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ABSTRACT

Title of Dissertation: CORTICAL PROTEINS ARE CHEMOKINETIC TO CELLS FROM THE MEDIAL GANGLIONIC EMINENCE

2nd Lt Michael J. Davis, Doctor of Philosophy, 2011

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During embryonic development, a majority of neocortical interneurons originate from the medial ganglionic eminence (MGE) and migrate into the cortex along a path tangential to existing cortical cytoarchitecture. While many cortical proteins are chemoattractant to MGE cells into the cortex, the chemokinetic nature of these factors has not been completely addressed. To better understand the influence of cortical proteins we searched for isoelectric fractions of neocortical extract from E14, E15, and E17 Sprague-Dawley rats that induced migratory activity in MGE cells of isochronic animals. Using multiwell migration assays, we found ganglionic eminence (GE) cell migration was most significant in the presence of pH 5.5 and 8.5 fractions for E14 and E15 animals, respectively. We also tested the migratory response of MGE cells to defined factors present in the neocortex during this stage of development; these included glial-derived neurotrophic factor (GDNF), neuregulin (NRG), reelin, and glutamate. Significant migration of E15 and E17 MGE cells occurred in the presence of glutamate and

NRG. In the multiwell migration assay we observed that E15 MGE cells have a significant chemokinetic reaction to pH 8.5, but not NRG1. Interestingly, imaging of GE explants again demonstrated that the pH 8.5 fraction induced MGE cells to significantly increase the velocity of migration. Therefore, kinesis may be a vital component of MGE cell migration into and within the cortex. These results indicate the likely value of kinetic factors in migration and the distribution of interneurons in the developing neocortex.

CORTICAL PROTEINS ARE CHEMOKINETIC TO CELLS FROM THE MEDIAL GANGLIONIC EMINENCE

by
2nd Lt Michael J. Davis

Thesis submitted to the Faculty of the
Neuroscience Graduate Program
Uniformed Services University of the Health Sciences
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy 2011

PREFACE

This thesis is based upon work performed in the laboratory of Dr. Sharon Juliano at the Uniformed Services University of the Health Sciences between September 18, 2008 and May 28, 2011.

AKNOWLEDGEMENTS

Dr. Sylvie Poluch, a friend, a motivator and a mentor. She kicked my butt, helped with my dissections and showed me how to fend for myself.

I want to thank my principle investigator and mentor, Dr. Sharon Juliano, for her patience, clarity of thought, and good nature. These characteristics allowed her to bear with me through a changing understanding of our data. I hope to currently mimic these qualities amid my colleges and with any luck, my own students in the future.

A special thanks to my wife, Aisha. She sacrificed time with me so I could work in the lab and she patiently listened to daily descriptions of my scientific endeavors.

To my parents, who planted a seed of intrigue in the world and instilled a strong moral compass to keep me on the path of truth.

To everyone I did not mention. It is through an experience like this that I can truly appreciate the proverbial “village” it takes to accomplish all that I have.

DEDICATION

To Aisha and my parents.

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LIST OF ABREVIATIONS

aCSF	Artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	One-way analysis of variance
BSA	Bovine serum albumin
CGE	Caudal ganglionic eminence
dPBS	Dulbecco's phosphate buffered saline
E15	Embryonic day 15 (E1= day vaginal plug is seen)
EGF	Epidermal growth factor
ErbB	EGF receptor
GABA	Gamma-aminobutyric acid
GE	Ganglionic eminence
HGF	Hepatocyte growth factor
LGE	Lateral ganglionic eminence
MGE	Medial ganglionic eminence
NB media	Neurobasal media (containing specified supplements)
NMDA	N-methyl-D-aspartate
NRG	Neuregulin
PBS	Phosphate buffered saline
pI	Isoelectric point
SDF-1	Stromal cell-derived factor-1
sIEF	Solution-phase isoelectric focusing

Chapter 1

INTRODUCTION

1.1 Background. The cerebral cortex is composed of six functionally and morphologically distinct layers, each having a specific ratio of excitatory and inhibitory signals. These opposing signals result from two principle cell types. Excitatory signals from projection neurons have a pyramidal morphology, while inhibitory signals are associated with a broad category of GABAergic interneurons (DeFelipe, 1993, Gupta et al., 2000). During the complex process of corticogenesis, progenitors of neocortical excitatory neurons arise from the neuroepithelium of the neocortical ventricular zone, while cortical interneurons originate from the ganglionic eminence (GE) (Rakic, 1982, Caviness et al., 1995, Marin and Rubenstein, 2003, Haubensak et al., 2004). Excitatory progenitor cells reach specific cortical layers via radial migration, while interneurons reach the cortex using a tangential mode of migration that is roughly perpendicular to radial glia (Anderson et al., 1997, Parnavelas, 2000, Marin and Rubenstein, 2001). Neocortical development involves complex processes including precisely regulated spatial and temporal distributions of molecules that guide migrating neurons. These patterns of interneuron tangential migration during development of the forebrain are highly conserved across mammalian species (Metin et al., 2007).

1.1.1 Origin and migration of cortical pyramidal neurons. Projection neurons predominantly originate from asymmetric division of radial glia in the neocortical ventricular zone and migrate radially to their final cortical destination

(Kozloski et al., 2001). Radial glia are a transient population of cells in the cerebral cortex that span from the ventricular zone to pial surface. Asymmetric cell division of progenitor cells in the neocortical ventricular zone produces radial glia and young projection neurons. The migrating cells from the neocortical ventricular zone migrate along the radial glia using somal translocation as a mode of locomotion (Samuels and Tsai, 2004). While many of the young projection neurons migrate along the radial glia to reach specific cortical layers (Malatesta et al., 2000, Miyata et al., 2001, Noctor et al., 2001, Noctor et al., 2004), these cells have been shown to also be highly motile that can migrate tangential to the glial scaffold (Shoukimas and Hinds, 1978). Deep layers of the cortex are first to form, followed by progressively superficial layers as neurons pass previously established layers, constituting an inside-out pattern of corticogenesis (Angevine and Sidman, 1961, Rakic, 1974). Projection neurons primarily use glutamate as their neurotransmitter and are responsible for long-range transmission between intracortical and extracortical regions of the central nervous system (DeFelipe and Farinas, 1992, O'Leary and Koester, 1993).

1.1.2 Origin and migration of neocortical GABAergic interneurons.

GABAergic interneurons comprise approximately 15-20% of all cortical neurons and play a vital role in modulating excitatory activities of projection neurons (Jones, 1986, Hendry et al., 1987, Meinecke and Peters, 1987, Markram et al., 2004). Interneurons are small, locally projecting neurons that provide inhibitory regulation of many cortical processes and can be classified by morphology, electrophysiological properties, and protein expression (Tsugorka et al., 2007,

Moore et al., 2010). Classification of GABAergic interneuron subpopulations based on protein expression includes calcium-binding proteins (such as parvalbumin, calretinin, and calbindin) and neuropeptides (somatostatin and neuropeptide Y) (Hendry et al., 1989, Markram et al., 2004, Ascoli et al., 2008). In rodents, all cortical interneurons arise from the ventricular zone of the GE. Three defined regions of the GE give rise to distinct subgroups of interneurons with distinct migratory pathways and fates in the forebrain. In rodents, these regions include the lateral and medial ganglionic eminence (LGE and MGE), which give rise to somatostatin and parvalbumin expressing interneurons, and the caudal GE (CGE), which produces calretinin and neuropeptide Y expressing interneurons (Walsh and Cepko, 1990, DeDiego et al., 1994, de Carlos et al., 1996, Anderson et al., 1997, Tamamaki et al., 1997, Lavdas et al., 1999, Marin and Rubenstein, 2001, Letinic et al., 2002, Xu et al., 2004). Cells leaving the GE are post-mitotic and express GAD65/67, the enzymes responsible for GABA production (Fishell et al., 1993). In addition to supplying most of the neocortical interneurons, the rodent MGE produces interneurons destined for various parts of the brain (Deacon et al., 1994, Pleasure et al., 2000, Wichterle et al., 2001, Stenman et al., 2003). In humans, cortical interneurons originate not only from the GE, but also from the neocortical ventricular neuroepithelium (Rakic, 2009).

In humans, a population of interneurons originating from the neocortical ventricular neuroepithelium migrates radially, ultimately giving rise to calretinin-positive interneurons, while the GE gives rise to tangentially migrating interneurons expressing neuropeptide Y and somatostatin (Fertuzinhos et al.,

2009, Petanjek et al., 2009a, Petanjek et al., 2009b). In humans, the percent of cortical interneurons arising from the GE is uncertain since the origin of the most common cortical interneurons, parvalbumin and calbindin-positive, have yet to be determined. Although the timing and extent that each region of GE contributes to cortical interneuron populations vary across mammalian species, all mammals have GABAergic precursors residing in the GE producing neurons that migrate along the same tangential route to the neocortex (Jones, 2009, Poluch and Juliano, 2010).

As indicated above, during embryonic development, cortical interneurons are produced in the proliferative zone of the GE and use a conserved process of tangential migration to reach the neocortex. Rat interneurons begin migrating from the subpallial GE toward the cerebral cortex at E14 (Yau et al., 2003, Metin et al., 2007, Jones, 2009). Migrating interneurons first reach the cortex by E15 and move along three cortical regions by E16; the ventricular zone, intermediate zone, and marginal zone (Anderson et al., 1997, Tamamaki et al., 1997, Lavdas et al., 1999, Wichterle et al., 1999, Brazel et al., 2003, Tanaka et al., 2003, Yau et al., 2003, Kriegstein and Noctor, 2004). Detailed observations show tangential movement of cortical interneurons into the cortex to be tortuous and complex (Ang et al., 2003, Tanaka et al., 2006, Lopez-Bendito et al., 2008) and may involve interaction with radial glia (Anton et al., 1997, Poluch and Juliano, 2007, Yokota et al., 2007). The tangential migration of GE cells from subpallium to pallium have characteristic complex branching dynamics (Yokota et al., 2007) involving a decision-making type of behavior (Martini et al., 2009). Eventually,

interneuron progenitors switch to radial migration and use the radial glia to reach their final cortical destinations (Anton et al., 1997, Nadarajah and Parnavelas, 2002, Ang et al., 2003, Tanaka et al., 2003, Poluch and Juliano, 2007, Yokota et al., 2007). The most dorsal cortex is invaded by E17 and these cells begin migrating radially within the neocortex by E18 and subsequently extend dendritic and axonal processes postnatally (Yau et al., 2003).

In addition to producing cortical interneurons, the GE supplies interneurons destined for the hippocampus, striatum and olfactory bulb (Deacon et al., 1994, Pleasure et al., 2000, Nery et al., 2002, Stenman et al., 2003). In addition to supplying the cortex, the CGE supplies interneurons to the bed nucleus of the stria terminalis, posterior nucleus accumbens, hippocampus, and specific nuclei of the amygdala (Nery et al., 2002). LGE is origin of striatal and olfactory interneurons (Deacon et al., 1994, Stenman et al., 2003). LGE and MGE give rise to interneurons destined for the hippocampus (Pleasure et al., 2000). This orchestrated process of tangential migration relies on an equally complex interplay of diffusible signaling factors and permissive corridors (Wichterle et al., 2003).

1.1.3 Mechanisms involved in the guidance of migrating cortical interneurons. The signals involved with migration of interneurons into the cortex are not completely understood (Rudolph et al., 2010). Whereas radial migration requires radial glia as a physical scaffold, tangential migration does not appear to require a cellular scaffold; at least none have been identified until this point. Instead, a combination of long-range diffusible factors and permissive corridors is

implicated in the guidance of tangentially migrating interneurons (Marin and Rubenstein, 2003, Wichterle et al., 2003).

Chemoattractant factors implicated in guiding interneurons to the cortex include NRG and GDNF (Flames et al., 2004, Pozas and Ibanez, 2005). Recent reviews note that excitatory neurotransmitters/factors may be involved with guidance of interneuron precursors into the cortex (Hernandez-Miranda et al., 2010). Along with being a neurotransmitter and influencing stem cell proliferation and synaptogenesis, evidence also points to GABA as influencing migration of neuron progenitors in the cortex (Behar et al., 1996, Wang and Kriegstein, 2009). On the other hand, repulsion of migrating interneurons occurs at the striatum, a location where semaphorin 3A is expressed. Migrating GE cells expressing neuropilin respond to semaphorin 3A by avoiding the striatum and continuing to the neocortex (Marin et al., 2001, Tamamaki et al., 2003). The cortical plate is a permissive territory for MGE-derived cells (Lopez-Bendito et al., 2008). Migration along permissive corridors may provide a way for interneurons to invade the developing cortex while preventing these cells from straying into other areas (Wichterle et al., 2003). Flow of cerebrospinal fluid is also implicated in providing guidance to migrating neurons (Sawamoto et al., 2006, Breunig et al., 2010). The spatial and temporal coordination of migratory cues most likely provide an important mechanism for interneuron precursors making the long tangential journey to the cortex.

1.1.4 Migratory effect of known factors. A recent and comprehensive review of cellular and molecular mechanisms for tangential migration in the

developing cortex (Nakajima, 2007) points to specific factors and mechanisms. While general understanding of guidance cues evolved around their chemoattractant and chemorepulsive properties (Marin and Rubenstein, 2003), recent findings suggest a more complex role for these molecules and their signaling pathways. Probably the most studied of these is stromal cell-derived factor-1 (SDF-1). SDF1 is a potent chemoattractant for interneuron precursors migrating to the cortex (Stumm et al., 2003). Distributed in the marginal zone and in a rostrally decreasing cortical gradient (Stumm et al., 2007, Lysko et al., 2010), SDF-1 has been shown to participate in a complex receptor feedback mechanism (Liapi et al., 2008) influencing branching and speed of interneuron migration (Lysko et al., 2010). When SDF-1 is present without a gradient, twice as many cells become mobile (Liapi et al., 2008), suggesting it also acts as a chemokinetic agent.

Hepatocyte growth factor (HGF) is considered a chemokinetic factor for interneurons migrating into the cortex. Mice lacking the activator for HGF present with abnormal migration from the GE and have reduced amounts of neocortical interneurons (Powell et al., 2001).

GDNF, NRG, reelin, GABA and glutamate also directly or indirectly affect neuronal migration in the developing cortex. Although, the complete mechanisms of action of these substances are continuously evolving (Behar et al., 1999, Flames et al., 2004, Cuzon et al., 2006, Paratcha et al., 2006).

Cells of the MGE respond to GDNF as a chemoattractant. Pozas and Ibanez (2005) placed GDNF-treated agarose beads in the embryonic cortex and

observed calbindin-expressing cells coalescing around the GDNF-treated beads, suggesting that GDNF attracts cells generated in the MGE. GDNF signaling occurs via binding the GDNF receptor alpha 1 co-receptor, which then binds the receptor tyrosine kinase RET (Airaksinen et al., 1999). Activated RET autophosphorylates and sends intracellular signals to the nucleus via mitogen-activated protein kinases and extracellular-signal-regulated kinases (Arighi et al., 2005, Choi et al., 2009). GDNF has been clearly shown to attract cells of the MGE (Pozas and Ibanez, 2005).

NRG is also an important factor in influencing migration. NRG is a chemoattractant for cells derived from the MGE and promotes directional guidance of these cells into the developing cortex (Flames et al., 2004). The membrane-bound form, NRG III, creates a permissive environment for migrating MGE cells, while the secreted form (NRG1) chemoattracts these cells (Flames et al., 2004). NRG1 contains an epidermal growth factor (EGF)-like motif that interacts with the EGF receptor ErbB-3 (Horan et al., 1995, Singer et al., 2001) and ErbB4 (Buonanno and Fischbach, 2001, Falls, 2003). The NRG receptor, ErbB4, colocalizes with E13 GE cells through the settlement of these interneurons within the postnatal cortex (Yau et al., 2003). Although NRG1 is shown to attract GE cells (Flames et al., 2004), the chemokinetic properties of NRG1 (Corbin and Butt, 2011) have not been investigated in relation to migrating interneurons.

Reelin is involved with the positioning and migration of radially migrating neurons (Schaefer et al., 2008). Although reelin classically associates with

signaling to radially migrating neurons from the neocortical ventricular zone, it may also provide cues for migrating interneurons from the subpallium to the developing cortex. In the canonical signaling of reelin, the central segment binds two neuronal cell surface receptors, apolipoprotein E receptor 2 and very-low-density-lipoprotein receptor (Hiesberger et al., 1999, Trommsdorff et al., 1999) and transduces its signal via Disabled-1 phosphorylation (Benhayon et al., 2003, Jossin et al., 2004). Although these signals clearly influence radial migration (Jossin et al., 2004, Poluch and Juliano, 2010), interneuron movement is disrupted when the cells that produce reelin are ablated, suggesting reelin signaling may influence interneuron tangential migration in the cortex (Caronia-Brown and Grove, 2010).

While neurotransmitters have classically been associated with neural transmission, some neurotransmitters have also been described as influencing neuronal migration, including glutamate and GABA (Corbin and Butt, 2011). Neurotransmitters can affect the tangential migration of GE cells into the cortical plate (Heng et al., 2007, Manent and Represa, 2007). Although not fully elucidated, glutamate signaling affects tangential migration of GE cells into the cortical plate (Manent and Represa, 2007). In the developing brain, a wave of glutamatergic expression occurs laterally-to-medially across the cortex, quickly followed by a similar expression gradient of GABAergic cells, suggesting that glutamate may influence interneuron tangential migration. Activation of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor selectively halts tangential migration within the cortex (Yozu et al., 2008) and causes neurite

retraction in these cells (Poluch et al., 2001). Further implicating the role of glutamate in tangential interneuron migration is the vesicular release of glutamate from thalamocortical fibers which could influence interneuron migration via the AMPA receptors expressed on tangentially migrating GE cells (Metin et al., 2000). Interestingly, AMPA activation causes GABA release from tangentially migrating GE cells in the cortex which may create a feedback mechanism by influencing migration of surrounding interneurons (Poluch and Konig, 2002). Less robustly, glutamate has also been shown to use N-methyl-D-aspartate (NMDA) receptors to influence migration of cells from the cortical ventricular zone to the cortical plate (Behar et al., 1999).

Interneuron tangential migration is a complex process and various novel molecules continue to be described. For example, necdin has been known to promote survival and differentiation of cells with a potential tumor suppressor role (Chapman and Knowles, 2009). Necdin is expressed in almost all postmitotic neurons and complexes with Dlx to affect GABAergic cell fate (Maruyama et al., 1991, Uetsuki et al., 1996, Kuwajima et al., 2004, Kuwajima et al., 2006). A recent study showed mice lacking the necdin gene had reduced migration of cells from the MGE and a reduced number of cortical interneurons, ultimately lowering the seizure threshold. Therefore, necdin is considered to be necessary for tangential migration of embryonic neurons from the MGE into the neocortex (Kuwajima et al., 2010). While many factors have been shown to participate in the guidance of cortical interneurons during development, other novel factors have been demonstrated to exist, but are not yet identified (Britto et al., 2006).

1.1.5 Further directions. GABAergic interneurons of the neocortex modulate excitatory activity required for normal brain function and optimize cortical circuit performance (Sohal et al., 2009). Disruption of interneuron migration can lead to improper distribution within the cortex and is associated with schizophrenia, autism, and epilepsy (Benes and Berretta, 2001, Levitt, 2005, Di Cristo, 2007, Gant et al., 2009). Since interneurons comprise a major class of neurons in the cortex, it is important to understand the signals involved with their migration into the cortex. By understanding these migratory cues we can better treat diseases when they are disturbed and/or use them to control the migration of transplanted interneuron progenitors. As indicated in the previous discussion, many factors have been identified that influence the migration of interneurons into the neocortex. The experiments proposed here attempt to identify novel proteins that impact the migration of interneurons and clarify the mechanisms affecting how these GABAergic cells move from one site to another.

1.2 Objectives.

Hypothesis: Factors in the neocortex that guide GABAergic cells, originating in the GE, can be isolated and their influence on GE cell migration characterized.

Specific Aim 1: **Do different isoelectric fractions of cortical extract cause varied amounts of migratory activity in GE cells?** To answer this question cortical extracts of embryonic day 14, 15, and 17 rats were used to identify cortical proteins that induce significant GE cell migration. *Experiment 1: Which isoelectric range(s) of neocortical extract most effectively influence GE cell*

migration? Using isoelectric focusing, proteins of neocortical extract from E14, E15, and E17 rats were separated by isoelectric point into five intervals (pH 3.0-4.6, 4.6-5.4, 5.4-6.2, 6.2-7.0, and 7.0-10.0). A multiwell migration assay identified the isoelectric fraction(s) that induced significant migration of isochronic GE cells.

