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**PROLIFERATION, DIFFERENTIATION, AND MIGRATION OF ENDOGENOUS
ADULT NEURAL STEM CELLS AND THEIR PROGENY *IN VIVO***

by Jason G. Emsley

**Submitted in partial fulfilment of the requirements for the degree of Doctor of
Philosophy in Anatomy and Neurobiology/ Neuroscience
at
Dalhousie University
Halifax, Nova Scotia**

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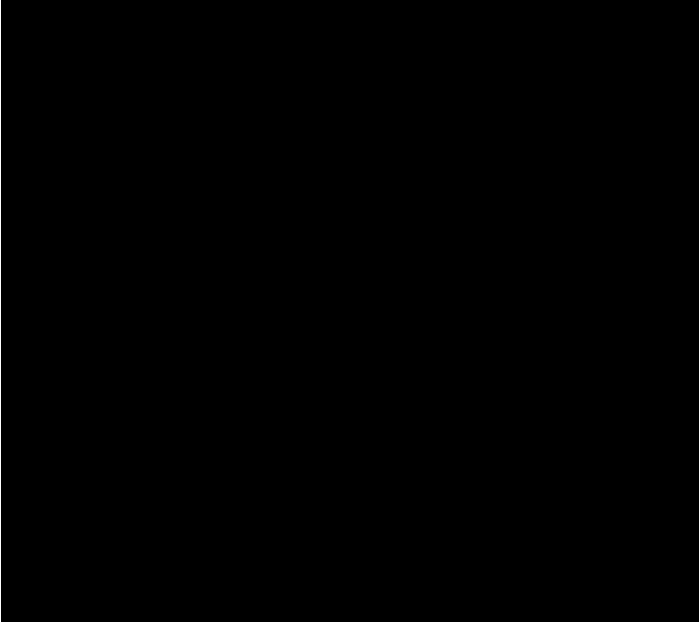
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Mater, requiescat en pace

TABLE OF CONTENTS

SIGNATURE PAGE	ii
COPYRIGHT AGREEMENT FORM	iii
DEDICATION PAGE	iv
LIST OF FIGURES	viii
ABSTRACT	ix
LIST OF ABBREVIATIONS AND SYMBOLS	x
ACKNOWLEDGEMENTS	xii
CHAPTER 1: GENERAL INTRODUCTION	1
Overview of central nervous system development	4
Discovery and isolation of mammalian neural stem cells	5
The definition of a neural stem cell	6
Overview of the major neurogenic regions	8
Proliferation of neural stem cells and their progeny	15
Factors influencing the differentiation of neural stem cells	17
Migration of neural stem cells and their progeny	19
The structure and function of ciliary neurotrophic factor	26
The structure and function of the laminins	30
The structure and function of the integrins	34
Overview of Parkinson's disease	38
The rodent nigrostriatal system as a model for Parkinson's disease	39
Rationale	41
Objectives of the thesis	43
CHAPTER 2: ENDOGENOUS CILIARY NEUROTROPHIC FACTOR ENHANCES FOREBRAIN NEUROGENESIS IN ADULT MICE	45
ABSTRACT	46
INTRODUCTION	47
MATERIALS AND METHODS	49
Animals and housing	49
Administration of CNTF and antibodies to CNTF	49
5-Bromo-2'-deoxyuridine (BrdU) administration	50
Histology and cell counts	51
RESULTS	54
CNTF enhances cell formation in the adult mouse subventricular zone	54
CNTF enhances cell formation in the dentate gyrus	59
CNTF predominantly promotes neuronal formation	63
CNTF accelerates normal migration of neurons in the dentate gyrus	71
CNTF receptor α is present in and around neurogenic regions	74
DISCUSSION	77
CNTF may enhance neural precursor proliferation and/or survival	77
CNTF enhances neuronal differentiation <i>in vivo</i>	80

CNTF may induce adult neurogenesis indirectly through activation of astroglia	82
Endogenous CNTF may mediate effects of other inducers of neurogenesis	84
Conclusions	85
CHAPTER 3: $\alpha 6\beta 1$ INTEGRIN DIRECTS MIGRATION OF NEURONAL PRECURSORS IN ADULT MICE	86
ABSTRACT	87
INTRODUCTION	88
MATERIALS AND METHODS	90
Animals and housing	90
Delivery of neutralizing antibodies against integrin subunits	90
Production and administration of a laminin peptide	91
Laminin tract injection	92
5-Bromo-2'-deoxyuridine (BrdU) administration	92
Histology and analysis	93
RESULTS	95
The $\alpha 6\beta 1$ integrin is expressed in neuroblasts of the rostral migratory stream	95
Laminin is expressed at low levels along the rostral migratory stream	95
Blockade of endogenous integrins disrupts the rostral migratory stream	98
Infusion of a peptide recognized by integrin $\alpha 6\beta 1$ redirects neuroblasts into the striatum	98
A tract of laminin is sufficient to divert cells from the rostral migratory stream	109
DISCUSSION	113
The $\alpha 6\beta 1$ integrin and laminin are associated with the rostral migratory stream	113
A peptide recognized by the $\alpha 6\beta 1$ integrin specifically draws neuroblasts into the neostriatum	115
The number of diverted neuroblasts, and their rate of travel, remains less than that in the rostral migratory stream	116
Laminin upregulation in CNS injury	118
Conclusions	118
CHAPTER 4: RETROGRADE TRACING TECHNIQUES INFLUENCE REPORTED DEATH RATES OF ADULT RAT NIGROSTRIATAL NEURONS	120
ABSTRACT	121
INTRODUCTION	122
MATERIALS AND METHODS	125
Retrograde labeling with Dil or Fluorogold	125

6-OHDA injection and axotomy	126
Histology and neuronal counts	127
RESULTS	129
More injection sites label more neurons in "non-injured" rats	129
Cell death appears to vary with retrograde tracing method and number of injection sites	135
DISCUSSION	143
Increased labeling with more neostriatal labeling sites suggests complex projection patterns	143
Neuronal death appears to be increased within comparable regions with 6 labeling sites	144
Neuronal death after 6-OHDA or axotomy is similar with Dil and Fluorogold labeling	145
Conclusions	146
CHAPTER 5: GENERAL DISCUSSION	147
Summary of results	148
Suggestions for future work based on Chapter 2	151
Suggestions for future work based on Chapter 3	153
Suggestions for future work based on Chapter 4	155
Future directions in neural stem cell research (suggestions, speculations, and cautions)	156
Neural stem cell behaviour (proliferation, differentiation, and migration)	156
The function of neural stem cells	158
The corollary of neural stem cells (cautions and speculations)	160
BIBLIOGRAPHY	162

LIST OF FIGURES

Figure 1.1	Schematic illustration of the cellular composition and architecture of the adult mammalian SVZ	11
Figure 1.2	Neurogenesis in the adult dentate gyrus	14
Figure 1.3	Neuroblasts migrate from the SVZ to the olfactory bulb	22
Figure 1.4	CNTF signaling pathway	28
Figure 1.5	The structure of laminin	33
Figure 1.6	Structure and signaling pathway of the integrins	36
Figure 2.1	Neural precursors proliferation is enhanced by CNTF and reduced by anti-CNTF	56
Figure 2.2	The number of neural precursors is increased by CNTF and reduced by anti-CNTF	58
Figure 2.3	Markers for cell death are present in regions of neurogenesis	62
Figure 2.4	CNTF enhances neurogenesis in the dentate gyrus	65
Figure 2.5	CNTF enhances neuronal differentiation and migration in the dentate gyrus	67
Figure 2.6	CNTF does not obviously influence astrocyte proliferation	70
Figure 2.7	CNTF does not obviously influence proliferation of oligodendrocytes or their progenitors	73
Figure 2.8	CNTFRα is present in and around neurogenic regions	76
Figure 3.1	Expression of the $\alpha 6\beta 1$ integrin and its substrate, laminin	97
Figure 3.2	Blockade of endogenous integrin subunits disrupts the rostral migratory stream	100
Figure 3.3	Infusion of a peptide or control substance enhances proliferation in the subventricular zone	103
Figure 3.4	Infusion of a peptide recognized by the $\alpha 6\beta 1$ integrin draws neuroblasts into the striatum	105
Figure 3.5	A peptide recognized by the $\alpha 6\beta 1$ integrin draws a significant number of neuroblasts towards and to the infusion site	107
Figure 3.6	A laminin tract is sufficient to draw neuroblasts away from the rostral migratory stream	111
Figure 4.1	Six injections of Dil in the neostriatum labels more neurons in the lateral regions of the SNc and VTA than two sites of Dil or Fluorogold	131
Figure 4.2	Six sites of Dil labels more neurons across the rostral to caudal extent of the SNc than does two sites of Dil or Fluorogold	134
Figure 4.3	The rates of neuronal loss following 6-OHDA appears more linear after pre-labeling with six sites of Dil	137
Figure 4.4	Reported neuronal survival varies depending on method of pre-labeling	140
Figure 4.5	Survival after two weeks within the middle one-third of the SNc is different depending on lesion and tracer	142

ABSTRACT

The adult CNS contains pluripotent, self-renewing stem cells which are capable of generating neurons, astrocytes, and oligodendrocytes. The factors controlling the proliferation, differentiation, and migration of neural stem cells and their progeny are currently the focus of intense study. This thesis is composed of three main projects. The first project assessed the role of the cytokine, ciliary neurotrophic factor (CNTF), on the proliferation and differentiation of neural stem cells in two major neurogenic regions of the forebrain, the subventricular zone (SVZ) and the dentate gyrus of the hippocampal formation. Injection of CNTF in adult C57BL/6 mouse forebrain induced proliferation in both neurogenic regions as assessed by bromodeoxyuridine (BrdU) incorporation. In the dentate gyrus, CNTF enhanced neuronal differentiation, and migration into the granule cell layer. Intraventricular injection of neutralizing anti-CNTF antibodies reduced proliferation. CNTF and anti-CNTF slightly decreased and increased, respectively, the number of apoptotic cells in the neurogenic regions. These results suggest that endogenous CNTF regulates adult neurogenesis by increasing proliferation and/or survival of newly-formed neurons. CNTFR α was most clearly present in astrocytes in neurogenic regions, and therefore the effects reported here could be indirect via neighbouring astroglia. The restricted expression of CNTF in the nervous system makes it a potential endogenous target for therapeutic cell replacement strategies.

New neuroblasts are constantly generated in the adult mammalian SVZ and migrate via a "rostral migratory stream" (RMS) to the olfactory bulb, where they differentiate into functional neurons. Little is known about molecules involved in the directed nature of this migration. The second major component of this thesis investigated the role of the $\alpha 6\beta 1$ integrin, and its ligand, laminin, in controlling guidance of migrating neuroblasts. Immuno-staining for both $\alpha 6\beta 1$ integrin and laminin was present within the RMS. Inhibition of the endogenous $\alpha 6$ or $\beta 1$ integrin subunits with locally injected antibodies disrupted the cohesive nature of the RMS. Local infusion of a 15 a.a. peptide, representing the E8 domain of the laminin α chain, and which is recognized by the $\alpha 6\beta 1$ integrin, redirected the neuroblasts away from the RMS into the neostriatum. Injection of a narrow tract of intact laminin also drew the neuroblasts away from the RMS, but in a more restricted localization. These results establish a critical role for integrins and laminin in adult SVZ-derived neuroblast migration.

The third component of this thesis examined retrograde cell tracing techniques in models of Parkinson's disease. Six sites of Dil injection labeled the substantia nigra more broadly and effectively than did 2 injection sites. Two injections of Fluorogold labeled fewer neurons, but their morphology was clearer. After injection of the neurotoxin 6-OHDA, neuronal survival was greater with 6 sites of Dil than with 2, but survival within the middle region was lower. Survival after 6-OHDA or axotomy was similar with Dil or Fluorogold. These results suggest that, because of a complex projection pattern of the nigrostriatal neurons, detailed quantification of neuronal survival should rely on extensive labeling.

LIST OF ABBREVIATIONS AND SYMBOLS

a.a.	amino acid(s)
µg	microgram
µL	microlitre
µm	micrometre
6-OHDA	6-hydroxydopamine (2,4,5-trihydroxyphenethylamine)
ABC	avidin biotin complex
BDNF	brain-derived neurotrophic factor
BMP	bone morphogenic protein
BrdU	5'-bromo-2'-deoxyuridine
CC	corpus callosum
CCD	charge-coupled device
ChAT	choline acetyltransferase
CLC	cardiotrophin-like cytokine
CLF	cytokine-like factor-1
CNPase	2'3'-cyclic nucleotide 3'-phosphodiesterase
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CNTRα	ciliary neurotrophic factor receptor alpha
CSPG	chondroitin sulphate proteoglycan
CTX	cerebral cortex
DAB	diaminobenzidene
DG	dentate gyrus
Dil	1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DV	dorsal-ventral
ECM	extracellular matrix
ED₅₀	effective dose-50%
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EtOH	ethanol
FG	fluorogold
FGF-2	fibroblast growth factor-2
GABA	gamma amino butyric acid
GC	granular cell layer (of dentate gyrus)
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
HCL	hydrochloric acid
Hi	hilus
HRP	horseradish peroxidase
IGF-1	insulin-like growth factor-1
IgG	immunoglobulin G
IgM	immunoglobulin M

IL-6	interleukin-6
JAK	janus kinase
kDa	kilodalton
kg	kilogram
LIF	leukaemia inhibitory factor
LPS	lipopolysaccharide
LV	lateral ventricle
M	Molar
mg	milligram
ML	medial-lateral
ML	molecular layer (of dentate gyrus)
ml	millilitre
mm	millimetre
mM	millimolar
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRNA	messenger ribonucleic acid
n	number of animals or specimens
NeuN	neuron specific nuclear protein
NGF	nerve growth factor
NS	neostriatum
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
OSM	oncostatin M
p	probability
PD	Parkinson's disease
PFA	paraformaldehyde
PSA-NCAM	polysialylated form of the neural cell adhesion molecule
RA	retinoic acid
RC	rostral-caudal
RMS	rostral migratory stream
SEM	standard error of the mean
SHH	sonic hedgehog
SNc	substantia nigra pars compacta
SNI	substantia nigra pars lateralis
SNr	substantia nigra pars reticulata
SOCS	suppressor of cytokine signalling
SSC	standard sodium citrate
STAT	signal transducer and activator of transcription
SVZ	subventricular zone
SVZa	subventricular zone, anterior region
TGFα	transforming growth factor alpha
TGFβ	transforming growth factor β
TN-C	tenascin-C
VTA	ventral tegmental area

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“The prize is the pleasure of finding the thing out, the kick in the discovery...”

—Richard Feynman

CHAPTER 1:

GENERAL INTRODUCTION

The central nervous system (CNS) is constantly developing. For some time, the cellular plasticity of the nervous system in response to its internal and external environment has been recognized as the hallmark of the brain's adaptability. Our understanding of this exciting phenomenon has been considerably expanded upon with recent definitive demonstrations that neural stem cells reside in the adult CNS, and that these unique cells are capable of generating new neurons. These findings question inherited dogmas about the CNS and its capacity for change, while challenging many fundamental concepts about neuronal development and degeneration. In addition, neural stem cell research brings with it the promise of novel therapeutic approaches to brain repair for Parkinson's and Alzheimer's Disease, spinal cord injury, and tumour growth. Clearly, more intense research is required into the behaviour of neural stem cells and their progeny.

This introductory chapter is presented as a background to the specific research projects that follow. Neural stem cells and their progeny may be characterized on the basis of three fundamental themes: those relating to proliferation, differentiation, and migration. Indeed, much of the work within this thesis, as it relates to neural stem cells, is organized on the basis of these three overarching themes. To provide a background to the work presented here, normal CNS development is briefly reviewed, and this section is followed by a discussion of the discovery and isolation of neural stem cells. A functional definition of neural stem cells follows, as well as a description of the major

neurogenic regions of the CNS. The phenomena of neural stem cell proliferation, differentiation, and migration are then reviewed.

A variety of protein factors have been employed within this thesis, and all of these have notable effects on neural stem cell behaviour. The first agent used was the neural cytokine, ciliary neurotrophic factor (CNTF), and its structure and function are considered here. In addition, the extracellular matrix molecule laminin, and a family of receptors for such molecules (the integrins) are similarly reviewed to provide background to the work described later in the thesis. This introduction then provides a brief description of Parkinson's disease, as well as a description of rodent models of this disease. This chapter concludes by outlining the rationale for the various studies presented here, and by providing a summary of the objectives for meeting those goals.

The work presented within this thesis focuses on three main projects. The first examines the role of CNTF in the proliferation, differentiation, and migration of neural stem cells and their progeny. The second major project studies the role of integrins and their ligand, laminin, in controlling the direction of migration of newly-generated neuroblasts. Finally, the thesis concludes with an examination of retrograde neuronal tracing techniques, and of the potential effects these methods have on assessing the efficacy of various models of neural degeneration or repair.

Overview of central nervous system development

Central nervous system development occurs in several discrete but rigidly coordinated phases. The vertebrate nervous system begins to form at the gastrula stage, during which the embryo is comprised of three distinct germinal layers (ectoderm, mesoderm, and endoderm) (Hall, 1992). The ectoderm, signalled by the mesoderm, then forms a neural groove (later a neural tube) along the rostral to caudal axis of the developing embryo. Along with the formation of the neural tube, such infolding gives rise to a closed ventricular system, the epithelial border of which comprises a germinal zone. It is at this germinal zone where the majority of neurons and glia are born. Postmitotic progeny of these cells undergo a series of overlapping developmental steps, namely differentiation, growth, and migration to a final position within the parenchyma (Ramon y Cajal, 1911; Shepherd, 1988).

Proliferation of neurons and glia is non-uniform along the rostral to caudal axis of the brain, and it is these discrepancies which produce major brain regions such as the telencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain) in vertebrates. A general rule is that cells born earlier tend to occupy regions closer to the ventricular germinal zones, whereas those born later migrate beyond these immature layers to more distal regions. Although the majority of neuronal development occurs prior to and just after an organism's birth and maturation, it is now clear that there are several regions, which will be discussed in more detail below, which retain a neurogenic capability throughout the life span of the animal.

Discovery and isolation of mammalian neural stem cells

The definitive isolation of neural stem cells began shortly after the isolation of stem cells from the hematopoietic system. Using a variety of phenotypic markers, Spangrude *et al.* (1988) were able to purify and characterize mouse hematopoietic stem cells, and demonstrate that these cells could reconstitute the hematopoietic system of irradiated mice. In 1992, Reynolds and Weiss showed that cells dissected from the adult mouse striatum could be dissociated into nestin positive spheres, or "neurospheres", and that these colonies could produce astrocytes and neurons *in vitro* (see also Reynolds, Tetzlaff, and Weiss, 1992). Similarly, Richards *et al.* (1992) reported the proliferation and generation of cells with neuronal morphology, which had come from dissected regions of the adult mouse brain.

Although these studies are often seen as a starting point in neural stem cell biology, earlier evidence suggesting the existence of adult neurogenesis is found throughout the literature. For example, Allen (1912) reports the persistence of mitosis in the lateral wall of the lateral ventricles (part of which is now known as the subventricular zone) of adult rats. Using thymidine autoradiography, Altman (1962) provided evidence for ongoing mitosis in the adult. In addition, increases in neuronal number have been reported in the dentate gyrus of the hippocampal formation (Altman and Das, 1965; Kaplan, 1981; Bayer, 1982), the olfactory epithelium (Graziadei and Monti Graziadei, 1978, 1979), and the subependymal zone (Boulder Committee, 1970). However, it was not until such cells could be isolated in culture that questions relating to

their growth factor responsiveness, function, localization and, indeed, their definition, could be truly approached.

The definition of a neural stem cell

There has been considerable debate as to what defines a neural stem cell. However, a working definition has been proposed which provides a framework for further characterization of the nature of neural stem cells (Weiss *et al.*, 1996). To be considered a stem cell (of any type), a cell must satisfy the following five criteria. It must (1) retain the ability to proliferate asymmetrically; (2) exhibit the ability for self-maintenance and renewal over the lifetime of the animal; (3) be capable of generating a large number of progenitors via transient amplification of a discrete population of progenitors; (4) retain its potential to form multiple cell types (i.e., have multi-lineage potential), and (5) be capable of generating new cells in response to disease or injury.

The complete lineage of the neural stem cell has not yet been elucidated, primarily because its exact phenotype has not been described. However, there have been some recent attempts to isolate neural stem cells based on phenotype (see, for example, Rietze *et al.*, 2001). In addition, there are suggestions as to what may constitute the neural stem cell lineage. It has been proposed that the neural stem cell itself is a slowly dividing, relatively quiescent population, which gives rise to transit amplifying cells. These transit amplifying cells give rise to constitutively proliferating cells, which are themselves precursors for neurons and glia (Morshead *et al.*, 1994). Related to this proposed lineage, it has further been

suggested that there is a primitive neural stem cell which arises from the totipotent embryonic stem cell, and developmentally precedes the existence of the relatively quiescent neural stem cell (Tropepe *et al.*, 2001).

There have been many challenges to such working definitions of neural stem cells and their associated lineages. It is unclear as to whether the definitions which can apply to, for example, the hematopoietic system, are equally applicable to the neural stem cell system. In addition, this definition does not necessarily contemplate the possibility that there can be overlap between cell lineages, and that stem cells from one system can give rise to progeny bearing the phenotype of a different system. For example, it has recently been suggested that cells from the hematopoietic system are capable of crossing the blood-brain barrier, and giving rise to neurons and glia (Brazelton *et al.*, 2000; Mezey *et al.*, 2000). As a corollary to those observations, it has also been demonstrated that neural stem cells can become skeletal or muscle cells (Galli *et al.*, 2000), and can also adopt a hematopoietic fate (Bjornson *et al.*, 1999). Further, it has been proposed that mature astrocytes are capable of becoming neural stem cells, and that they are capable of de-differentiating to take on this multipotent capability (Doetsch *et al.*, 1999; Seri *et al.*, 2001). It also remains unclear as to whether adult neural stem cells are remnants of development, or whether they constitute a *de novo* population of cells within the adult (van der Kooy and Weiss, 2000). Finally, the definitive source of neural stem cells remains controversial: some evidence suggests that they come from the ependymal layer itself (Johansson *et al.*, 1999; Momma *et al.*, 2000), whereas

others suggest that only the sub-ependymal layer exhibits truly "stem-like" properties (Chiasson *et al.*, 1999). In response to some of these challenges to the definition of a neural stem cell, some have proposed that the term neural stem cell describes more of a function than an entity (Blau *et al.*, 2001), and that the concept of tissue-specific adult stem cells needs further expansion.

Overview of the major neurogenic regions

Several neurogenic regions have been described in the adult CNS. These include the retina (Reh and Levine, 1998; Tropepe *et al.*, 2000; Fischer and Reh, 2001), the spinal cord (Weiss *et al.*, 1996), the cerebral cortex (Gould *et al.*, 1999; Magavi *et al.*, 2000; Gould *et al.*, 2001), the forebrain subventricular zone (SVZ) (Lois and Alvarez-Buylla, 1993; Morshead *et al.*, 1994; Gates *et al.*, 1995; Doetsch *et al.*, 1997; Luskin, 1998), and the dentate gyrus of the hippocampal formation (Altman and Das, 1965; Erikson *et al.*, 1998; Gage *et al.*, 1998; Kornack and Rakic, 1999). It is the latter two regions, the SVZ and the dentate gyrus, which are of primary interest for the work described in this thesis. The cellular architecture and the nature of the neurogenic process within these two regions will be briefly described.

The subventricular zone is the best described neurogenic region within the adult mammalian CNS, and it is the site from which most studies have isolated neural precursors, and neurospheres, *in vitro* (Reynolds and Weiss, 1992). During embryogenesis, the SVZ lies at the junction between the ventricular and intermediate zones (Boulder Committee, 1970), and continues to be a generative

source of cells into adulthood, where it is also commonly referred to as the subependymal layer (Smart, 1961; Hinds, 1968; Blakemore, 1969).

An analysis of the proliferation kinetics and ultrastructural morphology of this region reveals that it is composed of a variety of cell types, including neuroblasts, glial cells, and putative precursor cells. In addition, this zone contains ependymal cells, tanycytes, microglia, and mature neurons, as well as mitotic, pyknotic, and unidentified cell types (Doetsch *et al.*, 1997; Garcia-Verdugo *et al.*, 1998). The major cellular components of this region are the Type A cells (which are migrating neuroblasts), surrounded by two forms of astrocytes (Type B1 and B2 cells), as well as proliferating (Type C) cells. The architecture of the relationship among these cell types is illustrated in Figure 1.1. Migrating neuroblasts within this region are immuno-positive for the polysialylated form of the neural cell adhesion molecule (PSA-NCAM) (Bonfanti and Theodosis, 1994; Rousselot *et al.*, 1995; Doetsch and Alvarez-Buylla, 1996), beta III tubulin (Tuj1) (Menezes and Luskin, 1994; Gates *et al.*, 1995), and the intermediate filament protein, nestin (Morshead *et al.*, 1994; Gates *et al.*, 1995; Thomas *et al.*, 1996). Type B glial cells are GFAP positive (Gates *et al.*, 1995; Lois *et al.*, 1996; Jankovski and Sotelo, 1996), nestin positive, and positive for the intermediate filament protein vimentin (Doetsch *et al.*, 1997; Peretto *et al.*, 1997). However, they are PSA-NCAM and Tuj1 negative. Finally, type C cells are nestin positive, but are negative for all of the other markers discussed above. The migration of Type A cells, from the rostral portion of the SVZ to the olfactory bulb, will be described in greater detail later.

Figure 1.1. Schematic illustration of the cellular composition and architecture of the adult mammalian SVZ

This cross-section of the SVZ illustrates a chain of migrating neuroblasts (Type A cells), which are ensheathed by two ultrastructurally different types of glial cells (Type B1 and B2 cells). Type B1 and B2 cells form a barrier between the migrating neuroblasts and the ependymal layer (Type E cells). Proliferating cells (Type C cells) are not ensheathed by Type B cells, but are strongly associated with migrating neuroblasts. (After Doetsch *et al.*, 1997).

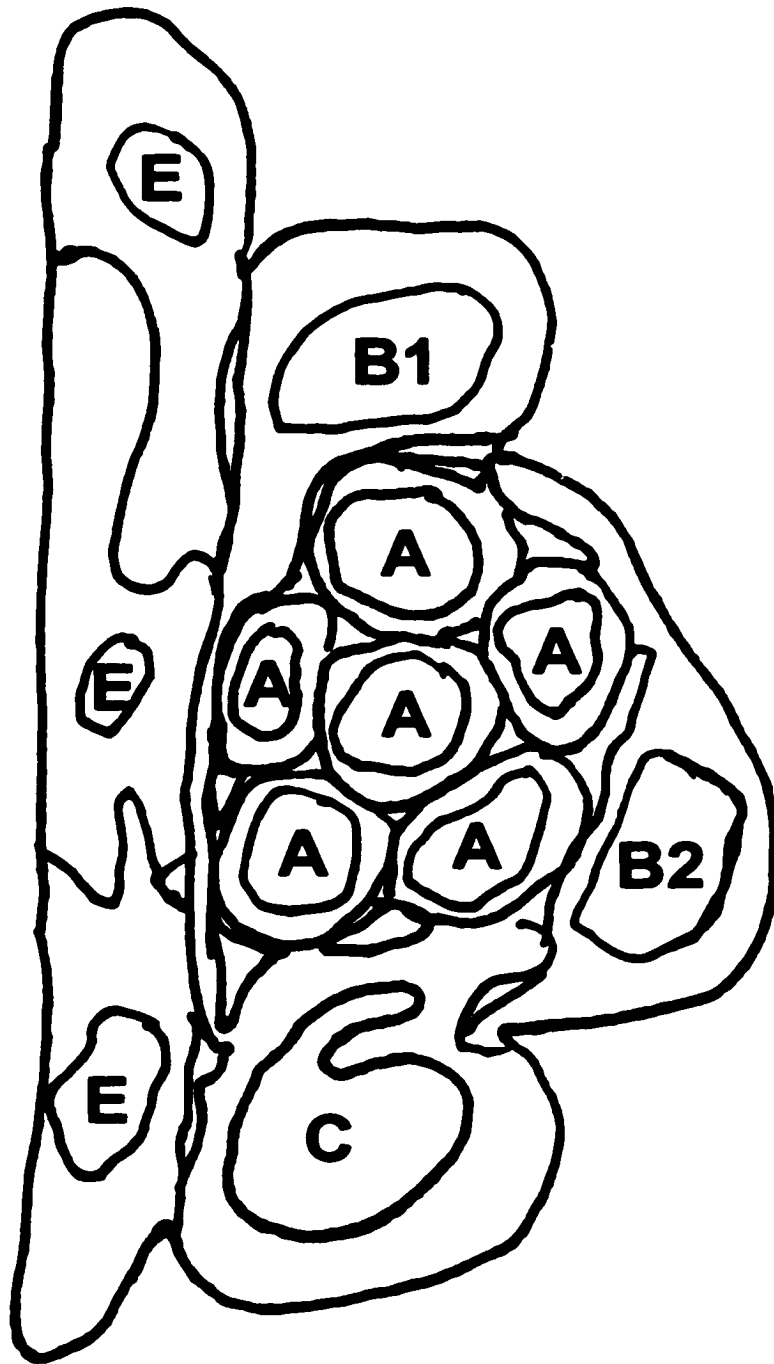


Figure 1.1

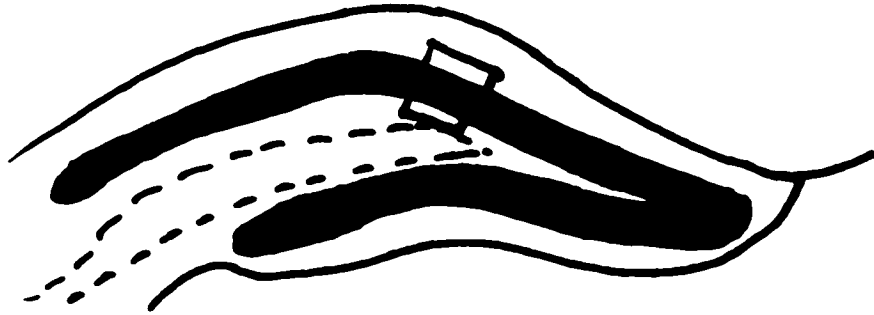
The dentate gyrus of the hippocampal formation is another neurogenic region of interest. Compared to the SVZ, however, less is known about the proliferation kinetics of neural stem cells in this region, or the functional significance of their neuronal progeny (Gage, 2000). The specific neurogenic region of the dentate gyrus is the subgranular zone, which is a continuous and extremely thin lamina between the hilus and the granular cell layer of the dentate gyrus (Figure 1.2). The population of progenitor cells which go on to become mature neurons is produced by asymmetric division, although it is unclear as to whether the progeny themselves are capable of further division (Gage *et al.*, 1998). Of the proliferating cells at the hilus-dentate gyrus border, approximately 50% develop a neuronal phenotype, become PSA-NCAM positive, possess growth cones, and migrate into deeper regions of the granule cell layer (in the direction of the molecular layer) (Figure 1.2) (Kuhn *et al.*, 1996). Once those cells reach these deeper granule cell regions, they become positive for the neuronal marker NeuN, as well as Calbindin (Kuhn *et al.*, 1996). The remaining 35% of these newly-generated cells take on a glial phenotype (Gage *et al.*, 1998), and the fate of the remaining approximately 15% is unknown, although these cells may die by apoptotic mechanisms (de Bilbao *et al.*, 1999). There have been few reports describing specific functions for the newly-generated neurons within the dentate gyrus, although it has been suggested that they may function in the formation of trace memories (Shors *et al.*, 2001).

