a; Miyama et al., 1997). Similarly, the current GF values are comparable to those obtained in other studies (Takahashi et al., 1993, 1995a; Miyama et al., 1997). Variations in $T_c$, $T_s$, and $T_{G1}$ values between studies may result from the use of different strains of mice. For example, C57BL/6 mice were used in the present study, whereas other studies have used CD1 mice. Interestingly, the control data presented in this study more closely resemble the values for individual cell cycle parameters measured in CD1 mice on E15 (Takahashi et al., 1995a). The value for $T_s$ calculated in this report is longer than the value determined for CD1 mice using cumulative BrdU labeling. However, this $T_s$ value is consistent with other reports of S-phase length in the rodent cortex (Atlas and Bond, 1965; Kauffman 1966, 1968; Shimada and Langman, 1970). Nonetheless, the data presented in this study clearly indicate a reduction in cell cycle length in Tg embryos on E14.

In the present study, an increase in MI (the proportion of mitotic figures in the VZ) was documented in Tg embryos on E14. The observed increase in MI in the VZ of Tg embryos on E14 resulted from the documented reduction in $T_c$ in Tg embryos with no corresponding decrease in $T_M$. The number of cells in M phase at a given instant is proportional to $T_M/T_C$. As $T_c$ is reduced in Tg embryos, $T_M$ accounts for a greater fraction of total cell cycle length. Consequently, at any given point in time the probability of detecting mitotic figures is
increased in Tg embryos, and the MI increases accordingly. Therefore, this observed increase in MI is due to the shortened cell cycle in Tg embryos.

Cumulative BrdU labeling also allowed for the measurement of the GF, which represents the proportion of actively proliferating cells within the VZ on E14. Neuron output from the VZ may be influenced by alterations in the GF. For example, a reduction in GF may result in diminished neuron production because fewer progenitor cells are actively proliferating (Haydar et al., 2000). Consistent with previously published observations (Takahashi et al., 1993, 1995; Miyama et al., 1997), essentially all cells in the VZ are proliferating in Tg and control embryos on E14. Thus, alteration in the GF does not appear to influence neuron output from the VZ in Tg embryos.

Neuron output from the proliferative zones of the developing cerebral wall is largely dependent on the proportion of cells that exits the mitotic cycle following any given round of cell division (Caviness et al., 1995, 1999; Takahashi et al., 1994, 1996, 1997). Early in neurogenesis, relatively few cells exit the cell cycle as post-mitotic neurons, and the progenitor population of the VZ expands accordingly. As neurogenesis progresses, the fraction of cells leaving the cycle exceeds 0.5, and the progenitor population becomes depleted as a consequence of increased neuron output (Takahashi et al., 1996). The present study shows that the proliferative fraction of cells (cells that re-enter the cell cycle) is significantly increased by 15% in Tg embryos on E14-E15 when compared to control embryos. As well, these data indicate that progression to the critical value of 0.5 that signals the shift from progenitor expansion to depletion is delayed in Tg embryos as approximately 58% of cells return to the cell cycle to undergo further rounds of division. In comparison, the proliferative fraction of cells in control embryos was found to be approximately 51%, while the exiting fraction was approximately 49%. These data indicate that neurogenesis in control animals was shifting to the phase of neuron output while the proliferative population in Tg embryos continued to expand.

The control values for BrdU+/Ki67- (exiting fraction) and BrdU+/Ki67+ (proliferative fraction) proportions obtained in this study were comparable to those obtained in previous studies using the same methods (Chenn and Walsh 2002), and to previously published cell cycle exit values determined using alternate methods (Takahashi et al., 1994, 1996; Haydar et al., 2000). The results of the present study support the findings of an earlier study from our lab showing that the combined volume of the VZ and SVZ was significantly increased by 26% in
Tg embryos at E16 (Popken et al., 2004). Taken together, these data suggest that cells are retained in the cell cycle during cortical neurogenesis in Tg embryos. As more cells re-enter the mitotic cycle in Tg embryos, the progenitor population is expanded and the potential for the production of greater numbers of neurons is increased.

Mathematical models of neurogenesis in the mouse cerebral cortex indicate that alterations in the proportion of cells exiting the cycle can have significant effects on total neuron output (Caviness et al., 1995, 1999, 2003; Takahashi et al., 1997). Model predictions show that decreases in the exiting fraction of cells lead to increased tangential expansion of the VZ, which in turn results in enhanced total neuron production from this zone (Caviness et al., 1995, 1999, 2003; Takahashi et al., 1997). Consequently, increases in cortical thickness and total cortical neuron number are predicted in the adult (Caviness et al., 2003). The finding of an increase in the proliferative fraction of cells at E14-E15, greater VZ/SVZ volume at E16, and increased cortical thickness (12%) and neuron number (27%) postnatally in Tg mice (Popken et al., 2004; Hodge et al., 2005) closely match the predictions of these mathematical models, lending further support to the conclusions presented in this study.

In the course of normal murine neurogenesis, neuron progenitors in the VZ complete an average of 11 cell cycles (Takahashi et al., 1995a). The increase in neuron number documented in the cerebral cortex of nestin/IGF-I Tg mice (Popken et al., 2004; Hodge et al., 2005) may be explained by an increase in the number of cell cycles completed by progenitors during neurogenesis and/or by the observed reduction in cell cycle exit. While cell cycle number has not been measured experimentally in Tg embryos, an increase in the number of cell cycles beyond the normal 11 cycles (Takahashi et al., 1995a) is theoretically possible given the 2.05 hr reduction in \( T_c \) and the retention of progenitors in the cell cycle documented in Tg embryos. For example, Haydar and collaborators (2000) have shown that delayed neurogenesis and microencephaly in embryos with trisomy 16 resulted from only 10 cell cycles, due to lengthening of \( T_s \) by 1.0 hr from E11-E17. Therefore, increased cell cycle number may act in conjunction with reduced cell cycle exit to produce the augmented cortical neuron number documented in these nestin/IGF-I Tg mice (Popken et al., 2004; Hodge et al., 2005).

The overall duration of the period of neurogenesis appeared to be normal in Tg embryos with no apparent differences from controls. Proliferating cells in the VZ were apparent at approximately E11 and disappeared by E17-18 in both Tg and control embryos.
The finding that neurogenesis in Tg embryos is of normal duration indicates that augmented IGF-I expression does not override intrinsic factors governing the developmental length of this interval, or extend the lifespan of progenitors in the VZ. Recently, it has been suggested that a proportion of radial glial progenitor cells from the VZ are retained at the ventricular surface into postnatal life, acting as neural stem cells (Tramontin et al., 2003). The methods employed in this present study did not permit an investigation of the effects of IGF-I on radial glial transformation and stem cell survival in the adult. More studies are required to determine if IGF-I influences progenitor cell numbers postnatally.

In conclusion, the results of this study indicate that IGF-I accelerates the cell cycle in the developing VZ underlying the cerebral cortex by decreasing $T_c$ and enhances progenitor cell re-entry into the cell cycle. As a result of these effects, measurable increases in cell number are apparent in the cortical plate and cerebral cortex of Tg mice during embryonic and postnatal development.
3.5 Bibliography


Chapter IV Effects of IGF-I on the development of specific cortical progenitor cell types

4.1 Introduction

The results presented in the previous chapter showed that IGF-I promotes cell cycle progression and cell cycle re-entry during embryonic development of the cerebral cortex (Hodge et al., 2004). Ultimately, increased cell cycle re-entry in nestin/IGF-I Tg embryos leads to augmented VZ and SVZ volume on E16 (Popken et al., 2004), indicating that the progenitor cell population is expanded in Tg embryos. However, several different types of progenitor cells are present in the cerebral wall during neurogenesis, and the previous data did not indicate which of these cells are influenced by IGF-I overexpression. To better understand how IGF-I effects different progenitor cell populations during cortical neurogenesis, the relative proportions of specific progenitor cells in the dorsomedial cerebral wall were determined on E14 in nestin/IGF-I Tg embryos and littermate controls.

On E14 the cerebral wall consists largely of a VZ as well as a smaller SVZ, both of which contain progenitor cells that give rise to cortical projection neurons (Miyata et al., 2001; Noctor et al., 2001, 2004; Heins et al., 2002; Englund et al., 2005). Several different types of cortical progenitor cells are known to exist within these proliferative zones on E14. The VZ consists largely of radial glial cells at this time. Radial glial cells are morphologically characterized by their radial processes, one of which contacts the ventricular surface and the other which extends to the pial surface of the telencephalic wall. Early studies indicated that radial glial cells acted as scaffolding used by newly generated neurons to guide migration to the cortical plate (Schmechel and Rakic, 1979). More recently, radial glial cells have been identified as mitotically active cortical projection neuron progenitor cells (Malatesta et al., 2000; Hartfuss et al., 2001; Miyata et al., 2001; Noctor et al., 2001, 2002; Heins et al., 2002). Radial glial cells in the cerebral VZ are characterized by their expression of the transcription factor Pax6, which is distinctively restricted to radial glia in the dorsal telencephalon (Gotz et al., 1998). Pax6 appears to be critically important for the proper differentiation of these cortical radial glial cells (Gotz et al., 1998) and is important for specifying the neuronal lineage of cortical radial glia (Heins et al., 2002). Therefore, immunohistochemical detection of Pax6 was used to determine the proportion of radial glial cells in the dorsomedial cerebral wall of Tg and control embryos on E14.

The division of radial glial cells may result in a number of outcomes for newly generated daughter cells. Three different types of cell division are generally recognized in the
developing cerebral wall. The first of these division types is referred to as symmetrical proliferative division which produces two progenitor cells. Symmetrical terminal divisions are also possible, and these types of divisions produce two post-mitotic cells. As well, division may be asymmetrical, producing one progenitor cell and one post-mitotic cell (Chenn and McConnell, 1995; Takahashi et al., 1996; Cai et al., 2002; Fishell and Kriegstein, 2003). Recent studies indicate that divisions of radial glial cells in the VZ produce both post-mitotic neurons and intermediate progenitor cells, which migrate to the SVZ (Noctor et al., 2004). These intermediate progenitor cells then undergo divisions in the SVZ, the majority of which produce two post-mitotic neurons (Noctor et al., 2004).

Intermediate progenitor cells in the cerebral SVZ are characterized by their random alignment with respect to the ventricular surface. Intermediate progenitor cells do not undergo the interkinetic nuclear migrations typical of radial glia in the VZ, and, as a result, divide at abventricular (non-surface) positions in the cerebral wall (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). These progenitors are characterized by the expression of Svetl (Tarabykin et al., 2001) and Tbr2 (Englund et al., 2005). Tbr2, a member of the T-box family of transcription factors, is abundantly expressed in the mouse brain during cortical neurogenesis and coincident with production of the SVZ (Bulfone et al., 1999; Englund et al., 2005). Interestingly, Pax6 expression is downregulated in intermediate progenitor cells shortly after their production by radial glia cell division and Tbr2 is upregulated (Englund et al., 2005). Following terminal division, Tbr2 expression is downregulated in newly post-mitotic neurons. Recent studies indicate that intermediate progenitor cells produce a significant number of the projection neurons populating the cerebral cortex, although the exact proportion of projection neurons produced by these cells has not yet been defined (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Englund et al., 2005). Therefore, Tbr2 was used as a marker for intermediate progenitor cells and the proportion of Tbr2 immunoreactive cells in the cerebral wall was determined in nestin/IGF-I Tg embryos and controls on E14.

As IGF-I promotes cell cycle re-entry on E14-E15, the relative number of post-mitotic neurons produced in Tg embryos on E14 may be expected to be less than that in controls, as more cells would be retained as progenitors in Tg mice. Projection neurons of the cortex express Tbr1, a T-box transcription factor related to Tbr2 (Hevner et al., 2001). Tbr1 is expressed specifically by glutamatergic projection neurons in the IZ, cortical plate, and marginal zone, with expression persisting into the mature cortex (Hevner et al., 2001). Using
Tbr1 as a marker of post-mitotic projection neurons in the cortex, the proportion of post-mitotic neurons in the dorsomedial cortex was determined on E14 in Tg and control embryos.

4.2 Materials and Methods

Embryo collection and tissue processing

Timed-pregnant dams were generated for the collection of embryos on E14, as described previously (see Chapter 3). Embryos were removed by hysterotomy and fixed in 70% ethanol at 4 °C overnight. Individual embryos were embedded in paraffin, sectioned at 4 μm in the coronal plane and mounted on Colorfrost Plus slides (Fisher Scientific).

Immunohistochemistry

Sections were processed for the immunohistochemical detection of antigens using the following antibodies: 1) Mouse monoclonal anti-Pax6 (1:200 dilution; Developmental Studies Hybridoma Bank, Iowa City, IA). 2) Rabbit polyclonal anti-Tbr1 (1:1000 dilution; a gift of Dr. Robert Hevner, University of Washington, Seattle, WA). 3) Rabbit polyclonal anti-Tbr2 (1:800 dilution; a gift of Dr. Robert Hevner, University of Washington, Seattle, WA). Prior to incubation with primary antibodies, sections were boiled in antigen unmasking solution (Vector Laboratories) for 10 min in a microwave. Primary antibody incubation was done overnight at 4 °C, and further processing was carried out using an avidin-biotin complex immunoperoxidase protocol (Vector Laboratories) for either mouse IgG or rabbit IgG according to the manufacturer’s protocol. Slides incubated without the addition of primary antibodies served as negative controls. Sections were counterstained with 0.1% aqueous basic fuchsin and coverslipped with Permount (Fisher Scientific).

Cell counting

Cell counts were conducted within a sector of the dorsomedial cerebral wall overlying the primordial primary somatosensory cortex that measured 150 μm (height) by 100 μm (width), where 150 μm was the average thickness of the dorsomedial cerebral wall in both Tg and control embryos. This sector was positioned over the same region of the dorsomedial cerebral wall as the sector used for the cumulative BrdU labeling and cell exit experiments (see Chapter 3). Cells were counted if they were located entirely within the sector or if they intersected its medial boundary. Cells intersecting the lateral margin of the sector were not included in the counts. Cell counts were conducted on 4 non-adjacent sections per brain (every second section in a series of 7 consecutive sections). For each animal, the proportion of labeled cells (Pax6, Tbr1, or Tbr2) relative to the total number of cells within the...
sector was determined. Counts were conducted on 3 embryos per group for each cell type. Statistical comparisons of data from control and Tg embryos were conducted using Student's t-test.

4.3 Results

*Pax6 immunoreactive cells in the dorsomedial cerebral wall*

Analyses of the relative proportions of different progenitor cell types were performed in nestin/IGF-I Tg and control embryos on E14, the approximate midpoint of cortical neurogenesis in normal mice. No signs of gross malformation or morphological abnormalities were evident in Tg embryos upon histological examination. On E14, the size and lamination pattern of the telencephalic wall were similar in Tg and control embryos, and the two groups were visually indistinguishable (Fig. 4-1). In both groups the thickness of the VZ was approximately 70 µm over the dorsomedial sector analyzed, and the overall thickness of the dorsomedial cerebral wall was approximately 150 µm in both Tg and control embryos.

In nestin/IGF-I Tg and control embryos, Pax6 immunoreactive cells were specifically localized to the VZ throughout all regions of the developing cerebral wall (Fig. 4-1 C, D). Pax6-positive cells were aligned radially with respect to the ventricular surface, consistent with the known morphology of radial glial cells. These Pax6 immunoreactive cells were typically found within the first 70-80 µm of cerebral wall thickness in both Tg and control embryos, consistent with their location in the VZ. In general, no difference in the distribution of Pax6 immunoreactive cells was noted between Tg and control embryos (Fig. 4-1 C, D).

Counts of Pax6-positive cells within a sector (150 µm x 100 µm) of the dorsomedial wall revealed no difference in the proportion of Pax6 immunoreactive cells between Tg and control embryos (t = 0.126; P > 0.05). The mean proportion of Pax6-positive cells within the dorsomedial cerebral wall was 0.567 ± 0.007 cells (mean ± SEM) in controls and 0.568 ± 0.005 cells in Tg embryos. Therefore, the proportion of Pax6 immunoreactive radial glia in the VZ of the dorsomedial cerebral wall did not differ between Tg and control animals on E14.