Specific Aim 2: **Can proteins in the most active cortical fractions be isolated and identified?** In Specific Aim 1, we were able to identify a specific isoelectric range of cortical fractions obtained from specific embryonic ages that differentially affected migration of interneurons. The aim was to identify specific proteins contained within the identified cortical fraction. *Experiment 2: What is the identity of proteins in the identified cortical fractions?* Using mass spectrometry analysis, the proteins that contribute to guiding cells leaving the GE into the neocortex and contained in the cortical fractions of interest from *Experiment 1* were identified and ranked according to prospective interest. Mass spectrometry analysis was accomplished by a method known as multi-dimensional protein identification technology (MudPIT). Protein samples were separated by charge and polarity, then directly analyzed by mass spectrometry.

Specific Aim 3: **How do the proteins in the identified cortical fraction influence migration of cells arising from the GE?** Many studies investigate the affect of specific proteins on the movement of cells leaving the GE, however very few experiments clarify the nature this migration. It is not completely known whether most of the identified proteins that encourage cellular movement are actually attracting GE cells or increase undirected movement. The experiments proposed here tested these ideas. *Experiment 3: Is the migration of GE cells to*

the identified factor(s) a function of chemoattraction or chemokinesis? To determine if factors identified in *Specific Aim 1* or *2* are chemokinetic or chemoattractant, migration of GE cell suspension were measured in a gradient-free setup of the multiwell migration assay. We assessed if significant GE cell migration occured in the presence of factor(s) without a gradient. *Experiment 4: Does the identified factor(s) affect migratory velocity of cells from MGE explant?* To determine if factors identified in *Specific Aim 1* or *2* can influence the speed of migration of GE cells, explants of GE were transplanted onto culture inserts with plain media (control) or media containing factor/fraction of interest or NRG1. Images were captured every 30 minutes for 20 hours. We assessed the difference in migration velocity of MGE cells exposed to factors compared to the control condition.

1.3 Overview of experimental design. These experiments aim to establish novel factors that guide interneurons to the neocortex during development and to increase understanding for the effect of these factors on GE cell migration. This study is part of a larger goal, to use interneuron progenitor transplantation for restoring GABAergic inhibition to specific brain regions. We hypothesize that during development, tangentially migrating interneurons from the GE are guided into the cortex by novel diffusible factors and these factors act to influence GE cell migration in complex ways.

1.3.1 Migration assay and methods. We confined our studies between embryonic days E14 - E17, as these times correspond to interneuron invasion of

the rat neocortex (Yau et al., 2003).

While many studies investigate the action of select proteins on migratory activity, we aimed to discover novel factors that influence this process by examining the entire neocortical proteome. To accomplish this task we separated neocortical extracts into isoelectric ranges and tested each range for its affect on GE cell migration.

To reduce the complexity of cortical protein samples, we used solution phase isoelectric fractionation (sIEF) (Smejkal and Lazarev, 2005). Modifications to existing protocols were required in order to retain protein activity. Because we made slight changes to standard protocols, we validated the separation of proteins by sIEF as outlined in the Methods.

The multiwell migration assay is an established method for *in vitro* study of neuronal migration, which we used to determine the ability of isoelectrically determined cortical factions to influence migratory potential (Behar, 2001). While the 48-well migration assay is well established, for a number of reasons, we concentrated on using the automated 96-well fluorescent-based migration assay for studying neural migration. Using a transformation to correct for assay variation, we performed validation of this method to ensure proper analysis of data from the 96-well assay (**Appendix C**).

1.4 Summary. In the following thesis, the developmental interaction of GE cells and neocortical signals are explored. Specifically, we assess the role of signals having an effect on the migration of GE cells. We evaluated activity in fractions

isoelectrically determined in the developing cortex that significantly influenced the migration of GE cells. To more clearly understand the role of these novel factors, we developed and used several innovative techniques, which required subsequent validations. We used multiwell migration assays in conjunction with isoelectric focusing to find groups of cortical proteins that significantly affect migration of GE cells from embryonic days E14, 15, or 17. We explored the nature of select positive control protein-cell interactions using the multiwall assay, while eliminating the protein gradient. We also observed how these factors influenced migration from MGE explants. We used MudPIT analysis to identify proteins in the most active isoelectric fractions. This procedure allowed us to take advantage of a mass spectrometry tool that identifies most proteins present in a complex proteome. Altogether, our study focused on isolating cortical proteins grouped through isoelectric focusing and understanding how they influence migration of GE cells for better understanding embryonic cortical development and the pathogenesis of certain human cortical dysplasias.

The activity of neocortical isoelectric fractions is age dependent, while NRG1 and glutamate remained consistently active for E15 and E17 MGE cells. Further analysis of the pH 8.5 fraction obtained at E15 revealed chemokinetic properties. The significant chemokinetic activity of the E15 pH 8.5 fraction is contrasted by the apparent chemoattractive properties observed for NRG1. Therefore, E15 neocortical extracts may contain novel factor(s) around the 8.5 isoelectric range that chemokinetically influence E15 MGE cell migration. Lastly, E17 cortex may contain novel factor(s) around the 3.0 isoelectric range that are

chemorepulsive for E15 MGE cells.

Chapter 2

MATERIALS AND METHODS

All animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee policies at USUHS, and procedures were performed at an institution approved by the Association for Assessment and Accreditation of Laboratory Animal Care.

2.1 Preparation of cell suspension and neocortical extracts. Cortical extract along with GE cell suspension and GE explants were prepared from embryonic Sprague Dawley rats (E14, E15, and E17; E1 = first day of vaginal plug; Charles River, Germantown MD or Taconic Farm, Rensselaer NY). Timed pregnant rats were deeply anesthetized using intraperitoneal injection of sodium pentobarbital (100-150 mg/kg). In a laminar flow hood, embryos were quickly removed from the uterine horn and collected in a Petri dish containing sterile-filtered ice-cold artificial cerebrospinal fluid (aCSF; containing NaCl 124mM, $MgSO_4$ 1.2mM, $NaHCO_3$ 26mM, $CaCl_2$ 2.4mM, NaH_2PO 1.2mM, Glucose 10mM and KCl 3.2mM) bubbled with 5% CO_2, 95% O_2. Embryonic age was confirmed by measuring crown-rump length (CRL) (**Appendix A**) (Torres et al., 2008). Using a dissecting microscope, the brains were rapidly extracted from embryos and dissected in ice-cold aCSF. Each brain was hemisected before microdissection of individual brain regions. Both the MGE and LGE were collected together from E14 rats, while the MGE was dissected from the telencephalic ventricular surface of E15 and E17 rats. Neocortical and subpallial explants were separately collected.

2.1.1 Preparation of cortical extracts. Immediately after each dissection, the fresh pieces of neocortex were transferred to a sterile 1.5mL Eppendorf tube, the aCSF replaced by 1 mL of ice-cold extraction buffer (protease inhibitor complete tablet by Sigma, in Dulbecco's phosphate buffered saline (dPBS) Mg^{2+} and Ca^{2+}) to wash and finally replaced with 200-300 µl of fresh ice-cold extraction buffer. The pieces of cortex were then triturated with a standard 200 µl pipette to maximize immediate exposure of proteins to the inhibitors, and placed on ice before sonicating (Cole-Parmer Ultrasonic Homogenizer 4710 series) three times for 5 seconds with 90% duty cycle and a tip limit of 4. The lysate was centrifuged at 4°C, 14,000 rcf, for 5 min after which the supernatant (i.e. extract) was moved to a sterile Eppendorf tube. The pellet was extracted for remaining protein with an additional 200 µl of ice-cold extraction buffer before sonicating, centrifuging, and removing supernatant again. Cortical extracts were immediately stored at -20°C until further processing by isoelectric fractionation.

2.1.2 Preparation of GE cell suspensions. GE from E14 or MGE from E15 or E17 animals were transferred to 15 mL centrifuge tubes; aCSF replaced with 1 mL of sterile-filtered dissociation buffer (2% glucose in dPBS containing no calcium, magnesium, or digestive enzymes). After incubating for 20 minutes at 37°C, GE or MGE explants were gently triturated with fire-polished glass pipettes of decreasing bore diameter until a single-cell suspension was achieved. Cells were centrifuged at 250 rcf, 4°C, for 5 minutes. Dissociation buffer was replaced with 1 mL of prewarmed NB media (Neurobasal medium supplemented with B27, N2, 10 mg/mL gentamycin, 0.6% glucose, and 2 mM GlutaMAX), gently

resuspended using a 1 mL pipette, and immediately incubated at 37°C and 5% CO_2 for no more than 30 minutes before use.

2.2 Solution phase isoelectric fractionation (sIEF). Cortical extracts from multiple isochronic collections were thawed, centrifuged to remove cryoprecipitate, and isochronically pooled. Pooled cortical extract was reduced to <360 µl by centrifuge concentration (VivaSpin 10kDa, GE Healthcare) at 4°C followed by fluorometric measure of protein concentration (Qubit, Invitrogen). Most formulations and methods for fractionation followed that of the Zoom sIEF (Invitrogen) protocol (Version C, 16 July 2004), except when indicated.

2.2.1. Preparing, running and storing sIEF samples. The isoelectric fractionator was loaded with a protein sample containing 360 µl cortical extract (if less, then brought up to 360 µl with culture grade water), 3.6 mL of sIEF denaturant, 40 µl of focusing buffer (pH 3-7), 40 µl of focusing buffer (pH 7-12). The protein sample was separated by sIEF into 5 isoelectric ranges using six pH boundaries (pH 3.0, 4.6, 5.4, 6.2, 7.0 and 10.0). We used Invitrogen's "Standard Format" for assembly and loading of the fractionator. sIEF was performed at 4°C using 2 mA and 2 W in four consecutive steps; 100 V for 30 minutes, 200 V for 1 hour, 400 V for 1 hour, and 600 V for 1 hour. Without bromophenol blue in our sample, we used formation of bubbles at the cathode as an indicator of electric current. After sIEF, the pH of each fraction was determined using pH-indicator strips (colorpHast, EMD, Gibbstown, NJ) (**Appendix B**).

We used these pH values to confirm the degree of fractionation and

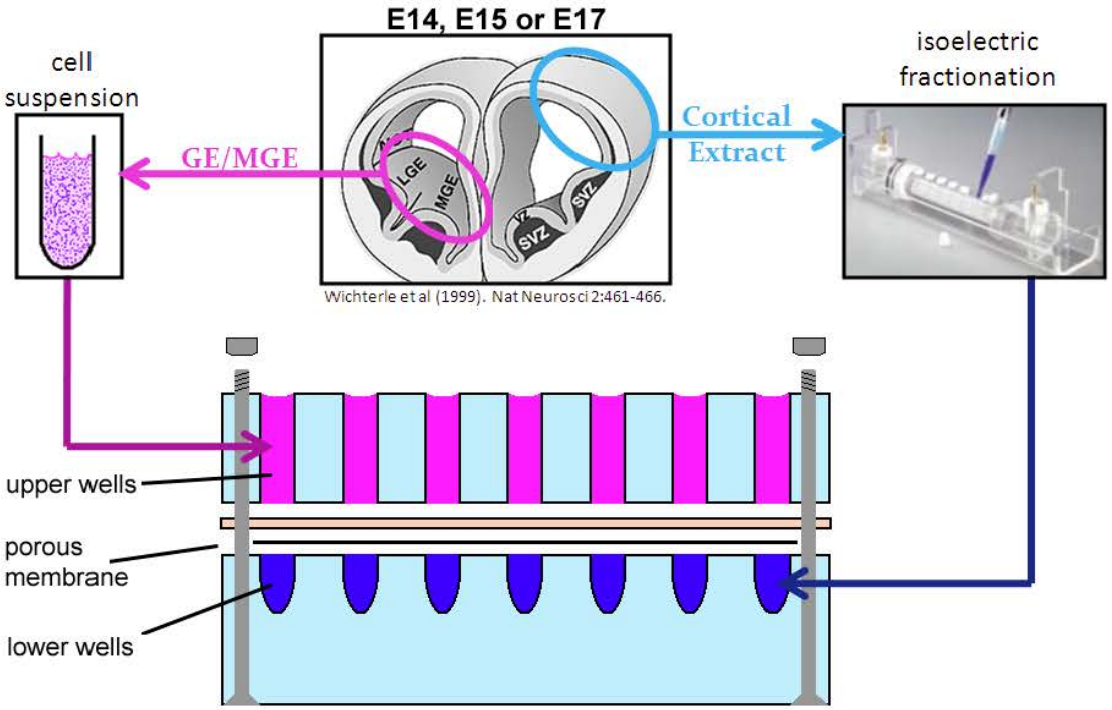
categorize fractions by pH (see results Figure 3). As a control against sIEF, we retained some of the original protein sample having not been fractionated, termed “total” fraction. To collect any remaining proteins, an additional 350 µl of sIEF denaturant was added to each chamber and transferred to their respective fraction. Fractions and total samples were dialyzed at 4°C for 6 hours using 10,000 molecular weight cutoff dialysis tubing (SnakeSkin, Thermo Scientific) in 5 L of 10 mM TrisHCl, neutralized to pH 7.0 by addition of NaOH. Buffer exchange occurred after 6 hours and dialysis continued at 4°C for an additional 8-12 hours. The extent of dialysis for each fraction was confirmed as pH-indicator strip readings of pH 7.0. The dialyzed fractions were concentrated to ~100 µl by centrifuge concentrating (VivaSpin 10 kDa, GE Healthcare) at 4°C. Final protein concentration was determined via fluorometric analysis (Qubit, Invitrogen) and aliquoted into 10 µg samples before storage at -80°C. Fractionation was confirmed by silver-staining (Silver BULLit, Amresco) isoelectric fractions separated by molecular weight on acrylamide gels using electrophoresis.

In summary, we performed sIEF with modifications to preserve protein activity. Specific procedural modifications included: exclusion of bromophenol blue and dithiothreitol from protein sample, low temperature fractionation and low temperature buffer exchange. Bromophenol blue, a pH indicator during fractionation, was not included in procedures as it significantly affected protein concentration readings made with a fluorometer (Qubit, Invitrogen). We determined this to be the case because the total protein coming out of the fractionator was greater than the amount going in. Dithiothreitol, a reducing

agent, was not included as it breaks sulfide bonds within protein complexes, possibly compromising biologic function of proteins. Isoelectric focusing was performed at 4°C to prevent protein denaturation caused by the fractionator unit warming. Although high molar urea and thiourea were used, we performed dialysis at a low temperature to allow proper refolding during protein renaturation.

2.3 Multiwell migration assays. Multiwell migration assays were used to determine migratory activity of dissociated GE cells in response to specific controls and a range of isoelectric fractions derived from each cortical extract (**Figure 1**).

Figure 1. Schematic layout for setting up the multiwell migration assay. Soluble protein extract is obtained from E14, E15, or E17 cortices and processed via sIEF (noted by blue oval, cortical extract). Fractionation products are placed in the lower wells of the migration assay (indicated by blue color). A porous membrane (indicated by a black line) is placed over the wells containing isoelectric fractions (blue). The porous membrane slows the diffusion of factors from the lower well, thereby creating a gradient. The assay is assembled with a gasket (beige bar) and top portion containing upper wells. After assembly, the upper wells (pink) are filled with fifty thousand GE cells and the whole unit is then placed in a humidified incubator for 20 hours.


E14, E15 or E17
cell
suspension
GE/MGE
LGE
MGE
VZ
SVZ
SVZ
Cortical
Extract
isoelectric
fractionation
Wichterle et al (1999). Nat Neurosci 2:461-466.
upper wells
porous
membrane
lower wells

A 48-well assay (AP48, NeuroProbe, Gaithersburg) with 8 μM pore membrane was used for testing E14 GE cells against isochronic cortical fractions at 10 μg/mL and controls. An automated 96-well assay (MBC96, NeuroProbe, Gaithersburg) with 10 μM pore membrane was used for testing E15 and E17 MGE cells against isochronic cortical fractions at 10 μg/mL and positive controls. For positive controls we tested recombinant mouse reelin (1pM and 1nM, 3820-MR/CF, R&D Systems), recombinant rat GDNF (3 nM, 512-GF, R&D Systems), recombinant human NRG-1-β1/HRG-β1 (1 nM, 396-HB/CF, R&D Systems), and L-glutamic acid (1 μM, G-6904, Sigma) diluted in NB media.

2.3.1 Validation of protein gradient across membrane of multiwell migration assay. The concept of a protein gradient across the membrane of the multiwell migration assay is central to our methods. To verify and characterize the protein gradient in the multiwell migration assay, we placed three concentrations of protein in the lower wells, assembled the assay and measured the protein concentration from the upper wells at fixed time intervals over a 40 hour period. Bovine serum albumin (BSA, Sigma, A7888) was dissolved in PBS at 0.5, 10 and 200 μM and loaded in the lower well of a multiwell migration assay. After assembly with an 8 μM pore membrane, PBS was loaded in the upper wells and the assay incubated at 37°C with 5% CO_2. Using a 10 μl pipette, samples were drawn from two upper wells of each protein concentration at 0, 0.5, 1, 2, 4, 13, 19, 25.5, 32 and 42 hours. Protein concentration of samples was measured in duplicate by fluorometric analysis (Qubit, Invitrogen). Data was averaged from duplicate readings of the two samples for each concentration and time point. For

analysis, the measured concentration was converted to a percent of the theoretical concentration if equilibrium were to be achieved (refer to *Figure 7* in Results).

2.3.2 Preparation of GE cells and assembly of 48-well migration assay. Preparation, assembly, and disassembly of the 48-well assay along with quantification of migration closely follow the manufacturer's manual (NeuroProbe, 05-2006) and the protocol described by Behar (Behar, 2001). Before assembly, the 8 μM pore membrane was precoated with poly-D-lysine (30,000-70,000 MW, 30 μg/ml; Sigma). In a laminar flow hood, 30 μl of cortical isoelectric fractions diluted in NB media were loaded in each of the lower wells of the 96-well plate. The first and last two columns of wells were not used because membrane processing disrupts cells in this area of the membrane. As a control, select lower wells were filled with plain NB media. The multiwell migration assay was assembled using the poly-D-lysine coated 8 μM pore membrane. Upper wells were loaded with 50 μl of GE cell suspension, at a concentration of 1 x 10^6 cells/mL. For chemokinetic analysis, the gradient was eliminated by placing equal concentration of factors in the upper and lower wells. Multiwell migration assays were assembled and placed in sterile 125 x 65 mm covered crystallization dishes, surround by ~10 mL of culture-grade water to prevent dehydration and incubated for 20 hours at 37°C with 5% CO_2. After incubation, the assays were disassembled and the upper portion of the membrane wiped to remove unmigrated cells. Migrated cells on the underside of the membrane were fixed, stained, and counted as described below.

2.3.3 Quantification of migration in 48-well migration assay. Cells that migrated through the membrane were fixed for 30 min with 4% paraformaldehyde in PBS, then stained in 0.1% cresyl violet for about 3 minutes. The membranes were washed and mounted cell side up onto 1 x 3 inch glass slides, allowed to slightly air dry, and covered with immersion oil (Immersol 518F, Zeiss). Areas of membrane corresponding to wells were mosaic imaged using bright field microscopy (Zeiss, Axiovert 200M) and an immersion 25x plan objective with focus corrections to capture only the migrating side of the filter. Images were coded for location to assist in blinded analysis. Only cells for which the nucleus was on the bottom side of the membrane were considered migrated cells. Images were processed in Photoshop (version 7.0.1) to mark relevant cells with a point. All points were quantified by using ImageJ particle analyzer (NIH, ImageJ, version 1.44).

***2.3.4 Analysis of 48-well Migration Data*.** Data in each run were normalized against the basal migration in the respective study by dividing each data point by the average migration seen in control conditions. Normalized data from studies of the same age were pooled.

2.3.5 Preparation of MGE cells and assembly of 96-well migration assay. Preparation, assembly, disassembly, fluorescent reading and cleaning of the 96-well migration assay adhered closely to those provided by the manufacturer (NeuroProbe, mb96 – rev. Mar-01) with guidance from the protocol described by Behar (Behar, 2001). Before assembly, a low volume 96-well plate (NeuroProbe, MP30) was precoated with poly-D-lysine (30,000-70,000 MW,

30µg/ml; Sigma), rinsed twice with culture-grade water, and allowed to dry. In a laminar flow hood, each well of the 96-well plate was loaded with 30 µl of isoelectric fractions or positive controls diluted in NB media. As a control, select lower wells were filled with plain NB media. The 96-well plate was assembled into the multiwell migration assay using a 10 µM pore membrane. Upper wells were loaded with 50 µl of bisbenzimide-stained MGE cells, at a density of 1 x 10^6 cells/mL. In each assay, background fluorescence was controlled for by loading select upper wells with cell-free NB media. For chemokinetic studies, the chemical gradient was abolished by placing an equal concentration of factors in the upper well with the MGE cells as was loaded in the corresponding lower well. The completed multiwell migration assay was placed in a covered sterile box, surround by about 25 mL of culture-grade water to prevent dehydration, and incubated for 20 hours at 37°C with 5% CO_2.