With a working definition of neural stem cells, and having described the two major neurogenic regions of the CNS, it is necessary to provide further

Figure 1.2. Neurogenesis in the adult dentate gyrus

(A) Coronal view of the hippocampal formation. The box indicates the granule cell layer, which is shown schematically in **(B)**. The numbers in **(B)** show the various stages of proliferation, migration, and differentiation of the newly-generated cells (see text for detailed description; After Gage, 2000).

A



B

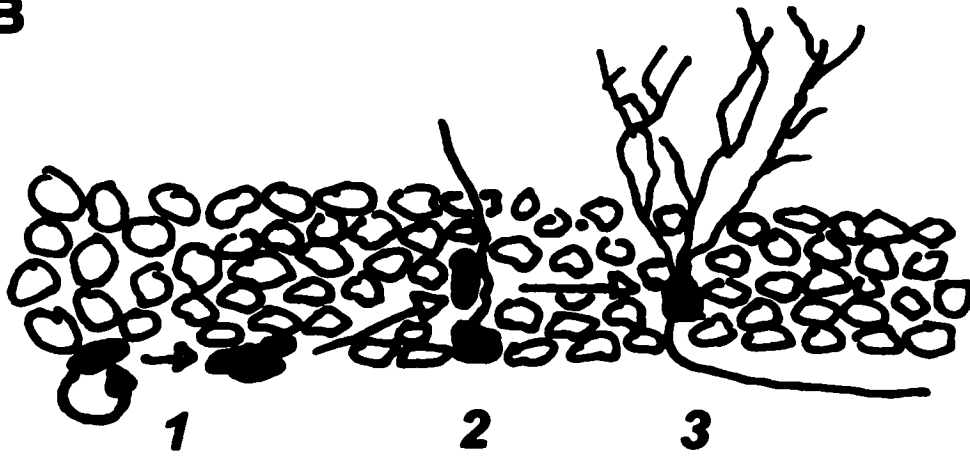


Figure 1.2

characterization of these unique populations of cells. Therefore, in addition to an understanding of the derivation and localization of neural stem cells and their progeny, one can characterize such cells on the basis of what factors influence their proliferation, differentiation, and migration. Specifically, what controls the kinetics of cell division? What factors regulate the differentiation of neural stem cells and their progeny to take on a neuronal or glial phenotype? How do these cells migrate to their final targets, and how does this form of migration differ from that seen during embryonic neural development? Finally, how might answers to these various biological questions be used for the development of therapeutic neural repair strategies? The following sections briefly examine some of the known influences on neural stem cell proliferation, differentiation, and migration.

Proliferation of neural stem cells and their progeny

A variety of factors are capable of regulating the proliferation of neural stem cells and their progeny, both *in vitro* and *in vivo*. Epidermal growth factor (EGF) can stimulate proliferation *in vitro* (Reynolds *et al.*, 1992, 1996; Gritti *et al.*, 1995) and *in vivo* (Craig *et al.*, 1996). Basic fibroblast growth factor (or fibroblast growth factor-2 (FGF-2)) has been shown to enhance proliferation of neural stem cells and their progeny *in vitro* (Gage *et al.*, 1995; Gritti *et al.*, 1995, 1996; Vicario-Abejon *et al.*, 1996). However, it remains unclear as to whether there are two distinct sub-populations of neural stem cells which are selectively responsive to EGF or FGF-2 (Ciccolini and Svendsen, 1998; Palmer *et al.*, 1999). In addition to EGF and FGF-2, there are a number of other factors which can

enhance cellular proliferation. These include thyroid hormone (T3) (Ben-Hur *et al.*, 1998) and transforming growth factor α (TGF α) (Fallon *et al.*, 2000). Inflammation, either by mechanical intervention or induced by lipopolysaccharide (LPS) also enhances SVZ cellular proliferation *in vivo*, as demonstrated by increases in PSA-NCAM labeling (Szele and Chesselet, 1996) or BrdU incorporation (Emsley and Hagg, unpublished observations). Exogenous brain-derived neurotrophic factor (BDNF) has been shown to enhance the number of newly-generated neurons in the olfactory bulb (Zigova *et al.*, 1998) and can increase cellular proliferation within the SVZ, and neurogenesis in regions proximal to the SVZ, such as the striatum, septum, and thalamus (Pencea *et al.*, 2001). Insulin-like growth factor (IGF-1) appears to be involved in increasing cellular proliferation *in vitro* (Arsenijevic *et al.*, 1998, 2001). IGF-1 also enhances cellular proliferation in the dentate gyrus (Åberg *et al.*, 2000; O'Kusky *et al.*, 2000; Trejo *et al.*, 2001), as does estrogen (Tanapat *et al.*, 1999).

In addition to the various growth factors and hormones which can influence stem cell proliferation, there are several external, environmental factors which can influence cellular proliferation within the dentate gyrus. Enriched environment (Kempermann *et al.*, 1997), exercise (van Praag *et al.*, 1999; Kempermann *et al.*, 2000), learning (Gould *et al.*, 1999), and seizure induction (Bengzon *et al.*, 1997; Nakagawa *et al.*, 2000), can all enhance cellular proliferation and subsequent neurogenesis. In contrast, prenatal stress (Lemaire *et al.*, 2000), opiate administration (Eisch *et al.*, 2000), and age (Kuhn *et al.*, 1996) can decrease the rate of neurogenesis. Although many external

influences upon adult neurogenesis have been identified, it is clear that further work is required to identify endogenous mechanisms underlying neural stem cell proliferation.

Factors influencing the differentiation of neural stem cells

Growth factors, neurotrophins, cytokines, signaling molecules, cell culture conditions, and the *in vivo* environment itself can all influence stem cell differentiation. Exposure of human ES cells to a panel of growth factors demonstrates that none of these factors *exclusively* directs differentiation, but rather alters the relative proportion of a specific cell type. For example, retinoic acid (RA), EGF, bone morphogenic protein-4 (BMP-4), and FGF-2 activate ectodermal and mesodermal markers, but nerve growth factor (NGF) allows differentiation of all three embryonic germ layers, including mesoderm (Schuldiner *et al.*, 2000). A number of neurotrophins and cytokines can influence neural stem cell differentiation and/or survival. These include BDNF (Ahmed *et al.*, 1995; Kirschenbaum and Goldman, 1995), and CNTF (Piquet-Pellorce *et al.*, 1994; Wolf *et al.*, 1994; Ip, 1998; Whittemore *et al.*, 1999).

A variety of signaling molecules and other molecules can influence the differentiation of neural stem cells and their progeny. Within cell cultures, the yield of dopaminergic neurons is enhanced by the vitamin ascorbic acid (Yan *et al.*, 2000), or by the homeodomain transcription factor Phox2 (Lo *et al.*, 1999). BMPs potently inhibit neurogenesis both *in vitro* and *in vivo*, but the protein Noggin can promote neurogenesis by inhibiting BMPs (Lim *et al.*, 2000).

Retinoic acid (RA) is also able to exert pro-neural and anti-mesodermal effects on mouse ES cells in culture (Bain *et al.*, 1996; Takahashi *et al.*, 1999). In addition, the protein Notch appears to limit neuronal differentiation by maintaining a cell's proliferative capacity (Faux *et al.*, 2001; Solecki *et al.*, 2001). The protein sonic hedgehog (SHH), a member of the TGF β superfamily, is also involved in patterning and growth in a variety of systems (Smith, 1994). SHH can induce the production of dopaminergic neurons *in vitro* (Hynes *et al.*, 1995; Wang *et al.*, 1995), and can induce spinal cord precursor cells to become neuronal (Dutton *et al.*, 1999).

Cell culture conditions themselves have a considerable influence on the resulting phenotype of ES and neural stem cells. For example, withdrawal of FGF-2 from a culture of ES cells can promote the production of dopaminergic neurons (Lee *et al.*, 2000). The yield of dopaminergic cells is also enhanced by culturing cells in low oxygen conditions (Studer *et al.*, 2000). Cell density also has an effect; multipotent neural stem cells normally give rise to neurons, astrocytes, and oligodendrocytes under high density culture conditions. However, when such cells are plated at a much lower density, they can differentiate into smooth muscle cells (Tsai and McKay, 2000). Finally, simply by exposing a neurosphere culture to FGF-2 along with glial cell conditioned media enhances the yield of tyrosine hydroxylase positive cells (Daadi and Weiss, 1999).

The environment into which neural stem cells are transplanted also exerts a strong influence on these cells' differentiation. Neuronal progenitors

transplanted into the striatum take on a GABAergic or dopaminergic phenotype (Zigova *et al.*, 1998), or express DARPP-32 (Armstrong *et al.*, 2000). The yield of dopaminergic cells transplanted to the striatum also appears to be further enhanced when these cells are transplanted into a dopamine-depleted rather than a normal striatum (Nishino *et al.*, 2000). In addition, neural precursor cells transplanted into the granule cell layer of the dentate gyrus take on a region-specific phenotype (Gage *et al.*, 1995). Finally, when neural stem cells are transplanted into the cortex, they not only take on the appropriate phenotype, but a subset of the transplanted cells are capable of making appropriate contralateral connections (Shin *et al.*, 2000). In some cases, however, neural restricted precursors maintain their default phenotype regardless of the environment into which they have been transplanted. For example, spinal cord-derived neuronal precursors continue to express choline acetyltransferase (ChAT) when grafted into the forebrain SVZ (Yang *et al.*, 2000).

Migration of neural stem cells and their progeny

Along with proliferation and differentiation, migration is a key feature of the progeny of neural stem cells. The following is an overview of the phenomenon of neuroblast migration within the adult CNS, and focuses on the location of these cells in the SVZ, a description of their normal pattern of migration, and their fate. Such migration will be further characterized by examining results from transplantation studies, as well as considering the effects of some potential guidance molecules.

One type of progeny of neural stem cells, as mentioned above, are the Type A cells (Figure 1.1). These are migrating neuroblasts which are PSA-NCAM, TuJ1, and nestin positive (Doetsch *et al.*, 1997). The normal migration pattern of these cells is reviewed in Luskin (1998): briefly, these neuroblasts migrate along a "rostral migratory stream" (RMS) from the anterior portion of the subventricular zone (SVZa) to the olfactory bulb (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Lois *et al.*, 1996) (Figure 1.3A). Neuroblasts migrate as chains of closely apposed elongated cells, which are connected by membrane specializations (Lois *et al.*, 1994; Wichterle *et al.*, 1997) (Figure 1.3B). The movement of these chains is tangential to the neuro-axis, as opposed to the more typical pattern of radial migration seen during development (Doetsch and Alvarez-Buylla, 1996). Tangential migration, at least in cell culture conditions, is approximately four times faster than radial migration (Wichterle *et al.*, 1997). As these cells move in chains, it is evident from slice preparations that occasionally they can reverse direction and move caudally along the stream (Kakita and Goldman, 1999). In addition, the chains do not simply begin their journey at the most rostral pole of the SVZ; rather, chains can be seen caudally along the SVZ of the lateral ventricles (Doetsch and Alvarez-Buylla, 1996).

Neuroblasts are capable of proliferating *en route* to the olfactory bulb (Lois *et al.*, 1994; Menezes *et al.*, 1995; Craig *et al.*, 1999). There may also be strain differences with respect to the speed with which such cells migrate, and the rate at which they migrate to the olfactory bulb (Lee *et al.*, 2001, Soc. Neurosci. Abstr. 248.2). Neuroblasts travel and proliferate within a "glial tube" made up of GFAP

Figure 1.3. Neuroblasts migrate from the SVZ to the olfactory bulb.

(A) Neuroblasts migrate tangentially in densely-packed chains along a rostral migratory stream (RMS) from the anterior portion of the SVZ (SVZa) to the olfactory bulb. Once they reach the olfactory bulb they migrate radially to the granule cell and glomerular layers of the bulb, where they differentiate into granule cell and periglomerular interneurons, respectively. CC = corpus callosum; CTX = cerebral cortex; LV = lateral ventricle. (After Goldman and Luskin, 1998; Luskin, 1998).

(B) Schematic diagram of neuroblast chain migration. Migrating Type A cells are ensheathed by GFAP positive astrocytes. In regions proximal to the SVZa, there are putative precursors, closely associated with the glial cells and the neuroblasts. (After Doetsch *et al.*, 1997).

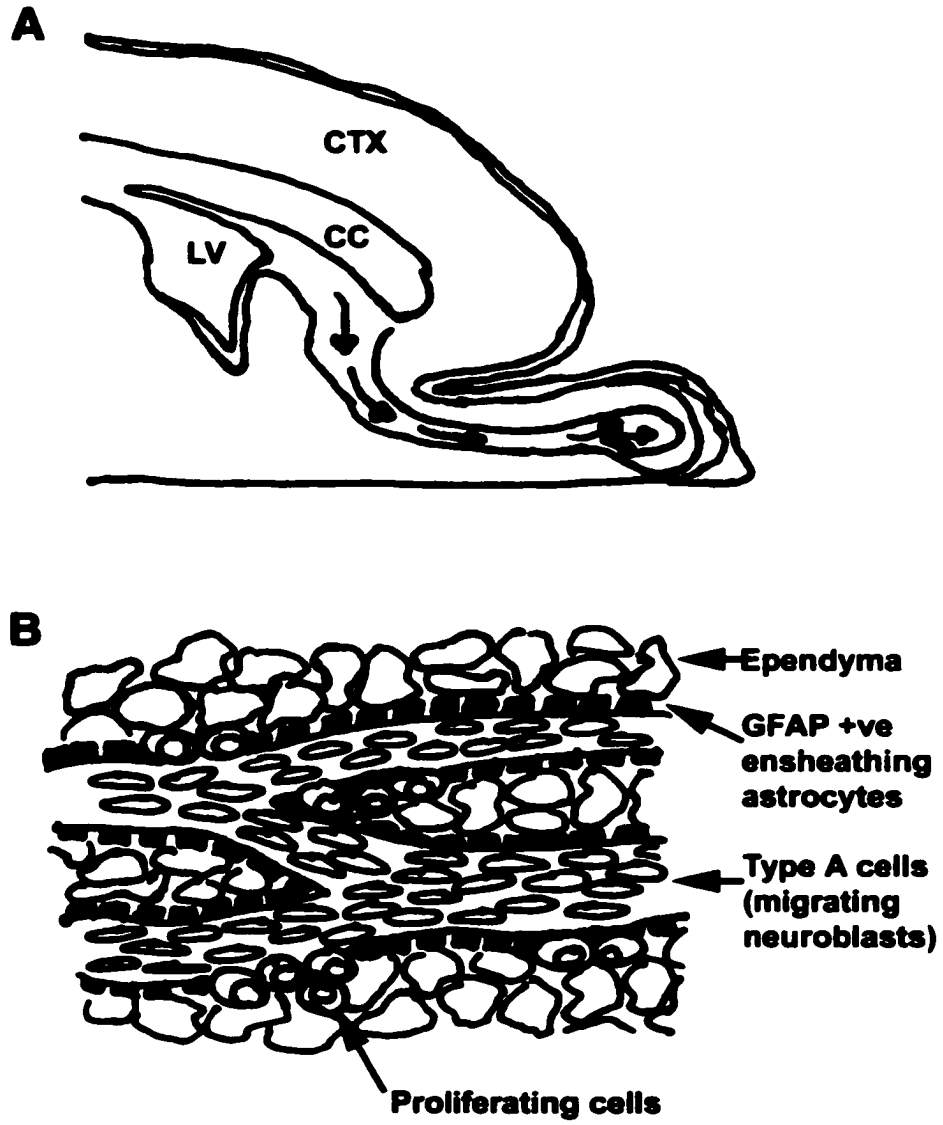


Figure 1.3

positive astrocytes and various extracellular matrix molecules such as Tenascin-C (TN-C) and chondroitin sulfate proteoglycans (CSPG) (Lois *et al.*, 1995; Thomas *et al.*, 1996; Peretto *et al.*, 1997). However, there is very little expression of astrocyte-specific genes in the neonatal (P0 - P7) SVZa, but high neuron-specific expression in the SVZa and RMS (for example TuJ1). These results would suggest that, at least in neonatal animals, the GFAP positive astrocytes are not necessary for neuroblast migration (Law *et al.*, 1999). Once cells reach their target area, the olfactory bulb, they migrate radially to become GABAergic granular neurons and, to a much lesser extent, tyrosine hydroxylase positive periglomerular interneurons (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Betarbet *et al.*, 1996).

Cellular migration within the rostral migratory stream is highly cohesive and persistent. Neuroblasts continue to migrate rostrally even if their terminal destination, the olfactory bulb, is removed (Kirschenbaum *et al.*, 1999). Cells of the rostral migratory stream are strongly positive for the polysialylated form of the neural cell adhesion molecule (PSA-NCAM), and the removal of the PSA moiety from NCAM disrupts cellular migration (Kiss, 1998; Chazal *et al.*, 2000).

A number of transplantation studies provide further characterization of the behaviour of migrating neuroblasts. For example, homotopically-transplanted cells (transplantation of SVZa-derived cells into the SVZa of a host animal) will incorporate into the host SVZa and RMS and will migrate along with the endogenous cells. However, following heterotopic transplantation (transplantation of ventricular zone cells into a host SVZa) the transplanted cells

will not undergo migration, but differentiate, suggesting that such cells are not responsive to local cues responsible for guiding migration (Zigova *et al.*, 1996). In addition, SVZa-derived cells transplanted to various brain regions demonstrate chain-like migration (even in the cortex and striatum), but only those cells transplanted to the olfactory bulb show evidence of proper migration and normal differentiation (Herrera *et al.*, 1999).

Some work has been performed to identify and characterize the various molecules which might be involved in controlling the migration of neuroblasts within the RMS. It has been shown that migration of neuroblasts continues even if the olfactory bulb is removed, suggesting that the olfactory bulb itself is not a source of chemo-attractant molecules (Kirschenbaum *et al.*, 1999). Further evidence suggests this possibility is likely, as it has also been demonstrated in culture that a piece of olfactory bulb tissue will not draw cultured SVZ cells toward it (Hu and Rutishauser, 1996). In addition, *in vivo* studies suggest that transplanted cell suspensions of olfactory bulb tissue fail to draw neuroblasts away from the RMS (Emsley and Hagg, unpublished observations). Although the olfactory bulb may not be a chemo-attractant source, other molecules have been identified which are critical for neuroblast migration. The best described is PSA-NCAM. For example, enzymatic removal of the PSA moiety from NCAM, or else targeted disruption of the NCAM gene itself, can lead to severe disturbances in normal neuroblast migration (Ono *et al.*, 1994; Hu *et al.*, 1996; Kiss, 1998; Chazal *et al.*, 2000). In addition to PSA-NCAM, the Ephrins are critical for neuroblast migration. Ephrins are receptor tyrosine kinases which include

EphB1-3 and EphA4, all of which are expressed within the SVZ. Targeted blockade of Eph receptors can decrease migration within the RMS (Conover *et al.*, 2000). In addition, an astrocyte-derived factor, called migration-inducing activity (MIA) is capable of guiding neuroblast migration from SVZ explants *in vitro* (Mason *et al.*, 2001). Finally, the protein Slit has been characterized as a potent chemo-repulsive agent for migrating neuroblasts. Slit is secreted by the septum, in the midline of the telencephalon and caudal to the SVZa. These proteins act directly to repel migrating neuroblasts away from the septal region, rather than simply acting as an inhibitor to migration (Hu and Rutishauser, 1996; Hu, 1999; Wu *et al.*, 1999). Not much has been learned about endogenous attractant molecules, even though it is likely that these molecules will prove to be more useful than chemo-repellents in a therapeutic setting. It is therefore evident that much more work is required to identify the signals involved in controlling migration of neuroblasts in the adult CNS.

After reviewing the phenomena of proliferation, differentiation, and migration of neural stem cells and their progeny, it is necessary to consider the various factors which have been used within the studies presented in this thesis. In general, much of the work within the thesis focuses on the role of endogenous CNTF in neural stem cell proliferation, differentiation, and migration, as well as the role of laminin and the integrins in neuroblast migration. Therefore, the following three sections consider the structure and function of CNTF, laminin, and the integrins, respectively.

The structure and function of ciliary neurotrophic factor

The following section reviews the localization, signaling pathway, and various functions of CNTF. CNTF is a member of a cytokine superfamily whose members include leukemia inhibitory factor (LIF), oncostatin M (OSM), and interleukin 6 (IL-6) (Manthorpe *et al.*, 1993; Sendtner *et al.*, 1994; Ip and Yancopoulos, 1996) (reviewed in Richardson, 1994). CNTF and its specific receptor, CNTFR α , are expressed throughout, and almost exclusively in, the nervous system (Stockli *et al.*, 1989; MacLennan *et al.*, 1994; Ip and Yancopoulos, 1996). This restricted expression makes CNTF a potential target for the development of selective pharmacological treatments. CNTF and CNTFR α have been localized to neurons and astrocytes (Henderson *et al.*, 1994; Rudge *et al.*, 1995; Alderson *et al.*, 1999). CNTFR α is expressed throughout the adult CNS, including the hippocampus and dentate gyrus, and during development is expressed in SVZ neuronal precursors (Ip *et al.*, 1993) and neurospheres (Lachyankar *et al.*, 1997).

CNTFR α is a soluble receptor which lacks a transmembrane component. It forms part of a tripartite receptor complex, the other members of which are LIF receptor β and gp130 (Ip and Yancopoulos, 1996). CNTF signals via the Janus-family tyrosine kinase/ Signal transducer and activator of transcription (JAK/STAT) signaling pathway (Bonni *et al.*, 1997; Kahn *et al.*, 1997) (Figure 1.4). Briefly, JAKs are constitutively associated with the receptor complex, and binding of CNTF to CNTFR α induces LIF receptor β and gp130 receptor dimerization, phosphorylating JAKs. STATs then bind to the receptor complex

Figure 1.4. CNTF signaling pathway

CNTF binds to the tripartite CNTFR α /LIF receptor β /gp130 receptor complex, activating JAK, leading to activation of STAT, which then translocates to the nucleus. STAT is then capable of leading to transcription of numerous growth factors and cytokines. STAT also induces transcription of the suppressor of cytokine signaling (SOCS), which is a negative regulator of the JAK/STAT pathway (Ip and Yancopoulos, 1996). (Modified from Cell Signaling Technology; after Heim, 1999).

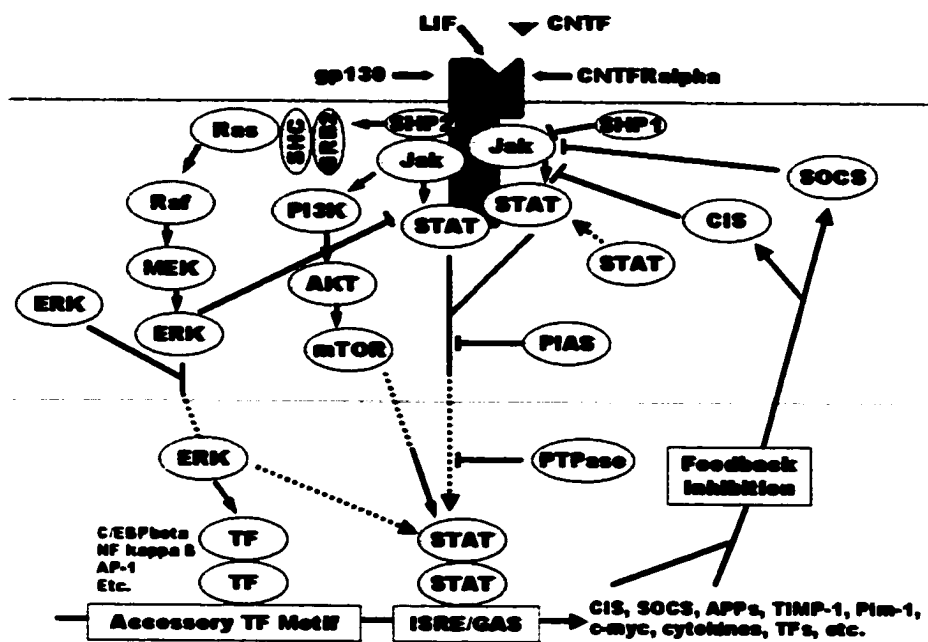


Figure 1.4

and are phosphorylated by JAKs. STATS are then able to translocate to the nucleus, and induce transcription of, for example, various growth factors and cytokines (Ip and Yancopoulos, 1996; Heim, 1999). Some overall effects of CNTF, acting via the JAK/STAT pathway, are reviewed here.

CNTF has survival-promoting effects *in vitro* on retinal pigment epithelium cells (Gupta *et al.*, 1997), embryonic catecholaminergic neurons (Coprav *et al.*, 1999), motoneurons (Zurn *et al.*, 1996), adrenal medulla cells (Tokiwa *et al.*, 1994), and hippocampal neurons (Semkova *et al.*, 1999). Its *in vivo* effects include enhancement of survival of embryonic motoneurons (Oppenheim *et al.*, 1991), hippocampal pyramidal neurons (Skaper *et al.*, 1992), rat facial motoneurons (Duberley and Johnson, 1996), degenerating substantia nigra dopaminergic neurons (Hagg and Varon, 1993), and medial septum neurons (Hagg *et al.*, 1992). CNTF has also been shown to prevent ischemia-induced neuronal loss (Wen *et al.*, 1995) and to promote the survival of several neuronal phenotypes in rodent and primate models of Huntington's Disease (Emerich *et al.*, 1996, 1997).

The reported effects of CNTF on cellular proliferation and differentiation are diverse. *In vitro* studies have implicated CNTF in promoting the proliferation or differentiation of oligodendroglia (Mayer *et al.*, 1994; Barres *et al.*, 1996; Lachyankar *et al.*, 1997; Marmur *et al.*, 1998; Rosano *et al.*, 1999), astrocytes (Lillien *et al.*, 1990; Yoshida *et al.*, 1993; Bonni *et al.*, 1997; Rajan and McKay, 1998; Park *et al.*, 1999; Rosano *et al.*, 1999; Whittemore *et al.*, 1999), and Müller glia (Ezzeddine *et al.*, 1997), as well as inhibiting proliferation of chick

sympathetic neurons (Ernsberger *et al.*, 1989). CNTF promotes the differentiation of bipolar neurons (Lachyankar *et al.*, 1997; Ezzeddine *et al.*, 1997), chick photoreceptors (Fuhmann *et al.*, 1995), amacrine cells (Ezzeddine *et al.*, 1997), and cholinergic neurons from midbrain cranial motor nuclei (Zurn and Warren, 1994). It can also promote tyrosine hydroxylase expression in noradrenergic neurons (Louis *et al.*, 1993) and enhance proliferation of GABAergic, cholinergic, and calbindin positive hippocampal neurons *in vivo* (Ip *et al.*, 1991). CNTF can induce sympathetic neurons, adrenergic superior cervical ganglia neurons, raphe neuronal precursors, and human neuroblastoma cells to express a cholinergic phenotype (Saadat *et al.*, 1989; Lawrance *et al.*, 1995; Rudge *et al.*, 1996; Matsuoka *et al.*, 1997).

CNTF has a variety of effects on differentiation of neuronal progenitors (Ip, 1998). For example, CNTF has been shown to maintain the pluripotency, and hence restrict the differentiation of, embryonic stem cells (Piquet-Pellorce *et al.*, 1994; Wolf *et al.*, 1994). Finally, *in vivo* studies demonstrate a role for CNTF in promoting the differentiation of sympathoadrenal progenitor cells (Ip *et al.*, 1994; Doering *et al.*, 1995). Because of its restricted expression and neurotrophic effects, CNTF may therefore be a potential target for influencing the proliferation and differentiation of neural stem cells and their progeny.