*The proportion of Tbr2 immunoreactive cells is increased in nestin/IGF-I Tg embryos*

To determine if augmented IGF-I expression alters the proportion of intermediate progenitor cells in the cerebral wall, counts of Tbr2 immunoreactive cells were conducted in the dorsomedial cerebral wall on E14. In both Tg and control embryos, Tbr2-positive cells were located within the SVZ, basal regions of the VZ, and lower regions of the IZ (Fig. 4-2). Some overlap of Tbr2-immunoreactivity with the VZ was apparent in both groups, consistent
Fig. 4-1. Representative photomicrographs illustrating Pax6 immunostaining in E14 control (A, C) and nestin/IGF-I Tg (B, D) embryos. Counts of Pax6-positive cells were conducted over the dorsomedial cerebral wall. Pax6-positive cells were identified by the accumulation of brown reaction product in cell bodies (C, arrows). Unlabeled cells appeared pink due to the use of a basic fuchsin counterstain. Control (A) and Tg (B) embryos could not be visually distinguished on E14, and Pax6 immunostaining patterns were similar in both groups. On E14, the cerebral wall consisted mainly of the VZ and SVZ in both groups (C, D). The thickness of the VZ was similar in Tg and control embryos, as was the thickness of the total cerebral wall. Pax6 immunoreactive cells were localized to the first 70 to 80μm of cerebral wall height in both groups, consistent with their position in the VZ. Scale bar in A = 100μm. Scale bar in C = 50μm.
Fig. 4-2. Photomicrographs illustrating Tbr2 immunostaining in E14 control (A, C) and nestin/IGF-I Tg (B, D) embryos. Counts of Tbr2-positive cells were conducted over the dorsomedial cerebral wall. Tbr2-positive cells were identified by the accumulation of brown reaction product in cell bodies (C, arrows), and unlabeled cells appeared pink. Low power photomicrographs illustrate that Tg (B) and control (A) embryos were not visually distinguishable on E14. Figures C and D illustrate the structure of the cerebral wall on E14, which was similar in Tg and control embryos. In both groups, Tbr2-positive intermediate progenitor cells were located in the basal VZ, SVZ, and lower IZ. Scale bar in A = 100μm. Scale bar in C = 50μm.
with studies showing measurable intermixing of the VZ and SVZ progenitor populations (Takahashi et al., 1995a, b; Haubensak et al., 2004; Englund et al., 2005). The distribution of Tbr2 immunoreactive cells was not notably different in Tg and control embryos, and the two groups could not be reliably distinguished based on visual observation of immunostaining patterns alone (Fig. 4-2 C, D).

Counts of Tbr2-positive cells in the dorsomedial cerebral wall revealed a significant increase of 27% (t = 3.182; P = 0.03) in the proportion of Tbr2 immunoreactive cells in Tg embryos as compared to littermate controls. In Tg embryos the mean proportion of Tbr2 immunoreactive cells in this region of the dorsomedial cerebral wall was 0.458 ± 0.03 cells, whereas the mean proportion was only 0.360 ± 0.007 cells in controls.

The proportion of Tbr1 immunoreactive cells is reduced in Tg embryos on E14

Immunohistochemical staining for Tbr1, a marker of post-mitotic cortical projection neurons, showed that Tbr1 immunoreactive cells were located in the IZ, cortical plate, and marginal zone in both Tg and control embryos (Fig. 4-3). Once again, the distribution of Tbr1-positive cells was similar in both groups and did not allow for visual identification of Tg and control embryos. Counts of Tbr1 immunoreactive cells showed that the proportion of Tbr1-positive cells was significantly decreased by 17% (t = 4.226; P = 0.01) in Tg embryos on E14. In control embryos the mean proportion of Tbr1 immunoreactive cells was 0.480 ± 0.017 cells. The mean proportion of Tbr1 immunoreactive cells in Tg embryos, by comparison, was only 0.398 ± 0.009 cells.

4.4 Discussion

The in vivo actions of IGF-I on specific cortical progenitor cell types have not been investigated previously. The present findings indicate that increased expression of IGF-I during embryonic development leads to a 27% increase in the proportion of Tbr2-positive intermediate progenitor cells in the cerebral wall on E14, during the period of cortical neurogenesis. However, IGF-I overexpression does not appear to alter the proportion of Pax6-positive radial glia in the VZ of the developing cerebral wall, at least on E14. In addition, the proportion of Tbr1-positive post-mitotic cortical projection neurons is reduced by 17% in Tg embryos on E14. These results indicate that IGF-I may differentially influence the development of specific cortical progenitor cells, and suggest a preferential effect of IGF-I on intermediate progenitor cells.
Fig. 4-3. Representative photomicrographs illustrating Tbr1 immunostaining in control (A, C) and nestin/IGF-I Tg (B, D) embryos at E14. Counts of Tbr1-positive cells were conducted over the dorsomedial cerebral wall. Tbr1-positive cells were identified by the accumulation of brown reaction product in cell bodies, and unlabeled cells appeared pink due to the use of a basic fuchsin counterstain. As illustrated in the low power photomicrographs, Tg (B) and control (A) embryos were not visually distinguishable on E14, and the general distribution of Tbr1-positive cells was similar in both groups. Figures C and D illustrate the structure of the cerebral wall on E14, which was not notably different between groups. In both groups, Tbr1 immunoreactive cells were concentrated in the IZ and cortical plate, consistent with the location of post-mitotic projection neurons in the developing cerebral wall. Scale bar in A = 100μm. Scale bar in C = 50μm.
In a previous study, augmented IGF-I expression in the cerebral wall from early embryonic development was shown to produce increases in the volume of the cortical plate (52%) and the combined volume of the proliferative VZ and SVZ (26%) in nestin/IGF-I Tg embryos at E16 (Popken et al., 2004). As well, IGF-I has been shown to augment proliferation in the cerebral wall by increasing cell cycle progression rate and promoting cell cycle re-entry (Hodge et al., 2004). Specifically, the proportion of cells re-entering the cell cycle on E14-E15 was increased by 15% in Tg embryos (Hodge et al., 2004). These data suggest that cells are retained as progenitors in the cell cycle during neurogenesis in Tg embryos, leading to an expansion of the proliferative zones. However, the volume measurements conducted at E16 were not specific to either proliferative zone, and the cell cycle re-entry experiments did not differentiate between cells re-entering the cycle within the VZ versus those dividing further within the SVZ. Therefore, it could not be said with certainty which progenitor population was affected by augmented IGF-I expression in Tg embryos.

The results of the present study are in agreement with those of previous studies showing that IGF-I promotes cell cycle re-entry (Hodge et al., 2004). IGF-I appears to promote the re-entry of cells into the cell cycle as Tbr2-positive intermediate progenitor cells, rather than Pax6-positive radial glia on E14. As a consequence of enhanced cell cycle re-entry on E14-E15, the population of Tbr1-positive post-mitotic projection neurons is reduced in Tg embryos. This reduction in Tbr1-positive projection neurons is consistent with data indicating that cells are retained within the proliferative zones of the cerebral wall during cortical neurogenesis in Tg embryos, which would result in decreased neuron output on E14. Ultimately, neuron output is increased in Tg embryos, as illustrated by the 54% increase in cortical plate cell number at E16 (Popken et al., 2004). Therefore, retention of cells as progenitors during earlier phases of neurogenesis leads to an initial decrease in neuron output, as illustrated by the reduction in Tbr1-positive neurons in Tg embryos on E14. Later in neurogenesis, however, neuron output is increased in Tg embryos as a consequence of expansion of the progenitor population resulting from increased cell cycle re-entry (Popken et al., 2004).

Recent studies indicate that Tbr2 is a marker for intermediate progenitor cells in the SVZ and basal VZ of the developing cerebral wall (Englund et al., 2005). The majority of Tbr2-positive intermediate progenitor cells have been shown to be actively proliferating on E14, as 94% of Tbr2-positive cells coexpress PCNA, a marker of proliferating cells, at this
time (Englund et al., 2005). However, Tbr2 is also expressed, to some extent, by newly generated neurons that arise from intermediate progenitor cells (Englund et al., 2005). The time required for Tbr2 to be downregulated in these cells is currently unknown. Therefore, some of the Tbr2 immunoreactive cells counted in the present experiment may have been post-mitotic neurons rather than intermediate progenitor cells. Further studies would be required to determine if the increase in Tbr2-positive cells documented in Tg embryos is entirely made up of mitotically active Tbr2-positive cells. Nonetheless, as most Tbr2 expressing cells are mitotically active on E14, the present data support a role for IGF-I in increasing the proportion of intermediate progenitor cells during embryonic development of the cerebral cortex.

The majority of intermediate progenitor cells appear to be produced by divisions of radial glia in the VZ (Miyata et al., 2004; Noctor et al., 2004; Englund et al., 2005). Most intermediate progenitor cells, in turn, undergo symmetrical terminal divisions to produce two neurons (Noctor et al., 2004). A small fraction (10.5%) of intermediate progenitor cell divisions within the SVZ consists of symmetrical proliferative divisions that produce two proliferative daughter cells (Noctor et al., 2004). The increase in Tbr2-positive cells documented in Tg embryos on E14 may result from enhanced production of intermediate progenitor cells from radial glia or from an increase in the proportion of intermediate progenitor cells undergoing symmetrical proliferative divisions. Further studies would be necessary to determine the exact mechanism responsible for enhanced Tbr2-positive cell numbers in Tg embryos.

The current findings indicate that the proportion of radial glial cells in the cerebral wall does not differ between Tg and control embryos on E14. Interestingly, analyses of the cell cycle kinetics of progenitor cells in the VZ on E14 indicated that this population of cells progresses through the cell cycle at a faster rate in Tg embryos (Hodge et al., 2004). Therefore, these data indicate that IGF-I may influence neuron output from the VZ by increasing the rate of radial glia proliferation rather than affecting the proportion of these cells remaining in the VZ. In accordance with this idea, the thickness of the VZ did not appear to change during the course of the cell cycle kinetics experiment conducted on E14, which was initiated at 9:00 A.M. on E14 and ended 14 hr later (Hodge et al., 2004). As well, the density of cells in the VZ of the dorsomedial cerebral wall did not differ between Tg and control embryos throughout E14 (Hodge et al., 2004). However, it is possible that the proportion of radial glia in the cerebral wall may differ between Tg and control embryos during earlier
phases of neurogenesis, before the appearance of the SVZ. Further analyses would be essential to determine how IGF-I effects radial glia development throughout cortical neurogenesis.

The exact proportions of cortical projection neurons produced by radial glia and intermediate progenitor cells are not clear at the present time (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Englund et al., 2005). As well, it is not presently known if radial glia and intermediate progenitor cells produce different subpopulations of cortical projection neurons. Therefore, it is difficult to determine exactly how the IGF-I-mediated increase in Tbr2-positive intermediate progenitor cells documented in the present analysis would affect total neuron output during cortical neurogenesis. Taken together, however, the current data strongly support a role for IGF-I in promoting neuronal proliferation during embryonic development. These results are in general agreement with various studies showing that IGF-I enhances neuronal proliferation in vitro (DiCicco-Bloom and Black, 1988; Torres-Aleman et al., 1990; Drago et al., 1991; Werther et al., 1993; Zackenfels et al., 1995), and in the hippocampus in vivo following peripheral administration of IGF-I (Aberg et al., 2000). The present findings also suggest a mechanism for the actions of IGF-I on cortical development, whereby IGF-I augments final neuron number in the cerebral cortex by promoting both cell cycle progression in the embryonic VZ and the retention of progenitors in the cell cycle as Tbr2-positive intermediate progenitor cells. Ultimately, neuron output is increased in Tg mice during neurogenesis by virtue of these IGF-I-mediated actions, resulting in augmented neuron number in the cortical plate during embryonic development and in the cerebral cortex during postnatal development (Hodge et al., 2004; Popken et al., 2004).
4.5 Bibliography


Chapter V  Effects of IGF-I on neuronal apoptosis in the cerebral cortex during embryonic and early postnatal development.

5.1 Introduction

Previous studies of nestin/IGF-I Tg mice showed that the total number of neurons in the cerebral cortex is increased beginning during embryonic development, such that final neuron number is 27% greater in Tg mice by P12 (Popken et al., 2004; Hodge et al., 2005). Studies of the cell cycle kinetics of precursor cells in the embryonic cerebral cortex indicated that augmented neuron number in Tg mice results, at least in part, from an IGF-I-mediated enhancement of neuronal proliferation (Hodge et al., 2004). As well, these studies showed that more cells return to the cell cycle during cortical neurogenesis in Tg mice, which results in expansion of the proliferative zones and an increase in the proportion of Tbr2-positive intermediate progenitor cells in these mice. Taken together, the results of these studies strongly indicate a role for IGF-I in promoting progenitor cell proliferation during embryonic cortical neurogenesis.

In addition to its ability to promote neuron proliferation, IGF-I has also been shown to act as an anti-apoptotic factor during CNS development (Aizeman and DeVellis, 1987; Torres-Aleman et al., 1990a, 1990b; Bozyczko-Coyne et al., 1993; Russell and Feldman, 1999; Chrysis et al., 2001; Yamada et al., 2001; Popken et al., 2004). Several studies indicate that IGF-I promotes the survival of cerebral cortical neurons in vitro (Takadera et al., 1999; Yamada et al., 2001). As well, the ability of IGF-I to promote neuron survival by inhibiting apoptosis has been demonstrated in the cerebellum of IGF-I Tg mice in which transgene expression is limited to postnatal development (Chrysis et al., 2001). As discussed previously (see Chapter 1), the majority of neuronal apoptosis in the cerebral cortex appears to occur during early postnatal development (Spreafico et al., 1995; Thomaidou et al., 1997; Verney et al., 2000), and estimates of the prevalence of neuronal apoptosis during embryonic cortical development vary widely among studies (Blaschke et al., 1996; Thomaidou et al., 1997; Haydar et al., 2000). Nonetheless, neuron elimination by apoptosis significantly contributes to the determination of final neuron number in the cerebral cortex.

Given that studies indicate a role for IGF-I in promoting neuron survival, elevated IGF-I expression in nestin/IGF-I Tg mice may decrease neuron apoptosis in the developing cerebral cortex. To test this hypothesis, the density of apoptotic cells was analysed in the cerebral cortex during embryonic development at E16 and during early postnatal development at P0 and
P5 in Tg mice and littermate controls. Apoptotic cells were detected using an antibody against activated caspase-3. Caspase-3 is expressed in the developing brain (Srinivasan et al., 1998; De Bilbao et al., 1999), and defects in neuronal apoptosis have been demonstrated in mice lacking the caspase-3 gene (Kuida et al., 1996). As well, IGF-I has been shown to prevent caspase-3-mediated death of cortical neurons in vitro (Takadera et al., 1999). Immunohistochemical detection of caspase-3 has been used to detect neuronal apoptosis in several studies (Srinivasan et al., 1998; DiCunto et al., 2000; Hu et al., 2000; Camarero et al., 2001), and provides results that are similar to those generated using the terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling (TUNEL) method (Srinivasan et al., 1998). Therefore, activated caspase-3 was determined to be an appropriate marker for the detection of cortical neuron death in Tg and control mice.

As noted previously, transgene expression in nestin/IGF-I Tg mice decreases to a steady-state level by P20, but remains elevated above control levels to at least P45 (Popken et al., 2004). However, it was not clear from previous studies if the increases in cortical neuron number that were documented at E16 and P12 represented a stable increase in neuron number that persisted into adulthood. To determine if neuron number remains elevated in the cerebral cortex after transgene expression has decreased to a steady-state level, stereological analyses of the cerebral cortex were conducted at P270 (equivalent to 9 months of age). Analyses were performed to determine total cortical volume, as well as the numerical density and total number of neurons in the cerebral cortex of nestin/IGF-I Tg and control mice.

5.2 Materials and Methods

Immunohistochemical detection of cleaved (activated) caspase-3 in the cerebral cortex at E16

Timed-pregnant dams were generated as described in previous sections. On E16, embryos were removed by hysterotomy and fixed overnight in 70% ethanol at 4°C. Brains were embedded in paraffin and serially sectioned at 20 μm in the coronal plane. Sections were mounted on Colorfrost Plus glass slides (Fisher Scientific) and air-dried overnight. Every tenth section in the series was selected for immunohistochemical processing. Briefly, sections were boiled in antigen unmasking solution for 10 min in a microwave and incubated with polyclonal rabbit anti-cleaved caspase-3 primary antibody overnight at 4°C (1:50 dilution; Cell Signaling Technologies, Beverly, MA). This antibody specifically detects cleaved caspase-3 and does not react with inactive forms of caspase-3 (e.g. procaspase-3) or with other caspases. Detection of primary antibody binding was accomplished using an ABC Elite Kit (Vector
Laboratories) according to the manufacturer’s protocol. Sections were counterstained with 0.1% aqueous basic fuchsin, differentiated in 95% ethanol and coverslipped with Permount (Fisher Scientific). Sections processed without the addition of primary antiserum were used as negative controls.

