THE ROLE OF α3β1 INTEGRIN IN CORTICAL DEVELOPMENT

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ABSTRACT

The Role of $\alpha_3\beta_1$ Integrin in Cortical Development
“(Under the direction of Eva S. Anton)”

Normal development of the mammalian cerebral cortex requires the coordinated migration of postmitotic neurons from the proliferative ventricular zone to the developing cortical plate. Of the two main classes of neurons in the cerebral cortex, glutamatergic long projection neurons arise from the ventricular zone of the dorsal telencephalon and migrate radially into the cortical plate. In contrast, GABAergic interneurons derive primarily from the medial ganglionic eminence in the ventral telencephalon, migrate tangentially into the cortex, and subsequently enter the cortical plate radially using the radial glial scaffold. Abnormalities in neuronal migration and layer formation lead to abnormal placement and connectivity of cortical neurons, an underlying cause of many congenital brain disorders such as epilepsy, microencephaly (small brain), schizencephaly (split brain hemispheres), lissencephaly (smooth cerebrum, without convolutions), macrogyria (large convolutions), polymicrogyria (small cerebral convolutions), and tuberous sclerosis. The aim of this dissertation is to elucidate the role of $\alpha_3$ integrin in the migration and differentiation of distinct classes of cortical neurons using conditional $\alpha_3$ null mouse models. We find that netrin1-$\alpha_3\beta_1$ integrin interactions promote and maintain the migration of GABAergic interneurons through the cortical marginal zone and, hence, are required for the proper integration of cortical interneurons into the postnatal cerebral cortex. In addition, the final
differentiation of pyramidal neurons and interneurons is dependent on α3β1 integrin signaling during cortical neuronal migration.
This dissertation is dedicated to Paride, Vera, Joey, Carmen, and Lee.
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<tbody>
<tr>
<td>AEP</td>
<td>Anterior entopeduncular area</td>
</tr>
<tr>
<td>ApoER2</td>
<td>Apolipoprotein E receptor type 2</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>CP</td>
<td>Cortical plate</td>
</tr>
<tr>
<td>DCC</td>
<td>Deleted in colorectal cancer</td>
</tr>
<tr>
<td>DiI</td>
<td>1,1'-dioctodecyl-3,3,3', 3'-tetramethylindocarbocyanine</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
</tr>
<tr>
<td>GE</td>
<td>Ganglionic eminence</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>Itgα3</td>
<td>α3 integrin</td>
</tr>
<tr>
<td>IZ</td>
<td>Intermediate zone</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
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<tr>
<td>LGE</td>
<td>Lateral ganglionic eminence</td>
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<td>MGE</td>
<td>Medial ganglionic eminence</td>
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<tr>
<td>MZ</td>
<td>Marginal zone</td>
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<tr>
<td>NJPA1</td>
<td>Neuron-glial junctional protein 1</td>
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<td>Semaphorin 3A</td>
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<tr>
<td>Sema3F</td>
<td>Semaphorin 3F</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinase</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
<tr>
<td>u-PAR</td>
<td>Urokinase-type plasminogen activator receptor</td>
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<tr>
<td>VLDLR</td>
<td>Very low-density lipoprotein receptor</td>
</tr>
<tr>
<td>VZ</td>
<td>Ventricular zone</td>
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CHAPTER 1
INTRODUCTION

Neuronal Migration and Cortical Layer Formation.

Construction of the six-layered cerebral cortex and establishment of the functional connectivity of neural networks within the cortex requires the long distance migration of neurons from their origins in proliferative zones of the telencephalon to their final position in the developing cerebral wall as well as appropriate neuronal differentiation. The cortex is primarily comprised of glutamatergic projection neurons and GABAergic interneurons, though the origins and modes of migration of these neuronal populations are distinct. Glutamatergic projection neurons arise from the ventricular zone of the dorsal telencephalon and migrate radially toward the surface of the brain to reach their final destinations within the cerebral wall, while GABAergic interneurons originate predominantly from the ventricular zone of the ganglionic eminence (GE) in the ventral telencephalon and migrate tangentially through the cortical marginal zone (MZ), subplate and lower intermediate/subventricular zones (IZ/SVZ) prior to entering the cortical plate (CP) using radial migration.