The structure and function of the laminins

Laminin is a major component of the extracellular matrix (ECM) of the basal lamina (Timpl *et al.*, 1979; Patterson, 1992; Burgeson *et al.*, 1994) It is a

cruciform molecule composed of a central long chain of approximately 400 kDa, and two short chains, each approximately 200 kDa (Palm *et al.*, 1985; Hunter *et al.*, 1989) (Figure 1.5). The long chain and its two short chains had previously been referred to as the A, B1, and B2 chains, respectively. A newer nomenclature has renamed these three laminin components as α , β , and γ , with various isoforms of laminin denoted as, for example, laminin-1 ($\alpha 1\beta 1\gamma 1$, formerly EHS laminin) or laminin-2 ($\alpha 2\beta 1\gamma 1$, formerly merosin) (Burgeson *et al.*, 1994). Laminin is produced by a variety of cell types, and its expression appears to be critical for CNS development and migration. During development, the expression pattern of laminin (in most of its isoforms), becomes progressively restricted to periventricular regions and vimentin-positive radial glial fibres (Liesi, 1985; Zhou, 1990). As was discussed earlier, the olfactory bulb is the final destination for neuroblasts migrating from the SVZa. Within the bulb, laminin- immunoreactivity persists (in a punctate staining pattern), and is restricted to the peripheral nervous system components such as the olfactory nerve and glomerular layers (Julliard and Hartmann, 1998; Kafitz and Greer, 1998). Laminin is present in a variety of isoforms, and it can have distinct patterns of regio-specific expression, such as in dendritic spines or neuronal somata. This broad range in the number of laminin isoforms, and the variety of regions in which these isoforms can be expressed, serve to enhance the selectivity of cell-matrix interactions (Liesi and Risteli, 1989; Hagg *et al.*, 1997; Tian *et al.*, 1997; Liesi *et al.*, 2001).

Laminin can stimulate migration of a variety of cell types, including macrophages, Schwann cells and tumour cells (Dubovy *et al.*, 2001; Fujiwara *et*

Figure 1.5. *The structure of laminin*

Laminin is an adhesive glycoprotein present in the basement membrane. It is composed of three distinct chains: one long ~400 kDa chain (the A or α chain), and two shorter chains (B1 and B2, or β and γ chains) each of which is ~200 kDa. The neurite outgrowth promoting region, termed the E8 fragment, contains the globular segment of the long chain that binds to integrin. (After Hunter *et al.*, 1989).

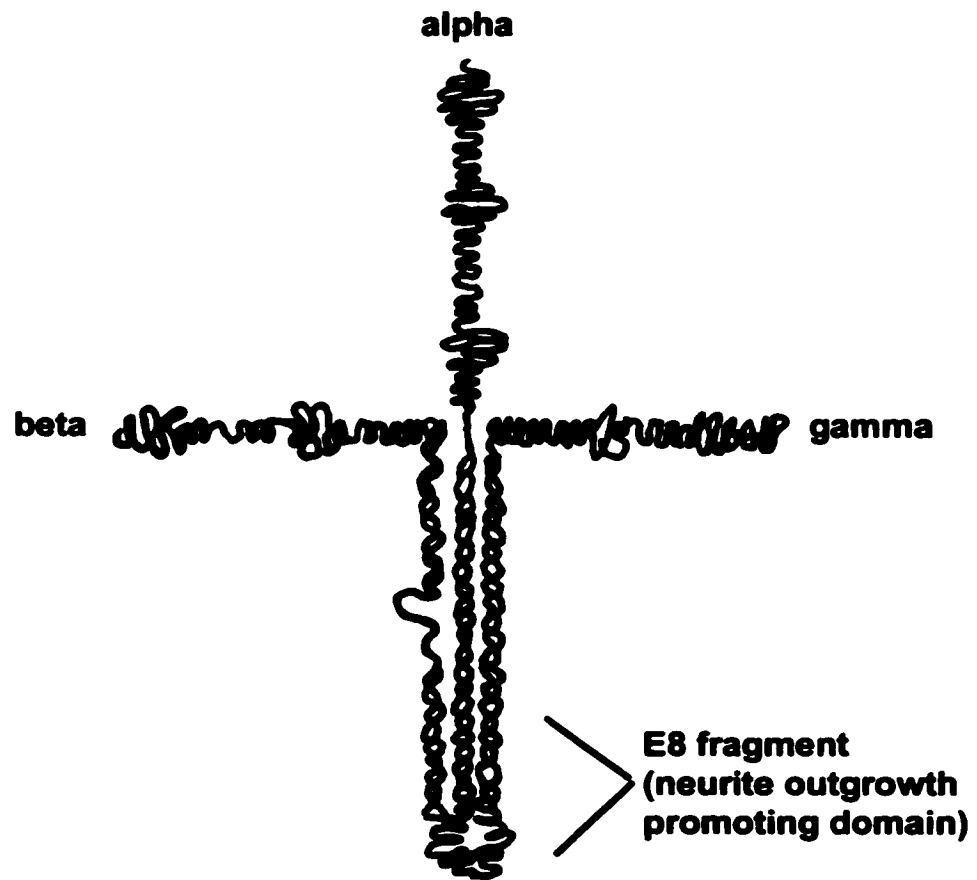


Figure 1.5

al., 2001). Laminin also influences migration of olfactory epithelial cells *in vitro* (Calof and Lander, 1991), is an essential component for immature fibre outgrowth (Zhou and Azmitia, 1988; Liesi, 1992), and may be critical for axonal growth and regeneration (Liesi, 1985). Laminin expression is upregulated in reactive astrocytes following neurotoxic injury (Liesi *et al.*, 1984), and its expression in medial septal neurons declines following septo-hippocampal disconnection, yet this can be restored via intraventricular infusion of nerve growth factor (NGF) (Hagg *et al.*, 1989). Exogenously supplied laminin or derivatives have proven useful in several CNS repair strategies, serving as a guiding scaffold in neuronal transplantation (Zhou and Azmitia, 1988; Zhou, 1990), neonatal corticospinal axon bridges (Schreyer and Jones, 1987), and within hydrogels in the developing optic tract and cerebral cortex (Plant *et al.*, 1997).

The structure and function of the integrins

The integrins are a class of membrane-bound receptors which function as heterodimers of α and β subunits (Hynes, 1992; Clarke and Brugge, 1995). The α subunits are approximately 120-180 kDa, and bind non-covalently to the β subunits, which themselves range from 90-110 kDa (Hynes, 1992) (Figure 1.6A). Integrins bind to the ECM, and thereby link this matrix to cytoskeletal proteins, which includes bundles of actin filaments. Therefore, integrins play a critical role in orientating cells within their environment (Figure 1.6B). The currently known total of 9 α and 14 β subunits are distributed throughout the adult CNS (Pinkstaff

Figure 1.6. Structure and signaling pathway of the integrins

(A) Schematic diagram showing the general structure of the integrin family of receptors. An integrin receptor is a heterodimer of linked α and β subunits. These subunits combine to form a ligand binding area, which includes several divalent cation binding sites.

(B) Integrins, via the β subunit, link the extracellular matrix to the internal cytoskeleton, especially via actin fibres. In addition, the integrins in general are linked to a variety of intracellular signaling pathways. (Modified from Eric Schaefer, Biocarta).

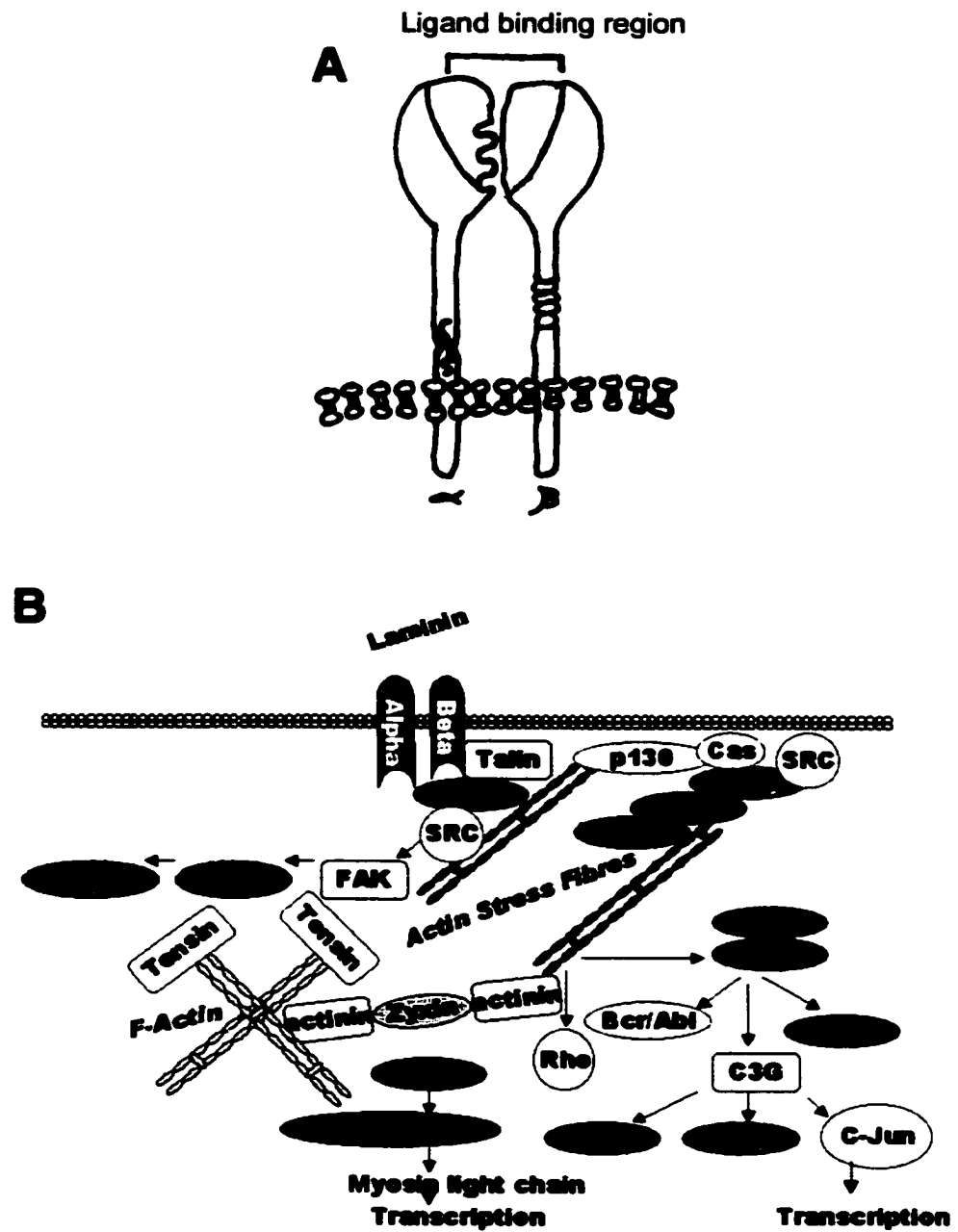


Figure 1.6

1998, 1999). Various combinations of integrins serve as selective receptors for specific regions of ECM molecules, such as fibrinogen, vitronectin, and laminin (Haas and Plow, 1994).

Integrins are important for controlling neural crest cell migration (Bronner-Fraser, 1993) and programmed cell death (Schwartz and Ingber, 1994). They can also regulate a variety of intracellular events, such as the level of intracellular calcium (Kanner *et al.*, 1993; Schwartz and Denninghoff, 1994), and gene expression (Yurochko *et al.*, 1992; Roskelley *et al.*, 1994). The integrins are also linked to several other intracellular signaling pathways (see Clarke and Brugge, 1995).

A specific receptor for laminin is the integrin $\alpha 6\beta 1$, which is present in mouse embryonic stem cells and undergoes a developmental switch from the $\alpha 6\beta 1$ to the $\alpha 6A\beta 1$ isoform (Cooper *et al.*, 1991; Thorsteinsdottir *et al.*, 1995). It is this latter $\alpha 6A\beta 1$ isoform which confers upon cells a more migratory phenotype (Shaw and Mercurio, 1994; Domanico *et al.*, 1997). This integrin combination specifically recognizes the E8 fragment on the terminal portion of the long (α) chain of laminin, either in the $\alpha 1$, $\alpha 4$, or $\alpha 5$ isoforms (see Figure 1.5B) (Aumailley *et al.*, 1990; Sonnenberg *et al.*, 1990; Engvall and Wewer, 1996; Kortessmaa *et al.*, 2000; Talts, *et al.*, 2000). A variety of integrin heterodimer combinations, such as $\alpha 5\beta 1$, $\alpha 6A\beta 1$, $\alpha v\beta 1$, $\alpha v\beta 5$ and $\alpha v\beta 8$, and low levels of $\alpha 6\beta 1$ are present in neural precursors from the neonatal rat forebrain (Jacques *et al.*, 1998). The $\beta 1$ integrin has been shown to be critical *in vivo* for neuroblast migration to the chick optic tectum (Galileo *et al.*, 1992). Further,

phage display libraries have been used to generate small peptides against the $\alpha 6 \beta 1$ integrin (Muruyama *et al.*, 1996), and these have been used *in vitro* to enhance chain migration, but not proliferation, of neural stem cell derived neuronal precursors (Jacques *et al.*, 1998). In conclusion, the integrins are critical regulators of cell migration, and it is therefore necessary to characterize their role in mediating migration of endogenous neuronal precursors.

Overview of Parkinson's disease

The research within this thesis concludes with a chapter on the influence of retrograde tracing techniques on reported cell loss in the nigrostriatal system. This research serves as a caution to future work which may employ such methods as neurotrophic factors or stem cell transplantation within a rodent model of Parkinson's disease. As background information, then, this introductory chapter concludes with a brief overview of Parkinson's disease, followed by a description of the rat nigrostriatal system, the methods used to label cells in this system, and the various models of nigrostriatal degeneration which are currently employed.

Parkinson's disease is a neurodegenerative disorder, the pathology of which is primarily characterized by progressive cell death of dopaminergic substantia nigra neurons which project to the striatum (reviewed in Calne *et al.*, 1992; Uitti and Calne, 1993). Clinical symptoms of Parkinson's disease include bradykinesia, cogwheel rigidity, and tremor. The etiology and pathogenesis of most forms of Parkinson's disease have not been completely elucidated. In

addition to a proposed genetic etiology, numerous environmental factors have been implicated, including pesticide exposure (Lozano *et al.*, 1998; Olanow and Tatton, 1999). The course of the disease, such as the rate at which dopaminergic neurons die, is not clear. It is not yet certain, for example, whether Parkinson's disease involves a linear cell loss, or if there is a massive cell loss immediately prior to or long before the onset of clinically detectable symptoms (Olanow and Tatton, 1999).

The rodent nigrostriatal system as a model for Parkinson's disease

The substantia nigra contains a complex collection of neuronal phenotypes with a variety of inputs and outputs. Very briefly, the substantia nigra is composed of three main components; the substantia nigra pars reticulata (SNr, containing GABAergic neurons), the substantia nigra pars lateralis (SNI, containing dopaminergic neurons), and the substantia nigra pars compacta (SNc, which contains dopaminergic neurons, and is the focus of the studies presented here) (Fallon and Loughlin, 1985; Paxinos, 1995). On each side of the rat brain there are as many as 10,000 – 12,000 dopaminergic neurons (Halliday and Tork, 1986), which project via the medial forebrain bundle to the striatum. In addition, projections from the SNc target the neostriatum and those of the medial ventral tegmental area target such areas as the nucleus accumbens, the olfactory tubercle, and prefrontal, perirhinal, entorhinal, and cingulate cortex (reviewed in Paxinos, 1995). Inputs to the SNc include the amygdala, hypothalamus, and the frontal and cingulate cortical areas, and dendrites of the SNc specifically receive

GABAergic input from the caudate, putamen, and the globus pallidus (Paxinos, 1995).

The rat nigrostriatal system is a commonly used model for Parkinson's disease. Various methods exist for inducing dopaminergic cell death in the SNc, but because such neurons often lose their phenotypic markers as part of the injury (Grafstein, 1975; Hagg *et al.*, 1988, 1989; Lams *et al.*, 1988), there is a requirement to "pre-label" neurons, using retrograde tracing compounds such as Fluorogold, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (Dil), or horseradish peroxidase (HRP) (for examples, see Victorin *et al.*, 1989; Morgan *et al.*, 1991; Hagg and Varon, 1993). Methods to induce dopaminergic cell death include medial forebrain bundle transection (Hagg and Varon, 1993; Brecknell *et al.*, 1995), or the administration of neurotoxins such as 6-hydroxydopamine (6-OHDA or 2,4,5-Trihydroxyphenethylamine; Javoy *et al.*, 1976) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Langston, 1987). There is, therefore, a broad range of methods used both to label cells and to induce their death experimentally; all of these methods relate to the aim of identifying potentially therapeutic agents for treating Parkinson's disease. Chapter 4 of this thesis therefore examines these various retrograde tracing techniques, and assesses the influence such techniques can have on reported dopaminergic cell death following mechanical or neurotoxic injury.

Rationale

Factors controlling the proliferation and differentiation of neural stem cells are only now being elucidated *in vitro* and *in vivo*. Very few agents have been described which have a clearly defined role in regulating or enhancing proliferation and subsequent neuro- or gliogenesis in the adult CNS. Although several influences upon adult neurogenesis have been described, the majority of these phenomena have been external, and often rather general. It is therefore necessary to identify and characterize the more immediate and endogenous regulators of this exciting phenomenon.

The phenomenon of neuroblast migration in the adult CNS has been well characterized, as discussed above. Although studies have identified, or ruled out, potential factors which might encourage or hamper neuroblast migration, much more work is still required to identify and characterize these factors. Specifically, more work is required to identify the role played by the extracellular matrix, and receptors for that matrix, in guiding migration. Further, to elucidate such factors may lead to a better understanding of normal neuroblast migration, as well as open the possibility to draw neuroblasts to a site of neuronal degeneration.

Finally, there has been considerable effort aimed at identifying potential therapeutic measures for ameliorating the symptoms of Parkinson's disease. Such means include transplantation of neural stem cells or their progeny, systemic administration of pharmaceuticals, and delivery of neurotrophic factors. A vast majority of these studies rely upon rodent models of Parkinson's disease,

which use pre-labeling techniques and the induction of dopaminergic cell death via mechanical or neurotoxic injuries. In order to test the efficacy of various potential treatments, it is necessary to have a model which might best reflect the pathogenesis of Parkinson's disease. Further, it is critical to be aware that the method one uses to pre-label such cells may in fact influence the reported cell death, thereby affecting the reported efficacy of the treatment being studied.

Objectives of the Thesis

To meet the goals arising from the rationale for performing this work, there are several objectives:

Objectives for Chapter 2: Endogenous ciliary neurotrophic factor enhances forebrain neurogenesis in adult mice

- To examine the role of exogenous and endogenous CNTF on cellular proliferation in neurogenic regions of the adult CNS
- To determine whether CNTF can enhance neurogenesis or gliogenesis in the dentate gyrus of the hippocampal formation
- To characterize the expression of the specific receptor for CNTF in the neurogenic regions
- To explore possible mechanisms for the action of CNTF on adult neurogenesis

Objectives for Chapter 3: $\alpha 6\beta 1$ integrin directs migration of neuronal precursors in adult mice

- To examine the expression of laminin, and its receptor (integrin $\alpha 6\beta 1$) within the subventricular zone and rostral migratory stream
- To characterize the endogenous role of the integrins in normal neuroblast migration
- To examine the effects of a tract of laminin in influencing neuroblast migration

- **To examine whether an infused synthetic peptide can be used to draw neuroblasts away from their normal migration pattern, and into the striatum**

Objectives for Chapter 4: Retrograde tracing techniques influence reported death rates of adult rat nigrostriatal neurons

- **To compare the effectiveness with which various tracing techniques label the substantia nigra**
- **To examine the effect that each tracing technique has on rates of reported cell death, following axotomy or neurotoxic injury**
- **To consider the extent to which different rates of reported neuronal death are a function of the existence of distinct sub-populations within the substantia nigra**

CHAPTER 2:

**ENDOGENOUS CILIARY NEUROTROPHIC FACTOR ENHANCES
FOREBRAIN NEUROGENESIS IN ADULT MICE**

The work presented within this chapter has been submitted for publication.

ABSTRACT

Adult neurogenesis occurs in the subventricular zone and dentate gyrus. A single injection of the neural cytokine ciliary neurotrophic factor (CNTF) in adult C57BL/6 mice forebrain induced proliferation in both neurogenic regions as assessed by BrdU incorporation. In the dentate gyrus, CNTF also enhanced neuronal differentiation, and migration into the granule cell layer. Intraventricular injection of neutralizing anti-CNTF antibodies reduced proliferation. CNTF and anti-CNTF slightly decreased and increased, respectively, the number of apoptotic cells in the neurogenic regions. These results suggest that endogenous CNTF regulates adult neurogenesis by increasing proliferation and/or survival of newly-formed neurons. As CNTFR α is most clearly present in astrocytes in neurogenic regions, this effect could be indirect via neighbouring astroglia. The restricted expression of CNTF in the nervous system makes it a potential endogenous target for therapeutic cell replacement strategies.

INTRODUCTION

Neurogenesis is subject to a variety of general growth factors and external influences. It is, however, necessary to characterize its more immediate and specific endogenous regulators. Expression of CNTF and its specific receptor, CNTFR α , is limited to the nervous system (Stockli *et al.*, 1989; MacLennan *et al.*, 1994; Ip and Yancopoulos, 1996), and thus CNTF is potentially useful for targeting neural-specific disorders. CNTF and CNTFR α have been localized to astrocytes, suggesting autocrine functions of CNTF (Henderson *et al.*, 1994; Rudge *et al.*, 1995; Alderson *et al.*, 1999). CNTFR α is expressed throughout the adult CNS, including the hippocampus and dentate gyrus, and during development is expressed in subventricular zone neuronal precursors (Ip *et al.*, 1993) and neurospheres (Lachyankar *et al.*, 1997).

CNTF may have varied roles in influencing differentiation and maturation of neural progenitors (Ip, 1998). CNTF can enhance the yield of astrocytes from subventricular zone cell cultures (Whittemore *et al.*, 1999), and can maintain the pluripotency, and hence restrict the differentiation of, embryonic stem cells *in vitro* (Piquet-Pellorce *et al.*, 1994; Wolf *et al.*, 1994). Thus, it is conceivable that CNTF can also regulate stem cells in adults and by virtue of its restricted expression is in a unique position to direct neurogenesis.

Here, the role of endogenous CNTF on the proliferation, migration, and differentiation of neural precursors in the subventricular zone and dentate gyrus

of adult mice was examined by single intracerebral injections of CNTF or neutralizing antibodies against CNTF.

MATERIALS AND METHODS

Animals and housing

All procedures were performed on adult (2 – 3 month old) male C57BL/6 mice (Charles River, St. Constant, Québec), and were conducted in accordance with Dalhousie University and Canadian Council on Animal Care guidelines. Efforts were undertaken to reduce the number of animals used in this study. Mice were housed in group cages (3-5 animals per cage), under a standard 12 hour light cycle, with food and water available *ad libitum*. For the CNTF administration experiments, 19 control and 10 experimental animals were used; for the anti-CNTF experiment, 10 control and 11 experimental animals were used; and in the migration-inducing experiment, 3 experimental animals were used.

Administration of CNTF and antibodies to CNTF

All surgical and euthanasia procedures were performed with an anaesthetic mixture of ketamine (60 mg/kg) and xylazine (12 mg/kg) in 0.9% saline. For the first experiment, animals received a single 1 μ L bolus of 0.1M PBS or CNTF (1 μ g in 0.1 M PBS, recombinant human CNTF, a gift from SCIOS, Inc., Mountain View, California) in the frontal cortex, above the rostral migratory stream. The coordinates for the injection sites (in mm from Bregma) were RC 2.0, ML -0.8 , and DV -3.6 , with Lambda and Bregma on a level plane (Franklin and Paxinos, 1997). Agents were delivered slowly via a thin (150 μ m diameter)

glass micropipette attached to a 1 μ L Hamilton syringe. A sham operated group was similarly anaesthetized, and received a burr hole in the same coordinates, without disrupting the dura. In a separate experiment, mice received PBS or CNTF injections. For the *in vivo* administration of anti-CNTF antibodies, animals received a single 1 μ L bolus injection of 3 μ g/ μ L anti-CNTF antibodies (a gift from Dr. Peter Richardson) or control rabbit IgG (Chemicon, Temecula, California) into the right lateral ventricle (RC -0.4, ML -1.0, DV -2.3) (Franklin and Paxinos, 1997). The anti-CNTF antibody was purified from rabbit serum by IgG binding on a protein A agarose column (Gibco BRL, Burlington, Ontario), followed by dialysis to 0.1 M PB, and its concentration was measured with a BioRad protein assay system (Richmond, California). The antibody neutralized the biological effects of 5 ng/mL CNTF with an ED₅₀ of 0.3 μ g/mL (1:10,000) in a chick ciliary ganglion neurite outgrowth assay, performed as described in Gupta *et al.* (1992). The IgG control did not block CNTF activity in this *in vitro* assay.

5-Bromo-2'-Deoxyuridine (BrdU) administration

To label proliferating cells in the CNTF injection experiment, beginning one day after surgery, mice received single daily intraperitoneal (i.p.) injections of BrdU (240 mg/kg; Sigma) dissolved in 0.9% saline (Miller and Nowakowski, 1988) for a total of 9 days. Animals in the CNTF antibody experiment received a more intense labeling paradigm than those in the previous experiment, receiving 240 mg/kg BrdU twice per day for three days prior to perfusion.

Histology and cell counts

On the day following the last BrdU administration (10 days after CNTF or control injection, and three or 10 days after CNTF or control antibody injection) animals were again anaesthetized and transcardially perfused with 20 mL cold phosphate-buffered saline and 20 mL cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were post-fixed for 24 hours, cryoprotected in 30% sucrose in 0.1 M phosphate buffer, and coronal sections of 30 μ m were cut on a freezing microtome and collected in sodium azide-containing Millonig's buffer. Every sixth section of tissue containing either the subventricular zone or the dentate gyrus of the hippocampal formation was used for each immunocytochemical procedure.

DNA denaturation for BrdU immunocytochemistry followed a modification of the procedure described in Kuhn *et al.* (1996). A monoclonal mouse anti-BrdU antibody was then used to label proliferating cells (1:5,000; Chemicon), followed by a secondary horse anti-mouse antibody (1:600; Vector, Burlingame, California). CNTFR α , was labeled with a mouse monoclonal antibody (1:500; PharMingen, Mississauga, Ontario), and oligodendrocytes (precursors or mature) were labeled with anti-NG2 (1:500; rabbit polyclonal, Chemicon) or CNPase (1:500; mouse monoclonal, Chemicon; Sprinkle, 1989), followed by appropriate secondary antibodies. Apoptotic cells were labeled with a rabbit polyclonal antibody against cleaved Caspase-3 (1:300; Cell Signaling Technology, Beverly, Massachusetts), or with Fluoro-Jade (Histo-Chem, Jefferson, Arkansas). For fluorescent double-labeling of proliferating cells, mouse monoclonal (1:1,000;

Chemicon) or sheep polyclonal (1:1,000; Research Diagnostics, Flanders, New Jersey) anti-BrdU antibodies were employed in conjunction with antibodies against NeuN (1:2,000; mouse monoclonal, Chemicon), GFAP (1:1,000; rabbit polyclonal, Chemicon), and CNPase (1:250; mouse monoclonal, Chemicon). Fluorescent secondary antibodies were conjugated with Alexa 546 (1:800; goat anti-mouse, Molecular Probes, Eugene, Oregon) or Alexa 488 (1:500; donkey anti-sheep; goat anti-mouse, Molecular Probes). For Fluoro-Jade labeling, mounted tissue sections were dehydrated in ethanol, soaked in 0.06% potassium permanganate, and stained in 0.001% Fluoro-Jade which included 0.00005% ethidium bromide as a counterstain (Schmued *et al.*, 1997).

Random start points were used to collect all sections used in cell counts. The number of BrdU positive cells in the subventricular zone was determined from a total of twelve DAB-stained sections per animal. Only the number of BrdU positive nuclei within 5 nuclear diameters from the ventricle wall was counted. For counts of proliferating cells within the dentate gyrus a total of 17 sections through the hippocampal formation per animal were analyzed, and only BrdU-positive nuclei within two nuclear diameters of the granule cell layer were counted. Proliferating cells within the hilus were counted from a randomly selected group of three sections containing the dorsal portion of the hippocampus. For assessment of double-labeled immunofluorescence, five sections per animal were imaged with a Spot CCD camera attached to a Zeiss Axioplan2 microscope. To determine double-labeling, images were overlaid and analyzed with Adobe Photoshop (Version 5.0). Approximately 50 BrdU-positive

nuclei within the subventricular zone and approximately 30 nuclei within the dentate gyrus were analyzed for BrdU/GFAP double-labeling, and an average of 125 nuclei were analyzed per animal for BrdU/NeuN double-labeling. To assess neuronal migration into the granule cell layer, five overlaid images from Photoshop were selected randomly from each group, and the distances from the hilus—dentate gyrus border into the granule cell layer were measured using Scion Image (Version Beta 3b). Fluoro-Jade stained cells were considered to be dying if they fluoresced significantly above baseline levels and simultaneously showed evidence of nuclear condensation (seen with ethidium bromide). Data are presented as means \pm SEM, and differences between groups were assessed using the non-parametric Mann Whitney U-test, with significance determined with α of .05.

