UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
F. EDWARD HÉBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

March 5, 2007

BIOMEDICAL GRADUATE PROGRAMS

Ph.D. Degrees

Interdisciplinary
-Emerging Infectious Diseases
-Molecular & Cell Biology
-Neuroscience

Departmental
-Clinical Psychology
-Environmental Health Sciences
-Medical Psychology
-Medical Zoology
-Pathology

Doctor of Public Health (Dr.P.H.)

Physician Scientist (MD/Ph.D.)

Master of Science Degrees

-Molecular & Cell Biology
-Public Health

Masters Degrees

-Comparative Medicine
-Military Medical History
-Public Health
-Tropical Medicine & Hygiene

Graduate Education Office
Dr. Eleanor S. Metcalf, Associate Dean
Janet Anastasi, Program Coordinator
Tanice Acevedo, Education Technician

Web Site
www.usuhs.mil/geo/gradpgm_index.html

E-mail Address
graduateprogram@usuhs.mil

Phone Numbers
Commercial: 301-295-9474
Toll Free: 800-772-1747
DSN: 295-9474
FAX: 301-295-6772

APPROVAL SHEET

Title of Dissertation: "Repair of Neocortex in a Model of Cortical Dysplasia"

Name of Candidate: Alisa Schaefer
Doctor of Philosophy Degree
27 March 2007

Dissertation and Abstract Approved:

Zygmunt Galdzicki, Ph.D. — 3/27/07 Date
Department of Anatomy, Physiology & Genetics
Committee Chairperson

Sharon Juliano, Ph.D. — 3/27/07 Date
Department of Anatomy, Physiology & Genetics
Committee Member

Diane Borst, Ph.D. — 03/27/07 Date
Department of Anatomy, Physiology & Genetics
Committee Member

Regina Armstrong, Ph.D. — 3/27/07 Date
Department of Anatomy, Physiology & Genetics
Committee Member

Michael Shamblott, Ph.D. — 3/27/07 Date
Department of OB/GYN, Johns Hopkins University
Committee Member

Copyright Statement

The author herby certifies that the use of any copyrighted material in the thesis manuscript entitled: Repair of Neocortex in a Model of Cortical Dysplasia

Is appropriately acknowledged and, beyond brief excerpts, is with the permission of the copyright owner.

Alisa W. Schaefer

Neuroscience Program

Uniformed Services University of the Health Sciences

Abstract

Title of Thesis: Repair of Neocortex in a Model of Cortical Dysplasia

Name: Alisa W. Schaefer

Thesis Directed by: Sharon Juliano, Ph.D.
Professor, Department of Anatomy, Physiology and Genetics

Our lab developed an animal model to elucidate factors associated with abnormal neocortical development and to attempt repair of cortical dysgenesis. We disrupt corticogenesis using an anti-mitotic methylazoxy methanol (MAM), which inhibits mitosis for several hours. The effects of MAM on neocortical development are assessed during early (embryonic day 24; E24) and late (E33) corticogenesis in ferrets. These animals have protracted cortical development with neurogenesis and migration continuing postnatally. MAM treatment on E24 leads to disorganized cortical laminae, abnormal radial morphology, precocious differentiation of radial glia, and dispersal of Cajal Retzius cells. MAM treatment on E33 leads to less severe effects including diminished layer 4, widespread termination of thalamocortical afferents, and abnormal distribution of $GABA_{A\alpha}$ receptors.

Reelin is a protein that plays a role in cortical layering and may also be a key factor in radial alignment. To assess the role of reelin in migration and radial morphology in our model, organotypic cultures were paired with wild type, heterozygous, or reeler mouse cortex. We observed that although reelin is necessary for cortical migration, other

factors in neocortex rescue radial morphology and reelin is not required for proper elongation of radial glia.

In attempts to repair E33 MAM treated cortex, we transplanted E27, E33 ferret and E14 mouse neural progenitor (NPs) cells into organotypic slices. Using a different paradigm, E27 and E33 fNPs were injected into the brains of E33 MAM treated and normal ferret kits. All donor cell types survive well in culture and differentiate into multiple cell phenotypes of neural origin. When transplanted into organotypic cultures, all donor cells survive and migrate into the cortical plate, although injections into the ventricular zone were significantly more likely to reach the cortical plate than transplants into the intermediate zone. *In vivo* transplants of fNPs into ferret kits also migrate into the cortex, differentiate, and become neurons, but not glia. The *in vivo* studies further revealed that the migration pattern of transplanted cells into MAM cortex varies from their distribution in normal cortex and that donor cells of different ages migrated into distinct layers in normal versus MAM cortex.

Repair of Neocortex in a Model of Cortical Dysplasia

by

Alisa W. Schaefer

Thesis/dissertation submitted to the faculty of the Neuroscience Program Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 2007.

Dedication

I dedicate this to my husband who has provided unwavering support over the past 7 years, to my daughter Isa who has been "a graduate school baby" independent, easy-going and happy, and to my unborn child.

Acknowledgements

"Never, Never Never Give Up" is the quote you will see on Sharon Juliano's computer. Her passion and dedication to science is exemplary. Thank you for being a role model as to how to persevere in a field that is filled with more failures than successes, but always fascinating.

It is an incredible feeling to have a group of committee members that have always shown a level of excitement and keen interest in this research project. Thank you for your time, feedback and support Regina Armstrong, Ph.D., Diane Borst, Ph.D., Zygmunt Galdzicki, Ph.D. and Michael Shamblott, Ph.D.

Donna Tatham and Sarah Dhandu were instrumental in the smooth execution of experiments. Their technical assistance, care of the ferrets, and help in many aspects of the research project ensured that things went according to plan. Sarah Dhandu was literally my hands for the *in vivo* experiments in the surgery suite, handling the ferrets and any non-sterile items once I was "scrubbed-in".

I would also like to acknowledge the people in the Veterinary Surgery Department who prepped the surgery suite, administered the anesthesia and taught me the basics of sterile surgery. Thanks to their expertise we never had any animal infections post-surgery and all our animals survived.

I would like to remember Cinda Helke, Ph.D., and John Sarvey, Ph.D., two amazing people that were incredibly committed to the graduate students at USUHS.

Janet Anastasi who is the backbone of the graduate education office and always has time for us and has the answer to all of our questions.

Regina Armstrong, Ph.D., the director of the Neuroscience program whose guidance was vital to the completion of this work.

Sylvie Poluch, Ph.D., and Debra McLaughlin, Ph.D., who not only provided an immeasurable amount of scientific assistance, but were there to support me emotionally as well. It is nice to know there are people out there who truly understand.

Maria Braga, Ph.D., and Boris Gafurov, Ph.D., for their patience and technical assistance with the electrophysiology.

Many of the students in the graduate education program: Clifton Dalgard, Ahmed Mohyeldin, Nathan Butler and Tara Romanczyk.We worked hard together. We remained each other's friends and support over the years.

John and Katie Pesce, Tammy Crowder, Doug Warner, Sam Vasilevsky, Sean Manion, Adam Vana for their friendship and understanding.

My grandfather and grandmother, for being incredibly strong, intelligent and motivated.

To my father for never judging and to my mother for instilling in me a devotion to education.

There are countless others who have contributed in some way to this dissertation and created a positive atmosphere at USUHS; I am incredibly grateful.

Table of Contents

APPROVAL SHEET ..... **I**

COPYRIGHT STATEMENT ..... **II**

ABSTRACT ..... **III**

REPAIR OF NEOCORTEX IN A MODEL OF CORTICAL DYSPLASIA ..... **V**

DEDICATION ..... **VII**

ACKNOWLEDGEMENTS ..... **VIII**

TABLE OF CONTENTS ..... **X**

CHAPTER 1 ..... **1**

INTRODUCTION **1**

CHAPTER 1 FIGURES **17**

CHAPTER 2 ..... **25**

REELIN IS NOT REQUIRED FOR RADIAL GLIAL ELONGATION IN NEONATAL FERRET CORTEX **25**

CHAPTER 2 FIGURES **50**

CHAPTER 3 ..... **58**

REPAIR OF NEONATAL CORTICAL DYSPLASIA USING EMBRYONIC NEURAL PROGENITOR CELLS IN FERRET **58**

CHAPTER 3 FIGURES **96**

CHAPTER 4.....111

DISCUSSION 111

REFERENCES.....126

LIST OF ABBREVIATIONS 135

LIST OF FIGURES 138

Chapter 1

Introduction

How the brain is formed

Proliferation and migration are the foundations of building a central nervous system (CNS). Only 19 days after fertilization, before a woman may have even realized she missed her menses, the neural plate, the core component of the embryonic CNS has formed. At this time, the embryo is highly sensitive to teratogens, such as alcohol, and ingestion by the mother can lead to severe developmental defects that include enlarged ventricles and a smaller forebrain, termed holoprosencepahly. Alcohol related neurodevelopment disorders (ARND) affect 1 in 100 live births (Sadler, 2004).

Understanding how the brain is formed is essential in the quest to repair it when things go wrong. Many events need to happen precisely and sequentially to produce normal cortical development. During the third to fourth week of embryonic development, neural folds fuse to form the neural tube containing a fluid filled ventricle (Sadler, 2004). Cells proliferate around the ventricles to expand their number and migrate away from their birth site to populate specific regions of CNS. Epigenetic insult can have mild to severe consequences on the proliferation and migration of CNS cells. By five weeks gestation, the cerebral hemispheres are formed and surround the lateral ventricles. The area lining the ventricles, known as the ventricular zone (VZ) and subventricular zone

(SVZ) are highly proliferative. The VZ/SVZ contains neural progenitor cells, which give rise to neurons, astrocytes, and oligodendrocytes. Early during development of the cerebral cortex, the neural progenitor cells increase their number by dividing symmetrically.

The initial stage of symmetric division is followed by a period of asymmetric division where one daughter cell remains mitotically active within the proliferative zone and the other daughter cell becomes post-mitotic and begins its journey into the cortex (Noctor et al., 2004). During this early period of development, the cortex has limited stratification. From dorsal to ventral, it is comprised of the marginal zone populated by Cajal Retzius cells and the subplate containing afferent fibers from the thalamus, these two layers are collectively known as the "preplate". Underneath the preplate is the pseudostratified epithelium or the birthplace of neurons.

Neurons born in this deep region migrate away from the VZ along radial glial guides to form the multi-layered cortex. Radial glial cells extend from the VZ to the pia, where they terminate with an end-foot process (Rakic, 1988; Noctor et al., 2002; Hartfuss et al., 2003). The earliest born cells leaving the VZ to migrate into the neocortex and split the primordial layer of cells known as the preplate into the marginal zone and subplate (Rakic, 1990; Parnavelas, 2000; Campbell, 2005). As the cerebral cortex continues to develop, cells migrating away from the VZ along radial glia guides pass through the intermediate zone, which also includes thalamocortical afferents and efferents. The migrating cells then bypass the subplate, essentially splitting the preplate to form the cortical plate. Once in the cortical plate, younger incoming cells migrate past the older cells already in position and stop just below the marginal zone leading to what is often

called “inside-out” pattern of cortical development (Angevine and Sidman, 1961; McConnell, 1988b; Rakic, 1988, 1995; Casanova and Trippe, 2006).

Importance of the marginal zone

The marginal zone, which is present during the earliest stages of cortical development, and the future layer 1, is the most external layer. Cajal Retzius cells are found in the marginal zone and have a distinctive horizontal bipolar morphology. They also have the important role of secreting a 350kD protein known as reelin. When reelin is absent, such as in the reeler mouse, cortical layering is turned upside down due to cells not being able to split the preplate; cells normally destined for the upper cortical regions are unable to migrate past earlier born cells. Neuronal heterotopias and disrupted radial glial morphology also occur in the reeler mouse (Dulabon et al., 2000; Rice and Curran, 2001; Hartfuss et al., 2003; D'Arcangelo, 2006). The exact role that reelin plays in cortical positioning is still unclear. It has been suggested that reelin provides a stop signal for migrating neurons, as a signal to detach from radial glial guides or as an attractant (Dulabon et al., 2000; Rice and Curran, 2001; Hartfuss et al., 2003; D'Arcangelo, 2006).

Role of radial glia

Radial glia have long been defined as the scaffolding for migrating neurons, albeit more recent findings establish that radial glia have a dual role in the developing cortex. Aside from their role as neuronal guides, they also act as neural precursors that predominantly give rise to pyramidal or excitatory neurons (Noctor et al., 2001b; Noctor et al., 2002).

Radial glial cells give rise to either intermediate progenitor cells or neurons by asymmetric division in the VZ. Each daughter neuron migrates away from the VZ to the

cortex while the intermediate progenitor moves into the SVZ, takes on a multipolar morphology, and then divides symmetrically producing two neurons that migrate radially to the cortical laminae (Chapt. 1, Fig. 1A) (Noctor et al., 2004).

Trajectory of the newborn neuron

Furthermore, the actual trajectory of the neuron can be subdivided into four distinct phases. First, the radial glia derived daughter cell moves rapidly from the VZ to the SVZ, where it pauses for approximately 24 hours. After this break it extends a process back into the VZ, then its cell body. The daughter cell then becomes bipolar, extending a leading process toward the pial surface before its ascent to the cortical plate with an axonal projection often trailing behind it (Chapt. 1, Fig. 1B) (Noctor et al., 2004).

Radial glial cells are mostly absent from the mature CNS. Their final round of division is asymmetric yielding an intermediate progenitor that undergoes a symmetric neurogenic division in the SVZ and a transforming radial glial cell that translocates to the pial surface and invoke its astrocytic fate (Chapt. 1, Fig. 1C) (Noctor et al., 2004).

Tangential Migration

Scientists were surprised to discover over the past 2 decades that cells populating the neocortex not only arise from the dorsal telencephalon, close to their ultimate destination within the cerebral cortex, but also from the ventral telencephalon. These neocortical cells migrate much greater distances than cells born in the VZ of the dorsal telencephalon and interestingly, make up for the increased migratory distance by moving much faster (50μm/hr) than their dorsal counterparts (10 μm/hr) (Kriegstein and Noctor, 2004). Cells migrating from this region are interneurons that arise from the ganglionic eminences of the ventral telencephalon (Chapt. 1, Fig. 2A, B) (Tamamaki et al., 1997;

Lavdas et al., 1999a; Marin and Rubenstein, 2003; Wonders and Anderson, 2005). After leaving the subpallium the cells migrate tangentially toward the cortico-striatal junction where they enter the neocortex and form several tangential streams to pass through the MZ, SVZ or the intermediate zone (IZ) to their final destination. Cells using the superficial route, through the MZ, dive down into the cortical plate and migrate radially towards the VZ. Cells traveling through the IZ migrate either obliquely or radially into the cortical laminae. Interneurons journeying through the SVZ may move into the VZ before ascending to the cortical plate (CP) (Chapt. 1, Fig. 2A, B) (Tamamaki et al., 1997; Lavdas et al., 1999b; Anderson et al., 2001; Zecevic and Rakic, 2001; Polleux et al., 2002; Ang et al., 2003; Kriegstein and Noctor, 2004; Tanaka et al., 2006). Interneurons or inhibitory cells from the ventral telencephalon work in conjunction with the excitatory pyramidal cells from the dorsal telencephalon to modulate information coming from the thalamus. Disruption in this balance of excitation to inhibition can result in epilepsy and other CNS electrophysiological defects (Jacobs et al., 1999; Miller et al., 2001; Kayser and Miller, 2002; Kriegstein and Noctor, 2004; Levitt, 2005; Miller, 2006).

Completion of migration

Once cells reach their final destination in the nervous system they comprise the six layers of the neocortex. As migration comes to an end, radial glia, which once spanned the entire thickness of the cortical wall, either degenerate or transform into astrocytes.

Timing of cortical development in different species

The timing of cortical development varies from species to species; it is almost complete by the time a mouse or rat is born after only 3 weeks of gestation. It continues

until the third trimester of gestation in humans and monkeys, but is protracted in ferrets lasting several weeks into the neonatal period (Jackson et al., 1989; Rosen et al., 1994; Noctor et al., 1997). In humans, the cerebral cortex takes shape during the third to fourth month of gestation, but refinement of cortical structure persists well into adolescence (Nissenkorn et al., 2001).

Malformations of cortical development

Epigenetic factors

During cortical development the precise timing and orchestration of events is imperative for proper migration and neural positioning. A variety of genetic and epigenetic factors can shift the ideal balance and lead to abnormal cortical formation.

Developmental defects of the CNS can have multiple causes ranging from environmental insults including drugs, alcohol, viruses and radiation, to metabolic and genetic anomalies (Nissenkorn et al., 2001). Defects in migration can range from mild to severe consequences such as dyslexia, intractable epilepsy, and schizophrenia which has been linked to abnormal reelin expression (Grayson et al., 2005; Brigman et al., 2006; Guerrini and Marini, 2006).

The ingestion of alcohol and use of drugs such as cocaine and heroine is reported to have drastic effects on cortical development. Each year in the United States approximately 10,000 babies are born with fetal alcohol syndrome (FAS) and alcohol related developmental disorders, which have a sequelae of signs and symptoms such as mental retardation, learning disabilities and behavioral problems due to anomalous migration (CDC, ; Olson et al., 1997). Of greater concern, is that 1 in 4 babies born in inner city hospitals are found to have cocaine in their meconium (Volpe, 1995).

Genetic defects of cortical development

Several genetic defects have been identified in human populations, which arise due to abnormalities in the migrational machinery. Disruptions in the reelin *(RELN)*, doublecortin *DCX)*, *lissencephaly* (*LIS1)* and filamin (*FLN)* genes all lead to varying degrees of aberrant cortical layering due to defects in the cytoskeletal architecture of the cell involved in translocation. *RELN* encodes for the protein reelin and gives rise to lissencephaly with cerebellar hypoplasia in humans. This defect leads to ataxia, mental retardation and epilepsy (Olson and Walsh, 2002). *DCX* encodes the protein doublecortin and if absent, results in dramatically altered cortical structure. Males tend to be more affected by defects in this gene than females, as its expression is X-linked. Females heterozygous for the defect will have a subset of affected cells that fail to migrate correctly (due to random inactivation of the X chromosome) and form a band in the subcortical white matter (a "double cortex") whereas males without the protection of a second X gene have a more severe migration deficit often leading to lissencephaly. Anomalies in the *LIS1* gene also lead to lissencephaly (both *DCX* and *LIS1* are microtubule-associated proteins). Much of what is known about the motor function of these genes comes from studying nuclear distribution mutant genes (*nud*s) in *Aspergillus nidulans* fungus with *nudF* and *LIS1* appearing to be orthologs (Feng and Walsh, 2001). The *LIS1* gene affects proper cortical migration through its influence on the dynein-dynactin structures associated with the microtubule assembly system, which gives structure to the cell and allows the dynamic movement of the cell necessary for migration (Gleeson et al., 1998; Morris et al., 1998; Gleeson et al., 1999; Gleeson and Walsh, 2000; Feng and Walsh, 2001).

Several spontaneous mutations and knockout animal models exist that have similarities to human neuropathologies. These animal models provide scientists with an opportunity to gain deeper insight into the mechanisms underlying the neurological disorders and possibilities for repair. The *reeler* and *LIS1* knockout mouse are an example of two animal models that have similarities to clinical populations with dysmorphic CNS due to epigenetic events.

The *reelin* gene in mice, which is the ortholog to the human *RELN* gene, encodes the glycoprotein reelin (Olson and Walsh, 2002). Similar to mutations in the *DCX* and *LIS1* genes, mutations in this gene also lead to cortical dysgenesis. Interestingly, the animal model for the *reeler* mutation is a spontaneous point mutation that was discovered in the 1950s and named for the lack of motor coordination in these mice that tend to walk like inebriated humans and fall over (Falconer, 1951).

Work by Assadi and colleagues (2003) indicates that reelin and lis1 may be part of the same signaling pathway. Reelin signals to the extracellular component of two lipoprotein receptors: the very low-density lipoprotein receptor (VLDL) and the apolipoprotein E receptor 2 (ApoER2). The Src family kinase phosphorylate disabled-1 (Dab1) located at the intracellular domain of the VLDL and ApoER2 receptors. Following this phosphorylation there is downstream signaling to phosphoinositide-3 kinase (PI3K) and Lis1, which interact with cytoskeletal motor elements such as dynein and dynactin which are essential components for migration (Assadi et al., 2003).

The high rate of developmental defects in the human population due to migrational anomalies is a fundamental reason why research in this field is imperative. An animal model that can illustrate the teratogenetic effects of an anti-mitotic substance

can lead to greater understanding of which elements of migration lead to abnormal development. Furthermore, understanding disruptions in cortical development are also helpful in finding ways to repair the developmental anomalies as well as revealing steps of normal development. Using animal models of cortical dysgenesis and elucidating several modes of repair is the foundation of this research project.

Cortical pathologies can range from mild to severe, to this end, our two animal models also range from mild to severe: injection of MAM early during gestation on embryonic day 24 (E24) leads neurological disruptions that are incompatible with life within 1 or 2 days after parturition. On the other hand, a similar dose of MAM on E33 results in a relatively normal functioning ferret that survives to adulthood and can reproduce. The model used here is also important because even though there are a number of genes that clearly cause migration disorders, the majority of cortical dysplasias in humans are not associated with specific genes, and are likely a result of environmental causes or a combination of genetic and environmental effects (Montenegro et al., 2002; Sisodiya, 2004). Additional evidence points to trauma and infectious diseases during pregnancy contributing to human cortical dysplasia (Marin-Padilla, 1999; Deukmedjian et al., 2004).

Ferret as a model for cortical repair

The ferret is an ideal animal model for testing methods that can be used to repair abnormal cortical development. The ferret has protracted neural development; neurons are still being born after birth (gestation is 41 days) (Noctor et al., 1997). There is continued presence of radial glial cells guiding neurons to the cortex until 3 weeks after birth (Voigt, 1989). This protracted development allows for selective disruption of

cortical development using an anti-mitotic. We use MAM, which stops successful division of cells in pregnant ferrets for ~ 8 hours (Cattaneo et al., 1995; Noctor et al., 2001a). This is accomplished by methylation of the 7' position of the guanine residue, which makes it impossible for DNA polymerase to copy the DNA, effectively inhibiting cell division (Zedeck et al., 1970; Cattabeni and Di Luca, 1997).

Administration of MAM on embryonic day 33 of the ferret; when cells destined for layer 4 of the somatosensory cortex are being generated, results in a reduction of cells predominantly in layer 4 (Noctor et al., 1997; Noctor et al., 2001a). The later born layers 2 and 3 appear normal, indicating that cell division and migration have resumed within several hours after the MAM injection. Although many aspects of cortical organization appear normal in E33 MAM treated ferrets, we observe several altered features. These include a widespread and diffuse pattern of thalamocortical afferents, which normally terminate in layer 4 (Palmer et al., 2001; McLaughlin and Juliano, 2005); $GABA_A$ receptors are also more widespread (Jablonska et al., 2004). Analysis of E24 MAM brains at P0 reveal abnormal migration, disrupted radial glia, ectopic presence of Cajal Retzius cells throughout the cortical thickness and precocious differentiation of radial glia into astrocytes (Hasling et al., 2003).

In the experiments described in this thesis, we evaluated different aspects of cortical dysgenesis and repair including neural migration, radial glial morphology, and transplantation of embryonic neural progenitor cells into organotypic cultures and perinatal host ferrets. We also assessed the ability of tissue from a different species to repair abnormal migration by using mouse tissue in our ferret model.

Stem cell therapy for cortical dysgenesis

Transplantation of stem cells for repair of the central nervous system

Stem cells hype or hope? Over the past 15 years we have made impressive strides in isolating neural progenitor cells and transplanting them into different models of neurodegenerative diseases such as amyotrophic lateral sclerosis (Lou Gehrigs's Disease), Alzheimer's Disease and Parkinson's Disease (Snyder et al., 1997; Vescovi et al., 1999a; Ourednik et al., 2002; Yang et al., 2002; Richardson et al., 2005; Vazey et al., 2006; Yasuhara et al., 2006), for review see two eloquent papers by Goldman and Conti (Goldman, 2005; Conti et al., 2006). One drawback of many previous studies is that stem cells are being transplanted into a host environment that is no longer conducive to migration and repair. Our research is specifically focused on repairing abnormal cortical development during the early perinatal period when factors are still present that are conducive to migration, differentiation and integration (Sheen et al., 1999).

Neural stem cells

Stem cells of the central nervous system, or more specifically "neural stem cells", are located in the region surrounding the lateral ventricles, and by definition: 1) self-renew, giving rise to clones 2) are multipotent and divide asymmetrically to produce neurons, astrocytes and oligodendrocytes. Stem cells derived from the embryonic blastocyst stage, on the other hand, are more flexible in their fate and can give rise to many cell types such as blood, bone or nervous tissue. A neural progenitor cell is a restricted neural stem cell that has a limited capacity for self-renewal, but is multipotent and capable of producing neurons, astrocytes and oligodendrocytes (Chapt. 1, Fig. 4 A, B) (McKay, 1997; Gage, 2000; Bjorklund and Svendsen, 2001).

We specifically use the term "ferret neural progenitor cells" when describing the cells isolated from embryonic ferret cortex. We demonstrate that: 1) the isolated cells form free-floating neural aggregates (Chapter 3, Fig 1 B), 2) the neural aggregates can be passaged several times and expanded, 3) the neural aggregates are a multipotent population immunoreactive for markers of neural progenitors, astrocytes and neurons, and 4) a single cell can give rise to clones and produce a sphere in a neural colony-forming cell assay (Chapter 3, Fig 1 A-D). We are nevertheless reticent to call these cells "stem cells" as several elements remain to be elucidated. For example: although we can passage the cells 5, 6 or 7 times, we have not been successful at continuous proliferation of the cells. Whether this is due to lack of appropriate mitogens in the medium, the age of the donor cells, or senescence because of a limited capacity of self-renewal is unclear. It is important to state that it has NOT been our goal to prove that the cells derived from the embryonic ferret brain are stem cells. This would entail testing a number of different mitogens, growth factors, hormones and so forth at a variety of concentrations and is one of the future directions of the project.

Deriving and culturing neural progenitor cells

Over the past two decades researchers have devised methods to obtain neural progenitor cells from the CNS and expanded them *in vitro*. One key goal of this work is to be able to expand the progeny of cells that can be used to replace or repair the missing or damaged neocortical cells, and limiting the number of cells taken from the donor. The CNS, unlike many organs of the human body, is limited in its ability to repair itself via the proliferation of new neurons once cortical development is complete. As a result, injury or insult to the brain leads to lifelong impairment (Horner and Gage, 2000).

Several protocols study, expand, and genetically modify or tag neural progenitor cells. Seminal work by the groups of Brent Reynolds and Samuel Weiss from the University of Calgary and Fred H. Gage at University of California at San Diego have developed standard approaches to deriving and culturing mouse neural progenitor cells (Reynolds et al., 1992; Gage et al., 1995). Furthermore, the teams of researchers from the laboratories of Brent Reynolds and Samuel Weiss have been instrumental in producing standardized media and reagents that can be purchased from a company Stem Cell Technologies (Vancouver, BC). We decided to purchase our media, growth factors, hormone mixture and clonal assay from this company as we feel it helps to eliminate human error and other variables involved in preparing the proliferation and differentiation reagents, which contain a variety of complex salt and hormone mixtures (Reynolds et al., 1992; Ray et al., 1993; Reynolds and Weiss, 1996).

The standard procedure entails removing the brain region of interest from an embryonic donor, which is microdissected and dissociated to form a single cell suspension. The cell suspension is placed in proliferation medium, which typically contains 10-20 ng/ml of epidermal growth factor (EGF), insulin, transferrin, progesterone, putrescine, sodium selenite, media, glucose, sodium bicarbonate and glutamine, and without any serum. Proliferation protocols may also call for 10-20 ng/ml of basic fibroblast growth factor (bFGF) in conjunction with heparin 2 μg/ml, and leukemia inhibitory factor (LIF) 1 ng/ml. The cells are cultured in flasks for approximately 7-10 days. During this period, single spheres become neural aggregates that enlarge over time. To minimize necrosis of cells at the center of the sphere, cells are passaged by spinning them down, removing the media, triturating them to form a single

cell suspension, and proliferating them once again in a flask. Cells should continue to expand and form spheres for many passages.

To allow cells to differentiate and assess their phenotype, they are removed from the proliferation medium and placed on an adherent substrate in the presence of serum, without growth factors (i.e., DMEM supplemented with a salt and hormone mixture, and 1% fetal bovine serum on poly-L-ornithine (15 μg/ml) coated glass coverslips in 24 well plates).

Self-renewal of a stem cell is tested by either placing a single cell in a well with proliferation media by itself or using a low density methyl-cellulose or collagen assay and watching the cell for three weeks to observe if there is clonal expansion of the single cell.

Methods of labeling donor cells

We attempted to find the optimal technique to label cell suspensions of donor cells, which would allow later localization in the host, the most effective incorporation, and the least damage to the donor cells in the process. We tried using CMTMR ((5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine) - mixed isomers), a dye containing a chloromethyl group that reacts with thiols, and is subsequently transformed into cell-impermeant fluorescent dye-thioether adduct. Although it labels the whole cell allowing for visualization of morphology and can withstand immunohistochemical processing, its labeling efficacy was low and it appeared to be toxic to the cells (Chapt. 1, Fig 5A).