2.3.6 Quantification of migration in 96-well migration assay. The NeuroProbe manual was followed (mb96 – rev. Mar-01); in brief, the framed membrane and 96-well plate were removed together from the multiwell migration assay, the supplied wiper was used to remove all non-migrated cells from the upper surface of the membrane, the 96-well plate cover was placed over the membrane/plate combination and held in place with lab tape, this was spun at 15°C, 450 rcf, for 5 minutes to release cells from the membrane and ensure the migrated cells were in the bottom of each corresponding well. Next, the plate lid was carefully removed and a razor used to cut along the inner edge of the membrane frame. Forceps were used to carefully peel the membrane away while

keeping the frame in place. The lid was placed back on the now frame/plate combination and spun again at 15°C, 450 rcf for 5 minutes to ensure cells were well lodged to the bottom of each well. After that, the lid and frame were removed and the 96-well plate carefully submerged for 20 minutes in a tray containing 4% paraformaldehyde in PBS. The plate was carefully removed and excess fixative solution blotted from the bottom and edges. The uncovered 96-well plate was read in a fluorescent plate spectrophotometer (Molecular Devices, Gemini XS - Spectra MAX) using 355 / 460 nM excitation/emission, with a 455 nM cutoff. Next, the plates were immediately viewed at 5x under a fluorescent microscope (Zeiss, Axiovert 200M) at 365 nM / 445 nM excitation/emission to detect wells containing fluorescent debris. Any data points corresponding to wells containing fluorescent debris were removed from analysis.

2.3.7 Analysis and statistics of 96-well migration data. Analysis of 96-well assay data was similar to analysis of 48-well data, except we had to correct for background fluorescent noise and positional biases. This was accomplished by subtracting the average fluorescence reading of all blank wells from all other fluorescence readings in that run. Positional biases were corrected by transforming the data to account for consistent changes in fluorescent plate readings based on location in the 96-well plate (**Appendix C**). Normalized data from studies of the same age were pooled.

2.4 Multi-dimensional Protein Identification Technology (MudPIT). The enormous complexity of each fraction posed a significant methodical challenge

for overall protein identification. Therefore, a 200 µg sample of E15 pH 8.5 fraction from E15 cortical extract was sent to the Keck Lab at Yale School of Medicine for analysis by MudPIT. Briefly, the sample was digested with trypsin and, separated into twenty fractions using strong cation exchange. Each fraction was separated by liquid chromatography with the elution product sent directly through a mass spectrometer. Mass spectrometry produced data representing the molecular weight of protein fragments. The spectrographic data was analyzed by Yale's Mascot MudPIT to determine the identity of proteins. Confidence scores were applied to each match, with higher scores representing higher confidence. We started with a list of more than 800 protein matches and by researching the most likely candidates; we identified four proteins that may influence tangential migration of interneurons.

2.5 Affect of sIEF products on viability and GABA expression of MGE cells.

To our knowledge, products from sIEF have not been used in live cell cultures to test protein function. To validate the effect of isolated neocortical fractions on GE cell migration, we assessed the effect of this treatment on cell viability and on other measures of the health of GE cell suspensions (i.e., GABA expression) in cell cultures.

2.5.1 Cell viability. We determined the potential toxicity of isoelectrically isolated fractions by evaluating cell viability. MGE cell suspensions taken from E15 neocortex were labeled with bisbenzamine and suspended in NB media. The cells were then seeded onto standard flat-bottom 96-well culture dishes

(Greiner bio-one) precoated with poly-D-lysine (30,000-70,000 MW, 30µg/ml; Sigma) at a density of $5x10^4$ cells per well. Either plain NB media or NB media containing one of the five fractions was immediately added to the cells in each well (at a final concentration of 10 µg/mL). Each condition was triplicated. Cells were incubated for 20 hr at 37°C and 5% CO_2, which is identical to the conditions for testing the migration of the isoelectric fractions, viability was then assessed using propidium iodide uptake (Aras et al., 2008). At the 19^{th} hour of incubation, we introduced propidium iodide (P4170, Sigma) at a final concentration of 5µg/mL into each condition and incubated the culture for 1 additional hour (20 hours total incubation). The cells were then washed with PBS, fixed with 4% paraformaldehyde in PBS, and immunostained as described below.

2.5.2 Percent of cells expressing GABA. Following a prior protocol (Brana et al., 2002), we immunostained propidium iodide-labeled cultures with antibodies against GABA. Fixed cells from the viability study were washed twice with tris-buffered saline, blocked at room temperature for one hour, then immunostained with rabbit-anti-GABA (Sigma, A2052, 1:200) on a rocker at 4°C for 3 hours and then at room temperature for another 30 minutes. We washed the cultures twice with TBS, then applied goat-anti-rabbit green secondary antibody (Invitrogen, A21068, 1:200) for 40 minutes at room temperature. After washing with TBS twice, cells were imaged. As a control, conditions without primary antibody resulted in only background signals.

2.5.3 Imaging and analysis. Red, green, and blue 10x fluorescent images from the center of each well were taken using an inverted fluorescence

microscope (Zeiss, Axiovert 200M). Images were converted to grayscale and then inverted using Photoshop (Adobe, V 7.0.1). We used Image J to (1) adjust linear threshold so that only cells of interest were highlighted and (2) to analyze particles with a pixel size greater than 17, 7, or 0 pixels^2 for images of propidium iodide, GABA and bisbenzimide, respectively. We choose a threshold for automated detection that matched results obtained by hand counting. Counts were made on all three wells for each condition. The percent viability was determined by dividing the number of non-propidium iodide-stained nuclei by the total number of bisbenzimide-stained nuclei in each well and multiplying by 100. The percent of cells expressing GABA for each condition was determined by dividing the total number of GABA-positive cells by the number of non-propidium iodide-stained nuclei and multiplying by 100.

2.6 Live imaging. We used live cell imaging and tracking software to determine the velocity of cells migrating from GE explants exposed to plain NB media (control), pH 8.5 fraction or NRG1 (1 nM). In each of two independent experiments, two cell culture inserts (0.4 μm, Millicell, Millipore) were presoaked with plain NB media, or with NB with 1 nM NRG1 or pH 8.5 fraction added in a sterile 6-well plate. MGE explants were transferred from ice-cold aCSF to each of the presoaked inserts and incubated at 37°C with 5% CO_2 for 30 minutes before live imaging. The 6-well plate was transferred to a prewarmed incubation chamber on an inverted microscope (Zeiss, Axiovert 200M). Axiovision software (Zeiss, version 4.8.2.0) was used to capture a phase contrast image at 5x or 10x

every 30 minutes for at least 7 hours to 24 hours. Axiovision images were analyzed with MTrackJ (version 1.4.1) in ImageJ (NIH, ImageJ, version 1.44) to track 6 cells for each explant. While no single cell was tracked for an entire twenty hours, tracking of all cells for each condition covered a 20 hour culture period. Therefore, we averaged the migration velocity (μm/hour) between track points for all cells in each explant. The average velocity of each explant was then used as a data point with a total of four explants representing each condition across two independent experiments.

2.7 Statistics. Statistical significance of mean differences were examined by one-way analysis of variance (ANOVA) followed by a post hoc Dunnett's test for multiple comparisons (SPSS software, version 12.0.1). Statistical significance was set at p-values < 0.05.

Chapter 3

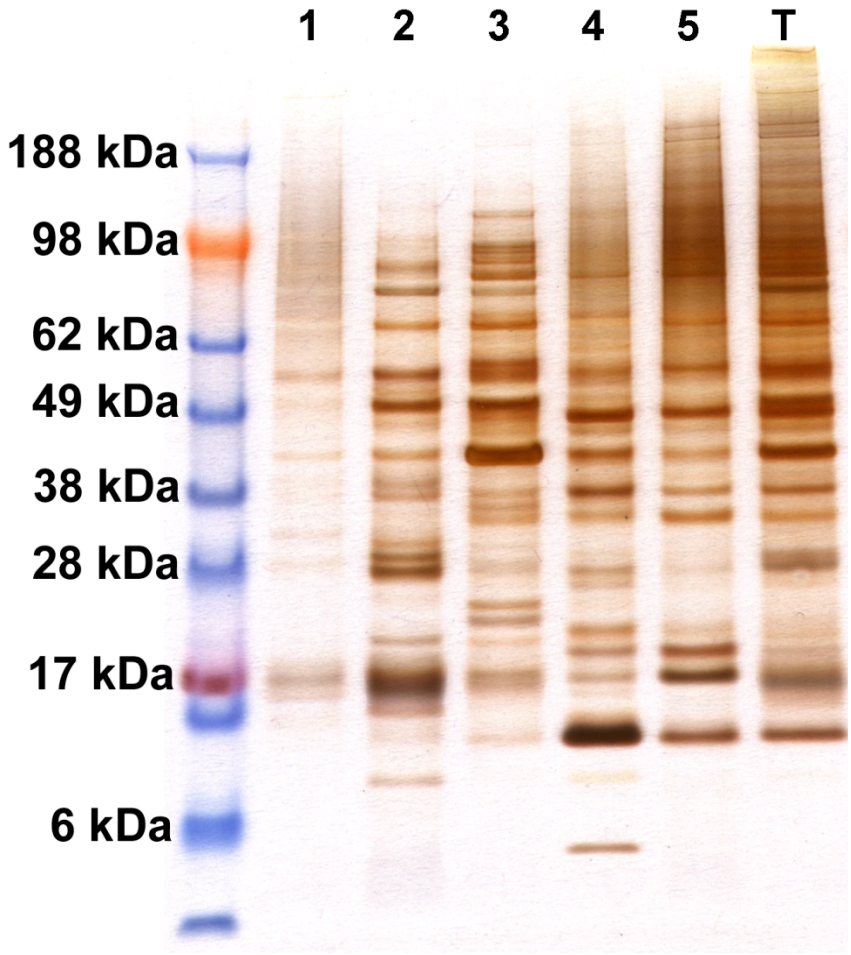
RESULTS

3.1 Verification/Validation. We found standardizing and verifying many features of these experiment to be important for understanding our data. To do this, we standardized each feature of the experiments for consistency. What follows is a summary of the logic, process, and result for the verification or validation of each component.

3.1.1 Verifying the isoelectric fractionation of cortical extract.

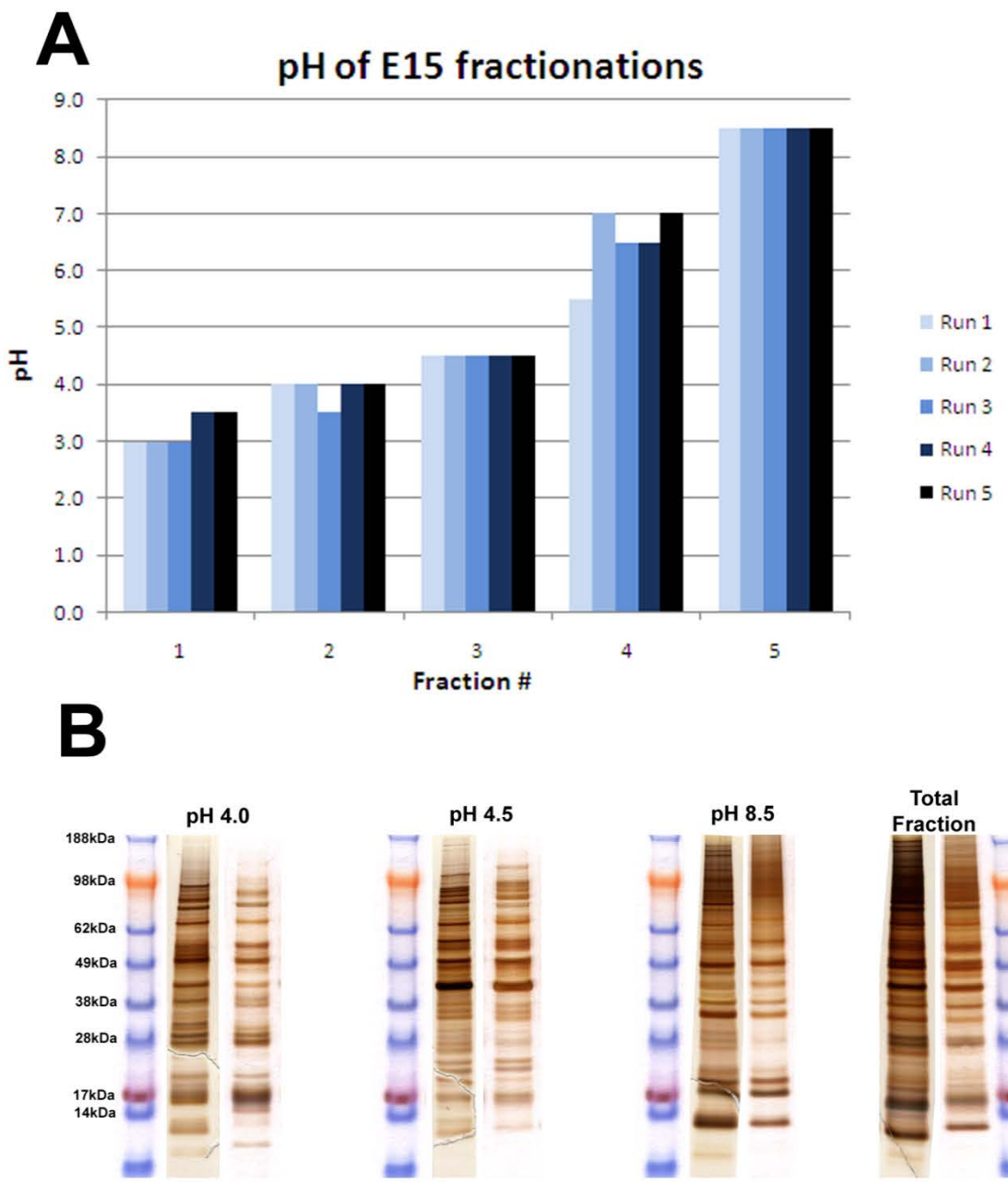
Isoelectric separation of proteins was verified by differences in pH between the five fractions (**Appendix B**). We compared the protein profiles of each fraction on silver stained gels of electrophoretically separated fractions. Figure 2 demonstrates that although there is some overlap, each cortical fraction contains an array of proteins, which as a group; differ from those displayed in the others. Each fraction contains proteins spanning a wide range of molecular weights.

Figure 2. Silver stain gel of isoelectric fractions 1-5 and the total fraction (T) (1 µg each). Each fraction contains a different group of proteins from the cortical proteome as sIEF focusing seperated proteins into 5 fractions and a total containing a pH of 3.0, 4.0, 4.5, 7.0, 8.5, and 6.5 respectively. The protein profile of each fraction is different, with a wide representation of molecular weights.


1
2
3
4
5
T
188 kDa
98 kDa
62 kDa
49 kDa
38 kDa
28 kDa
17 kDa
6 kDa

We also tested whether different runs of sIEF resulted in fractions of similar pH. Figure 3A shows that although slight variations of pH occurred in different runs, fractions from different sIEF processes generally resulted in similar pH values for comparable fractions. We also used silver stained one-dimensional electrophoresis to determine the reproducibility of fraction protein profiles from different isoelectric focusing procedures that have the same pH (**Figure 3B**). Although the pH of fractions may vary slightly, the protein profile of fractions from different sIEF processes that have the same pH also have consistently similar protein profiles. Therefore, results from experiments using fractions of the same pH can be compared across different sIEF processes.

Figure 3. Isoelectric fractions have a specific pH. (A) Graph showing the pH of each fraction obtained from 5 different sIEF processes involving E15 cortical extracts. Fraction numbers 1-5 represent samples from the anode to cathode ends, respectively. Note the general trend of increasing pH across sIEF processes and that pH values obtained from different samples were similar. (B) Silver staining shows replication in overall protein profiles of independent fractions having the same pH. Although there are slight differences in the banding pattern, the sliver stained protein profile for each fraction is remarkably similar. The molecular weight references are shown to the left of each sliver stain, except for the silver stain shown for the total fraction (T), which is placed to the right of the gel.


A
pH of E15 fractionations
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
pH
1
2
3
4
5
Fraction #
Run 1
Run 2
Run 3
Run 4
Run 5
B
pH 4.0
pH 4.5
pH 8.5
Total Fraction
188kDa
98kDa
62kDa
49kDa
38kDa
28kDa
17kDa
14kDa

3.1.2 Verifying and characterizing MGE primary cell cultures. To ascertain if exposure to the isoelectrically obtained cortical extracts affected survival of neocortical cell suspensions used in the migration assay, we determined cell viability after 20h in culture as described in the Methods. Figure 4 demonstrates that more than 60% of the E15 MGE cells exposed to each fraction were alive after 20 hours in culture, with no significant difference in survivability among the fractions. We also characterized the morphology and expression of GABA and parvalbumin in cultures of dissociated MGE cells used in the migratory assays, since these substances are typically expressed in cells arising from the MGE. Examples of immunostained cells positive for GABA and parvalbumin can be seen in Figure 5. Further, we found 70% of surviving MGE cells were immunoreactive for GABA after 20 hours of incubation with various neocortical fractions, with no significant differences in survival among the fractions (**Figure 6**). The percent of cells expressing GABA in our culture is comparable to other studies that evaluated the relative proportion of GABAergic cells residing in the MGE (Cuzon et al., 2006). These analyses demonstrate that inclusion of isoelectric fractions derived from embryonic neocortex in the culture medium does not significantly alter the viability of the cell suspensions or the percent of MGE cells expressing GABA compared to the control situation (plain NB media). Therefore, differences in cell migration seen in our experiments are not likely to be caused by differences in cell viability caused by the neocortical fractions, but rather by the chemokinetic or chemoattractant properties of each isoelectric fraction.

Figure 4. Graph depicting the percent of viable cells from cultures of E15 MGE cells after 20 hours of incubation with isoelectric fractions that spanned the entire isoelectric range. No significant difference in the percent of cell viability was observed among all tested conditions. The lack of difference in survivability between any of the fractions indicates that our isoelectric fractions do not adversely affect viability of GE cells. Each condition was tested in triplicate. Percent viability was calculated by subtracting the total percent of cells labeled with propidium iodide from 100. Data are mean ±SEM. One-way ANOVA with significant p-value set at 0.05.

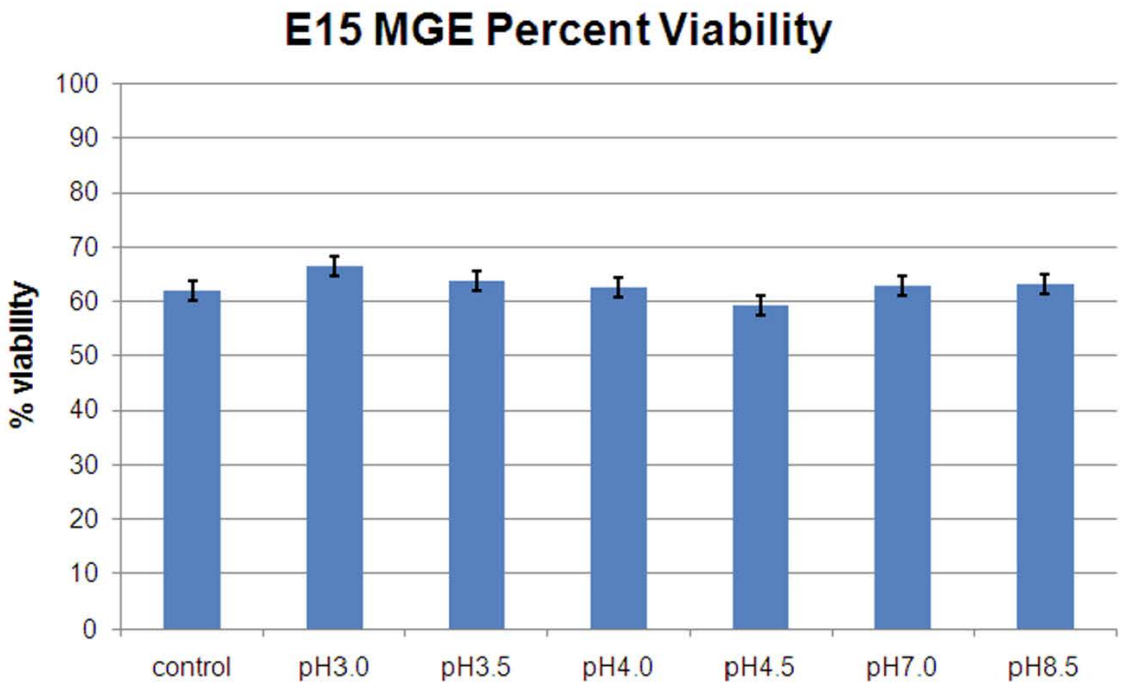

E15 MGE Percent Viability
% viability
100
90
80
70
60
50
40
30
20
10
0
control
pH3.0
pH3.5
pH4.0
pH4.5
pH7.0
pH8.5

Figure 5. MGE cell suspensions were immunologically characterized. Cell suspensions from E15 MGE with bisbenzimide-labeled nuclei (blue) were cultured in plain NB media for 20 hours. Cells were placed in fixative and immunostained for GABA (green) and parvalbumin (red). Coexpression of the inhibitory neurotransmitter, GABA, and the calcium-binding protein, parvalbumin verify the MGE origin of the cell suspensions (Xu et al., 2004). Arrowheads indicate cultured MGE cells with a fusiform morphology characteristic of tangentially migrating interneurons (Marin and Rubenstein, 2001, Xu et al., 2004). Scale bars = 50 μm.