RESULTS

CNTF enhances cell formation in the adult mouse subventricular zone

Compared to normal, sham operated (burr hole in the skull only), and PBS-injected controls, a single injection of a bolus of CNTF in the frontal cortex 10 days later led to a noticeable increase in the number of BrdU labeled cells within the SVZ (Figure 2.1A vs. B). Cells included in the analysis were limited to the SVZ only, and did not include those cells traveling within the rostral migratory stream. In the 12 sections analyzed per animal, the total number of BrdU-positive nuclei within the SVZ on both the left and right sides combined was greater after CNTF injection (272 ± 32 , SEM, $n = 10$ from two separate experiments, $p < 0.05$, Mann Whitney U-test) than in normal untreated animals (144 ± 24 , $n = 5$), PBS-injected (167 ± 10 , $n = 9$ from two separate experiments), and sham operated controls (192 ± 32 , $n = 5$) (Figure 2.2A). There were no statistically significant differences among any of the control groups.

To evaluate the role of endogenous CNTF, we injected a neutralizing antibody against CNTF into the lateral ventricles to ensure distribution throughout the brain. Proliferating cells were labeled with an intense three-day BrdU paradigm. Three or 10 days after antibody injection there was an apparent reduction in the cellular proliferation within the SVZ compared to mice injected with a control IgG (Figure 2.1C vs. D; Figure 2.2B). Three days after antibody injection, the total number of BrdU-positive nuclei in anti-CNTF injected mice in the SVZ on both the left and right sides (187 ± 29 , $n = 6$, $p < 0.05$) was

Figure 2.1. Neural precursor proliferation is enhanced by CNTF and reduced by anti-CNTF

Proliferating cells were labeled with i.p. administration of BrdU and show up dark in the differential interference contrast microscopy images. Normal levels of proliferation in the subventricular zone were not affected by intracerebral injection of a single bolus of control PBS (A) but were enhanced by injection of CNTF (B). Intraventricular administration of an antibody directed against CNTF led to marked reduction in subventricular zone proliferation (D) compared to IgG-injected controls (C). Note the unstained cluster of cells in the subventricular zone in (D) (→). The apparent increase in cells in the IgG-injected compared to PBS-injected mice is the result of a more intense BrdU labeling procedure. Proliferation in the dentate gyrus of the hippocampal formation was unaffected by PBS injection (E) but was similarly enhanced by CNTF (F). Administration of anti-CNTF did not significantly alter levels of proliferation (H) compared to IgG controls (G). Cc = corpus callosum, Gc = granule cell layer, Hi = hilus, Lv = lateral ventricle, MI = molecular layer, Ns = neostriatum. Scale bar in (A), 50 μm for all panels.

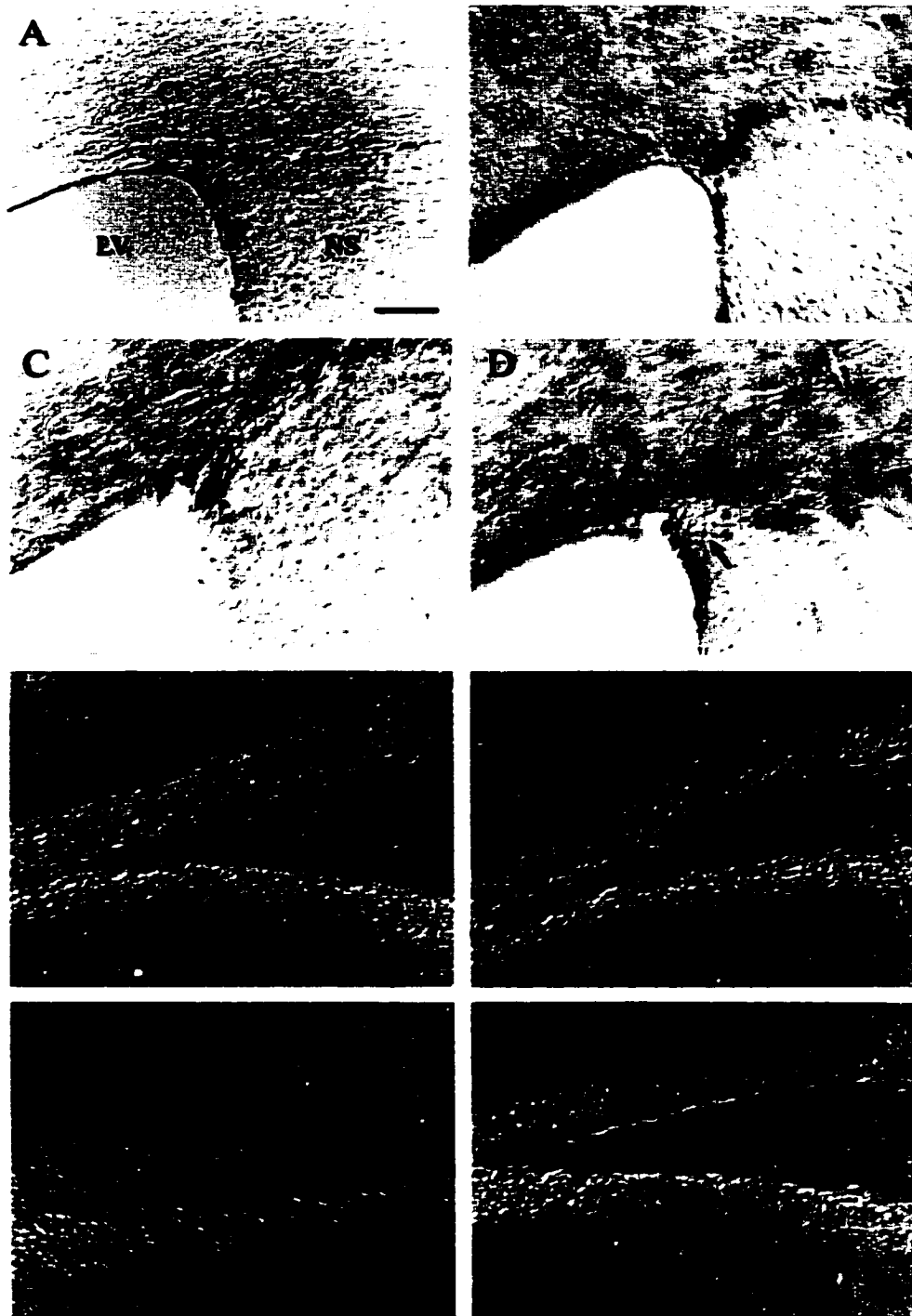


Figure 2.1

Figure 2.2. The number of neural precursors is increased by CNTF and reduced by anti-CNTF

A) Intracerebral injection of CNTF increases the number of BrdU-positive (proliferated) nuclei in the subventricular zone compared to normal, PBS-injected, and sham-operated controls ($p < 0.05$). **(B)** Intraventricular injection of an antibody against CNTF caused a reduction in BrdU-positive cells in the subventricular zone after three days, compared to IgG-injected controls ($p < 0.05$). After ten days proliferation was not reduced compared to IgG controls ($p < 0.10$). **(C)** Within the dentate gyrus, CNTF enhanced cellular proliferation by approximately 50% compared to normal, sham-operated, and PBS-injected controls ($p < 0.05$). **(D)** Proliferation within the dentate gyrus was not significantly reduced by anti-CNTF injection.

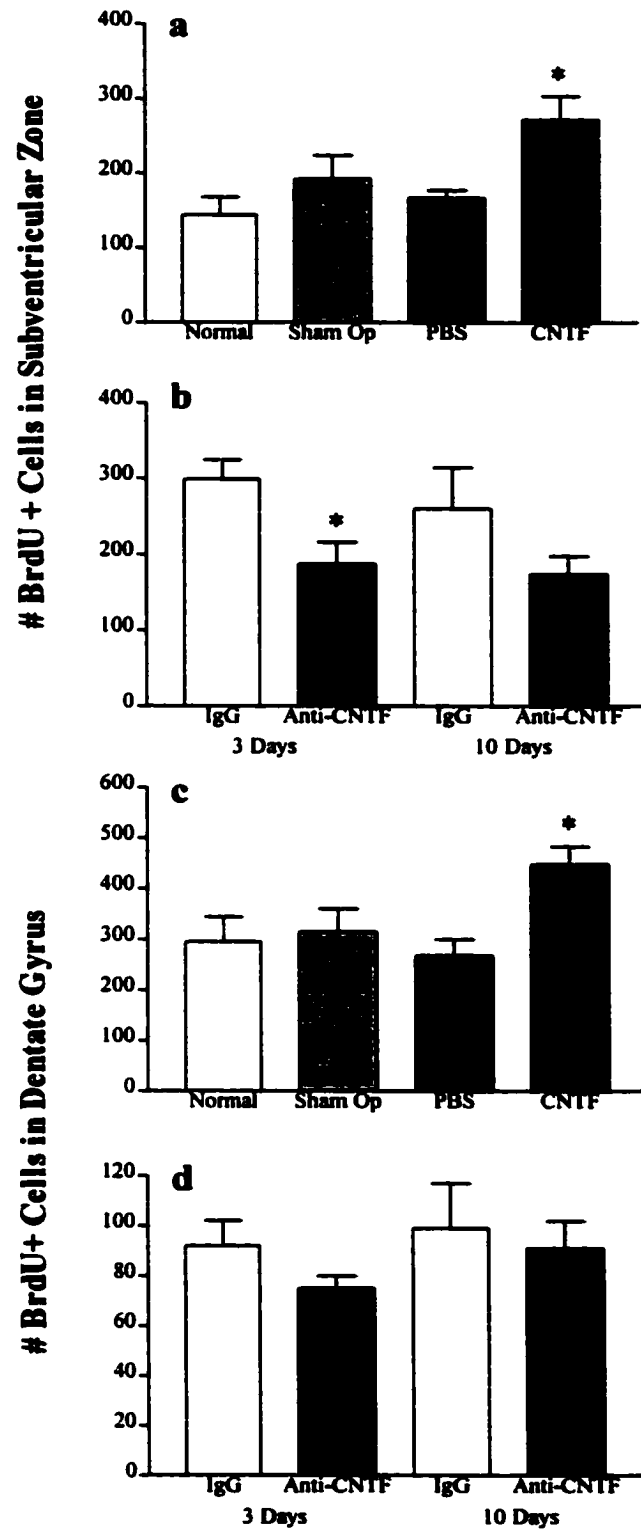


Figure 2.2

significantly lower than the number in IgG-injected controls (299 ± 25 , $n = 5$).

Ten days after antibody administration, the number of BrdU-positive cells was not significantly lower with CNTF antibodies than with purified IgG controls (174 ± 24 versus 260 ± 54 , $n = 5$ each, $p < 0.10$). There were no differences in the number of proliferating cells on the ipsilateral or contralateral sides.

CNTF enhances cell formation in the dentate gyrus

A single injection of CNTF in the forebrain cortex increased the number of BrdU-positive nuclei within the border of the hilus and the dentate gyrus (the neurogenic region) compared to controls (Figure 2.1E vs. F). Within CNTF-treated animals there was an approximately 50% increase in the number of BrdU-positive cells (448 ± 35 , $n = 10$), versus that in normal (295 ± 49 , $n = 5$), sham operated (315 ± 45 , $n = 5$), or PBS-injected (268 ± 32 , $n = 9$) controls (left and right sides combined, $p < 0.05$) (Figure 2.2C). There were no statistically significant differences among the three control groups, nor were there differences between the number of BrdU-positive nuclei on the left and right sides within each of the control or experimental groups.

We examined the possibility that CNTF was merely increasing the rate of migration of proliferating cells from the hilus to the hilar—dentate gyrus border (which would result in an emptying out of the hilus) by counting the number of BrdU-positive nuclei within the hilus of three sections of each animal. There were no statistically significant differences between any of the groups, with

averages of 7 ± 2 , 6 ± 2 , and 6 ± 1 BrdU-labeled hilar cells in normal, PBS-treated, or CNTF-treated groups, respectively.

Three or ten days after intraventricular injection of antibodies against CNTF, the appearance of BrdU-positive nuclei within the dentate gyrus was not noticeably different from controls (Figure 2.1G vs. H; Figure 2.2D). The number of cells was also not different (not shown). The lack of a clear effect of intraventricular antibodies in the dentate gyrus was most likely caused by a reduced diffusion into the parenchyma. No differences were seen in cell number between the ipsilateral and contralateral sides.

We considered the possibility that CNTF played a role in enhancing cellular formation by improving the survival of normally-generated cells. We counted the number of cells which expressed the activated (cleaved) form of Caspase-3, a putative apoptotic marker (Figures 2.3A and B). In control or CNTF-injected animals there were very few Caspase-3 positive cells in the three sections per animal analyzed containing either the SVZ or the dentate gyrus. In the SVZ of CNTF-injected animals there were on average 2.3 Caspase-3 positive cells versus 3 cells in PBS-injected controls, whereas in the dentate gyrus there were 2.3 or 2.5 Caspase-3 positive cells in CNTF or PBS-injected controls, respectively. None of these differences was statistically significant.

We also assessed the number of nuclei within the dentate gyrus with staining for the apoptotic marker Fluoro-Jade (Schmued *et al.*, 1997) (Figures 2.3C - E). High levels of background staining within the SVZ did not allow for a thorough analysis of nuclei in that region. Fluoro-Jade stained cells were

Figure 2.3. Markers for cell death are present in regions of neurogenesis

Immunostaining for activated Caspase-3 in normal mice suggests an ongoing process of apoptosis within the rostral migratory stream adjacent to the subventricular zone (▶ in A, and inset), and in the granular cell layer of the dentate gyrus (▶ in B). High magnification confocal micrograph of an apoptotic cell in the granule layer of the dentate gyrus (▷) labeled with the death marker Fluoro-Jade (C), and nuclear marker ethidium bromide (D), and the merged image in (E). Note the nuclear condensation detected with ethidium bromide (D). Scale bar for a and b, 50 μm; for c – e scale bar is 20 μm.

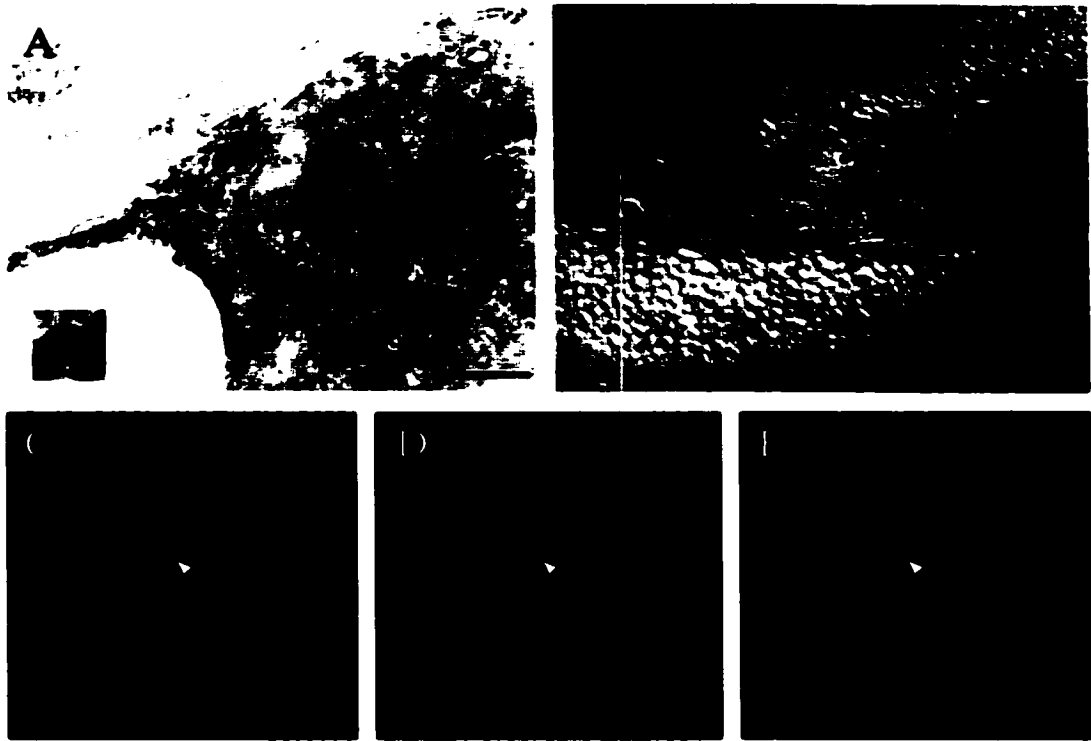


Figure 2.3

counted if they fluoresced significantly above baseline levels and simultaneously showed nuclear condensation (seen with ethidium bromide, another sign of apoptosis). The number of Fluoro-Jade stained cells in the dentate gyrus of PBS-injected controls (10 ± 1 , $n=9$) was not significantly greater than that in CNTF-injected animals (7 ± 1 , $n = 9$, $p < 0.10$). Injection of antibodies against endogenous CNTF did not lead to a significant increase in markers for cell death after three (n.s) or ten ($p < 0.01$) days in comparison to IgG-injected controls.

CNTF predominantly promotes neuronal formation

Most proliferating cells within the dentate gyrus normally become neurons (Gage *et al.*, 1998) and we assessed whether CNTF would also affect neurogenesis in this region. Sections from control and experimental groups were examined for simultaneous expression of BrdU and the neuronal marker NeuN. Double-labeling of these recently-proliferated neurons was only seen within, or at the border of, the dentate gyrus granule cell layer (Figure 2.4). Of the newly-formed cells, a higher percentage of neurons was generated within the CNTF-treated animals, with an average of $29 \pm 3\%$ BrdU/NeuN labeled cells versus that in normal ($14 \pm 0\%$, $p < 0.005$), sham operated ($13 \pm 2\%$, $p < 0.005$) or PBS-injected ($17 \pm 2\%$, $p < 0.01$) controls ($n = 5$ in all groups; Figure 2.5A). The number of newly-generated neurons was approximately 90% higher than that for the average of the three control groups. We did not assess neuronal differentiation within the SVZ because cells in this region do not express mature neuronal markers until they reach the olfactory bulb.

Figure 2.4. CNTF enhances neurogenesis in the dentate gyrus

Confocal images of nuclei simultaneously labeled with anti-BrdU (green) and anti-neuron specific nuclear protein (NeuN, red). BrdU-labeling shows that cellular proliferation is enhanced within CNTF versus PBS-injected controls. The merged image and close-up inset from a CNTF-treated mouse demonstrates that newly-proliferated cells express the neuronal marker NeuN in the granule cell layer (→). Note the distance from the hilus—dentate gyrus border of the co-labeled cell. Scale bar, 50 μm .

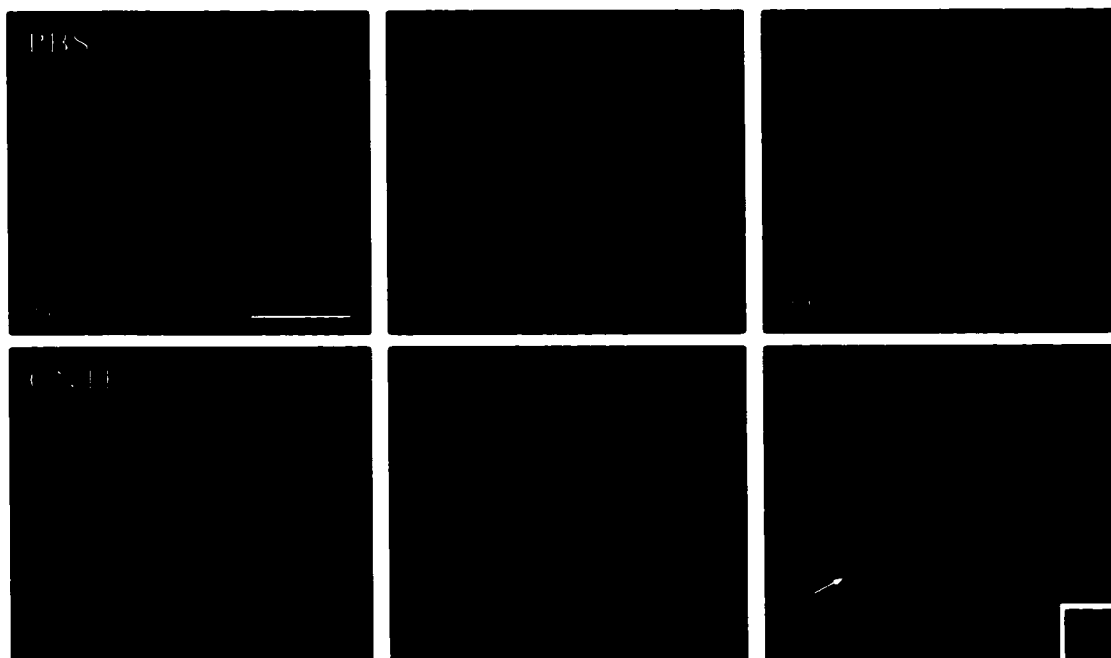


Figure 2.4

Figure 2.5. CNTF enhances neuronal differentiation and migration in the dentate gyrus

(A) CNTF significantly increased the number of cells which were both BrdU and NeuN positive, compared to normal or sham operated ($p < 0.005$) or PBS-injected controls ($p < 0.01$). **(B)** CNTF enhanced the average distance by which these new neurons had migrated from the hilus—dentate gyrus border into the granule cell layer.

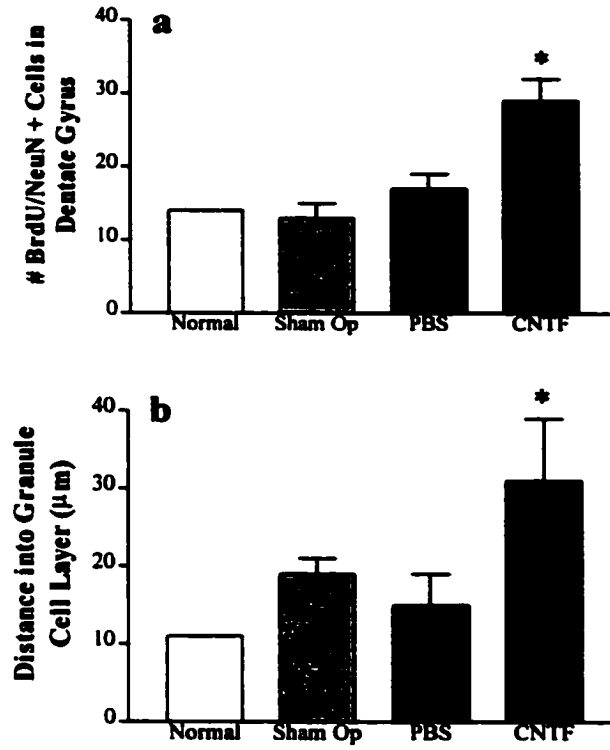


Figure 2.5

To assess a potential role of CNTF in glial formation, we assessed the number of cells within the SVZ that were positive both for BrdU and for the astrocytic marker GFAP. In all of the groups, there were very few cells which were clearly double-labeled for BrdU and GFAP (Figures 2.6A and C), with an average of 6 cells identified in any group, from an average of five sections per animal. The number of GFAP-positive BrdU-positive cells in the CNTF group (8 ± 1) was greater than in the normal, sham operated, and PBS groups (3 ± 1 , 3 ± 1 , and 4 ± 1 , respectively $p < 0.01$). Surprisingly, CNTF injections appeared to decrease GFAP staining in the neurogenic region and rostral migratory stream (Figures 2.6A and C). GFAP expression throughout the striatum was relatively low within both the normal and sham operated groups. In contrast, there was a moderately higher overall level of GFAP expression around the injection site in the PBS or CNTF-injected groups (not shown).

As was observed in the SVZ, there were very few proliferating cells in the region of the dentate gyrus which were clearly identifiable as GFAP-positive (Figures 2.6B and D). Within a total of five dorsal hippocampal sections per animal, there were anywhere from zero to six cells (an average of two) in the dentate gyrus which could be clearly identified as both BrdU and GFAP positive. There were no statistically significant differences among the various groups. As was seen in the SVZ, GFAP expression appeared reduced in the neurogenic region at the hilus—dentate gyrus border in the CNTF-injected group. The general level of GFAP expression in the regions within and around the

Figure 2.6. CNTF does not obviously influence astrocyte proliferation

Compared to overall cell proliferation (BrdU = green), proliferation of GFAP-positive astrocytes (red) did not appear markedly different after CNTF injection. (A, B) Single injection of PBS or CNTF (C, D) in the subventricular zone (left) or dentate gyrus (right). Cellular proliferation was greater in the CNTF-injected animals, most notably in the subventricular zone (C). GFAP expression was reduced in CNTF-injected animals in the rostral migratory stream (C) and in the hilus—granule cell region of the dentate gyrus (D). Scale bar in A, 50 μm .

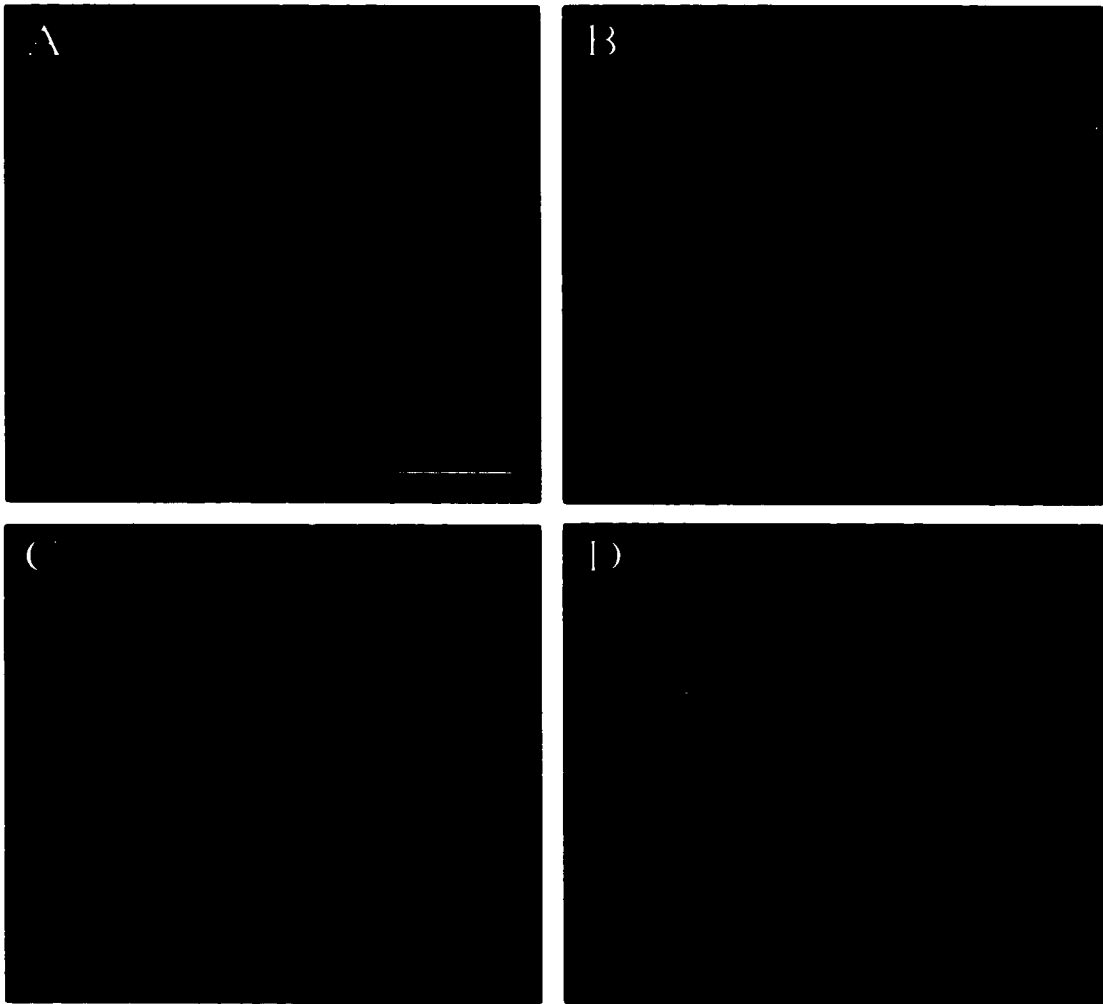


Figure 2.6

hippocampus was assessed, and there were no appreciable differences among any of the control or experimental groups.

We assessed the effect of injected CNTF on oligodendrocyte proliferation by double-labeling with BrdU and CNPase (2',3'-cyclic nucleotide 3'-phosphodiesterase, an intracellular oligodendrocyte marker; Figures 2.7A and B). CNPase most strongly labels oligodendrocyte processes, and therefore it was difficult to count with certainty if cells were clearly double-labeled. However, CNTF appeared to have no effect on the level of oligodendrocyte staining in either the SVZ or the dentate gyrus (not shown). NG2 is a marker for oligodendrocyte precursors (Figures 2.7C and D). CNTF had no noticeable effects on the expression of the oligodendrocyte marker NG2 compared to PBS-injected controls.

CNTF accelerates normal migration of neurons in the dentate gyrus

During adult hippocampal neurogenesis, newly-proliferated cells migrate from the hilus—dentate gyrus border into the granule cell layer. In the CNTF-injected mice, newly-generated neurons were located within deeper regions of the granule cell layer (Figure 2.5B; $31 \pm 8 \mu\text{m}$, 11 ± 0 , 19 ± 2 , 15 ± 4) than in the normal, sham operated, and PBS groups respectively ($p < 0.05$). Analysis of sagittal sections from an additional experiment showed that injection of CNTF above the rostral migratory stream had no chemoattractant effect on this population of neuroblasts ($n = 3$; data not shown).

Figure 2.7. *CNTF does not obviously influence proliferation of oligodendrocytes or their progenitors*

Examples of double-labeling for BrdU and CNPase (representative sections shown in **A, B**) or by labeling for NG2, a marker for oligodendrocyte precursors (**C, D**). Very few oligodendrocytes were associated with the subventricular zone, but in the region of the dentate gyrus their processes (**B**) and cell bodies (**D**) were evident. Scale bar in **A**, 50 μm .