Attempts were made to quantify the number of apoptotic cells in the developing cerebral cortex using stereological methods. However, in both groups the number of apoptotic cells in the cortex was not sufficient to allow for the use of the optical disector method. This method requires that 200 immunoreactive cells be counted on representative sections for each animal (Gundersen et al., 1988b). It was found that, on average, the number of apoptotic cells in the cortex of a given animal would be less than 200 even if all sections in the series (on average 135 sections per animal) were processed. Therefore, the number of apoptotic cells/mm² was determined in the cortex on E16. Cell counts were conducted on every tenth section from the serial paraffin sections (approximately 13 sections per animal). On each section, the area of the cortex was measured using established boundary criteria (Alvarez-Bolado and Swanson, 1996) and the number of cleaved caspase-3 immunoreactive cells was determined. The number of cells/mm² was determined as the total number of caspase-3 positive cells divided by the sum of the area measurements. Apoptotic cellular profile areas were determined by measuring the areas of 20 cleaved caspase-3 immunoreactive cells per animal in μm², and mean profile areas were determined for each group. Statistical comparisons between control and Tg mice were conducted using Student’s t-test.

Cleaved caspase-3 immunohistochemistry at P0, P5

At P0 and P5, nestin/IGF-I Tg and littermate control animals were anesthetized by i.p. injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). Animals were perfused through the left ventricle with 4% paraformaldehyde in 0.1M phosphate buffer for 20 min at a perfusion pressure of 50-60 mm Hg for P0 animals and 60-70 mm Hg for P5 animals. Brains were removed, weighed, and transferred to the same 4% paraformaldehyde fixative solution for 40 min. Brains were then transferred to 0.1M phosphate buffer to rinse out any remaining fixative solution.

Prior to sectioning, tissue blocks were cryoprotected by immersion in 30% sucrose in 0.1M phosphate buffer overnight at 4°C. Serial frozen sections (50 μm) were cut in the coronal plane through the entire extent of the cerebral hemispheres and transferred to 0.1M phosphate buffer. Every fourth section in the series was collected on glass slides and stained
for the detection of Nissl substance using 0.1% thionine in acetate buffer. Adjacent sections were processed for the detection of cleaved caspase-3 immunoreactivity as follows. Free-floating sections were incubated in polyclonal rabbit anti-cleaved caspase-3 primary antiserum (1:1500 dilution; Cell Signaling Technologies) overnight at room temperature. Sections were then incubated in biotinylated goat anti-rabbit IgG secondary antibody (1:200) for 1 hr at room temperature. Further processing was carried out using a Vector Elite ABC kit (Vector Laboratories) according to the manufacturer's instructions, with DAB as the chromogen. Sections were mounted on glass slides and lightly counterstained with 0.1% aqueous basic fuchsin. Sections incubated without the addition of primary antiserum were used as negative controls.

Stereological analyses were conducted to determine the total volume of the cerebral cortex and the \( N_v \) and total number of cortical neurons for each animal using the Nissl stained sections and methods outlined in previous sections (see the methods described for stereology conducted at P12 in Chapter 2), with the following modifications. For P0 animals, the dimensions of the disector box used for determining neuronal \( N_v \) were 15 \( \mu m \times 15 \mu m \times 10 \mu m \), where 10 \( \mu m \) was the z-axis height. For P5 animals, the dimensions of the disector box used were 25 \( \mu m \times 25 \mu m \), and cells were counted if they fell within 10 \( \mu m \) of z-axis height. As well, the \( N_v \) and total number of cleaved caspase-3 immunoreactive cells in the cerebral cortex were determined at each age. The \( N_v \) of cleaved caspase-3 immunoreactive cells was determined using the optical disector method (Gundersen et al., 1988b), as described previously (see Chapter 2). The total number of cleaved caspase-3 immunoreactive cells was calculated for each animal using the cortical volume and caspase-3 \( N_v \) measurements. For P0 animals, studies were carried out on 3 animals per group. For P5 animals, studies were carried out on 3 Tg animals and 5 control mice. For all variables, statistical comparisons between control and Tg mice were conducted using 2 x 2 (groups x ages) ANOVA.

**Stereological analyses of the adult cerebral cortex**

Nine month old (approximately P270) nestin/IGF-I Tg and normal littermate control mice (N = 3 per group) were deeply anaesthetized with an i.p. injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Individual mice were perfused through the ascending aorta with a fixative solution containing 4% paraformaldehyde and 1% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 45 minutes at a perfusion pressure of 100-120 mm Hg. After determining brain weights, brains were transferred to additional fixative solution and stored at...
4°C for 24 hr. Tissue blocks were then transferred to a solution of 15% sucrose in 0.1M phosphate buffer and stored at 4°C overnight. Serial frozen sections cut at 30 μm in the coronal plane through the entire rostrocaudal extent of the cerebral hemispheres were mounted on glass slides and air-dried overnight. Every 4th section in the series (P = 4) was collected and stained for Nissl substance with 0.1% thionine in acetate buffer, dehydrated in ascending grades of ethanol, cleared in xylene and mounted with Permount.

Stereological analyses were performed to measure the total volume of the cerebral neocortex, as well as the Nv and total number of cortical neurons. The cerebral neocortex was defined as all six-layered regions of the cerebral cortex as previously described (see Chapter 2). Total neocortex volume was measured using Cavalieri's direct estimator (Gundersen et al., 1988a), described in detail in earlier sections. The Nv of neurons in the cerebral neocortex was determined using the optical disector method (Gundersen et al., 1988b). Neuron counts were conducted on seven sections that were equally spaced along the caudal-to-rostral extent of the cerebral hemispheres. The dimensions of the disector counting frame and the z-axis height used for counting were identical to those used for the stereological analyses of the cortex at P12. Statistical analyses of all variables were conducted using Student’s t-test.

5.3 Results

Apoptosis in the cerebral wall at E16

As noted in the methods section, the number of apoptotic cells in the developing cortex at E16 was very low in both Tg and control embryos. In fact, these numbers were so low that stereological methods could not be used to quantify the Nv of apoptotic cells in the cortex at this age. Therefore, the number of apoptotic cells (cleaved caspase-3 immunoreactive) per mm² of cerebral wall was determined in Tg and control embryos (N = 2 per group). The number of apoptotic cells/mm² did not differ between groups at this age (t = 0.152, P > 0.05). Mean values (± SEM) were 1.007 ± 0.273 cells/mm² for Tg mice and 1.824 ± 0.237 cells/mm² for controls, indicating that very few cells were undergoing apoptosis in either group. However, while this difference did not reach statistical significance, the mean number of apoptotic cells/mm² was 45% lower in Tg embryos as compared to controls. In both groups, apoptotic cells were observed in the VZ, SVZ, IZ, and cortical plate. In general, more apoptotic cells were located in the IZ and cortical plate, where post-mitotic neurons are known to reside, than in the proliferative zones (VZ, SVZ).
The mean profile area of cleaved caspase-3 immunoreactive cells did not differ between groups on E16 ($t = 0.480, P > 0.05$). For Tg mice the mean profile area of apoptotic cells was $22.54 \pm 0.90 \ \mu m^2$, and for controls this value was $23.00 \pm 0.33 \ \mu m^2$. As cell profile areas were similar in Tg and control mice, the probability of cells being intersected by the plane of section was the same in both groups. Differences in cell profile areas can bias measurements of the number of cells/mm$^2$ because larger cells have a greater probability of being intersected by the plane of section. Therefore, the present results indicate that the number of apoptotic cells/mm$^2$ in the cortex was a valid estimate of apoptosis at E16, as differences in cellular profile areas did not bias this measurement. As profile areas did not differ between groups, the estimates of apoptotic cells/mm$^2$ generated at E16 are approximately equivalent to the $N_v$ of apoptotic cells in the cerebral wall at this age.

**Apoptosis in the cerebral cortex during early postnatal development**

Apoptotic cells were observed in all layers and cytoarchitectonic regions of the cerebral cortex in Tg and control mice at P0 and P5. At P0, a number of cleaved caspase-3 immunoreactive cells were observed in layer II/III of the cingulate and retrosplenial cortices (Fig. 5-1). At P5, apoptotic cells were noted in layer II/III in the cingulate, retrosplenial, frontal, and primary motor regions of the cortex. As well, cleaved caspase-3 immunoreactive cells were observed in layer V in the primary motor and primary somatosensory regions, and these cells had the morphological appearance of large pyramidal neurons. In general, the distribution pattern of apoptotic cells within the cerebral cortex was similar in Tg and control mice. A number of cleaved caspase-3 immunoreactive cells in the cerebral cortex displayed morphological characteristics of neurons, including dendritic arborizations (Fig. 5-2A, B). Many cells displayed obvious neuronal characteristics, but had degenerated to the point where cellular processes had begun to break down (Fig. 5-2C). A small proportion of labeled cells exhibited only short processes or no processes at all, and may have been either apoptotic neurons or glia. In some cases, the labeled cells had degenerated to the point where the cell body and processes had broken down into smaller compartments, indicating that apoptosis was quite advanced (Fig. 5-2D). This type of cell was always scored as a single cell in the present analyses.

The $N_v$ of cleaved caspase-3 immunoreactive cells in the cerebral cortex was analyzed at P0 and P5 using $2 \times 2$ ANOVA comparison of the data between Tg and control mice. This analysis revealed a significant main effect for groups ($F = 45.994, P < 0.001$), indicating that
Fig. 5-1. Representative photomicrographs illustrating the distribution of cleaved caspase-3 immunostaining in the cerebral cortex of control (A) and nestin/IGF-I Tg (B) mice at P0. The distribution of cleaved caspase-3 positive cells is demonstrated by circles outlining individual cleaved caspase-3 immunoreactive cells in figures A and B. The morphologies of individual cleaved caspase-3 positive cells are illustrated in Figure 5-2. Cleaved caspase-3 positive cells were identified by the accumulation of brown-black reaction product in cell bodies. Sections were counterstained with basic fuchsin. As shown in these low magnification photomicrographs, the number of apoptotic cells per section was relatively low in both control and Tg mice. However, as illustrated by the circles outlining individual immunoreactive cells, an increase in the density of these caspase-3 positive cells was apparent in controls (A) as compared to Tg mice (B). Immunoreactive cells were particularly abundant in layer II/III of the cingulate and retrosplenial cortices at this age. As well, cleaved caspase-3 positive cells were noted in layers V and VI of the frontal cortex. The general distribution pattern of immunoreactive cells through different cortical regions was similar in both groups. Scale bar = 250μm.
Fig. 5-2. Photomicrographs of cleaved caspase-3 immunoreactive cells in the cerebral cortex at P5. These sections were processed without counterstaining to enhance photographic contrast. The majority of cleaved caspase-3 immunoreactive cells in the cerebral cortex at P0 and P5 exhibited morphological hallmarks that were characteristic of neurons (A, B), such as dendritic arborizations. Some neurons had degenerated to the point where their processes had begun to break down (C). As well, some labeled cells were observed in which apoptosis appeared to be quite advanced (D). These cells were characterized by cell bodies and processes that had broken down into smaller compartments. Scale bar in A = 10\(\mu\)m and applies to all components of the figure.
the \( N_v \) of cleaved caspase-3 immunoreactive cells was decreased in Tg animals. A main effect of ages was also apparent (\( F = 202.23 \, P < 0.001 \)), indicating that in both Tg and control mice the \( N_v \) of apoptotic cells in the cortex was greater at P0 than at P5. The interaction term (groups \( \times \) ages) was not significant (\( F = 4.034, \, P = 0.07 \)), so further analysis of the data was not warranted. However, it was noted that the \( N_v \) of apoptotic cells was reduced in Tg mice by 31% at P0 and by 39% at P5 (Table 5-1). Using the total cortical volume data and estimates of the \( N_v \) of apoptotic cells at P0 and P5, estimates of the total number of apoptotic cells in the cortex were calculated for each mouse. Analysis of the data using 2 \( \times \) 2 ANOVA showed that the main effect for groups was not significant (\( F = 3.812, \, P = 0.08 \)), indicating that the total number of apoptotic cells in the cortex did not differ between groups. As well, the main effect for ages was not significant (\( F = 0.430, \, P > 0.05 \)), indicating that the total number of apoptotic cells did not differ significantly between P0 and P5 (Table 5-1).

**Cortical volume, neuronal \( N_v \) and total neuron number at P0 and P5**

The effect of elevated IGF-I expression on cerebral cortex development was assessed in Tg mice and their normal littermate controls on P0 and P5 using stereological methods. Morphometric variables of the cerebral cortex measured in Tg and control mice are summarized in Table 5-2. Analysis of the total cortical volume data using 2 \( \times \) 2 ANOVA revealed a significant main effect for groups (\( F = 6.913, \, P = 0.03 \)), indicating that cortical volume was significantly greater in Tg mice (Table 5-2). The \( N_v \) of neurons in the cerebral cortex did not differ significantly between groups at either age studied (\( F = 0.483, \, P > 0.05 \)). However, analysis of the total cortical neuron number data using 2 \( \times \) 2 ANOVA revealed a significant main effect for groups (\( F = 28.826, \, P < 0.001 \)), indicating that total neuron number was increased Tg mice. Consistent with studies at P12 (Popken et al., 2004; Hodge et al., 2005), the total number of neurons in the cortex was increased in Tg mice by 29% at both P0 and P5.

**Histology and stereological analyses of the cerebral cortex at nine months of age**

Stereological analyses were performed to measure the total volume, \( N_v \) of neurons, and total number of neurons in the cerebral cortex of nestin/IGF-I Tg and control mice at nine months of age. No signs of gross malformation or pathological abnormalities in the brains of Tg mice were apparent upon examination of histological sections. The brains of nestin/IGF-I Tg mice appeared larger than those of littermate controls (Fig. 5-3), consistent with studies of Tg mice at P12 (Popken et al., 2004). Brain weights of Tg mice were significantly greater
Table 5-1
Stereological analyses of the numerical density ($N_v$) and total number of cleaved caspase-3 immunoreactive neurons in the cerebral cortex at postnatal day 0 and postnatal day 5

<table>
<thead>
<tr>
<th></th>
<th>Postnatal Day 0</th>
<th></th>
<th>Postnatal Day 5</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Nestin/IGF-I Tg</td>
<td>Control</td>
<td>Nestin/IGF-I Tg</td>
</tr>
<tr>
<td>$N_v$ (cells/mm$^3$)</td>
<td>430.73 ± 18.39</td>
<td>297.45 ± 8.09*</td>
<td>184.66 ± 12.34</td>
<td>112.29 ± 19.13*</td>
</tr>
<tr>
<td>Total number of</td>
<td>5827 ± 500</td>
<td>5217 ± 288</td>
<td>7099 ± 696</td>
<td>4891 ± 972</td>
</tr>
<tr>
<td>cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All values are listed as the mean ± SEM. *P < 0.001 for comparisons between Tg and control mice using 2x2 ANOVA.