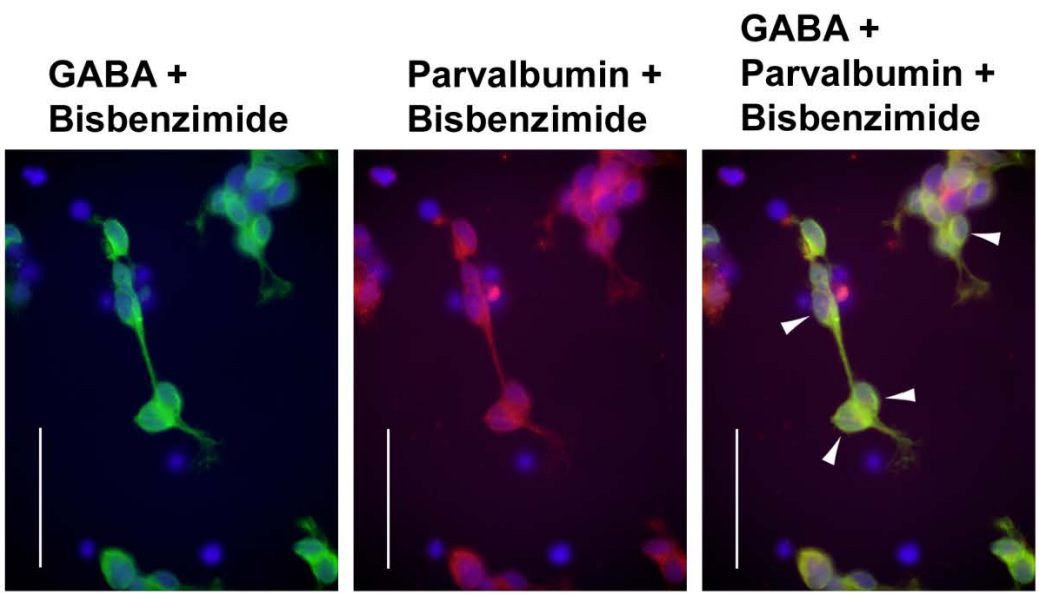
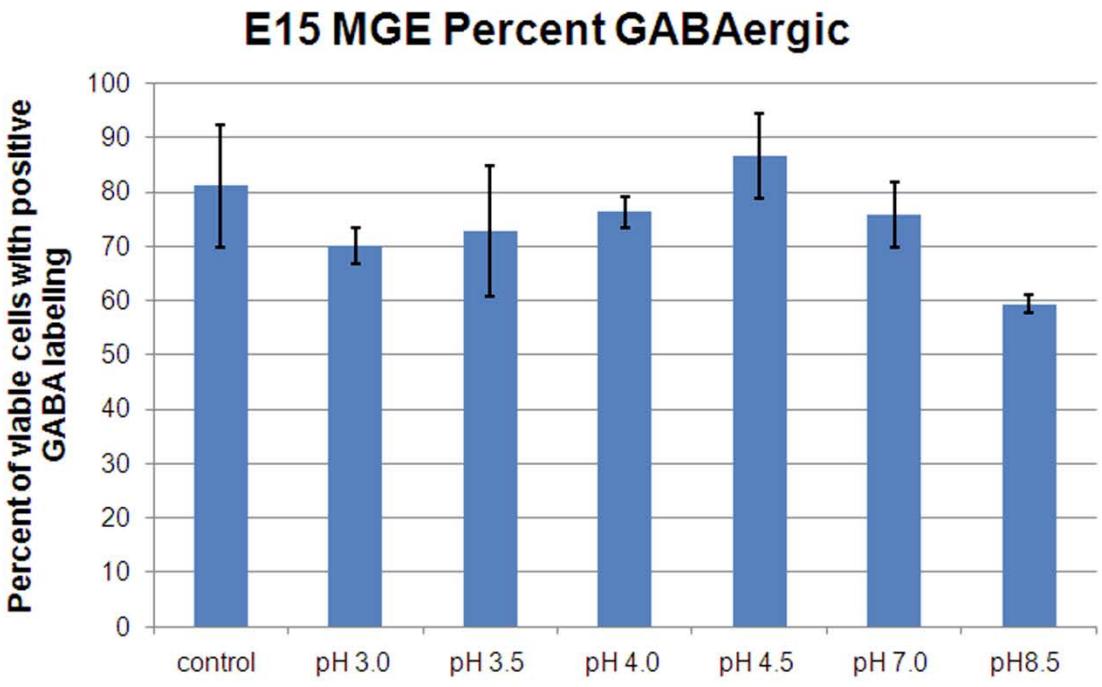

GABA +
Bisbenzimide
Parvalbumin +
Bisbenzimide
GABA +
Parvalbumin +
Bisbenzimide

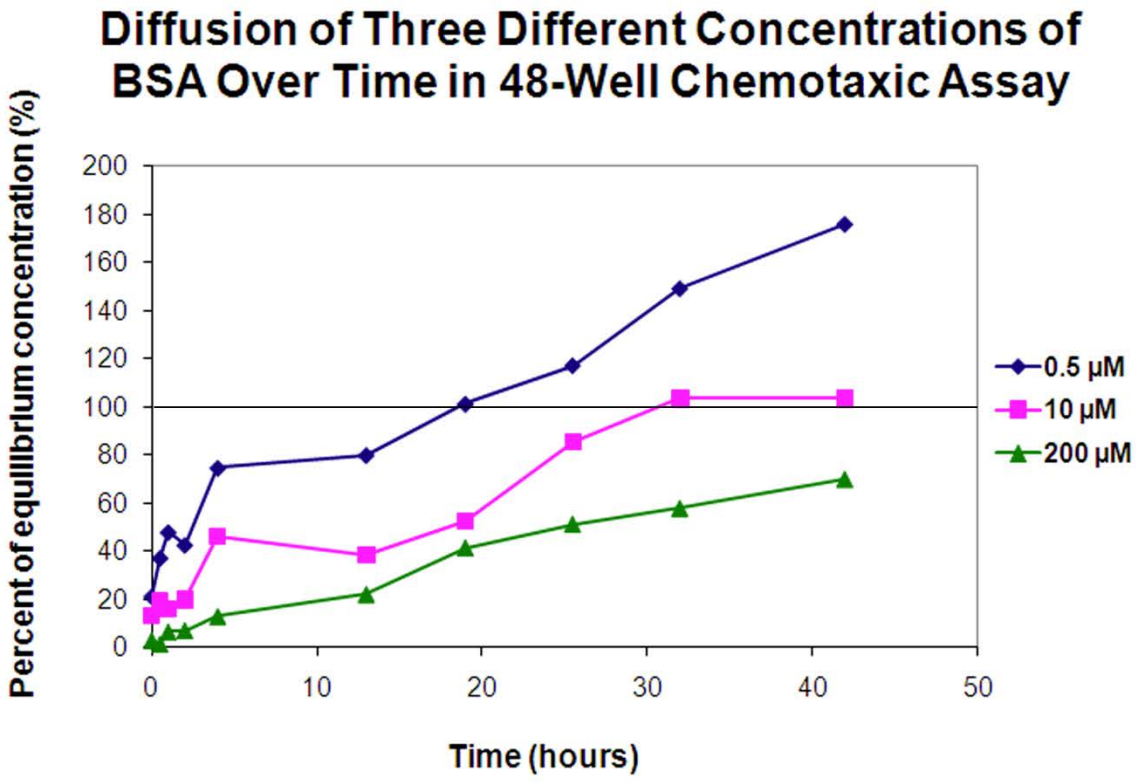
Figure 6. The percent of MGE cells in cell suspension expressing GABA after 20 hours of incubation with fractions spanning the entire isoelectric range. There is no significant difference in the percent of cells immunoreactive for GABA between all tested conditions. Therefore, the isoelectric fractions do not appear to significantly affect the GABAergic nature of our MGE cell cultures. Each condition was tested in triplicate. Data are mean ±SEM. One-way ANOVA with significant p-value set at 0.05.


E15 MGE Percent GABAergic
Percent of viable cells with positive GABA labelling
100
90
80
70
60
50
40
30
20
10
0
control
pH 3.0
pH 3.5
pH 4.0
pH 4.5
pH 7.0
pH8.5

3.2 Multiwell migration assays: validation of protein gradient. A protein gradient is crucial for studies of chemoattraction in the multiwell migration assay. Gradients form in the multiwell migration assay when a concentration difference exists across the porous membrane. Proteins use Brownian motion to preferentially move across the porous membrane to the upper well, until equilibrium is achieved, at which point movement in both directions is equal. A gradient of chemoattractant factors will cause cells in the upper well to migrate through the pores, towards an area of higher concentration.

To characterize the equilibrium of proteins across the membrane we loaded lower wells with three different concentrations of BSA diluted in PBS (0.5, 10 and 200 μM), assembled the assay, loaded the upper wells with plain PBS and drew two samples from the upper wells of the three different concentrations at 10 fixed time intervals (0, 0.5, 1, 2, 4, 13, 19, 25.5, 32 and 42 hours). The protein concentration of each sample was tested in duplicate and the concentration was converted to a percent of the concentration if equilibrium were achieved. We found the lowest concentration of BSA (0.5 μM) equilibrated across the membrane at around 20 hours. The intermediate concentration of BSA (10 μM) equilibrated across the membrane at around 30 hours. The highest concentration of BSA (200 μM) did not equilibrate across the membrane after 40 hours of incubation (**Figure 7**). This confirmed the ability of the multiwell migration assay to maintain a protein concentration gradient up to and beyond 20 hours. We found equilibrium was achieved in less time with smaller concentration differences across the membrane.

Figure 7. Chronological graph of protein concentration in the upper wells of a 48-well migration assay when the bottom wells were loaded with three different concentrations of BSA. Values are the concentration of each condition, set as a percent relative to the theoretical concentration that would be achieved at equilibrium (100%, the black bar). BSA concentration of 0.5 μM equilibrated around 20 hours, the BSA concentration of 10 μM equilibrated around 30 hours and the BSA concentration of 200 μM did not equilibrate after 40 hours. In general, lower concentration gradients came to equilibrium faster. Each point is represented by the average of the protein concentrations from two independent samples measured in duplicate.


Diffusion of Three Different Concentrations of BSA Over Time in 48-Well Chemotaxic Assay
Percent of equilibrium concentration (%)
200
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
Time (hours)
0.5 µM
10 µM
200 µM

3.3 Multiwell migration assays: cresyl violet 48-well. Migration of E14 GE cells in response to E14 isoelectric fractions was measured using a 48-well migration assay in four independent experiments. Each experiment used isoelectric fractions from independent sIEF processes. The fractions tested spanned the entire isoelectric range. Isoelectric fractions of different pH were placed in the lower wells of the migration assay with cell suspensions obtained from E14 GE placed in the upper wells. For this set of studies, cells were counted as they migrated through the pores of the membrane of a multiwell migration assay. An example of cells that migrated through the membrane pores of the multiwell migration assay can be seen in Figure 8. The quantification procedure is described in the Methods. We found the isoelectric pH 5.5 and 8.0 fractions to induce significant migration of E14 GE cells (**Figure 9**).

Figure 8. Morphology of the cultured GE cells. Images are of cresyl violet stained GE cells that migrated through the pores of a 48-well migration assay and adhered to the underside of the membrane. (A) The migrated cells clearly have interneuron-like fusiform morphology indicative of migrating GE cells (Marin and Rubenstein, 2001). (B) GE cells display a complex array of processes which may be involved with migration and cell-to-cell interactions. The pores can be seen in these images, which are 8 μm in diameter. Scale bars = 50 μm.

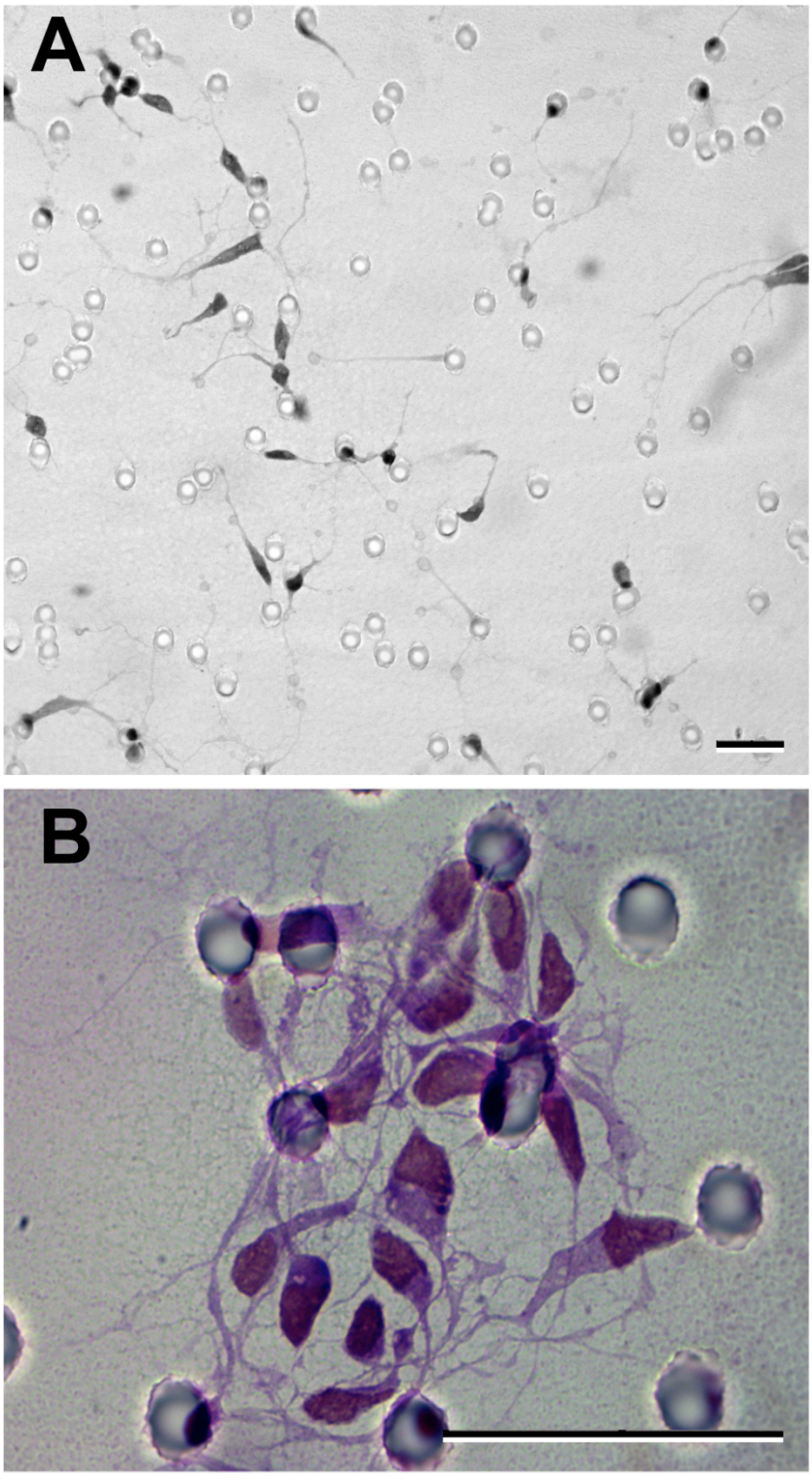
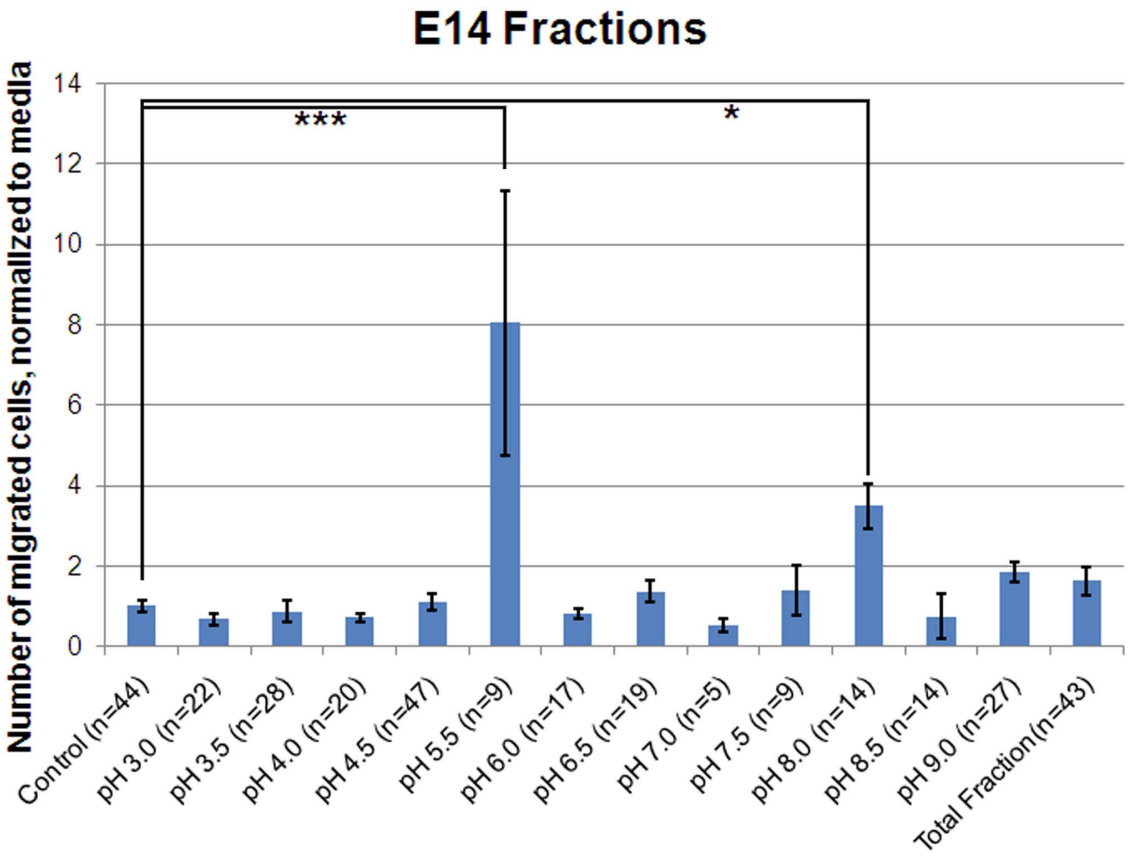
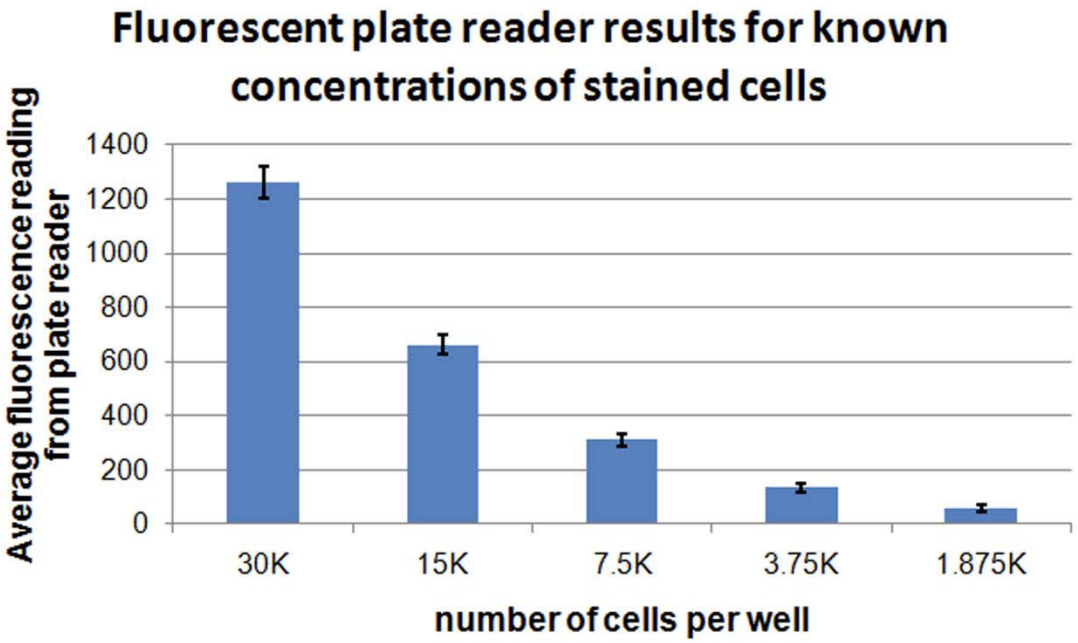

A
B

Figure 9. Graph of migration from E14 GE cell suspensions in response to isoelectric fractions of E14 cortical extract. For ease of interpretation and to pool data, all results were normalized to the control condition. Statistical analysis demonstrates the pH 5.5 fraction was most active in causing migration of E14 GE cells with the pH 8.0 fraction also inducing significant migration of E14 GE cell suspensions. All other isoelectric fractions did not significantly influence migration of E14 GE cells. The number of wells analyzed for each condition is indicated. A one-way ANOVA was conducted, followed by a two-tailed Dunnett's post hoc test of each condition compared to the control (media alone). Data are mean ±SEM. *p<0.05 and ***p<0.001.