Cortical layer formation begins with the radial migration of postmitotic cells from the cortical ventricular zone, a proliferative region lining the lateral ventricles of the dorsal telencephalon. These cells move towards the pial surface to form a neuronal layer known as the preplate. Next, another cohort of postmitotic neurons migrate from the ventricular zone and through the subventricular and intermediate zones, splitting the preplate into a superficial
marginal zone layer (layer I, postnatally) and a deeper subplate. Henceforth, the neuronal layers established between the marginal zone and subplate (layers II-VI, postnatally) are known collectively as the cortical plate. As development proceeds, each successively generated cohort of postmitotic neurons moves radially outwards to form a neuronal layer directly beneath the marginal zone. In this manner, early-born neurons constitute the deep layers of the cortical plate, while later-born neurons comprise the more superficial layers.

Two forms of radial migration, termed somal translocation and locomotion, exist (Nadarajah et al., 2001; Nadarajah et al., 2002; Nadarajah and Parnavelas et al., 2002). During early stages of cortical development, while the cerebral wall is thin, neurons utilize somal translocation to generate and split the preplate. Cells within the ventricular zone of the dorsal telencephalon extend and attach a long leading process to the pial surface and migrate continuously towards the pia by shortening their leading process (Brittis et al., 1995; Miyata et al., 2001; Morest, 1970; Nadarajah et al., 2001). As the cerebral wall expands, locomotion becomes the predominant form of radial migration. The locomotion of cortical neurons depends on close interaction with the processes of radial glial cells, which extend from the ventricular zone into the pial basement membrane. The pial basement area is rich in extracellular matrix (ECM) components, including collagen IV, collagen XVIII, nidogen, perlecan, agrin, and laminin isoforms (Timpl and Brown, 1996; Erickson and Couchman, 2000). These radial glial processes provide a substrate for the locomotion of cortical neurons. The locomotion is characterized by brief, rapid movements followed by long, stationary periods (Nadarajah et al., 2001). Somal translocation is used as cortical neurons reach the top of the cerebral cortex (Nadarajah et al., 2001).
In contrast to the radial migration of glutamatergic projection neurons, GABAergic interneurons migrate tangentially, orthogonal to the radial glial scaffold, into the emerging cerebral wall (Anderson et al., 1997; Letinic and Rakic, 2001; Maricich et al., 2001; Tamamaki et al., 1997; Wichterle et al., 2001). Evidence for the tangential migration of cortical neurons was provided by observations of a tangential dispersion of clonally related cortical neurons (O’Rourke et al., 1992; Walsh and Cepko, 1992; Fishell et al., 1993; Tan and Breen, 1993; O’Rourke et al., 1995; Tan et al., 1995; de Carlos et al., 1996). In addition, the virtual absence of GABAergic interneurons from the neocortex of mice deficient in Dlx1/2, homeobox transcription factors expressed in the ventricular and subventricular zones of the ganglionic eminence, revealed that cortical interneurons originate and migrate from the ganglionic eminence of the ventral telencephalon into the developing cerebral cortex (Anderson et al., 1997). The ganglionic eminence can be divided into medial, lateral, and caudal subdivisions based on differential transcription factor expression patterns (Anderson et al., 1999; Marin et al., 2000; Nery et al., 2002; Nery et al., 2003). Of these regions, the ventricular zone of the medial ganglionic eminence (MGE) produces the majority of cortical GABAergic interneurons as has been demonstrated by 1,1'-dioctodecyl-3,3,3', 3'-tetramethylindocarbocyanine (DiI) tracing and GE transplantation experiments (Lavdas et al., 1999; Anderson et al., 2001; Wichterle et al., 2001).

Although the medial ganglionic eminence is the primary source of cortical GABAergic interneurons, cells tangentially migrating into the cortex also originate from the lateral ganglionic eminence (LGE) and anterior entopeduncular area (AEP) (Anderson et al., 2001; Cobos et al., 2001; Wichterle et al., 2001). In the ventral telencephalon, GABAergic interneurons, prevented from entering the developing striatum by repulsive
guidance cues, migrate superficial or deep to the striatum prior to invading the cerebral wall (Marin et al., 2001). During early stages of cortical development (mouse embryonic day 11.5, E11.5), GABAergic interneurons principally arise from the MGE and AEP, migrating through the cortical marginal zone and subplate (Lavdas et al., 1999; Anderson et al., 2001; Marin et al., 2001). At mid-embryonic stages (E12.5-E14.5), the majority of tangentially migrating cells derive from the MGE, traversing the subplate and lower intermediate zone/subventricular zone (Lavdas et al., 1999; Marin et al., 2001; Wichterle et al., 2001; Anderson et al., 2001). At late stages of cortical development (E14.5-E16.5), the LGE and MGE are the principal sources of cortical GABAergic interneurons (Anderson et al., 2001).