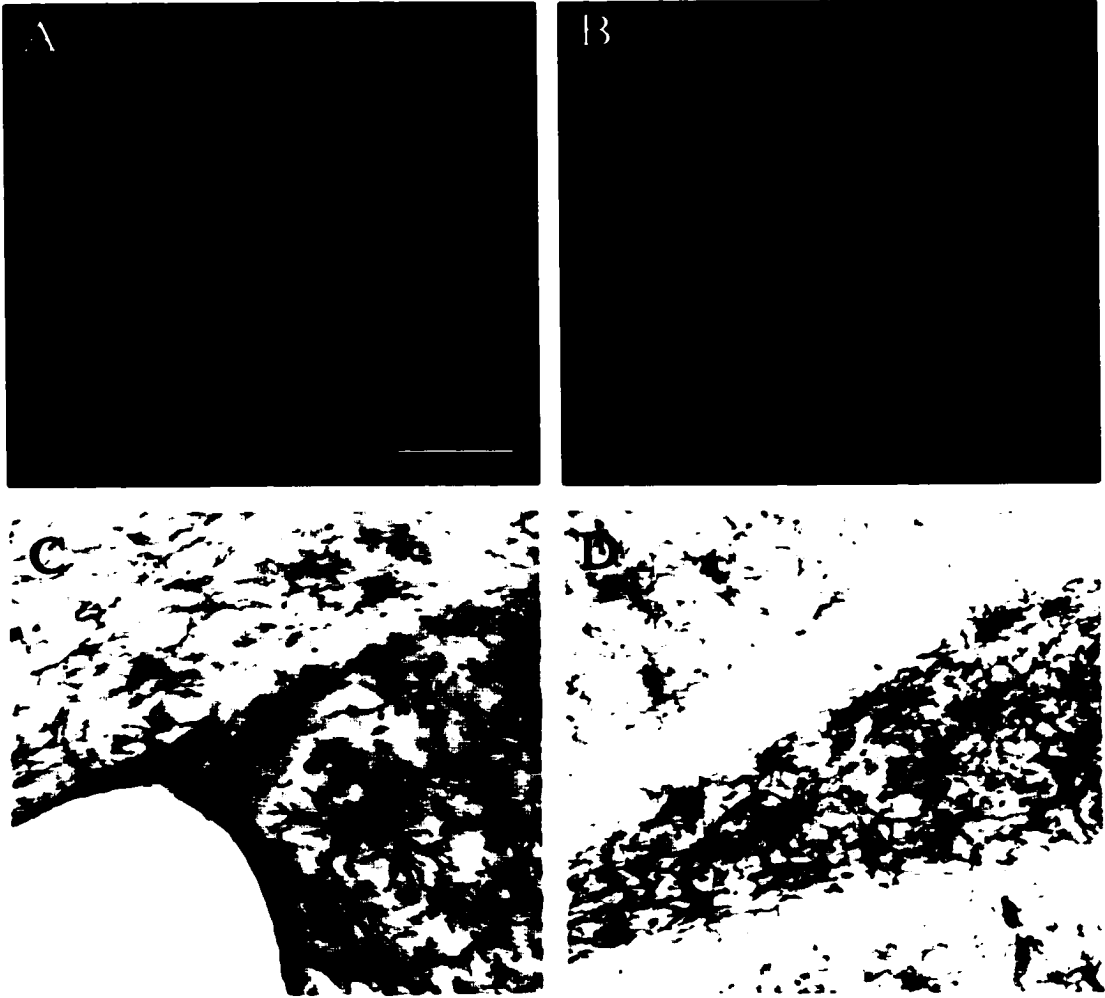


Figure 2.7

CNTF receptor α is present in and around neurogenic regions

We used immunohistochemistry to assess expression of the specific receptor for CNTF, CNTFR α , within the subventricular zone and dentate gyrus (Figure 2.8). Faint staining was detected around the subventricular zone, with most of the staining seen in the processes of astrocytes enclosing the rostral migratory stream (Figure 2.8A). Expression levels were considerably higher within the hippocampal formation, with strong neuronal expression within the hilus, and moderate cell body staining in neurons throughout the granule cell layer of the dentate gyrus. Small, darkly stained cell bodies were seen at the hilus—dentate gyrus border, with the intensity of such staining tapering off in deeper regions of the granule cell layer (Figure 2.8B). There were no detectable differences in the overall levels of expression of CNTFR α between CNTF and PBS-injected controls.

Figure 2.8. *CNTFR α* is present in and around neurogenic regions

Immunostaining for the specific CNTF receptor, CNTFR α , revealed faint labeling of astrocytic processes (\rightarrow) around the subventricular zone and rostral migratory stream (A). (B) CNTFR α labeling was more prominent in neurons of the dentate gyrus, and on cell bodies within the hilus—dentate gyrus border and granule cell layer (\blacktriangleright). Note that the staining density of the small cells appears to taper off in deeper regions of the granule cell layer. Scale bar in A, 50 μ m.



Figure 2.8

DISCUSSION

The current findings that CNTF induces, and antibodies against CNTF reduce, neurogenesis in the adult mouse subventricular zone and dentate gyrus provide strong evidence that CNTF is a critical endogenous regulator of this process. Our results suggest that i) this may result from a combination of increased proliferation of neural precursors and reduced death of newly formed neuroblasts; ii) CNTF selectively enhances proliferation in the normal neurogenic regions, predominantly caused an increase in neuronal formation and increased the speed of migration, but did not affect the direction of migration, suggesting that the normal process of neurogenesis is enhanced and not altered, and iii) in the neurogenic regions, CNTFR α was present most clearly in astrocytes, which are known to produce CNTF, suggesting that the effects of endogenous CNTF on neurogenesis may be indirect and autocrine.

CNTF may enhance neural precursor proliferation and/or survival

CNTF may normally enhance adult neurogenesis *in vivo* by stimulating proliferation of neural stem cells in the neurogenic regions. CNTF could also stimulate proliferation of their progeny, including the neuroblasts, which reportedly continue to proliferate *en route* to the olfactory bulb (Lois and Alvarez-Buylla, 1994). The effects of the CNTF antibodies were seen at early times after injection (3 days) and in the subventricular zone, close to the origin of the rostral migratory stream. This suggests that CNTF plays a role in the proliferation of

stem cells or early neural progenitors. The effects of the bolus injection of anti-CNTF were also apparent 7 and 10 days later (the time of the BrdU labeling), suggesting that early interruptions of CNTF signaling have long-lasting effects on the entire process of neurogenesis. This also raises the possibility that the turnover of extracellular levels of endogenous CNTF is very slow, consistent with the observations *in vitro* that CNTF is not readily released by astrocytes (Sendtner *et al.*, 1994; Rudge *et al.*, 1995). Along the same lines, the effects of a single injection of CNTF probably were also long lasting, although we cannot conclude this because of the chronic labeling protocol (0-10 d). Additional evidence for a longer-lasting effect of CNTF comes from the fact that Levison *et al.* (1996) report no increases in subventricular zone proliferation relative to controls 24 hours after cortical CNTF injection. It should also be noted, however, that such results were obtained in rats, not mice, and used a 10-fold smaller dose of CNTF combined with a less concentrated and shorter BrdU injection paradigm.

The neurogenic effects of CNTF reported here appear to differ from the observation that transgenic mice which chronically over-express the CNTF-related cytokine IL-6, show a reduced neurogenesis in the dentate gyrus (Vallieres *et al.*, 2000, Soc. Neurosci. Abstr. 24.20). However, such mice may not have normal adult neurogenesis, as they could undergo abnormal development of the nervous system, including compensatory mechanisms such as reductions in CNTF expression. Our results were obtained with a normal strain of mice. Moreover, CNTF reportedly can induce proliferation or differentiation of neural precursors (Ip, 1998), astrocytes (Lillien *et al.*, 1990;

Yoshida *et al.*, 1993; Bonni *et al.*, 1997; Rajan and McKay, 1998; Park *et al.*, 1999; Rosano *et al.*, 1999), oligodendroglia (Mayer *et al.*, 1994; Barres *et al.*, 1996; Lachyankar *et al.*, 1997; Marmur *et al.*, 1998; Rosano *et al.*, 1999), and Müller glia (Ezzedine *et al.*, 1997). It has recently been demonstrated that CNTF can enhance EGF-induced SVZ proliferation *in vivo* (Shimazaki *et al.*, 2001).

The process of neurogenesis in the adult forebrain is characterized by apoptosis of a sub-population of newly formed cells, including that in the rostral migratory stream (Brunjes and Armstrong, 1996), and to a lesser extent in the subgranular region of the dentate gyrus (de Bilbao *et al.*, 1999). We have found more evidence for this process in the neurogenic regions by the staining of cells with Fluoro-Jade, which is believed to be a marker for cell death, and nuclear condensation of such cells, a sign of apoptosis. A few cells in these regions also had staining for the activated form of Caspase-3, suggesting that this pathway is involved in the death of neural precursor cells, as it is in apoptosis of other cell types (Friedlander and Yuan, 1998).

CNTF reduced the number of cells with Fluoro-Jade-positive, condensed nuclei. This may suggest that endogenous CNTF plays a role also in promoting the survival of neural precursors and newly formed neurons (in the dentate gyrus). Others have suggested that IGF-1 plays a similar role (O'Kusky *et al.*, 2000). Such a trophic role for CNTF in these regions is conceivable as it has survival-promoting effects *in vitro* on a variety of cell types, including hippocampal neurons (Semkova *et al.*, 1999). In addition, CNTF enhances the

survival of a number of neuronal types *in vivo* (Oppenheim, 1991; Hagg et al., 1992; Skaper *et al.*, 1992; Hagg and Varon, 1993; Emerich *et al.*, 1996, 1997).

CNTF enhances neuronal differentiation in vivo

CNTF injection not only increased the number of newly generated cells but also increased the proportion of cells in the dentate gyrus that were also labeled for the neuronal marker NeuN. Thus, CNTF appears to have increased the speed of normal differentiation into neurons. CNTF also caused an apparent increase in migration speed, as new neurons (BrdU/NeuN positive cells) had migrated farther into the granule cell layer than with control injections. CNTF injections generated numerous new cells in the subventricular zone and dentate gyrus, and numerous new neurons in the dentate gyrus (neuroblasts from the subventricular zone differentiate in the distant olfactory bulb). In contrast, only very few new cells had astroglial markers (GFAP) even after CNTF injection, and none appeared to have markers for oligodendrocytes (CNPase) or oligodendrocyte precursors (NG2). Thus, CNTF predominantly enhances differentiation into the neuronal phenotype, i.e., speeds up the normal process of differentiation or promotes the survival of new neurons.

These results are in apparent contrast to the described role of CNTF in the majority of *in vitro* studies, which suggest that CNTF directs differentiation of neural stem cells towards an astroglial phenotype (Wolf *et al.*, 1994; Park *et al.*, 1999; Whitemore *et al.*, 1999). Furthermore, precursor cells with a null mutation of the suppressor of cytokine signaling gene exhibit decreased neuronal

differentiation in conjunction with an increased generation of astrocytes *in vitro* (Turnley and Bartlett, 2000 Soc. Neurosci. Abstr. 214.5). Neurogenin has been shown to promote neurogenesis while inhibiting glial differentiation *in vitro* via suppression of the JAK/STAT signaling pathway (Sun *et al.*, 2001), which is also activated by CNTF (Bonni *et al.*, 1997; Kahn *et al.*, 1997). On the other hand, others have shown that CNTF causes differentiation of precursors that are committed to the neuronal lineage (Ip *et al.*, 1991, 1994; Fuhrmann *et al.*, 1995; Lachyankar *et al.*, 1997). Thus, it is possible that the effects of CNTF *in vivo* reflect further differentiation of newly formed neuronal precursors. This is consistent with the observation that transgenic mice, which chronically over-express the CNTF-related cytokine IL-6, do not show increased astrocyte formation, although their neurogenesis is reduced (Vallieres *et al.*, 2000 Soc. Neurosci. Abstr. 24.20). It is possible that the effects of neural cytokines seen *in vitro* are overruled by other mechanisms in the neurogenic regions, which cause early differentiation into a neuronal lineage. A clear example of such a mechanism is the finding that Noggin from the ventricular ependyma inhibits the gliogenic properties of endogenous BMP, leading to neuroblast formation (Lim *et al.*, 2000). Here, CNTF injection did affect astroglial responses around the more distant injection site. Thus, the behaviour of the neural stem cells and their progeny is dependent on the cellular context, something that needs to be taken into account much more when conducting *in vitro* experiments.

CNTF may induce adult neurogenesis indirectly through activation of astroglia

The CNTF specific receptor, CNTFR α , has been detected in the developing subventricular zone (Ip *et al.*, 1993). In the adult, only astroglia had clear staining for CNTFR α in this region. In the dentate gyrus CNTFR α staining was present on, besides normal neurons, small cells that could represent new cells or astroglial cell bodies. Technical difficulties prevented us from performing double-labeling with BrdU. Thus, it is possible that the neurogenic effects of CNTF resulted indirectly through activation of surrounding astroglia.

It has recently been proposed that astrocytes within the subventricular zone as well as the dentate gyrus exhibit neural stem cell-like qualities (Doetsch *et al.*, 1999; Seri *et al.*, 2001). It is possible that in our *in vivo* experiments CNTF enhanced proliferation of astrocyte-derived neural stem cells within the subventricular zone and dentate gyrus. Our results demonstrate that, although the numbers were very small compared to the bulk of proliferated cells, there was also an increase in the number of newly generated cells with GFAP staining in the neurogenic regions after CNTF injection. However, despite our use of confocal microscopy, it was not possible to determine conclusively that such cells were BrdU and GFAP-positive, as this would require analysis via electron microscopy (Doetsch *et al.*, 1997). The CNTF injection also caused a reduction of GFAP staining in the neurogenic regions (in contrast to an induction close to the injection site) suggesting that the astrocytes dedifferentiate, and further

raising the possibility that CNTF promotes neurogenesis by stimulating astroglial transformation into neural stem cells which subsequently proliferate.

CNTF may also have contributed indirectly to survival of newly formed cells by stimulating astrocytes to produce other survival-promoting factors. For instance, astrocytes can produce FGF-2 (Hatten *et al.*, 1988), LIF (Banner *et al.*, 1997), and NGF (Lu *et al.*, 1991; Schwartz *et al.*, 1993). Another factor could be BDNF, which can be produced by astrocytes (Zafra *et al.*, 1992) and which supports neurogenesis *in vivo* (Buckland and Cunningham, 1999). TrkB receptors are found in the neurogenic regions (Yan *et al.*, 1997), and may act synergistically with receptors for CNTF (Hashimoto *et al.*, 1999). The finding that cell death was more clearly affected 10 days after injection of anti-CNTF, but less so after 3 days, suggests that withdrawal of neurotrophic support for the new cells occurs long after neutralizing endogenous CNTF. This again raises the possibility of an indirect effect involving astrocyte-released neurotrophic factors.

A role for IGF-1 in neurogenesis has recently become evident, as injection of IGF-1 promotes whereas antibodies inhibit neurogenesis (Åberg *et al.*, 2000; O'Kusky *et al.*, 2000; Trejo *et al.*, 2001). There may be a direct link between the actions of CNTF and IGF-1, as CNTF administration *in vivo* can lead to increased astrocytic IGF-1 levels (Guthrie *et al.*, 1997), upregulation of IGF binding proteins (Wood *et al.*, 1995), and increased IGF-1 receptor expression in oligodendrocytes (Jiang *et al.*, 1999).

Endogenous CNTF may mediate effects of other inducers of neurogenesis

CNTF may mediate seizure-induced neurogenesis. Several studies have shown increased neurogenesis in models of epilepsy (Bengzon *et al.*, 1997; Nakagawa *et al.*, 2000; Scharfman *et al.*, 2000), while others have demonstrated increases in astroglial CNTF following kainate-induced hippocampal synaptic reorganization (Lowenstein *et al.*, 1993) and pilocarpine-induced seizure (Jankowsky and Patterson, 1999).

Various CNS lesions can induce proliferation in the subventricular zone (Szele and Chesselet, 1996; Weinstein *et al.*, 1996 Kirschenbaum *et al.*, 1999). Such lesions also cause an increase in CNTF synthesis by surrounding astrocytes (Ip *et al.*, 1993). It is conceivable that such lesions also lead to changes in the astrocytes of the neurogenic regions and a concomitant release of CNTF, which then mediates neurogenesis.

Estrogen treatments (Tanapat *et al.*, 1999) and prenatal stress (Lemaire *et al.*, 1999), both involving steroid hormones, affect hippocampal neurogenesis. The TR4 orphan receptor is a member of the steroid receptor superfamily, and is expressed in the developing nervous system (van Schaick *et al.*, 2000) and adult brain, including the granule cell layer of the hippocampus (Chang *et al.*, 1994). TR4 and its close family member, TR2, can induce transcription of CNTFR α (Young *et al.*, 1997, 1998). Thus, steroid activation of TR2 or TR4 could lead to modified expression of CNTFR α and thus regulate neurogenesis.

Conclusions

CNTF is expressed almost exclusively in the nervous system (Stockli et al., 1989; Ip and Yancopoulos, 1996). Together with its proposed endogenous and critical role in promoting neurogenesis, CNTF may be an advantageous target for the development of strategies for neuronal replacement therapies.

CHAPTER 3:

**$\alpha6\beta1$ INTEGRIN DIRECTS MIGRATION OF
NEURONAL PRECURSORS IN ADULT MICE**

The work presented within this chapter has been submitted for publication.

ABSTRACT

New neuroblasts are constantly generated in the adult mammalian subventricular zone (SVZ), and migrate via the restricted rostral migratory stream (RMS) to the olfactory bulb, where they differentiate into functional neurons. Little is known about molecules involved in the directed nature of this migration. Laminins and their receptor, the integrins, are known to regulate migration of other cell types. The role of the $\alpha6\beta1$ integrin, and its ligand, laminin, in controlling guidance of the migrating neuroblasts in adult mice was investigated. Immuno-staining for the $\alpha6\beta1$ integrin was present in the neuroblasts and their processes in the rostral SVZ and the RMS. Inhibition of the endogenous $\alpha6$ or $\beta1$ subunit with locally injected antibodies disrupted the cohesive nature of the RMS. Local infusion of a 15 a.a. peptide, representing the E8 domain of the laminin α chain, and which can bind the $\alpha6\beta1$ integrin, redirected the neuroblasts away from the RMS into the neostriatum. Injection of a narrow tract of intact laminin also drew the neuroblasts away from the RMS, but in a more restricted localization. Taken together, these results establish a critical role for integrins and laminin in adult SVZ-derived neuroblast migration. They also suggest that integrin-based strategies could be used to direct and restrict neuroblasts to CNS regions where they are needed for cell replacement therapies in the nervous system.

INTRODUCTION

Neuroblasts migrate within the adult CNS from the SVZ to the olfactory bulb. This phenomenon of neural stem cell progeny is likely under the control of a variety of factors. The work within this chapter focuses on identifying some of the endogenous regulators of this migration.

Integrin $\alpha6\beta1$, present in mouse embryonic stem cells, undergoes a developmental switch from the $\alpha6B\beta1$ to the $\alpha6A\beta1$ isoform (Thorsteinsdottir *et al.*, 1995; Cooper *et al.*, 1991). Expression of $\alpha6A\beta1$ isoform is associated with a more migratory phenotype of cells (Shaw and Mercurio, 1994; Domanico *et al.*, 1997). This $\alpha6\beta1$ integrin specifically recognizes the E8 globular domain of the long α chains of laminin (Aumailley *et al.*, 1990; Sonnenberg *et al.*, 1990; Kortessmaa *et al.*, 2000; Talts *et al.*, 2000). The $\beta1$ integrin is upregulated in seizures (Pinkstaff *et al.*, 1998) and is critical *in vivo* for neuroblast migration to the chick optic tectum (Galileo *et al.*, 1992; Zhang and Galileo, 1998). Further, a small peptide that binds the $\alpha6\beta1$ integrin (Muruyama *et al.*, 1996) enhances chain migration, but not proliferation, of neural stem cell derived neuronal precursors *in vitro* (Jacques *et al.*, 1998). In addition, the $\alpha6\beta1$ integrin is involved in migration of Schwann cells (Dubovy *et al.*, 2001) and lymphocytes (Gimond *et al.*, 1998).

Laminin is an adhesive glycoprotein present in basement membranes, and is produced by a variety of cell types (Timpl *et al.*, 1979; Burgeson *et al.*, 1994). Laminin expression is prominent during CNS development and migration, and as

development proceeds its pattern of expression becomes more limited and localized to periventricular regions and vimentin-positive radial glial fibres (Liesi, 1985; Zhou, 1990). Within the adult olfactory bulb, a more punctate pattern of laminin-immunoreactivity persists, which is restricted to the olfactory nerve and glomerular layers (Julliard and Hartmann, 1998; Kafitz and Greer, 1998). A variety of other CNS laminin isoforms have been described but not in any detail in the SVZ or RMS. Of interest is that although laminin is best known for promoting neurite outgrowth, it also has a role in migration of olfactory epithelial cells *in vitro* (Calof and Lander, 1991), and many other cell types (Fujiwara *et al.*, 2001).

The endogenous role of $\alpha 6 \beta 1$ integrins in the rostral migratory stream and inhibition by subunit specific antibodies was characterized. The ability of laminin peptides or whole laminin to redirect these neuroblasts was also tested.

MATERIALS AND METHODS

Animals and housing

All procedures were performed on adult (2 – 3 month old) male C57BL/6 mice (Charles River, St. Constant, Québec) and were conducted in accordance with Dalhousie University and Canadian Council on Animal Care guidelines. Efforts were undertaken to reduce the number of animals used in this study. Mice were housed in group cages (3-5 animals per cage), under a standard 12 hour light cycle, with food and water available *ad libitum*. All surgical and euthanasia procedures were performed with an anaesthetic mixture of ketamine (60 mg/kg) and xylazine (12 mg/kg) in 0.9% saline. A total of 20 mice were used for the integrin subunit disruption experiment (14 control, 6 experimental); 13 mice for the peptide infusion experiment (7 control, 6 experimental); and 18 mice for the laminin tract experiment (6 control, 6 experimental).

Delivery of neutralizing antibodies against integrin subunits

Biologically active neutralizing antibodies were used to block the activity of either the $\alpha 6$ or $\beta 1$ integrin subunits (Sonnenberg *et al.*, 1987, 1988; Gao *et al.*, 1995; Wilkins *et al.* 1996). A 1 $\mu\text{g}/\mu\text{L}$ bolus of rat anti-human $\alpha 6$, mouse anti-human $\beta 1$, or purified control rabbit, rat, or mouse IgG (all from Chemicon, Temecula, California) was delivered directly above the rostral migratory stream at the following coordinates, in mm from Bregma: RC 1.7, ML, -0.7, and DV -4.0 (Franklin and Paxinos, 1997). Antibodies were infused over a two minute period

via a 470 μm diameter needle of a 1 μL Hamilton syringe, which was left in the brain for an additional two minutes before retraction. These mice were euthanized after three days.

Production and administration of a laminin peptide

A 15 amino acid polypeptide (designated P3, with amino acid sequence VSWFSRHRYSPPFAVS), representing the E8 domain of the laminin α chain and capable of binding $\alpha 6\beta 1$ integrin (Muruyama *et al.*, 1996) was commercially synthesized (Sigma Genosys, The Woodlands, Texas). The peptide was dissolved in vehicle containing 0.02% acetic acid to ensure solubility (BDH, Toronto, Ontario), rat serum albumin to protect the peptide (1 mg/mL; Sigma, Oakville, Ontario), and gentamicin antibiotic (25 $\mu\text{g}/\text{mL}$; Sigma). Peptide (2.5 $\mu\text{g}/\text{day}$) or control vehicle was loaded into Alzet 1002 mini-osmotic pumps (0.25 $\mu\text{L}/\text{hour}$; Alza, Palo Alto, California) and was delivered for 14 days via a 300 μm diameter metal infusion cannula (Plastics One, Roanoke, Virginia), stereotactically placed into the striatum to a depth of 3.0 mm from the skull surface, 1.1 mm rostral and 1.3 mm lateral to Bregma (Franklin and Paxinos, 1997; Figure 3.3A). The cannula was affixed to the skull with cyanoacrylate glue (Loctite 454, Loctite Corp, Hartford, Connecticut), and the pump was inserted subcutaneously between the scapulae. The outside of the pump and cannula were then rinsed with 0.1 $\mu\text{g}/\text{mL}$ gentamicin in 0.9% saline.

Laminin tract injection

Using a modification of the method described in Zhou and Azmitia (1988), a narrow cannula tract containing 1 μ L of purified mouse laminin (isolated from Engelbreth-Holm-Swarm (EHS) mouse sarcoma; Chemicon; 0.025 μ g in 0.05 M Tris HCl, pH 7.4 with 2 mM EDTA (Sigma)) or vehicle was injected above the rostral migratory stream in adult mice. The cannula was inserted, in mm from Bregma, to the following coordinates: RC 1.7, ML -0.7, and DV -4.0 (Franklin and Paxinos, 1997), and left for three minutes. The cannula was drawn dorsally, over a three minute period to DV -2.0 mm, while dispensing either laminin or vehicle, thereby leaving a narrow, 2 mm long tract (Figure 3.6A). After two more minutes, the cannula was slowly retracted. These mice were analyzed after eight days.

5-Bromo-2'-deoxyuridine (BrdU) administration

To label proliferating cells, mice received intraperitoneal (i.p.) injections of BrdU (200 mg/kg; Sigma) dissolved in 0.9% saline (Miller and Nowakowski, 1988). Animals receiving peptide or vehicle infusion via the osmotic pumps received single daily BrdU injections on days three to five after surgery, two daily injections on days six through 13, and six hourly injections on the day prior to sacrifice. Animals receiving a single bolus of blocking antibodies received daily BrdU injections for three days after surgery, and those receiving an injected laminin tract received BrdU once per day for five days, starting three days after surgery.

Histology and analysis

On the day following the last BrdU administration animals were again anaesthetized and transcardially perfused with 20 mL cold phosphate-buffered saline and 20 mL cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were post-fixed for 24 hours, cryoprotected in 30% sucrose in 0.1 M phosphate buffer, and coronal, horizontal, or sagittal sections of 30 μ m were cut on a freezing microtome and collected in sodium azide-containing Millonig's buffer. Every sixth section of tissue was used for each immunocytochemical procedure.

DNA denaturation for BrdU immunocytochemistry followed the procedure described in Kuhn *et al.* (1996). A monoclonal anti-BrdU antibody was used to label proliferating cells (1:1,000; Chemicon) followed by a secondary horse anti-mouse antibody (1:600; Vector). Labeling of α 6 β 1 (1:3,000, mouse monoclonal, Chemicon), Laminin A-B chain (1:100, Chemicon), Laminin α 2 chain (1:100, Alexis, San Diego, California), PSA-NCAM (1:5; monoclonal supernatant; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa), and TuJ1 (1:3,000; mouse monoclonal, Babco, Richmond, California) was followed with appropriate secondary antibodies (horse anti-mouse IgG or goat anti-rat IgG, 1:400 (Vector)), or goat anti-mouse IgM for PSA-NCAM (1:400; Cappel, West Chester, Pennsylvania).

All tissue was blindly analyzed with the observer unaware of control or experimental group. Cell profiles were counted at 400X total magnification (40X objective), and total neuroblast number was estimated as the number of profiles

counted \times [section thickness/(section thickness + average neuroblast diameter)] \times section interval (Abercrombie, 1946). Photomicrographs were produced with a Spot CCD camera attached to a Zeiss Axioplan 2 microscope. Data are presented as means \pm SEM, and differences between groups were assessed using the non-parametric Mann Whitney U-Test with a minimum significance level of $p < 0.05$, unless otherwise indicated.

RESULTS

The $\alpha6\beta1$ integrin is expressed in neuroblasts of the rostral migratory stream

A monoclonal antibody was used to assess the expression of the $\alpha6\beta1$ integrin. The entire length of the stream, from the subventricular zone to the olfactory bulb, was positive for $\alpha6\beta1$, with small cell bodies and thin, faintly labeled processes (Figure 3.1A). Fewer cells appeared to express $\alpha6\beta1$ than would express immature neuronal markers such as TuJ1 (Figure 3.1B). In addition, cells found on the more outer regions of the RMS more strongly expressed $\alpha6\beta1$ than did those in the central core of the RMS. Expression of the $\alpha6\beta1$ integrin was also present in astrocytes within the striatum, particularly in those astrocytes most closely associated with insertion of a cannula. In addition, there were occasionally $\alpha6\beta1$ positive astrocytes within the cerebral cortex, and this was also evident in untreated tissue.

Laminin is expressed at low levels along the rostral migratory stream

Laminin is the ligand for the $\alpha6\beta1$ integrin, and its expression levels peak developmentally, and rapidly decline in most CNS regions by the onset of adulthood (Liesi, 1985). Levels of laminin A-B chain expression were extremely faint along the length of the rostral migratory stream, but these levels of expression were higher than that in the surrounding tissues (Figure 3.1C). Expression of the $\alpha2$ chain of laminin was slightly more prominent along the RMS

Figure 3.1. Expression of the $\alpha6\beta1$ integrin and its substrate, laminin

(A) The $\alpha6\beta1$ integrin is expressed within cells of the rostral migratory stream. Note that the expression of $\alpha6\beta1$ integrin is less dense than that of other markers for migrating immature neurons, such as TuJ1 (B). (C) Laminin A-B is not found at significant levels in the adult CNS, but expression of laminin $\alpha2$ is more notable in the RMS (D). Staining for $\alpha2$ laminin is evident with an antibody which recognizes rabbit. Shown in (E) is the rostral migratory stream labeled with the 2G9 antibody against $\alpha2$ laminin, from Hagg *et al.* (1997). Scale bar in A for A, B and D, 100 μm . Scale bar in C, 100 μm .