E14 Fractions
Number of migrated cells, normallzed to media
14
12
10
8
6
4
2
0
***
*
Control (n=44)
pH 3.0 (n=22)
pH 3.5 (n=28)
pH 4.0 (n=20)
pH 4.5 (n=47)
pH 5.5 (n=9)
pH 6.0 (n=17)
pH 6.5 (n=19)
pH 7.0 (n=5)
pH 7.5 (n=9)
pH 8.0 (n=14)
pH 8.5 (n=14)
pH 9.0 (n=27)
Total Fraction (n=43)

3.4 Multiwell migration assays: fluorescent 96-well. For further analysis we used an automated 96-well assay to quantify cell migration towards specific factors. This assay uses a 96-well plate as the lower wells for protein samples. Cell suspensions obtained from E14, E15, and E17 MGE are labeled with bisbenzimide to allow measurement of fluorescence and the amount of cells that migrate into the lower well is quantified by total fluorescence in each well of the 96-well plate. To understand the dynamic range of fluorescent signals and correlate fluorescent intensity to quantity of stained cells, we determined the fluorescent plate spectrophotometer readings for known concentrations of bisbenzimide-labeled MGE cells. We loaded a poly-D-lysine coated, low-volume 96-well culture plate with a serial dilution of bisbenzimide stained E15 MGE cells. Wells were filled with 30, 15, 7.5, 3.75 or 1.875 thousand cells. Each dilution was represented in 16 different wells. After 20 hours of incubation the cells were fixed and fluorescence of each well was measured in a fluorescent plate spectrophotometer (**Figure 10**). A near linear relationship exists between readings from the spectrophotometer and the number of cells per well. The large signal-to-noise ratio validates use of the fluorescent spectrophotometer to detect bisbenzimide-labeled cells.

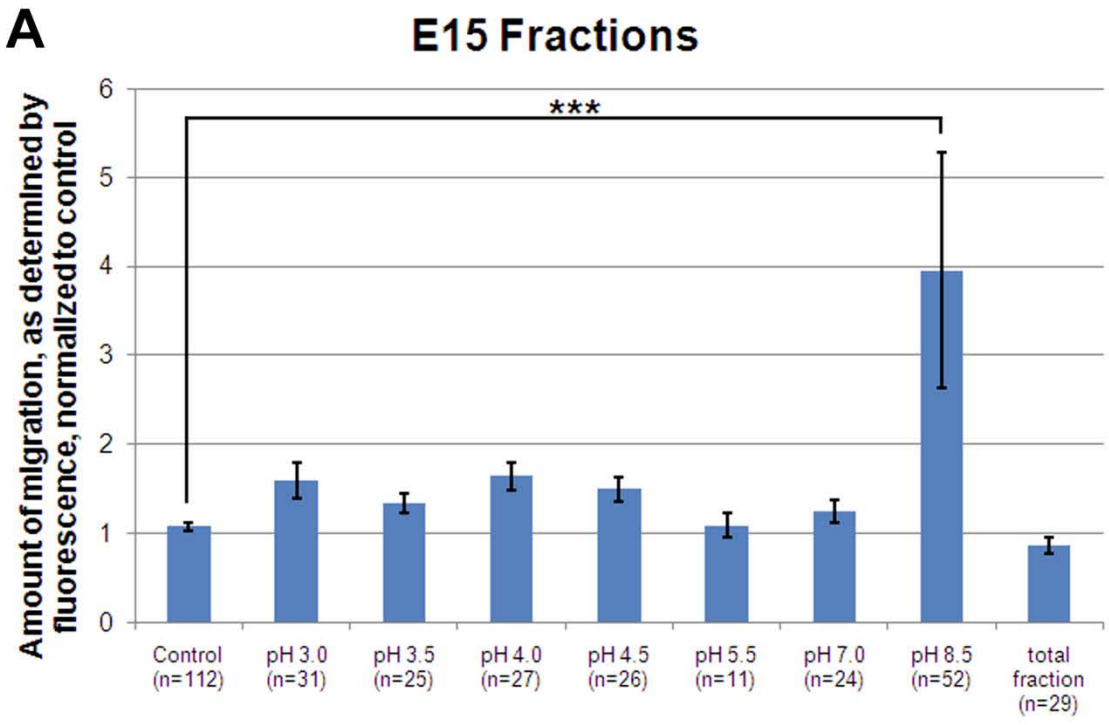
Figure 10. Bar graph displays fluorescence plate spectrophotometer readings for bisbenzimide-stained E15 MGE cell suspension dilutions loaded in the low-volume 96-well plate used in this multiwell migration assay. A proportional relationship between cell density and fluorescence is reflected in proportionally higher readings from wells containing more cells. A total of 12 separate wells were analyzed for each condition. Error bars are SEM.

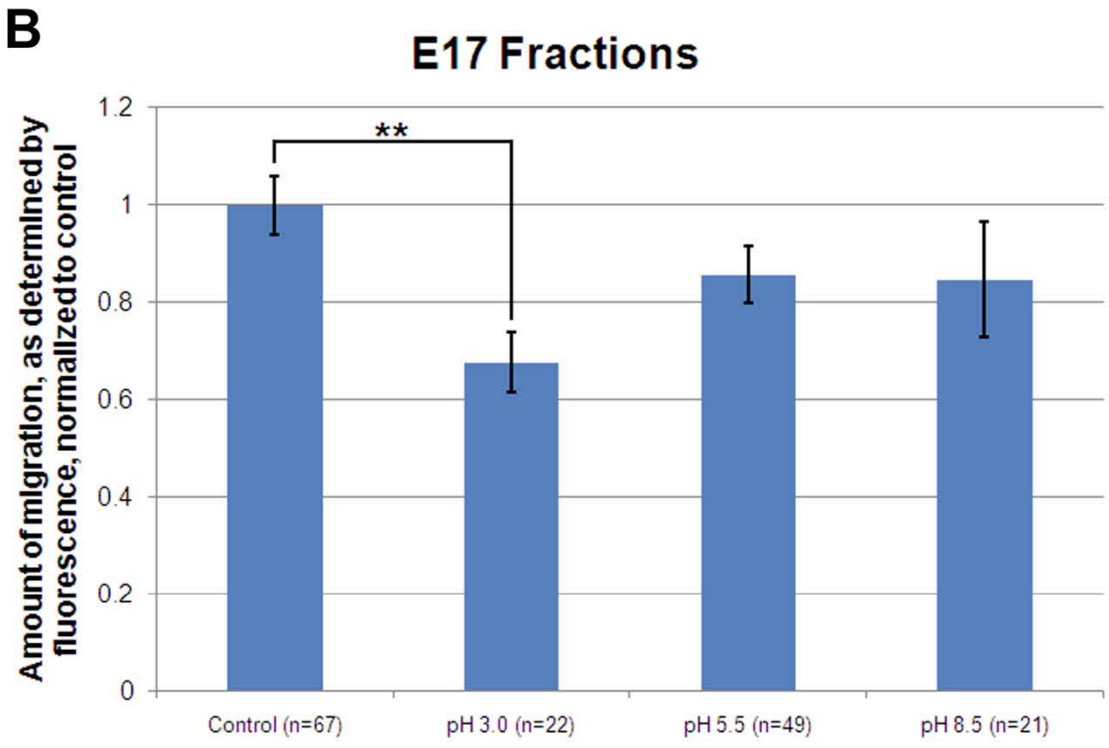

Fluorescent plate reader results for known concentrations of stained cells
Average fluorescence reading from plate reader
1400
1200
1000
800
600
400
200
0
30K
15K
7.5K
3.75K
1.875K
number of cells per well

3.4.1 Effect of E15 and E17 cortical isoelectric fractions on isochronic MGE cell migration.

During the development of the neocortex on embryonic days E15 and E17, cells leaving the MGE migrate into the cortical plate (Anderson et al., 1997, Tamamaki et al., 1997, Lavdas et al., 1999, Wichterle et al., 1999, Brazel et al., 2003, Tanaka et al., 2003, Yau et al., 2003, Kriegstein and Noctor, 2004). We used the 96-well fluorescent assay to evaluate the influence of E15 and E17 isoelectric fractions on MGE cell migration. When cortical fractions obtained at E15 were tested, the E15 pH 8.5 fraction caused a significant increase in migration of E15 MGE cell suspensions, while the other fractions did not induce significant migration (**Figure 11A**). When cell suspensions were obtained from E17 MGE and tested with isochronic fractions obtained from E17 neocortex, a significant increase in migration in response to any of the fractions was not observed, but migration was significantly reduced in the presence of E17 pH 3.0 fraction (**Figure 11B**).

Figure 11. Graph of results from E15 and E17 fractions using the 96-well fluorescence migration assay. (A) Significantly more E15 MGE cells migrated in the presence of E15 pH 8.5 fraction in comparison to the control (plain media). (B) Significantly fewer cells obtained from E17 MGE cells migrated in the presence of E17 pH 3.0 neocortical fraction compared to the control (plain media). For each condition, data was analyzed from at least two independent experiments. The number of wells analyzed for each condition is indicated on the x-axis. For each age, significance was determined with a one-way ANOVA followed by a two-tailed Dunnett's post hoc test of each pH fraction compared to the control condition. Data are mean ±SEM. **p<0.01 and ***p<0.001.


A
E15 Fractions
Amount of migration, as determined by fluorescence, normalized to control
***
0
1
2
3
4
5
6
Control (n=112)
pH 3.0 (n=31)
pH 3.5 (n=25)
pH 4.0 (n=27)
pH 4.5 (n=26)
pH 5.5 (n=11)
pH 7.0 (n=24)
pH 8.5 (n=52)
total fraction (n=29)


B
E17 Fractions
Amount of migration, as determined by fluorescence, normalized to control
**
0
0.2
0.4
0.6
0.8
1
1.2
Control (n=67)
pH 3.0 (n=22)
pH 5.5 (n=49)
pH 8.5 (n=21)

3.4.2 Chemoattractant effect of positive controls on E15 and E17 MGE cells.

We tested the influence of glutamate, reelin, GDNF, and NRG1 in our model; all of which have been shown to effect migration of cells into the neocortex (Behar et al., 1999, Flames et al., 2004, Cuzon et al., 2006, Paratcha et al., 2006). We used the 96-well migration assay to test the response of E15 and E17 MGE cells to gradients of these four substances (**Figure 12**).

We found 1 μM glutamate to induce a significant migration of E15 and E17 MGE cells. At a concentration of 1 μM, glutamate has been shown to positively influence migration of E13, E14, E15, E16, E17 and E18 cortical neurons (Behar et al., 1999). This effect may be caused by activation of the NMDA receptor as migrating interneurons express functional NMDA receptors (Soria and Valdeolmillos, 2002, Manent and Represa, 2007). The same mechanisms that drive cortical neuronal migration via NMDA activation may also influence interneuron migration via receptor activation.

We found 3 nM GDNF did not significantly influence migration of E15 or E17 MGE cells. Calbindin-positive cells have been shown to migrate towards sources of GDNF (Pozas and Ibanez, 2005). Since the majority of calbindin-positive interneurons originate from the MGE (Xu et al., 2004), GDNF has been understood to be a chemoattractant for MGE cells. Although others have demonstrated chemoattraction by GDNF (Pozas and Ibanez, 2005), we did not detect a significant amount of GE cell migration in relation to GDNF.

We also test the ability for reelin to influence MGE cell migration. Reelin was tested at 1 pM and 1 nM, which are near biologically active concentrations

(Weeber et al., 2002, Sinagra et al., 2005). Our studies used a recombinant reelin which represented the central fragment of the reelin protein. This fragment is required for binding the two types of reelin receptors (Hiesberger et al., 1999, Trommsdorff et al., 1999) and transducing a signal that is likely required for interneuron movement (Caronia-Brown and Grove, 2010). We found both concentrations of reelin did not significantly affect migration of E15 or E17 MGE cells.

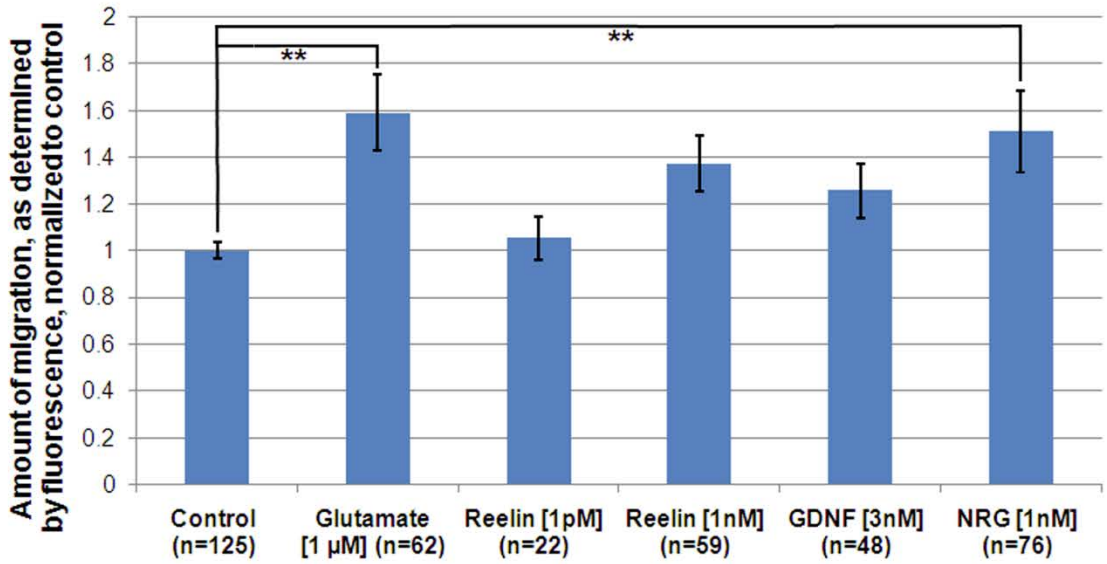
NRG influences migration of cells into the neocortex (Rio et al., 1997, Gierdalski et al., 2005). The different NRG subtypes affect cell migration in different ways, with the secreted form (NRG1) acting as a chemoattractant for MGE-derived neurons (Flames et al., 2004). NRG1 interacts with the EGF receptor ErbB4 found on migrating interneurons (Buonanno and Fischbach, 2001, Yau et al., 2003). We used NRG1 at 1 nM as this is near the described biologically active range for this protein and has been successfully used in other studies (Karey and Sirbasku, 1988, Gierdalski et al., 2005, Poluch and Juliano, 2010). We found NRG1 significantly increases migration of E15 and E17 MGE cells.

We found E15 and E17 MGE cells had similar response profiles to the tested factors. Both E15 and E17 MGE cells significantly migrated in a gradient of 1 μM glutamate or 1 nM NRG1 (**Figure 12**). Although a gradient was present, the increased migration of MGE cells may be due to contact with chemokinetic agents. Therefore, the increased migration in the presence of glutamate and NRG1 may have been due to chemoattraction or chemokinesis. We performed

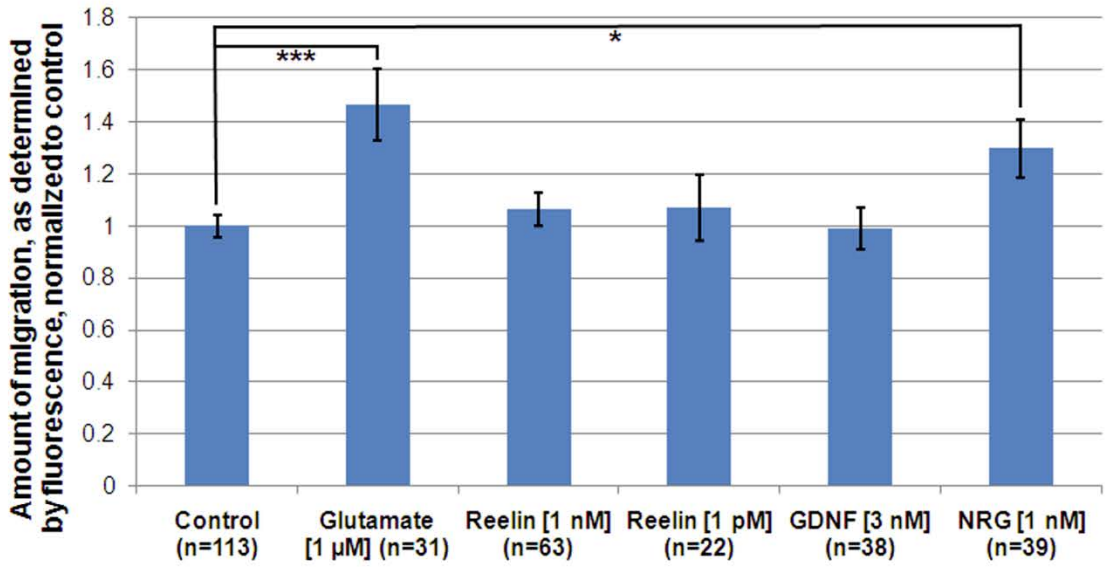
chemokinetic studies to better understand the attractant nature of NRG1.