BrdU was added to the culture media during proliferation prior to differentiation of the cells. Although double labeling with other markers was efficacious only a few cells

were labeled using this approach. This method only labels mitotically active cells and because we transplanted neural progenitor cells as well as a subpopulation of cells that are post-mitotic, we preferred a marker that was not dependent on mitosis (Chapt. 1, Fig 5B). Bisbenzimide was tested *in vitro*, but when it was used to label cells prior to transplantation onto organotypic slices, it was not stable during the immunohistochemical processing and donor cells could not be visualized (Chapt. 1, Fig 5C).

We also used pNIT green fluorescent protein (GFP) (kind gift of Fred Gage) and infected ferret progenitor cells. We attained approximately 10% rate of infection. In culture, cells retained the label in the cytoplasm following differentiation and could be double labeled (Chapt. 1, Fig 5D, Fig 6A-D). We also purchased lentiviral GFP from Biogenova (Rockville, MD), which is reported to be highly efficient at cell labeling; our rate of infection was approximately 10% (Zufferey et al., 1997). Following *in vivo* transplantation into host animals, very few GFP+ cells could be identified at the injection site and almost no cells were visualized in the cortex. It is unclear whether this is due to the low number of GFP+ cells, or perhaps the GFP was suppressed following *in vivo* transplantation and differentiation (Chapt. 1, Fig. 7A).

Vybrant CM-DiI (Vybrant lipophilic dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlora) (Molecular Probes, Eugene, Oregon) worked the best of all the methods we tested. It was easy to label cells, the labeling efficacy was greater than 90%, the cells remained healthy, the dye was retained by the cell membrane throughout immunohistochemical processing. The lipohilic CM-DiI is incorporated into the cell membrane and allows for visualization of cellular morphology; it also remains following

standard fixation methods (4% paraformaldehyde) (Chapt. 1, Fig. 7B and Chapter 3, Fig. 7A-E)

Results from the transplantation of the cells onto organotypic slices and into perinatal ferret brains are detailed in Chapter 3.

Chapter 1 Figures

Chapter 1, Figure 1

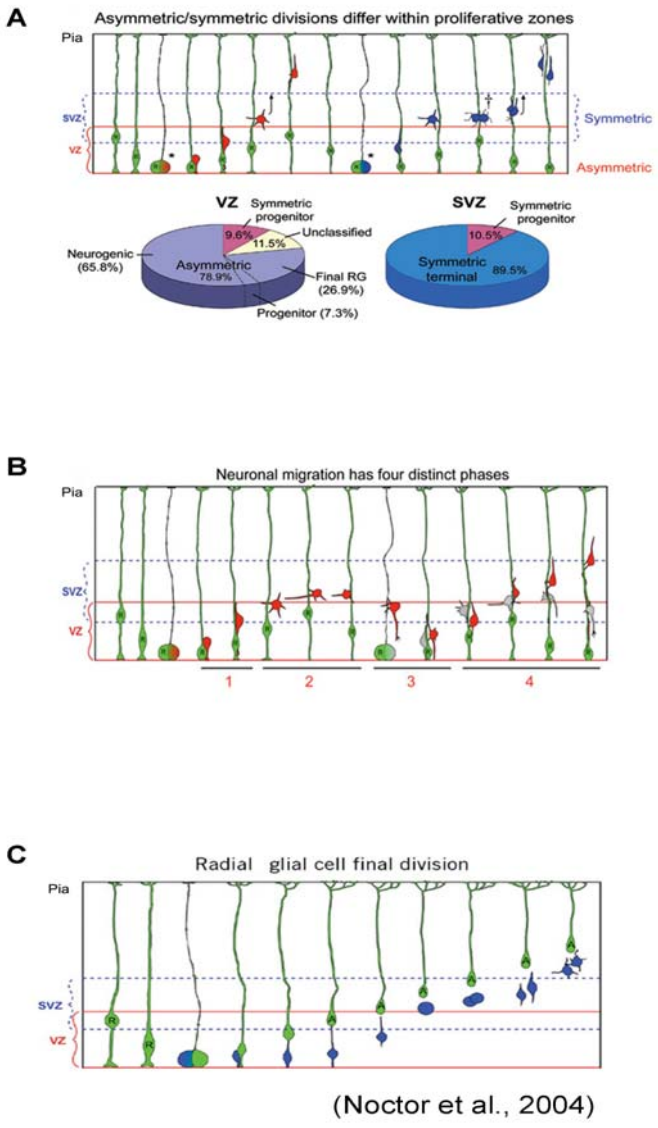


Figure 1A. Radial glia as neuronal progenitors: Radial glial cells give rise to neuronal progenitors through asymmetric division in the VZ. The red daughter cell is either a neuron that migrates to the cortex or a blue intermediate progenitor that migrates to the SVZ. The blue intermediate progenitor will symmetrically divide in the SVZ to give rise to two neurons.

Figure 1B. Neurons born in the VZ undergo four phases of migration before arriving at their final destination. 1. After being generated in the VZ, the cell 2. moves to the SVZ where it remains for approximately 24 hours. 3. A process is extended ventrally, followed by the cell body. 4. The neuron becomes bipolar with a process leading its ascent to the cortex and a trailing axon.

Figure 1C. The radial glial cell undergoes an asymmetric division at the ventricle surface. Pursuant to this, the radial glia begins its ascent towards the pial surface and transforms into an astrocyte. The blue daughter cell is the intermediate progenitor that undergoes symmetric neurogenic division in the SVZ.

Chapter 1, Figure 2

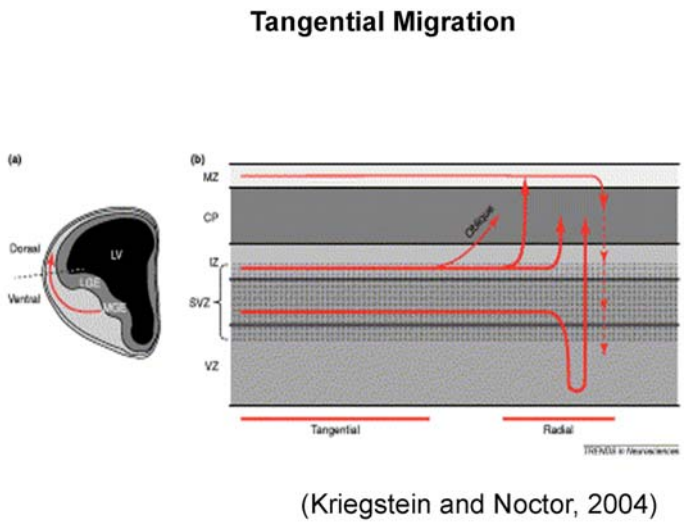


Figure 2. Cells from the ganglionic eminences migrate tangentially to the dorsal cortex. The broken line indicates that some cells descend radially from the marginal zone to the ventricular zone, The solid lines indicate cells that have migrated either obliquely or radially from deeper to more superificial cortical regions.

Chapter 1, Figure 3

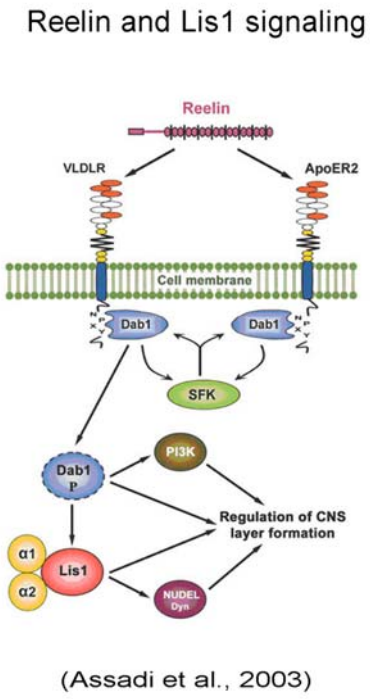
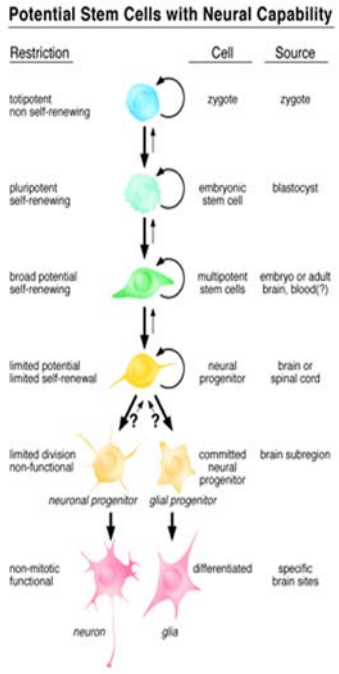


Figure 3. Reelin binds to the VLDL and ApoER2 lipoprotein receptors. The cytoplasmic domains of these receptors bind to disabled-1 (Dab1), which results in upregulated tyrosine phosphorylation of Dab1. Lis1 may either be a downstream effector of Dab1 or work in a parallel pathway with Dab1. Lis1 interacts with the Nudel/cytoplasm dynein complex in the CNS to regulate lamination.

Chapter 1, Figure 4

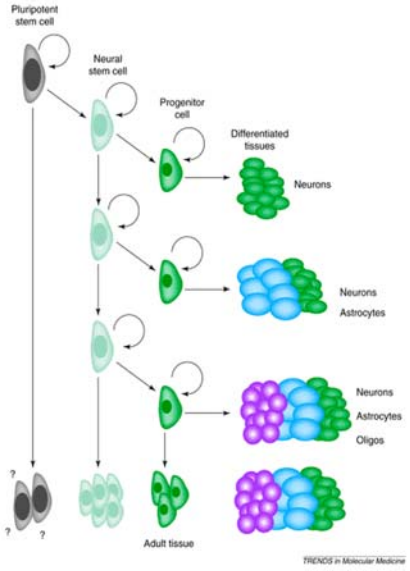
From stem cell, to progenitor cell, to neurons and glia

A



(Gage, 2000)

B



(Bjorklund and Svendsen, 2001)

Figure 4 A, B. These two diagrams help elucidate the definition of stem cells, their potential and the terminology associated with each stage. A totipotent stem cell can become any tissue and has unlimited capacity for self-renewal. We believe that the tissue we have dissected from the ferret and mouse CNS contains uncommitted neural progenitors, committed glial and neuronal progenitors as well as already differentiated neurons and glia.

Chapter 1, Figure 5

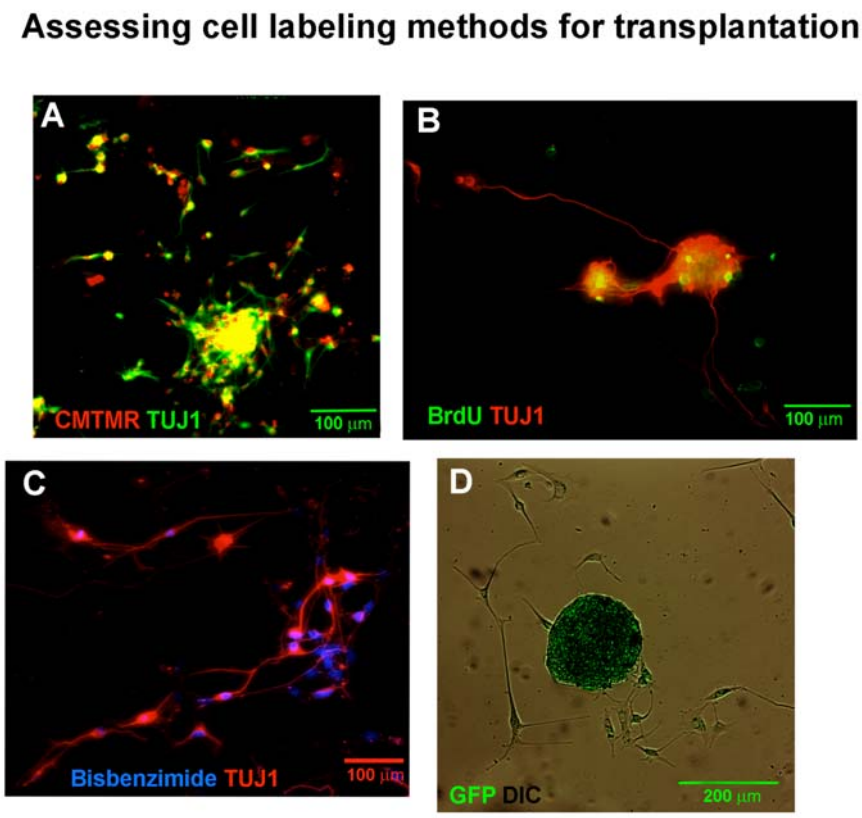


Figure 5 A, B, C, D. Different labeling methods were assessed to determine which method would be most appropriate for labeling donor cells prior to transplantation into host tissue *in vitro* and *in vivo*. Although many labeling methods worked well in culture, these approaches had several limitations when transplanted into host tissue (see text for details).

Chapter 1, Figure 6

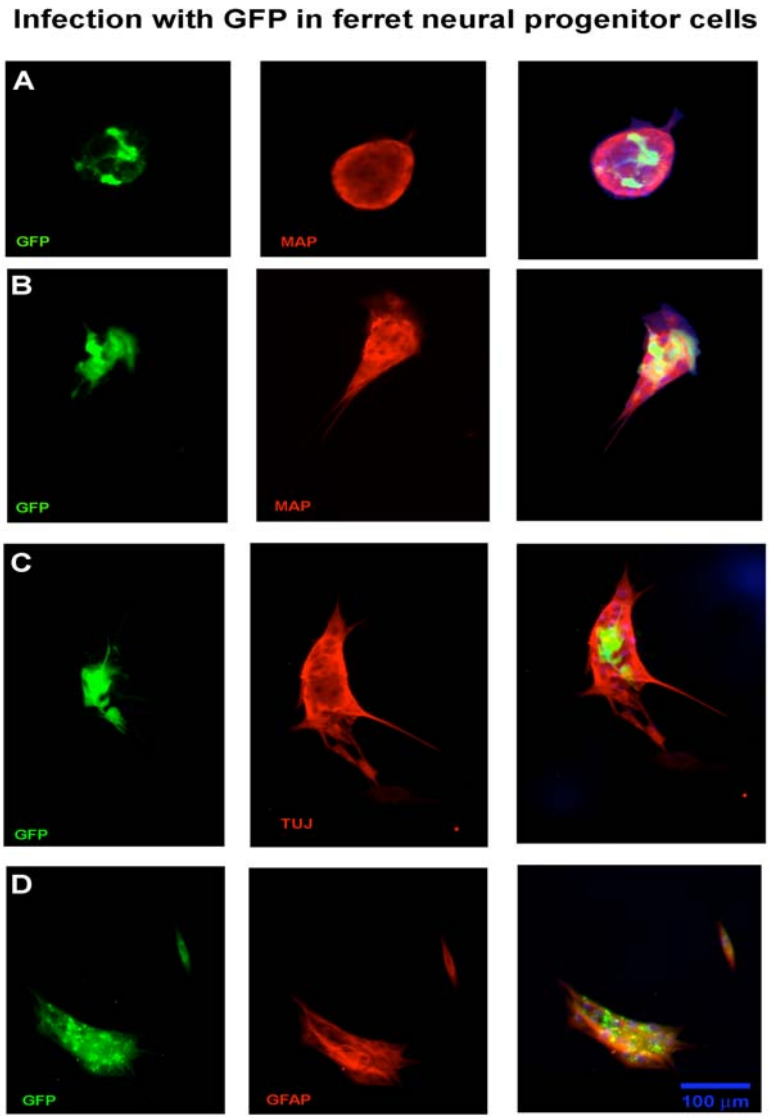


Figure 6 A, B, C, D. E33 ferret progenitor cells can be infected with GFP *in vitro* during proliferation. Cells are then differentiated as whole spheres on poly-L-ornithine coated glass coverslips. The GFP remains active and cells can be double labeled with neuronal markers such as MAP2abc and TUJ1, and the astrocytic marker GFAP.

Chapter 1, Figure 7

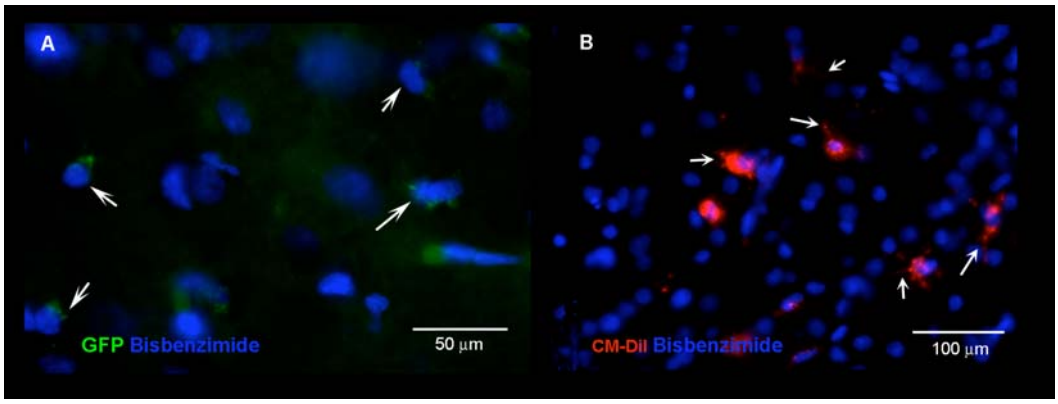


Figure 7. In (**A**) E33 ferret progenitor cells infected with GFP and proliferated were transplanted into ferret brains *in vivo.* Relative to CM-DiI (**B**), labeling efficacy is low and fluorescence is weak. These images were taken near the injection site.

Chapter 2

Reelin is not required for radial glial elongation in neonatal ferret cortex

Alisa Schaefer, Sylvie Poluch, Sharon Juliano

Program in Neuroscience, USUHS, Bethesda, MD 20814

"Reelin not essential for radial glial elongation "

Corresponding author:

Sharon L. Juliano

USUHS

4301 Jones Bridge Rd

Bethesda MD 20814

sjuliano@usuhs.edu

5 Figures

25 Pages

Key words:

Cortical dysplasia

MAM

Migration disorder

Neocortical development

Cerebral cortex

Acknowledgements:

We thank Sarah Dhandu for excellent technical assistance and animal husbandry. This work was supported by PHS NIH NIMH RO1 MH 62721 (SLJ).

Abstract

Numerous functions related to neuronal migration are linked to the extracellular glycoprotein reelin. Reelin also elongates radial glia, which are disrupted in mutant reeler mice. Our lab developed a model of cortical dysplasia in ferrets, which shares features with the reeler mouse, including impaired migration of neurons into the cerebral cortex and disrupted radial glia. Explants of normal ferret cortex in coculture with dysplastic ferret cortex restore the deficits in this model. To determine if reelin is integral to the repair, we used explants of wild type (WT) and heterozygous (het) P0 mouse cortex (containing reelin), as well P0 reeler cortex (not containing reelin), in coculture with organotypic cultures of dysplastic ferret cortex. This arrangement revealed that all types of mouse cortical explants (WT or het and reeler) elongated radial glia in the ferret model of cortical dysplasia, indicating that reelin is not required for proper radial glial morphology. Migration of cells into ferret neocortex, however, did not improve with explants of reeler cortex, but was almost normal after pairing with WT or het explants. We also placed an ectopic source of reelin in ferret cultures at the pial surface to reveal that migrating cells move toward the reelin source in dysplastic cortex. The morphology of radial glia in these cultures was also improved toward normal. Our results demonstrate that reelin is important for neuronal positioning, probably by attracting neurons migrating into the cerebral cortex, and that reelin is capable of elongating radial glial cells but is not the only radialization factor.

Introduction

Improper neuronal migration during development of neocortex results in disorders ranging from lissencephaly to epilepsy. To study mechanisms involved in neocortical migration we developed an animal model mimicking features of human cortical dysplasia. Development of normal ferret cortex was disrupted by injecting a short acting antimitotic (methylazoxy methanol, MAM) into pregnant ferrets on the 24th day of gestation (E24). This disrupts the birth of cells destined to populate the subplate, resulting in specific effects including: poorly formed cortical layers, disorganized radial glial cells, and Cajal Retzius cells scattered throughout the cortical wall, rather than their normal superficial position (Noctor et al., 1999; Hasling et al., 2003).

We further observed that factors present in normal ferret cortex improved radial glial morphology and neuronal migration into the neocortex. A specific candidate present in normal cortex that improved radial glial morphology is neuregulin (Hasling et al., 2003; Gierdalski et al., 2005). Although elements of normal cortex restore many of the disrupted features of E24 MAM treated cortex, it is not clear neuregulin is the only factor involved, or that it improves migration of neurons in addition to repairing radial glial morphology.

Another substance that improves or elongates radial glia is reelin (Forster et al., 2002; Frotscher et al., 2003; Hartfuss et al., 2003). Several roles have been proposed for reelin during neocortical development including functioning as: a chemoattractant, a stop or detachment signal, and for its ability to directly elongate radial glia. (Frotscher, 1997; Soriano et al., 1997; Dulabon et al., 2000; Hartfuss et al., 2003) E24 MAM treatment shares several features with the reeler mouse including deformed radial glial

cells and aberrant reelin cues. In reeler mice, reelin signaling is absent, while after MAM-treatment, reelin signals arise from abnormally located Cajal Retzius cells. Although the exact role that reelin plays in neuronal positioning and radial glial morphology is still unclear, overall evidence suggests that it influences both elongation of radial glia and positioning of migrating cells (Soriano et al., 1997; Forster et al., 2002; Frotscher et al., 2003; Hartfuss et al., 2003).

To further clarify the role of reelin in cortical development, we used wild type, heterozygous, and reeler mouse explants in culture with E24 MAM treated organotypic slices. If reelin *is needed* for normal cortical layering and proper radial cell alignment, then reeler mouse cortex would neither improve radial glia or cortical migration of MAM treated slices, while wild type or heterozygous cortex would improve both, as shown previously (Hasling et al., 2003). If reelin *is not needed* for proper migration or radial glial alignment, both migration and radial glial morphology would improve with the reeler explants. We report here that radial glial morphology was improved with all types of explants, including reeler, but that migration of neurons into the neocortex was only marginally improved by the presence of reeler explants. We then tested the ability of an exogenous source of reelin to influence neuronal migration in MAM-treated ferret cortex. Our findings indicate reelin is not required for proper alignment of radial glia, but is key for normal neuronal migration.

Materials and Methods

Experimental Design

To assess the effect of reelin on radial glial cell morphology and neocortical migration we injected pregnant ferrets with MAM on E24 and prepared organotypic cultures of P0 ferret cortex with P0 wild type, heterozygous and reeler mouse cortical explants and cocultured them together for 2 days *in vitro*. Most ferret kits were injected with BrdU prior to preparation of the cultures. Following the coculture period, several techniques determined radial glial cell morphology and neocortical migration. To assess radial glial cell morphology we injected fluorescently labeled dextrans into the intermediate zone (IZ) of the cultured slice or placed a small crystal of DiI in the slice after fixation with paraformaldehyde; neural migration was assessed independently using BrdU immunoreactivity.

Injections

Timed pregnant ferrets were obtained from Marshall Farms (New Rose, N.Y.). Each received an injection of MAM (methylazoxy methanol) on embryonic day 24. Each ferret was briefly anesthetized with isofluorane (5%) and the MAM injected i.p. (14-17 mg/kg dissolved in 5ml saline; Midwest Research Institute). P0 ferret kits received i.p. injections of 50 mg/kg of BrdU (Sigma) dissolved in saline at a volume of 0.1 cc; each injection was repeated 3 times at 45 minute intervals.

Organotypic Cultures

P0 ferrets were injected i.p. with 50 mg/kg of sodium pentobarbital. When insensitive to pain, the brain was quickly removed and maintained in ice-cold oxygenated artificial cerebral spinal fluid (aCSF) (containing H_2O, and in mM amounts, NaCl, 124;

$NaHCO_3$, 26; Glucose, 10; NaH_2PO, 1.2; KCl, 3.2; MgSO4, 1.2; $CaCl_2$, 2.4). After removal of the anterior and posterior poles, slices were made at 500 μm using a tissue slicer. Each slice was placed in a 70 μm insert (Millipore Corporation Billerica, MA) in a 6 well tissue culture plate and 500 μl of Minimal Essential Medium with 10% normal horse serum supplemented with gentamicin, L-glutamine, and glucose. These were cultured for 2 days at standard conditions in an incubator (37ºC, 95% air, 5% CO_2). After the ferret slices were prepared, P0 B6C3Fe mice (*a/a* $Reln^{rl}/Reln^{rl}$, *a/a* $Reln^{rl}$/+, +/+, stock number 000235, The Jackson Laboratory, Bar Harbor, ME) were treated in the same manner as the ferrets and the brains removed and sliced. The neocortex was dissected from each slice and placed adjacent to the cortical surface of an E24 MAM treated slice, with the deepest cortical layer of the mouse explant adjacent to the pia of the ferret slice. To determine the genotype of each mouse explant, we either used all the mice and genotyped them later (n=5 dams) or determined the genotype prior to the culture preparation using PCR (n=3 dams).

Coculture of organotypic ferret slices with 293T cells

To assess the effect of an exogenous source of reelin, we used HEK 293T cells transfected with the full length *reelin* encoding cDNA plasmid (pCRl) kindly provided by Gabriella D'Arcangelo (Houston, TX, USA) (Arnaud et al., 2003). 293T cells (control or reelin+ cells) were cultured in Dulbecco's modified Eagle's medium (MEDIATECH Inc., Herndon, VA). 293T cells (25,000 control or reelin+ cells) were diluted in Matrigel (BD Biosciences, Bedford, MA). E24 MAM treated slices (prepared as described above) were transferred to an insert and then 2 μl of Matrigel (containing the 293T cells or Matrigel alone) were placed next to the pial surface (Chapt. 2, Figure 4A). The cocultures were

incubated for 1 hour in a medium supplemented with BrdU (100 µg/ml), then were washed twice and incubated with fresh medium. After 2 days in culture, the slices were fixed overnight (paraformaldehyde 4%) and immunoreacted against BrdU.

Fluorescent Dextran and DiI Injections

After 2 days in culture (DIC), slices were removed from the incubator and placed into a slice chamber perfused with ACSF (Hasling et al., 2003). Each slice was injected with a fluoresecent dextran (Fluororuby; Molecular Probes, Eugene, OR) into the intermediate zone using glass micropipettes with a tip size of 20-30 µm. The dextran was iontophoresed for 3 min at 4 µA with alternating positive current. Slices were left in the chamber for 5-8 hrs hours subsequent to the injection to allow time for the cell marker to be transported throughout the cells. The tissue was then placed in 4% paraformaldehyde for at least 24 h. Alternatively, a small crystal of DiI was placed on the fixed slice to visualize the radial glia. Slices were stained with bisbenzimide trihydrochloride (Sigma, St Louis, MO) prior to being mounted and coverslipped with Vectashield Mounting Medium for Fluorescence (Vector Laboratories, Burlingame CA).

BrdU immunoreactivity

To reveal the presence of BrdU in the ferret slices, the cocultures were immunoreacted according to the protocol described by Roche (http://www.roche-applied-science.com/pack-insert/1170376a.pdf). Following coculture with the mouse cortex and fixation in 4% buffered paraformaldehyde for a minimum of 48 hours, immunohistochemistry was conducted to reveal the presence of BrdU in the ferret slices. The cocultures were placed in absolute methanol for 10 minutes at 4ºC, followed by 1 hour in 2N HCl at 37ºC. The slices were then placed in borate buffer (pH 8.5) changing

twice over 10 minutes, then washed in 0.1 M PBS pH 7.4, three times, 15 minutes each at room temperature. After this, the slices were incubated in anti-rat BrdU (Becton Dickinson, Franklin Lakes, NJ) at 1:100 with 0.5% bovine serum albumin diluted in 0.1 M PBS pH 7.4 at 4ºC for 48-72 hours, and washed in 0.1 M PBS pH 7.4, three times at 15 minute intervals. The secondary antibody Cy2 (Jackson ImmunoResearch West Grove, PA) or Alexa 488 (Molecular Probes) anti-rat was diluted 1:500 in PBS and slices incubated for 1 hour at room temperature.

Quantification of BrdU IR cells

To determine the ability of cells to migrate in either normal, E24 MAM treated cortex alone, or after coculture with mouse cortical plate or HEK 293T cells embedded in Matrigel, we plotted the distribution of BrdU+ cells after 2 DIC. Each slice was imaged using a Zeiss Axiovert 200 microscope (Göttingen, Germany) and a montage reconstructed using Metamorph Imaging Software (Downington, PA); the position of each BrdU+ cell was plotted. Boundaries were drawn indicating the pia of the ferret slice and the outer edge of the VZ. This region was divided into 3 equal bins for each coculture and the number of cells per bin was counted in a slab 500 μm in width. The bins included a region in the intermediate zone close to the VZ (i.e., the deep part of the intermediate zone, IZd), a region in the IZ, but closer to the cortical plate (i.e., the superficial part of the intermediate zone, IZs), and the region including the cortical plate (CP). Histograms were made to indicate the position of BrdU+ cells across animals in each condition. See diagram in Figure 4 (Chapt. 2). To compare across samples, the number of cells/bin were calculated as a percent of the total number of cells in each slice. To determine statistical

significance, a 2 way ANOVA was conducted, followed by a Tukey test for comparisons between groups. Data is expressed as the mean ± SEM

Quantification of radial glial morphology

Each dextran injection labeled a cluster of radial glial cells as described previously (Juliano et al., 1996). MAM treatment led to abnormal positioning of radial glia. To quantify the change in morphology, the angle of deviation for each cluster in the treated and untreated groups was measured as described previously (Hasling et al., 2003; Gierdalski et al., 2005). The mean angular deviation was compared for statistically significant differences between conditions using the Mann Whitney Rank Sum test.