Table 5-2
Summary of morphometric variables of the cerebral neocortex measured in nestin/IGF-I transgenic (Tg) and control mice at postnatal day 0 and postnatal day 5

<table>
<thead>
<tr>
<th></th>
<th>Postnatal Day 0</th>
<th></th>
<th>Postnatal Day 5</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Nestin/IGF-I Tg</td>
<td>Control</td>
<td>Nestin/IGF-I Tg</td>
</tr>
<tr>
<td>Brain weight (mg)</td>
<td>77 ± 3</td>
<td>107 ± 3**</td>
<td>161 ± 6</td>
<td>186 ± 7**</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>1.29 ± 0.10</td>
<td>1.39 ± 0.05</td>
<td>3.14 ± 0.26</td>
<td>2.52 ± 0.16</td>
</tr>
<tr>
<td>Cortical volume (mm$^3$)$^a$</td>
<td>13.484 ± 0.702</td>
<td>17.576 ± 1.158*</td>
<td>38.245 ± 1.985</td>
<td>43.187 ± 1.195*</td>
</tr>
<tr>
<td>$N_v$ of neurons (neurons/mm$^3$)</td>
<td>611,546 ±</td>
<td>604,068 ± 8,036</td>
<td>275,512 ±</td>
<td>311,897 ±</td>
</tr>
<tr>
<td>Total number of</td>
<td>8,228,041 ±</td>
<td>10,608,325 ±</td>
<td>10,397,285 ±</td>
<td>13,407,626 ±</td>
</tr>
<tr>
<td>neurons</td>
<td>285,864</td>
<td>638,927***</td>
<td>351,864</td>
<td>775,809***</td>
</tr>
</tbody>
</table>

All values are listed as the mean ± SEM. $^a$Volume, $N_v$ of neurons, and total number of neurons were measured bilaterally. *P < 0.05, **P = 0.001, ***P < 0.001 for comparisons between Tg and control mice using 2x2 ANOVA.
Fig. 5-3. Representative sections (30 μm thick) stained with thionine for Nissl substance, through the cerebral hemispheres of nestin/IGF-I Tg (A) and non-Tg littermate control (B) mice at nine months of age (P270). Scale bar = 1mm.
than those of control mice, but body weights did not differ significantly between groups (Table 5-3). The total volume of the cerebral neocortex was increased by 23% in Tg mice as compared to controls. The $N_v$ of neurons in the neocortex did not differ significantly between groups. However, total neuron number was significantly increased by 32% in nestin/IGF-I Tg mice.

5.4 Discussion

The results of the present study show that elevated IGF-I expression in nestin/IGF-I Tg mice promotes neuron survival in the cerebral cortex during early postnatal development. These data are in agreement with previous studies showing that IGF-I promotes neuron and glia survival in vitro (Aizeman and DeVellis, 1987; Torres-Aleman et al., 1990a, b; Pons et al., 1991; Barres et al., 1992; Bozyczko-Coyne et al., 1993; Sortino and Canonico, 1996; Russell and Feldman, 1999; Ye and D’Ercole, 1999; Yamada et al., 2001; Ness et al., 2002; Ness and Wood, 2002). As well, these data are consistent with a previous report showing that IGF-I overexpression during postnatal development reduced the density of apoptotic cells in the cerebellum in vivo on P7 by decreasing procaspase-3 and cleaved caspase-3 protein levels in the cerebellum (Chrysis et al., 2001). Therefore, the present findings suggest that the increased neuron number documented previously in nestin/IGF-I Tg mice (Popken et al., 2004; Hodge et al., 2005) results, at least in part, from an IGF-I-mediated enhancement of neuron survival.

The results of the present study indicate that augmented expression of IGF-I during embryonic development does not appear to influence apoptosis in the developing cerebral wall at E16. Previous studies have shown varying amounts of apoptosis in the embryonic cortex, with some studies indicating that 50-70% of the cells in the embryonic cortex are eliminated by apoptosis (Blaschke et al., 1996). However, other reports indicate that apoptosis is a rare event in the developing cortex, with only approximately 9% of cells in the cortex undergoing apoptosis on E16 (Thomaidou et al., 1997). The current findings appear to be consistent with those analyses showing that a relatively small number of cells undergo apoptosis in the embryonic cortex (Thomaidou et al., 1997; Verney et al., 2000). In both Tg and control mice, apoptotic cells were observed so rarely in the cerebral wall that stereological methods could not be used to generate an estimate of the $N_v$ of apoptotic cells. Consistent with earlier reports, the majority of neuronal apoptosis was documented during early postnatal development in the present analysis (Spreafico et al., 1995; Verney et al., 2000).
Table 5-3
Summary of morphometric variables measured in the cerebral neocortex in control and nestin/IGF-I transgenic mice at nine months of age (P270)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Nestin/IGF-I Tg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>37.4 ± 5.2</td>
<td>32.7 ± 6.1</td>
</tr>
<tr>
<td>Brain weight (mg)</td>
<td>438 ± 9</td>
<td>620 ± 20**</td>
</tr>
<tr>
<td>Cortical volume (mm(^3))(^a)</td>
<td>83.22 ± 2.93</td>
<td>102.69 ± 4.68*</td>
</tr>
<tr>
<td>(N_v) of neurons (neurons/mm(^3))</td>
<td>180,733 ± 12,674</td>
<td>193,411 ± 11,334</td>
</tr>
<tr>
<td>Total number of neurons</td>
<td>14,976,229 ± 677,653</td>
<td>19,781,603 ± 871,864*</td>
</tr>
</tbody>
</table>

All values are listed as mean ± SEM. \(^a\)Volume, \(N_v\) of neurons, and total number of neurons were measured bilaterally. *\(P < 0.05\), **\(P < 0.01\) for comparisons between Tg and control mice using Student's t-test.
In previous studies, the number of cells in the cortical plate was shown to be increased by 54% in Tg embryos on E16 (Popken et al., 2004). The results of the present analysis indicate that an IGF-I-mediated decrease in apoptosis likely does not contribute significantly to this increase in cortical plate cell number at E16. These data suggest that increased cortical plate cell number in Tg mice during embryonic development results from increased neuron progenitor proliferation rather than decreased apoptosis. Therefore, it is likely that increased neuron number in the embryonic cerebral cortex results from the enhanced cell cycle progression and increased cell cycle re-entry documented previously in Tg embryos (Hodge et al., 2004). However, decreased neuron apoptosis in response to elevated IGF-I does appear to contribute to the determination of final neuron number during postnatal development of the cortex.

IGF-I overexpression in Tg mice during postnatal development significantly decreases the $N_v$ (number per mm$^3$) of apoptotic cells on P0 and P5, as judged using an antibody that specifically detects activated (cleaved) caspase-3. This reduction in the $N_v$ of apoptotic cells was slightly greater in Tg mice at P5 (39%) than at P0 (31%). At these postnatal ages, the $N_v$ of cortical neurons does not differ significantly between groups, indicating that the density of neurons in the cortex is similar in both groups. Therefore, the documented reduction in the $N_v$ of apoptotic cells in the cortex, without a parallel change in the $N_v$ of cortical neurons, corresponds to a significant decrease in the number of cells undergoing apoptosis in the Tg cortex. However, as total cortical volume is significantly increased in Tg mice, estimates of the overall total number of apoptotic cells in the cortex do not differ significantly between groups. Nonetheless, these data strongly support a role for IGF-I in promoting neuron survival in the developing cerebral cortex. Previously, it has been shown that the $N_v$ of apoptotic cells in the cortex continues to be reduced by 26% in Tg mice at P7 (Popken et al., 2004), indicating that elevated IGF-I expression persistently augments neuron survival throughout the peak period of neuron apoptosis in the cerebral cortex.

Neuron apoptosis was more prevalent in the cerebral cortex during early postnatal development than during embryonic development in both Tg and control mice, consistent with a number of other studies (Spreafico et al., 1995; Thomaidou et al., 1997; Verney et al., 2000). However, the peak time of cortical neuron apoptosis documented in the present study appears to differ slightly from other reports of cortical apoptosis in rodents. The present report found that the $N_v$ of apoptotic neurons was greater at P0 than at P5 in both groups. Other studies
have shown low levels of apoptosis in the mouse cortex at birth, and indicated that apoptosis increases to a peak at approximately P4 (Verney et al., 2000). Similarly, the peak of neuron apoptosis in the cerebral cortex of rats has been shown to occur between P5 and P8 (Spreafico et al., 1995). The present data likely differ from other reports of apoptosis in the mouse cortex because these studies were confined to several regions of the parietal cortex (Verney et al., 2000), whereas the current analysis encompasses all cytoarchitectonic regions of the cortex. In both Tg and control mice, the density of apoptotic cells was lowest at E16, rose to a peak at P0, and declined thereafter. These data indicate that, while IGF-I decreases the density of apoptotic neurons during postnatal development, the temporal pattern of apoptosis in the cortex is not altered in Tg mice.

Transgene expression in nestin/IGF-I Tg mice decreases to a steady-state level by P20 (Popken et al., 2004), raising the possibility that neurons may be eliminated in Tg mice during subsequent development in response to lowered transgene expression. To address this issue, stereological analyses of the cerebral cortex were performed in adult Tg and control mice at nine months of age (P270), well after the time at which transgene expression levels off. Consistent with stereological analyses conducted at P12 (Popken et al., 2004; Hodge et al., 2005), brain weights remained significantly elevated in Tg mice by 28% at P270. This increase in brain weight is comparable to the 23% increase documented in Tg mice at P12 (Popken et al., 2004; Hodge et al., 2005). Body weights were unaffected by IGF-I overexpression in adult nestin/IGF-I Tg mice, consistent with transgene expression being restricted to the brain. The total volume of the cerebral cortex was increased in Tg mice (23%) at P270 to a similar degree as that documented in Tg mice at P12 (31%). The \( N_v \) of neurons was not altered in Tg mice at P270, but total neuron number was increased by 32% in Tg mice as a result of increased cortical volume. Therefore, the increase in cortical neuron number documented in Tg mice during early postnatal development persists as a stable increase well into adult life. These data indicate that neurons are not eliminated in Tg mice as a result of decreased transgene expression during later phases of postnatal development.

Interestingly, the \( N_v \) of neurons did not differ between nestin/IGF-I Tg and control mice at P270. A number of studies have shown that increased expression of IGF-I in different lines of mice during postnatal development results in decreased neuronal \( N_v \) and increased process outgrowth in various regions of the brain (Gutierrez-Ospina et al., 1996; Dentremont et al., 1999; O'Kusky et al., 2000). The current results, therefore, suggest that elevated IGF-I
expression in nestin/IGF-I Tg mice does not augment process outgrowth sufficiently to reduce neuronal packing density. This may be due to the fact that peak IGF-I overexpression in nestin/IGF-I Tg mice occurs during early postnatal development, before extensive process outgrowth has occurred. Alternatively, the cellular sites of IGF-I transgene expression in nestin/IGF-I Tg mice may limit the availability of IGF-I at outgrowing processes. In any case, this finding clearly differentiates nestin/IGF-I Tg mice from previously studied lines of IGF-I Tg mice.

In conclusion, the results of the present study indicate that IGF-I promotes neuron survival in the cerebral cortex during early postnatal development by decreasing the numerical density of activated caspase-3 immunoreactive neurons. However, the density of apoptotic cells in the cerebral wall was similar in Tg and control embryos at E16, suggesting that elevated IGF-I may not augment cellular survival at this age. These results indicate that increased neuron number in the cortex of nestin/IGF-I Tg mice results from both enhanced neuron progenitor proliferation during embryonic development and decreased neuron apoptosis during early postnatal development.
5.5 Bibliography


efficient stereological methods and their use in pathological research and diagnosis. APMIS 96:379-394.


Chapter VI  Discussion and conclusions

6.1 Summary of results

The present studies indicate that IGF-I promotes growth and development of the cerebral cortex. Augmented IGF-I expression in nestin/IGF-I Tg mice results in increased cortical volume and a 27% increase in total neuron number, without a corresponding change in the numerical density of neurons. The studies presented in this thesis have shown that increased neuron number in the cortex results from an IGF-I-mediated enhancement of neuron progenitor proliferation in combination with reduced neuronal death by apoptosis during postnatal development. Increased neuron output in the developing cortex of nestin/IGF-I Tg mice is evident by E16 (Popken et al., 2004) and results from an increase in the rate of progenitor progression through the cell cycle, as well as enhanced cell cycle re-entry. As a result of increased cell cycle re-entry, cells are retained in a proliferative state in Tg mice, which leads to increased volume of the VZ/SVZ at E16 (Popken et al., 2004). As well, the proportion of Tbr2-positive cells in the cerebral wall is increased in Tg mice, indicating that cells are retained in the cell cycle as intermediate progenitors.

In addition to the increase in cortical neuron number documented in Tg mice at P12, the total number of glia in the cortex is increased by 37% in Tg mice at this age. Analyses of the effects of IGF-I on the development of distinct cytoarchitectonic regions of the cortex indicated that IGF-I differentially augments the growth of the primary motor and primary somatosensory cortices, with overgrowth of the primary motor cortex augmented to a relatively greater extent than that of the somatosensory cortex. Additionally, the neurons of layer I appear to be particularly sensitive to the effects of IGF-I. The density of reelin-expressing cells in the marginal zone of the developing cortex is increased in Tg mice on E14, shortly after the normal time of production of these cells. These results indicate that an initial amplification in output contributes, at least in part, to the increase in layer I neurons documented on P12.

Increased neuron number in the cortex is apparent by as early as E16 in Tg embryos (Popken et al., 2004). The present studies indicate that apoptosis is a rare event in the E16 cerebral wall, and suggest that an IGF-I-mediated decrease in neuron apoptosis may not contribute to increased cell number in the cortical plate at E16. During embryonic development, the incidence of apoptosis in progenitor cells of the cerebral wall is estimated to be as low as 0.14% per day in normal mice (Haydar et al., 2000). Consistent with these findings, very low levels of apoptosis were noted in the developing cortex of Tg and control
embryos at E16, and the density of apoptotic cells did not differ between groups. Therefore, inhibition of apoptosis by IGF-I likely makes a relatively small contribution to neuron output from the proliferative zones of the cerebral wall.

Given that increased cell number in the cortical plate occurs well before peak neuron elimination by apoptosis in the cerebral cortex, enhanced neuron progenitor proliferation must contribute to the observed increase in neuron number. This thesis has documented that IGF-I overexpression during embryonic development regulates neuron progenitor proliferation in the VZ by increasing the rate of progenitor cell proliferation through the cell cycle, which results in a 2.05 hr decrease in total cell cycle length in Tg embryos at E14. As well, IGF-I promotes the re-entry of neuron progenitors into the cell cycle, resulting in expansion of the proliferative zones by E16 (Popken et al., 2004). Neuron output is enhanced as a result of increased cell cycle re-entry, as illustrated by the 54% increase in cortical plate cell number documented on E16 (Popken et al., 2004). IGF-I appears to promote progenitor cell re-entry into the cell cycle as intermediate progenitor cells, and does not alter the proportion of Pax6-positive radial glia in the VZ of the cerebral wall on E14. These results strongly support a role for IGF-I in promoting neuron progenitor proliferation during cortical neurogenesis and suggest a differential effect of IGF-I on distinct progenitor cell populations.

In addition to its ability to promote neuron progenitor proliferation, the results presented herein indicate that IGF-I also decreases neuron apoptosis in the cerebral cortex during early postnatal development. The $N_v$ of apoptotic cells in the cortex is significantly reduced in Tg mice on P0 and P5, and previous studies have shown that the $N_v$ of apoptotic cells continues to be reduced in Tg mice on P7 (Popken et al., 2004). These results show that increased neuron number in the cerebral cortex of nestin/IGF-I Tg mice results from a combination of enhanced neuron output during cortical neurogenesis and inhibited neuron death during postnatal cortical development. Augmented IGF-I expression during embryonic and postnatal development produces a stable increase in cortical neuron number that persists well into adult life. However, a corresponding increase in process outgrowth for neurons in the cerebral cortex is not apparent in this particular line of IGF-I Tg mice, as the $N_v$ of neurons in the cortex is similar in adult Tg and control mice. This result differentiates nestin/IGF-I Tg mice from other lines of mice in which significant increases in process outgrowth in response to elevated IGF-I levels have been demonstrated (Gutierrez-Ospina et al., 1996; Dentremont et al., 1999; O'Kusky et al., 2000).
6.2 Effects of IGF-I on neuronal proliferation

The in vivo role of IGF-I in regulating the proliferation of neuron progenitors in the developing cerebral cortex has not been investigated previously. The results of the present in vivo series of experiments strongly support a role for IGF-I in promoting the growth and development of the cerebral cortex. These studies have shown that elevated IGF-I expression in nestin/IGF-I Tg mice beginning during embryonic development produces neocortical overgrowth and a measurable increase in the total number of neurons in the cerebral cortex. Increased cell number in the developing cortex is apparent in Tg mice by E16 (Popken et al., 2004), well before the major period of neuron elimination by apoptosis in the cortex, suggesting that increased neuron number in the cortex results, at least in part, from an IGF-I-dependent increase in cortical neuron progenitor proliferation.