Figure 12. Graph of E15 (A) and E17 (B) positive control results from multiwell fluorescence migration assays. Results represent plate reader data transformed for position in the assay and normalized to control conditions. Only glutamate (1 μM) and NRG1 (1 nM) induced significant migration of E15 and E17 MGE cells. Although not significant, reelin (1 nM) induced migration of E15 MGE cells (p=0.084) (A). For each age, significance was determined with one-way ANOVA and a two-tailed Dunnett's post hoc test between all conditions and control. Data are mean ±SEM. *p<0.05, **p<0.01, ***p<0.001

A


E15 Positive Controls
Amount of migration, as determined by fluorescence, normalized to control
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
Control (n=125)
Glutamate [1 µM] (n=62)
Reelin [1pM] (n=22)
Reelin [1nM] (n=59)
GDNF [3nM] (n=48)
NRG [1nM] (n=76)

B


E17 Positive Controls
Amount of migration, as determined by fluorescence, normalized to control
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
*
Control (n=113)
Glutamate [1 µM] (n=31)
Reelin [1 nM] (n=63)
Reelin [1 pM] (n=22)
GDNF [3 nM] (n=38)
NRG [1 nM] (n=39)

3.4.3 Chemokinetic response of E15 MGE cells to NRG1 and pH 8.5.

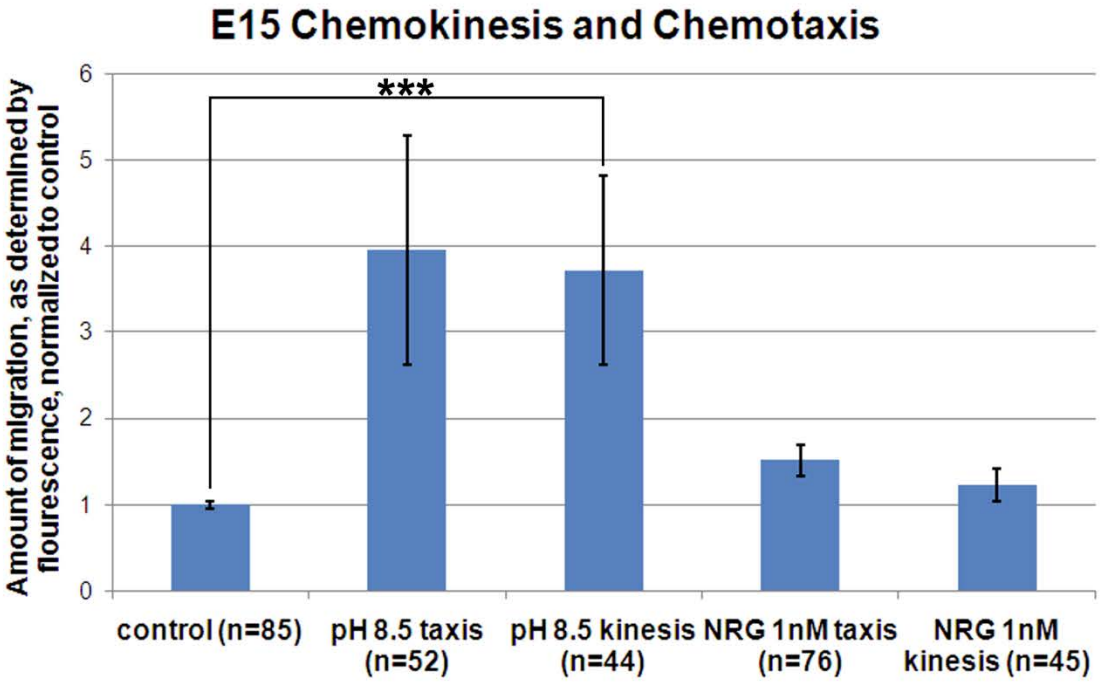
To further assess the precise influence of the factors that cause significant movement of MGE cells, we tested pH 8.5 fraction and NRG1 in the multiwell migration assay without a protein gradient. This allowed us to differentiate between cell migration towards a gradient of chemoattractant agents or the random undirected movement of cells in a non-gradient environment of chemokinetic factors. This is particularly appealing considering that NRG1 has not been explicitly shown to act as a chemokinetic factor for cortical interneurons.

Previously, we used a chemoattractant setup in our multiwell migration assays by having proteins diffuse from the lower well to the upper well, thereby creating a gradient. Cells loaded in the upper well presumably migrated to the lower well by chemoattraction. It is possible that mere contact with proteins diffusing from the lower well could have initiated chemokinetic activity in cells from the upper well. In the multiwell migration assay, a protein gradient of either chemokinetic or chemoattractant factors could result in similar amounts of cells migrating across the membrane. Therefore, significant migration in the presence of a gradient suggests chemoattraction, but requires validation in conditions without a gradient.

We used a chemokinetic setup to distinguish the chemokinetic or chemoattractant behavior of GE cells in the presence of both the pH 8.5 fraction and NRG1, since they elicited migration of E15 neocortical cell suspensions. Using the multiwell migration assay, we eliminated the protein gradient by placing an equal concentration of either pH 8.5 fraction or NRG1 across the membrane.

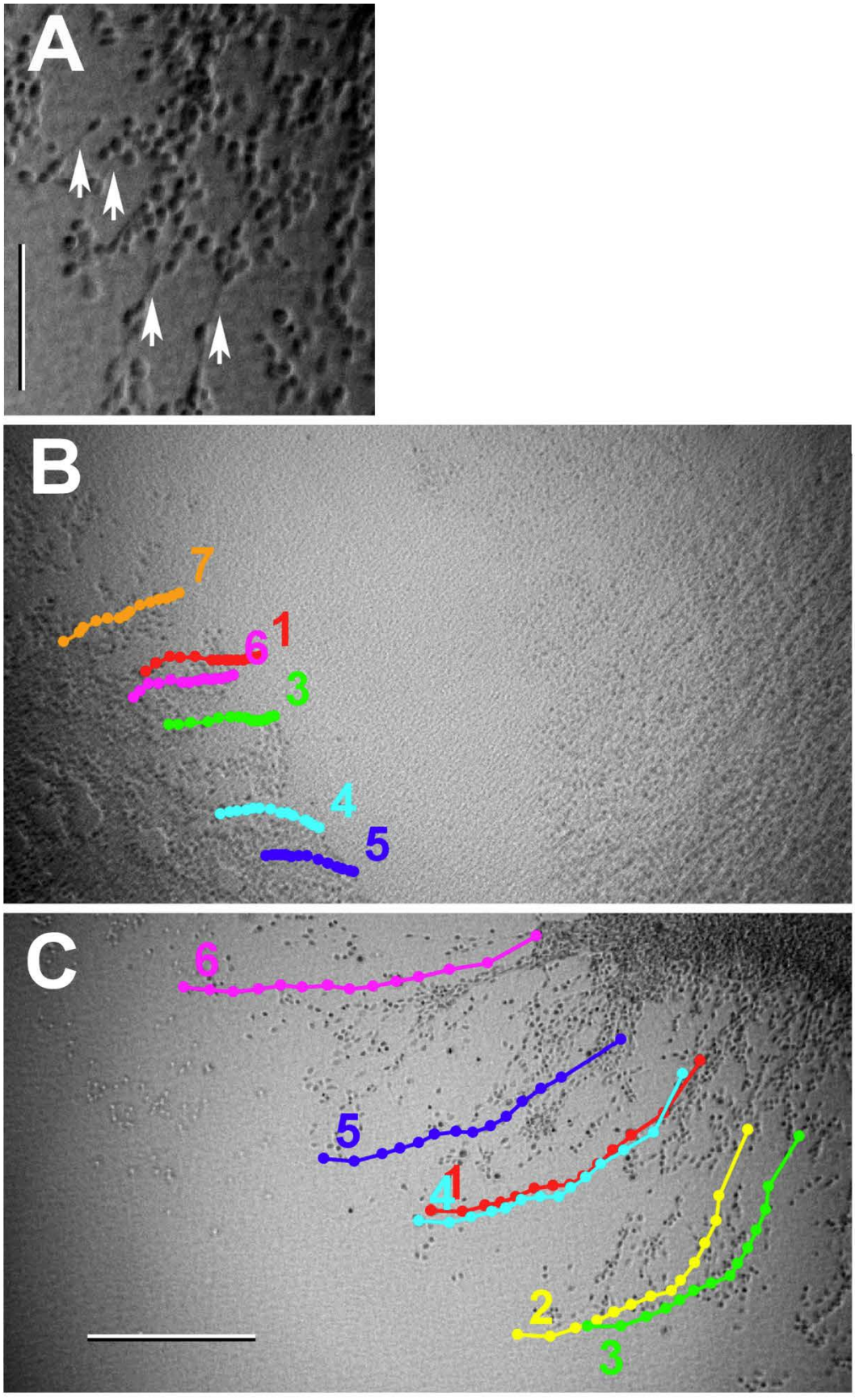
Therefore, migration induced by the presence of pH 8.5 fraction or NRG1 would be considered chemokinesis. By eliminating the gradient in our 96-well migration assay in a chemokinetic setup, we found increased migration was maintained for pH 8.5 fraction (**Figure 13**). Cell migration of E15 MGE was significantly greater in a gradient-free environment of pH 8.5 fraction compared to the control condition, suggesting that factor(s) existing in this isoelectric fraction are chemokinetic for cells of the MGE. The migration of E15 MGE cells in a gradient-free environment of NRG1 was increased, but not significantly different from the control condition. Although this suggests the migration we observed for E15 MGE cells in a gradient of NRG1 was chemoattraction, increased migration in a gradient-free environment of NRG1suggests partial chemokinetic activity. Based on the results in this study, we conclude that pH 8.5 fraction influences E15 MGE cells chemokinetically while NRG1 acts like a chemoattractant factor while displaying some chemokinetic properties.

Figure 13. Quantification of E15 MGE cell migration in a gradient-free environment demonstrates chemokinesis. A significant difference in cell migration exists between the control condition and chemokinetic condition for the pH 8.5 fraction. The comparable amount of E15 MGE cell migration in the presence of a gradient or in a gradient-free environment suggests that pH 8.5 fraction is chemokinetic to these cells. The amount of cells migrating in the chemokinetic condition for NRG1 is not significantly different from the control. NRG1 can be considered chemoattractant since MGE cells significantly migrate when a gradient is present. The increased migration seen for the chemokinetic condition suggests NRG1 may have some chemokinetic properties. Each condition represents data from at least two independent experiments. Significance was determined with one-way ANOVA and a two-tailed Dunnett's post hoc test between control and the two kinesis conditions. Data are mean ±SEM. ***p<0.001.


E15 Chemokinesis and Chemotaxis
Amount of migration, as determined by flourescence, normalized to control
6
5
4
3
2
1
0
***
control (n=85)
pH 8.5 taxis (n=52)
pH 8.5 kinesis (n=44)
NRG 1nM taxis (n=76)
NRG 1nM kinesis (n=45)

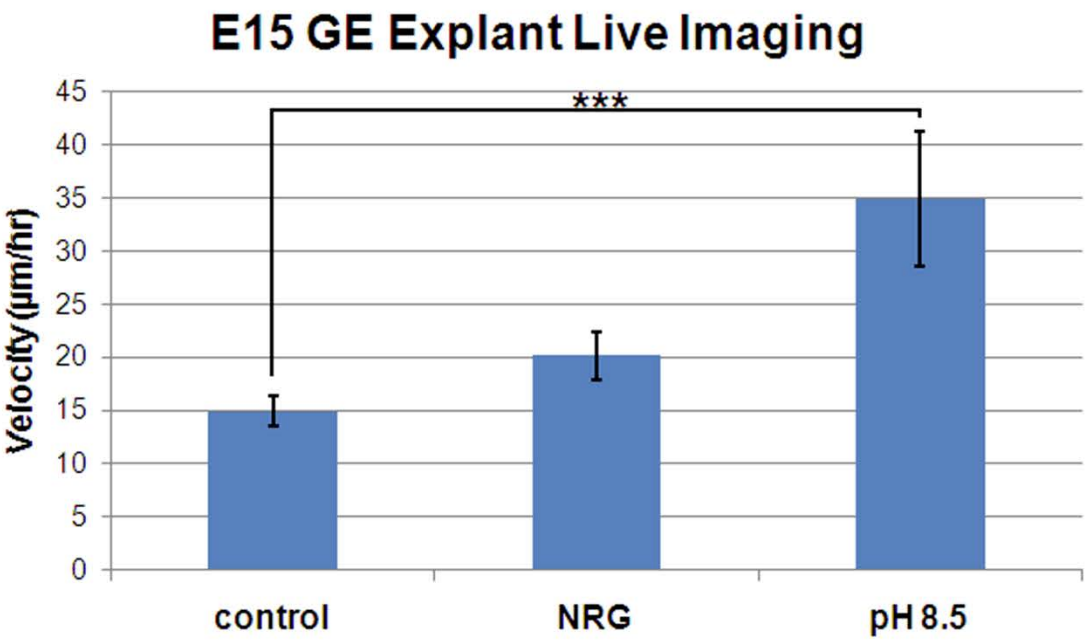
3.5 Live imaging. Next, we directly observed cellular movement over time to confirm our observations of pH 8.5 fraction and NRG1 from the multiwell migration assay. While our previous results showed the chemokinetic and chemoattractant properties of pH 8.5 fraction and NRG1, we were interested in knowing the effect of these agents on velocity of migration. Since results from the multiwell migration assay represent the total number of cells that migrate after 20 hours, it provides us with an end-state result. To better characterize the behavior of MGE cells, we used live imaging to gather information during the process of MGE cell movement when exposed to a gradient-free environment of the neocortical fraction pH 8.5 or NRG1. This was accomplished by placing MGE explants on culture inserts in wells containing plain NB media (control), pH 8.5 fraction (10 μg/mL) or NRG1 (1 nM) and placing them in a live imaging culture system; images of the MGE explants were captured every 30 minutes for at least 7 hours. After all images were captured, we processed each video with ImageJ to track the position of cells over time (**Figure 14**). With no existing protein gradient, cells were in contact with each factor of interest. This suggests that if migration velocity is significantly greater in one of the experimental conditions than the control, then the substance causes generalized increased movement, therefore behaving as a chemokinetic agent.

Figure 14. E15 MGE explants were cultured on 6-well inserts with either plain NB media (control), NRG1 (1 nM), or pH 8.5 fraction (10 µg/mL). The inserts were placed in an incubator attached to a Zeiss Axiovert 200 microscope. These images are of migrating cells from E15 MGE explants on culture inserts taken every 30 minutes for 7 hours. (A) An enhanced image from one of the many captured time points reveals leading processes (white arrows) characteristic of tangentially migrating GE cells. ImageJ cell tracking is superimposed on images from cells exposed to (B) NRG1 or (C) pH 8.5 fraction. Each color represents the path of a single cell, with each dot representing a position the cell occupied in each 30 minute interval. Cells exposed to the pH 8.5 fraction have substantially longer track lengths than those exposed to plain media or NRG1 for the same period of time. Scale bar for A = 100 µm and for B and C = 500 µm.


A
B
7
6
1
3
4
5
C
6
5
1
4
2
3

Within each condition, tracking represented migration spanning at least a 7 hour culture period. The average velocity of cell migration from E15 MGE explants significantly increases from the control when exposed to pH 8.5 fraction (**Figure 15**). Additionally, exposure to NRG1 did not significantly increase the average velocity of MGE cell migration. The increased velocity of E15 MGE cells suggests a chemokinetic response to agent(s) in the E15 pH 8.5 fraction. Although the MGE cells exposed to NRG1 moved faster than the control cells, the difference was not significant. Results from NRG1 live imaging support the view that NRG1 acts mostly as a chemoattractant, but retains some chemokinetic properties for E15 MGE cells. These live imaging results correspond with and support our results from the multiwell migration assay. Altogether, we found E15 pH 8.5 fraction induces significant chemokinetic activity of E15 MGE cells. While our results also support the existing view that NRG1 is a chemoattractant for cells from the MGE, they also indicate that NRG1 may have chemokinetic actions on migrating MGE cells.

Figure15. Quantification of cell migration velocity from E15 MGE explants cultured in the presence of plain NB media (control), 1 nM NRG1, or 10 µg/mL pH 8.5 fraction. The migration velocity of cells from MGE explants is significantly increased in the presence of pH 8.5 fraction. This suggests that E15 pH 8.5 fraction has a chemokinetic effect on cells leaving the E15 MGE. Interestingly, NRG1 also induces increased migration velocity, although it is not significant (p = 0.495). Therefore, NRG1 again acts as a chemoattractant while displaying some chemokinetic properties. Data points are the average velocity between all track points for each cell in one explant. Each graph bar represents the average of data points from all explants exposed to the same condition. Significance was determined with a one-way ANOVA followed by a two-tailed Dunnett's post hoc test between control and the two test conditions. Data are mean ±SEM. ***p<0.001.


E15 GE Explant Live Imaging
Velocity (µm/hr)
45
40
35
30
25
20
15
10
5
0
***
control
NRG
pH 8.5

3.6 Multi-dimensional Protein Identification Technology (MudPIT). We identified the E15 pH 8.5 fraction as capable of chemokinetically influencing the migration of E15 MGE cells. After having characterized the effects on cell migration, we wanted to know which proteins were present in the pH 8.5 fraction. Knowledge of the protein contents could provide us with candidate proteins involved with the potent chemokinetic effect of E15 pH 8.5 fraction. Therefore, the E15 pH 8.5 fraction was analyzed by MudPIT to confirm origin of sample and identify novel proteins in the embryonic cortex that influence interneuron migration. MudPIT analysis revealed at least 4 molecules of interest to interneuron migration, 2 examples of predicted cortical proteins, and 8 unnamed proteins (**Table 1**).

The four proteins we identified as potential signals influencing interneuron cortical migration are Neuromedin-B, TUC-4b, unc-5 homolog C, and cadherin EGF LAG seven-pass G-type receptor 2 (**Table 1A**). Neural migration is affected by each of these four proteins. (i) Based on location and effect on neurite outgrowth (Quinn et al., 2003), TUC-4b may be involved with GE migration to the cortex. (ii) Neuromedin-B may theoretically enhance interneuron migration via GABA release (Lee et al., 1999, Lopez-Bendito et al., 2003). (iii) Unc-5 homolog C binds netrin, which promotes interneuron guidance in the developing cortex (Kennedy, 2000, Kruger et al., 2004, Stanco et al., 2009). (iv) Theoretically, interaction of the cadherin EGF LAG seven-pass G-type receptor 2 with interneuron membrane-bound forms of cadherins or reelin may influence tangential migration (Katso et al., 2006, Talmud et al., 2009). Although these four

proteins cover a broad range of migration mechanisms, each may have a unique role in tangential migration of interneurons.

To ensure our sample represented E15 cerebral cortex, we identified cortical proteins normally during this stage of development. MudPIT analysis revealed the presence of vimentin and alpha-tubulin in our sample (**Table 1B**). These cytoskeletal proteins are strongly represented in the embryonic cortex (Hutchins and Casagrande, 1989, Gloster et al., 1994). Presence of these proteins confirms the cortical origin of our sample.

We identified 8 unnamed protein products (**Table 1C**). These are unnamed because they were either present in very low concentrations or the sequence is not associated with any known proteins. Four of the unnamed proteins (protein IDs of 1334163, 56107, 55628 and 56200) were identified in more than one ionic fraction, thereby increasing the probability of their presence in the pH 8.5 fraction. These 8 unnamed proteins may represent novel cortical factors involved with tangential interneuron migration.

Table 1. Table of relevant MudPIT results. (A) Tuc-4b, Neuromedin B, unc-5, and cadherin EGF LAG seven-pass G-type receptor 2 may influence tangential migration of cortical interneuron. All four of these proteins are directly or indirectly involved with neural migration. (B) Beta-tubulin and vimentin are proteins of the developing cortex, presence of which validates the cortical origin of our sample. (C) Six separate unnamed proteins were generated by MudPIT analysis. These unnamed proteins may be novel sources of interneuron guidance in the developing cortex. Results were organized by match score within each category. Bolded rows designate proteins that were detected in more than one ionic fraction, indicating higher concentrations and more reliable identifications.

	Score	Protein ID	Protein Name	MW	% Coverage
A	**146**	**gi|21666559**	**TUC-4b [Rattus norvegicus]**	**73853**	**12.3**
	26				
	24	gi|157820747	neuromedin-B [Rat]	13136	17.1
	23	gi|149069502	unc-5 homolog C (C. elegans)-like, isoform CRA_c [Rat]	42694	6.2
	22	gi|22095545	Cadherin EGF LAG seven-pass G-type receptor 2	233333	2.1
B	**48**	**gi|93277119**	**tubulin-folding cofactor B [Rat]**	**27230**	**10.2**
	30	gi|14389299	vimentin [Rat]	53700	4.1
C	130	gi|57429	unnamed protein product [Rat]	49931	6.7
	127				5.6
	54	gi|56643	unnamed protein product [Rat]	35633	4.4
	95	**gi|1334163**	**unnamed protein product [Rat]**	**39134**	**6.9**
	94	**gi|56107**	**unnamed protein product [Rat]**	**47086**	**9.9**
	68	**gi|55628**	**unnamed protein product [Rat]**	**68674**	**4.9**
	67	**gi|56200**	**unnamed protein product [Rat]**	**61389**	**6.3**
	48	gi|55775	unnamed protein product [Rat]	16220	12.4
	23	gi|56878	unnamed protein product [Rats]	13201	36.1

Chapter 4

DISCUSSION

Depending on embryonic age, we found different isoelectric fractions of cortical extract to significantly influenced migration of GE cells. Migration of GE cells from E14 rats are significantly influenced by E14 neocortical fractions of pH 5.5 and 8.0. The E15 pH 8.5 fraction acts as a chemokinetic agent for E15 MGE cells. The E17 pH 3.0 fraction significantly inhibited migration of cells from E17 MGE. Overall, we identified that specific isoelectric fractions influence migrating GE cells and the effect varies with embryonic age.