Genotyping of mice using PCR

Mouse genotype was determined via PCR of tail snips following the protocol published by D'Arcangelo et al., with a slight modification (D'Arcangelo et al., 1996). Mouse genotype was determined via PCR of tail snips. Tail digestion was accomplished by placing the tissue in digestion buffer with 0.1mg/ml proteinase K and incubating overnight at 55ºC. For the PCR reaction, PCR reagents from Promega were mixed together along with a reeler reverse TGCATTAATGTGCAGTGTTG), a reeler forward (TAATCTGTCCTCACTCTGCC) and a wild-type reverse primer (ACAGTTGACATACCTTAATC). Forty nanograms of genomic DNA was added to each reaction. The initial denaturation lasted for 5 minutes at 94ºC, followed by a 1 minute 94ºC denaturation, followed by a 2 minute 55ºC primer annealing phase and then a 3 minute 72ºC primer extension phase for 30 cycles. These were completed with a final 10 minute 72ºC phase. The PCR product was mixed with Blue Juice (Promega) and loaded into a 2% agarose gel with 5mg/ml ethidium bromide and run at 100 volts for

1hour in TBE. Genotype was determined by the wild-type amplificate at 280 bp, the reeler amplificate at 380 bp and the heterozygous amplificate has both bands.

Results

Do mouse cortical explants realign MAM treated radial glia?

Our previous experiments indicated that normal ferret cortex explants cocultured with E24 MAM treated ferret slices restored radial morphology and cortical migration (Hasling et al., 2003). In this study, mouse cortical explants were cocultured with E24 MAM treated cortical slices to determine if a factor was present in mouse cortex capable of restoring normal radial glial morphology. We paired mouse explants taken from wild type or heterozygous cortex with slices of E24 MAM treated cortex and allowed them to remain in culture for 2 days. Following the culture period, injections of fluorescent dextrans were made into the intermediate zone of the MAM treated slices to label radial glia.

The morphology of radial glia in E24 MAM treated organotypic slices cocultured with wild type or heterozygous mouse explants was elongated and their fibers extended toward the pial surface, as opposed to the fractured and disrupted appearance of E24 MAM-treated cortical cultures alone (Chapt. 2, Figure1A-C). This appearance is similar to normal P0 ferret slices with no MAM treatment (i.e. normal) or to E24 MAM treated slices paired with normal ferret cortex (Hasling et al., 2003). To quantify the effect of the cortical explants, the angular deviation of the labeled radial glial cells was calculated. The angular deviation of E24 MAM treated radial glia paired with WT or heterozygous cortical explants is not significantly different from the deviation of normal P0 ferret cortex (Chapt. 2, Figure 2D). This suggests that mouse cortex is capable of restoring the MAM treated radial glial morphology toward normal. We found no differences between

culture with either wild type or heterozygous (WT or het) cortical explants and combined the data from these two genotypes together.

Does reeler mouse cortex realign MAM treated radial glia?

After establishing that WT or het mouse cortex realigned the disordered radial glia, we tested the influence of reelin by using explants of reeler cortex with the E24 MAM treated cortical slices. We reasoned that if reelin was essential to the process of elongating radial glia, explants taken from reeler mice would not realign the disrupted radial glia in MAM treated cultures. If it were not necessary, however, the reeler cortical explant would be able to elongate the disrupted radial glia. After coculture of E24 MAM treated cortex with reeler cortical explants, the angle of deviation of the dextran labeled radial glia was similar to the normal ferret or after coculture of E24 MAM treated cortex with WT or het cortical explants (Chapt. 2, Figure 2A-D). This suggests that reelin is not a required element for elongating radial glia.

Can normal or reeler mouse cortical explants restore neuronal positioning after MAM treatment?

When P0 E24 MAM treated cortex was cocultured with normal ferret cortical explants, cells migrated into the cortical plate more effectively than in E24 MAM treated cortex (Hasling et al., 2003). To determine if mouse cortex was also capable of restoring proper neural positioning, WT or het, and reeler mouse cortical explants were cocultured with E24 MAM treated organotypic cortical slices for 2 days. Prior to culture preparation, ferret kits received injections of BrdU. In normal ferret cortex, after 2 days in culture many BrdU+ cells migrate into the cortical plate, as reported previously (Chapt. 2, Figure 3C) (Hasling et al., 2003). BrdU+ cells in the E24 MAM treated cultures alone are

relatively evenly distributed throughout the cortical wall (Chapt. 2, Figure 3C). When the E24 MAM treated cultures are paired with WT or het mouse explants, quantification of the distribution of BrdU+ cells indicates that significantly more neurons navigate into the cortical plate. We saw no differences in the migration pattern between WT or heterozygous explants, so these conditions were grouped together. The WT or het pattern was similar to that seen in normal ferret cortex, cultured alone (Noctor et al., 1999; Hasling et al., 2003). These data support the idea that WT or heterozygous cortical plate also contains diffusible factors conducive to migration of neurons.

When reeler explants were paired with MAM treated cortex, the ability of cells to migrate into the cortical plate diminished compared to the WT or het cultures. The BrdU+ cells did not preferentially migrate into the cortical plate, but remained distributed throughout the cortical wall. Both the distributions of E24 MAM treated cultures alone and the E24 MAM treated cortex paired with a reeler cortical explant were significantly different from the normal distribution, whereas the cultures of E24 MAM treated cortex paired with WT or het cortical explants did not differ from the normal pattern of migration. These results indicate that even though the radial glia were realigned by coculture with reeler cortical explants, the improved radial morphology is not sufficient to restore neural migration, suggesting that reelin signaling is a key to normal migration.

The influence of exogenous reelin on neuronal migration and radial glial morphology

To further evaluate the effect of reelin on migration of cortical neurons, we used an exogenous source of reelin under the following conditions. HEK 293T cells transfected with the reelin gene (see Methods) or control HEK 293T cells were positioned adjacent to the pia of an E24 MAM treated slice in order to mimic the normal

source of reelin. BrdU was added to the media as described in the Methods. After two days in culture, the slices were fixed and immunoreacted to reveal BrdU, disclosing the position of labeled cells migrating during the culture period. Many BrdU+ cells migrated toward the cortical plate and clustered near the reelin source. When HEK 293T control cells were placed next to the pia of the E24 MAM slices, migrating cells remained scattered throughout the cortical plate; their ability to migrate to the upper cortical region was not restored (Chapt. 2, Figure 4). This suggests that normotopic reelin attracts migrating cells in ferret cortex at P0. The number of BrdU+ migrating cells was significantly greater in the cortical plate of cultures with the reelin+ cells placed near the pia. The ability to migrate successfully implies that the radial glial scaffolding was also repaired. To investigate this possibility, a small crystal of DiI was placed in the VZ or IZ of the E24 MAM treated organotypic cultures with reelin+ or control cells at the pial surface. After several days of incubation in the cultures grown with reelin HEK 293T cells at the pial surface, the DiI labeled radial glia were organized and elongated, similar to the normal pattern. In the slices cultured with control (non reelin producing) cells, the radial glia were disoriented and less likely to extend toward the pia. Evaluation of the angular deviation revealed that it was high in the organotypic cultures with control cells, and low in the cultures with reelin+ cells, i.e. a high value representing more disorganization. The positioning of reelin+ cells near the pial surface not only improves the migration of cells into the cortical plate, but also elongates radial glia in E24 MAM treated slices.

Discussion

Similarities and differences between MAM and reeler models

MAM is a short acting anti-mitotic that methylates guanine nucleotides of single stranded DNA during S-phase. This methylation prevents DNA polymerase from replicating the DNA, therefore cells born within approximately 8 hrs following the administration of MAM are arrested in S-phase and effectively removed from the cell cycle. Normal cell division resumes after the effect of MAM has worn off (Noctor et al., 1999). The E24 MAM model results in a constellation of effects including disorganized radial glia, disrupted cortical migration and aberrant location of Cajal Retzius cells (which secrete the reelin protein) (Noctor et al., 1999). These effects are highly specific to the day of treatment, since injections of MAM on other gestational days do not lead to such dramatic consequences, but result in more subtle changes that do not include disrupted radial glia or Cajal Retzius cells (Noctor et al., 1999).

The reeler mouse shares several features with the E24 MAM treated ferret including disorganized radial glia, failure of cells to migrate into the neocortex properly, and disrupted thalamic projections (data not shown). Reelin signaling is also abnormal in both models, although in the MAM treated cortex, Cajal Retzius cells are disorganized and scattered throughout the cortical wall, producing an ectopic source of reelin, whereas in the reeler mouse, reelin is absent. The correlation between the absence of reelin with disrupted radial glia and inverted cortical lamination influenced a number of researchers to look for the role of reelin in producing these effects. The idea that reelin is important for both the elongation of radial glial cells and the proper positioning of migrating neurons is supported by a number of studies (Hunter and Hatten, 1995; Ogawa et al.,

1995; Frotscher, 1997; Soriano et al., 1997; Marin-Padilla, 1998; Howell et al., 1999; Dulabon et al., 2000; Forster et al., 2002; Magdaleno et al., 2002; Frotscher et al., 2003; Hartfuss et al., 2003; Luque et al., 2003; Beffert et al., 2004).

Factors involved in radial glial morphology

A diffusible factor present in normal cortical plate restores radial glia toward their normal morphology in E24 MAM treated cortex, and also improves migration into the cortical plate (Gierdalski and Juliano, 2002, 2003; Hasling et al., 2003; Gierdalski et al., 2005). Several studies implicate reelin as one of the factors generally involved in maintaining glial morphology (Soriano et al., 1997; Forster et al., 2002; Frotscher et al., 2003; Hartfuss et al., 2003). There is a direct effect on radial glia in dissociated cultures and also when radial glia are present in organotypic cultures. Using radial glia derived from the hippocampus, reelin causes elongation of these cells by signaling through Disabled 1 and integrin receptors (Forster et al., 2002). In a slice culture model of the cerebellum, reelin alters the morphology of Bergmann glia (Soriano et al., 1997). Exogneously applied reelin also causes elongation of radial glia in the hippocampus; when the ectopic source is positioned in a normotopic fashion, the migration of granule cells improves (Frotscher et al., 2003; Zhao et al., 2004). We also observed in the current study that an exogenous but normotopic source of reelin causes radial glia to realign in E24 MAM treated slices. The capability of reelin to produce this change in phenotype is not in question; it appears from our results, however, that although reelin is capable of altering the radial glial phenotype, it is not *required* for altering and/or maintaining radial glia elongation. Our study reports that reeler cortex, without reelin present, contains a factor capable of positively altering the radial glia morphology. Reelin may therefore

play a redundant role in radial glial elongation. Similar findings were reported by Soriano et al., who demonstrated that transplants of reeler cortical cells elongated Bergmann glia in the mouse cerebellum (Soriano et al., 1997).

Many substances influence radial glial morphology. We observed that an apparently soluble factor in normal cortex improved the morphology of disrupted radial glia in MAM treated cortex (Gierdalski and Juliano, 2002, 2003; Hasling et al., 2003; Gierdalski et al., 2005). A likely candidate for this factor was neuregulin 1 (NRG1), which can independently elongate radial glia, and in ferret cortex and acts through erbB receptor signaling (Gierdalski et al., 2005). Several other factors are also important in mediating radial glial morphology including FGF, and BLBP by acting on Notch signaling (Gaiano and Fishell, 1998; Yoon et al., 2004; Anthony et al., 2005; Dang et al., 2006). A number of influences therefore alter the morphology of radial glia, including NRG1 and reelin. In this study, we evaluated radial glial morphology and migration of neurons into the cortical plate during later stages of corticogenesis. It may be that different substances act at different points during development and that each factor plays a distinct role in maintaining or altering radial glial phenotype in a time dependent manner.

The influence of reelin on neuronal migration

Despite the improved radial glial morphology after coculture with reeler cortical explants, migration of neurons into the neocortex was only very slightly improved. Since this suggested an influence of reelin on the ability to migrate effectively, we conducted additional experiments to test the idea that reelin has an attractive effect on the migrating cells of neocortex. Reelin is a large glycoprotein secreted into the extracellular matrix

(Ogawa et al., 1995; D'Arcangelo et al., 1999). During corticogenesis, it is produced by Cajal Retzius cells located in the marginal zone. This protein is absent from the mutant reeler, and is the presumptive cause for their classic migration deficit. Although reelin clearly appears to be involved in neuronal migration, its precise role in producing normal movement is not obvious and substantial evidence supports assorted potential functions for this protein. Various authors report that reelin operates as a stop signal (Dulabon et al., 2000), a signal to detach migrating cells from radial glia (Hack et al., 2002), or as a permissive factor that directs cells toward the cortical surface (D'Arcangelo, 2006). In the studies reported here, neocortical explants of WT or het mice improved migration of neurons toward the cortical surface in E24 MAM treated cortex, while neocortical explants lacking reelin did not. Our experiment using an exogenous source demonstrated that migrating neurons in ferret cortex are attracted toward the cells producing reelin, supporting the idea that reelin in a normotopic position can act as a permissive factor for migrating neurons. An additional confounding factor in the MAM treated organotypic cultures, however, is that in this model of cortical dysplasia, the Cajal Retzius cells producing reelin are scattered throughout the cortical plate and intermediate zone, resulting in an ectopic source of reelin (Noctor et al., 1999; Hasling et al., 2003). Coculture of MAM treated cortex with normal cortical explants realigns the Cajal Retzius cells to a their superficial position in the marginal zone (Hasling et al., 2003), which undoubtedly allows for improved migration. In the case of the reeler cortical explant paired with MAM treated cortex in culture, the migration is not restored, either because of a lack of reelin in the cortical explant, or because the Cajal Retzius cells remained scattered through the cortical wall, or both.

These experiments also demonstrate the dichotomy between improved radial glial morphology and the ability of cells to migrate into the cortical plate. The improved radial glial morphology shown here was not a factor strong enough to also improve migration. This suggests that the missing substance (reelin) was necessary to promote normal migration as an instructive factor, or that it interfered with normal migration by the presence of the abnormally placed Cajal Retzius cells. It seems likely that relatively normal morphology of radial glia is necessary for proper migration of cortical cells and that reelin signaling is also necessary for correct positioning of migrating cells. It is not clear, however, whether the location of a reelin source is important. That is, is it a directional or attractive signal? Although this study cannot be conclusive as to whether reelin acts as an attractant, several pieces of evidence suggest that it does. We observed that (i) migrating cells move dramatically toward a reelin producing source cells and (ii) despite relatively normal radial glia, migration is not restored in E24 MAM treated slices when the cortical explant does not produce a source of reelin. Both findings indicate that ectopic sources of reelin attract migrating cells. This evidence is supported by a study reporting that in reeler hippocampal organotypic cultures, granule cells improved migration only when reelin was placed in its normal position and not when it was diffusely distributed in the media (Zhao et al., 2004). In contrast, Magdaleno et al. (2002) found that ectopically expressed reelin in the ventricular zone of transgenic reeler mice rescues both their behavior and features of cortical and cerebellar architecture. Although reelin in the ventricular zone did not restore proper cortical lamination, it did allow migration of neurons past the preplate and partially restored migration of Purkinje cells in the cerebellum. Magdaleno et al (2002) suggest that reelin operates in a complex manner

and may rely on other molecules that provide additional positional cues (Magdaleno et al., 2002).

Chapter 2 References

Anthony TE, Mason HA, Gridley T, Fishell G, Heintz N. 2005. Brain lipid-binding protein is a direct target of Notch signaling in radial glial cells. Genes Dev 19:1028-1033.

Arnaud L, Ballif BA, Cooper JA. 2003. Regulation of protein tyrosine kinase signaling by substrate degradation during brain development. Mol Cell Biol 23:9293-9302.

Beffert U, Weeber EJ, Morfini G, Ko J, Brady ST, Tsai LH, Sweatt JD, Herz J. 2004. Reelin and cyclin-dependent kinase 5-dependent signals cooperate in regulating neuronal migration and synaptic transmission. J Neurosci 24:1897-1906.

D'Arcangelo G. 2006. Reelin mouse mutants as models of cortical development disorders. Epilepsy Behav 8:81-90.

D'Arcangelo G, Homayouni R, Keshvara L, Rice DS, Sheldon M, Curran T. 1999. Reelin is a ligand for lipoprotein receptors. Neuron 24:471-479.

D'Arcangelo G, Miao GG, Curran T. 1996. Detection of the reelin breakpoint in reeler mice. Brain Res Mol Brain Res 39:234-236.

Dang L, Yoon K, Wang M, Gaiano N. 2006. Notch3 signaling promotes radial glial/progenitor character in the Mammalian telencephalon. Dev Neurosci 28:58-69.

Dulabon L, Olson EC, Taglienti MG, Eisenhuth S, McGrath B, Walsh CA, Kreidberg JA, Anton ES. 2000. Reelin binds alpha3beta1 integrin and inhibits neuronal migration. Neuron 27:33-44.

Forster E, Tielsch A, Saum B, Weiss KH, Johanssen C, Graus-Porta D, Muller U, Frotscher M. 2002. Reelin, Disabled 1, and beta 1 integrins are required for the

formation of the radial glial scaffold in the hippocampus. Proc Natl Acad Sci U S A 99:13178-13183.

Frotscher M. 1997. Dual role of Cajal-Retzius cells and reelin in cortical development. Cell Tissue Res 290:315-322.

Frotscher M, Haas CA, Forster E. 2003. Reelin controls granule cell migration in the dentate gyrus by acting on the radial glial scaffold. Cereb Cortex 13:634-640.

Gaiano N, Fishell G. 1998. Transplantation as a tool to study progenitors within the vertebrate nervous system. J Neurobiol 36:152-161.

Gierdalski M, Juliano SL. 2002. Influence of radial glia and Cajal-Retzius cells in neuronal migration. Results Probl Cell Differ 39:75-88.

Gierdalski M, Juliano SL. 2003. Factors affecting the morphology of radial glia. Cereb Cortex 13:572-579.

Gierdalski M, Sardi SP, Corfas G, Juliano SL. 2005. Endogenous neuregulin restores radial glia in a (ferret) model of cortical dysplasia. J Neurosci 25:8498-8504.

Hack I, Bancila M, Loulier K, Carroll P, Cremer H. 2002. Reelin is a detachment signal in tangential chain-migration during postnatal neurogenesis. Nat Neurosci 5:939-945.

Hartfuss E, Forster E, Bock HH, Hack MA, Leprince P, Luque JM, Herz J, Frotscher M, Gotz M. 2003. Reelin signaling directly affects radial glia morphology and biochemical maturation. Development 130:4597-4609.

Hasling TA, Gierdalski M, Jablonska B, Juliano SL. 2003. A radialization factor in normal cortical plate restores disorganized radial glia and disrupted migration in a model of cortical dysplasia. Eur J Neurosci 17:467-480.

Howell BW, Herrick TM, Cooper JA. 1999. Reelin-induced tryosine phosphorylation of disabled 1 during neuronal positioning. Genes Dev 13:643-648.

Hunter KE, Hatten ME. 1995. Radial glial cell transformation to astrocytes is bidirectional: regulation by a diffusible factor in embryonic forebrain. Proc Natl Acad Sci U S A 92:2061-2065.

Juliano SL, Palmer SL, Sonty RV, Noctor S, Hill GF, 2nd. 1996. Development of local connections in ferret somatosensory cortex. J Comp Neurol 374:259-277.

Luque JM, Morante-Oria J, Fairen A. 2003. Localization of ApoER2, VLDLR and Dab1 in radial glia: groundwork for a new model of reelin action during cortical development. Brain Res Dev Brain Res 140:195-203.

Magdaleno S, Keshvara L, Curran T. 2002. Rescue of ataxia and preplate splitting by ectopic expression of Reelin in reeler mice. Neuron 33:573-586.

Marin-Padilla M. 1998. Cajal-Retzius cells and the development of the neocortex. Trends Neurosci 21:64-71.

Noctor SC, Palmer SL, Hasling T, Juliano SL. 1999. Interference with the development of early generated neocortex results in disruption of radial glia and abnormal formation of neocortical layers. Cereb Cortex 9:121-136.

Ogawa M, Miyata T, Nakajima K, Yagyu K, Seike M, Ikenaka K, Yamamoto H, Mikoshiba K. 1995. The reeler gene-associated antigen on Cajal-Retzius neurons is a crucial molecule for laminar organization of cortical neurons. Neuron 14:899-912.

Soriano E, Alvarado-Mallart RM, Dumesnil N, Del Rio JA, Sotelo C. 1997. Cajal-Retzius cells regulate the radial glia phenotype in the adult and developing cerebellum and alter granule cell migration. Neuron 18:563-577.

Yoon K, Nery S, Rutlin ML, Radtke F, Fishell G, Gaiano N. 2004. Fibroblast growth factor receptor signaling promotes radial glial identity and interacts with Notch1 signaling in telencephalic progenitors. J Neurosci 24:9497-9506.

Zhao S, Chai X, Forster E, Frotscher M. 2004. Reelin is a positional signal for the lamination of dentate granule cells. Development 131:5117-5125.

Chapter 2 Figures

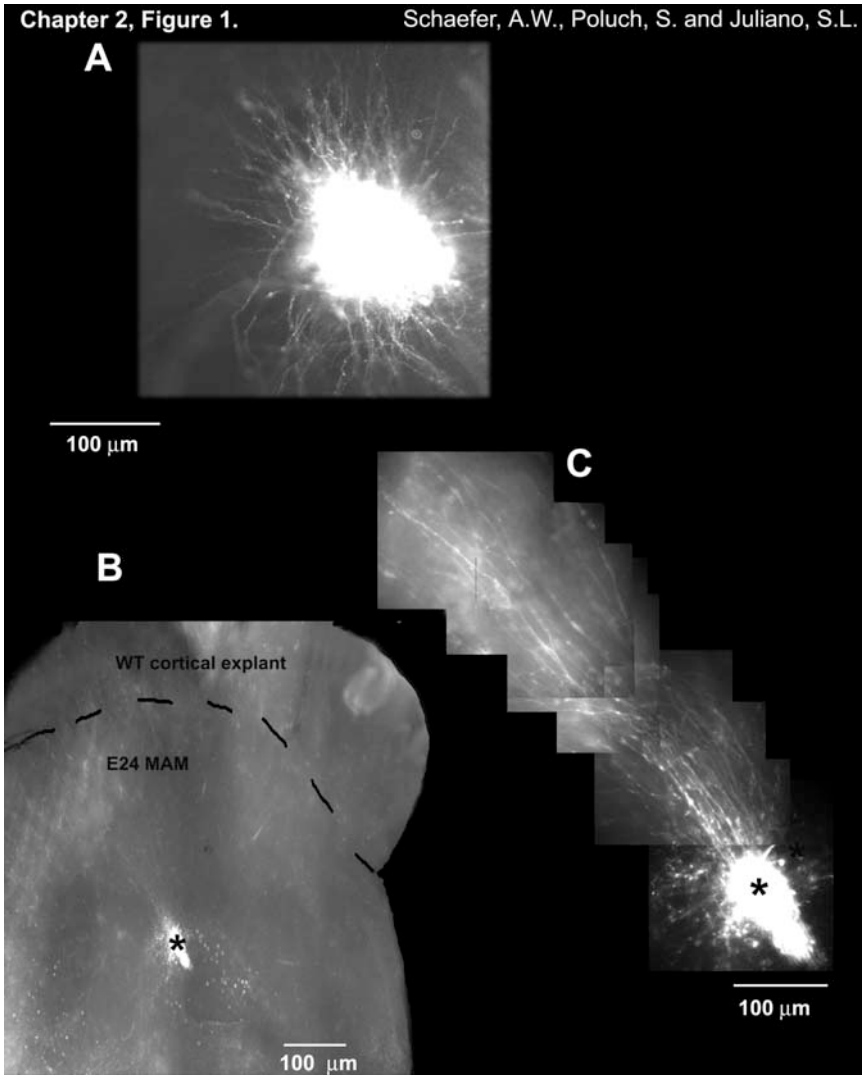


Figure 1. A. Example of radial glia in E24 MAM treated cultures. After 2 days in culture, this organotypic slice was injected with fluororuby, which selectively labeled a cohort of radial glial cells. The radial glia extend their processes in a fan shaped manner, losing their usual elongated, spoke-like appearance. **B**. An organotypic culture of an E24 MAM treated slice with a cortical explant from a wild type (WT) mouse. The asterisk

indicates the site of an injection of fluororuby, which can be seen at higher power in **C.** The labeled radial glial fibers are elongated and extend toward the pial surface.

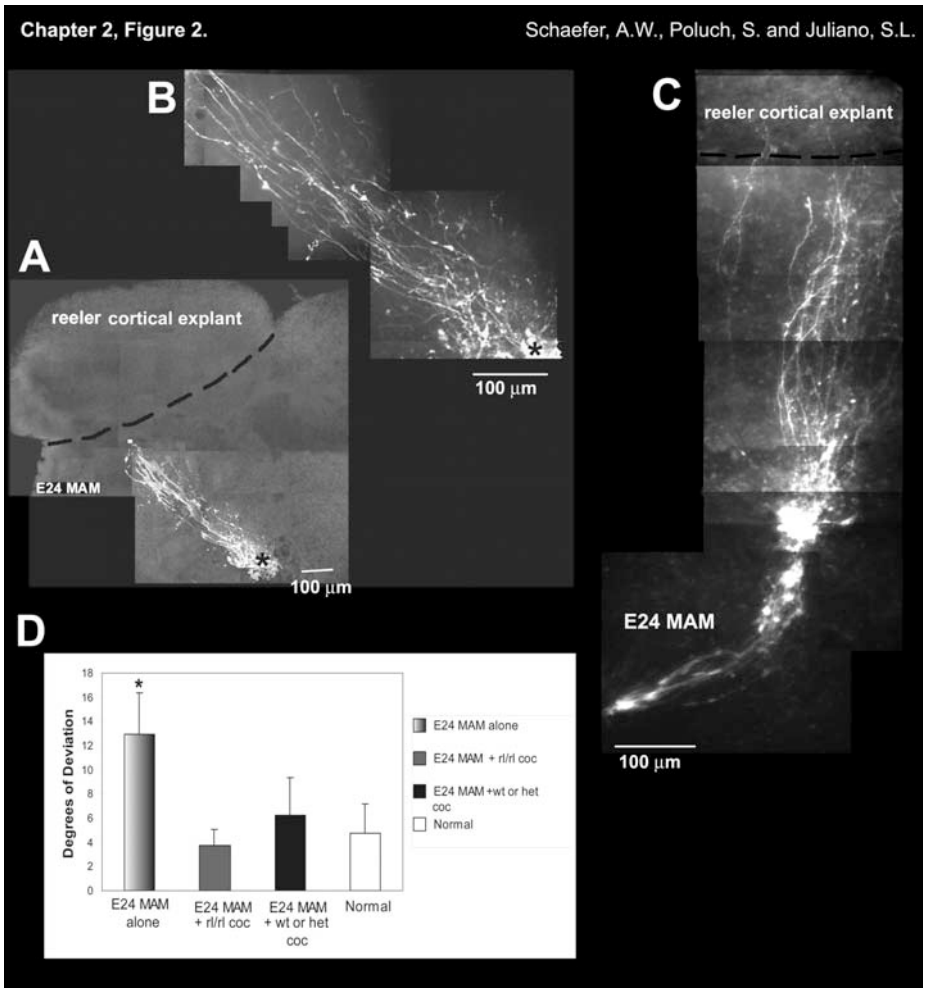


Figure 2. Examples of E24 MAM treated cortex cocultured with reeler cortical explants. **A.** shows an organotypic culture of an E24 MAM treated slice cocultured with an explant obtained from reeler cortex. After 2 days in culture the intermediate zone was injected with fluororuby. **B.** is a higher power view of the same injection. The asterisks indicate the injection site. **C.** is a different culture of an E24 MAM treated slice, also cocultured with a reeler cortical explant that received a fluorescent dextran injection. In both examples, the radial glia are elongated and extend toward the pia, suggesting that reelin is not necessary for fiber extension. **D.** contains a graph of angles of deviation in radial glia under different culture conditions. A large degree of angular deviation occurs in the radial processes in the E24 MAM treated cortex (n=6). However, coculture with either wild type or heterozygous

mouse cortical explants (n=6) or reeler cortex explants (n=6) restores radial morphology so that the angle of deviation is similar to the angle of deviation in normal ferret cortex (n=6). The only set of angles significantly different from the normal values are the E24 MAM treated alone ($* p< 0.006$, Mann Whitney Rank Sum test). Error bars = standard deviation.