The capacity of IGF-I to promote neuron proliferation has been documented by a number of in vitro studies (DiCicco-Bloom and Black, 1988; Torres-Aleman et al., 1990; Drago et al., 1991; Werther et al., 1993; Zackenfels et al., 1995; Arsenijevic et al., 2001). These studies indicate that IGF-I promotes proliferation of neuroepithelial cells derived from E10 mice (Drago et al., 1991), as well as neuron precursors cultured from E15-E16 mice (Lenoir and Honegger, 1983). Neuron progenitor cells isolated from the ganglionic eminences of E14 mice require IGF-I for cell division, and will not proliferate in the presence of only EGF or FGF-2 (Arsenijevic et al., 2001). IGF-I also appears to promote the proliferation of cultured sympathetic neuroblasts (DiCicco-Bloom and Black, 1989) and rat olfactory bulb explants (Torres-Aleman et al., 1992).

Relatively few in vivo studies have examined the ability of IGF-I to promote neuron progenitor proliferation, largely because many of these studies have used IGF-I Tg mice with transgene expression restricted to postnatal development. IGF-I has been shown to moderately increase neuron proliferation in vivo in the cerebellum of IGF-II/IGF-I mice during early postnatal development (Ye et al., 1996). In these mice, BrdU labeling showed that the total number of proliferating granule cell progenitors in the external granule cell layer of the cerebellum is modestly increased in Tg mice during early postnatal development (Ye et al., 1996). Total neuron number in the granule cell layer of the cerebellum was augmented by 82% in IGF-II/IGF-I Tg mice at this age. Growth of the hippocampal dentate gyrus is also augmented in IGF-II/IGF-I mice. A significant increase of 50-61% in the total number of neurons has been documented in the dentate gyrus of IGF-II/IGF-I Tg mice from P28 to P130
(O'Kusky et al., 2000). Given that neurogenesis in the dentate gyrus persists during postnatal development, this increase in neuron number in the dentate gyrus may result, in part, from increased neuron proliferation in response to elevated IGF-I levels, although this effect has not been demonstrated experimentally (O'Kusky et al., 2000). Interestingly, peripheral infusion of IGF-I has been shown to enhance neurogenesis in the dentate gyrus in adult mice (Aberg et al., 2000).

As illustrated in the cerebellum and dentate gyrus of IGF-II/IGF-I Tg mice, the magnitude of IGF-I-mediated increases in neuron number tends to be greater in regions of the brain where transgene expression coincides with both neurogenesis and neuronal elimination by apoptosis. For example, the number of Purkinje cells is augmented to a lesser degree (20%) than the total number of granule cells (82%) in the cerebellum of IGF-II/IGF-I Tg mice (Ye et al., 1996). As Purkinje cell generation occurs during early embryonic development, this increase in Purkinje cell number likely results only from enhanced cell survival in response to elevated IGF-I levels (Ye et al., 1996). As well, in regions such as the hypoglossal and facial nuclei, where transgene expression occurs after both the period of neuron production and the period of neuron elimination, total neuron number in Tg mice does not differ from controls.

Studies of IGFBP-1 Tg mice provide some additional support for an effect of IGF-I on neuron proliferation. In IGFBP-1 Tg mice the number of BrdU-labeled proliferating progenitor cells is decreased by 41% in the granule cell layer of the dentate gyrus and by 19% in the SVZ of the lateral ventricle on P3 (Ni et al., 1997), indicating a significant reduction in progenitor proliferation in the absence of IGF-I.

The findings presented herein extend the results of the above studies by examining the mechanisms by which IGF-I acts to promote neuron progenitor proliferation in the developing cerebral cortex. The present experiments show that IGF-I promotes neuron proliferation by increasing the rate at which progenitors progress through the cell cycle. Specifically, elevated IGF-I expression in Tg mice decreases the total length of the cell cycle on E14 by 2.05 hr, owing to a reduction in G1 phase length. As well, IGF-I promotes the re-entry of progenitor cells into the cell cycle on E14-E15, with 15% more cells returning to undergo further rounds of division in Tg mice as compared to littermate controls. Ultimately, this increase in cell cycle re-entry results in expansion of the proliferative zones of the cerebral wall in Tg mice by 26% on E16 (Popken et al., 2004). As a consequence of increased cell-cycle re-entry, the potential for generating neurons is increased in Tg embryos due to expansion of the progenitor
cell population. Correspondingly, neuron output is increased in Tg embryos as evidence by a significant 54% increase in the total number of cells in the cortical plate at E16 (Popken et al., 2004).

In addition to showing that IGF-I promotes neuron progenitor proliferation during cortical neurogenesis, alterations in the proportion of a specific progenitor cell type in the cerebral wall have been demonstrated in nestin/IGF-I Tg mice on E14. Immunohistochemical markers defining individual progenitor cell populations in the developing cortex have only recently been defined (Heins et al., 2002; Englund et al., 2005). Therefore, the effects of IGF-I on these distinct, in vivo progenitor cell types had not been analysed previously. In the present study, IGF-I was found to differentially influence the proportions of Pax6-positive radial glial cells and Tbr2-positive intermediate progenitor cells in Tg embryos on E14. The proportion of Pax6-positive radial glia in the cerebral wall on E14 did not differ between Tg and littermate control embryos. However, a significant (27%) increase in the proportion of Tbr2-positive intermediate progenitor cells was documented in Tg embryos on E14, and a corresponding decrease in the number of post-mitotic projection neurons was noted.

Taken together with data showing that IGF-I augments cell cycle re-entry on E14-E15, these results suggest that IGF-I promotes retention of cells in the proliferative zones of the cerebral wall as intermediate progenitor cells. The exact effect of enhanced intermediate progenitor cell number on cortical development in Tg mice remains to be determined. For example, future studies could be designed to determine the laminar fates of neurons generated by intermediate progenitor cell divisions and the exact proportion of total cortical projection neurons produced by intermediate progenitor cells. Furthermore, it would be of interest to determine if IGF-I increases the proportion of Tbr2-positive intermediate progenitor cells at other times during cortical neurogenesis, such as on E16-E17 when the thickness of the SVZ begins to exceed that of the VZ. Clearly, the finding that IGF-I increases the proportion of intermediate progenitor cells in the cerebral wall on E14 raises many interesting questions about the influence of IGF-I on the development of different cell types in the embryonic brain.

In conclusion, the results of the present set of experiments clearly support a role for IGF-I in promoting neuron progenitor proliferation in the embryonic cortex. As well, several mechanisms by which IGF-I augments progenitor proliferation in vivo have been demonstrated. These experiments are among few that have documented the ability of IGF-I to
promote progenitor proliferation in vivo, and suggest that IGF-I is an important regulator of cortical development.

6.3 General overview of the mammalian cell cycle

The mammalian cell cycle consists of four phases: G1 (gap 1), S (DNA synthesis), G2 (gap 2), and M (mitosis). Upon exit from the cell cycle, cells enter a quiescent state known as G0. During G1 phase cellular size increases and many components required for the initiation and completion of S phase are synthesized. Progression through G1 is controlled by a number of molecules, including the D-type cyclins (D1, D2, and D3). Expression of D cyclins is known to be induced by growth factors during early G1 phase (Sherr, 1996; Sherr and Roberts, 1999). Cyclin D forms complexes with two cyclin dependent kinases (CDKs), CDK4 and CDK6. Cyclins form the regulatory components of these subunits, while CDKs act as catalytic components. Cyclin D/CDK4/6 complexes translocate to the nucleus where they function to phosphorylate retinoblastoma protein (Rb). Rb acts as a negative regulator of G1 progression when in a hypophosphorylated state. In this hypophosphorylated state, Rb binds to and inactivates the transcription factor E2F, which is known to promote transcription of a number of genes required for DNA synthesis. CyclinD/CDK4/6 activity can be inhibited by members of the Ink4 family of cyclin kinase inhibitors, which includes p15, p16, p18, and p19. Later in G1 phase cyclin E expression is upregulated, and cyclin E complexes with its partner CDK2. Cyclin E/CDK2 further phosphorylates Rb, leading to hyperphosphorylation of Rb and release of E2F (see Dyson, 1998 for review). Cyclin E/CDK2 also phosphorylates p27, an inhibitory protein of the CIP/KIP family of cyclin kinase inhibitors that also includes p21 and p57. Phosphorylation of p27 relieves p27-mediated cell cycle inhibition and promotes G1 to S phase progression (reviewed in Reed, 1997; Sherr and Roberts, 1999; Ekholm and Reed, 2000).

Following activation of E2F and cyclin E/CDK2 in late G1, cells pass through the G1 restriction point and progress into S phase. When cells pass through the G1 restriction point they are no longer dependent upon growth factors to complete the cell cycle; rather, they rely on intracellular signals to execute subsequent phases of the cell cycle (Pardee, 1974, 1989). Progression through S phase is dependent upon the activity of cyclin A, which complexes with CDK2. Upon completion of DNA synthesis, cells pass into G2 phase where the components necessary for cells to undergo mitosis are produced. Progression from G2 to M phase is regulated by the activity of cyclin B in complex with CDK1. At the onset of mitosis, cyclin B translocates to the nucleus where the cyclinB/CDK1 complex is known to phosphorylate a
number of components that participate in the mitotic process. For example, cyclin B/CDK1 activity is necessary for activation of proteins responsible for construction of the bipolar spindle. Inactivation of cyclin B/CDK1 during anaphase and telophase results in cellular exit from mitosis (reviewed in Jackman and Pines, 1997; Smits and Medema, 2001).

6.4 IGF-I regulates cell cycle progression in a variety of cell types

The in vivo role of IGF-I in governing the cell cycle kinetics of proliferation during cortical neurogenesis has not been investigated previously. To determine if IGF-I influences the rate of progenitor progression through the cell cycle, the cell cycle kinetics of progenitors in the VZ of the cerebral wall were examined on E14. These findings indicate that IGF-I significantly increases progenitor cell progression rate through the G1 phase of the cell cycle, which results in an overall decrease in total cell cycle length in Tg embryos. This decrease in $T_c$ is due solely to the reduction in G1 phase, without a corresponding change in any other phase of the cell cycle.

IGF-I is known to promote mitosis in various cell types. Mounting evidence indicates that this ability to promote mitosis is associated with IGF-I-mediated regulation of the cell cycle. A number of studies have shown that IGF-I regulates progression through one or more phases of the cell cycle. Consistent with the present findings showing that IGF-I promotes G1 progression, the majority of these studies indicate that IGF-I enhances G1 to S phase progression (Chen and Rabinovitch 1989, 1990; Leof et al., 1992; Lu and Campisi, 1992; Chakravarthy et al., 2000; Dupont et al., 2000; Stull et al., 2002; Frederick and Wood, 2004; Hu et al., 2004). The ability of IGF-I to promote G1/S phase progression has been demonstrated in a number of cell types including mammary epithelial cells (Stull et al., 2002), granulosa cells of the ovarian follicle (Hu et al., 2004), fibroblasts (Chen and Rabinovitch, 1989, 1990; Lu and Campisi, 1992), myoblasts (Chakravarthy et al., 2000), and oligodendrocyte precursors (Frederick and Wood, 2004).

IGF-I has been shown to act in a synergistic or additive manner with other growth factors to effect G1/S progression in several of these cell types (Dupont et al., 2000; Stull et al., 2002; Frederick and Wood, 2004). For example, in oligodendrocyte precursors in vitro, IGF-I and FGF-2 synergistically promote G1/S progression (Frederick and Wood, 2004). In mammary epithelial cells, both EGF and IGF-I are required for control of early G1 phase events, while IGF-I alone is required for these epithelial cells to complete late G1 and progress into S phase (Stull et al., 2002). In other cell types IGF-I by itself has pronounced effects on
cell cycle progression. *In vitro* studies of satellite cells (myoblasts that give rise to skeletal muscle cells) extracted from Tg mice that overexpress IGF-I show that IGF-I both promotes G1/S phase transition and increases the number of cell divisions that satellite cells are capable of undergoing in culture (Chakravarthy et al., 2000).

While the present studies have clearly demonstrated that IGF-I promotes G1 phase progression in VZ cortical progenitors, the molecular mechanisms responsible for the IGF-I-mediated decrease in G1 phase length are not clear from these experiments. The molecular mechanisms by which IGF-I promotes G1/S phase progression have been the subject of a number of recent studies, which show that IGF-I regulates components of the cell cycle that are crucial determinants of cell cycle progression. IGF-I appears to regulate the expression of cyclin D1, an early G1 phase cyclin, in many cell types (Furlanetto et al., 1994; Rosenthal and Cheng, 1995; Duforny et al., 1997; Reiss et al., 1997; Dupont et al., 2000; Hamelers et al., 2002; Stull et al., 2002). However, this effect does not hold true for all cell types as IGF-I has no effect on cyclin D1 expression in oligodendrocyte precursors (Frederick and Wood, 2004). In some cases, IGF-I increases cyclin D1 protein levels and promotes an increase in the rate of transcription of cyclin D1 in addition to increasing cyclin D1 expression (Furlanetto et al., 1994). As well, IGF-I increases cyclin D1 accumulation in the nuclei of breast cancer cells (MCF-7 cell line) during G1 phase (Hamelers et al., 2002).

IGF-I also regulates the expression of a number of other G1 phase proteins, including cyclin D2, cyclin D3, CDK2, CDK4, cdc2, cyclin E, and p27 (Furlanetto et al., 1994; Rosenthal and Cheng, 1995; Reiss et al., 1997; Chakravarthy et al., 2000; Dupont et al., 2000; Stull et al., 2002). In general, proteins that promote G1 phase progression, such as the cyclins and CDKs, are upregulated by IGF-I (Furlanetto et al., 1994; Rosenthal and Cheng, 1995; Reiss et al., 1997; Dupont et al., 2000; Hamelers et al., 2002; Stull et al., 2002). Conversely, inhibitors of G1 phase, such as p27, are generally downregulated by IGF-I (Chakravarthy et al., 2000; Dupont et al., 2000). IGF-I has also been shown to either maintain the phosphorylation of Rb or stimulate hyperphosphorylation of Rb (Rosenthal and Cheng, 1995; Duforny et al., 1997). In its hyperphosphorylated state Rb cannot inactivate the E2F transcription factor, and cell cycle progression occurs as a result.

The signaling pathways through which IGF-I acts to influence the expression of G1 phase regulatory proteins have been determined in some cell types. In many cases, IGF-I-mediated activation of the PI3-K signaling pathway, via interaction with IGF1R, appears to be
critical in regulating cell cycle progression (Duforny et al., 1997; Chakravarthy et al., 2000; Hamelers et al., 2002; Hu et al., 2004). PI3-K activation leads to the activation of Akt. The mechanisms by which Akt acts to regulate cyclin D expression are not yet clear (Dupont et al., 2003). However, some evidence indicates that the FKHR transcription factor, a downstream target of Akt, may regulate transcription of cyclins D1 and D2 (Ramaswamy et al., 2002; Dupont et al., 2003). IGF-I is also known to activate the MAPK pathway. Evidence collected to date suggests that activation of the MAPK pathway by IGF-I pathway is not critical for regulation of G1 phase proteins in breast cancer cells (Duforny et al., 1997). However, the MAPK pathway has been associated with cyclin D regulation in studies of other cell types (Lavoie et al., 1996; Weber et al., 1997; Cheng et al., 1998). As well, Ras proteins, which act upstream of MAPK, appear to be required for IGF-I-stimulated G1 phase progression in BALB/c 3T3 fibroblasts (Lu and Campisi, 1992). Therefore, current evidence indicates that IGF-I-mediated regulation of G1 phase proteins may result from activation of either the PI3-K or MAPK signaling pathways, depending upon the cell type under study.

While much evidence indicates that IGF-I is involved in regulation of G1 phase progression, some studies have found that IGF-I has a role in regulating other phases of the cell cycle. For example, IGF-I has been shown to increase the re-entry of some types of quiescent (G0) cells into the cell cycle and promote the progression of these cells into S-phase (Stiles et al., 1979; Clemmons et al., 1980; DiCicco-Bloom and Black, 1988; Lorenzo et al., 1993; Reiss et al., 1997). IGF-I has been shown to upregulate expression of cyclin A2, an S phase cyclin, in mammary epithelial cells (Stull et al., 2002), myoblasts (Chakravarthy et al., 2000), and ventricular myocytes (Reiss et al., 1997) supporting a role for IGF-I in S phase regulation. A slight decrease (12 min) in S phase length was documented in the present studies of Tg embryos on E14, which may represent a subtle effect of IGF-I on S phase progression in cortical progenitors. However, the 1.85 hr reduction in G1 phase length documented in Tg embryos indicates a more pronounced effect of IGF-I on G1 progression during cortical neurogenesis, suggesting that IGF-I acts predominantly to regulate G1 phase in cortical neuron progenitors.