Figure 3.1

(Figure 3.1D). Although antibody staining of the $\alpha 2$ chain of laminin in mouse was fairly weak, it was noted that a different anti- $\alpha 2$ antibody does label laminin $\alpha 2$ chain in rabbit. This 2G9 antibody, however, does not recognize mouse laminin, and therefore a representative photomicrograph from Hagg *et al.* (1997) is also presented (Figure 3.1E).

Blockade of endogenous integrins disrupts the rostral migratory stream

We assessed the endogenous role of integrins in guiding neuroblast migration to the olfactory bulb by injecting, above the rostral migratory stream, biologically active antibodies against the $\alpha 6$ or $\beta 1$ integrin subunits (Figure 3.2A). Compared to IgG controls (Figure 3.2B), antibodies against either integrin subunit led three days later to a significant disruption in the integrity and direction of the stream, as shown by TuJ1 immunoreactivity. Infusion of antibodies against the $\alpha 6$ integrin subunit appeared to cause a lack of stained neuroblasts below the injected area (above the rostral migratory stream) (Figure 3.2C). Blocking the $\beta 1$ integrin subunit led to a dispersal of the neuroblasts as well as the loss of cohesive patterns of chains within the leading portion of the stream (Figure 3.2D).

Infusion of a peptide recognized by integrin $\alpha 6\beta 1$ redirects neuroblasts into the striatum

We infused a 15 amino acid peptide (designated P3; Muruyama *et al.*, 1996), which can bind the $\alpha 6\beta 1$ integrin, to draw migrating neuroblasts away

Figure 3.2. Blockade of endogenous integrin subunits disrupts the rostral migratory stream

Antibodies against specific integrin subunits were infused into the parenchyma above the rostral migratory stream, as shown in the schematic diagram in (A). Tissue was analyzed seven days after injections. TuJ1 labeling shows that a bolus injection of control IgG failed to disrupt the integrity of the stream (B), whereas blocking antibodies against the $\alpha 6$ integrin subunit altered distribution of neuroblasts within the stream (C). Blocking antibodies against the $\beta 1$ integrin subunit produced marked disruption of the stream, with chains of neuroblasts losing their cohesion, and cells drifting off from their normal migration route (D). Scale bar in B for B-D, 50 μm . Figure 3.2A after Franklin and Paxinos (1997).

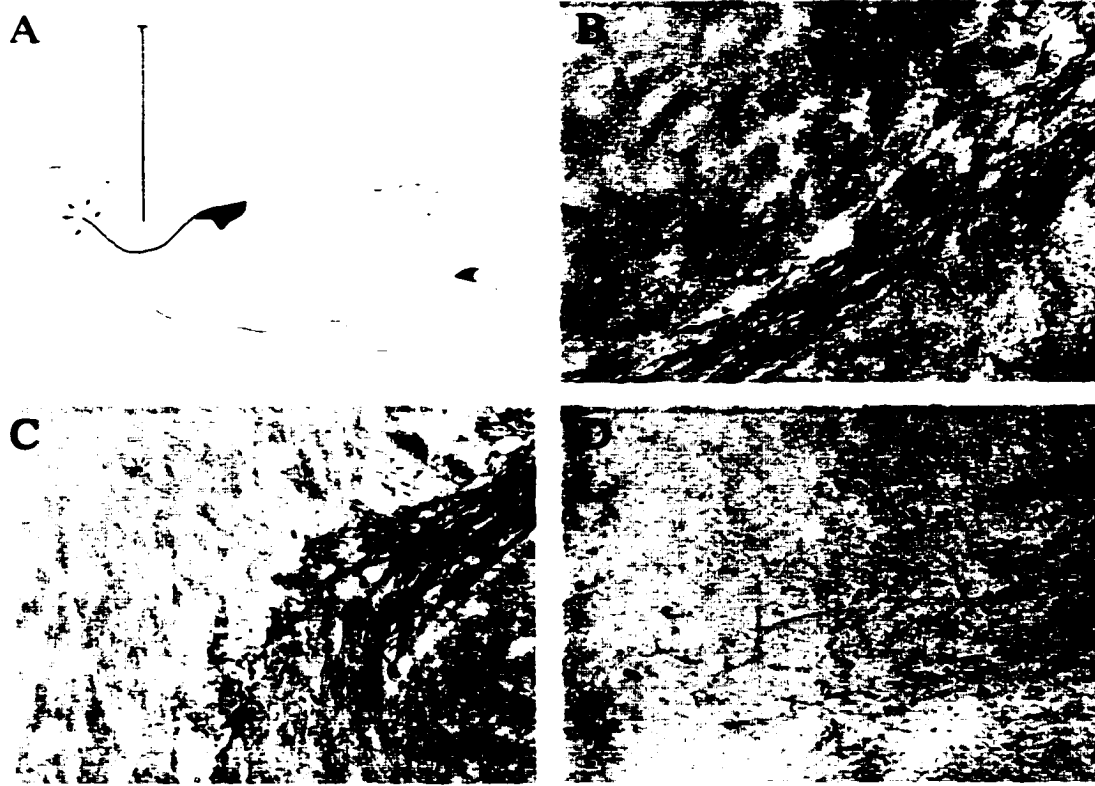


Figure 3.2

from their normal course of movement to the olfactory bulb (schematically illustrated in Figure 3.3A). Compared to control infusions, the P3 peptide was capable of stimulating migration of newly formed neuroblasts into the striatum (Figure 3.4).

Assessment of immunostaining for the polysialylated form of the neural cell adhesion molecule (PSA-NCAM) on newly-formed neuroblasts indicated that in both vehicle and P3-infused animals there was a slight thickening of chains of cells on the infused (Figure 3.4A) versus the non-infused side (not shown). These chains appeared thicker in P3-infused animals. There was also a general trend, seen more prominently in P3-infused animals, towards a thinning of those portions of the RMS which were considerably rostral to the striatal infusion site. In contrast to vehicle-infused animals, numerous PSA-NCAM positive cells were noted between the subventricular zone and the infusion site in P3-infused animals (Figures 3.4B and C). In some cases chains of cells were evident, either apparently drifting away from the subventricular zone or actually connecting portions of the RMS with the infusion site. In addition, many PSA-NCAM positive cells were localized to the infusion site (lateral to the RMS), and were only found on the medial, and never the lateral, side of the infusion site.

The number of PSA-NCAM positive cells between the subventricular zone and infusion site was greater after P3 infusion (44 ± 15 , SEM, $n = 6$, $p < 0.001$, one-tailed Mann Whitney U-Test) than with vehicle (3 ± 2 , $n = 7$) (Figure 3.5A). There were 77 ± 17 PSA-NCAM positive cells in the region of the infusion site in P3-infused animals ($n = 6$) compared to 3 ± 2 cells in vehicle-infused animals (n

Figure 3.3. *Infusion of a peptide or control substance enhances proliferation in the subventricular zone*

A peptide recognized by the $\alpha 6\beta 1$ integrin was infused into the striatum for a total of two weeks, as shown in the schematic diagram (A). Low power horizontal photomicrograph (B), with rostral at the top and caudal at the bottom. Infusion of control vehicle or peptide led to slightly increased cellular proliferation throughout the striatum on the infusion side (shown with labeling for BrdU). In both control and experimental animals there was a slight thickening of the rostral migratory stream on the infusion side (arrows), concomitant with a thinning of extremely rostral portions of the rostral migratory stream, especially in the peptide-infused group. Scale bar for B, 500 μm . Figure 3.3A after Franklin and Paxinos (1997).

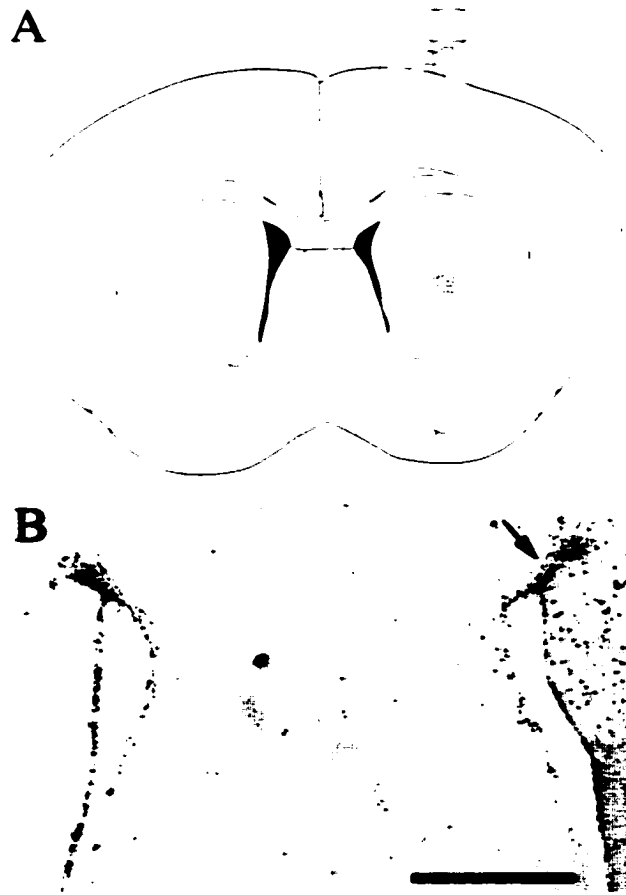


Figure 3.3

Figure 3.4. Infusion of a peptide recognized by the $\alpha6\beta1$ integrin draws neuroblasts into the striatum

Migrating neuroblasts were immunostained with antibodies to either PSA-NCAM (A-C) or TuJ1 (D-F). Compared to vehicle infused controls (A and D), infusion of a 15 amino acid peptide into the striatum led two weeks later to the apparent movement of PSA-NCAM (B) or TuJ1 (E) positive neuroblasts towards the infusion site (marked with asterisks in each figure). Such cells were found both between the subventricular zone or the rostral migratory stream and the infusion site. Neuroblasts *en route* from the rostral migratory stream towards the infusion site extended their processes over considerable distances (seen in higher magnification photomicrographs C and F). Scale bar in A for A, B, D and E, 50 μm ; scale bar in C, for C and F, 20 μm .

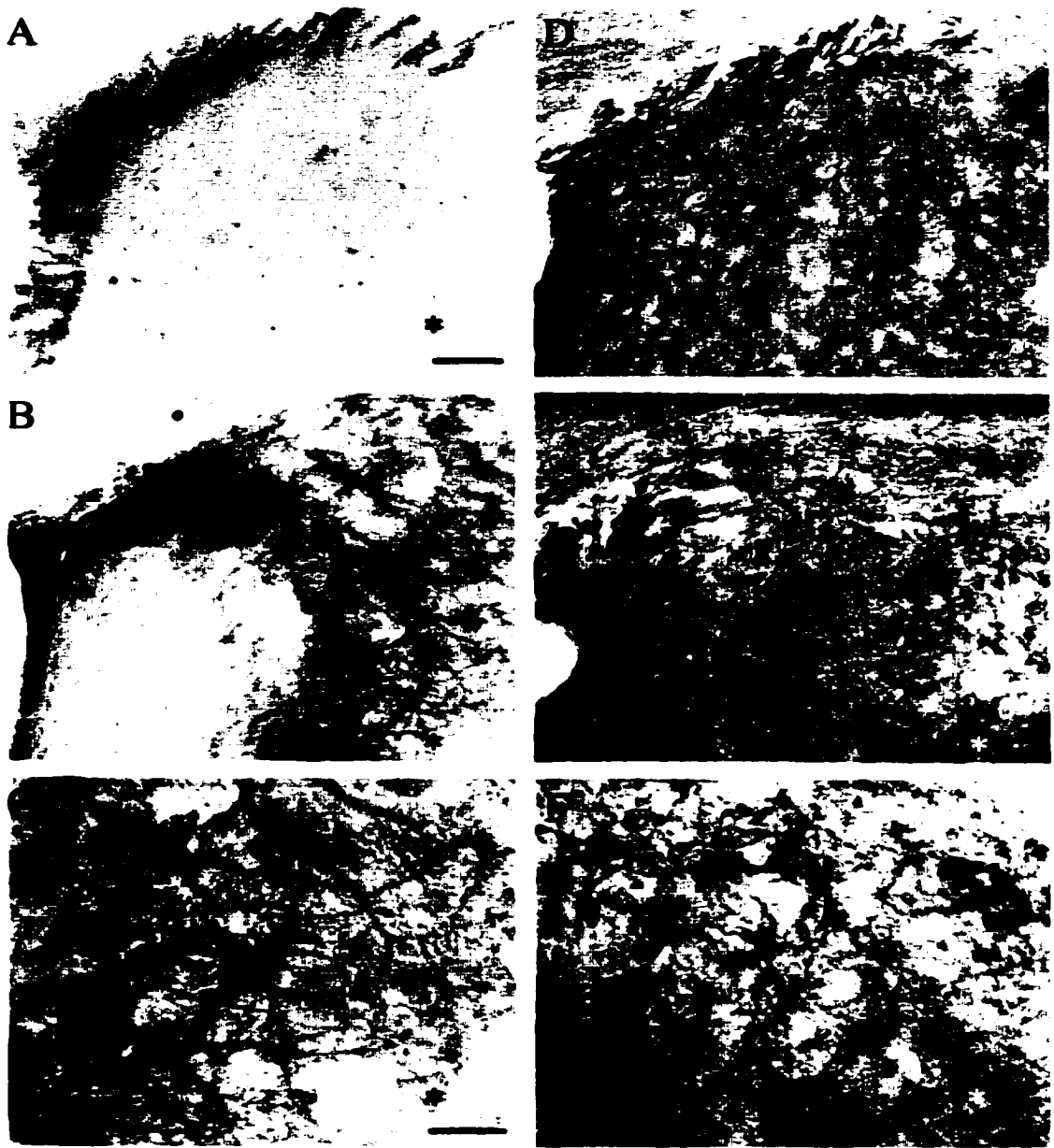


Figure 3.4

Figure 3.5. A peptide recognized by the $\alpha6\beta1$ integrin draws a significant number of neuroblasts towards and to the infusion site

(A) After two weeks, significantly more PSA-NCAM positive neuroblasts were found between and at the subventricular zone/rostral migratory stream and the infusion site with P3 peptide compared to vehicle-infused controls ($p < 0.001$). **(B)** The number of TuJ1 positive cells either between or at the infusion site was similarly greater than in controls ($p < 0.001$).

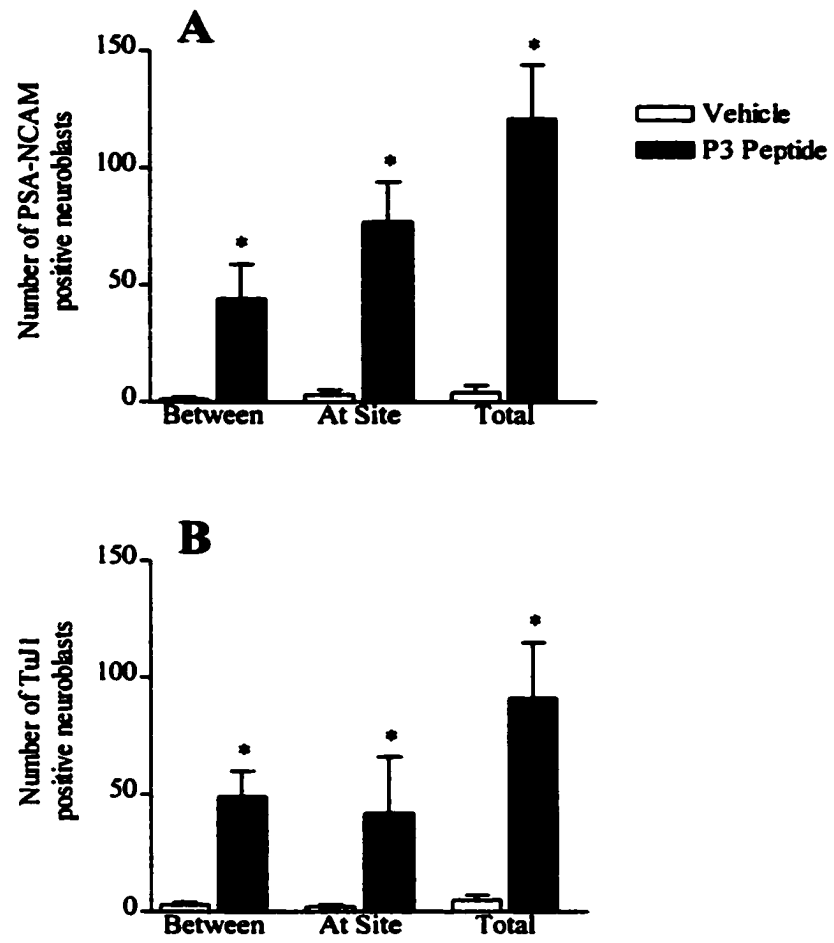


Figure 3.5

= 7, $p < 0.001$). The overall number of PSA-NCAM positive cells drawn away from the subventricular or RMS was greater after P3 infusion (121 ± 23 , $n = 6$, $p < 0.001$) than with infusions of vehicle (4 ± 3 , $n = 7$).

Labeling for the immature neuronal marker TuJ1, directed against class III β -tubulin (Lee *et al.*, 1990; Alexander *et al.*, 1991; Menezes and Luskin, 1994; Gates *et al.*, 1995), revealed a pattern of staining very similar to that seen with antibodies against PSA-NCAM. Briefly, chains of TuJ1 positive cells were evident between the subventricular zone/rostral migratory stream and the infusion site in P3 (Figure 3.4E) but not vehicle-infused animals (Figure 3.4D). As with PSA-NCAM labeling, TuJ1 positive cells were only seen at the medial, and never the lateral, side of the P3 infusion site. Similarly, there was a thickening of chains around the rostral migratory stream on the infusion side in both groups of animals, but thinning of the very rostral portions of the stream was only evident in P3-infused animals.

A significantly greater number of TuJ1 positive cells were found between the subventricular zone and infusion site in P3-infused (49 ± 11 , $n = 6$, $p < 0.001$) versus vehicle-infused controls (3 ± 1 , $n = 7$) (Figure 3.5B). There were 42 ± 24 ($n = 6$) versus 2 ± 1 TuJ1 positive cells at the infusion site in P3 and vehicle-infused animals, respectively ($p < 0.001$). The overall number of TuJ1 positive cells between the subventricular zone, rostral migratory stream, and the infusion site was greater in P3-infused (91 ± 24 , $n = 6$, $p < 0.001$) than in vehicle-infused controls (5 ± 2 , $n = 7$). There were no significant differences between the number of TuJ1 and PSA-NCAM positive neuroblasts.

Bromodeoxyuridine (BrdU) incorporation was used to assess the general level of cellular proliferation. A moderate amount of proliferation was noted within the striatum on both the infused and non-infused sides within both the experimental and vehicle-infused groups. Increased levels of proliferating cells were found clustered around the injection sites in both P3 and vehicle-infused animals (Figure 3.3B). In both groups, there was a slight thickening of the rostral migratory stream on the infusion side, as well as a slightly higher general level of proliferation within the subventricular zone on the infusion side. However, the rostral migratory stream appeared to be thinner on the infusion side at points considerably more rostral to the striatal infusion site. This thinning of the stream was apparent in both horizontal and coronal tissue sections, and occurred more often, and more prominently, in P3-infused animals.

A tract of laminin is sufficient to divert cells from the rostral migratory stream

Laminin is the ligand for the $\alpha 6\beta 1$ integrin, and we assessed whether a narrow, 2 mm tract of laminin could guide migrating neuroblasts away from the rostral migratory stream (Figure 3.6A). BrdU incorporation revealed that in vehicle and laminin-tract injected animals there was cellular proliferation around the injection site, and associated with the length of the cannula tract (Figure 3.6B). In addition, proliferating cells were seen dorsal to the tract, and some thickening of the rostral migratory stream was noted on the injection side. This

Figure 3.6. A laminin tract is sufficient to draw neuroblasts away from the rostral migratory stream

A thin tract of laminin was injected above the rostral migratory stream, shown in the schematic drawing in (A). (B) Immunostaining for BrdU illustrates the position of the tract relative to the rostral migratory stream, and shows a moderate number of proliferated cells within the laminin tract (arrows). PSA-NCAM staining reveals that, compared to vehicle injected controls (C), laminin can cause neuroblasts to be diverted from their normal migration pathway (D, tract position outlined with broken lines). Similar results were noted with TuJ1 immunohistochemistry in control (E) and laminin infused animals (F). Scale bar in B, 100 μm ; scale bar in C for C-F, 50 μm . Figure 3.6A after Franklin and Paxinos (1997).

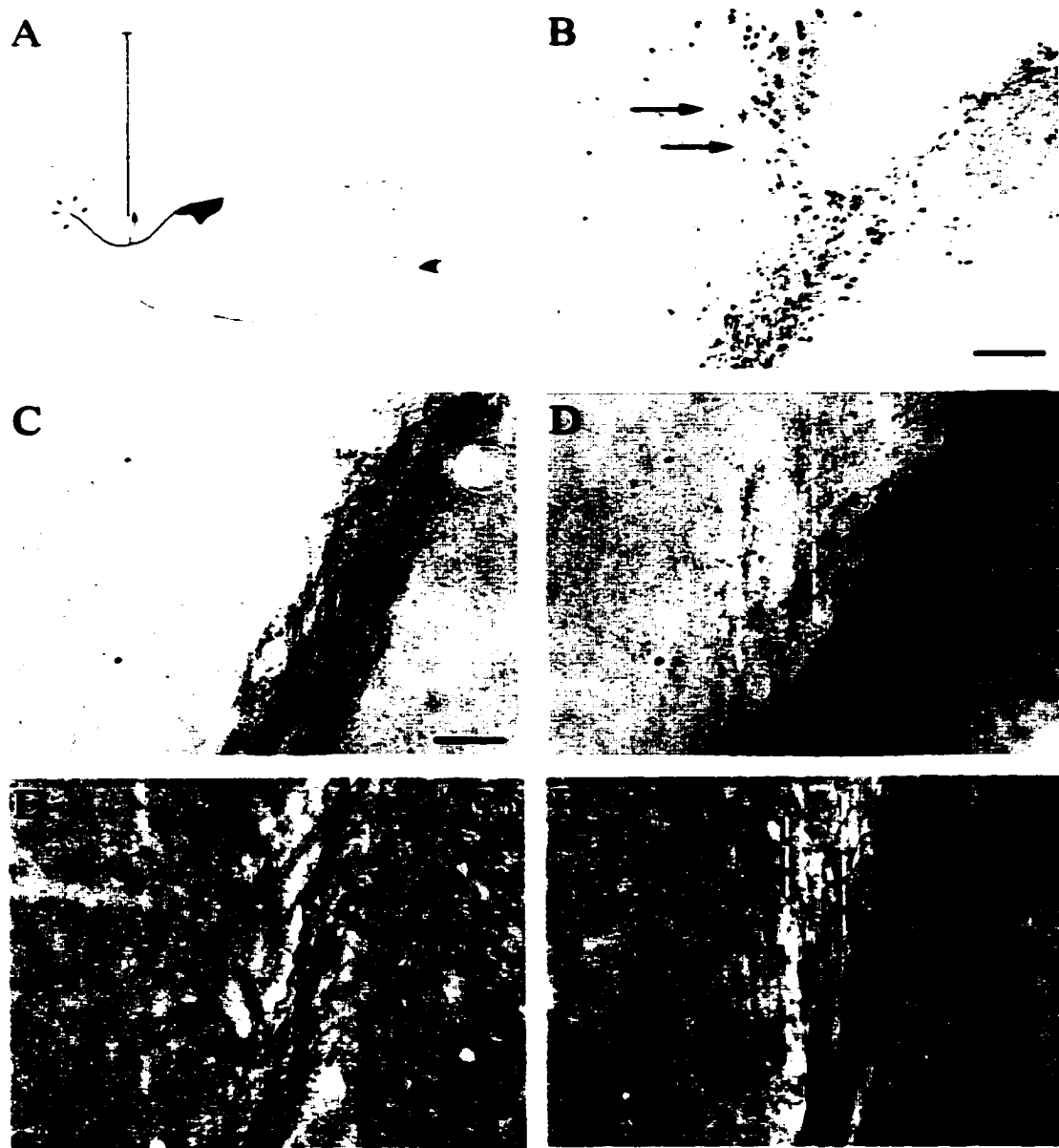


Figure 3.6

thickening was only slightly more pronounced in laminin versus vehicle-injected controls.

The ability of laminin to guide neuroblasts into the tract was assessed with PSA-NCAM and TuJ1 immunoreactivity. In PSA-NCAM stained tissue there were anywhere from 15 to 108 positive cells seen within the laminin tract of each animal (Figures 3.6C and D). TuJ1 labeling in five out of seven laminin-injected brains indicated that there were as few as 15 and as many as 303 TuJ1 positive neuroblasts seen within the tract (Figures 3.6E and F). The greatest distance migrated by neuroblasts within the laminin tract ranged from 300 to 830 μm ($550 \pm 109 \mu\text{m}$, SEM, $n = 4$ sagittally-sectioned specimens), over a course of seven days after delivery of laminin. In one of the six vehicle-injected controls, there were 180 TuJ1 positive neuroblasts diverted into the tract, and those had migrated as far dorsally as 200 μm . However, none of the other control animals had diverted neuroblasts in its tract. Finally, PSA-NCAM and TuJ1 labeling showed thickening of the rostral migratory stream near the injection site, and this was evident only in laminin tract injected animals.

DISCUSSION

The work presented here outlines a critical role for the $\alpha6\beta1$ integrin and its ligand, laminin, in controlling the migration of neuroblasts in the adult CNS. Specifically, we have shown that (1) $\alpha6\beta1$ integrin may be expressed by migrating neuroblasts of the rostral migratory stream, and that the $\alpha2$, but not the $\alpha1$, isoform of laminin is expressed along the length of the RMS; (2) blocking antibodies directed against the $\alpha6$ or $\beta1$ integrin subunits can significantly disrupt neuroblast migration, and (3) a tract of laminin is capable of drawing neuroblasts away from their normal course of migration in a restricted fashion, and that a synthetic peptide recognized by the $\alpha6\beta1$ integrin can be used to redirect migrating neuroblasts into the neostriatum in a global manner.

The $\alpha6\beta1$ integrin and laminin are associated with the rostral migratory stream

More work is required to examine the role of other extracellular matrix molecules besides laminin, and to consider their relative contribution to guiding, enhancing, or inhibiting RMS neuroblast migration. Immunohistochemistry showed here that the $\alpha1$ isoform of laminin is not abundantly present along the RMS. Further work could consider the relative abundance of other isoforms, such as $\alpha3$, $\alpha4$, and $\alpha5$. The RMS travels within a glial tube which is highly enriched with various extracellular adhesion molecules, including Tenascin-C (TN-C), and chondroitin sulfate proteoglycan (CSPG) (Thomas *et al.*, 1996;

Peretto *et al.*, 1997), and therefore the relative contributions of such molecules requires further study.

Staining for the $\alpha 6 \beta 1$ integrin demonstrated that it is present in more external regions of the RMS. This would suggest that neuroblasts in the outer "shell" of the RMS are more involved in controlling the stream's interaction with laminin in the ECM, and that those cells within the inner "core" of the RMS rely upon one another for a migratory substrate. Finally, it has been noted that a number of integrin subunit combinations are present in newly generated neuroblasts (Jacques *et al.*, 1998). Further work might characterize the relative importance of these subunit combinations *in vivo*.

A number of observations within this study present opportunities for further characterization of the role of the extracellular matrix and the integrins in guiding adult neuroblast migration. To examine the endogenous role of the integrins, we blocked individual integrin subunits with biologically active antibodies. Inhibition of the endogenous $\alpha 6$ subunit disrupted migration and inhibition of the $\beta 1$ subunit disrupted the cohesive nature of the RMS. The β subunit is most closely associated with the intracellular cytoskeleton, and it has been suggested that it is most closely involved in aligning cells with the matrix, whereas the α subunit may be more involved in conferring flexibility in ligand binding via the various subunit combinations (Chan *et al.*, 1992). Clearly both subunits are required for interaction with the extracellular matrix, but our results suggest that each subunit plays a unique role in controlling migration. Further, because the $\alpha 6$ and $\beta 1$ subunits appear to have differing roles in migration, it may also be possible that

different integrin subunit combinations which recognize other ECM molecules, such as fibronectin, may be involved in controlling neuroblast migration.

Related to the differences in subunit involvement, it has also been suggested that, in contrast to the $\alpha6\beta1$ integrin isoform, the $\alpha6A\beta1$ isoform is associated with a more migratory phenotype of cells (Shaw and Mercurio, 1994; Domanico *et al.*, 1997). Additional work might address the relative amounts of these two $\alpha6\beta1$ integrin isoforms within the normal RMS, and whether laminin or peptide administration alters their relative abundance.

A peptide recognized by the $\alpha6\beta1$ integrin specifically draws neuroblasts into the neostriatum

The pattern of migration shown with peptide infusion into the neostriatum differs from recent results showing a role for the growth factor TGF α in inducing migration of SVZ-derived cells (Fallon *et al.*, 2000). That study demonstrated that intrastriatal infusion of TGF α leads to extensive proliferation and migration of cells away from the subventricular zone. Specifically, TGF α induced extensive proliferation of cells along the SVZ from its dorsal to ventral plane, and these cells migrated as a wave towards the infusion site. A subset of those cells went on to express neuronal markers and served to improve function in rats with 6-OHDA lesions of the nigrostriatal system. Within our study, it is worth noting that although the peptide generated against $\alpha6\beta1$ integrin did not draw all of the proliferating population from the SVZ, it was more selective in its ability to draw migrating neuroblasts from the rostral SVZ (the SVZa). Clearly, more work is

required to examine the mode of migration of proliferating cells in the Fallon *et al.* (2000) study, and to test the functional significance of neuroblasts drawn into the neostriatum in the study presented here.