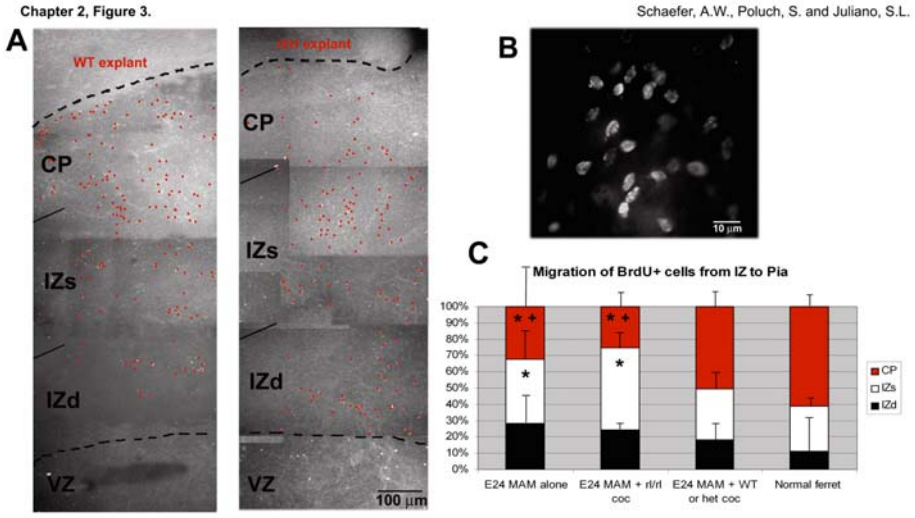


Figure 3. BrdU immunoreactive cells. **A** illustrates two examples of E24 MAM treated slices that were cocultured with a WT or reeler cortical explant. Prior to culture preparation the P0 kits were injected with BrdU and migration of the BrdU+ cells was observed after 2 days in culture. Each BrdU+ cell is indicated with a red dot. **B** shows a higher power view of BrdU+ cells. **C** shows histograms indicating the positions of BrdU+ cells. The space between the VZ and pial surface was divided into 3 equal regions as described in the Methods. The number of BrdU+ cells in each region was counted and expressed as a percent of the total number of cells. The bins labeled IZd (Intermediate zone deep) are closest to the VZ, the bins labeled IZs represent the cells in the intermediate zone closest to the cortical plate, the bins labeled CP are in the cortical plate. The distributions of the E24 MAM treated cultures alone (n=6) and the E24 MAM treated cultures with reeler cortical explants (n=5) were significantly different from the normal distribution (n=6) in the IZs and CP, whereas the E24 MAM treated cultures paired with WT or het explants (n=5) were similar to the normal pattern at all levels. In the CP, the E24 MAM treated cultures alone and the E24 MAM treated cultures paired with reeler cortex were significantly different from the normal and E24 MAM paired with WT or het explants. Error bars indicate standard deviation. Significance was determined using a 2 way ANOVA followed by a Bonferroni test for pairwise comparisons.
* $p \leq 0.05$, + $p \leq 0.006$, # $p < 0.001$.

Chapter 2, Figure 4. Schaefer, A.W., Poluch, S. and Juliano, S.L.

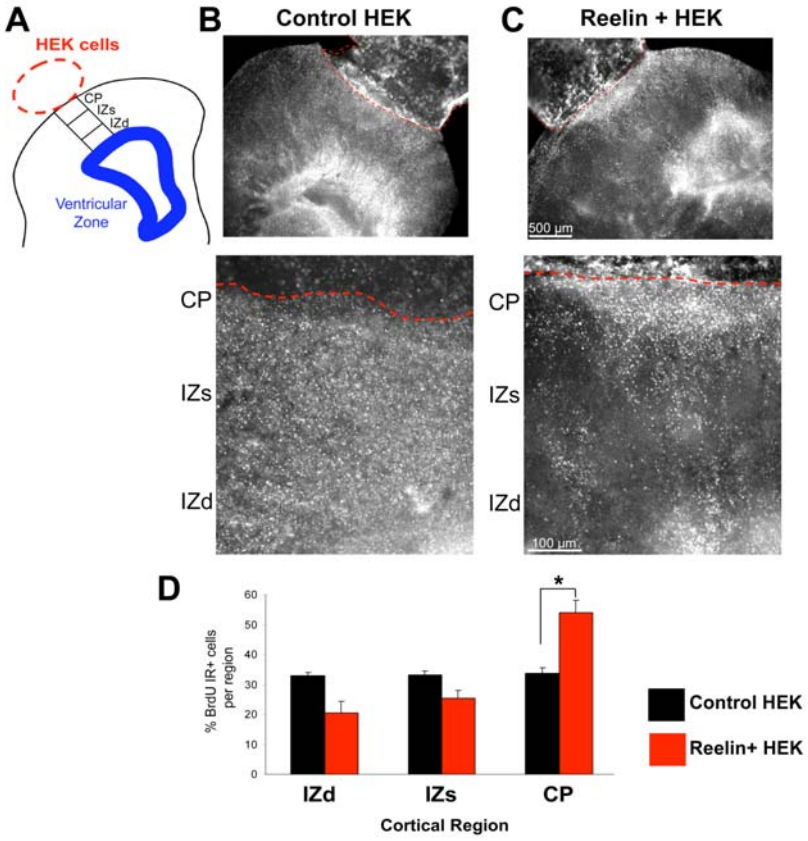


Figure 4. Position of migrating cells in E24 MAM treated organotypic cultures after coculture with control or reelin+ cells. **A** HEK cells transfected with the gene encoding reelin, or control cells without the gene, were placed into a drop of matrigel (red oval) that was positioned near the pial surface of E24 MAM treated organotypic cultures of cortex. **B** Control cells were placed in the matrigel, BrdU+ cells can be seen in a typical pattern for an E24 MAM treated slice in that they are spread throughout the VZ and cortical plate and do not migrate effectively. **C** An example of an E24 MAM treated cortical culture with reelin+ cells in matrigel placed at the pial surface. In this case, the cells migrate successfully to the cortical plate and many more of them are positioned near the cortical surface. **D** Quantification of the number of migrating BrdU+ cells found in different regions within the cortical wall. The space between the VZ and pial surface was

divided into 3 equal regions as described in the Methods and as indicated in **A**. The number of BrdU+ cells in each region was counted and expressed as a percent of the total number of cells. The bins labeled IZd (Intermediate zone deep) are closest to the VZ, the bins labeled IZs represent the cells in the intermediate zone closest to the cortical plate, and the bins labeled CP are located in the cortical plate. The red dashed lines indicate the border between the placement of the HEK cells in matrigel and the brain. There was a significant difference between the percent of cells reaching the CP in cultures with the reelin+ (n=6) versus control cells (n=8). Error bars indicate standard deviation. Significance was determined using a 2 way ANOVA followed by a followed by a Tukey test. ***** $p < 0.05$.

Chapter 2, Figure 5. Schaefer, A.W., Poluch, S. and Juliano, S.L.

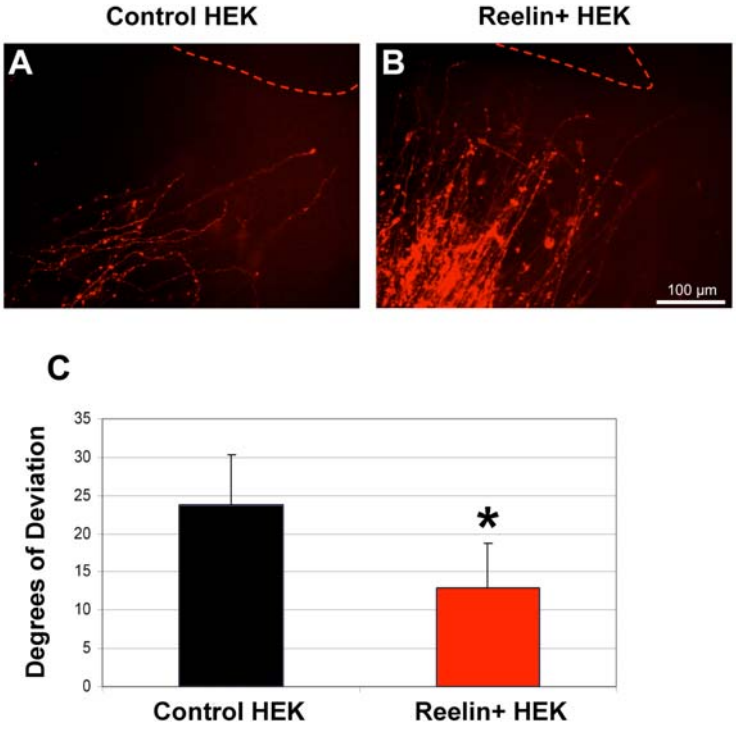


Figure 5. Morphology of E24 MAM treated radial glia after coculture with control or reelin producing HEK cells. Small crystals of DiI were placed in the ventricular zone of E24 MAM treated organotypic cultures to label radial glial fibers. **A.** The slices cultured with control HEK cells (no reelin) displayed disorganized glial fibers that generally did not extend to the pial surface (n=4). **B.** The cultures paired with reelin+ HEK cells contained radial glial fibers that were elongated and extended to the pial surface (n=4). **C**. The angular deviation was computed for these radial glial fibers and found to be significantly different between the two culture conditions. Mann Whitney Rank Sum Test, P=0.006. The red dashed lines delineate the HEK cells included in Matrigel.

Chapter 3

Repair of neonatal cortical dysplasia using embryonic neural progenitor cells in ferret.

A.W. Schaefer and S.L. Juliano

ABSTRACT

Although altered gene expression clearly causes failure of the neocortex to form properly, many causes of neocortical dysplasia arise from environmental or unknown factors. Our lab studies a model of cortical dysplasia induced by injection of methylazoxy methanol (MAM) into pregnant ferrets on embryonic day 33 (E33), which shares many features of neocortical dysplasia in humans. E33 MAM treatment results in characteristic deficits that include dramatic reduction of layer 4 in somatosensory cortex, widespread termination of thalamic afferents and altered distribution of GABAergic elements. We attempted to repair MAM treated cortex using ferret neural progenitor cells obtained at E27 and E33 and mouse neural stem cells obtained at E14. When these cells were transplanted into organotypic cultures of normal and MAM treated ferret cortex obtained on postnatal day 0 (P0), all progenitor cells migrated similarly in both hosts, preferentially residing in the upper cortical plate. The site of transplantation was significant, however, so that injections into the ventricular zone were more likely to reach the cortical plate than transplants into the intermediate zone. When similar cells were transplanted into ferret kits ~P7-9 and allowed to survive for 2-4 weeks, the donor cells migrated differently and also reached distinct destinations in normal and MAM treated hosts. This suggests that the organotypic cultures may be too immature to support normal migration of the transplanted cells, while transplants into the ferret kits indicate that MAM treated brains serve as more receptive hosts, and E27 donor cells are less restricted than E33 donor cells.

INTRODUCTION

During development of the cerebral cortex, progenitor cells line the lateral ventricles, in a region known as the ventricular zone (VZ). Pyramidal cells arise from the dorsal telencephalon and migrate radially along a glial scaffold towards the pia. Interneurons on the other hand, are born in the VZ of the ganglionic eminences in the ventral telencephalon. These cells migrate tangentially from the ventral to the dorsal telencephalon and into the cortical plate (Anderson et al., 2001; Marin and Rubenstein, 2001). The migrating cells populate the cerebral cortex in an "inside–out" manner so that the later born cells bypass earlier born cells to populate more superficial layers (Rakic, 1990; Parnavelas, 2000; Kriegstein and Noctor, 2004). In the ferret, which has a gestation period of 41 days, corticogenesis is relatively protracted and continues until approximately 2 weeks after birth, depending on the cortical region (Jackson et al., 1989; Noctor et al., 1997).

This complex process of cortical development can be disrupted due to genetic and environmental factors. Defects in genes such as *LIS1, DCX,* and *FLN* identified in humans result in cortical malformations including lissencephaly, doublecortex, and ectopic neuronal clusters (Gleeson et al., 1999). Epigenetic factors such as alcohol, drugs of abuse, methyl mercury, cigarette smoke and radiation can produce a constellation of cortical defects consisting of abnormal proliferation, and migration, cell death, and other central nervous system (CNS) changes. For example, fetal alcohol syndrome can result in altered neuronal distribution and migration, as well as neuronal heterotopias (Miller, 1993, 1996; Ikonomidou et al., 2000; Rice and Barone, 2000; Mendola et al., 2002; Bailey et al., 2004; Miller, 2006).

Often, cortical dysplasias in humans are not associated with specific genes, and usually the result of environmental causes or a combination of genetic and environmental effects (Montenegro et al., 2002; Sisodiya, 2004). Additional evidence points to trauma and infectious diseases during pregnancy contributing to human cortical dysplasias (Marin-Padilla, 1999; Deukmedjian et al., 2004). As a result we developed a model of cortical dysgenesis that mimics many features of human disorders (Noctor et al., 2001a; Palmer et al., 2001; McLaughlin and Juliano, 2005).

The research reported here focuses on repairing congenital neural migration deficits during the neonatal period. To do this we use an animal with a highly convoluted cortex, the ferret. The ferret is an ideal model to study cortical migration and repair partly because it has a protracted period of corticogenesis; cells continue to divide and migrate until several weeks after birth. In addition, radial glia, which guide migrating cells from the VZ to the cortical plate (CP), persist until 2-3 weeks after birth (Voigt, 1989). The presence of these glial guides may facilitate repair if transplanted cells are delivered during the window of time when they are still guiding endogenous neurons to the CP. In our model, an antimitotic (methylazoxy methanol; MAM) is delivered to pregnant ferrets, which temporarily disrupts the birth of cortical neurons (Cattaneo et al., 1995; Cattabeni and Di Luca, 1997; Noctor et al., 2001a; Jablonska et al., 2004; McLaughlin and Juliano, 2005). When MAM is injected on embryonic day 33 (E33) the birth of cells populating layer 4 of the somatosensory cortex is interrupted, leading to a set of characteristic effects. While many features of the E33 MAM treated ferret appear normal, including the topographic maps of somatosensory and visual cortex, the general architecture of the cortex outside of layer 4 in somatosensory cortex, and the distribution of excitatory

amino acid receptors, other features are abnormal (Noctor et al., 1997; Noctor et al., 2001a). These include alteration of thalamocortical projections, which normally terminate in layer 4, the distribution of $GABA_{A\alpha}$ receptors and GABAergic neurons, and the neuronal responses recorded through the cortical layers (Noctor et al., 2001a; Palmer et al., 2001; Jablonska et al., 2004; McLaughlin and Juliano, 2005). The E33 MAM treated ferret model makes an excellent host to attempt repair by transplanting neural progenitor cells from embryonic ferrets and mice. The animals have characteristic migratory deficits, allowing us to assess differences between normal and MAM treated cortex after receiving donor transplants. In the current study we used donor cells from 3 sources to determine the potential of distinctive donors to repair damaged cortex *in vivo* and *in vitro*. We included donor cells from i) embryonic day 27 (E27) ferrets, which are young, theoretically multipotential, and able to migrate to different cortical layers (Bohner et al., 1997), (ii) ferret embryos at E33, which corresponds to the population of cells missing from the treated ferrets, and (iii) E14 mouse neural stem cells to demonstrate the capability of cells from another species to survive, migrate, and respond to cues after transplantation.

METHODS

MAM injection

On the 33rd day of gestation pregnant ferrets (Marshall Farms, New Rose, N.Y.) were injected ip with methylazoxy methanol (14-17 mg/kg; Midwest Research Institute dissolved in 5ml of 0.9% sodium chloride); MAM inhibits mitosis for approximately 8 hours (Cattaneo et al., 1995; Noctor et al., 2001a; Palmer et al., 2001; Jablonska et al., 2004; McLaughlin and Juliano, 2005).

Organotypic cultures

P0 ferrets kits were anesthetized with either 50 mg/kg euthasol or Na pentobarbital. When the animal was unresponsive to painful stimuli, the brain was quickly removed and placed in ice-cold aCSF. Coronal slices (500 μm) were cut using a Stoetling tissue chopper and placed on a 0.4 μm culture plate insert (Millicell-CM, Bedford, MA) in a 6 well plate with enough media to form a meniscus above the slice. We used minimum essential medium (MEM) with Earle's salts without L-Glutamine (Gibco) supplemented with 10% normal horse serum (Gibco), 0.001% gentamicin, L-glutamine-200mM 100x (Gibco) and 0.6% glucose. The slices were placed in an incubator (37ºC, 5% CO_2) and maintained for 3-5 days. Most of these cultures received injections of ferret neural progenitor cells or mouse cells within 24 hours of slice preparation.

Preparation of ferret neural progenitor cells

Under sterile conditions, the uterine horns containing either E27 or E33 embryos were removed from a pregnant ferret under 1-2% isofluorane anesthesia. The uterine horns were immediately placed in ice-cold artificial cerebral spinal fluid (aCSF)

(containing H_2O, and in mM amounts, NaCl, 124; $NaHCO_3$, 26; Glucose, 10; NaH_2PO, 1.2; KCl, 3.2; MgSO4, 1.2; $CaCl_2$, 2.4), the embryos removed and the brains isolated. To obtain donor cells, the meninges, the rostral and caudal poles were removed from the telencephalon and the cerebral cortices dissected from the brains, and placed in ice-cold oxygenated aCSF. The tissue was coarsely chopped using micro-dissection scissors, and then transferred to a 15 ml centrifuge tube containing PBS without calcium or magnesium (Gibco) and 0.6% glucose (Sigma). The solution was then mechanically triturated to form a single cell suspension using a series of 9" fire polished Pasteur pipettes. Cell density was calculated and the cells were prepared for labeling and acute transplantation either into organotypic slices or into host animals. Preparation of cells for transplantation was completed within 2 hours. Injection into either slices or into host brains took an additional 2-4 hours. Hence cells were injected into host tissue within 6 hours from the time the uterine horns were removed from the pregnant ferret. Cells were also proliferated, passaged and differentiated to determine their potential fates both *in vivo* and *in vitro*. We refer to these cells as ferret neural progenitor cells (fNPs) with the *caveat* that may contain post-mitotic cells. Our experiments showed that single cells placed in a flask with media or at low density in a collagen matrix gave rise to spheres indicating that calling them ferret neural progenitor cells is an appropriate definition.

Cell culture

Ferret cells were isolated as described above and placed into Complete Neurocult™ Proliferation Medium composed of: NeuroCult® NSC Basal Medium (mouse) 900 ml supplemented with NeuroCult® NSC Proliferation Supplements (mouse) 100 ml and recombinant human epidermal growth factor 20ng/ml (rhEGF) (Stem Cell

Technologies, Vancouver, Canada); they were allowed to proliferate 7-9 days before passaging. Cells went through a minimum of 2 passages before being used for differentiation. They were differentiated either as whole spheres or as single cells for 3-7 days on poly-L-ornithine 15 μg/ml (Sigma, St. Louis, MO) coated glass coverslips, Complete Differentiation Medium® containing NeuroCult® NSC Basal Medium (mouse) 900 ml supplemented with NeuroCult® NSC Differentiation Supplements (mouse) 100 ml (Stem Cell Technologies). The proliferative potential of ferret donor cells was assessed using the Neurocult® Neural Colony Forming Cell (NCFC) assay procedure using the Neurocult® NCFC Assay kit (mouse) from Stem Cell Technologies. The cells were proliferated as free floating neurospheres and passaged 2x before being used for the clonal assay. Cells were dissociated into a single cell suspension and plated at a very low density in a collagen matrix supplemented with Neurocult® NCFC serum free medium without cytokines, NeuroCult® NSC Proliferation Supplements (mouse) and rhEGF 10 μg/ml (Stem Cell Technologies). Cultures were replenished with Complete Replenishment Medium: NeuroCult® NSC Basal Medium (mouse) 9 ml supplemented with NeuroCult® NSC Proliferation Supplements (mouse) 1 ml and rhEGF 0.5 μg/ml (Stem Cell Technologies) once every 7 days. The single cell was followed for 21 days by using a Sharpie pen® to draw a circle around the individual cell and giving it a reference number. The same cells were digitally imaged at regular intervals to capture their expansion.

Mouse neural progenitor cells

Embryonic day 14 (E14) mouse cells were taken from the cerebral cortices of CD-1 (Charles River Laboratories, Wilmington, MA) timed-pregnant mice using aseptic

technique. The uterine horns with the embryos were removed from the anesthetized mother and derived in the same manner as described above. Once the cells were made into a single cell suspension, they were placed in NeuroCult® NSC Basal Medium supplemented with NeuroCult® NSC Proliferation Supplements and rhEGF (Stem Cell Technologies) and allowed to proliferate 6-8 days before passaging. The cells were passaged twice prior to injection into organotypic slices. We use the term mouse neural stem cells (mNSCs) to describe this procedure because the tissue was prepared according to the protocol of Reynolds and Weiss, who characterize these cells as "stem cells" (Reynolds et al., 1992; Reynolds and Weiss, 1996).

Labeling of neural cell suspensions

Five microliters of Vybrant CM-DiI cell labeling solution 1mM in solvent (Molecular Probes, Eugene, OR) was added to 1 million cells suspended in 1 ml of 37ºC PBS (Gibco) with 0.6% glucose. The cells were incubated for 15 minutes at 37ºC and mixed every few minutes. They were then washed 3 times by spinning the cell suspension at 1500 rpm for 5 minutes to form a pellet, removing the supernatant, and adding 12 more ml of 37ºC PBS with 0.6% glucose. After the washes, the cells were counted again and prepared for transplantation.

Transplants into organotypic slices

One hundred thousand cells were injected into specific regions of the cultured slice using a Nanoliter 2000 controlled mini-pump injector (WPI, Sarasota, FL) and a pulled micropipette with ~30 μm tip. Five injections of 50 nl each were placed on the surface of the slice resulting in a final injection volume of 250 nl. The slices in 6 well trays were placed back in the incubator (5% CO_2, 37°C) for 2 hours before more media

was added. Each injection took 2-3 minutes; therefore all slices received injections within a 2-3 hour period. A total of 65 slice cultures were used, taken from 36 kits obtained from 18 litters. The exact number of cultures analyzed for each condition is indicated in the graphs.

Transplants in host animals

In a sterile surgery suite (postnatal day) P7–9 ferret kits were anesthetized using 1% isoflurane and a nose cone and kept warm with a temperature controlled heating pad. Using a stereotaxic device, 500,000 cells in 1-2 µl of media were injected into the VZ of somatosensory cortex using the coordinates 3mm lateral and 3mm anterior of bregma at depths of 2 and 3 mm. This was done with a Hamilton syringe after making a small hole in the skull using a Dremel drill. The needle was left in place for 2 minutes before injecting the cells; the cells were injected over a 10 minute period and allowed to settle for 2 minutes prior to slowly retracting the needle. The skin of the skull was closed with NEXABAND® Liquid topical tissue adhesive (Abbott Laboratories, Chicago, IL). The surgery was completed within 20 minutes, allowing us to isolate and transplant donor cells into 4-6 host animals within 6 hours. Once surgery was concluded and the animal awoke from anesthesia, it received a 0.03 mg/kg injection of buprenorphine and was returned to the mother, where it was watched to ensure it remained awake and nursing normally. Kits that underwent surgery were compared to the non-surgical siblings for weight gain, ambulation, and nursing. All animals were normal in weight gain and behavior. A total of 24 kits were used, taken from 12 different litters. The exact number of animals and histologic sections used for each analysis is indicated on the graphs.

Tissue processing

Approximately 2-4 weeks after surgery each ferret was anesthetized using 50 mg/kg of either Na pentobarbital or euthasol. For immunohistochemistry each animal was perfused with 4% paraformaldehyde. The brain was removed from the skull and immersed in paraformaldehyde for several more days. Tissue was cut at 75 μm thickness on a vibratome or cryoprotected and cut at 30 μm thickness on a cryostat.

Immunohistochemistry

Tissue was washed in 0.1M PBS pH 7.4 three times and subsequently blocked for 1 hour in PBS serum and 0.1% triton-X. The primary antibody was prepared in the blocking solution, applied in the following dilutions: GFAP 1:500, Nestin 1:100 (Chemicon, Temecula, CA), TUJ1 1:100, and MAP2abc 1:100 (Sigma, St. Louis, MO) for 1 hour at room temperature on a shaker and then left 48 hours at 4ºC. The appropriate secondary antibody Alexa 488 or Alexa 546 (Molecular Probes) was applied for 1 hour and the slice washed 3 times with PBS. Each slice was incubated in a 2 μg/ml solution of bisBenzimide for 5 minutes to label nuclei which was removed prior to be being mounted in Vectashield mounting medium for fluorescence (Vector labs, Burlingame, CA) and coverslipped.

Analysis

Organotypic cultures

To assess the distribution of cells leaving the injection site, only those that migrated away from the areas of injection were counted. We estimate that 30% of the injected cells migrated away from the location of injection. Since slices were made at P0, prior to the completion of cortical lamination, it is not possible at this early age in the ferret to clearly delineate individual layers, so to maintain consistency across slices and

experiments we considered a depth of 300 μm starting at the pia to be the cortical plate. The cortical plate was further subdivided into 3 equal laminar regions of 100 μm thickness that corresponded to the upper, middle, and lower cortical plate. The next 300 μm below the cortical plate corresponded to the intermediate zone, which was superficial to the deep IZ injection. Cells that migrated away from the site of injection and had at least one process were included in the cell count. This was done after imaging slices using a 10x or 20x objective and creating a montage of the slice. Using Adobe Photoshop (San Jose, CA) a new layer was added over the montage of the slice, the cortical depths were delineated and the zoom feature was used to determine morphology. Once the cells in each region were counted they were expressed as a percentage in order to compare across slices and experiments. Data is expressed as the mean ± SEM

In vivo sections

As in the organotypic cultures, only cells that migrated away from the injection site into the cortical plate were counted. We estimated that 10-20% of the transplanted cells migrated away from the injection sites. Using the nuclear stain bisBenzimide, six cortical layers were identified, which were combined into 3 regions that included: layers 1 and 2, layers 3 and 4, and layers 5 and 6. The location of the injection was targeted to the VZ immediately below the somatosensory cortex. One to three slices from the somatosensory cortex of each brain were used for analysis. The exact number of animals and slices for each condition are indicated on the graphs. The cortex was divided into lateral, middle, and medial regions and all the cells in each region were counted (Chapt. 3, Supplemental Figure 1). The labeled cells were recognized by the dye and by their

morphology; only cells that had clear processes were included in our analysis. Data is expressed as the mean ± SEM.

Later study using immunohistochemical markers for neurons and glia revealed that many of the CM-DiI labeled cells were neurons. Once all the cells were counted, the number in each region was expressed as a percentage of all the cells counted within a slice to reduce the variability that might occur between experiments.

RESULTS

Cultures of neocortical ferret cell suspensions

To determine the practicality and viability of using acute and/or proliferated embryonic ferret and mouse cells for transplantation into organotypic and *in vivo* hosts, we first examined the cells *in vitro* to assess their ability to proliferate and to differentiate into different cell phenotypes. To do this we assessed different culture conditions that included: (i) preparation of neurospheres, which were subsequently passaged and differentiated, and (ii) a clonal analysis to determine the potential of a single cell to proliferate and differentiate into different cell fates. These studies were not designed to conclude that we were creating ferret "stem cells", but that we obtained a population of cells from ferret embryos that could be expanded and differentiated into multiple cell fates.

This set of experiments established that cells obtained from E27 and E33 ferret cortex could proliferate into free floating spheres, survive, expand a minimum of 2 passages, and differentiate to give rise to neurons and astrocytes. We first determined that single free-floating cells could give rise to neurospheres, subsequently passaged and continue to form new spheres after multiple passages. Figure 1A shows an example of an E27 derived cell forming a clonal sphere after 3 weeks in culture in a collagen matrix. Figure 1B illustrates E33 derived cells that proliferate into spheres, that can be passaged multiple times and continue to form neurospheres. Figure 1C and D are examples of E33 neurospheres that were differentiated and immunoreacted to reveal neurons (TUJ1+), glia (GFAP+), and immature pluripotent cells (Nestin+). These examples demonstrate that E27 and E33 ferret donor cells can be handled similarly to mouse neural stem cells

(mNSC) (Reynolds and Weiss, 1996). Our analysis led us to conclude that cells we obtained from developing ferret cortex (E27 and E33) are capable of proliferating into clonal spheres and differentiating into multiple cell fates.

Organotypic culture experiments

Control experiments

Several experiments were used to control for specific aspects of our label and injection procedure. Because the cells were labeled with CM-DiI, there was a possibility that the dye could leak out or be incorporated by cells in the vicinity of the injection. To check these problems, two control experiments were conducted. First, acute suspensions of cells were killed by leaving them out of the incubator at room temperature in the tissue culture hood for 2 days; they were subsequently injected into the VZ of organotypic slices. The CM-DiI fluorescence was visible at the site of injection, but no labeled cells were identified in the cortical plate (see Supplemental Figure 2A, Chapt. 3). In a second control study, supernatant was removed from the labeled cells after the second wash and also injected into the VZ of organotypic slices. Although there was a minimal amount of fluorescent label at the site of injection, there were no labeled cells in the cortex (not shown). These two experiments suggest that leaking of the dye from dead cells or CM-DiI in the supernatant do not contribute to the observations in our analysis and that the presence of labeled cells in the cortex represents migration of the injected cells to the target neocortex.