Effects of IGF-I on regulation of the G2 and M phases of the cell cycle have also been demonstrated. In IGF-I−/− mice, uterine epithelial cells stimulated with estradiol appear to be arrested in G2 phase, suggesting that IGF-I is critical for G2/M phase progression in these cells (Adesanya et al., 1999). IGF-I has also been shown to influence the expression of G2/M phase
regulatory molecules. In osteosarcoma cells, IGF-I increases the level of cyclin B1 mRNA, a protein that is known to be a critical regulator of the G2/M checkpoint (Furlanetto et al., 1994). IGF-I has also been shown to increase cyclin B1 protein levels in myocytes (Reiss et al., 1997) and mammary epithelial cells (Stull et al., 2002). However, these myocytes were not able to progress into M phase and undergo cell division in the presence of IGF-I by itself, indicating that IGF-I alone was not sufficient to promote G2/M phase progression in these cells (Reiss et al., 1997). Studies of fibroblasts lacking IGF1R support a role for IGF-I in regulating the cell cycle (Sell et al., 1994). In these cells, all phases of the cell cycle are lengthened when compared to wildtype fibroblasts (Sell et al., 1994); however, these results may be representative of loss of both IGF-I and IGF-II signaling through the IGF1R. The lengths of the G2 and M phases of the cell cycle were found to be similar to controls in nestin/IGF-I Tg mice on E14, indicating that IGF-I does not appear to regulate these phases of the cell cycle in cortical neuron progenitors. Taken together, the results of these studies suggest that IGF-I may differentially influence specific phases of the cell cycle in distinct cell types. As well, IGF-I may effect cell cycle regulation differently when combined with other growth promoting factors, such as estrogen and FGF-2.

6.5 Studies of IGF-I-mediated cell cycle regulation in the central nervous system

Relatively few studies have addressed the ability of IGF-I to promote cell cycle progression in the CNS. The capacity of IGF-I to regulate the cell cycle has been examined in vitro in several studies of neural cells. For example, IGF-I has been shown to promote entry of cultured sympathetic neuroblasts into the cell cycle, and to augment DNA synthesis in these cells (DiCicco-Bloom and Black, 1988). Much of the evidence supporting a role for IGF-I-mediated cell cycle regulation in the CNS has come from the study of oligodendrocyte progenitor cells. In vitro studies indicate that IGF-I is capable of increasing the number of oligodendrocyte progenitors that enter S phase of the cell cycle (Jiang et al., 2001). FGF-2 and IGF-I, in combination, act synergistically to recruit oligodendrocyte progenitor cells into S phase (Frederick and Wood, 2004); however, IGF-I alone does not seem to affect the rate of progression through the mitotic cycle in these cells (Jiang et al., 2001). IGF-I does promote oligodendrocyte progenitor progression through G1 phase when combined with FGF-2 (Frederick and Wood, 2004). Additionally, IGF-I appears to be required for oligodendrocyte progenitors to progress through G2/M, as cells treated with FGF-2 alone are not capable of passing through G2/M until IGF-I is added to the culture medium (Frederick and Wood, 2004).
These results suggest that IGF-I acts in conjunction with other growth factors to regulate the cell cycle in oligodendrocyte precursors.

At the molecular level, addition of IGF-I alone to cultured oligodendrocyte progenitors does not affect cyclin D1 expression. However, when combined with FGF-2, IGF-I controls several components of the cell cycle regulatory machinery in oligodendrocyte precursors (Frederick and Wood, 2004). For example, IGF-I and FGF-2 upregulate cyclin D1 expression in oligodendrocyte precursors and enhance cyclin D1 association with CDK4. IGF-I also appears to maintain the level of cyclin E protein in oligodendrocyte precursor cells, and the combination of IGF-I and FGF-2 promotes association of cyclin E with its complementary cyclin dependent kinase, CDK2. As well, IGF-I and FGF-2 act to decrease expression of p27 and augment Rb hyperphosphorylation, thereby enhancing cell cycle progression (Frederick and Wood, 2004). Taken together, the above results indicate a role for IGF-I-mediated cell cycle regulation in the CNS.

The findings of the present studies indicate that IGF-I may differentially affect the cell cycle in neuron and oligodendrocyte progenitors. Oligodendrocytes appear to require both IGF-I and FGF-2 for progression through G1 phase, and IGF-I seems to be necessary for G2/M passage in these cells. Overexpression of IGF-I alone in nestin/IGF-I Tg mice promotes progression through G1, but has no apparent effect on the G2 and M phases of the cell cycle. Given that an in vivo model has been used in the present studies, it is difficult to determine which of the many growth factors that are likely present in the developing cortex IGF-I may interact with to effect G1 progression.

To our knowledge, this study represents the first attempt to characterize the effects of IGF-I on cell cycle progression in the CNS in vivo. While the techniques used to characterize cell cycle kinetics in vivo are labour intensive, they do offer some benefits when analysing cortical neuron progenitors. These techniques allowed for the determination of the cell cycle kinetics of precursors located specifically within the VZ. Cell cycle kinetics are known to vary between the VZ and SVZ (Takahashi et al., 1995a, b), and it may be difficult to accurately separate these two populations in vitro. As well, cell cycle length is known to vary between regions of the cerebral wall, due to the rostrolateral to caudomedial gradient of cortical neurogenesis. Use of an in vivo analysis allowed for concentration on a specific region of the cerebral wall, in which cell cycle length is known to be homogenous among different cells in
the VZ (Cai et al., 1997). Therefore, the analysis used to determine cell cycle kinetics in the VZ on E14 produced an accurate estimate of cell cycle lengths in control and Tg embryos.

While the in vivo model used in these experiments allows for the study of specific cell populations and preserves the complex environment of the cells under study, it does make the molecular mechanisms by which IGF-I effects cell cycle progression more difficult to assess. The current experiments did not determine how IGF-I interacts with different cell cycle components to promote G1 progression. Relatively little is known about factors that control the progression of T\textsubscript{G1} during cortical neurogenesis, although it has been shown that the cell cycle modulators p27\textsuperscript{Kip1} and cyclin E are important regulators of T\textsubscript{G1} (Delalle et al., 1999; Mitsuhashi et al., 2001). As noted above, IGF-I, in combination with FGF-2, has been shown to decrease p27 expression and increase cyclin E association with CDK2 (Frederick and Wood, 2004). Therefore, IGF-I may interact with these proteins to increase G1 progression rate in cortical neuron progenitors, but further studies would be required to determine if this is the case. Nonetheless, IGF-I clearly is an important factor involved in controlling G1 phase progression during neurogenesis in the developing cerebral cortex.

6.6 General overview of apoptosis signaling pathways

In addition to promoting neuron progenitor proliferation during embryonic development of the cortex, IGF-I also reduces neuron death by apoptosis in the cortex during early postnatal development. These mechanisms act in combination to promote increased neuron number in the cerebral cortex of nestin/IGF-I Tg mice. The ability of IGF-I to inhibit apoptosis in the nervous system has been much more extensively documented in the literature than the capacity of IGF-I to promote neuron progenitor proliferation. Therefore, the present observations are in general agreement with a number of studies showing that IGF-I is a potent anti-apoptotic factor regulating neural cell death. The results of the present study are discussed below in relation to current evidence suggesting that IGF-I promotes neural cell survival in vitro, in vivo, and in experimental models of disease and injury.

Apoptosis signaling pathways can be separated into two general categories, intrinsic cell death pathways and extrinsic cell death pathways. Intrinsic cell death pathways are activated in response to changes in cellular homeostasis. Mitochondria play an important role in intrinsic cell death signaling. Apoptotic signaling is initiated upon release of cytochrome c from the mitochondria. Cytochrome c in the cytosol interacts with the apoptotic protease-activating factor-1 (Apaf-1) in the presence of dATP/ATP, causing a conformational change in
Apaf-1 (Li et al., 1997; Saleh et al., 1999). This conformational change allows Apaf-1 to form a complex with procaspase-9, which facilitates the cleavage of procaspase-9 to catalytically active caspase-9. Caspase-9 is a member of the caspase family of cysteine proteases, which are synthesized as inactive precursor peptides that must be proteolytically cleaved before becoming activated (reviewed in Troy and Salvesen, 2002). Caspase-9 is typically considered to be an initiator caspase because it is involved in the initiation of the apoptotic signaling cascade. Activation of caspase-9 results in downstream cleavage and activation of effector caspases, such as caspase-3 and caspase-7. Ultimately, activation of effector caspases results in cleavage of a number of intracellular targets and activation of the caspase-activated DNase, which leads to the DNA fragmentation characteristic of apoptotic cell death (reviewed in Roth and D’Sa, 2001; Troy and Salvesen, 2002; Liou et al., 2003).

The Bcl-2 family of proteins, a group of structurally related proteins known to mediate apoptosis, are thought to act upstream of caspase activation to affect apoptotic signaling pathways. Anti-apoptotic members of the Bcl-2 family (e.g. Bcl-2 and Bcl-X\textsubscript{L}) have been shown to prevent the release of cytochrome c from the mitochondria by interacting with and inactivating pro-apoptotic Bcl-2 family members, such as Bax (Green and Reed, 1998; Roth and D’Sa, 2001). One way in which pro-apoptotic Bcl-2 proteins like Bax promote apoptosis is to oligomerize and form channels in the mitochondria that allow for the release of pro-apoptotic proteins, such as cytochrome c (Antonsson et al., 2000; Saito et al., 2000).

In addition to intrinsic cell death signaling, neuronal apoptosis may also be mediated by extrinsic death pathways that function through death receptors. These death receptors typically belong to the tumour necrosis factor receptor superfamily. Fas is a well characterized member of this family that has been implicated in neuronal apoptosis during normal development (Felderhoff-Mueser et al., 2000; Raoul et al., 2000). Fas is a transmembrane receptor with an extracellular cysteine rich domain involved in ligand binding and receptor self-association (Siegel et al., 2000). The intracellular domain of Fas consists of a death domain that is involved in transmitting apoptosis signals (Tartaglia et al., 1993; Huang et al., 1996). Binding of FasL, the key ligand for Fas, to the Fas receptor results in recruitment of a cytosolic protein known as Fas-associated death domain protein (FADD) and formation of complex containing Fas, FasL, and FADD (Liou et al., 2003). Formation of this complex results in recruitment of either caspase 8 or 10, both of which are initiator caspases, to the complex, at which time the caspase is activated as a result of proteolytic cleavage. The activated initiator caspase then
cleaves and activates effector caspases as described above, leading to apoptotic cell death (Liou et al., 2003).

Fas/FasL signaling may also lead to activation of an alternate apoptosis signaling pathway that acts independently of caspases. Association of FasL with Fas has been shown to result in binding of Daxx, a Fas-binding protein, with the death domain of Fas. Upon association with the Fas death domain, Daxx activates the Jun N-terminal kinase (JNK) pathway (Yang et al., 1997). Activated JNK promotes apoptosis by phosphorylating and inactivating Bcl-2 (Yamamoto et al., 1999). JNK also mediates apoptosis by activating the c-Jun transcription factor, which, in turn, upregulates FasL expression thereby promoting FADD-dependent apoptosis (Morishima et al., 2001).

6.7 IGF-I promotes neural cell survival in vitro

Various in vitro studies indicate that IGF-I promotes the survival of both neurons and glia. IGF-I has been shown to promote the survival of cerebral cortical (Aizeman and DeVellis, 1987; Bozyczko-Coyne et al., 1993; Yamada et al., 2001), sympathetic (Russell and Feldman, 1999), hypothalamic (Torres-Aleman et al., 1990a, b; Pons et al., 1991; Sortino and Canonico, 1996), and retinal neurons (Bozyczko-Coyne et al., 1993). In cultured cortical neurons, IGF-I decreases apoptosis by reducing activation of caspase-3 (Takadera et al., 1999). As well, IGF-I inhibits apoptosis in spinal cord motoneurons (Ang et al., 1992; Hughes et al., 1993; Neff et al., 1993). Vincent and collaborators (2004) showed that IGF-I-mediated spinal cord motoneuron survival following glutamate toxicity results from activation of both the PI3-K and MAPK signalling pathways. IGF-I also promoted motoneuron survival by inhibiting caspase-3 activation (Vincent et al., 2004). However, IGF-I-mediated activation of the MAPK pathway does not appear to be involved in the prevention apoptosis in all neural cell types (Russell et al., 1998; Ryu et al., 1999). For example, in embryonic dorsal root ganglion neurons, the PI3-K pathway mediates the anti-apoptotic functions of IGF-I without contribution from MAPK signaling (Russell et al., 1998).

The anti-apoptotic functions of IGF-I on neural cells have been extensively studied using cultured cerebellar granule neurons. Various studies indicate that IGF-I prevents cerebellar granule neuron apoptosis in vitro (D’Mello et al., 1993; Galli et al., 1995; Tanaka et al., 1998; Leski et al., 2000). Several different signaling pathways appear to be involved in IGF-I-mediated cerebellar granule neuron survival. Several studies show that the anti-apoptotic functions of IGF-I in cerebellar granule neurons are mediated by activation of PI3-K
signaling (Dudek et al., 1997; Yamagashi et al., 2003). Activation of Akt by the PI3-K pathway appears to be essential for the actions of IGF-I on cerebellar granule neurons (Dudek et al., 1997; Blair et al., 1999; Zhong et al., 2004). Initiation of PI3-K signalling has been shown to suppress the activation of c-Jun pathway members in cerebellar granule neurons, thereby inhibiting apoptosis (Yamagashi et al., 2003).

The anti-apoptotic effects of IGF-I on cerebellar granule neurons may also be regulated by direct interaction of IGF-I signaling with components of the mitochondrial death pathway (Gleichmann et al., 2000; Linseman et al., 2002b). Gleichmann and coworkers (2000) showed that IGF-I protects against serum starvation-induced apoptosis by stimulating the phosphorylation of BAD, a pro-apoptotic member of the Bcl-2 family of proteins. Other studies have shown that IGF-I both blocks cytochrome c translocation from mitochondria and effects additional components of the mitochondrial death pathway. IGF-I appears to inhibit expression of Bim, a pro-apoptotic Bcl-2 family protein that regulates cytochrome c release via interaction with Bax (Linseman et al., 2002b). The effects of IGF-I on these components of the mitochondrial death pathway are dependent upon Akt activation, which results in suppression of FKHRL1 activation. FKHRL1 is a member of the forkhead family of transcription factors that has been shown to regulate Bim transcription (Linseman et al., 2002b). IGF-I also appears to affect apoptosis in cerebellar granule neurons by inhibiting activation of both caspase-9 and caspase-3 (Linseman et al., 2002b; Zhong et al., 2004), and by preventing the caspase-dependent cleavage of myocyte enhancer factor 2D, a pro-survival transcription factor (Butts et al., 2003).

Studies of cultured cerebellar granule neurons from weaver mutant mice further support a role for IGF-I in promoting cerebellar granule neuron survival (Zhong et al., 2002). Cerebellar granule neurons from weaver mice undergo apoptosis in culture, and this increased apoptosis is associated with a reduction in IGF1R phosphorylation and Akt activation. As well, IGFBP-5 expression is increased in cerebellar granule neurons derived from weaver mice, and treatment with an antibody against IGFBP-5 enhanced cerebellar granule neuron survival in culture. Treatment with IGF-I also promoted cerebellar granule neuron survival through a reduction in caspase-3 activity (Zhong et al., 2002). Evidence indicates that cerebellar granule neurons may rely on IGF-I derived from Purkinje cells for their survival (Linseman et al., 2002a). In Purkinje cells, IGF1R signaling appears to suppress death receptor signaling via interaction with FADD (Linseman et al., 2002a), suggesting that IGF-I may
directly affect Purkinje cell survival as well. Further evidence of a role for IGF-I in Purkinje cell survival comes from Purkinje cell degeneration mice in which IGF-I expression is reduced in cultured Purkinje cells undergoing apoptosis (Zhang et al., 1997).