Whereas glutamatergic projection neurons use radial glial fibers as a substratum for migration, the substrate utilized for tangential migration remains unresolved. The close interaction of interneurons migrating tangentially through the intermediate zone with corticofugal axons suggests that GABAergic interneurons may use corticofugal axons as a substrate for migration (O’Rourke et al., 1995; Metin and Godement, 1996; Metin et al., 2000; Denaxa et al., 2001). Moreover, blocking the function of TAG-1, a neural adhesion molecule expressed on corticofugal axons, decreases the number of interneurons migrating into the cortex of embryonic slices in culture (Denaxa et al., 2001). However, the migration patterns, number, and morphology of GABAergic interneurons are unaffected in Tag-1 deficient mice (Denaxa et al., 2005). Furthermore, a substantial number of interneurons migrate through axon-sparse cortical regions, including the lower intermediate zone and subventricular zone (Marin and Rubenstein, 2003).
Adhesive Interactions During Neuronal Migration.

Specific adhesive interactions between neurons, glia, and the surrounding extracellular matrix (ECM) are critical for the appropriate migration and placement of cortical neurons. Astrotactin, neuron-glial junctional protein 1 (NJPA1), gap junctions, and integrin receptors have been shown to regulate adhesive interactions between migrating neurons and radial glial cells. Astrotactin, a glycoprotein expressed by migrating cortical and cerebellar neurons, mediates adhesive interactions between migrating granule cells and cerebellar glia. Inhibiting the function of astrotactin in vitro and in vivo reduces neuron-glial association and decreases the rate of granule cell migration along glial fibers (Edmondson et al., 1998; Adams et al., 2002). NJPA1, a radial glial membrane protein situated at the junction between migrating cortical neurons and radial glial fibers also regulates neuron-glial adhesion and migration. The application of antibodies against NJPA1 reduces neuron-glial interactions and the rate of neuronal migration on glial substrates (Anton et al., 1996). Surprisingly, gap junctions, which form an aqueous pore between the cytoplasm of two adjacent cells for cell-cell communication, also serve an adhesive function during neuronal migration along radial glial fibers. Gap junction subunits, connexin 26 and connexin 43, are expressed in both migrating neurons and along radial glial fibers at contact points between these cells (Elias et al., 2007). During migration, gap junction adhesion sites promote stabilization of the neuronal leading process along radial fibers and subsequent nuclear translocation (Elias et al., 2007).
Integrins Regulate the Adhesion and Migration of Cortical Neurons.

Integrins are heterodimeric cell surface ECM receptors that serve as structural links between extracellular ligands and the internal cytoskeleton (Hynes, 2002). 18 α and 8 β integrin subunits, non-covalently associated single transmembrane glycoproteins, have been identified. These subunits can combine to form 24 distinct heterodimeric integrin receptors. With the exception of the large vertebrate β4 integrin cytoplasmic tail, both subunits have short cytoplasmic tails, between 20-70 amino acid residues, and large extracellular domains of several hundred amino acids in length (Hynes, 1992). The extracellular domains of both the α and β subunits are required for binding to ECM molecules (Xiong et al., 2002), whereas the β integrin cytoplasmic tail interacts with actin-binding proteins, establishing a mechanical link to the cytoskeleton (Calderwood et al., 2000; Critchley, 2000). While integrins primarily bind to components of the ECM, they also interact with other transmembrane receptor systems, thereby regulating a wide range of cellular processes including adhesion, migration, proliferation, survival and differentiation (Giancotti and Ruoslahti, 1999; Miranti and Brugge, 2002).