Where is the best location to transplant donor cells?

We first asked whether the injection site influenced the ability of the cells to survive and migrate effectively. We chose two different loci for injection. The VZ, where

cells are born and normally begin migrating to the neocortex, and the IZ, a site closer to the target and containing the radial glial scaffold. These injection sites received ferret donor cells acquired at different times in development. When donor cells were injected into the ventricular zone, significantly more cells reached the cortical plate compared with transplantations into the intermediate zone, even though the IZ injections were closer to the cortical plate. Figure 2 shows examples of both E27 and E33 donor cells injected into either the VZ or the IZ (Chapt. 3, Figure 2A-F). The two donor cell types have a similar pattern of distribution in the host tissue, which is influenced by the location of injection (Chapt. 3, Figure 2G,H).

Of the cells that reached the cortical plate, we observed a differential distribution depending on the injection site. At the time of injection into the host slice (P0), cells destined for layers 2 and 3 are still being born and have not yet reached the cortical plate. The future layer 4 is migrating to the cortical plate and not completely organized into a compact layer. To analyze in more detail the distribution of transplanted cells that reached the cortical plate, this region was subdivided into upper, middle and lower CP as indicated in the Methods. As described above, most cells that were injected into the intermediate zone remained there. Of the cells that reached the CP after an IZ transplant, most were found in the lower cortical plate (LCP), the closest cortical area to the site of injection (Chapt. 3, Figure 2G,H). Cells injected into the VZ, however, migrated past the IZ to the most superficial region of the cortical plate, predominantly the upper cortical plate (UCP).

In summary, cells injected into the VZ of organotypic cultures of ferret cortex at P0 fare better than cells injected into the IZ. More cells reach the neocortex, and once there, a greater number migrate into the upper layers of the cerebral cortex.

The influence of cell donor type on migration pattern in normal and MAM treated organotypic cultures

We assessed whether the age of donor cells affects where they reside after transplantation. Earlier work by McConnell and colleagues found that depending on the mitotic cycle, younger cells are multipotential and under certain conditions capable of residing in many cortical layers, whereas later born cells become restricted in their laminar fate (McConnell, 1988a; Frantz and McConnell, 1996; Bohner et al., 1997; Desai and McConnell, 2000). In the ferret somatosensory cortex, E27 cells populate the deep cortical layers whereas cells born on E33 are destined for layer 4, which is the layer with dramatically fewer cells in our MAM model (Noctor et al., 1997). We also tested the ability of cells from another species; E14 mNSC to survive, migrate and differentiate in ferret cortex.

Each cell type (E27, E33 acute cell suspensions or E14 mNSC) was injected into the VZ of normal and MAM treated host organotypic cultures and incubated for 3 days. All cell donor types displayed similar patterns of migration in normal and MAM treated cortex. As indicated above, after VZ injections, the majority of cells migrated into the CP in both normal and MAM treated cortex. For cells that entered the cortical plate, a significant proportion of these reached the UCP (Chapt. 3, Figure 3A-G). This position corresponds to the target of endogenous cells migrating toward the cortical plate at the time the cultures received injection. The cells obtained from E27 embryos did not

preferentially reside in the lower layers (the normal location of ferret cells in somatosensory cortex born on E27). McConnell and colleagues reported that if E29 donor cells from the visual cortex undergo S-phase before being transplanted into the host tissue they migrate to their typical deep layer target, but if they undergo S-phase in the host tissue they will assume the laminar fate of the endogenous cells (McConnell and Kaznowski, 1991; Bohner et al., 1997). At P0, the age of the host cultures, cells born on E33 would normally be completing their migration into the UCP, as layers 2-3 of the somatosensory cortex are being generated and beginning their migration into the upper cortical plate at this time (Noctor et al., 1997). Figure 3A-E (left panels, Chapt. 3,) illustrates the distribution of different donor cells in the cortical plate after 3 DIC. In the right column of Figure 3A-E (Chapt. 3) high power views of typical labeled cells are shown. Figure 3F and G (Chapt. 3) demonstrates that for E27 and E33 donor cells, significantly more cells reach the upper part of the cortical plate in both normal and MAM host tissue. The mouse donor cells also migrated in a similar pattern as can be seen in Figure 3E and H (Chapt. 3). Figure 3H (Chapt. 3) shows that there were no significant differences in the migration pattern between any of the donor cells transplanted into MAM treated organotypic cultures. These observations may indicate that in our study, the transplanted cells respond to cues from the local environment, since neurons generated in the host are migrating to the superficial cortex at this time.

In summary, cells obtained from E27, E33 ferret or E14 mouse embryos and injected into the VZ of P0 organotypic cultures of ferret cortex preferentially migrated into the UCP, despite their date or species of origin. This was true for both normal and

MAM treated host organotypic cultures. These results suggest that the environment of the slice influences the ultimate distribution of the transplanted cells.

In vivo experiments

Although much information can be garnered from organotypic slice cultures, we also assessed the ability of the donor cells to survive, migrate, and integrate when transplanted *in vivo*. This approach has stronger clinical relevance and can provide greater information regarding the potential of transplanted cells to treat disorders *in vivo*. We assessed the viability and integration of donor cells prepared in different ways, since the fate of transplanted cells may be influenced by treatment prior to injection. Donor cells for *in vivo* transplantation were used in two ways: (i) labeled and transplanted acutely, or (ii) placed in Complete Neurocult™ Proliferation Medium (Stem Cell Technologies) for 2-8 days, without passaging, then dissociated, labeled, washed and transplanted. The second treatment involved exposure to rhEGF as described in the Methods (Reynolds et al., 1992a; Reynolds and Weiss, 1996). In all cases, MAM treated and normal ferret kits aged P7-9 days received donor cell transplants and were sacrificed 2-4 weeks later. No differences were noted between the ages and survival times. The injections were targeted to the VZ and 1 mm superficial to the VZ (i.e. the site of the subventricular zone, SVZ) in the region of the somatosensory cortex.

Control experiments

Control injections of dead, CM-DiI labeled cells were injected into host brains. The cells were killed by incubation in 4% paraformaldehyde in PBS for 2 hours, and then washed three times in buffer before injection. They were injected in the same volume as

live cells. Although fluorescent cells could be seen at the site of injection, none migrated to the cortex (Chapt. 3, Supplemental Figure 2B).

Overall pattern of migration

Cells were counted as described in the Methods and in Supplemental Figure 1A and B. Figure 4 shows examples of transplant sites into normal and E33 MAM treated hosts and the overall migration pattern. We observed that in the normal animals, both E27 and E33 donor cells migrated to a restricted middle region in the somatosensory cortex (Chapt. 3, Figure 4D-F). In contrast, the overall distribution of the transplanted cells in the MAM treated hosts was more diffuse, with cells migrating into the medial, middle, and lateral cortical regions (Chapt. 3, Figure 4A-C). Higher power views of cells that migrated into the cerebral cortex can be seen in Figure 4G-I (Chapt. 3). For both the E27 and E33 donor cells, significantly more cells were located in the middle region of cortex in normal hosts, whereas there were no significant differences in the distribution of either donor type in MAM treated hosts (Chapt. 3, Figure 5A-B).

Donor cell type: proliferated vs acute

Acute and proliferated cells were taken from E33 normal donors and either transplanted within 6 hours after retrieval of the embryo containing uterine horns or post proliferation as described in the Methods. After assessing the distribution of cells that migrated into the cortex, we found no difference between the proliferated cells and the acutely transplanted cells. Figure 6A (Chapt. 3) demonstrates donor cells obtained at E33, proliferated as neurospheres, and transplanted into MAM treated brains distribute similarly to those transplanted acutely. Comparable results emerged for E33 donor cells either proliferated or acutely transplanted into normal animals (Chapt. 3, Figure 6B).

Since there were no significant differences between the distributions of acute or proliferated donor cells, the data from the two conditions were combined for subsequent analyses.

MAM vs normal host for E27 and E33 fNPs

E27 donor cells migrated differently in normal and MAM treated hosts (Chapt. 3, Figure 6C). In normal animals, the cells reached all layers in relatively equal proportions. In the MAM treated brains, significantly more E27 donor cells moved into the superficial layers 1-2. Transplants of E33 donor cells in normal and MAM treated brains, however, did not distribute differently in the two hosts, but existed in relatively equal proportions through all cortical layers (Chapt. 3, Figure 6D). Although there was slight variability in the overall laminar pattern of the E33 donor cells between the normal and MAM treated hosts, there were no significant differences between the two groups. We also compared the distribution of E27 and E33 donor cells transplanted into E33 MAM treated host cortex. As in the other comparisons, more E27 donor cells reached the superficial layers 1-2 than the number of cells that settled in layers 3-4 and 5-6. The E33 donor cells were more evenly distributed and showed no significant differences in the laminar pattern (Chapt. 3, Figure 6E).

Transplanted cell phenotype

We used neuronal and glial markers to determine if we could ascertain the phenotype of donor cells that migrated into the neocortex. A number of the fluorescently labeled transplanted cells that migrated into the cortex were reactive for the neuronal marker MAP2abc (Chapt. 3, Figure 7A, B, C). However, we did not find any CM-DiI labeled cells that expressed the astrocytic marker GFAP (Chapt. 3, Figure 7D, E).

Nevertheless, many GFAP+ cells were found in the region where cells were injected, possibly indicating the presence of reactive cells around the injection site (Yang et al., 2002).

DISCUSSION

MAM treatment and the ferret model

Methylazoxy methanol (MAM) is an anti-mitotic that inhibits cell birth for a limited period of time. In our model, we specifically target layer 4 of ferret somatosensory cortex by injecting MAM on embryonic day 33 (E33) into pregnant ferrets. MAM prevents DNA synthesis in dividing cells, which recovers in 8-12 hours (Evans and Jenkins, 1976; Noctor et al., 1999). Several groups demonstrated that administration of MAM during gestation results in selective neuronal loss, due to the prevention of DNA synthesis during a restricted window of time (Matsumoto et al., 1972; Evans and Jenkins, 1976; Noctor et al., 1999). MAM is also selective for interfering with the proliferation of neurons, but not astrocytes (Cattaneo et al., 1995). Using appropriately timed BrdU injections, we previously demonstrated that DNA synthesis recommences within 24 hours after MAM injection and that relatively normal birth of cells and migration of neurons after MAM treatment in ferrets (Noctor et al., 1999). In rodents, other studies report resumption of normal cell birth after about 12 hours (Evans and Jenkins, 1976).

Ferrets are an excellent animal to study for this model. They have a relatively long period of corticogenesis (Noctor et al., 1999), allowing precise targeting of a specific cortical layer with an injection of MAM. In addition, they have a highly convoluted cerebral cortex, typical of higher order mammals, which makes research more relevant to humans. Finally, we have described very precise characteristics that result from E33 MAM injections into pregnant ferrets, which establishes this treatment as an ideal model for attempts at cortical repair.

Why do cells migrate more efficiently when injected into the VZ vs IZ?

We found that transplants of cells into the VZ were significantly more effective in reaching the neocortex than injections of cells into the IZ. These findings suggest that the neocortical VZ provides cues to cells migrating into the cerebral cortex. Several reports support this notion. As cells migrate from the VZ into the neocortex, a period of retrograde migration often occurs in which migrating cells turn back into the VZ and then finally reverse and continue into the neocortex, suggesting cues are being received from the VZ (Kriegstein and Noctor, 2004; Noctor et al., 2004). In addition, spontaneous Ca^{++} fluctuations occur in radial glia and have been suggested to play a role in neuronal migration and cell division (Komuro and Rakic, 1996; Owens and Kriegstein, 1998; Weissman et al., 2004). The fluctuations originate in the VZ (LoTurco et al., 1991) and appear to be spontaneous, but also occur in response to electrical or mechanical stimulation (Weissman et al., 2004). Blocking the Ca^{++} currents causes failure of neurons to migrate into the cortical plate, substantiating the idea that calcium transients are necessary for proper migration (Komuro and Rakic, 1998). Kriegstein and colleagues suggest that calcium increases may assist neuronal migration by coordinating specific events as neurons leave the VZ en route to the cortical plate (Weissman et al., 2004). Since the calcium waves originate in the VZ, this notion validates the idea that the VZ contains key signaling information for migrating neurons.

Another recent study suggests that Slit is a protein secreted by the choroid plexus and may diffuse through the VZ and act on migrating neurons by repelling them away from their source (Sawamoto et al., 2006). Slit1 and Slit2 are present in the CNS and act on the Robo class of receptors (Nguyen-Ba-Charvet et al., 2004). The action of Slit may

provide a mechanism that repels certain classes of neurons away from their origin in the VZ and toward the neocortex or other targets (Ward et al., 2003).

Laminar distribution of cells after transplantation into organotypic cultures.

Our studies found very little difference between donor cell type (E27, E33, mNSC) or host type (normal vs MAM) and the ultimate laminar distribution of the cells transplanted into organotypic cultures. After transplantation into either normal or MAM treated slices, all donor cell types tended to migrate into the upper cortical plate. The organotypic cultures used in our study were relatively young in terms of the overall laminar fate; they were prepared at P0 and remained in culture for 3 days. We used young brains because they survive better in organotypic culture conditions, but this compromised the ultimate laminar fate of the migrating transplants.

The finding of all donor types in the upper cortical plate suggests that all the transplanted cell types were influenced by the environment of the host slice, which would be generating neurons migrating into the cortical plate at the time of the injection (P0). Our findings agree with the earlier work of McConnell and colleagues who demonstrated that environmental cues are important in deciding the laminar fate of migrating neurons (McConnell and Kaznowski, 1991; McConnell, 1995; Frantz and McConnell, 1996). These researchers determined that when cells were transplanted into the brains of developing ferrets, neurons forming the earliest generated cortical layers (i.e. young neurons) were more flexible in their ability to respond to the environment (the host) than older neurons, which form later generated cortical layers. Therefore, a laminar fate restriction occurred as the animals matured (Bohner et al., 1997; Desai and McConnell, 2000). Moreover, the fate of the younger, more adaptable neurons was determined by the

location (i.e. the environmental cues) in which they underwent their last cell division (Bohner et al., 1997; Desai and McConnell, 2000). The studies conducted here were within the time frame identified by McConnell and colleagues to allow the last cell division within the host tissue. This reinforces the idea that all the donor cells (E27, E33, and mNSCs) responded to cues in the slice, because cells migrating at this time P0-P3 would all be either in the upper cortical region or migrating to that site.

A recent study by Temple and colleagues, however, reports that proliferating cortical cells do not use external environmental cues to decide their fate, but rely on an intrinsic program to determine cell phenotype and their probable laminar location (Shen et al., 2006). Although this study is very interesting, it did not establish the laminar position of migrating cells, but used clonal analyses to assess cell fate, as determined by laminar immunocytochemical markers. A migrating cell in a relatively normal environment after transplantation (such as those observed by McConnell and associates *in vivo*) may be influenced by factors not available to cells in a clonal analysis. The same is true for our experiments in an organotypic environment, which allows us to assess actual laminar fate.

Location of transplanted cells in vivo

In contrast to the situation in organotypic cultures, transplants to ferret kits *in vivo* revealed distinctions between normal and MAM treated hosts and the age of the donor cells. After injections of donor cells into MAM treated host kits, the transplants migrated from the middle to the medial and lateral extents of our target area in somatosensory cortex. In contrast, the transplants into normal host kits migrated to a limited region in the middle part of the somatosensory cortex, just superficial to the injection site. This was

true for both E27 and E33 donor cells. These observations suggest that MAM treated cortex was a more permissive substrate for the donor cells in that they distributed widely throughout the cortex, whereas the donor cell distribution was very limited in normal cortex. Many studies indicate differences in the ability of cells to migrate in a damaged brain versus healthy host tissue, suggesting that damaged cortex may release a homing signal to transplanted cells (Ourednik et al., 2002). Interestingly, Sheen et al. (Sheen et al., 1999) indicated that the HiB5 multipotent neural precursor line integrates into host tissue when transplanted *in utero*, but not into the adult brain undergoing induced apoptotic degeneration. Findings such as these indicate that research done in this study holds more promise for translation into clinical cures versus studies that transplant progenitor cells in animal models of disease that induce cell death. The neonatal MAM treated ferrets provide a permissive environment for transplanted cells, (although we have a diminished layer 4 from an acute teratogenic insult), at the time the donor cells are transplanted endogenous cells are still migrating and the cortical layers are starting to form. Another possibility suggested by McConnell and colleagues and relevant to our study, is that constant feedback exists from the developing cortex to cells leaving the VZ (Desai and McConnell, 2000). They suggest that signals from neurons recently arrived in the cortex communicate to VZ cells that a layer is complete and no new cells are needed in that specific site. This idea may account for fewer transplanted cells migrating into to "normal" cortex as these brains had a full complement of neurons in each layer at P7-9. In E33 MAM treated brains, however, layer 4 remains incomplete, and feedback signals may encourage the migrating cells.

We additionally found that the transplanted cells migrated differently depending on the age of the donor cells and in normal vs MAM treated animals. E27 donor cells, which would normally reside in the lower cortical layers, migrated preferentially to the upper cortical layers in MAM treated cortex, while they distributed evenly through the cortical layers in normal animals. E33 donor cells distributed relatively evenly through the cortical layers in both types of hosts. The E27 donor cells, however, appeared to respond to the environment of the host, suggesting they were more flexible in their fate. One possibility is that the MAM treated brains may actually lack specific cues that inform cells to stop migrating when they reach a target layer, therefore allowing the more flexible E27 cells to keep migrating.

Differentiation of donor cells

When the cells transplanted *in vivo* were assessed to see if they displayed different phenotypes, we found a number of CM-DiI labeled transplanted cells were immunoreactive to neuronal antibodies. No fluorescently labeled donor cells were positive for GFAP, even though the same population of cells grown in culture differentiated into neurons and astrocytes. Indicating the different influences on phenotype *in vitro* versus *in vivo*. Albeit, it is possible that our markers were not sensitive enough to detect specific proteins, it is also possible that most transplanted cells in our experiments differentiated into neurons. Several studies suggest that cues in the host environment can direct grafted cells to become neurons (Macklis, 1993; Sheen et al., 1999; Ogawa et al., 2002; Yang et al., 2002). The host environment may specifically encourage neuronal differentiation of transplanted cells in several disease models such as

Huntington's and Parkinson's. Transplanting neural progenitor cells into MAM treated animals neonatally may drive them towards a neuronal fate.

Summary

Over the past decade the possibility of using progenitor cells and stem cells to repair damaged brain has become a reality (Horner and Gage, 2000; Lindvall et al., 2004; Goldman, 2005; Conti et al., 2006). Much of the previous research focused on neurodegenerative diseases. Our research targets cortical neuropathology at an early time point, when the brain contains factors and cues that support cell survival, migration, differentiation and integration. This has important clinical relevance as younger brains are plastic and more likely to respond positively to treatment with stem cells (Sheen et al., 1999). Taken together, our data indicate that progenitor cells are able to survive, migrate and differentiate in host cortex. In young animals, the host cortex influences laminar fate of the cells.

Chapter 3 References

Anderson SA, Marin O, Horn C, Jennings K, Rubenstein JL (2001) Distinct cortical migrations from the medial and lateral ganglionic eminences. Development 128:353-363.

Bailey CD, Brien JF, Reynolds JN (2004) Chronic prenatal ethanol exposure alters the proportion of GABAergic neurons in layers II/III of the adult guinea pig somatosensory cortex. Neurotoxicol Teratol 26:59-63.

Bohner AP, Akers RM, McConnell SK (1997) Induction of deep layer cortical neurons in vitro. Development 124:915-923.

Cattabeni F, Di Luca M (1997) Developmental models of brain dysfunctions induced by targeted cellular ablations with methylazoxymethanol. Physiol Rev 77:199-215.

Cattaneo E, Reinach B, Caputi A, Cattabeni F, Di Luca M (1995) Selective in vitro blockade of neuroepithelial cells proliferation by methylazoxymethanol, a molecule capable of inducing long lasting functional impairments. J Neurosci Res 41:640-647.

Conti L, Reitano E, Cattaneo E (2006) Neural stem cell systems: diversities and properties after transplantation in animal models of diseases. Brain Pathol 16:143-154.

Desai AR, McConnell SK (2000) Progressive restriction in fate potential by neural progenitors during cerebral cortical development. Development 127:2863-2872.

Deukmedjian AJ, King MA, Cuda C, Roper SN (2004) The GABAergic system of the developing neocortex has a reduced capacity to recover from in utero injury in experimental cortical dysplasia. J Neuropathol Exp Neurol 63:1265-1273.

Evans LA, Jenkins EC (1976) PHA response and methylazoxymethanol acetate. Chem Biol Interact 14:135-140.

Frantz GD, McConnell SK (1996) Restriction of late cerebral cortical progenitors to an upper-layer fate. Neuron 17:55-61.

Gleeson JG, Lin PT, Flanagan LA, Walsh CA (1999) Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. Neuron 23:257-271.

Goldman S (2005) Stem and progenitor cell-based therapy of the human central nervous system. Nat Biotechnol 23:862-871.

Horner PJ, Gage FH (2000) Regenerating the damaged central nervous system. Nature 407:963-970.

Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovska V, Horster F, Tenkova T, Dikranian K, Olney JW (2000) Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. Science 287:1056-1060.

Jablonska B, Smith AL, Palmer SL, Noctor SC, Juliano SL (2004) GABAA receptors reorganize when layer 4 in ferret somatosensory cortex is disrupted by methylazoxymethanol (MAM). Cereb Cortex 14:432-440.

Jackson CA, Peduzzi JD, Hickey TL (1989) Visual cortex development in the ferret. I. Genesis and migration of visual cortical neurons. J Neurosci 9:1242-1253.

Komuro H, Rakic P (1996) Intracellular Ca2+ fluctuations modulate the rate of neuronal migration. Neuron 17:275-285.

Komuro H, Rakic P (1998) Orchestration of neuronal migration by activity of ion channels, neurotransmitter receptors, and intracellular Ca2+ fluctuations. J Neurobiol 37:110-130.

Kriegstein AR, Noctor SC (2004) Patterns of neuronal migration in the embryonic cortex. Trends Neurosci 27:392-399.

Lindvall O, Kokaia Z, Martinez-Serrano A (2004) Stem cell therapy for human neurodegenerative disorders-how to make it work. Nat Med 10 Suppl:S42-50.

LoTurco JJ, Blanton MG, Kriegstein AR (1991) Initial expression and endogenous activation of NMDA channels in early neocortical development. J Neurosci 11:792-799.

Macklis JD (1993) Transplanted neocortical neurons migrate selectively into regions of neuronal degeneration produced by chromophore-targeted laser photolysis. J Neurosci 13:3848-3863.

Marin-Padilla M (1999) Developmental neuropathology and impact of perinatal brain damage. III: gray matter lesions of the neocortex. J Neuropathol Exp Neurol 58:407-429.

Marin O, Rubenstein JL (2001) A long, remarkable journey: tangential migration in the telencephalon. Nat Rev Neurosci 2:780-790.

Matsumoto H, Spatz M, Laqueur GL (1972) Quantitative changes with age in the DNA content of methylazoxymethanol-induced microencephalic rat brain. J Neurochem 19:297-306.

McConnell SK (1988) Fates of visual cortical neurons in the ferret after isochronic and heterochronic transplantation. J Neurosci 8:945-974.

McConnell SK (1995) Constructing the cerebral cortex: neurogenesis and fate determination. Neuron 15:761-768.

McConnell SK, Kaznowski CE (1991) Cell cycle dependence of laminar determination in developing neocortex. Science 254:282-285.

McLaughlin DF, Juliano SL (2005) Disruption of layer 4 development alters laminar processing in ferret somatosensory cortex. Cereb Cortex 15:1791-1803.

Mendola P, Selevan SG, Gutter S, Rice D (2002) Environmental factors associated with a spectrum of neurodevelopmental deficits. Ment Retard Dev Disabil Res Rev 8:188-197.

Miller MW (1993) Migration of cortical neurons is altered by gestational exposure to ethanol. Alcohol Clin Exp Res 17:304-314.

Miller MW (1996) Limited ethanol exposure selectively alters the proliferation of precursor cells in the cerebral cortex. Alcohol Clin Exp Res 20:139-143.

Miller MW (2006) Effect of prenatal exposure to ethanol on glutamate and GABA immunoreactivity in macaque somatosensory and motor cortices: critical timing of exposure. Neuroscience 138:97-107.

Montenegro MA, Guerreiro MM, Lopes-Cendes I, Guerreiro CA, Li LM, Cendes F (2002) Association of family history of epilepsy with earlier age at seizure onset in patients with focal cortical dysplasia. Mayo Clin Proc 77:1291-1294.

Nguyen-Ba-Charvet KT, Picard-Riera N, Tessier-Lavigne M, Baron-Van Evercooren A, Sotelo C, Chedotal A (2004) Multiple roles for slits in the control of cell migration in the rostral migratory stream. J Neurosci 24:1497-1506.

Noctor SC, Scholnicoff NJ, Juliano SL (1997) Histogenesis of ferret somatosensory cortex. J Comp Neurol 387:179-193.

Noctor SC, Palmer SL, Hasling T, Juliano SL (1999) Interference with the development of early generated neocortex results in disruption of radial glia and abnormal formation of neocortical layers. Cereb Cortex 9:121-136.

Noctor SC, Palmer SL, McLaughlin DF, Juliano SL (2001) Disruption of layers 3 and 4 during development results in altered thalamocortical projections in ferret somatosensory cortex. J Neurosci 21:3184-3195.

Noctor SC, Martinez-Cerdeno V, Ivic L, Kriegstein AR (2004) Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci 7:136-144.

Ogawa Y, Sawamoto K, Miyata T, Miyao S, Watanabe M, Nakamura M, Bregman BS, Koike M, Uchiyama Y, Toyama Y, Okano H (2002) Transplantation of in vitro-expanded fetal neural progenitor cells results in neurogenesis and functional recovery after spinal cord contusion injury in adult rats. J Neurosci Res 69:925-933.

Ourednik J, Ourednik V, Lynch WP, Schachner M, Snyder EY (2002) Neural stem cells display an inherent mechanism for rescuing dysfunctional neurons. Nat Biotechnol 20:1103-1110.

Owens DF, Kriegstein AR (1998) Patterns of intracellular calcium fluctuation in precursor cells of the neocortical ventricular zone. J Neurosci 18:5374-5388.

Palmer SL, Noctor SC, Jablonska B, Juliano SL (2001) Laminar specific alterations of thalamocortical projections in organotypic cultures following layer 4 disruption in ferret somatosensory cortex. Eur J Neurosci 13:1559-1571.

Parnavelas JG (2000) The origin and migration of cortical neurones: new vistas. Trends Neurosci 23:126-131.

Rakic P (1990) Principles of neural cell migration. Experientia 46:882-891.

Reynolds BA, Weiss S (1996) Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol 175:1-13.

Reynolds BA, Tetzlaff W, Weiss S (1992) A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J Neurosci 12:4565-4574.

Reynolds BA, Tetzlaff W, Weiss S (1992a) A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J Neurosci 12:4565-4574.

Rice D, Barone S, Jr. (2000) Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ Health Perspect 108 Suppl 3:511-533.

Sawamoto K, Wichterle H, Gonzalez-Perez O, Cholfin JA, Yamada M, Spassky N, Murcia NS, Garcia-Verdugo JM, Marin O, Rubenstein JL, Tessier-Lavigne M, Okano H, Alvarez-Buylla A (2006) New neurons follow the flow of cerebrospinal fluid in the adult brain. Science 311:629-632.

Sheen VL, Arnold MW, Wang Y, Macklis JD (1999) Neural precursor differentiation following transplantation into neocortex is dependent on intrinsic developmental state and receptor competence. Exp Neurol 158:47-62.

Shen Q, Wang Y, Dimos JT, Fasano CA, Phoenix TN, Lemischka IR, Ivanova NB, Stifani S, Morrisey EE, Temple S (2006) The timing of cortical neurogenesis is encoded within lineages of individual progenitor cells. Nat Neurosci 9:743-751.

Sisodiya SM (2004) Malformations of cortical development: burdens and insights from important causes of human epilepsy. Lancet Neurol 3:29-38.

Voigt T (1989) Development of glial cells in the cerebral wall of ferrets: direct tracing of their transformation from radial glia into astrocytes. J Comp Neurol 289:74-88.

Ward M, McCann C, DeWulf M, Wu JY, Rao Y (2003) Distinguishing between directional guidance and motility regulation in neuronal migration. J Neurosci 23:5170-5177.

Weissman TA, Riquelme PA, Ivic L, Flint AC, Kriegstein AR (2004) Calcium waves propagate through radial glial cells and modulate proliferation in the developing neocortex. Neuron 43:647-661.

Yang M, Stull ND, Berk MA, Snyder EY, Iacovitti L (2002) Neural stem cells spontaneously express dopaminergic traits after transplantation into the intact or 6-hydroxydopamine-lesioned rat. Exp Neurol 177:50-60.