In addition to its ability to promote neuron survival, IGF-I also prevents apoptosis in glial cells. IGF-I has been shown to promote oligodendrocyte survival following growth factor withdrawal (Barres et al., 1992), glutamate-induced toxicity (Ness et al., 2002; Ness and Wood, 2002) and tumour necrosis factor-α-mediated injury (Ye and D’Ercole, 1999). IGF-I appears to regulate glutamate toxicity-induced apoptosis via activation of the PI3-K pathway, which leads to persistent activation of Akt (Ness et al., 2002; Ness and Wood, 2002). IGF-I also regulates the survival of Schwann cells, the myelinating cells of the peripheral nervous system, through activation of PI3-K signaling (Delaney et al., 1999, 2001; Cheng et al., 2001). IGF-I-mediated PI3-K activation in Schwann cells has been shown to downregulate the activity of the c-Jun N-terminal protein kinases JNK1 and JNK2, resulting in inhibition of apoptosis (Cheng et al., 2001).

A role for IGF-I as an anti-apoptotic factor in the nervous system is further supported by studies of cultured cell lines derived from nervous system tumours. In particular, cell lines derived from neuroblastoma tumours (pediatric tumours that occur in the peripheral nervous system) have been commonly used to study the effects of IGF-I on apoptosis in a variety of experimental conditions. For example, IGF-I has been shown to regulate glucose-induced apoptosis in neuroblastoma cell lines (Leinninger et al., 2004; Russo et al., 2004). Studies have shown that IGF-I prevents glucose-induced apoptosis both by interacting with Bcl-2 (Beierle et al., 2002; Leinninger et al., 2004; Russo et al., 2004) and by decreasing caspase activation (Saeki et al., 2002; Leinninger et al., 2004). As well, IGF-I appears to prevent apoptosis in neuroblastoma cells by inhibiting glucose-mediated mitochondrial membrane depolarization (Leinninger et al., 2004). Once again, IGF-I activation of PI3-K pathway signaling is critically important for IGF-I regulation of apoptosis in these cells (Saeki et al., 2002). IGF-I acts in a similar manner to prevent hyperosmotic-induced apoptosis in neuroblastoma cells (Matthews and Feldman, 1996; Matthews et al., 1997; Van Golen and Feldman, 2000). In the SH-SY5Y neuroblastoma cell line, IGF-I has been shown to inhibit hyperosmotic-induced apoptosis by preventing caspase-3 activation and inhibiting mitochondrial membrane depolarization (Van Golen and Feldman, 2000).
Taken together, the above results suggest that IGF-I is a potent inhibitor of apoptosis in the nervous system. IGF-I appears to act via several different signaling pathways, including both the MAPK and PI3-K pathways, to regulate apoptosis in neurons, glia, and tumour cells. However, activation of the PI3-K signaling pathway and Akt seems to be particularly important for controlling IGF-I’s anti-apoptotic actions. The studies presented in this thesis did not address the molecular mechanisms by which IGF-I promotes neuron survival in the cerebral cortex; rather, they were conducted to assess the contribution of IGF-I-mediated inhibition of neuron death to the documented increase in cortical neuron number in Tg mice. Current evidence indicates that IGF-I interacts with many different regulators of apoptosis including the caspases, the Bcl-2 family of proteins, and the JNKs. As well, IGF-I may affect cytochrome c translocation from the mitochondria into the cytosol, and IGF-I appears to regulate mitochondrial membrane potential. The effects of IGF-I on these regulators of apoptosis vary with cell type and may also be affected by the mechanisms used to induce apoptosis in the cells under study. Therefore, it is difficult to speculate about the apoptosis pathways effected by IGF-I overexpression during cortical development in nestin/IGF-I Tg mice. Clearly, more studies would be required to precisely determine the molecular pathways responsible for the observed reduction in neuron apoptosis in the present studies.

6.8 IGF-I prevents apoptosis in mutant mouse models of IGF-I function

In vivo studies of the effects of IGF-I on CNS development support a role for IGF-I as an anti-apoptotic factor in the nervous system. For example, IGF-I prevents optic nerve axon elimination when administered to neonatal rats and may inhibit retinal ganglion neuron death in these animals (Gutierrez-Ospina et al., 2002). However, much of the in vivo evidence demonstrating anti-apoptotic actions of IGF-I in the brain comes from studies of IGF-I Tg mice. In IGF-II/IGF-I Tg mice, high transgene expression is apparent in the cerebellum, and the number of granule cells in this region is greatly increased (82%) as a result (Ye et al., 1996). This increase in cerebellar granule neurons has been shown to result from a slight increase in proliferation (Ye et al., 1996), as discussed in a previous section, and a greater increase in neuron survival (Chrysis et al., 2001). The number of Purkinje cells, which are generated before the onset of transgene expression, is also increased in IGF-II/I Tg mice likely as a result of enhanced survival (Ye et al., 1996).

The ability of IGF-I to prevent cerebellar granule neuron apoptosis in vivo was illustrated by counts of TUNEL-positive cells during early postnatal development (Chrysis et
al., 2001). At P7 the number of TUNEL-positive cells per mm² was significantly reduced in IGF-II/IGF-I Tg mice, with the majority of apoptotic cells localized to the internal and external granule cell layers of the cerebellum. This decrease in apoptotic cells was associated with reduced levels of procaspase-3 and caspase-3 proteins, as well as decreased PARP expression in IGF-II/IGF-I Tg mice. PARP is a caspase substrate that is cleaved during the apoptotic process (Chrysis et al., 2001). Additionally, IGF-I overexpression resulted in increased expression of the anti-apoptotic proteins Bcl-X₅ and Bcl-2 and decreased expression of the pro-apoptotic proteins Bad and Bax (Chrysis et al., 2001). Increased Bcl-X₅ protein levels and enhanced Bcl-2 immunoreactivity have also been demonstrated in these mice (Baker et al., 1999; Chrysis et al., 2001). These results suggest that IGF-I acts to regulate neuron survival in vivo through interactions with multiple apoptosis effector proteins.

In vivo studies of IGF-I⁻¹ mice further support a role for IGF-I in regulating apoptosis in the developing rodent brain. Development of the inner ear is delayed in IGF-I⁻¹ mice and the number of auditory neurons in the inner ear is reduced (Camarero et al., 2001). This reduction in neuron number results from increased neuron death by apoptosis, as judged by counts of both TUNEL and activated caspase-3 positive cells (Camarero et al., 2001). Increased apoptosis is also evident in IGFBP-1 Tg mice (Ni et al., 1997). In these mice, the number of TUNEL positive cells is increased by 55% in the hippocampus (Ni et al., 1997). These results suggest that apoptosis is increased in the absence of IGF-I and confirm that IGF-I regulates cellular survival in the developing nervous system.

The findings presented in this thesis are in general agreement with the above studies showing that IGF-I regulates neuron apoptosis in the developing CNS. Thus, the current analyses are among few that have directly documented the anti-apoptotic actions of IGF-I in vivo. While these experiments focused on the anti-apoptotic actions of IGF-I during cortical development, it is likely that IGF-I overexpression in nestin/IGF-I Tg mice reduces neuron apoptosis in many other brain regions. Consistent with this idea, experiments from our lab have shown that the Nv of apoptotic neurons in the caudate-putamen is reduced in Tg mice at P5 (J.R. O’Kusky, personal communication). Similar to the in vitro experiments discussed above, IGF-I appears to interact with multiple apoptosis effector proteins in vivo. Therefore, further studies would be required to determine the effector proteins acting in response to elevated IGF-I expression in nestin/IGF-I Tg mice.
6.9 Future directions

The present set of studies has examined the in vivo role of IGF-I in regulating neuron progenitor proliferation during cortical neurogenesis and established that IGF-I promotes neuron survival during early postnatal development of the cortex. These studies have raised several questions about the effects of IGF-I on cerebral cortex development that may warrant further analysis. The experiments discussed in this report have focused on the effects of IGF-I on the generation of cortical projection neurons produced from the VZ and SVZ of the dorsal telencephalon. However, the effects of IGF-I on cortical interneurons, which are produced by progenitors in the ventral telencephalon and subsequently migrate into the developing cerebral wall, have not been directly addressed (Anderson et al., 1997, 1999, 2002). Interestingly, the volume of the ventrolateral telencephalic wall is not increased by IGF-I overexpression at E16, whereas the volume of the dorsolateral telencephalic wall is significantly augmented (Popken et al., 2004). Therefore, IGF-I may be expected to differentially augment the production of interneurons and projection neurons, respectively, from these regions of the telencephalon. Studies using molecular markers for interneurons, such as Dlx and Lhx6 (Anderson et al., 1997), may be useful in determining the specific effects of IGF-I on interneuron production. It would also be of interest to determine which classes of projection neurons are affected by elevated IGF-I expression. The molecular characteristics of cortical projection neurons are currently under study, and these studies support the existence of various subpopulations of cortical projection neurons (Hevner et al., 2003). Determining the effects of IGF-I on different classes of projection neurons would lead to a better understanding of the functional significance of increased cortical neuron number that has been documented in Tg mice.

The cell cycle kinetics analysis conducted at E14 revealed that IGF-I significantly increases neuron progenitor progression rate through the cell cycle. However, it is unclear at the present time if this represents a unique effect of IGF-I on cortical projection neuron progenitors or if IGF-I generally promotes cell cycle progression in many different types of CNS progenitor cells. The present studies have shown that IGF-I regulates cell cycle progression in VZ cells of the cerebral wall, which include radial glia. However, the types of cells generated by radial glia differ between regions of the developing brain (Malatesta et al., 2003). In the cortex, these cells have been shown to generate neurons (Miyata et al., 2001; Noctor et al., 2001, 2002; Malatesta et al., 2003; Englund et al., 2005), while in the ventral telencephalon radial glia have been shown to give rise to glial cells rather than neurons.
(Malatesta et al., 2003). It would be interesting to determine if IGF-I promotes cell cycle progression in the gliogenic radial glial progenitors of the ventral telencephalon as it does in the neurogenic radial glia of the cerebral wall. Analyses of this type would be useful in determining the effects of IGF-I on specific progenitor populations and would provide insight into the actions of IGF-I during neurogenesis of regions other than the cortex.

The finding that IGF-I promotes cell cycle re-entry in the dorsal telencephalon during cortical neurogenesis indicates that IGF-I may act to retain cells in a progenitor cell state. Additionally, evidence has been presented showing that IGF-I enhances the proportion of Tbr2 positive intermediate progenitor cells in the cerebral wall of Tg embryos. These findings suggest that IGF-I has a role in regulating cortical progenitor cell fate determination. However, it is unclear if IGF-I promotes the retention of other types of CNS progenitor cells in a proliferative state. In fact, some studies suggest that IGF-I promotes the neuronal differentiation of progeny derived from mouse striatal progenitors without affecting progenitor proliferation or survival (Arsenijevic and Weiss, 1998). As well, IGF-I appears to increase neuron production from stem cells derived from the forebrain of adult mice rather than increasing the number of stem cells themselves (Brooker et al., 2000). Therefore, IGF-I may differentially regulate progenitor cell proliferation and differentiation in distinct progenitor cell populations and at different times during development. Further studies would be required to determine the potential of IGF-I in controlling progenitor fate determination in diverse regions of the developing CNS. Knowledge of the effects of IGF-I on different progenitor types would be helpful in assessing the potential of IGF-I to generate specific classes of neurons. This may prove useful in the treatment of neurodegenerative disorders characterized by loss of specific neuronal populations.

The finding that IGF-I promotes cell cycle progression and progenitor cell re-entry in to the cell cycle during embryonic cortical neurogenesis raises many interesting questions about the actions of IGF-I during normal adult neurogenesis. Neurogenesis in the adult rodent brain is normally restricted to two proliferative zones, the SVZ of the lateral ventricle and the subgranular zone of the hippocampal dentate gyrus (reviewed in Alvarez-Buylla and Lim, 2004; Kempermann et al., 2004). Precursor cells located within these zones have astrocytic characteristics and give rise to immature neurons that develop electrical activity and are capable of forming synaptic contacts (Markakis and Gage, 1999; Seri et al., 2001; van Praag et al., 2002; Carleton et al., 2003; Kempermann et al., 2003). SVZ progenitors produce neurons
destined for the olfactory bulb, while subgranular zone progenitors give rise to granule cells of
the dentate gyrus (Alvarez-Buylla and Lim, 2004). Some evidence suggests that IGF-I
promotes neurogenesis in the adult rodent dentate gyrus, as peripheral infusion of IGF-I
appears to enhance progenitor proliferation in this region (Aberg et al., 2000; Trejo et al.,
2001). The actions of IGF-I on neurogenesis in the adult dentate gyrus appear to be dependent
upon interaction of IGF-I with estradiol (Perez-Martin et al., 2003).

While these studies suggest that IGF-I promotes progenitor proliferation, it is still
unclear by which mechanisms IGF-I acts to augment proliferation. Does increased progenitor
proliferation in the dentate gyrus reflect an increase in cell cycle progression rate or an
enhancement in the recruitment of additional progenitor cells into the proliferative cycle?
What are the molecular characteristics of the progenitor cells affected by IGF-I treatment, and
what types of neurons are they producing in response to elevated IGF-I levels? Additionally,
the effect of IGF-I on the survival of newly generated neurons has not been assessed. Given
that IGF-I is a potent inhibitor of neuronal apoptosis, as discussed above, it may be expected
that IGF-I will promote the survival of neurons produced during adult neurogenesis. It has
been shown that newly generated neurons persist for extended periods in both the olfactory
bulb (Winner et al., 2002) and dentate gyrus (Kempermann et al., 2003), and this persistence of
new neurons may be necessary in order for the cells to integrate into and function within
existing systems. Therefore, IGF-I may influence neurogenesis in the adult brain both by
promoting the generation of new neurons and by enhancing neuronal survival, each of which
may have significant functional consequences. Given, as well, that IGF-I promotes
synaptogenesis in IGF-I Tg mice during postnatal development (O’Kusky et al., 2000, 2003)
and that newly generated neurons likely must integrate into synaptic networks in order to
function, it would be interesting to determine the effects of IGF-I on the synaptic integration of
newly generated neurons.

Determining the answers to the above questions would provide greater insight into the
functional importance of IGF-I’s actions during adult neurogenesis in the dentate gyrus.
Neurogenesis in the adult dentate gyrus has been associated with several different functions
including hippocampus-dependent learning and memory formation (Kempermann et al., 2004).
However, studies of the functional importance of adult neurogenesis are in the early stages and
many studies have documented conflicting findings on the capacity of newly generated
neurons to functionally integrate into existing systems (reviewed in Kempermann et al., 2004).
The functional significance of neurogenesis in the adult mammalian cerebral cortex is even less defined than that in the dentate gyrus. The occurrence of neurogenesis in the adult primate cerebral cortex has been reported in some studies (Gould et al., 1999). However, others have not been able to replicate this finding (Kornack and Rakic, 2001), indicating that further studies are necessary before the normal physiological role of neurogenesis in the mammalian cortex can be defined. Therefore, the involvement of IGF-I in this process is unclear at the present time.

The behavioural and functional consequences of the IGF-I-mediated increases in neuron number documented in various brain regions of nestin/IGF-I Tg mice have yet to be determined. Studies of other lines of IGF-I Tg mice suggested that these mice performed no better and no worse than controls in the Morris water maze test, a hippocampus-dependent learning task (J.R. O’Kusky, personal communication). Thus, Morris water maze performance does not appear to be altered in IGF-I Tg mice even though total neuron number is greatly augmented, for example, in the hippocampal dentate gyrus (29-61%) of IGF-I Tg mice (O’Kusky et al., 2000). However, IGF-I Tg mice may be expected to perform better in such a task following a clinical challenge. For example, following a brain injury or lesion, elevated IGF-I expression in Tg mice may protect neurons from apoptotic cell death. Alternatively, as IGF-I Tg mice already have greater neuron numbers in the dentate gyrus, they may be able to lose more neurons following injury before the effects of this loss result in behavioural changes. Some evidence for an effect of IGF-I on performance in behavioural testing exists in the literature. For example, IGF-I administration has been shown to improve Morris water maze performance in diabetic rats, suggesting that IGF-I may be useful for ameliorating the effects of certain diseases on cognitive function (Lupien et al., 2003). Further studies would be required in order to fully understand the consequences of enhanced neuron number in nestin/IGF-I Tg mice at a behavioural and systems level of function.