The number of diverted neuroblasts, and their rate of travel, remains less than that in the rostral migratory stream

We have shown that a peptide directed against the $\alpha 6\beta 1$ integrin is capable of drawing migrating neuroblasts into the neostriatum. However, it is worth noting that the RMS continues to carry cells from the SVZa to the olfactory bulb. Although the number of cells drawn to this ectopic site is significantly greater than that seen in any of the control conditions, the number of neuroblasts drawn by this peptide is much lower than the overall number of neuroblasts which may be in the RMS at any one time. It may be possible that the number of cells drawn into the striatum would increase over time, because these cells use one another as a migration-inducing substrate (Wichterle *et al.*, 1997). Similarly, the lower number of neuroblasts drawn into the laminin tract may only be the start of a persistent stream into the parenchyma. It may also be possible that a greater number of cells could be drawn into a tract composed of more concentrated laminin.

Related to the observations about overall cell number, it was also noted that the distance by which the diverted neuroblasts moved within the laminin tract was approximately 500 μm . A normal migrating neuroblast within the RMS can travel up to 720 μm per day (Lois and Alvarez-Buylla, 1994). Therefore, over the

course of this 7 day experiment it is possible that a normal neuroblast could travel up to ten times the distance it actually did. There are a few potential explanations for this lesser distance. It may simply be that there was a considerable lag time for any cells to be receptive to the laminin tract, and thus to begin their diverted course of travel. Second, it may have been that the concentration of laminin was not sufficient to afford faster migration. In addition, these cells, like those drawn by the peptide into the striatum, may have been faced with extracellular matrix molecules which work to inhibit (either actively or passively) the course of neuroblast migration. Finally, migrating neuroblasts are capable of reversing their direction of movement *en route* to the olfactory bulb (Kakita and Goldman, 1999). It may therefore be possible that neuroblasts drawn away from the RMS are capable of changing their direction, and could return to their normal route of migration.

It has already been mentioned that diverted neuroblasts move through the parenchyma at a rate which is considerably lower than their normal rate along the RMS. This observation also highlights an apparent discrepancy between the migrational capacity of endogenous cells and those which have been transplanted. It appears that the endogenous neuroblasts drawn to novel sites with either laminin or with integrin-specific peptides are less capable of traversing the CNS parenchyma than are transplanted stem cells. Transplanted neural stem cells or their progeny have a high capacity for migration, either in response to epidermal growth factor (EGF) (Fricker-Gates *et al.*, 2000) or in crossing the blood-brain barrier and migrating through the CNS to surround a tumour mass

(Aboody *et al.*, 2000). The significance of such an apparent discrepancy requires further study. It may be that the environment within the CNS actively inhibits migration via signals to which transplanted neural stem cells are not responsive. In addition, it may be that transplanted cells, raised *in vitro*, have a greater inherent propensity for migration.

Laminin upregulation in CNS injury

Both integrins and laminins can be upregulated as a result of CNS injury. It is possible that integrins might play a role in endogenous CNS repair, as it has been shown that $\beta 1$ integrin mRNA levels rise during seizure (Pinkstaff *et al.*, 1998). In addition, reactive astrocytes can significantly increase the production of laminin in response to a mechanical injury (Liesi *et al.*, 1984). Within one control animal receiving a vehicle injection above the RMS (instead of laminin), it was noted that some neuroblasts were diverted along this mechanically produced tract. It may be possible that the mechanical stimulation induced laminin expression by reactive astrocytes, and that this was sufficient to draw cells a short distance.

Conclusions

We have demonstrated here that laminin and the integrins are critical for guiding neuroblast migration *in vivo*. Specifically, the $\alpha 6\beta 1$ integrin and its ligand, laminin, appear to be involved in maintaining the integrity of the RMS as it travels from the SVZa to the olfactory bulb. Further, we provide evidence that

exogenous sources of laminin, or peptides specific to the $\alpha6\beta1$ integrin, may be employed in drawing endogenous neuroblasts to degenerating CNS regions. These findings might prove highly effective within therapeutic neural repair strategies. Specifically, one could use a peptide to redirect neuroblasts in a global fashion, or use laminin either to guide endogenous cells in a restricted manner, or to sequester transplanted neural stem cells within a particular CNS region.

CHAPTER 4:

RETROGRADE TRACING TECHNIQUES INFLUENCE REPORTED DEATH RATES OF ADULT RAT NIGROSTRIATAL NEURONS

The following chapter has been published as:

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ABSTRACT

Injury often causes loss of neuronal markers and prior retrograde labeling can circumvent this problem of identification. We have previously used a time-consuming protocol for labeling all dopaminergic substantia nigra pars compacta neurons in adult rats by injecting the fluorescent tracer Dil into 6 sites throughout each neostriatum. Here, two weeks after injection of Dil into 2 central locations, only half of these nigrostriatal neurons were labeled. With 6 sites, more medial and lateral neurons were labeled, and also more in the midportion along the medial-lateral extent of the pars compacta. Less than 0.5% of the contralateral neurons were labeled. Two injections of Fluorogold also labeled fewer neurons, but their morphology was clearer. Two to four weeks after injection of the neurotoxin 6-OHDA into the 2 neostriatal sites, the total number of surviving neurons appeared greater with 6 sites of Dil than with 2. However, within the middle region of the nigra, survival was lower with the 6 sites. This suggests that neurons that project outside the 2 central striatal tracer and 6-OHDA injection regions may be spared initially, but that those in the midportion that project to the central region are more vulnerable with the 6 site protocol. Some reports suggest that Fluorogold pre-labeling increases neuronal death. Here, survival after 6-OHDA or axotomy was similar with Dil or Fluorogold. These results suggest that because of a complex projection pattern of the nigrostriatal neurons, detailed quantification of neuronal survival should rely on extensive labeling. However, for drug screening purposes, faster labeling with Fluorogold using 2 sites is more suitable and should provide reliable data.

INTRODUCTION

Dopaminergic neurons of the substantia nigra pars compacta (SNc) project to the neostriatum (caudate-putamen), and degenerate in Parkinson's disease. The majority of these neurons project unilaterally, but there are reports that in adult rats between 1 and 10% project to the contralateral neostriatum (Fass and Butcher, 1981; Gerfen *et al.*, 1982; Loughlin and Fallon, 1982; Berger *et al.*, 1991; Brecknell *et al.*, 1995). Various animal models exist for mimicking some of the cellular and behavioural effects of Parkinson's disease, including axotomy by nigrostriatal (medial forebrain bundle) transection (Hagg and Varon, 1993; Brecknell *et al.*, 1995; Lu and Hagg, 1997), and administration of the neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Langston, 1987; Mokry, 1995) or 6-hydroxydopamine (6-OHDA or 2,4,5-Trihydroxyphenethylamine; Javoy *et al.*, 1976; Sauer and Oertel, 1994; Przedborski *et al.*, 1995; Schwarting and Huston, 1996). After axotomy or neurotoxin injuries, neurons lose their phenotypic markers by which they are normally recognized, a process that in various systems is often not followed by neuronal cell death (Hagg *et al.*, 1988, 1989; Lams *et al.*, 1988). Most nigrostriatal neurons die after these injuries (Hagg and Varon, 1993; Sauer and Oertel, 1994; Winkler *et al.*, 1996) but some treatments that result in improved survival do not prevent the injury-induced reduction in phenotypic markers (Hagg and Varon, 1993; Lu and Hagg, 1997; Hagg, 1998). More classical histological stains such as cresyl violet, which labels ribosomes, are not reliable, as neurons

undergo chromatolysis with ribosome dispersion, making identification of neurons difficult.

To circumvent this problem of neuronal identification, many investigators have relied on retrograde labeling of neuronal cell bodies with injections of tracers into the innervation territory. Thus, all nigrostriatal neurons can be labeled by stereotaxic injections of the fluorescent tracer Dil (1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine) into 6 sites throughout the neostriatum of adult rats (Hagg and Varon, 1993). However, the injection procedure is very time-consuming, Dil is insoluble in aqueous media, and because Dil is transported passively within the plasma membrane, it requires 10-14 days after injection before sufficiently clear labeling of cell bodies is achieved. Others have used the faster tracer Fluorogold, typically injected into one or two neostriatal sites, to pre-label the nigrostriatal neurons (Victorin *et al.*, 1989; Sauer and Oertel, 1994; Brecknell *et al.*, 1995; Nikkah *et al.*, 1995), or else have employed horseradish peroxidase (HRP) to label subpopulations of nigral neurons (Morgan *et al.*, 1991; Mendez and Hong, 1997). The total number of neurons that are labeled with the 6-site method (Hagg and Varon, 1993; Lu and Hagg, 1997) appears to be greater than that seen with fewer sites of Fluorogold (Sauer and Oertel, 1994). This is understandable, as dopaminergic projections from the SNc to the neostriatum tend to be arranged in both medial-to-lateral and anterior-to-posterior topographical patterns. For example, neurons in medial and anterior regions of the SNc would project to more medial and anterior regions of the neostriatum (Fallon and Moore, 1978). Therefore, it would be expected that

injection into a small region within the neostriatum would only label a discrete and topographically corresponding subpopulation of neurons within the SNc. There have been a few anecdotal and published reports suggesting that Fluorogold pre-labeling is toxic and increases the extent of neuronal loss after injury (Schmued and Fallon, 1986; Garrett *et al.*, 1991; Wessendorf, 1991).

Models of Parkinson's disease are often employed to examine the efficacy of various potential treatments. Such treatments could include administration of neurotrophic factors, or transplantation of neural stem cells or their progeny. It is therefore critical to establish the effects that retrograde tracing techniques, as described above, can have on the reported efficacy of various therapeutic strategies. To determine the effect of labeling technique on reported cell death, the extent of labeling of normal SNc cells using the more time-consuming 6-site Dil injection protocol was compared with one involving injection of Dil into 2 central sites of the neostriatum. The labeling efficiency of 2 sites of Dil with 2 sites of Fluorogold was also examined. These retrograde labeling methods were evaluated for their effects on SNc neuronal survival after injury by 6-OHDA or axotomy.

MATERIALS AND METHODS

All animal procedures were conducted in accordance with protocols approved by Dalhousie University and Canadian Council on Animal Care guidelines. Efforts were undertaken to minimize the number of animals used in this study. For all invasive procedures, female Sprague-Dawley rats (225-250 g; Charles River, St. Constant, Québec) were anaesthetized with a mixture of ketamine (62.5 mg/kg), xylazine (3.25 mg/kg), and acepromazine (0.62 mg/kg) in 0.9% saline. A total of 62 rats were used in this study (n = 9 for Dil dissolving tests; n = 12 for assessing normal labeling patterns; n = 29 for 6-OHDA studies; and n = 12 for medial forebrain bundle transection studies).

Retrograde labeling with Dil or Fluorogold

Dil is not water-soluble, and previous studies have employed combinations of alcohols, DMSO, and phosphate buffered saline to dissolve Dil prior to injection. To compare the effectiveness of different solvents for Dil, a few rats received 6 injections into the neostriatum of 0.3% Dil (2 μ L/site; Molecular Probes, Eugene, Oregon) dissolved in 0.1 M PBS/10% ethanol, 100% ethanol, or analytical grade DMSO (Sigma). The 470 μ m diameter injection needle was inserted to a predefined ventral-most coordinate, and Dil was slowly released while pulling up the cannula to the dorsal-most predefined coordinate. The coordinates (in mm from Bregma; (Paxinos and Watson, 1982) for the six sites were (1) RC 1.6, ML -1.7, DV -5.8/-4.4; (2) RC 1.3, ML -3.0, DV -6.4/-4.2; (3)

RC 0.3, ML 2.3, DV -7.0/-3.7; (4) RC 0.1, ML 4.0, DV -6.8/-4.4; (5) RC -1.2, ML -4.5, DV -7.0/-5.0; and (6) RC -2.8, ML -5.0, DV -6.6/-6.0) (Hagg and Varon, 1993). In most animals, labeling was performed on both sides. For the remainder of the rats with the 6-site injection protocol, Dil was dissolved in 100% ethanol.

For the 2 site injection method, 0.3% Dil was diluted in 2 μ l 100% ethanol per site and injected over two minutes per site at coordinates (1) RC 0.5, ML -2.5, DV -4.5; and (2) RC -0.5, ML -3.5, DV -4.5, as described elsewhere (Nikkah *et al.*, 1995). Fluorogold injections (2% in 0.9% saline, 0.2 μ L/site; Fluorochrome, Inc., Denver, Colorado) were at the same 2 coordinates.

6-OHDA injection and axotomy

Two weeks after retrograde tracer injection, a set of rats was again anaesthetised and received unilateral injections of 6-OHDA (Sigma), dissolved in 0.3% L-ascorbic acid (Sigma) in saline, at 20 μ g/site (2 μ L/site) at coordinates (1) RC 0.5, ML -2.5, DV -5.0; and (2) RC-0.5, ML -4.2, DV -5.0 (Lee *et al.*, 1996).

Two weeks after retrograde tracer administration, another set of rats was again anaesthetized and received a complete transection of the right medial forebrain bundle, including the nigrostriatal pathway. A Scouten knife (Kopf Instruments, Tujunga, California) was stereotactically guided to 3.6 mm caudal from Bregma, 2.8 mm lateral, and 6.2 mm ventral, with the tooth bar set at -3.3 mm. The blade was expressed to a length of 2.5 mm and then lowered to 8.7 mm ventral from Bregma, via a slight dorso-ventral sawing action. The blade

was retracted, expressed again, and pulled up to 6.2 mm ventral from Bregma. The blade was then fully retracted, and the assembly retracted from the brain.

Histology and neuronal counts

Two weeks after the tracer injections (non-injured rats) or two, four, or six weeks after the lesion, animals were again anaesthetized and transcardially perfused with 100 mL of cold phosphate-buffered saline and 250 mL of cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Brains were post-fixed for 24 hours, cryoprotected in 30% sucrose in 0.1 M phosphate buffer, and coronal sections of 30 μm were cut on a freezing microtome and collected in sodium azide-containing Millonig's buffer. Every sixth section throughout the substantia nigra was mounted on gelatin-covered slides and coverslipped in Fluoromount (BDH, Toronto, Canada). To quantify the relative number of SNc neurons labeled with the various techniques, two independent investigators counted the number of cell body profiles larger than 9 μm in longest diameter along the rostral to caudal extent of the SNc (Hagg and Varon, 1993). The total number of counted neurons (profiles) was calculated by summing the numbers from all sections. The percentage of surviving neurons on the ipsilateral side was calculated by normalizing with the number of labeled neurons on the non-lesioned contralateral side. To analyze a portion of the substantia nigra which appeared to be labeled similarly with all tracing methods, a 500 μm stretch along the medial to lateral extent of the compacta region (~ one third), half-way along the rostral to caudal extent of the nigra (540 μm from the rostral end) was

selected. To assess the extent of contralateral nigrostriatal projections, neuronal cell bodies were counted on the contralateral side of four "non-lesioned" control animals which had received six unilateral striatal Dil injections. The numbers were compared for statistically significant differences using the one tailed, non-parametric, Mann-Whitney U test with a significance level of p smaller than 0.05. To confirm completeness of the transections, three sections rostral to the injury from each transected animal were processed for immunostaining with a monoclonal antibody against tyrosine hydroxylase (1:40,000; Chemicon, Temecula, California) combined with a sensitive ABC-DAB method (Hagg and Varon, 1993; Lu and Hagg, 1997).

RESULTS

More injection sites label more neurons in “non-injured” rats

The efficacy of various media for dissolving Dil was examined. Dil dissolved most readily in 100% ethanol, followed by 100% analytical grade DMSO, followed by 10% ethanol in 0.1 M phosphate buffered saline. With the latter method, Dil tends to precipitate, which may result in uneven distribution of the injection in the neostriatum. The precipitates can also clog the injection needle, if the needle is not flushed often. Following the injections of the various solvents, the animals did not appear to behave abnormally, even with the 100% ethanol injections. Histological examination of the neostriatum did not reveal observable differences around the injection sites, i.e., with all solvents a small (~150 μm) diameter cavity had formed around each of the sites. There were no appreciable differences in the total number of counted cell profiles across the SNc or the morphology of the neuronal cell bodies.

Labeling with 6 sites of Dil was more extensive than with 2 sites, especially in the lateral portion of the SNc and in the ventral tegmental area (VTA) (Figure 4.1A vs. 4.1B and 4.1C). The morphology of the substantia nigra neuronal cell bodies and processes was more clearly defined after Fluorogold labelling (Figures 4.1C and 4.1F) than with either 2 (Figures 4.1B and 4.1E) or 6 (Figures 4.1A and 4.1D) Dil sites. The morphology of neurons observed with the 2 or 6 sites of Dil appeared similar.

Figure 4.1. Six injections of Dil in the neostriatum labels more neurons in the lateral regions of the SNc and VTA than two sites of Dil or Fluorogold

All labeling shown here is in non-injured rats. (A) SNc labeling with six sites of Dil. Medial is to the left in all figures. (B) SNc labeling with two sites of Dil. Note the fewer neurons, especially in the VTA (asterisk) and lateral region of the SNc. (C) SNc labeling with two sites of Fluorogold. Note that Fluorogold is superior to Dil for labeling fine neuronal processes. (D,E,F) Higher magnification of cells shown in (A, B, and C), respectively. Scale bar in A = 200 μm for A-C; scale bar in D = 50 μm for D-F.

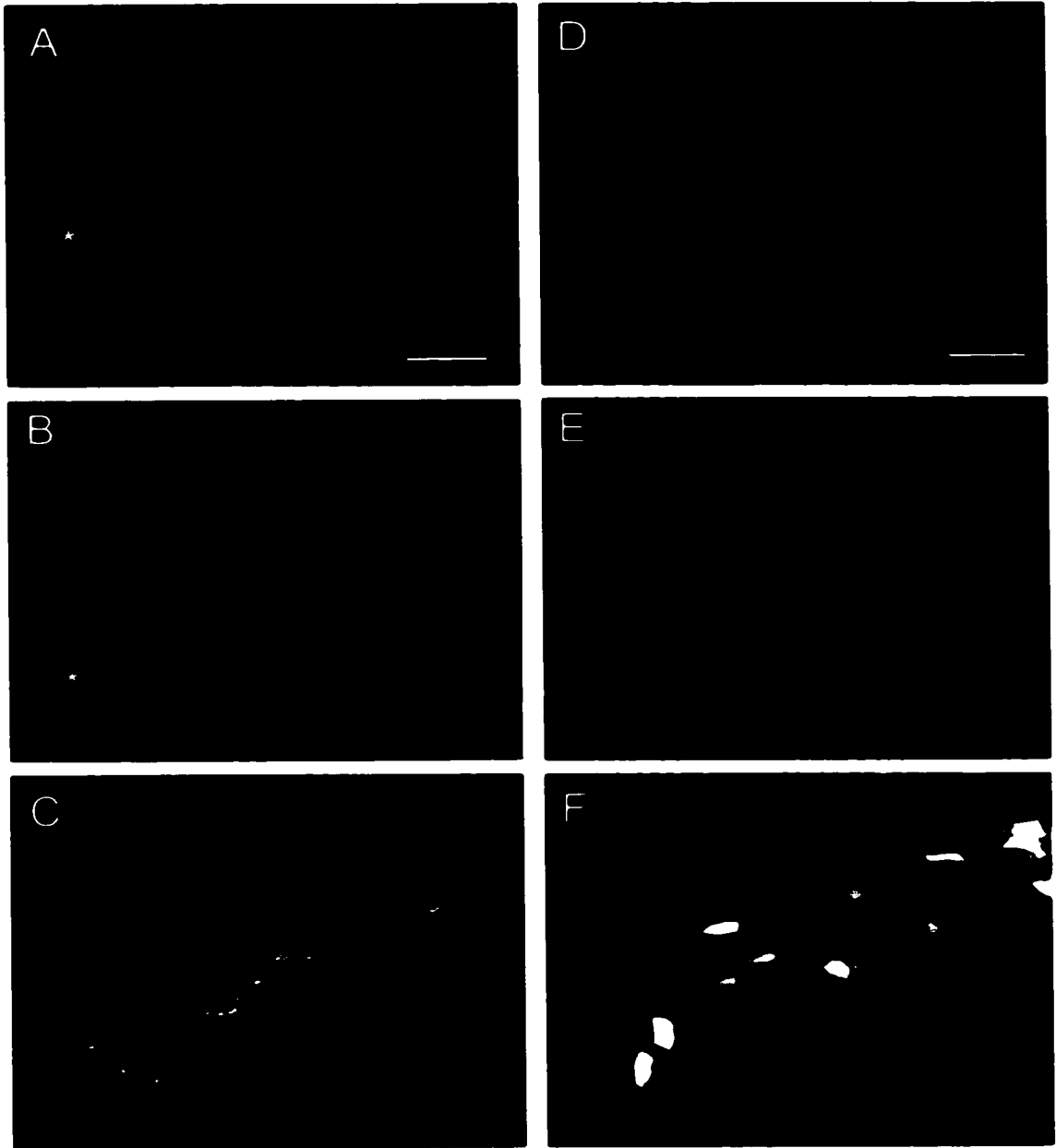


Figure 4.1

With the 6-site Dil injection protocol (in 100% ethanol), the distribution of labeled neurons was similar to that reported previously (Figure 4.2A; (Hagg and Varon, 1993)). An average of 4 SNc neurons (individual values: 1, 3, 5, 6; ~0.4% of the total) were found on the contralateral side of the unilateral Dil- injected control rats. These sparse neurons were located throughout the substantia nigra. This number is consistent with a previous report (Fass and Butcher, 1981), but notably lower than that suggested in other reports (Loughlin and Fallon, 1982; Brecknell *et al.*, 1995). A similar low number of labeled neurons was seen in the contralateral ventral tegmental area. The rostro-caudal distribution of labeled neurons per section seen with 2 sites of Dil or 2 sites of Fluorogold appeared similar as with the 6 sites, but the number of neurons per section was always smaller (Figure 4.2A). When the number of neurons was calculated as a percentage of those labeled with the 6-site method, the amount of labeling with 2 sites of Dil appeared to be less midway between the rostral and caudal regions of the substantia nigra (Figure 4.2B). The total number of neurons labeled with 6 sites of Dil pre-labeling was 995 ± 45 , SEM, $n = 4$, which was more than twice the number labeled with 2 sites Dil (440 ± 21 , $n = 4$ $p < 0.025$) or with 2 sites FG (339 ± 4 , $n=4$ $p < 0.025$)

The number of neurons within the middle one-third region of the SNc (along its medial to lateral extent) was examined in a specific section part way along the rostral to caudal aspect of the SNc (section number 18). The number of neurons labeled with the 6-site method was significantly greater than that in the 2 site Dil method or the 2 site Fluorogold method (52 ± 3 , $n=4$ vs. 32 ± 2 , $n=4$

Figure 4.2. Six sites of Dil labels more neurons across the rostral to caudal extent of the SNc than does two sites of Dil or Fluorogold

(A) Presented are number of neurons per section (\pm SEM) along the rostral to caudal extent of the SNc for 6 site Dil (closed circles), 2 site Dil (open circles), and 2 site FG (triangles). Section number 0 = rostral pole of the SNc, section number 54 = caudal pole of the SNc. (B) The number of 2 site labeled SNc neurons calculated as a percentage of the number of 6 site labeled SNc neurons.

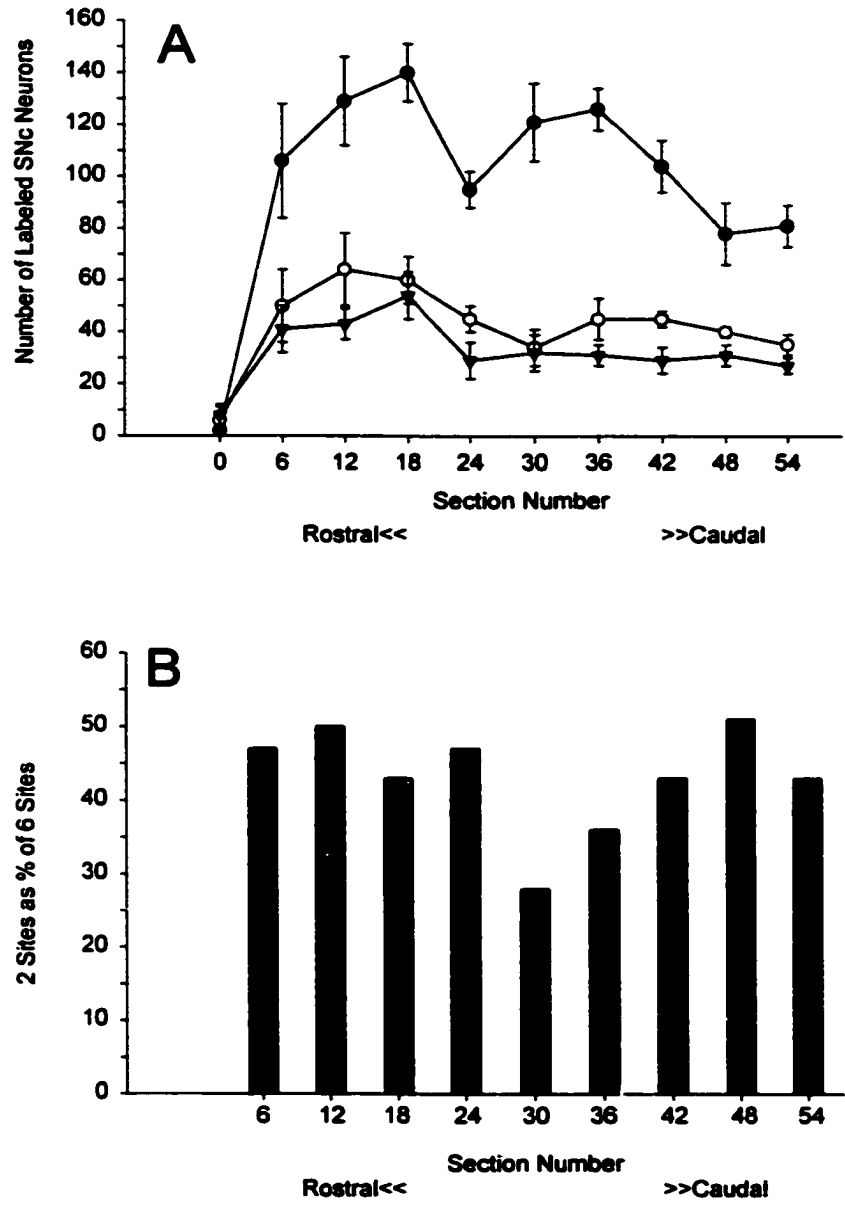


Figure 4.2

$p < 0.025$ and 28 ± 4 , $n=4$; $p < 0.025$). There was no statistically significant difference between the number of neurons labeled with 2 sites of Dil versus those labeled with 2 sites of Fluorogold in this region.

Cell death appears to vary with retrograde tracing method and number of injection sites

Two weeks after 6-OHDA administration, the percentage of neurons that was detectable in rats that had been pre-labeled with 6 sites of Dil was greater than that of rats pre-labeled with 2 sites of Dil ($79 \pm 7\%$, $n = 4$ vs. $65 \pm 2\%$, $n = 4$; $p < 0.05$; Figure 4.3). After four weeks, the survival with the 6 site method was also greater compared to the 2 site method ($57 \pm 5\%$, $n = 7$ vs. $44 \pm 5\%$, $n = 3$; $p < 0.05$). The survival seen with 2 sites of Dil was not statistically different from 2 sites of Fluorogold at two or four weeks after 6-OHDA administration ($65 \pm 2\%$, $n = 4$ vs. $69 \pm 1\%$, $n = 3$; and $44 \pm 5\%$, $n = 3$ vs. $51 \pm 3\%$, $n = 4$; Figure 4.3).

The rate of neuronal loss over the 6 weeks after 6-OHDA appeared linear in the rats with the 6 sites of Dil (Figure 4.3). With the 2 labeling sites (Dil or Fluorogold) the rate of neuron loss appeared greater over the first two weeks than at later times. The rate of cell death after 6-OHDA seen with one site of Fluorogold labeling in a previous report (Sauer and Oertel, 1994) was slightly greater than that seen here with 2 sites.

Because the 6 site method labeled more medial and lateral neurons, we analyzed a smaller mid-region at a set distance along the rostral to caudal extent of the SNc. The neurons in that region presumably all project to the mid-portion

Figure 4.3. *The rates of neuronal loss following 6-OHDA appears more linear after pre-labeling with six sites of Dil*

Presented are the percentages (total number compared to the contralateral side) of surviving neurons labeled by six sites Dil (closed circles), two sites Dil (open circles), or two sites Fluorogold (triangles). The survival values seen after a one site FG injection (open squares) were taken from Sauer and Oertel, 1994.