Supplemental Information

Papain vs no enzyme

The use of enzymes to dissociate cells may remove the extracellular receptors, affecting how cells respond to cues from the extracellular environment. In order to assess if the dissociation method affected the migratory fate of cells; we mechanically dissociated cells using a Pasteur pipette or dissociated cells from the same litter by first digesting the tissue in Earl's Balanced Salt Solution (EBSS) (Gibco) with the enzyme 1 mg/ml papain (Roche, Indianopolis, IN), 0.2 mg/ml cysteine (Sigma), 0.2 mg/ml EDTA (Sigma) 0.1mg/ml DNase and incubated for 30 minutes in a 37°C water bath with manual rocking every few minutes. The solution was centrifuged, the supernatant discarded and the cell rich solution transferred to EBSS containing 10mg/ml of albumin, bovine fraction V (Sigma) and 10mg/ml trypsin inhibitor Type II-O, chicken egg white (Sigma), the solution was triturated then centrifuged. Following centrifugation, the supernatant was discarded and the cells resuspended in PBS without calcium or magnesium (Gibco) and 0.6% glucose (Sigma) and labeled with CM-DiI (see Methods), prior to being injected into host organotypic cortex (Gritti et al., 2001). We found that cells had a similar pattern of migration with either type of dissociation method (Supplemental figure 1A). When cells were injected into the ventricular zone (VZ) of MAM treated slices the papain-dissociated cells, as well as the mechanically dissociated cells, migrated to the cortical plate and once in the cortical plate, were located in the upper cortical region. There were no significant differences between groups.

Type of media

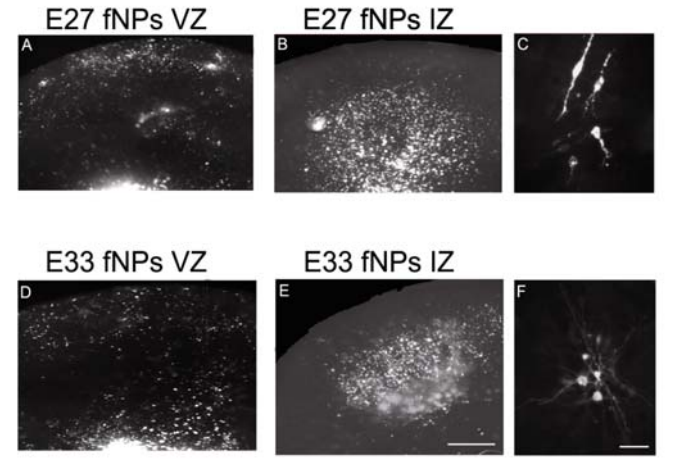
We also assessed whether the type of media and the presence or absence of serum (which contains undefined components) influence laminar fate. We found that laminar fate was not affected by the type of media. For all types of media, more cells were found in the UCP vs the MCP or LCP after injections into the VZ. The following paradigms were used: i) MEM with Earle's salts without L-Glutamine (Gibco) supplemented with 100ml (10%) normal horse serum (NHS) (Gibco), 10mg gentamicin, 10ml L-glutamine-200mM 100x (Gibco) and 6g of glucose (Sigma) per 1000ml of media. (ii) Neurobasal media (Gibco) with 20ml B-27 50x (Gibco), 10ml N2 100x (Gibco), 10mg gentamicin, 10ml L-glutamine-200mM 100x (Gibco) and 6g of glucose per 1000ml. (iii) Exposure to the MEM + 10% NHS serum containing media for 3 hours, which was then removed and replaced with the Neurobasal media.

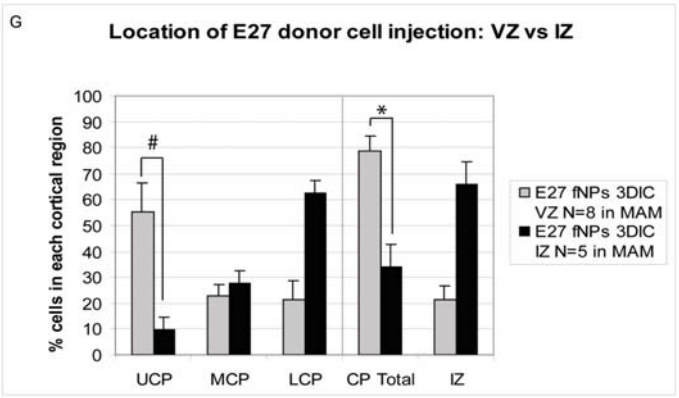
Chapter 3 Figures

Figure 1. Cell culture models of ferret embryonic cells. **A** is a clonal assay of E27 ferret neural progenitor cells proliferated as free floating neurospheres and passaged 2x before being used for the clonal assay. Cells were dissociated into a single cell suspension and plated at a very low density in a collagen matrix. The single cells were followed for 21 days. Individual cells were identified by drawing a circle around them on the bottom of the dish and allocating a reference number. The cells were imaged every 3 days with a digital camera to capture their clonal expansion. The grid underneath the sphere was a 2x2 mm scoring dish used to determine the size of the sphere. Scale bar = 1000 μm. **B** shows E33 free floating ferret neural progenitor cells. After 1 DIC, cells had yet to form spheres. Within 5 days spheres began to form. At 6 DIC spheres were about 200 μm in

diameter. Spheres were continuously passaged (approximately every 8 days) and they gave rise to new spheres. Scale bar = 200 μm. **C and D** show that E33 ferret neurospheres were differentiated in a manner similar to mouse neurospheres. The proliferation media containing EGF was removed and the cells were exposed to differentiation media containing serum. Whole spheres or single cells that had been dissociated from whole spheres were plated on a glass poly-L-ornithine coated coverslip and allowed to differentiate over several days. Ferret neurospheres gave rise to different cell types including neurons (TUJ1+) and astrocytes (GFAP+), or remained undifferentiated (Nestin+). Scale bar = 100 μm.

Chapter 3, Figure 2. Schaefer, A.W. and Juliano, S.L.





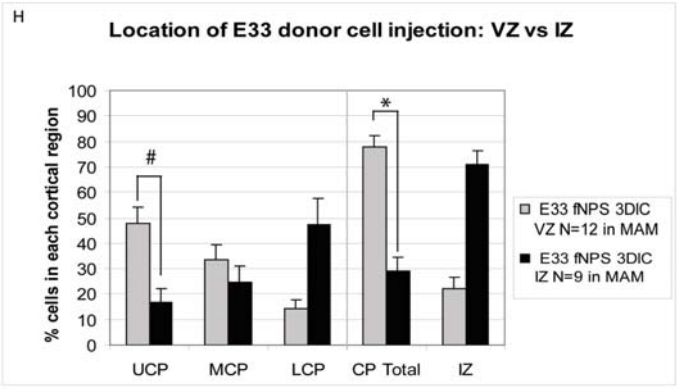


Figure 2. Location of donor cell injections into organotypic host slices. Images of E27 (**A, B, C**) or E33 (**D, E, F**) ferret neural progenitor cells (fNPS) injected into the ventricular zone (VZ) or intermediate zone (IZ) of ferret slices. Donor cells were labeled with the lipophilic fluorescent dye CM-DiI. After injections into the VZ, many cells could be seen in the CP, with a paucity of cells in the IZ. When cells were injected in the IZ, they remained there and few cells migrated away from the site of injection. Higher power views show the neuron-like morphology of donor cells (**C, F**). **A, B, D, E** Scale bar = 600 μm **C, F** Scale bar = 100 μm **G, H.** The right side of each graph demonstrates that significantly more cells reached the cortical plate after injections into the VZ, compared with IZ injections (Mann Whitney, * $P \leq 0.001$). The left side of each graph is a break-down of the cells that migrated into the CP. Within the CP, significantly more cells reached the upper cortical plate (UCP) when the injections were made into the VZ. This is true for both E27 (**G**) and E33 (**H**) donor cells. A two-way ANOVA revealed significant differences between VZ and IZ groups. A subsequent Holm-Sidak pairwise comparison test indicated significant differences between the percentage of cells that reached UCP in slices receiving either E27 or E33 injections (# $P \leq 0.050$). Error bar values represent the standard error of the mean (SEM). MCP, middle cortical plate; LCP, lower cortical plate. The number of organotypic slices analyzed for each condition is indicated in the graph legend.

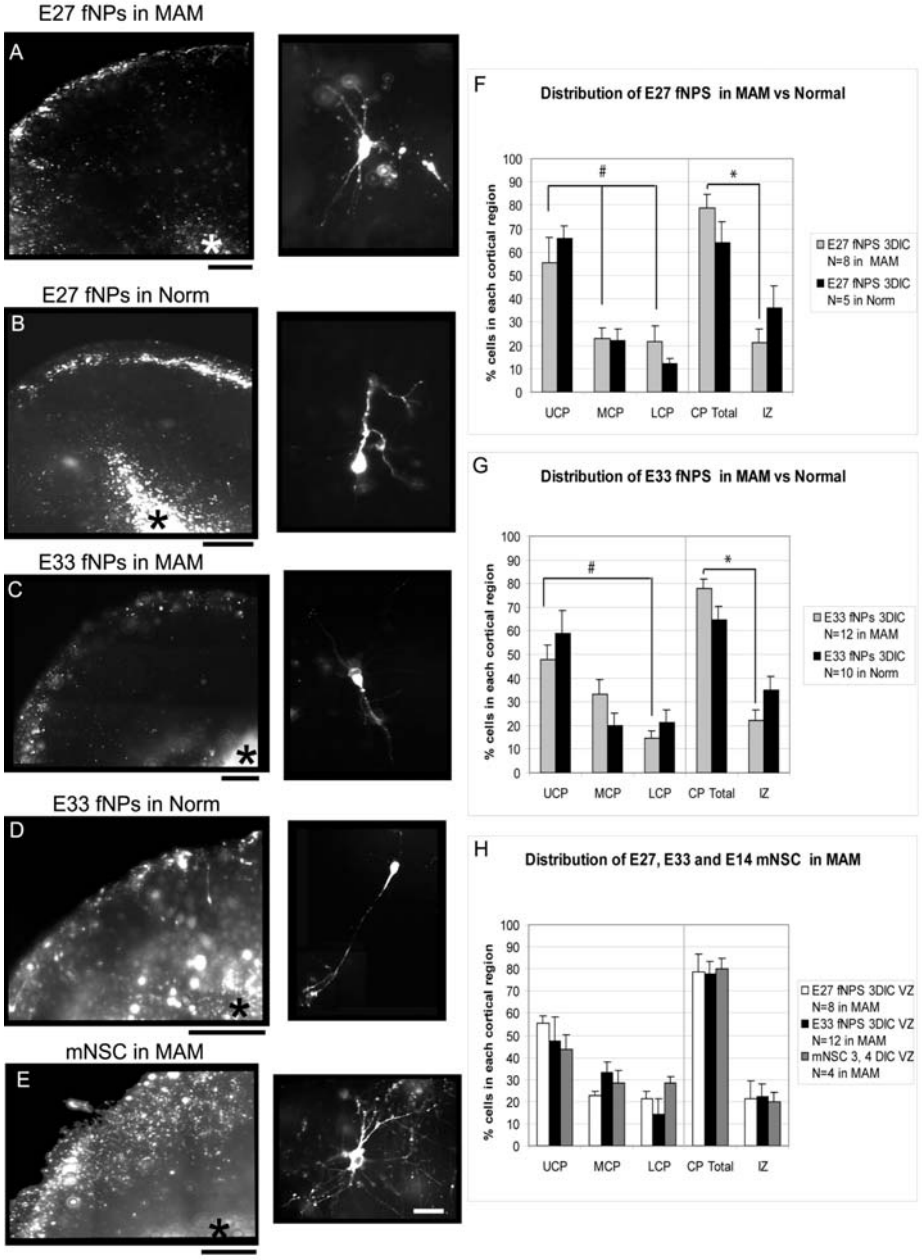

E27 fNPs in MAM
A
E27 fNPs in Norm
B
E33 fNPs in MAM
C
E33 fNPs in Norm
D
mNSC in MAM
E
F
Distribution of E27 fNPS in MAM vs Normal
% cells in each cortical region
UCP
MCP
LCP
CP Total
IZ
E27 fNPS 3DIC N=8 in MAM
E27 fNPS 3DIC N=5 in Norm
G
Distribution of E33 fNPS in MAM vs Normal
E33 fNPs 3DIC N=12 in MAM
E33 fNPs 3DIC N=10 in Norm
H
Distribution of E27, E33 and E14 mNSC in MAM
E27 fNPS 3DIC VZ N=8 in MAM
E33 fNPS 3DIC VZ N=12 in MAM
mNSC 3, 4 DIC VZ N=4 in MAM

Figure 3. Distributions of transplanted cells after injection into normal and MAM treated organotypic hosts. Figure **A-E** demonstrate examples of injections into organotypic cultures obtained from normal (**B, D**) and MAM treated (**A, C, E**) animals. They received injections of either E27 (**A, B**), E33 (**C, D**) or mouse neural stem cells (**E**). Higher power views of transplanted cells with neuronal morphology can be seen in the right hand columns. Asterisks indicate the injection site. The images in the right hand column show differentiated, neuronal morphology of the donor cells. Scale for the left hand images of **A, B, C, D, E** is 500 μm; for the higher power images in the right hand column, 10 μm. Graphs **F-H** represent the distributions of cells in the organotypic cultures after transplantation. **F** and **G** show that for E27 (**F**) and E33 (**G**) donor cells in both normal and MAM cortex, more cells reached the CP (cortical plate) as indicated in the right side of the graph using a paired t-test (*$P \leq 0.050$). The left side of the graph indicates that of the cells reaching the CP, a greater number of these cells migrated into the upper cortical plate (UCP) after injection in the VZ. This is true for both E27 and E33 donor cells and for normal and MAM treated hosts. This was determined using the two-way ANOVA followed by the Holm-Sidak test, (# $P \leq 0.050$). **H** demonstrates that all the three donor types of cells transplanted into organotypic slices migrated in a similar pattern, including the E27 and E33 fNPs, as well as the mNSCs (mouse neural stem cells). MCP, middle cortical plate; LCP, lower cortical plate. The number of organotypic cultures for each condition is indicated. Error bar values equal SEM.

Chapter 3, Figure 4. Schaefer, A.W. and Juliano, S.L.

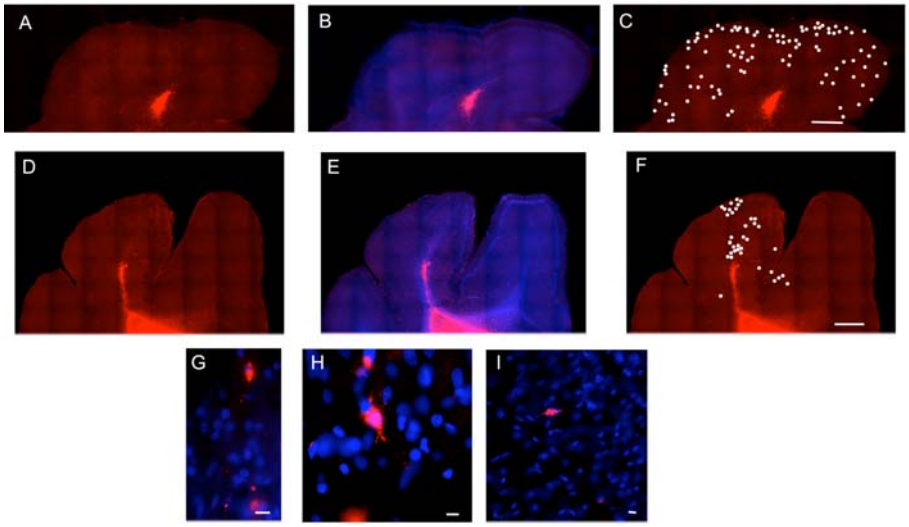


Figure 4. Injections of CM-DiI labeled donor cells into MAM treated and normal animals *in vivo*. **A-C** is an example of a donor cell injection into a MAM treated host, while **D-F** is an example of a transplant into a normal host. **A** and **D** demonstrate the CM-DiI labeled injection and **B** and **E** are an overlay of the injection with a bisBenzimide nuclear stain of the same image. Positions of the migrating cells are indicated in **C** and **F** with white dots. **G-I** are higher power images that show CM-DiI labeled cells that have migrated into the neocortex. **G** is from the MAM treated cortex and **H** and **I** are from the normal cortex. Scale bar = 1000 μm for **A-F** and 10 μm for **G-I.**

Chapter 3, Figure 5. Schaefer, A.W. and Juliano, S.L.

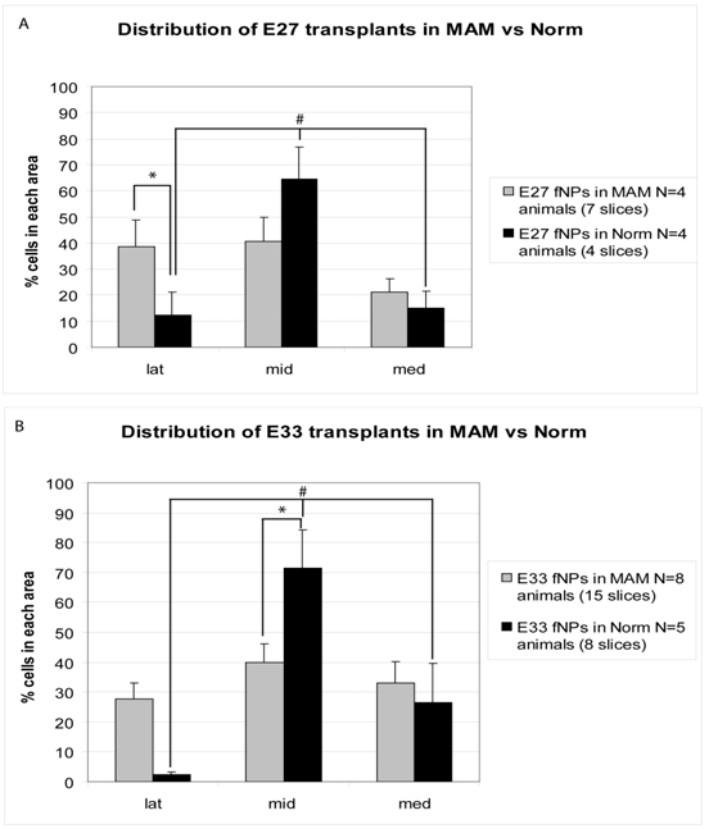


Figure 5. Graphs demonstrating the overall distribution pattern after injection into normal and MAM treated host kits. **A** shows that injections of E27 fNPs (ferret neural progenitor cells) were relatively evenly distributed throughout the mediolateral aspect of the somatosensory cortex after injection into MAM treated cortex, whereas they remained clustered to the middle region, superficial to the injection site in normal host kits. A two-way ANOVA, followed by Holm-Sidak indicated significantly more cells in the lateral aspect of MAM cortex versus normal cortex (* $P \leq 0.050$). Using the same statistical tests, comparisons within the normal cortex showed significantly more cells in the middle aspect of the cortex for E27 (**A**) and E33 (**B**) transplanted cells (# $P \leq 0.050$). Graph **B** shows significantly more cells in the middle region of normal cortex compared to MAM (* $P \leq 0.050$). Error bar values equal SEM. The number of animals (N) and total number of slices used for each condition are indicated.

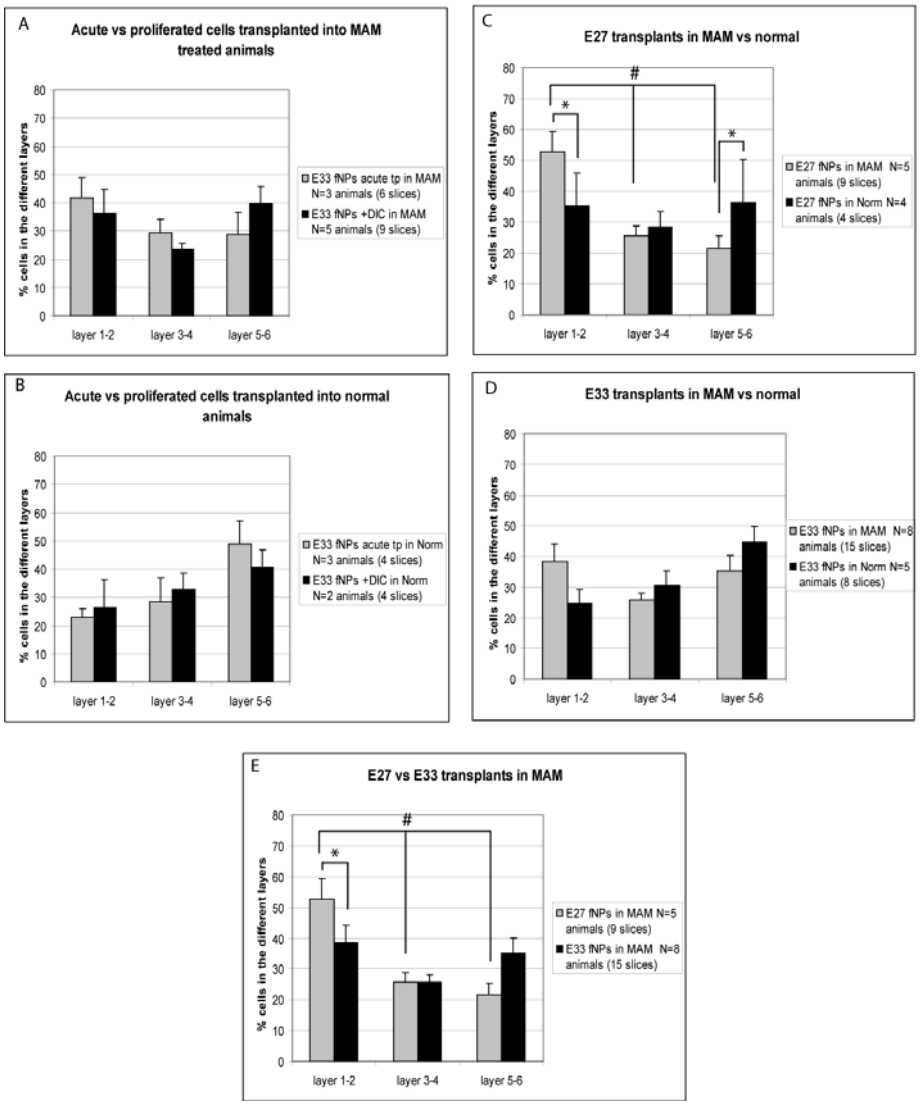


Figure 6. Graphs of the cortical distribution of donor cells transplanted into ferret kits. **A** and **B** compare the distribution of cells that were either proliferated or transplanted acutely. For E33 fNPs (ferret neural progenitor cells), the distributions were similar when comparing the distributions in MAM treated animals (**A**) or assessing the pattern in normal hosts (**B**). No significant differences were observed. **C** and **D** compare the cortical distributions of transplanted E27 versus E33 donor cells. When E27 fNPs were injected

into MAM treated hosts (**C**), the donor cells preferentially migrated into layers 1-2. Transplants of E33 fNPs into normal and MAM hosts, however, were distributed relatively equally among cortical layers (**D**). **E** compares the distribution of E27 versus E33 donor cell transplants in MAM treated hosts. There were significantly more E27 vs E33 donor cells in layers 1-2 (two-way ANOVA followed by Holm-Sidak pairwise comparisons; (*$P \leq 0.05$). The same statistical tests indicated that there are significantly more E27 cells in layers 1-2 vs layers 3-4 and 5-6 (# $P \leq 0.05$). Error bar values equal SEM. The number of animals (N) and slices used in each condition are indicated in the graphs.

Chapter 3, Figure 7. Schaefer, A.W. and Juliano, S.L.

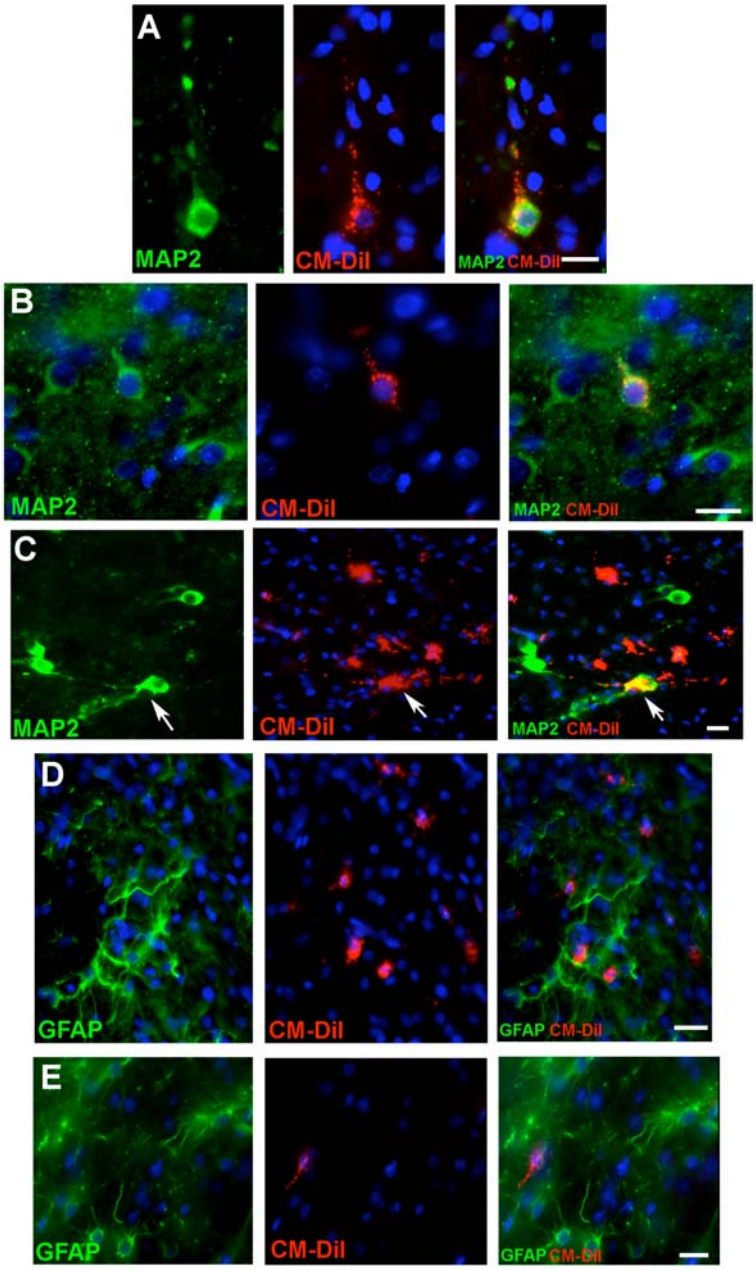
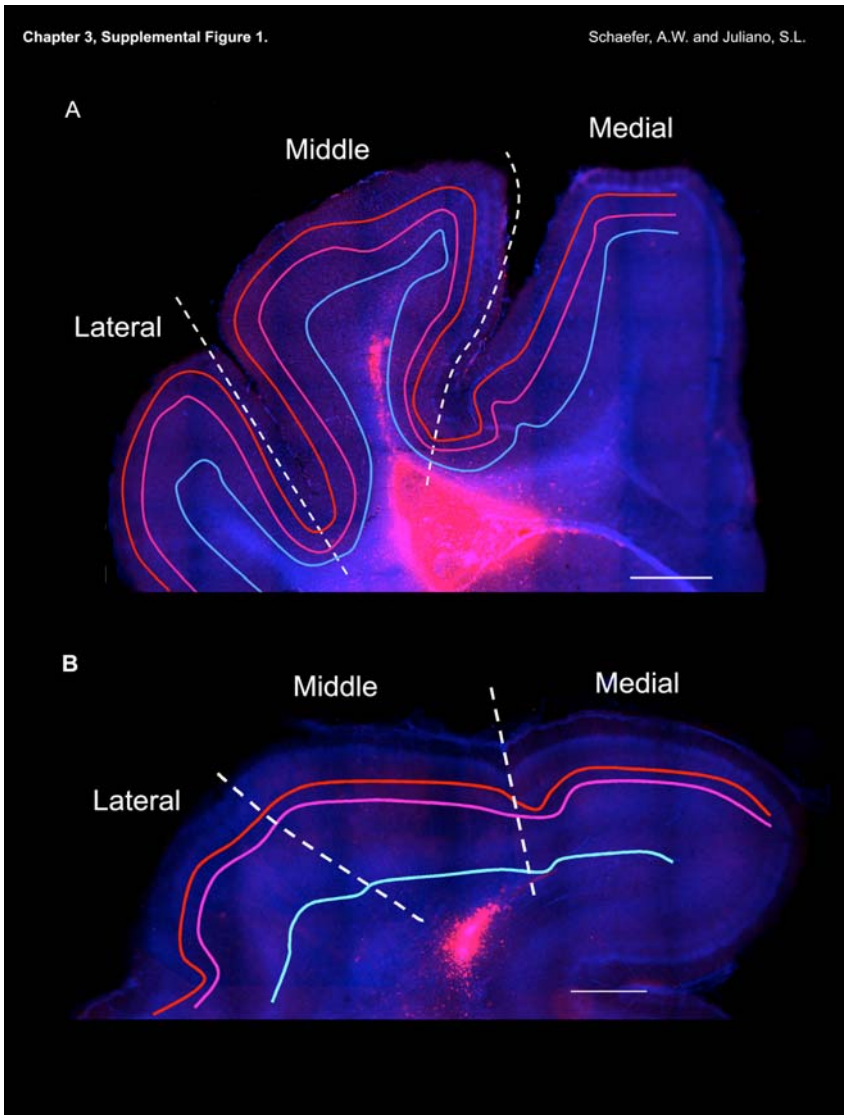
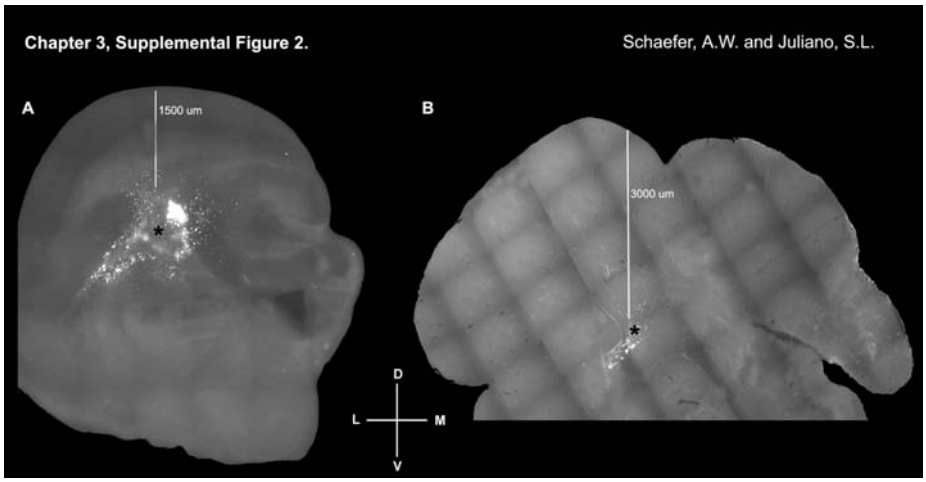