Knowledge of the role of IGF-I in regulating normal neurogenesis in adult mammals may be of therapeutic interest in the treatment of a number of CNS diseases. There is currently much interest in exploiting the potential of endogenous stem cells in the adult brain to replace neurons lost in different CNS disorders (reviewed in Lie et al., 2004). Given that the functional consequences of neurogenesis in the normal adult brain are not well understood, this research is in very early stages of development. However, studies have demonstrated that neurogenesis does occur in the SVZ and subgranular zone of the dentate gyrus following
hypoxic-ischemic brain injury (Jin et al., 2001; Kee et al., 2001) and seizure (Parent et al., 2002), suggesting that new neurons may be generated in response to injury. The pro-mitotic effects of IGF-I on neurons that our lab and others have demonstrated suggest that IGF-I may be useful in enhancing neurogenesis following injuries such as these. In fact, IGF-I has been shown to be upregulated in reactive microglia and astrocytes following hypoxic-ischemic insults (Beilharz et al., 1998), suggesting a possible involvement of IGF-I in the recovery process. However, it remains to be seen if increased neurogenesis following brain injury directly results in enhanced recovery (Lie et al., 2004). Nonetheless, the characteristics of IGF-I certainly make it a candidate for use in regulating neurogenesis in the injured adult brain.

Given that the functions and potential uses of endogenous stem cells in the adult brain are uncertain at the present time, a number of studies have focused on the feasibility of using a variety of other cell types to replace neurons lost as a consequence of disease processes. These cell types include human embryonic stem cells, fetal neural precursors, and differentiated neurons. Cell transplantation has been studied extensively in models of Parkinson’s disease, a progressive neurological disease characterized by loss of dopamine-releasing nigrostriatal neurons (reviewed in Sayles et al., 2004). One such example of these studies is the transplantation of embryonic nigral neurons into the affected regions of rat brains, which resulted in some integration of the transplanted cells into the host brains and limited functional recovery (Dunnett et al., 1981, 1983). However, very few of these cells survived after transplantation (Sayles et al., 2004). Therefore, the neuron survival promoting actions of IGF-I could be exploited in these types of models to increase the number of cells persisting in the brain following transplantation. The pro-mitotic actions of IGF-I may also be useful in cell transplant experiments. For example, the ability of IGF-I to promote neuronal differentiation from certain progenitor cells (Arsenijevic and Weiss, 1998; Brooker et al., 2000) may be useful in directing the production of specific types of neurons from stem cells prior to transplantation, and may also prove useful in increasing the proportion of neurons produced from stem cells. Alternatively, the pro-mitotic effects of IGF-I on neuron progenitors may be used to expand cell populations in vitro prior to transplantation. Clearly, more studies are required to determine the potential uses of IGF-I in cell transplantation therapies.

The ability of IGF-I to promote both neuronal and glial survival during normal development suggests that IGF-I may be useful in the treatment of many different CNS injuries and diseases that are characterized by apoptotic cell death. Various studies have focused on
the role of IGF-I in promoting cellular survival following experimental brain injury. For example, a significant number of studies indicate that IGF-I effectively reduces apoptosis following hypoxic-ischemic brain injury (Guan et al., 1996, 2000a, 2001; Johnston et al., 1996; Tagami et al., 1997; Wang et al., 2000; Liu et al., 2001a, 2001b; Mackay et al., 2003). In fact, treatment with only the N-terminal tripeptide of IGF-I appears to prevent neuronal apoptosis following hypoxic-ischemic brain injury (Guan et al., 2004). IGF-I and associated IGF binding proteins are upregulated in the brain following hypoxic-ischemic injury, suggesting that endogenous production of IGF system components may function to prevent neuronal loss in the injured brain (Gluckman et al., 1992; Beilharz et al., 1998; O'Donnell et al., 2002; Hwang et al., 2004). Similarly, IGF-I prevents apoptotic motor neuron death following spinal cord (Nakao et al., 2001) and retinal (Seigel et al., 2000) ischemia. In the injured spinal cord the proportion of TUNEL positive motor neurons is decreased in injury sites treated with IGF-I (Nakao et al., 2001). The anti-apoptotic effects of IGF-I following spinal cord ischemia appear to result from IGF-I-mediated upregulation of the anti-apoptotic protein Bcl-X\textsubscript{L} and downregulation of the pro-apoptotic protein Bax, indicating that IGF-I may act to preserve intracellular homeostasis following hypoxic-ischemic insult (Nakao et al., 2001).

Neuroprotective effects of IGF-I have also been demonstrated using several other models of experimental brain injury. For example, treatment with IGF-I reduces apoptosis following a penetrating stab injury to the brain (Kazanis et al., 2003). Similar to studies of hypoxic-ischemic brain insult, expression of IGF system proteins is upregulated in models of penetrating brain injury (Garcia-Estrada et al., 1992; Walter et al., 1997; Kazanis et al., 2004). As well, IGF-I treatment inhibits apoptosis following olfactory axotomy, as judged by a decrease in the number of TUNEL positive cells following administration of IGF-I (Mathonnet et al., 2001). IGF-I has also been shown to inhibit neuron apoptosis in models of olivocerebellar pathway injury (Fernandez et al., 1999). In some cases, combining IGF-I with other factors, such as glycosaminoglycans (Vergani et al., 1999; Di Giulio et al., 2000) and estradiol (Azcoitia et al., 1999), enhances neuroprotection following experimental injury.

In addition to its neuroprotective effects, IGF-I appears to be effective at promoting glia survival in experimental disease models. Acute demyelination is associated with apoptotic death of mature oligodendrocytes and a subsequent increase in IGF-I expression (Mason et al., 2000a). Elevated expression of IGF-I in the brains of MT-I/IGF-I Tg mice has been shown to prevent mature oligodendrocyte apoptosis and promote remyelination (Mason et al., 2000b).
Furthermore, IGF-I and IGFBP-2 are upregulated during demyelination in experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis (Liu et al., 1994; Yao et al., 1995). Subcutaneous application of IGF-I also appears to reduce lesion severity in this animal model (Yao et al., 1996), suggesting that IGF-I may be useful in the treatment of demyelinating disorders.

Taken together, the above studies suggest that IGF-I is capable of preventing both neuronal and glial apoptosis in models of experimental injury and disease. Therefore, current evidence indicates that IGF-I both promotes neuron proliferation and inhibits apoptosis during normal development of the CNS and in models of CNS disease and injury. These findings raise the possibility of using IGF-I as a therapeutic agent in the treatment of a number of CNS diseases, including neurodegenerative diseases that are characterized by the death of specific neuronal populations. In accordance with this idea, IGF-I treatment has been shown to prevent neuron loss by apoptosis in animal models of Parkinson’s, Huntington’s, and Alzheimer’s disease (Dore et al., 1997; Alexi et al., 1999; Guan et al., 2000b; Offen et al., 2001). In Huntington’s disease, the neuroprotective effects of IGF-I appear to be mediated by activation of Akt, which then phosphorylates the huntingtin protein (Humbert et al., 2002).

Neuroprotective actions of IGF-I have also been demonstrated in studies of amyotrophic lateral sclerosis, a progressive disorder characterized by motor neuron death. Retrograde viral delivery of IGF-I to motor neurons has been shown to effectively prevent motor neuron death and delay disease progression in mutant mouse models of amyotrophic lateral sclerosis (Kaspar et al., 2003). However, clinical trials in which IGF-I was delivered subcutaneously demonstrated little effect of IGF-I treatment on disease outcome (Lai et al., 1997; Borasio et al., 1998). Nonetheless, these studies suggest that further research into the therapeutic applications of IGF-I in the treatment of neurodegenerative disorders is warranted.

In addition to the potential clinical usefulness of IGF-I in treating neurodegenerative diseases, some studies suggest that IGF-I signaling may be linked to the pathogenesis of certain neurodegenerative disorders. IGF-I appears to be particularly important in the pathogenesis of Alzheimer’s disease (reviewed in Carro and Torres-Aleman, 2004). It has been shown that IGF-I stimulates β-amyloid clearance from the brain, suggesting that disrupted IGF-I function may be involved in the formation of amyloid plaques (Carro et al., 2002). As well, IGF-I has been shown to regulate the phosphorylation of tau protein, which is a component of the neurofibrillary tangles characteristic of Alzheimer’s disease. IGF-I appears to regulate tau
phosphorylation through inhibition of tau hyperphosphorylation by glycogen synthase kinase 3β, thereby preventing neurofibrillary tangle production (Carro and Torres-Aleman, 2004). These studies suggest that disrupted IGF-I signaling may be a contributing factor in the development of Alzheimer’s disease and clearly indicate a need for further research on the association of IGF-I signaling with the pathogenesis of Alzheimer’s disease.

As discussed above, the ability of IGF-I to promote neurogenesis and prevent neuron death within the context of brain injury and disease clearly requires further research. However, several potential problems may arise when attempting to use IGF-I as a therapeutic agent. One such issue is the efficacy of systemic IGF-I administration in treating CNS disorders. In order to reach the affected area, sufficient amounts of IGF-I must cross the blood brain barrier and travel into the brain parenchyma. It has been established that IGF-I crosses the blood brain barrier, as discussed in Chapter 1 (Frank et al., 1986; Rosenfeld et al., 1987; Duffy et al., 1988; Reinhardt and Bondy, 1994, Pan and Kastin, 2000, Pulford and Ishii, 2001). As well, peripheral IGF-I administration has been sufficient to promote neurogenesis in the dentate gyrus (Aberg et al., 2000), inhibit cognitive decline in diabetic rats (Lupien et al., 2003), and improve neurological functioning after brain injury (Hatton et al., 1997; Saatman et al., 1997; Fernandez et al., 1998; Pulford et al., 1999). However, it remains to be seen if systemic IGF-I administration significantly promotes neuron survival following insults such as stroke where rapid neuron death is a characteristic feature.

Alternative routes of IGF-I administration have been demonstrated and may offer more efficient delivery of IGF-I to affected brain regions. Intraventricular infusions of IGF-I have proved successful in reducing infarct volume following cerebral artery occlusion (Guan et al., 2000, 2001; Mackay et al., 2003). However, this technique is invasive and requires surgical placement of a cannula, making rapid delivery of IGF-I difficult to achieve. Consequently, intraventricular cannulation likely has limited applicability in a clinical setting. Recently, intranasal administration of IGF-I has been used to deliver IGF-I to the brain following experimental cerebral artery occlusion (Liu et al., 2001a, b). Intranasal administration was shown to significantly reduce infarct volume by 60% (Liu et al., 2001b), indicating a notable effect of IGF-I on the injured brain region. The direct effects of IGF-I on neuron survival following injury were not assessed in this study and further analyses would be required in order to determine the capacity of intranasally administered IGF-I to reach the injury site and rescue
cells from apoptotic death. Nonetheless, intranasal administration represents a viable and clinically feasible alternative to systemic and intraventricular routes of administration.

The biological availability of therapeutically administered IGF-I represents another area of concern that must be addressed in order for IGF-I to be effectively applied in a clinical setting. The majority of circulating IGF-I exists in complexes with IGFBPs, suggesting that the biological availability of systemically administered IGF-I may be limited. IGFBPs are also expressed in the brain (see Chapter 1), and are upregulated in the brain following hypoxic-ischemic injury (Gluckman et al., 1992; Guan et al., 1996; Beilharz et al., 1998). Little information is available regarding the normal functions of IGFBPs in the brain, but it has been suggested that they may either modulate or inhibit the functions of IGF-I (reviewed in Jones and Clemmons, 1995). Some studies have shown that IGFBPs modulate the effects of IGF-I on neuronal loss following hypoxic-ischemic injury (Guan et al., 1996). These analyses indicate that des (1-3)-N-IGF-I, an IGF-I analogue that does not bind effectively to IGFBPs, is ineffective at preventing neuronal loss following carotid artery ligation. IGF-I was found to effectively reduce neuronal loss in this model, suggesting that the ability of IGF-I to promote neuron survival is enhanced by the ability of IGF-I to interact with IGFBPs (Guan et al., 1996). However, a similar study showed that administration of high affinity IGFBP ligand inhibitors, which displace IGF-I from IGFBPs, effectively reduced infarct volume in in vitro and in vivo models of hypoxic-ischemic brain injury (Mackay et al., 2003). These results suggest that the bioavailability of IGF-I may be limited by IGFBPs and indicate that IGFBPs may interfere with the therapeutic application of IGF-I. Clearly, the in vivo roles of the different IGFBPs during normal development and in the context of disease processes must be better defined in order to determine if IGFBPs represent a significant obstacle to the clinical use of IGF-I.

The effective use of IGF-I in the treatment of CNS diseases requires that the actions of IGF-I are specifically targeted to affected brain regions. General administration of IGF-I in the brain may have unwanted side-effects related to the pro-mitotic and anti-apoptotic functions of IGF-I. IGF-I, like many growth promoting factors, has been linked to cancer progression in a number of cell types (reviewed in Pollak, 2000; LeRoith and Roberts, 2003). IGF-I also appears to play in role in the formation of different types of brain tumours, including gliomas and meningiomas (reviewed in Glick et al., 1997). IGF-I has been shown to stimulate the proliferation of primary cultures of glioma cells derived from tumour biopsies and promote the survival of these cells in vitro (Glick et al., 1989). As well, inhibition of IGF1R function using
antisense RNA has been shown to reduce the proliferation of C6 glioma cells in culture (Resnicoff et al., 1994). Therefore, the use of IGF-I in treating neurodegenerative diseases and brain injuries could potentially exacerbate cancer progression. However, we have not detected any evidence of CNS tumours or an increase in the incidence of tumour formation in the several hundred adult IGF-I Tg mice used in our studies over the years (A.J. D’Ercole, J.R. O’Kusky, personal communications). It is likely that multiple genetic mutations are necessary for the development of CNS tumours and that IGF-I overexpression alone cannot induce tumour formation in the CNS. Nonetheless, treatment with IGF-I may promote the progression of existing cancers and this must be taken into account when testing the feasibility of using IGF-I as a treatment for CNS diseases.

In addition to the link between IGF-I and cancer progression, treatment with IGF-I could potentially cause several other unwanted side-effects in the brain. Reactive gliosis is a feature of a number of traumatic brain injuries and can result in the formation of glial scars that inhibit CNS regeneration and repair (reviewed in Streit et al., 1999; McGraw et al., 2001). Both astrocytes and microglia appear to contribute to glial scarring. The effects of IGF-I on glial scar formation are uncertain. However, IGF-I has been shown to promote increases in glial cell numbers in various IGF-I Tg mouse models, likely resulting, at least in part, from an IGF-I-mediated increase in glial proliferation (Ye et al., 1995a, 2004; Popken et al., 2004). As well, IGF-I has been shown to promote microglia proliferation following hypoxic-ischemic brain injury (O’Donnell et al., 2002). These results suggest that IGF-I could potentially exacerbate glial scar formation following brain injury. However, some studies indicate that treatment with IGF-I significantly inhibits reactive gliosis following neurotoxic injury (Fernandez et al., 1997), and treatment with the N-terminal tripeptide of IGF-I has been shown to inhibit microglia proliferation (Guan et al., 2004). Therefore, the role of IGF-I in glial scar formation needs to be further defined in order to assess the side-effects that may occur during therapeutic treatment of brain injuries with IGF-I.

In summary, the studies presented in this thesis have shown that IGF-I promotes progenitor cell proliferation during cortical neurogenesis and decreases neuron death by apoptosis during early postnatal development of the cerebral cortex in vivo. These findings strongly support an important role for IGF-I in regulating the development of the cortex and have illustrated several mechanisms by which IGF-I acts to influence cortical development. The pro-mitotic and anti-apoptotic functions of IGF-I documented during normal development
in vivo raise many questions about the potential therapeutic uses of IGF-I in the treatment of CNS diseases and injuries characterized by neuron loss. IGF-I may be used to promote the proliferation of endogenous neuron progenitor cells in the adult brain, which may provide a mechanism by which neurons lost during disease progression could be replaced. Alternatively, the anti-apoptotic functions of IGF-I could be exploited to prevent neuron loss in neurodegenerative diseases and traumatic brain injuries. However, a number of issues must be addressed before IGF-I can be realistically applied in a clinical setting. These factors include the biological availability of IGF-I, routes of administration to the brain and the link between IGF-I and cancer progression. Nonetheless, the therapeutic applications of IGF-I in the treatment of CNS diseases certainly warrant further research.
6.10 Bibliography


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