4.1 GABAergic interneurons. We confirmed the MGE origin of cells used in our assay by morphology, GABA/parvalbumin coexpression, and the high percent of cells expressing GABA. Although we focused our attention on cells residing in the MGE, different regions within the GE predict the physiological subtype of interneuron (Butt et al., 2005). We did not investigate the CGE, which produces late-born interneurons expressing reelin and vasoactive intestinal peptide and comprises about 30% of cortical interneurons (Nery et al., 2002, Miyoshi et al., 2010). We measured the percent of cells in our culture that express GABA after exposure to different cortical isoelectric fractions. We found the various isoelectric fractions did not significantly affect the percent of cells expressing GABA. Interestingly, Trinh et al. (2006) used a reversed setup to show exposure of cortical cell cultures to GE-secreted factors induces GABA, calretinin, and calbindin expression in cortical cells. Our results are more translatable to cell-

based therapies because we determined the GABAergic disposition of these cells when exposed to fractions and confirmed parvalbumin expression, consistent with MGE cells that have invaded the cortex (Wonders et al., 2008).

4.2 Multiwell migration assays. We used the multiwell migration assay as a high throughput screening method to determine the ability of various factors to attract cells of interest. Depending on the assembly of this migration assay, it can be used to test chemoattraction or chemokinesis. The following discusses results from experiments we performed to verify maintenance of a protein gradient and to determine optimal run time of the multiwell migration assay.

4.2.1 Protein gradient in migration assay. We measured the changing protein concentration of the upper well in the multiwell migration assay and found a protein gradient is formed and can be maintained past 40 hours. In general, we found lower concentration gradients equilibrated in less time than higher concentration gradients. The lowest tested concentration of 0.5 μM BSA, equilibrated across the porous membrane of the migration assay in 20 hours, whereas the highest concentration of 200 μM BSA maintained a gradient past 40 hours.

In addition, the protein gradient study lacked cells in the upper well. The presence of cells may reduce the speed of equilibrium by blocking pores in the membrane. Therefore, protein gradients are likely maintained for even longer in our migration studies. Second, the protein gradient study involved repeated movement of the assay when samples were drawn. Agitation of the assay during

collection of samples may have increased the rate that molecules crossed the membrane. Therefore, our migration studies likely maintain a protein gradient even longer then calculated here because they are minimally agitated. Overall, the dynamic concentration curve we measured suggests reliable protein gradients in the multiwell migration assays.

4.2.2 Optimizing run time of migration assay. We optimized the multiwell migration assay for run time. Optimal run time in the multiwell migration assay balances cell migration and equilibration of protein gradients. We chose a 20 hour run time because our testing of BSA gradients in the multiwell migration assay indicated that lengthier incubations could lead to equilibration of protein gradients across the porous membrane. Our live imaging of MGE cell migration confirms that 20 hours is sufficient time for interneurons to migrate through the 8 μM pore size membrane. Other protocols typically use between 18 -20 hours to run neurons in the multiwall migration assay (Behar, 2001, Flames et al., 2004).

4.3 Chemokinetic agents. Cells from the GE can undergo unstimulated random locomotion, migrate toward chemoattractant factors, or respond to chemokinetic agents (Wichterle et al., 1999, Powell et al., 2001, Flames et al., 2004). Chemokinetic agents are those that induce increased random movement of cells (Becker, 1977). We discuss the meaning of our results in relation to chemokinetic migration of interneurons.

4.3.1 E15 pH 8.5 fraction is chemokinetic for E15 MGE cells. The strongest migratory activity in our E15 samples was observed for E15 MGE cells

exposed to a gradient of E15 pH 8.5 fraction. We initially could not distinguish between migratory activity caused by chemoattraction or chemokinesis when there was a gradient of proteins in the multiwell migration assay. Although a gradient was present, the increased migration of MGE cells may have been due to contact with chemokinetic agents. Therefore, the increased migration in a gradient of E15 pH 8.5 fraction may have been a result of chemoattraction or chemokinesis. To determine if the response of the E15 MGE cell suspension was due to chemokinesis or chemoattraction we created a chemokinetic assay by abolishing the protein gradient by placing an equal concentration of the pH 8.5 fraction both above and below the membrane. Without a gradient, the pH 8.5 fraction caused significant migration of E15 MGE cells. Ultimately, this raised the likeliness that the E15 cortical pH 8.5 fraction may contain a novel chemokinetic agent, or agents, for E15 MGE cells.

A likely candidate for chemokinesis around this isoelectric point is HGF. HGF is located in the developing cortex and acts as a chemokinetic agent in the migration of interneurons from GE to cerebral cortex (Powell et al., 2001, Powell et al., 2003). HGF also plays an important role as a paracrine agent that mediates wound healing, tissue regeneration, and carcinogenesis (Nakamura, 1991). Although HGF has a theoretical pI of 8.1 (protein ID 170172522, Scripps Protein Calculator v3.3), the MudPIT analysis did not identify it in our cortical pH 8.5 fraction, this reduces the likelihood that HGF is involved with the response of E15 MGE cells to the pH 8.5 fraction.

4.3.2 NRG1 displays chemoattractant and chemokinetic properties

for E15 MGE cells. Using a chemoattractant setup of the multiwell migration assay, we found recombinant NRG1 induced migration of E15 and E17 MGE cells. A chemokinetic setup, without a gradient of NRG1, failed to induce significant migration of E15 MGE cells. Live imaging confirmed this by demonstrating that velocity of E15 MGE cell migration did not significantly increase above the control when exposed to NRG1. Our evidence coincides with the current view that NRG1 is a chemoattractant for MGE-derived neurons (Flames et al., 2004). Although increased cell velocity is a key component of chemokinesis, we cannot rule out NRG1 as a chemokinetic agent for MGE cells. Although exposure to NRG1 to MGE cells did not significantly affect cell movement, the increased amount of migration and velocity of movement suggests NRG1 has some chemokinetic properties for migrating interneurons. These results coincide with the observation of NRG having similar function as the chemokinetic factor HGF (Corbin and Butt, 2011).

NRG1 has an isoelectric point within the pH 8.5 fraction, making it a second candidate for the increased migratory response of GE cells. As have others, we demonstrated NRG1 to be chemoattractant for MGE cells. The migratory response of E15 MGE cells to the pH 8.5 fraction, however, was consistently greater than that from NRG1. This increased response was observed in the multiwell migration assay and live imaging experiments. This suggests that a component in the pH 8.5 fraction may be acting independently or in conjunction with NRG1 to increase GE cell migration.

4.3.3 Gradients and chemokinesis. Although migration of E15 MGE

cells in our assay is not based on a gradient of the pH 8.5 fraction, gradients are important for interneuron migration in the cortex. While the chemotaxic chamber used here isolates specific properties of cortical migration, the nature of *in vitro* experiments may exclude possible elements necessary to properly model tangential migration. For example, the multiwell migration assay lacks extracellular matrix proteins (e.g., chondroitin sulfate proteoglycans) likely involved with maintaining and supporting the formation of gradients important for migration of interneurons (Ramos-Moreno et al., 2006, Ishii and Maeda, 2008). Another extracellular element this assay lacks is the polysialic acid-neural cell adhesion molecule, an important regulator of cell-cell interaction in the nervous system (Durbec and Cremer, 2001) and regulator of neural migration (Seki and Arai, 1993). While not directly implicated in tangential migration of interneurons, the expression of polysialic acid-neural cell adhesion molecule in the intermediate zone around E14-E17 (Jimenez et al., 2002) suggests an important role in the migration of interneurons from the GE. Therefore, extracellular matrix proteins may serve as both permissive/restrictive corridors and for maintenance of fixed gradients.

While testing these agents in isolated *in vitro* environments can provide information on specific ligand-cell interactions, these conditions may also lack *in vivo* components required for proper ligand-environment interaction (Pankonin et al., 2005). Therefore, other assays including matrigel, organotypic cultures, or *in vivo* analysis should also be used to verify the chemoattractant or chemokinetic nature of these factors.

4.3.4 Role of chemokinesis in cortical migration of interneurons.

Along with many other migratory mechanisms, chemokinetic agents facilitate movement of interneurons into the developing cortex (Corbin and Butt, 2011). Permissive corridors confine migration of GE cells while chemoattractant and chemokinetic factors influence the direction and velocity of movement. Permissive corridors are created by the combination of repulsive cues and extracellular matrix. Repulsive factors promote movement out of certain regions and shape the boundaries of migration for interneurons. Slit is a repulsive molecule that is expressed in the ventricular zone of the LGE and initiates subpallial interneuron migration (Zhu et al., 1999). Colocalization of the receptor for Slit, Robo, with migrating calbindin-positive cells in the subpallium suggests that Slit/Robo signaling may be involved with preventing entry of specific interneurons into the striatum (Marin et al., 2001, Marin et al., 2003, Andrews et al., 2006). Slit/Robo interaction also prevents medial entry during tangential movement (Zhu et al., 1999, Andrews et al., 2007). Semaphorin 3a is a chemorepellant that keeps cortical interneurons out of the developing striatum (Marin et al., 2001, Zimmer et al., 2010, Hernandez-Miranda et al., 2011). Together, interneurons are pushed out of the GE en route to the cortical plate (the role of slit), while at the same time repelling them from moving toward the striatum (the role of semaphorin 3a). Extracellular matrix factors provide an environment permissive to migrating cells. Polysialic acid neural cell adhesion molecule is expressed along the migratory route of interneurons (Jimenez et al., 2002). The extracellular matrix can also participate in the formation and

maintenance of chemical gradients by binding diffusible factors and changing their activity. Laminin, matrigel, and heparin sulfate proteoglycans bind to and increase the activity of NRG1 (Li and Loeb, 2001, Miralem and Avraham, 2003). In addition to binding semaphorin 3a, chondroitin sulfate proteoglycans act as a repulsive factor for migrating cortical interneurons (Zimmer et al., 2010). Together the repulsive cues and extracellular matrix proteins confine and direct interneuron migration.

Chemoattractants for cortically migrating interneurons include the neurotrophins; the growth factors NRG1 and GDNF, the chemokine, SDF-1 and neurotransmitters, glutamate, GABA, and dopamine (Stumm et al., 2003, Flames et al., 2004, Pozas and Ibanez, 2005, Represa and Ben-Ari, 2005, Manent et al., 2006, Crandall et al., 2007, Sanchez-Alcaniz et al., 2011, Wang et al., 2011). Also see Corbin and Butt (2011) for a recent and comprehensive review.

Chemokinetic factors are important for tangential migration of cortical interneurons, with HGF as the most investigated example (Powell et al., 2001, Powell et al., 2003). Considering the different effects HGF may have on specific interneuron subpopulations (Corbin and Butt, 2011), HGF may participate as a guidance cue for tangentially migrating interneurons. In conjunction with other guidance mechanisms for interneuron migration, chemokinetic activity from novel factor(s) with a pI around 8.5 deserves further investigation.

4.4 Chemorepulsive agents. When cells respond to a factor by moving away from an area of higher concentration, then the factor is considered a

chemorepulsive agent (Becker, 1977). We discuss our findings that suggest E17 cortex contains repulsive agents for E17 MGE cells.

4.4.1 E17 pH 3.0 fraction is chemorepulsive for E17 MGE cells. The E17 pH 3.0 fraction has apparent chemorepellant effects on E17 MGE cells. Using the 96-well migration assay, we found significantly reduced E17 MGE migration in the presence of E17 pH 3.0 fraction. Although this suggests chemorepulsive effects, further analysis will be needed to resolve this interaction. A way to test for chemorepulsive effects is to switch the gradient in our multiwell migration assay. In this setup, chemorepulsion would cause an increased migration of cells to the lower well. Semaphorin 3a is the most studied chemorepellant signal for GE cells migrating to the cortex, as it prevents striatal invasion via neuropilin signaling (Marin et al., 2001). We estimate semaphorin 3a has a pI of 7.41 (protein ID 31543681, Scripps Protein calculator v. 3.3), therefore it was likely not a factor in the response of MGE cells to pH 3.0 fraction. Of the investigated ages (E14, 15, and 17), only E17 MGE cells showed a chemorepulsive response. This underlies the temporal complexity of cortical development as demonstrated by Britto et al. (2006). By switching the neocortex of E12.5 mouse organotypic slice cultures with E13.5 neocortex, Britto et al. showed that interneurons from the MGE and LGE switch from being repelled by E12.5 medial neocortex to being attracted by E13.5 medial neocortex. This showed that permissive and repulsive cues of the neocortex can switch in just 24 hours. Ultimately, a novel chemorepellant(s) for E17 MGE cells may be present in the 3.0 pI range of E17 cortex, which substantiates further investigation.

4.5 Neurotransmitters. Current understanding holds that cortical interneuron migration may require excitatory neurotransmitters for guidance (Hernandez-Miranda et al., 2010). We used glutamate as an example of a neurotransmitter that may affect migration of MGE cells in our multiwell migration assays.

4.5.1 Glutamate significantly influences E15 and E17 MGE cell migration. We found an increased migration of E15 and E17 MGE cells in response to a gradient of glutamate. Glutamatergic receptors may be important for tangentially migrating interneurons in the cortex (Manent and Represa, 2007). Migrating cortical interneurons have functional AMPA receptors, while existence of functional NMDA receptors on these cells remains controversial (Soria and Valdeolmillos, 2002, Manent and Represa, 2007). The AMPA receptors on interneurons lack the GluR2 subunit, thereby making these cells calcium permeable (Metin et al., 2000) and more likely to transduce a signal affecting migration. Interestingly, the activation of AMPA receptors by glutamate causes release of GABA from tangentially migrating neurons, possibly leading to a chemoattraction feedback mechanism of tangentially migrating GE cells (Poluch and Konig, 2002, Manent and Represa, 2007). GABA regulates migration of MGE cells once they move passed the corticostriate junction and tangentially within the cortex (Cuzon et al., 2006). The cells in the MGE and those that exit the MGE express different $GABA_A$ subunits, which partially accounts for the increased potency and affinity of GABA as MGE cells advance into the cortex (Cuzon Carlson and Yeh, 2010). Having shown that our cultures express GABA

and knowing glutamate can cause GABA release via AMPA activation (Poluch and Konig, 2002), it is possible that indirect release of GABA from our cell cultures was the underlying cause for increased migration in the presence of glutamate. The complex interaction of glutamate and GABA on migrating interneurons may be central to guidance of GE cells to their proper location in the developing cortex. Further investigation is needed to determine if glutamate acts as a chemokinetic or chemoattractant for E15 MGE cells.

4.6 MudPIT. The MudPIT analysis identified at least four proteins that may directly or indirectly influence interneuron cortical migration; Neuromedin-B, TUC-4b, unc-5 homolog C, and cadherin EGF LAG seven-pass G-type receptor 2. These four proteins all have an effect on neuronal migration. (i) TUC-4b is strongly expressed along interneuron migratory routes in rat embryonic cortex and regulates neurite outgrowth (Quinn et al., 2003). GABAergic interneurons generated in the adult neocortex strongly express TUC-4, also called CRMP4 (Dayer et al., 2005). CRMP4 inhibits neurite outgrowth by interacting with RhoA in a NOGO-dependent manner to regulate F-actin bundling (Luo et al., 1993, Rosslenbroich et al., 2005, Alabed et al., 2007). Since CRMP4 is part of a family that is involved in collapsing/semaphorin 3 signaling (Goshima et al., 1995, Lee et al., 2002) and interacts with chondroitin sulfate (Franken et al., 2003), it is likely to have an important role in regulating cortical migration of interneurons. (ii) Neuromedin B depolarizes hippocampal interneurons (Lee et al., 1999). Although it is not definitively known whether Neuromedin-B causes depolarization of MGE

cells en route to the neocortex, release of GABA does influences tangential migration of interneurons. Cuzon et al. (2006) showed $GABA_A$ receptor signaling is required for cortical entry of MGE cells and Lopez-Bendito et al. (2003) demonstrated the importance of $GABA_B$ receptor signaling for tangential migration within the cortex (Lopez-Bendito et al., 2003). The changing characterization of the $GABA_A$ subunit expression and electrophysiological properties in migrating MGE cells reveals a dynamic expression pattern that may allow Neuromedin-B to modify tangential migration (Cuzon Carlson and Yeh, 2010). (iii) Unc-5 homolog C is a receptor for netrin-1 (Kennedy, 2000, Kruger et al., 2004), and netrin-1 promotes guidance of interneurons in the developing cortex (Stanco et al., 2009). Netrin is expressed in the GE (Metin et al., 1997) and Unc-5 interacts with netrin-1 to promote axonal repulsion (Hong et al., 1999). Therefore, if Unc-5 is expressed on migrating interneurons then netrin signaling may promote interneuron migration out of the GE. (iv) Cadherin EGF LAG seven-pass G-type receptor 2 expression is related to lipoprotein regulation (Talmud et al., 2009) and may indirectly affect reelin signaling through apolipoprotein E receptor 2 (Hiesberger et al., 1999, Trommsdorff et al., 1999). Cadherins bind the cadherin EGF LAG seven-pass G-type receptor 2 receptor and the EGF motif interacts with Rac1 to regulate cell-to-cell contact (Ray et al., 2007) and cell migration (Katso et al., 2006). While expression of the receptor may remain constant, different forms of cadherin can specify spatiotemporal signals in the developing cortex (Wu and Maniatis, 1999). Also, the EGF domain of this protein may be similar to the EGF-like motif of NRG (Falls, 2003). Although this is a

receptor, characterizing its expression on tangentially migrating cells would help us understand the role of cadherin signaling on migrating interneurons.

Further investigation can provide better understanding for the role of Neuromedin-B, TUC-4b, unc-5 homolog C, and cadherin EGF LAG seven-pass G-type receptor 2 in migration of cortical interneurons. Deficient or faulty tangential migration may be observed in mutant mice or when blocking the receptor for these proteins. Although we look for cortical proteins, our fractions may contain special sugars, fats or nucleic products that influence cell migration. To cross-check for the presence of specific analytes, namely the four listed above, MudPIT should be repeated on an independent sample of pH 8.5 fraction.

The unbiased nature of MudPIT allows for equal sensitivity in detecting proteins of low abundance, integral membrane proteins, or proteins of extreme hydrophobicity, pI or molecular weight (Washburn et al., 2001). We demonstrate the unbiased nature of MudPIT analysis by detecting cadherin EGF LAG seven-pass G-type receptor 2 receptor, an integral membrane protein. Our MudPIT results include a wide range of molecular weight proteins. Proteins having a molecular weight around 10 kDa to over 200 kDa were identified by MudPIT. Sensitivity is optimized by interfacing the protein fractionation with mass spectrometry (Washburn et al., 2001). The sensitivity of MudPIT analysis does not explain the lack of predicted proteins in our isoelectric sample.

Our MudPIT results did not include specific cortical proteins that have a predicted pI within the range of our tested sample. The E15 pH 8.5 fraction theoretically contains proteins with a pI greater than and around 8.5, but MudPIT

did not detect the presence of NRG or GDNF. NRG and GDNF have predicted pI's of 8.3 and 9.0, respectively (protein IDs 123793119 and 20269903, Scripps Protein Calculator v3.3). Knowing the sensitivity of MudPIT, NRG and GDNF may not have been present in the E15 pH 8.5 fraction. Therefore, isoelectric focusing may not have separated proteins into theoretically predicted fractions. Possibly, posttranslational modifications could have altered the charge of proteins in our sample before isoelectric fractionation, ultimately changing the pI of these proteins. Therefore, postranslational modifications may cause proteins to focus into fractions with a different pI than that predicted from an unmodified protein sequence. We may conclude that predicted proteins have unpredictable pI's, causing them to focus into unpredictable isoelectric fractions. Although we may not be able to predict the proteins that exist in each fraction from isoelectric focusing, we are able to separate the proteome into predictable and reproducible quanta.

4.7 Summary. We started by successfully verifying the separation and reproducibility of cortical protein fractionation from the sIEF procedure used to preserve protein function. Next, we successfully verified the immunological and morphological characteristics of cells used in our culture assay. We determined that twenty hours of incubation with the isoelectric fractions does not significantly affect percent viability or percent of cells expressing GABA in our culture. We successfully validated the presence of a dynamic protein gradient in the multiwell migration assay. We determined that smaller concentration differences

equilibrate is less time, with the lowest measurable concentration difference maintaining a gradient up to 20 hours.