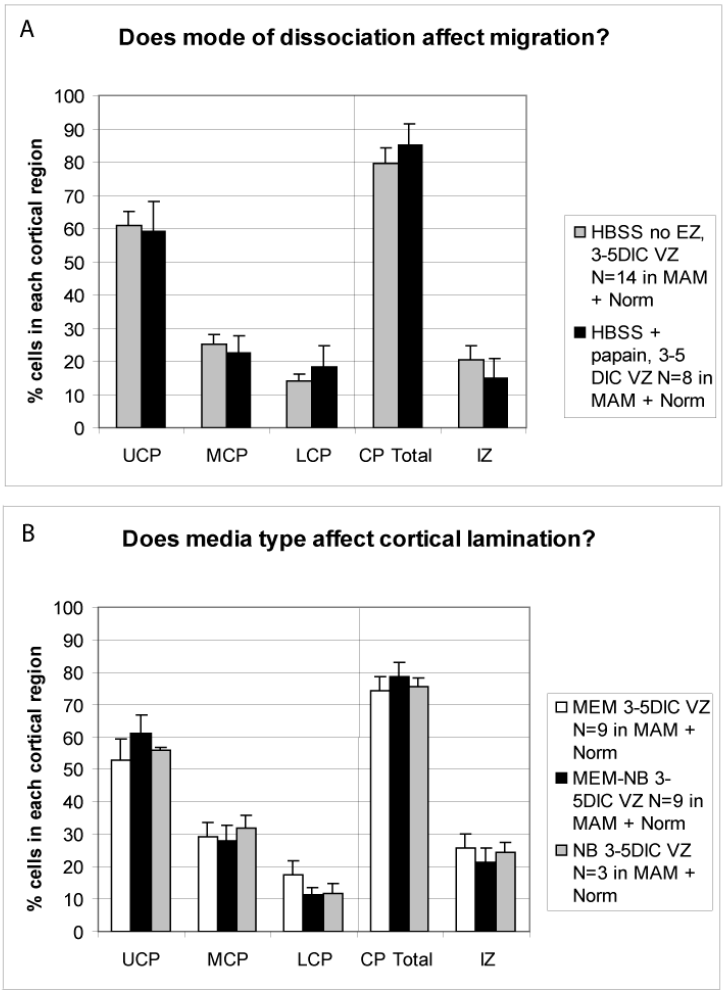
Figure 7. Images of double labeled cells after transplantation into ferret kits. Figure **A-C** show donor cells of different morphology that were double labeled for neuronal markers (MAP). Shown are images of immunoreactivity for MAP in green, the CM-DiI label in red and a merged image. **D-E** show examples of immunoreactivity for the astrocytic marker GFAP in green, and CM-DiI in red. Merged images are also shown. No GFAP-CM-DiI double-labeled cells were observed. Scale bar = 10 μm.



Supplemental Figure 1. Parcellation of cortex for quantification**.** The neocortical layers were outlined using the nuclear stain of bisbenzimide to determine the laminar boundaries in the somatosensory cortex of normal (**A**) and MAM treated cortex (**B**). The red line delineates layer 2 and the pia; the pink line delineates layers 3 and 4; the light blue line borders layers 5 and 6. The white dashed lines divide the outlined cortex into lateral (to the left), middle, and medial segments (to the right). The MAM treated cortex (**B**) displays a different sulcal pattern than the normal cortex (**A**). Scale bar = 1000 μm.



Supplemental Figure 2. Images of control experiments. Both the organotypic cultures (**A**) and ferret kits (**B**) received control injections of dead, CM-DiI labeled cells to assess the possibility that extraneous dye might lead to cellular label that was not from the transplanted cells. Although CM-DiI fluorescence could be seen at the injection site, there were no labeled cells in the cortex or far from the injection site. Scale bar in **A** = 1500 μm, **B** = 3000 μm.



Supplemental Figure 3. The effect the mode of dissociation and the type of media on the ability of donor cells to migrate into the neocortex on organotypic slices. **A** demonstrates that the mode of dissociation (using enzymes versus no enzymes) did not affect the migratory pattern of donor cells. Transplants were made into the ventricular zone (VZ) of both normal and MAM treated cortex. **B** shows that different types of media did not affect the migratory pattern of donor cells. Error bar values equal SEM. Three different types of media were used: MEM, NB (neurobasal), or a short exposure to MEM followed by culture in NB. Transplants were made into normal and MAM treated cortex. See text for details. DIC, days in culture.

Chapter 4

Discussion

The overarching goal of this research is to elucidate the mechanisms of cortical development and eventually provide a cure or a repair mechanism for cortical dysgenesis. At risk mothers could be followed prenatally, pathogenesis detected early and allogeneic stem cells transplanted into a baby's brain during the perinatal period when there is still a fair amount of plasticity in the central nervous system. This would hopefully result in children that are less severely affected by their neural pathogenesis; they would reach their developmental milestones on time and function normally within society. To develop these possibilities we developed two animal models of cortical dysgenesis that mimic some of the neurological disorders seen in the human population and we developed a variety of approaches to garner information on the potential for cortical repair.

Rescue of the E24 MAM phenotype

Use of MAM on E24 has a greater teratogenic effect than injection of the anti-mitotic later on in gestation. Injection of MAM on E33 leads to diminished layer 4. These animals live to adulthood and function normally. Use of MAM on E24 produces ferret kits that die within the first few days after birth. Our first study expounds upon the role of reelin in radial glial morphology as well as its importance in neuronal layering. We used a coculture system where mouse cortex is cultured next to ferret cortex. We found that the diffusion of factors is sufficient to improve both migration and radial glial morphology when wild type mouse tissue was used. When reeler tissue was used, we were able to improve radial glial morphology but not cortical migration. This elucidated the dichotomy between normal radial morphology and cortical migration. Just improving radial morphology, without the presence of reelin was not sufficient to induce cells to migrate to their proper laminae. The phenotype could be rescued by the normotopic presence of reelin. Reelin expressing HEK 293T cells placed at the pial surface of the E24 MAM treated cortex restored normal migration and radial morphology. This finding indicates that although reelin is not required for improved radial morphology (as another factor in the reeler cortex was able to restore radial glial alignment), it is capable of rescuing the E24 MAM phenotype. The mouse and human *reelin* genes are orthologous. Mutations to this gene in the human produce ataxia, mental retardation and epilepsy (Olson and Walsh, 2002). The ability to rescue the phenotype in mouse holds promise for managing defective reelin signalling related symptoms in humans.

Migration of ferret embryonic donor cells on organotypic slices

In our second study, we used the organotypic culture model to show that exogenous cells injected onto organotypic slices could survive, migrate, and differentiate on tissue from the same or different species. We determined that location of injection had an important consequence on destination. We hypothesized that injecting the cells in the IZ, just below the CP (the projected target of the injections) would increase the number of cells that invaded the CP as they would have to migrate over a short distance. Cells were also injected into the VZ, where endogenous cells are born. Significantly more cells migrated to the CP when injected in the VZ versus the IZ. We postulate that, since cells are normally born in the VZ, and donor cells are transplanted when endogenous cells are migrating to the cortex, that the VZ contains signals, not present in the IZ, to leave the VZ and invade the CP. (See Chapter 3 for more information). This finding may be important for clinical studies, where the precise site of transplantation can influence the ultimate distribution of injected cells.

Migration of mouse embryonic donor cells on organotypic slices

Knowing from our first study and previous work in the laboratory that diffusion of factors from the mouse cortex could affect radial morphology and cell migration in the ferret cortex (Hasling et al., 2003) we tested the ability of mouse cells to migrate in E33 MAM cortex. Mouse cells had a migration pattern similar to ferret cells (Chapter 3, Figure 3 E, H). This confirms findings from our first study that cells from other species

are capable of responding to exogenous cues from another animal, and supports the notion of conservation of signaling mechanisms involved in brain development (Schuurmans and Guillemot, 2002; Casanova and Trippe, 2006).

Migration and differentiation of ferret donor cells in vivo

The results from the culture of the ferret neural progenitor cells and their survival, migration and differentiation in the organotypic slice model led us to pursue the ability of the progenitor cells to survive, migrate, differentiate and integrate in the disrupted and normal cortex *in vivo*. Compared to the organotypic cultures, *in vivo* transplanation could provide signaling cues, molecules and factors in a 3D environment. Organotypic cultures degrade over time, *in vivo* transplantation would allow more time for cells to migrate and integrate into host tissue and this approach is more clinically relevant.

We asked whether the migration pattern of donor cells differed if they were proliferated in the presence of EGF prior to transplantation or if they were acutely transplanted. This was important to assess, as transplantation studies generally use either acute transplantations or proliferated cells, and not both methods to assess the ability of donor cells to repair the dysgenic nervous system (Sheen et al., 1999; Yang et al., 2000; Englund et al., 2002b; Englund et al., 2002a; Ourednik et al., 2002; Yang et al., 2002; Eriksson et al., 2003; Wernig et al., 2004). Our study did not find any significant differences in the migration pattern of the acutely transplanted versus proliferated cells in MAM treated or normal animals, suggesting that the fate of the donor cells was not significantly altered during its exposure to the proliferation media containing EGF and a hormone/salt mixture (Stem Cell Technologies) (Reynolds and Weiss, 1996). A possible reason for this is that the cells we transplanted include a mixed population of cells, when

used acutely as well as following proliferation. In both cases we most likely have mitotically active neural precursors and committed neuronal and glial precursors, as well as post-mitotic neurons and glia. Proliferating the donor cells also maintains a portion of the progenitor cells mitotically active and does not inhibit survival of differentiated cells at the early passages (Gritti et al., 2001), thus we can infer that the ratio of mitotic to post-mitotic cells is similar in the acutely transplanted versus cells that were transplanted following several days of proliferation. Transplantation studies by McConnell in the ferret cortex indicate that "only terminally post-mitotic daughters inherit a laminar commitment, whereas daughters that retain a proliferative potential also retain a multipotent laminar fate" (McConnell and Kaznowski, 1991). The distribution of our donor cells to different layers in the host cortex is indicative of their mixed origins.

Electrophysiology of the E33 MAM treated Ferret

The good survival, migration and differentiation of donor cells *in vivo* is promising in terms of the ability of progenitor cells to improve cortical deficits. One of our goals was to assess the functional potential of transplanted cells using multi-electrode array (MEA) recordings (Ayanda Biosystems, Lausanne, Switzerland). We knew from previous work in the lab that MAM treated cortex has an abnormal current source density profile (McLaughlin and Juliano, 2005). This abnormal pattern probably arises from widespread thalamocortical afferents, which normally terminate in layer 4, as well as alterations in the laminar position of $GABA_{A\alpha}$ receptors. These structural changes appear to result in a shift in the normal balance of excitation and inhibition (Noctor et al., 2001a; Palmer et al., 2001; Jablonska et al., 2004). The MEA allows us to record from the entire cortex simultaneously, when all conditions of a region of cortex are the same.

Using the MEA, we obtained preliminary data supporting improved functional responses in MAM treated animals that received transplants (Chapt. 4, Figure 1, 2 A, B). This work needs to be further elucidated and remains as an exciting future direction for this project.

Another goal was to assess integration of the donor cell in the host tissue using patch clamping techniques in collaboration with Maria Braga, Ph.D. Preliminary data indicates that the donor cells are integrated into the host tissue (Chapt. 4, Figure 2 C, D, E), but technical challenges limited the ability to record from a large number of cells. One of the challenges is that transplanted donor cells are much smaller than endogenous cells making them difficult to patch successfully. Also, the transplanted cells appear to be very fragile, perhaps due to the presence of CM-DiI in the cell membrane, leading to poorly formed seals with the patch pipettes. Finding a way to efficiently infect ferret donor cells with green fluorescent protein would be one avenue that we would like to pursue to determine if this method reduces the number of cells that do not form effective seals.

Culturing cortical cells from embryonic ferrets: a new source of neural stem cells?

Cell culture allowed us to assess the potential of ferret neural progenitor cells, to determine if it is possible to culture ferret progenitor cells, expand them several times, and differentiate them. These cells are phylogenetically closer to human cells compared to mouse progenitor cells. There are serious moral and ethical concerns with using human embryonic neural progenitor cells, thus using ferret neural progenitor cells provides a relatively inexpensive, accessible source of progenitor cells for scientific research and transplantation into other species (such as a monkey) as opposed to using mouse stem cells. We found ferret donor cells could be expanded and differentiated using protocols

intended for mouse culture. Cells proliferated as free-floating spheres in proliferation media could be passaged several times, and differentiated into neurons and astrocytes (Chapter 3, Figure 1 B, C, D). Using the colony forming cell assay and placing single cells at low density in a collagen matrix we found that a single cell could give rise to clones measuring more than 1000 μm over a period of three weeks (Chapter 3, Figure 1 A). These observations suggest that the potential use of ferret neural stem cells could be a powerful tool for use in future studies to repair damaged cerebral cortex.

Future directions

There are many aspects of this research that can be further pursued. Several questions still remain unanswered.

Cell Culture

We would like to continue optimizing the protocols for proliferating and passaging ferret neural progenitor cells in culture and to determine specific concentrations of mitogens and other factors that promote ferret proliferation and differentiation. There are numerous publications reflecting diverse approaches to culturing mouse progenitor cells for optimal proliferation. They also report components that can be added to influence cell fate such as: leukemia inhibitory factor (LIF), platelet-derived growth factor (PDGF), ciliary neurotrophic factor (CNTF), brain-derived growth factor (BDNF), or nerve growth factor (NGF) (Reynolds et al., 1992; Gage et al., 1995; Johe et al., 1996; Reynolds and Weiss, 1996; McKay, 1997; Marmur et al., 1998; Svendsen et al., 1999; Vescovi et al., 1999b; Caldwell et al., 2001; Erlandsson et al., 2001; Tropepe et al., 2001).

It would also be useful to quantify the percentage of mitotically active to post - mitotic neurons, astrocytes and oligodendrocytes immediately following dissociation and at different times during proliferation. Furthermore, layer specific antibody markers such as reelin, which labels cells from layer 1, Cux1 which is a marker for layer 2/3 cells, or the layer 5 marker ER81, could be used to assess whether cell phenotype changes over time (Shen et al., 2006).

Cell Labeling

Second, we would like to infect ferret progenitor cells with GFP at a high rate of efficiency. Currently the rate of efficiency using a lentiviral vector (Biogenova, Rockville, MD) or pNIT GFP remains at ~10%. A longer cell cycle in the ferret and other differences at the cellular level may impact on the ability of ferret progenitor cells to be transfected or infected with virus relative to mouse progenitor cells. Although we injected GFP labeled ferret progenitor cells *in vivo* after 7 days of proliferation, to allow time for the GFP to be expressed, we found only a few fluorescent GFP cells near the site of injection. Efficient infection of ferret donor cells with GFP would improve the possibility of being able to patch clamp a GFP+ cell in host tissue to better evaluate functional integration into host cortex.

Transplantation of donor cells from the ganglionic eminences

In the E33 MAM treated brain we know that GABAergic elements are specifically disturbed (Jablonska et al., 2004; Poluch et al., 2007). GABAergic cells arise from the ganglionic eminences and migrate tangential to the cortical plate in superficial and deep streams. Once they reach they cortical pate, they migrate radially to their final destination (Poluch et al., 2007). Due to the alterations of GABAergic elements in MAM treated cortex, it will be important to assess the transplantation of cells obtained specifically from the ganglionic eminences. One question to test is whether the migration pattern is similar to the population of cells taken from the cerebral cortex. A second question could assess if transplantation of donor cells from the ganglionic eminences either themselves or transplanted in conjunction with donor cells from the dorsal telencephalon alter the functional response profile in host tissue.

Behavioral Analysis

Another question of interest is the distinction in behavior between the E33 MAM treated and normal ferrets. E33 MAM treated ferrets appear relatively normal. For example, they grow at the same rate as normal ferret kits, they socialize with other ferrets, and do not appear overtly abnormal. From day-to-day care of the ferrets, many of the caretakers indicate that the adolescent male MAM treated ferrets have less refined social skills than their untreated counterparts. This type of behavior can be related to human clinical diagnoses of hyperactivity or attention deficit disorder more predominant in males versus females subsequent to an *in utero* neurotoxic event (Rice and Barone, 2000). We hope to initiate a series of studies to evaluate behavioral deficits in MAM treated ferrets that may help us correlate subtle behavioral alterations with clear clinical correlates and whether treatment with cell transplantation alters their behavioral skills.

Conclusion

This thesis adds to the scientific body of knowledge in several ways:

i) We used an animal model that is higher up on the phylogenetic scale, and is closer to humans with its protracted corticogenesis, convoluted cortex, and complex social behavior in comparison to the rodent.

ii) We used MAM treatment at two different time points, on E24 which has a severe constellation of defects and results in postnatal mortality as well as on E33 with more subtle cortical abnormalities and survival to adulthood.

iii) We used several approaches to elucidate factors associated with cortical dysgenesis and the potential for repair.

a. Cocultures of E24 MAM treated tissue with cortex from the reeler mouse provides evidence that reelin is not necessary for radial alignment, but is crucial for cortical lamination and that cocultures with reelin secreting cells rescues this phenotype
b. Injection of E27 and E33 donor cells onto organotypic slices demonstrated that the cells could survive, migrate, and differentiate *in vitro*.
c. Injecting the cells in the ventricular zone versus the intermediate zone provided evidence that location of injection is important with significantly more cells migrating to the upper cortical region following injection into the VZ versus the IZ *in vitro*.
d. Cells from a different species, i.e., mouse neural stem cells, could also survive, migrate, and differentiate in ferret tissue, indicating that cells

from another species can respond to factors appropriate for migration and survival.

e. Ferret neural progenitor cells were injected into perinatal ferrets via a bore hole in the skull and into the brain. All the animals recovered immediately from the surgery, there were no incidences of infection or death due to surgery, animals grew in length and gained weight similar to their non-surgery treated littermates, indicating minimal physiological consequences to the animal after undergoing brain surgery.

f. Donor cells survived, migrated from the VZ to the CP, and differentiated into MAP2abc+ neurons, demonstrating the potential of progenitor cells to replace lost neurons.

g. After *in vivo* injections, MAM cortex exhibited an environment more conducive to migration as a greater number of donor cells were found in the superficial layers compared to the pattern of migration in normal cortex, which was more evenly distributed. In addition, the distribution of transplanted cells in normal cortex was very restricted compared to the widespread and diffuse tangential dispersal after injection in MAM treated cortex.

h. We are pioneers in the field of ferret progenitor cell culture as we are the only laboratory to proliferate and culture ferret neural progenitor cells.

The results conveyed in this thesis provide one more step on the way to improving the lives of children born with cortical malformations. By culturing E24 MAM treated tissue for just 2 days with wild type or heterozygous mouse tissue or reelin expressing

cells we were able to restore cortical migration and radial morphology. In the E33 MAM model, exogenously transplanted cells survived, migrated, and had differentiated morphology within 3 days when transplanted onto organotypic slice. *In vivo*, transplanted cells also survived, migrated to the cortex and differentiated into neurons within 2-4 weeks. Furthermore, the observation that young ferrets awoke quickly from anesthesia, immediately began nursing when returned to the jill, did not incur any infections, gained weight and ambulated similarly to their non-surgery treated siblings, is evidence that brain surgery in young mammals does not have serious physiological repercussions. In addition, the mortatlity rate was the same as their littermates that had not undergone surgery. The positive outcome from this research provides the groundwork for promising future research that will hopefully one day lead to the improvement of lives for children born with neural defects.

Chapter 4 Figures

Chapter 4, Figure 1. Schaefer, A.W.

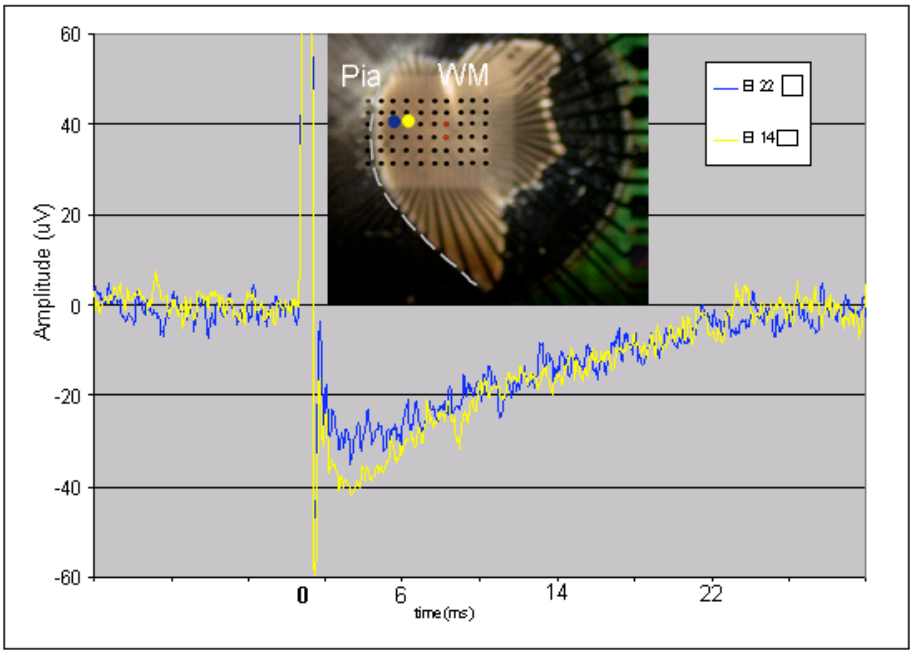


Figure 1. This figure shows an example of two traces recorded using a multi-electrode array (MEA). The array with a slice situated on the electrodes is shown in the center of the image. Placement of the electrodes is indicated with black dots. The outer surface of the slice is on the left and indicated with a white dotted line (Pia); the inner surface of the slice is on the right (white matter, WM). The stimulating electrodes are shown as red dots. All the electrodes recorded activity, but only two traces are shown, which correlate to the activity recorded at the blue and yellow dots. Time to peak amplitude for both traces was approximately 3.5 ms after the stimulus. The stimulus artifact can be seen at t=0 ms. The activity recorded in the traces returns to baseline after about 23.5 ms. The recordings were conducted under conditions when excitatory activity in the slice was enhanced using: depolarizing KCl 10 mM, the glutamatergic agonist glutamate 100 µM, and the inhibition of $GABA_A$ receptors with bicuculline 4 µM to diminish GABAergic drive.
MEA (Ayanda Biosystems, Lausanne, Switzerland) Custom made 3D, 6x10 array of electrodes with 400µm spacing between electrodes. The platinum electrodes have a 70 µm diameter, they are 80-95 µm in height and have a resistance of 200-250 kΩ.

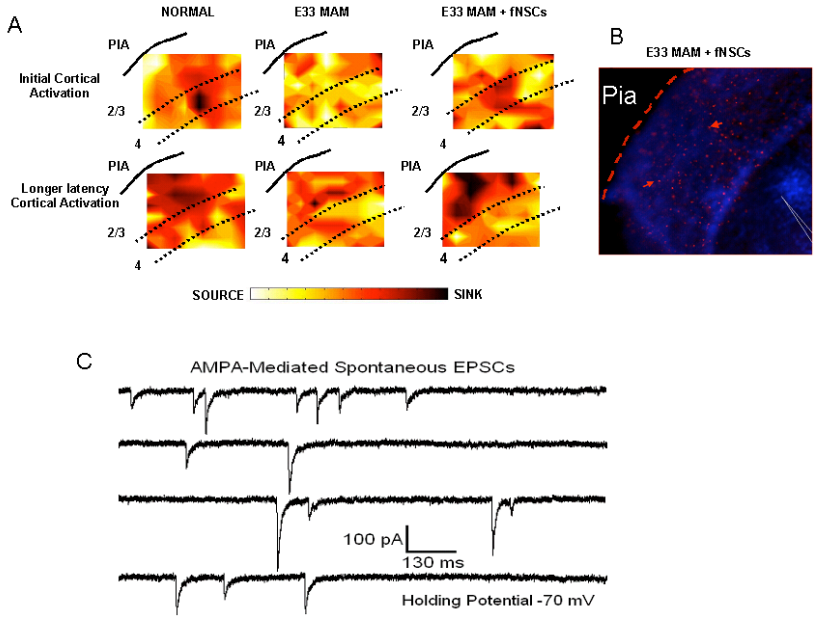


Figure 2. A. Illustrated here are contour maps of responses during recordings from a P25 E33 MAM treated ferret that received a transplant of E27 ferret neural progenitor cells on P9. Recordings were made using a 6x10, 3D micro-electrode array (Ayanda Biosystems). The responses are presented as contour maps at two time points in response to white matter stimulation. The initial sinks in response to stimulation (bipolar microelectrode) occur in layer 4 (left image) and progress to the upper layers 5 msec later (bottom column) for normal cortex & MAM cortex with transplants (fNPs on the right). In MAM treated cortex alone, the sinks do not occur in an organized manner (central column). The precise site of recording for **A** is illustrated in **B**. Transplanted CM-DiI labeled cells can be seen in the cortical plate. **C.** Illustrates patch clamp recordings from a cell transplanted into a P7 ferret kit surviving until P25. The cell was recorded with 10 µM BIC, 100 µM APV, and 10 µM picrotoxin in the extracellular solution. Traces of AMPA receptor-mediated spontaneous excitatory postsynaptic currents in the whole-cell voltage-clamp mode at a holding potential of –70 mV. Mean amplitude 208 ±42 pA, frequency 1.2 Hz, resting membrane potential -64 mV.

References

Anderson SA, Marin O, Horn C, Jennings K, Rubenstein JL (2001) Distinct cortical migrations from the medial and lateral ganglionic eminences. Development 128:353-363.

Ang ES, Jr., Haydar TF, Gluncic V, Rakic P (2003) Four-dimensional migratory coordinates of GABAergic interneurons in the developing mouse cortex. J Neurosci 23:5805-5815.

Angevine JBJ, Sidman RL (1961) Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. Nature 192:766-768.

Anthony TE, Mason HA, Gridley T, Fishell G, Heintz N (2005) Brain lipid-binding protein is a direct target of Notch signaling in radial glial cells. Genes Dev 19:1028-1033.

Arnaud L, Ballif BA, Cooper JA (2003) Regulation of protein tyrosine kinase signaling by substrate degradation during brain development. Mol Cell Biol 23:9293-9302.

Assadi AH, Zhang G, Beffert U, McNeil RS, Renfro AL, Niu S, Quattrocchi CC, Antalffy BA, Sheldon M, Armstrong DD, Wynshaw-Boris A, Herz J, D'Arcangelo G, Clark GD (2003) Interaction of reelin signaling and Lis1 in brain development. Nat Genet 35:270-276.

Bailey CD, Brien JF, Reynolds JN (2004) Chronic prenatal ethanol exposure alters the proportion of GABAergic neurons in layers II/III of the adult guinea pig somatosensory cortex. Neurotoxicol Teratol 26:59-63.

Beffert U, Weeber EJ, Morfini G, Ko J, Brady ST, Tsai LH, Sweatt JD, Herz J (2004) Reelin and cyclin-dependent kinase 5-dependent signals cooperate in regulating neuronal migration and synaptic transmission. J Neurosci 24:1897-1906.

Bjorklund A, Svendsen CN (2001) Chimeric stem cells. Trends Mol Med 7:144-146.

Bohner AP, Akers RM, McConnell SK (1997) Induction of deep layer cortical neurons in vitro. Development 124:915-923.