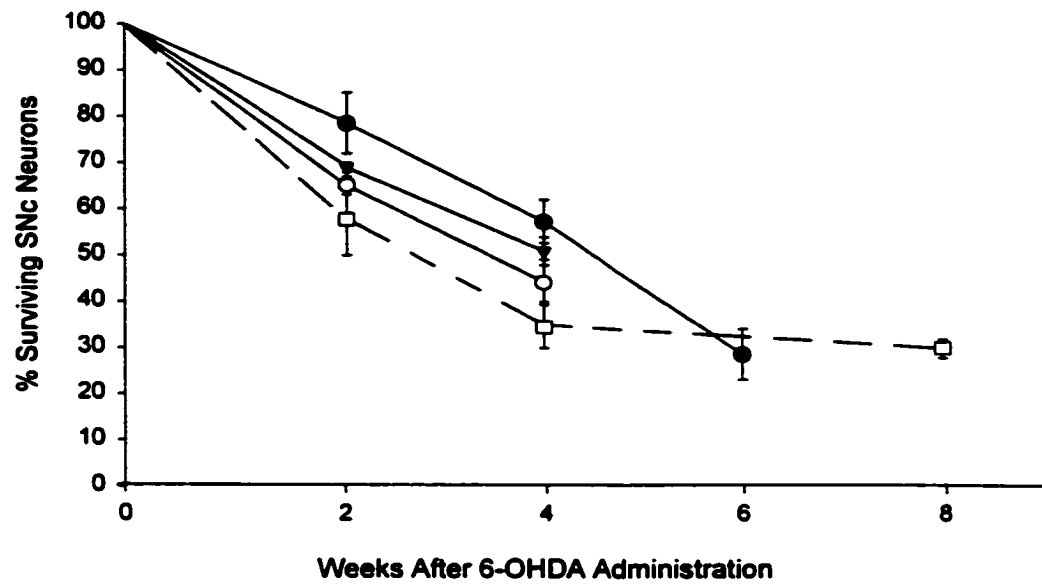


Figure 4.3

of the neostriatum which received the tracing in all cases and which was injected with 6-OHDA. After two weeks, the survival in this middle region was significantly lower for neurons pre-labeled with 6 sites of Dil compared to those pre-labeled with 2 sites of Dil or Fluorogold ($p < 0.05$; Figure 4.4A). The absolute number of surviving neurons with the 6 site method (25 ± 2 , $n = 4$), did not differ significantly from the number of neurons with the 2 site method (22 ± 3 , $n = 4$). The morphology of the surviving neurons also appeared similar in this mid-region, two weeks after the 6-OHDA lesion (Figure 4.5A vs. 4.5B), and labeling with FG was superior in that it showed the processes of dying neurons.

Two weeks after medial forebrain bundle transection, the survival of neurons labeled with 2 sites of Dil was less ($28 \pm 3\%$, $n = 4$) than with 2 sites of Fluorogold ($45 \pm 4\%$, $n = 4$; $p < 0.05$; Figure 4.4B), but comparable to that reported with the 6 site method ($33 \pm 6\%$, $n = 4$) (Lu and Hagg, 1997). Survival within the middle one-third region of the SNc was not significantly different between the rats with 2 sites of Dil or Fluorogold ($50 \pm 3\%$, $n=4$ vs. $43 \pm 3\%$, $n=4$; Figure 4.4B). As with cells treated with 6-OHDA, FG was especially effective in showing processes of dying cells. A greater amount of debris was also noted within the FG labeled tissue.

Figure 4.4. Reported neuronal survival varies depending on method of pre-labeling

(A) Survival two weeks following 6-OHDA administration appears greater for the entire SNc pre-labeled with six sites of Dil (solid bars) than for those labeled with two sites of Dil (open bars) or Fluorogold (gray bars). However, survival within the middle one-third of the SNc appears lower for neurons pre-labeled with six sites of Dil. **(B)** Neuronal survival two weeks after medial forebrain bundle transection. Overall survival is lower for neurons labeled with two-site Dil (open bars) than for neurons labeled with Fluorogold (gray bars), but neuronal survival is similar within the middle one-third of the SNc.

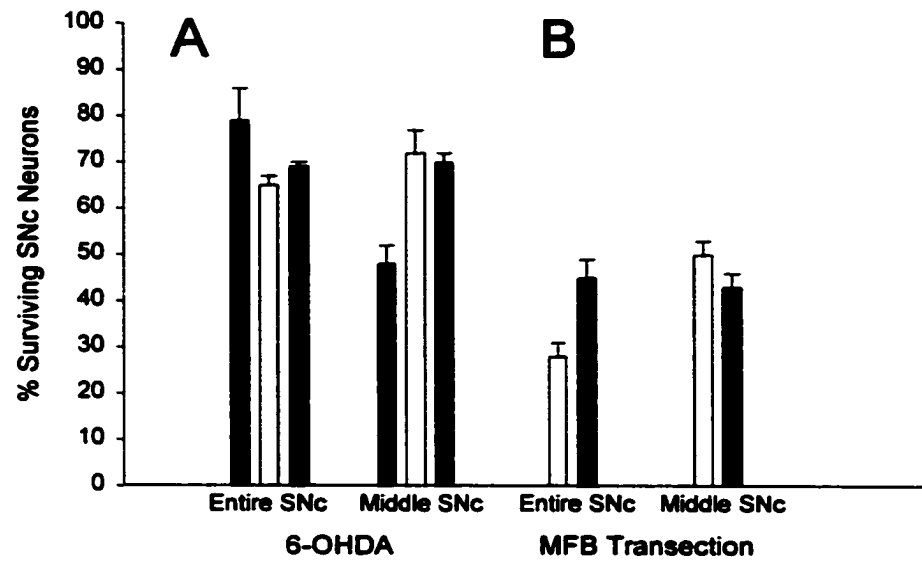


Figure 4.4

Figure 4.5. Survival after two weeks within the middle one-third of the SNc is different depending on lesion and tracer

Photomicrograph of 6-OHDA treated neurons labeled with six sites Dil (A) showing that degeneration within the middle region of the SNc is greater than in the SNc labeled with two sites of Dil (B). (C) After Fluorogold labeling, survival is similar to two sites Dil, but neuronal processes and degenerating cells are more easily recognized. (D) Photomicrograph of middle one-third of a non-injured SNc labeled with 6 sites Dil. Two weeks after medial forebrain bundle transection survival is similar for neurons labeled with two sites Dil (E) or two sites Fluorogold (F). Note that Fluorogold labeling of debris after transection is more prominent than with Dil or after 6-OHDA. Scale bar for all figures = 100 μm .

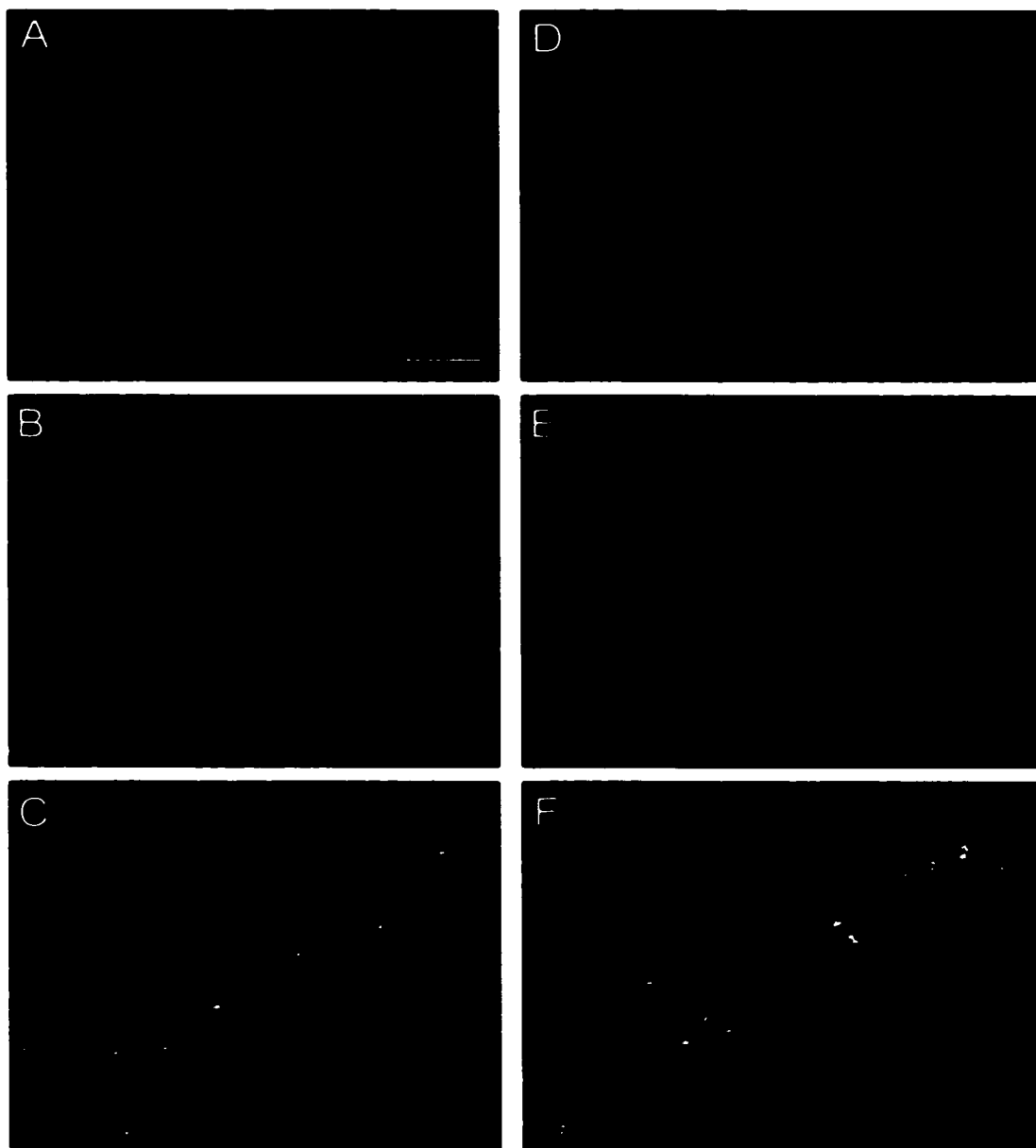


Figure 4.5

DISCUSSION

We set out to find a more efficient neuronal pre-labeling method for studies involving models of nigrostriatal degeneration. A comparison of three different retrograde labeling methods revealed that: i) compared to 2 sites, 6 injection sites throughout the neostriatum results in a more extensive distribution of labeled neurons in the substantia nigra pars compacta, as well as in more labeled neurons within sub-regions, ii) the extent and pattern of 6-OHDA induced neuronal loss with 6 labeling sites differs from that seen after 2 sites, and iii) Fluorogold labels slightly fewer neurons than Dil and does not affect the extent of cell death after 6-OHDA neurotoxicity or axotomy.

Increased labeling with more neostriatal labeling sites suggests complex projection patterns

As expected, a greater number of injection sites more broadly labeled the SNc from its medial to lateral extent, and proportionally labeled more neurons from its anterior to posterior extent. The increased labeling seen in the six-site model within the middle one-third region at a set point along the rostral to caudal extent (at SN30), however, is unexpected. This difference is likely not due to differences in concentration or volume of tracer injected because the intensity of individual neuronal labeling did not vary between the two conditions. Instead, it suggests that a subgroup of neurons within the middle region project to neostriatal regions which would only be labeled by the 6 site method. These

results may be a further indication that the SNc is not a homogenous collection of neurons, but that it contains definable subpopulations. Subpopulations of nigrostriatal neurons have been characterized by virtue of their topographical projection patterns (Fallon and Moore, 1978), their localization in phenotypically distinct dorsal and ventral tiers (Gerfen *et al.*, 1987), and by the presence or absence of neurotrophins such as BDNF (Seroogy *et al.*, 1994; Conner *et al.*, 1997).

Neuronal death appears to be increased within comparable regions with 6 labeling sites

Because 6 sites labeled a more widespread area, including regions that may not have been exposed to similar concentrations of 6-OHDA injected into the central region of the neostriatum, we focused on the central region. Neuronal death (expressed as a percentage of the "non-injured" contralateral control side) was greater with 6 sites than with 2 sites (either Dil or FG). This suggests that those cells labeled only by the 6 site method may be more vulnerable than those labeled in the 2 site model. Thus, it could be that those cells that project outside the central regions of the neostriatum may be more vulnerable. Alternatively, labeling with 6 sites may predispose more of the centrally projecting SNc neurons to die after 6-OHDA administration than with the 2 site labeling. This is conceivable, as the total volume of ethanol delivered per striatum was greater in the 6 site method. The 6 sites also could have caused a greater amount of inflammation or ischemic damage.

Overall neuronal death after 6-OHDA in the 6 site method was slower, and displayed a more linear rate. It is unclear which animal model, if any, reflects the true course of pathogenesis of PD, as the latter is not known (Olanow and Tatton, 1999). Therefore, it is not clear whether the 6 site model, with its more linear pattern of cell death, is better than the other models in mimicking the pattern of cell death that exists in PD. Cell loss in the central region was more rapid than in more outlying regions of the SNc. This may be because it takes longer for 6-OHDA to reach the processes of the outlying neurons or that, as discussed above, centrally located SNc neurons may be more predisposed to die. After the initial central loss, the total number was reduced over longer times. This suggests that neuronal death in the central region contributes to the later loss of other neurons.

Neuronal death after 6-OHDA or axotomy is similar with Dil and Fluorogold labeling

Neuronal death within the middle region of the SNc, compared to the “non-injured” contralateral controls, in rats labeled with either Dil or FG, was similar two weeks after 6-OHDA or medial forebrain bundle transection. More neurons appeared to die in the Dil than in the FG labeled animals, which may be because 2 sites of Dil labels more broadly than 2 sites of FG. It is possible that those additional neurons labeled by Dil are more susceptible to axotomy-induced death, and that they die more quickly than those in the middle of the SNc (where cell death was equivalent regardless of tracer used). If so, this would mean that

the pattern and course of death differs depending on whether transection or 6-OHDA was used, and may suggest different vulnerabilities to different lesioning methods. In this system, FG did not therefore appear to contribute to 6-OHDA or axotomy induced neuronal death. However, it is still possible that in other systems, or when used within this model at higher concentrations, FG could contribute to cell death (Schmued and Fallon, 1986; Garrett *et al.*, 1991; Wessendorf, 1991). Because of this potential problem with FG, it may be difficult to label more SNc neurons without increasing the overall amount. FG, however, has clear advantages over Dil, including the speed with which it retrogradely labels neurons (Schmued and Fallon, 1986), its solubility in aqueous media, and its effectiveness for showing detailed cellular morphology.

Conclusions

These results demonstrate that the method of retrograde labeling can substantially influence the reported cell loss. Such variance in reported neuronal death likely results from the existence of topographically different neuronal subpopulations with varying degrees of vulnerability. For accuracy, all neurons should be labeled, and such labeling can be achieved through the use of multiple sites of Dil. For efficiency and thus for rapid screening of the biological effects of various agents or treatments, 2 sites, using FG, are sufficient. It is also likely that these nuances of labeling techniques can be generalized to other systems.

CHAPTER 5:

GENERAL DISCUSSION

The work described above provides insight into the nature of proliferation, differentiation, and migration of neural stem cells and their progeny, and offers a cautionary tale about studies which employ tracing techniques within models of neurodegeneration. The following general discussion starts with a brief summary of the results presented within this thesis. Following that, suggestions are made about future research which would directly arise from each section of the thesis. This chapter concludes with speculations about future directions for neural stem cell research.

Summary of results

There is a critical need to describe the factors controlling endogenous proliferation and differentiation of neural stem cells. The work presented in Chapter 2 showed that:

- CNTF enhances, whereas antibodies against endogenous CNTF reduce, cellular proliferation
- CNTF enhances neurogenesis in the dentate gyrus
- The migration of newly generated neurons into deeper layers of the granule cell layer of the dentate gyrus is enhanced by CNTF
- CNTF does not alter the proliferation of oligodendrocytes or their progenitors
- The number of newly generated GFAP positive astrocytes is enhanced by CNTF, but remains low

- **The intensity of GFAP staining in the neurogenic regions is reduced by CNTF**
- **The receptor for CNTF, CNTFR α , is expressed within and around the neurogenic regions**
- **The effects of CNTF on neurogenesis may be via increased survival of newly generated cells and/or by stimulating GFAP positive astrocytes to dedifferentiate and become proliferative**

Taken together, these results suggest that CNTF is a critical endogenous regulator of the phenomenon of adult forebrain neurogenesis.

Work on the effects of CNTF on adult neurogenesis was followed by a characterization of factors controlling migration of neuroblasts in the adult CNS. In Chapter 3 of the thesis, I provided evidence that:

- **The extracellular matrix molecule laminin is expressed along the RMS, and a specific receptor for laminin, the $\alpha 6\beta 1$ integrin, is expressed by neuroblasts**
- **Blocking the $\alpha 6$ or $\beta 1$ subunits can significantly disrupt endogenous neuroblast migration**
- **A laminin tract above the RMS is sufficient to draw neuroblasts away from their normal course of migration, in a localized fashion**
- **Infusion of a small peptide recognized by the $\alpha 6\beta 1$ integrin can be used to draw migrating neuroblasts into the neostriatum, in a global fashion**

From this work, it can be concluded that integrins and the extracellular matrix are crucial endogenous components of normal adult neuroblast migration. Further, laminin and specific peptides may be used to draw neuroblasts into a novel site, and may therefore be useful within CNS repair strategies.

The final research chapter of the thesis serves as a cautionary note about the use of retrograde tracing techniques within models of neurodegeneration.

The work presented within Chapter 4 demonstrated the following:

- A six site labeling method labels more neurons, and labels the substantia nigra more broadly and effectively, than does a two site labeling system**
- The rate of cell death (either via 6-OHDA or medial forebrain bundle transection) is influenced by the retrograde tracing method used**
- Fluorogold, at least within this paradigm, is not toxic, as was previously suggested**
- Different rates of cell death within specific regions of the substantia nigra provide further evidence for the existence of sub-populations within the substantia nigra**

It is evident from the results above that the labeling method has a strong influence on reported cell death. It is therefore important to keep such conclusions in mind when testing the efficacy of a neural repair strategy (such as stem cell transplantation), or when examining the course of neurodegeneration.

Suggestions for future work based on Chapter 2

From the results describing the endogenous role of CNTF in adult forebrain neurogenesis, the following suggestions for further work are especially aimed at elucidating the mechanism of action of CNTF, as well as its possible role in other described instances of neurogenesis. It was suggested within this thesis that CNTF might enhance cellular proliferation and subsequent neuronal number by increasing the survival of newly generated cells. Further studies might provide more concrete evidence for this assertion. One could examine if, over a specified time course, CNTF or anti-CNTF significantly reduces or increases, respectively, the number of apoptotic cells within the neurogenic regions. Such an analysis might also be used *in vitro* with neurospheres and their progeny, and could use labeling for a variety of cell death markers, including Fluorojade, Caspase-3, or TUNEL. Further work could also examine whether CNTF is enhancing the number of newly generated cells via a very indirect method, such as by increasing the uptake of BrdU.

One could also focus more intensively upon the mechanism of action of CNTF, and examine the apparent discrepancy between the results reported here and those previously reported using cell culture systems. For example one might ask how endogenous signaling via the JAK/STAT pathway could lead to increases in the number of neurons. In *in vitro* systems, this pathway is normally seen as a gliogenic one. How is this pathway, or the behaviour of this pathway, different *in vivo*, and what might be the definable influences *in vivo* (such as the protein Noggin, perhaps) which are assisting CNTF in pushing such cells towards

a neuronal phenotype? More generally, what might this discrepancy say about other signaling pathways?

Further work on the role of CNTF in adult neurogenesis would benefit from studies involving CNTF knockout mice. One might simply ask if there is a reduction in cellular proliferation and subsequent neurogenesis within these animals. Further, one could then examine whether exogenously applied CNTF can compensate for a possible paucity in cell number. However, because CNTF knockout mice are viable, it is likely that there are compensatory mechanisms and redundancies built into the regulation of neural stem cell proliferation. This is quite likely, not only because CNTF knockout mice are viable, but also because during normal development CNTF is not present in biologically significant amounts (Stockli *et al.*, 1991), yet CNTFR α is responsive to another ligand, the complex of cardiotrophin-like cytokine (CLC) and cytokine-like factor-1 (CLF) (Elson *et al.*, 2000).

Finally, future work based on the CNTF results could examine in more detail its potential within other described instances of neurogenesis. It was mentioned earlier that it may have a role in cellular proliferation and neurogenesis induced by seizure, estrogen, and lesions, yet one could look more closely at the specific role of CNTF in these instances. For example, is CNTF released in significant amounts in neurogenic regions during seizure? In addition, one could investigate the potential role of CNTF in other potential external regulators of adult neurogenesis. For example, one could examine whether CNTF or CNTFR α expression is upregulated during learning or

exposure to enriched environments, and whether these expression levels decline, along with the rate of neurogenesis, during aging.

Suggestion for future work based on Chapter 3

The results presented within this chapter demonstrated a role for laminin and the integrins in controlling adult neuroblast migration. Ideas for further work are presented here, and these ideas primarily relate to characterizing additional ECM molecules and their receptors, examining the role of integrins in cell signaling, and using peptides or ECM molecules in CNS repair. Some ECM molecules intrinsically associated with the RMS have been well described (Thomas *et al.*, 1996; Peretto *et al.*, 1997). Further work could examine in detail the composition of the ECM along the RMS and, as an extension of those studies, assess what integrin subunit combinations or other receptors are expressed by migrating neuroblasts.

Future studies might focus on how integrins are regulated along the way to the olfactory bulb. For example, are they downregulated in cells once they reach the olfactory bulb and begin a radial form of migration? Or do their levels increase as they reach a greater source of laminin? Related to those studies, one could also examine the involvement of various integrin subunit combinations in other signaling pathways (Clarke and Brugge, 1995). For example, besides simply controlling migration and aligning these cells to the ECM, one could examine whether integrins play a role in the proliferation which occurs *en route* to the olfactory bulb (Lois and Alvarez-Buylla, 1994).

The role of astrocytes in relation to integrin/laminin mediated guidance requires further study. It has been shown that astrocytes can convert to radial glia in response to cellular transplantation (Leavitt *et al.*, 1999). One might examine if astrocytes which form the glial tube along the RMS secrete laminin at considerably higher levels than they do in other CNS regions. Another area for further research involves examining the relative importance of integrin mediated guidance of neuroblasts. For example, what is the nature of the interplay between the roles of PSA-NCAM and the $\alpha 6\beta 1$ integrin?

Finally, more work is required to examine the potential for the integrins and laminins in CNS repair strategies. For example, how far can one draw cells away from the RMS or the SVZa? Is it possible to create a thin laminin tract from the SVZa to the substantia nigra in a model of Parkinson's disease? Further, what is the resultant phenotype of these cells if drawn to the substantia nigra or the neostriatum? Another area for studies of the integrins does not specifically focus on neural stem cells or their progeny, but may be useful within studies of spinal cord repair. It is clear that the integrins are useful for cell and fibre guidance within the CNS, and that a host of small peptides can be generated for recognition by specific integrin subunit combinations. Therefore, in axonal injuries (such as in the spinal cord), one could screen for integrins specifically expressed in cut ends of spinal axons, and potentially use synthetic peptides to encourage fibre tract regeneration.

Suggestion for future work based on Chapter 4

Chapter 4 demonstrated that retrograde tracing techniques can influence the reported death rate of nigrostriatal neurons. Based on that main result, the following suggestions for future studies are presented. Because the tracing method can influence reported rates of cell death, it might be advisable to examine the literature for cases reporting different rates of neuronal death. This would be a critical exercise especially if studies are at odds with respect to the efficacy of a potential neural repair strategy, such as the administration of a neurotrophic factor, the transplantation of neural stem cells, or the use of some other pharmacological agent. It may be that the discrepancy has less to do with controversy about the effectiveness of a proposed treatment than it has to do with the effect of the method used to pre-label those neurons. Related to this suggestion for further analysis, one may also examine whether discrepancies in reported cell death rates can be generalized to other pre-labeling systems used to study cell death (such as labeling of retinal ganglion cells by applying Fluorogold to the superior colliculi).

The results presented in Chapter 4 also provided further evidence for the existence of discrete neuronal subpopulations within the substantia nigra. Such subpopulations have been described, for example, based on the presence or absence of BDNF (Seroogy *et al.*, 1994; Conner *et al.*, 1997). Our results suggest that certain neurons may be less susceptible to cell death. Further work could examine whether this difference in cell death is merely due to the fact that some cells project to regions which did not receive a neurotoxic insult.

Alternatively, one could assess whether such discrepancies in cell death are the result of differences in expression of trophic factors or their receptors. Further, one could ask if there are discrete genetic differences between such subpopulations of cells, and if those differences can be directly related to their susceptibility to degeneration.

Future directions in neural stem cell research (suggestions, speculations, and cautions)

The growing field of neural stem cell research brings with it exciting challenges to our fundamental understanding of neurobiology. It also brings the promise of therapeutic strategies for neural repair. This thesis concludes with a brief discussion of what I believe should be the foci for future neural stem cell work. Specifically, the field of neural stem cell research must work intensively to study the mechanisms underlying stem cell behaviour, their function and, finally, their relationship to aberrant cellular development and proliferation.

Neural stem cell behaviour (proliferation, differentiation, and migration)

Although there has been a multitude of studies in recent years, a considerable amount of work remains to be done on the mechanisms controlling neural stem cell proliferation, differentiation, and migration. The genes controlling proliferation are only now being elucidated and, as was described in more detail in the introduction, so are the potential lineage relationships among hematopoietic stem cells, neural stem cells, and their progeny. Further work

describing the nature of the stem cell lineage, or lineages, is, however, critical for the years ahead if we are to understand the behaviour of these cells *in vivo*. This is important not just to be able to explain the biological mechanisms underlying this lineage, but such studies would also lend insight into an understanding of aberrant stem cell proliferation or differentiation. In addition, there remains much work to be done to identify whether neural stem cells are a holdover of development, or if they represent a new population of cells within the adult CNS. If neural stem cells are a holdover of development, why is it that certain regions, but not others, retain such a proliferative capacity? On the other hand, how would our basic conception of neural development have to change if neural stem cells represented a newly generated population within the adult brain? Further, we must ask what controls these cells' entry into a proliferative state, and similarly deduce what intrinsic and extrinsic signals maintain neural stem cells in a relatively quiescent state. Finally, more work is required to determine if neural stem cells in each neurogenic region are genetically similar in their proliferative capacity or if there exists various forms of neural stem cells in each described (and perhaps yet to be described) region of adult neurogenesis.

With respect to advancing our understanding of the mechanisms controlling differentiation of neural stem cells, most studies have focused on driving cells towards a dopaminergic phenotype, or simply enhancing the yield of neurons relative to glia. A great deal of effort is still required to determine whether neural stem cells can always give rise to all phenotypes (neuronal and glial), and what the intrinsic and extrinsic cues are that control or regulate such

differentiation. Further, there is considerable discrepancy between what controls differentiation *in vitro* versus *in vivo*; this discrepancy was made apparent with results presented here on the endogenous role of CNTF in promoting neurogenesis. More generally, defining the factors controlling differentiation of neural stem cell progeny is a critical biological question. Such an undertaking is also clinically important, as any proposed therapeutic cell replacement strategy must be based upon a solid understanding of the cues necessary to increase the yield of one phenotype while simultaneously suppressing other phenotypes.

Adult neuroblast migration is a fascinating and by now well-described phenomenon. However, very few studies have shed light on the mechanism by which these cells traverse such a long distance in the forebrain. Although the nature of such migration, and some of the factors controlling it, have been described, much work remains to be done. Very few guidance molecules (be they conducive or repellent to migration) have been discovered and characterized in the RMS. In addition, the role of various ECM molecules and their receptors is only now being uncovered. Future work on the migration of neuroblasts will undoubtedly focus on the nature of these cells' homotypic interactions, and will require a thorough analysis of the guidance molecules regulating their migration from the SVZa to the olfactory bulb.

The function of neural stem cells

Why make new neurons? This is a fundamental biological and philosophical problem. It is well established that new neurons are made in the

SVZ, the dentate gyrus of the hippocampal formation, the retina, and the spinal cord, but it remains unclear as to what purpose is served by these newly generated cells. Indeed, the function and importance of these cells may differ from species to species. For example, it is clear that rodents would benefit more concretely than would humans from replacement or addition of olfactory bulb neurons. Similarly, there may be differences between humans and mice with respect to the functional significance of newly generated neurons in the dentate gyrus.

The existence of self-renewing pluripotent cells has been seen as a potential source of neurons for CNS repair. Although much effort has been focused in that direction, there has been little effort thus far to determine the normal, endogenous function of such cells. Some studies have examined the physiological properties of neural precursors (Liu *et al.*, 1999), and some have postulated that new neurons in the dentate gyrus are responsible for certain types of memory formation (Shors *et al.*, 2001). However, much more work remains to answer the following general, yet critical, questions: Why do we need to replace neurons in the olfactory bulb? Do extant cells become desensitized to odours? To what extent do new granule cells in the dentate gyrus contribute to learning and memory? Is the age-related decline in dentate gyrus cell proliferation related to memory loss? And, perhaps most importantly, how does the addition of new neurons alter our understanding of neural plasticity?

The corollary of neural stem cells (cautions and speculations)

The discovery and isolation of neural stem cells was rightfully greeted with excitement and optimism. Neural stem cells bring with them the hope of neuronal replacement (either with endogenous or exogenous sources). Many studies, including those described within this thesis, have been undertaken with the assumption that proliferation, differentiation, and migration of these cells and their progeny is an inherently positive phenomenon. Although we know very little about the resultant function of neural stem cells, we most often assume that their role in the CNS is either benevolent at best or benign at worst. As studies continue, we must also keep in mind that cellular proliferation, differentiation, and subsequent migration, when uncontrolled, is cancer. Ultimately, it may be that the fledgling field of neural stem cell biology and the more experienced domain of cancer cell biology may merge, and that genes involved in unchecked cellular proliferation are common to those seen in neurogenic regions of the adult CNS.

The possibility that these proliferative regions in the adult CNS are themselves a potential source of neural cancers, be they gliomas or neuroblastomas, is indeed worthy of our cautious consideration and intense study. Should such a possibility be true, however, one may conclude optimistically that, with a better understanding of neural stem cells' mechanisms and functions, we may not only be more able to deal with neurodegenerative diseases, but may also be in a position to understand and fight neural cancers. Thus, even in the event that the proliferation, differentiation, and migration of neural stem cells proves to be malevolent, further understanding of these

mechanisms and functions may well provide hope.

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