We used multiwell migration assays to determine if isochronic GE cells migrate significantly in response to a gradient of each cortical isoelectric fraction. We found a gradient of E14 pH 5.5 and 8.0 fractions significantly increases E17 GE cell migration, E15 pH 8.5 significantly increases E15 MGE cell migration, and E17 pH 3.0 significantly reduces cell migration of E17 MGE cells. In testing positive controls we found both glutamate and NRG1 significantly enhance migration of E15 and E17 MGE cells. Next, we next used the multiwell migration assay to determine if the migratory activity for pH 8.5 fraction and NRG1 was chemoattractive or chemokinetic in nature. By eliminating a concentration gradient, we determined that MGE cells respond to pH 8.5 fraction as a chemokinetic agent and NRG1 as a chemoattractant with some chemokinetic properties. We used live imaging of MGE explants incubated with pH 8.5 fraction or NRG1 to further understand the relationship of these factors on migration of MGE cells. By comparing the average velocity of cell migration for each condition to the control, we again observed that MGE cells responded to the pH 8.5 fraction and NRG1 as a chemokinetic and chemoattractant agent, respectively. We processed the E15 pH 8.5 fraction to determine which proteins may be contained in this chemokinetic cortical sample. MudPIT analysis identified at least four proteins that may be involved with interneuron cortical migration; Neuromedin-B, TUC-4b, unc-5 homolog C, and cadherin EGF LAG seven-pass G-type receptor 2. Each of these identified factors and signaling pathways

warrant further investigation to help us understand how the cortical proteins of pH 8.5 fraction influences tangential interneuron migration in the developing cortex.

While the activity of isoelectric fractions appears age dependent, NRG1 and glutamate remained consistently active for inducing E15 and E17 MGE cell migration. The chemokinetic activity of E15 pH 8.5 appears independent from the chemoattractant behavior seen for NRG1. Therefore, the E15 cortex may contain novel factor(s) around the 8.5 isoelectric range that are chemokinetic for E15 MGE cells. Lastly, E17 cortex may contain novel factor(s) around the 3.0 isoelectric range that are chemorepulsive for E15 MGE cells.

The complex assignment of interneuron subtypes to specific layers and locations within the developing cortex requires an equally complex interplay of diffusible factors and permissive corridors. Our study looked at the most prominent migration of GE cells in response to factors from the developing cortex. The response to factors in the cortex is likely due to the modifiable combination of receptors expressed on individual interneurons. Thus, dynamic receptor expression on migrating interneurons can quickly modify cellular responses to diffusible cortical signals.

Our most substantial findings demonstrate that response of GE cells to cortical proteins may dramatically change during discrete stages of cortical development. We also demonstrate that the E15 cortical proteins responsible for significantly affecting E15 MGE cell migration appear to be chemokinetic in nature. One predicted role of chemokinetic agents at this stage of development is to drive interneurons deep into the cortex. The candidates we identified to be

involved with the chemokinetic migration of MGE cells are appealing and substantiate further investigation.

APPENDIX A: CONFIRMING EMBRYONIC AGE USING CROWN-RUMP LENGTH (CRL)

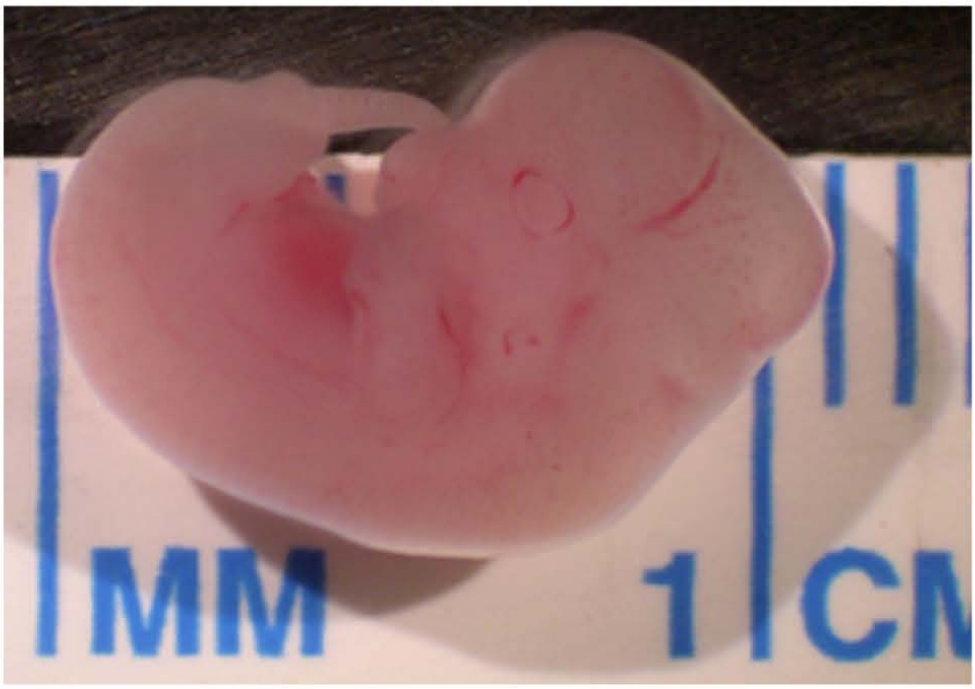
Purpose: Compare CRL of the E14, E15 and E17 embryos used in our studies to that stated in the literature.

Reasoning: The accurate determination of embryonic age is important, especially in the context of results specific to stages of embryonic development. As embryonic dating systems can vary, we confirmed embryonic age by measuring CRL (Torres et al., 2008). The accuracy of our stated embryonic age was determined by how well the CRL from our dissections corresponded with that in the literature.

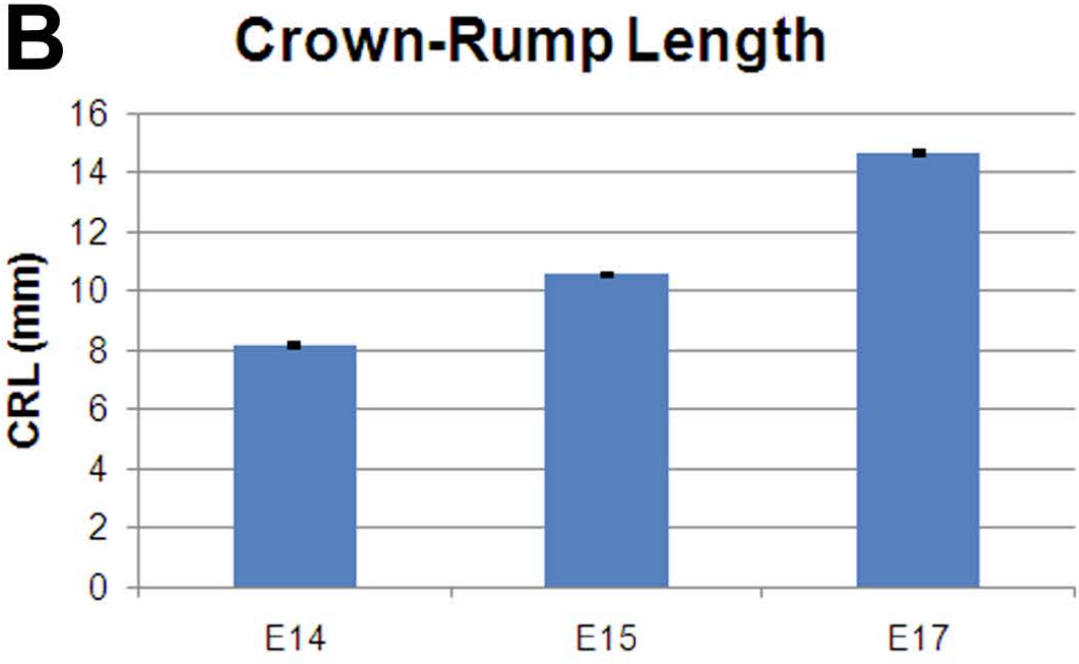
Methods: Immediately following removal of embryos from the uterine horn, CRL was measured in a 150mm petri dish containing ice-cold aCSF. We used a Nikon SMZ-U dissecting microscope with 7.5x magnification to determine CRL of each embryo using a metric scale ruler placed under the petri dish (**Figure 16A**).

Figure 16. CRL was measured from freshly dissected embryos. (A) Photograph demonstrating the measurement technique we used to determine embryonic CRL. This is an E15 Sprague-Dawley rat having a CRL of 11 cm. (B) Graph of CRL for E14, E15 and E17 rats. Error bars are SEM.

A



B



Results: We measured CRL in 61 E14 rats, 385 E15 rats and 158 E17 rats. The average CRL was 8.19 mm for E14, 10.56 mm for E15 and 14.67 mm in E17 (**Figure 16B**).

Conclusion: Correcting for their use of E0 dating system, our average CRL for each embryonic stage was very similar to those determined by Torres et al. (2008). The difference in CRL between what we determined and Torres et al. (2008) is 0.12 mm for E14 rats, 0.04 mm for E15 rats and 0.86 mm for E17 rats. Therefore our stated embryonic age, verified by CRL, is accurate as it corresponds to previously publish parameters.

APPENDIX B: VALIDATION OF PROTEIN SEPERATION BY sIEF

Purpose: Confirm our sIEF procedure leads to proper isoelectric separation of proteins.

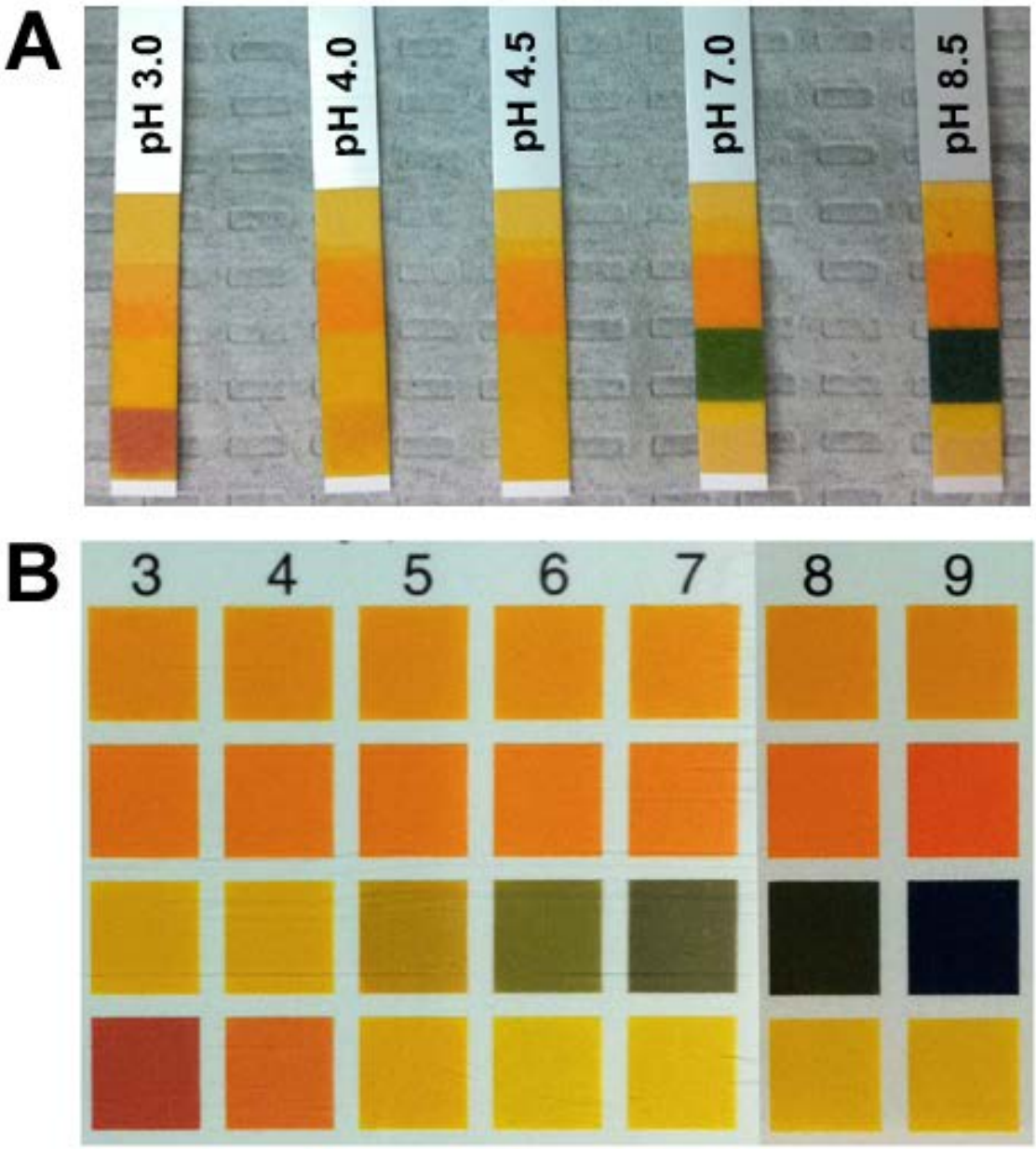
Reasoning: Having performed sIEF with slight modifications in order to preserve protein activity, we needed to confirm proteins were isoelectrically separated.

Methods: Test pH of each fraction using pH indicator strips (see the Methods).

Results: Isoelectric focusing was successful as determined by pH indicator strips having different color profiles for different fractions from the same focusing procedure (**Figure 17**). Isoelectric separation did occur, as determined by differences in protein profiles of fractions across the isoelectric range (**see results, Figure 1**). Of note, the pH of fractions vary slightly between sIEF processes, therefore it was important for us to group results by pH, rather than by fraction number.

Conclusion: The procedure we used for sIEF successfully separated proteins by pI.

Figure 17. We used pH indicator strips to find pH of fractions and determine success of isoelectric fractionation. (A) Photo of pH-indicator strips wetted with fractions 1-5 from left to right, from a single isoelectric focusing. (B) The pH was determined by comparing pH strips to the manufacturer's colorimetric chart. The pH for each fraction is different and increases in respect to fractions collected from the anode (left strip) to cathode (right strip) end of the fractionator.


A
pH 3.0
pH 4.0
pH 4.5
pH 7.0
pH 8.5
B
3
4
5
6
7
8
9

APPENDIX C: VALIDATING TRANSFORMATION OF 96-WELL MIGRATION DATA

Purpose: Remove noise caused by consistent change in fluorescence plate readings based on location in the 96-well migration assay.

Reasoning: We observed differences in the average fluorescent values for different columns of the 96-well migration assay even when the test conditions were the same. Therefore, we transformed the data to correct for this variability.

Procedure:

Obtaining correction values:

1. We performed two 96-well migration assays as per the Methods. We placed plain NB media in all lower wells loaded E15 MGE cells in the upper wells. After culturing for 20 hours, fluorescent values were collected as indicated in the Methods. Values for each well were averaged across the two runs. Lastly, we determined a correction value for each well by dividing the average of all wells by the average fluorescent values of each well.

Verify Effectiveness of correction:

1. Transform data from two randomly picked and independent 96-well migration assays. Transformation was accomplished by dividing the average of each well from the two experiments by the correction

values determined above.

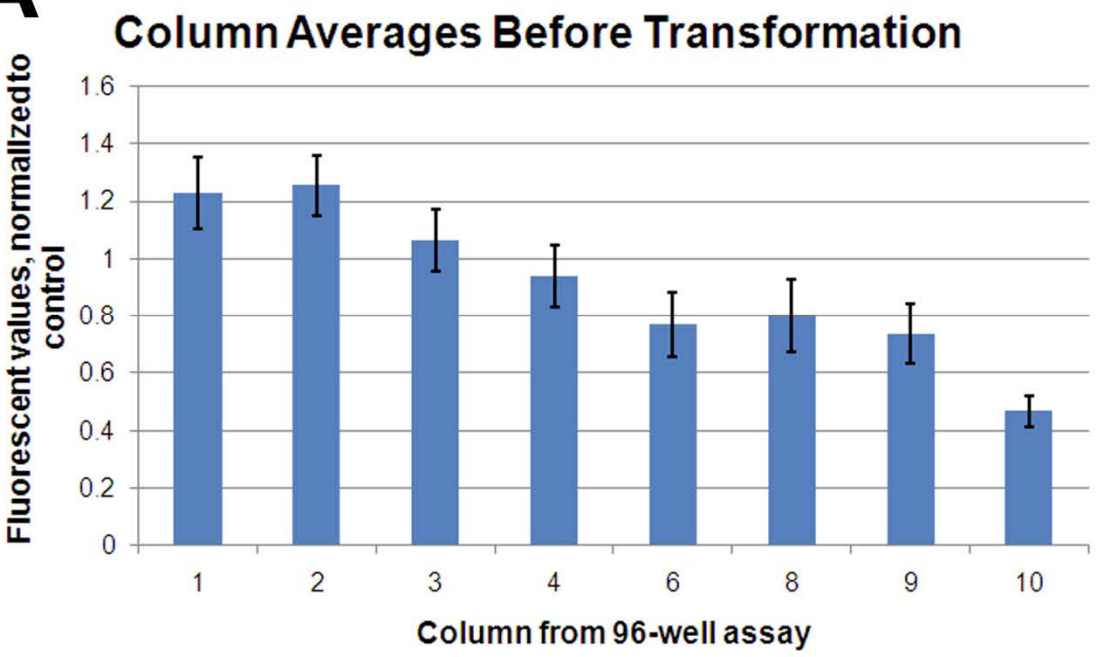
2. Analyze by averaging transformed data from each column and running separate one-way ANOVAs for original and transformed data.

Results: Significant differences exist between average fluorescent values within columns of 96-well migration assay before normalization (**Figure 18A**). After transformation, average column values are not significantly different (**Figure 18B**).

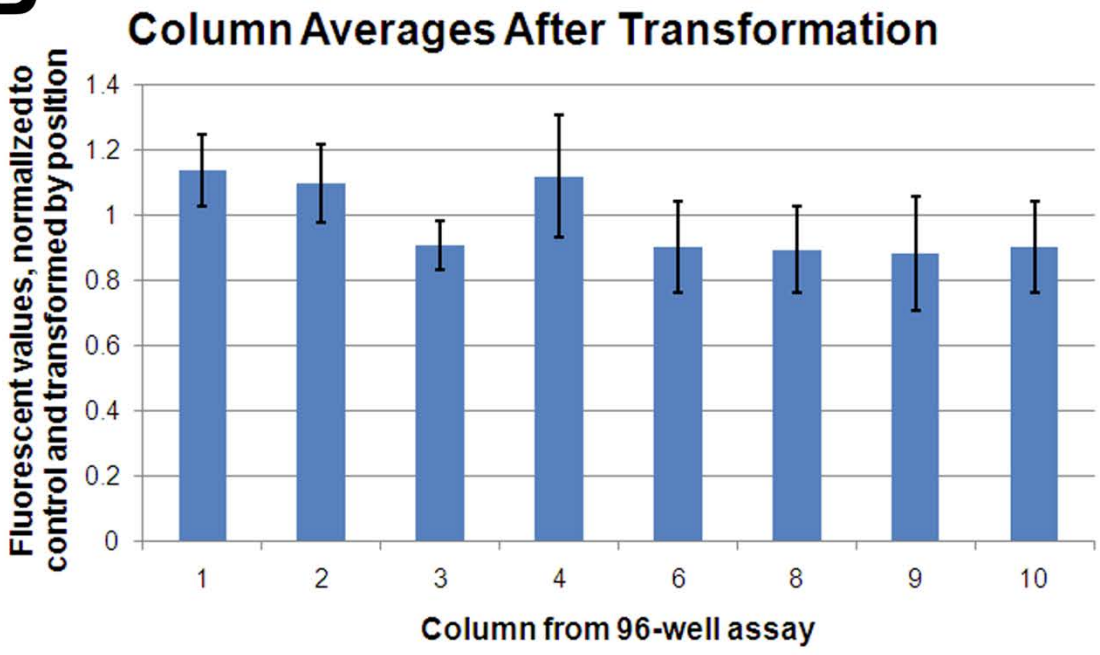
Conclusion: Transformation with our correction values removes noise caused by a consistent change in fluorescence plate readings based on location in the 96-well migration assay, leading to results with better reproducibility.

Figure 18. Graph of average fluorescence readings from each column of the 96-well migration assay before (A) and after transformation (B) to account for positional in the assay. Significant differences exist among the averages of original data within columns. After transformation no significant difference exists among the average data between columns. One-way ANOVA used to test significance among all conditions with significant p-value set at 0.05.

A


Column Averages Before Transformation
Fluorescent values, normallzed to control
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
6
8
9
10
Column from 96-well assay

B


Column Averages After Transformation
Fluorescent values, normallzed to control and transformed by posltion
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
6
8
9
10
Column from 96-well assay

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