Brigman JL, Padukiewicz KE, Sutherland ML, Rothblat LA (2006) Executive functions in the heterozygous reeler mouse model of schizophrenia. Behav Neurosci 120:984-988.

Caldwell MA, He X, Wilkie N, Pollack S, Marshall G, Wafford KA, Svendsen CN (2001) Growth factors regulate the survival and fate of cells derived from human neurospheres. Nat Biotechnol 19:475-479.

Campbell K (2005) Cortical neuron specification: it has its time and place. Neuron 46:373-376.

Casanova MF, Trippe J, 2nd (2006) Regulatory mechanisms of cortical laminar development. Brain Res Brain Res Rev 51:72-84.

Cattabeni F, Di Luca M (1997) Developmental models of brain dysfunctions induced by targeted cellular ablations with methylazoxymethanol. Physiol Rev 77:199-215.

Cattaneo E, Reinach B, Caputi A, Cattabeni F, Di Luca M (1995) Selective in vitro blockade of neuroepithelial cells proliferation by methylazoxymethanol, a molecule capable of inducing long lasting functional impairments. J Neurosci Res 41:640-647.

CDC Birth Defects http://0-www.cdc.gov.library.csuhayward.edu:80/ncbddd/bd/. In.
Conti L, Reitano E, Cattaneo E (2006) Neural stem cell systems: diversities and properties after transplantation in animal models of diseases. Brain Pathol 16:143-154.
D'Arcangelo G (2006) Reelin mouse mutants as models of cortical development disorders. Epilepsy Behav 8:81-90.
D'Arcangelo G, Miao GG, Curran T (1996) Detection of the reelin breakpoint in reeler mice. Brain Res Mol Brain Res 39:234-236.
D'Arcangelo G, Homayouni R, Keshvara L, Rice DS, Sheldon M, Curran T (1999) Reelin is a ligand for lipoprotein receptors. Neuron 24:471-479.
Dang L, Yoon K, Wang M, Gaiano N (2006) Notch3 signaling promotes radial glial/progenitor character in the Mammalian telencephalon. Dev Neurosci 28:58-69.
Desai AR, McConnell SK (2000) Progressive restriction in fate potential by neural progenitors during cerebral cortical development. Development 127:2863-2872.
Deukmedjian AJ, King MA, Cuda C, Roper SN (2004) The GABAergic system of the developing neocortex has a reduced capacity to recover from in utero injury in experimental cortical dysplasia. J Neuropathol Exp Neurol 63:1265-1273.
Dulabon L, Olson EC, Taglienti MG, Eisenhuth S, McGrath B, Walsh CA, Kreidberg JA, Anton ES (2000) Reelin binds alpha3beta1 integrin and inhibits neuronal migration. Neuron 27:33-44.
Englund U, Fricker-Gates RA, Lundberg C, Bjorklund A, Wictorin K (2002a) Transplantation of human neural progenitor cells into the neonatal rat brain: extensive migration and differentiation with long-distance axonal projections. Exp Neurol 173:1-21.
Englund U, Bjorklund A, Wictorin K, Lindvall O, Kokaia M (2002b) Grafted neural stem cells develop into functional pyramidal neurons and integrate into host cortical circuitry. Proc Natl Acad Sci U S A 99:17089-17094.
Eriksson C, Bjorklund A, Wictorin K (2003) Neuronal differentiation following transplantation of expanded mouse neurosphere cultures derived from different embryonic forebrain regions. Exp Neurol 184:615-635.
Erlandsson A, Enarsson M, Forsberg-Nilsson K (2001) Immature neurons from CNS stem cells proliferate in response to platelet-derived growth factor. J Neurosci 21:3483-3491.
Evans LA, Jenkins EC (1976) PHA response and methylazoxymethanol acetate. Chem Biol Interact 14:135-140.
Falconer DS (1951) Two congenital defects of the limb bones in the mouse. Acta Anat (Basel) 13:371-378.
Feng Y, Walsh CA (2001) Protein-protein interactions, cytoskeletal regulation and neuronal migration. Nat Rev Neurosci 2:408-416.
Forster E, Tielsch A, Saum B, Weiss KH, Johanssen C, Graus-Porta D, Muller U, Frotscher M (2002) Reelin, Disabled 1, and beta 1 integrins are required for the formation of the radial glial scaffold in the hippocampus. Proc Natl Acad Sci U S A 99:13178-13183.
Frantz GD, McConnell SK (1996) Restriction of late cerebral cortical progenitors to an upper-layer fate. Neuron 17:55-61.

Frotscher M (1997) Dual role of Cajal-Retzius cells and reelin in cortical development. Cell Tissue Res 290:315-322.
Frotscher M, Haas CA, Forster E (2003) Reelin controls granule cell migration in the dentate gyrus by acting on the radial glial scaffold. Cereb Cortex 13:634-640.
Gage FH (2000) Mammalian neural stem cells. Science 287:1433-1438.
Gage FH, Ray J, Fisher LJ (1995) Isolation, characterization, and use of stem cells from the CNS. Annu Rev Neurosci 18:159-192.
Gaiano N, Fishell G (1998) Transplantation as a tool to study progenitors within the vertebrate nervous system. J Neurobiol 36:152-161.
Gierdalski M, Juliano SL (2002) Influence of radial glia and Cajal-Retzius cells in neuronal migration. Results Probl Cell Differ 39:75-88.
Gierdalski M, Juliano SL (2003) Factors affecting the morphology of radial glia. Cereb Cortex 13:572-579.
Gierdalski M, Sardi SP, Corfas G, Juliano SL (2005) Endogenous neuregulin restores radial glia in a (ferret) model of cortical dysplasia. J Neurosci 25:8498-8504.
Gleeson JG, Walsh CA (2000) Neuronal migration disorders: from genetic diseases to developmental mechanisms. Trends Neurosci 23:352-359.
Gleeson JG, Lin PT, Flanagan LA, Walsh CA (1999) Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. Neuron 23:257-271.
Gleeson JG, Allen KM, Fox JW, Lamperti ED, Berkovic S, Scheffer I, Cooper EC, Dobyns WB, Minnerath SR, Ross ME, Walsh CA (1998) Doublecortin, a brain-specific gene mutated in human X-linked lissencephaly and double cortex syndrome, encodes a putative signaling protein. Cell 92:63-72.
Goldman S (2005) Stem and progenitor cell-based therapy of the human central nervous system. Nat Biotechnol 23:862-871.
Grayson DR, Jia X, Chen Y, Sharma RP, Mitchell CP, Guidotti A, Costa E (2005) Reelin promoter hypermethylation in schizophrenia. Proc Natl Acad Sci U S A 102:9341-9346.
Gritti A, Galli R, Vescovi AL (2001) Cultures of Stem Cells of the Central Nervous System. In: Protocols for neural cell culture, 3rd Edition (Fedoroff S, Richardson A, eds), pp 173-197. Totowa, New Jersey: Humana Press.
Guerrini R, Marini C (2006) Genetic malformations of cortical development. Exp Brain Res 173:322-333.
Hack I, Bancila M, Loulier K, Carroll P, Cremer H (2002) Reelin is a detachment signal in tangential chain-migration during postnatal neurogenesis. Nat Neurosci 5:939-945.
Hartfuss E, Forster E, Bock HH, Hack MA, Leprince P, Luque JM, Herz J, Frotscher M, Gotz M (2003) Reelin signaling directly affects radial glia morphology and biochemical maturation. Development 130:4597-4609.
Hasling TA, Gierdalski M, Jablonska B, Juliano SL (2003) A radialization factor in normal cortical plate restores disorganized radial glia and disrupted migration in a model of cortical dysplasia. Eur J Neurosci 17:467-480.
Horner PJ, Gage FH (2000) Regenerating the damaged central nervous system. Nature 407:963-970.

Howell BW, Herrick TM, Cooper JA (1999) Reelin-induced tryosine phosphorylation of disabled 1 during neuronal positioning. Genes Dev 13:643-648.
Hunter KE, Hatten ME (1995) Radial glial cell transformation to astrocytes is bidirectional: regulation by a diffusible factor in embryonic forebrain. Proc Natl Acad Sci U S A 92:2061-2065.
Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovska V, Horster F, Tenkova T, Dikranian K, Olney JW (2000) Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. Science 287:1056-1060.
Jablonska B, Smith AL, Palmer SL, Noctor SC, Juliano SL (2004) GABAA receptors reorganize when layer 4 in ferret somatosensory cortex is disrupted by methylazoxymethanol (MAM). Cereb Cortex 14:432-440.
Jackson CA, Peduzzi JD, Hickey TL (1989) Visual cortex development in the ferret. I. Genesis and migration of visual cortical neurons. J Neurosci 9:1242-1253.
Jacobs KM, Kharazia VN, Prince DA (1999) Mechanisms underlying epileptogenesis in cortical malformations. Epilepsy Res 36:165-188.
Johe KK, Hazel TG, Muller T, Dugich-Djordjevic MM, McKay RD (1996) Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev 10:3129-3140.
Juliano SL, Palmer SL, Sonty RV, Noctor S, Hill GF, 2nd (1996) Development of local connections in ferret somatosensory cortex. J Comp Neurol 374:259-277.
Kayser AS, Miller KD (2002) Opponent inhibition: a developmental model of layer 4 of the neocortical circuit. Neuron 33:131-142.
Komuro H, Rakic P (1996) Intracellular Ca2+ fluctuations modulate the rate of neuronal migration. Neuron 17:275-285.
Komuro H, Rakic P (1998) Orchestration of neuronal migration by activity of ion channels, neurotransmitter receptors, and intracellular Ca2+ fluctuations. J Neurobiol 37:110-130.
Kriegstein AR, Noctor SC (2004) Patterns of neuronal migration in the embryonic cortex. Trends Neurosci 27:392-399.
Lavdas AA, Grigoriou M, Pachnis V, Parnavelas JG (1999a) The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. J Neurosci 19:7881-7888.
Lavdas AA, Grigoriou M, Pachnis V, Parnavelas JG (1999b) The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. J Neurosci 19:7881-7888.
Levitt P (2005) Disruption of interneuron development. Epilepsia 46 Suppl 7:22-28.
Lindvall O, Kokaia Z, Martinez-Serrano A (2004) Stem cell therapy for human neurodegenerative disorders-how to make it work. Nat Med 10 Suppl:S42-50.
LoTurco JJ, Blanton MG, Kriegstein AR (1991) Initial expression and endogenous activation of NMDA channels in early neocortical development. J Neurosci 11:792-799.
Luque JM, Morante-Oria J, Fairen A (2003) Localization of ApoER2, VLDLR and Dab1 in radial glia: groundwork for a new model of reelin action during cortical development. Brain Res Dev Brain Res 140:195-203.

Macklis JD (1993) Transplanted neocortical neurons migrate selectively into regions of neuronal degeneration produced by chromophore-targeted laser photolysis. J Neurosci 13:3848-3863.
Magdaleno S, Keshvara L, Curran T (2002) Rescue of ataxia and preplate splitting by ectopic expression of Reelin in reeler mice. Neuron 33:573-586.
Marin-Padilla M (1998) Cajal-Retzius cells and the development of the neocortex. Trends Neurosci 21:64-71.
Marin-Padilla M (1999) Developmental neuropathology and impact of perinatal brain damage. III: gray matter lesions of the neocortex. J Neuropathol Exp Neurol 58:407-429.
Marin O, Rubenstein JL (2001) A long, remarkable journey: tangential migration in the telencephalon. Nat Rev Neurosci 2:780-790.
Marin O, Rubenstein JL (2003) Cell migration in the forebrain. Annu Rev Neurosci 26:441-483.
Marmur R, Mabie PC, Gokhan S, Song Q, Kessler JA, Mehler MF (1998) Isolation and developmental characterization of cerebral cortical multipotent progenitors. Dev Biol 204:577-591.
Matsumoto H, Spatz M, Laqueur GL (1972) Quantitative changes with age in the DNA content of methylazoxymethanol-induced microencephalic rat brain. J Neurochem 19:297-306.
McConnell SK (1988a) Fates of visual cortical neurons in the ferret after isochronic and heterochronic transplantation. J Neurosci 8:945-974.
McConnell SK (1988b) Development and decision-making in the mammalian cerebral cortex. Brain Res 472:1-23.
McConnell SK (1995) Constructing the cerebral cortex: neurogenesis and fate determination. Neuron 15:761-768.
McConnell SK, Kaznowski CE (1991) Cell cycle dependence of laminar determination in developing neocortex. Science 254:282-285.
McKay R (1997) Stem cells in the central nervous system. Science 276:66-71.
McLaughlin DF, Juliano SL (2005) Disruption of layer 4 development alters laminar processing in ferret somatosensory cortex. Cereb Cortex 15:1791-1803.
Mendola P, Selevan SG, Gutter S, Rice D (2002) Environmental factors associated with a spectrum of neurodevelopmental deficits. Ment Retard Dev Disabil Res Rev 8:188-197.
Miller KD, Pinto DJ, Simons DJ (2001) Processing in layer 4 of the neocortical circuit: new insights from visual and somatosensory cortex. Curr Opin Neurobiol 11:488-497.
Miller MW (1993) Migration of cortical neurons is altered by gestational exposure to ethanol. Alcohol Clin Exp Res 17:304-314.
Miller MW (1996) Limited ethanol exposure selectively alters the proliferation of precursor cells in the cerebral cortex. Alcohol Clin Exp Res 20:139-143.
Miller MW (2006) Effect of prenatal exposure to ethanol on glutamate and GABA immunoreactivity in macaque somatosensory and motor cortices: critical timing of exposure. Neuroscience 138:97-107.

Montenegro MA, Guerreiro MM, Lopes-Cendes I, Guerreiro CA, Li LM, Cendes F (2002) Association of family history of epilepsy with earlier age at seizure onset in patients with focal cortical dysplasia. Mayo Clin Proc 77:1291-1294.
Morris NR, Efimov VP, Xiang X (1998) Nuclear migration, nucleokinesis and lissencephaly. Trends Cell Biol 8:467-470.
Nguyen-Ba-Charvet KT, Picard-Riera N, Tessier-Lavigne M, Baron-Van Evercooren A, Sotelo C, Chedotal A (2004) Multiple roles for slits in the control of cell migration in the rostral migratory stream. J Neurosci 24:1497-1506.
Nissenkorn A, Michelson M, Ben-Zeev B, Lerman-Sagie T (2001) Inborn errors of metabolism: a cause of abnormal brain development. Neurology 56:1265-1272.
Noctor SC, Scholnicoff NJ, Juliano SL (1997) Histogenesis of ferret somatosensory cortex. J Comp Neurol 387:179-193.
Noctor SC, Palmer SL, Hasling T, Juliano SL (1999) Interference with the development of early generated neocortex results in disruption of radial glia and abnormal formation of neocortical layers. Cereb Cortex 9:121-136.
Noctor SC, Palmer SL, McLaughlin DF, Juliano SL (2001a) Disruption of layers 3 and 4 during development results in altered thalamocortical projections in ferret somatosensory cortex. J Neurosci 21:3184-3195.
Noctor SC, Martinez-Cerdeno V, Ivic L, Kriegstein AR (2004) Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci 7:136-144.
Noctor SC, Flint AC, Weissman TA, Dammerman RS, Kriegstein AR (2001b) Neurons derived from radial glial cells establish radial units in neocortex. Nature 409:714-720.
Noctor SC, Flint AC, Weissman TA, Wong WS, Clinton BK, Kriegstein AR (2002) Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia. J Neurosci 22:3161-3173.
Ogawa M, Miyata T, Nakajima K, Yagyu K, Seike M, Ikenaka K, Yamamoto H, Mikoshiba K (1995) The reeler gene-associated antigen on Cajal-Retzius neurons is a crucial molecule for laminar organization of cortical neurons. Neuron 14:899-912.
Ogawa Y, Sawamoto K, Miyata T, Miyao S, Watanabe M, Nakamura M, Bregman BS, Koike M, Uchiyama Y, Toyama Y, Okano H (2002) Transplantation of in vitro-expanded fetal neural progenitor cells results in neurogenesis and functional recovery after spinal cord contusion injury in adult rats. J Neurosci Res 69:925-933.
Olson EC, Walsh CA (2002) Smooth, rough and upside-down neocortical development. Curr Opin Genet Dev 12:320-327.
Olson HC, Streissguth AP, Sampson PD, Barr HM, Bookstein FL, Thiede K (1997) Association of prenatal alcohol exposure with behavioral and learning problems in early adolescence. J Am Acad Child Adolesc Psychiatry 36:1187-1194.
Ourednik J, Ourednik V, Lynch WP, Schachner M, Snyder EY (2002) Neural stem cells display an inherent mechanism for rescuing dysfunctional neurons. Nat Biotechnol 20:1103-1110.

Owens DF, Kriegstein AR (1998) Patterns of intracellular calcium fluctuation in precursor cells of the neocortical ventricular zone. J Neurosci 18:5374-5388.
Palmer SL, Noctor SC, Jablonska B, Juliano SL (2001) Laminar specific alterations of thalamocortical projections in organotypic cultures following layer 4 disruption in ferret somatosensory cortex. Eur J Neurosci 13:1559-1571.
Parnavelas JG (2000) The origin and migration of cortical neurones: new vistas. Trends Neurosci 23:126-131.
Polleux F, Whitford KL, Dijkhuizen PA, Vitalis T, Ghosh A (2002) Control of cortical interneuron migration by neurotrophins and PI3-kinase signaling. Development 129:3147-3160.
Poluch S, Jablonska B, Juliano SL (2007) Alteration of interneuron migration in a ferret model of cortical dysplasia. Cerebral Cortex In press.
Rakic P (1988) Specification of cerebral cortical areas. Science 241:170-176.
Rakic P (1990) Principles of neural cell migration. Experientia 46:882-891.
Rakic P (1995) A small step for the cell, a giant leap for mankind: a hypothesis of neocortical expansion during evolution. Trends Neurosci 18:383-388.
Ray J, Peterson DA, Schinstine M, Gage FH (1993) Proliferation, differentiation, and long-term culture of primary hippocampal neurons. Proc Natl Acad Sci U S A 90:3602-3606.
Reynolds BA, Weiss S (1996) Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol 175:1-13.
Reynolds BA, Tetzlaff W, Weiss S (1992) A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J Neurosci 12:4565-4574.
Reynolds BA, Tetzlaff W, Weiss S (1992a) A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J Neurosci 12:4565-4574.
Rice D, Barone S, Jr. (2000) Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ Health Perspect 108 Suppl 3:511-533.
Rice DS, Curran T (2001) Role of the reelin signaling pathway in central nervous system development. Annu Rev Neurosci 24:1005-1039.
Richardson RM, Broaddus WC, Holloway KL, Fillmore HL (2005) Grafts of adult subependymal zone neuronal progenitor cells rescue hemiparkinsonian behavioral decline. Brain Res 1032:11-22.
Rosen GD, Sherman GF, Galaburda AM (1994) Radial glia in the neocortex of adult rats: effects of neonatal brain injury. Brain Res Dev Brain Res 82:127-135.
Sadler TW (2004) Langman's Medical Embryology, 9th Edition. Baltimore, MD: Lippincott Williams and Wilkins.
Sawamoto K, Wichterle H, Gonzalez-Perez O, Cholfin JA, Yamada M, Spassky N, Murcia NS, Garcia-Verdugo JM, Marin O, Rubenstein JL, Tessier-Lavigne M, Okano H, Alvarez-Buylla A (2006) New neurons follow the flow of cerebrospinal fluid in the adult brain. Science 311:629-632.
Schuurmans C, Guillemot F (2002) Molecular mechanisms underlying cell fate specification in the developing telencephalon. Curr Opin Neurobiol 12:26-34.

Sheen VL, Arnold MW, Wang Y, Macklis JD (1999) Neural precursor differentiation following transplantation into neocortex is dependent on intrinsic developmental state and receptor competence. Exp Neurol 158:47-62.
Shen Q, Wang Y, Dimos JT, Fasano CA, Phoenix TN, Lemischka IR, Ivanova NB, Stifani S, Morrisey EE, Temple S (2006) The timing of cortical neurogenesis is encoded within lineages of individual progenitor cells. Nat Neurosci 9:743-751.
Sisodiya SM (2004) Malformations of cortical development: burdens and insights from important causes of human epilepsy. Lancet Neurol 3:29-38.
Snyder EY, Park KI, Flax JD, Liu S, Rosario CM, Yandava BD, Aurora S (1997) Potential of neural "stem-like" cells for gene therapy and repair of the degenerating central nervous system. Adv Neurol 72:121-132.
Soriano E, Alvarado-Mallart RM, Dumesnil N, Del Rio JA, Sotelo C (1997) Cajal-Retzius cells regulate the radial glia phenotype in the adult and developing cerebellum and alter granule cell migration. Neuron 18:563-577.
Svendsen CN, Caldwell MA, Ostenfeld T (1999) Human neural stem cells: isolation, expansion and transplantation. Brain Pathol 9:499-513.
Tamamaki N, Fujimori KE, Takauji R (1997) Origin and route of tangentially migrating neurons in the developing neocortical intermediate zone. J Neurosci 17:8313-8323.
Tanaka DH, Maekawa K, Yanagawa Y, Obata K, Murakami F (2006) Multidirectional and multizonal tangential migration of GABAergic interneurons in the developing cerebral cortex. Development 133:2167-2176.
Tropepe V, Hitoshi S, Sirard C, Mak TW, Rossant J, van der Kooy D (2001) Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. Neuron 30:65-78.
Vazey EM, Chen K, Hughes SM, Connor B (2006) Transplanted adult neural progenitor cells survive, differentiate and reduce motor function impairment in a rodent model of Huntington's disease. Exp Neurol 199:384-396.
Vescovi AL, Gritti A, Galli R, Parati EA (1999a) Isolation and intracerebral grafting of nontransformed multipotential embryonic human CNS stem cells. J Neurotrauma 16:689-693.
Vescovi AL, Parati EA, Gritti A, Poulin P, Ferrario M, Wanke E, Frolichsthal-Schoeller P, Cova L, Arcellana-Panlilio M, Colombo A, Galli R (1999b) Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. Exp Neurol 156:71-83.
Voigt T (1989) Development of glial cells in the cerebral wall of ferrets: direct tracing of their transformation from radial glia into astrocytes. J Comp Neurol 289:74-88.
Volpe J (1995) Neurology of the Newborn. In, 3rd Edition, pp 811-842: W.B. Saunders Company.
Ward M, McCann C, DeWulf M, Wu JY, Rao Y (2003) Distinguishing between directional guidance and motility regulation in neuronal migration. J Neurosci 23:5170-5177.
Weissman TA, Riquelme PA, Ivic L, Flint AC, Kriegstein AR (2004) Calcium waves propagate through radial glial cells and modulate proliferation in the developing neocortex. Neuron 43:647-661.

Wernig M, Benninger F, Schmandt T, Rade M, Tucker KL, Bussow H, Beck H, Brustle O (2004) Functional integration of embryonic stem cell-derived neurons in vivo. J Neurosci 24:5258-5268.
Wonders C, Anderson SA (2005) Cortical interneurons and their origins. Neuroscientist 11:199-205.
Yang H, Mujtaba T, Venkatraman G, Wu YY, Rao MS, Luskin MB (2000) Region-specific differentiation of neural tube-derived neuronal restricted progenitor cells after heterotopic transplantation. Proc Natl Acad Sci U S A 97:13366-13371.
Yang M, Stull ND, Berk MA, Snyder EY, Iacovitti L (2002) Neural stem cells spontaneously express dopaminergic traits after transplantation into the intact or 6-hydroxydopamine-lesioned rat. Exp Neurol 177:50-60.
Yasuhara T, Matsukawa N, Hara K, Yu G, Xu L, Maki M, Kim SU, Borlongan CV (2006) Transplantation of human neural stem cells exerts neuroprotection in a rat model of Parkinson's disease. J Neurosci 26:12497-12511.
Yoon K, Nery S, Rutlin ML, Radtke F, Fishell G, Gaiano N (2004) Fibroblast growth factor receptor signaling promotes radial glial identity and interacts with Notch1 signaling in telencephalic progenitors. J Neurosci 24:9497-9506.
Zecevic N, Rakic P (2001) Development of layer I neurons in the primate cerebral cortex. J Neurosci 21:5607-5619.
Zedeck MS, Sternberg SS, Poynter RW, McGowan J (1970) Biochemical and pathological effects of methylazoxymethanol acetate, a potent carcinogen. Cancer Res 30:801-812.
Zhao S, Chai X, Forster E, Frotscher M (2004) Reelin is a positional signal for the lamination of dentate granule cells. Development 131:5117-5125.
Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D (1997) Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotechnol 15:871-875.

List of abbreviations

aCSF	artificial cerebral spinal fluid
ApoER2	apolipoprotein E receptor 2
APV	2-Amino-5-phosphonopentanoic acid
ARND	alcohol related neurodevelopment disorders
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BIC	bicuculline
bp	base pairs
BrdU	5-bromo-2-deoxyuridine
CM-DiI	Vybrant lipophilic dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlora
CMTMR	((5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine) - mixed isomers)
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CP	cortical plate
Dab1	disabled-1
DIC	days in culture
DMEM	Dulbecco's Modified Eagle Medium
EGF	epidermal growth factor

FAS	fetal alcohol syndrome
fNPs	ferret neural progenitor cells
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
het	heterozygous
ip	intraperitoneal
IZ	intermediate zone
IZd	intermediate zone, deep
IZs	intermediate zone, superficial
KCl	potassium chloride
LCP	lower cortical plate
LIF	leukemia inhibitory factor
MAM	methylazoxy methanol
MCP	middle cortical plate
MEA	multi-electrode array
MEM	minimum essential medium
mNSC	mouse neural stem cells
MZ	marginal zone
NB	neurobasal
NGF	nerve growth factor
NHS	normal horse serum
nl	nanoliter

NRG1	neuregulin 1
PDGF	platelet-derived growth factor
PI3K	phosphoinositide-3 kinase
SVZ	subventricular zone
UCP	upper cortical plate
VLDLR	very low-density lipoprotein receptor
WM	white matter
WT	wild type
VZ	ventricular zone

Genes:

DCX	doublecortin gene
FLN	filamin gene
LIS1	lissencephaly gene
RELN	reelin gene

List of Figures

Chapter 1

Chapter 1- Figure 1- Elements of cortical development

Chapter 1- Figure 2- Tangential migration

Chapter 1- Figure 3- Reelin and Lis1 signaling

Chapter 1- Figure 4- From stem cell, to progentior cell, to neurons and glia

Chapter 1- Figure 5- Assessing cell labeling methods for transplantation

Chapter 1- Figure 6- Infection with GFP in ferret neural progenitor cells

Chapter 1- Figure 7- Comparing GFP to CM-DiI labeled cells following transplantation *in vivo*

Chapter 2

Chapter 2- Figure 1-Example of radial glial morphology in E24 MAM treated cultures alone or cocultured with WT mouse explants

Chapter 2- Figure 2- Example of radial glial morphology in E24 MAM treated cultures cocultured with reeler mouse explants and quantification of the angles of deviation in radial glia under different culture conditions

Chapter 2- Figure 3- Migration and quantification of distribution of BrdU+ cells in E24 MAM treated cultures cocultured with WT and reeler mouse explants

Chapter 2- Figure 4- Migration and quantification of distribution of BrdU+ cells in E24 MAM treated cultures cocultured with either control or reelin expressing HEK 293T cells

Chapter 2- Figure 5- Example of radial glial morphology in E24 MAM treated cultures cocultured with either control or reelin expressing HEK 293T cells and quantification of the angular deviation of the radial glia in each condition

Chapter 3

Chapter 3- Figure 1- Proliferation, clonal assay and differentiation of ferret neural progenitor cells

Chapter 3- Figure 2- Migration and quantification of the distribution of ferret neural progenitor cells injected into the VZ vs IZ of MAM treated and normal organotypic slices

Chapter 3- Figure 3- Migration and quantification of the distribution of E27, E33 fNPs and E14 mNSC in MAM treated organotypic slices

Chapter 3- Figure 4- Lateral to medial distribution of donor fNPs in MAM treated and normal host cortex following *in vivo* transplantation

Chapter 3- Figure 5- Quantification of the lateral to medial distribution of donor fNPs in MAM treated and normal host cortex following *in vivo* transplantation

Chapter 3- Figure 6- Quantification of the layer location of donor cells from different conditions following *in vivo* transplantation into MAM treated and normal host ferret kits

Chapter 3- Figure 7- Immunohistochemistry of CM-DiI labeled donor cells following *in vivo* transplantation into host ferret kits

Chapter 3- Supplemental Figure 1- Parcellation of cortex for quantification

Chapter 3- Supplemental Figure 2- Injection of dead CM-DiI labeled cells into an organotypic slice and *in vivo* to test for leakage of dye

Chapter 3- Supplemental Figure 3- Quantification of distribution of donor cells in organotypic slices depending on mode of dissociation and type of culture medium

Chapter 4

Chapter 4- Figure 1- Example of two traces recorded from an acute slice made from a ferret kit with transplanted donor cells. Recordings were done using a multi-electrode array.

Chapter 4- Figure 2- Contour maps of cortical responses following recordings from ferret kits using the multi-electrode array, and an example of a patch clamped labeled donor cell following transplantation into a host ferret kit.