The activity of integrin receptors is regulated by bidirectional signaling across the plasma membrane, referred to as inside-out and outside-in signaling. In their quiescent state, integrins bind ligands with low affinity. Inside-out signaling provides an intracellular stimulus to begin the activation process, whereby talin, a cytoskeletal protein, undergoes a conformational change to expose its head domain and binds to the cytoplasmic tail of the β integrin subunit (Calderwood, 1999). Interaction of talin with the cytoplasmic tail of the β integrin subunit, unclips the C-terminal domains of the α and β subunits and extends the extracellular domains. This conversion of the extracellular domains from a bent to an
extended conformation, allows for high affinity ligand binding (Xiong et al., 2001; Vinogradova et al., 2002; Tadokoro et al., 2003; Vinogradova et al., 2004; Qin et al., 2004; Tanentzapf and Brown, 2006; Wegener et al., 2007). Subsequently, the transmembrane domains of the activated receptors may undergo homotypic oligomerization, in which each α or β subunit self-associates, resulting in integrin receptor clustering. Outside-in signaling is initiated upon ligand binding and integrin receptor clustering, leading to a separation of α and β subunit transmembrane domains, thereby recruiting and activating signal transduction proteins linked to the actin cytoskeleton (Miranti and Brugge, 2002; Zhu et al., 2007). This permits integrins to form focal adhesions, stable connections to the extracellular matrix mediated by the association of integrins and actin stress fibers through intermediate adaptor proteins, and allows integrins to modulate cellular processes such as migration.

Integrin receptor subunit expression patterns in the developing cortex demonstrate the relevance of integrins to cerebral cortical development. β1 and β5 integrin as well as α1 and α3 integrins are expressed across the developing cerebral cortex (Cousin et al., 1997; Gardner et al., 1999; Dulabon et al., 2000). αv integrin is expressed by radial glial fibers of the developing cerebral wall, while α6 integrin is expressed in the ventricular zone and cortical plate of the developing cerebral cortex (Hirsch et al., 1994; Georges-Labouesse et al., 1998; Anton et al., 1999). In addition, α8 integrin expression is concentrated on the spines and postsynaptic densities of dendritic processes in layer V of the cerebral cortex by embryonic day 16 (Einheber et al., 1996). Developmental changes in cell surface integrin expression alter the adhesive interactions and the ligand preferences of developing neurons, thereby regulating neuronal cell migration and placement in the developing cerebral cortex.
The relevance of integrins to cortical development is exemplified by the phenotypes of integrin deficient mice. Mice deficient in \( \alpha_v, \alpha_3, \alpha_6, \beta_1, \) and \( \beta_4 \) integrins exhibit distinct cortical malformations. \( \alpha_v \) null mice, 80% of which die at E11.5 and the rest at birth, display disrupted cellular organization in the cerebral wall and lateral ganglionic eminence (LGE) (Bader et al., 1998). Conditional deletion of \( \alpha_v \) integrin in neurons and glia leads to motor deficits due to axonal degeneration in the spinal cord and cerebellum as well as cerebral hemorrhage (McCarty et al., 2005). \( \alpha_6 \) deficient mice die at birth with aberrant laminar organization of the cerebral cortex in which ectopic neuroblasts extend out of the pial surface and produce wavy neurite outgrowth within the cortical plate (Georges-Labouesse et al., 1998). Mice deficient in \( \beta_4 \) integrin, which only associates with \( \alpha_6 \), exhibit the same cortical phenotype (Murgia et al., 1998). Although \( \beta_1 \) integrin deficient mice die at E5.5, conditional deletion of \( \beta_1 \) integrin in neurons and glia results in radial glia endfeet and pial basement membrane defects as well as disrupted cortical laminar organization (Graus-Porta et al., 2001).

\( \alpha_3 \beta_1 \) is a major integrin expressed by neurons in the developing cortex (Anton et al., 1999). Outside the nervous system, \( \alpha_3 \beta_1 \) is primarily expressed on the basal surface of epithelial cells. Although early work on this integrin demonstrated binding to fibronectin, laminin, entactin/nidogen, and collagen (Elices et al., 1991; Dedhar et al., 1992), it is now generally accepted that it mainly serves as a receptor for certain isoforms of laminin found in basement membranes, including Laminin-5 and 10/11 (Delwel et al., 1994; Kikkawa et al., 1998). Recently, \( \alpha_3 \beta_1 \) integrin has been identified as a receptor for netrin-1 in epithelial cells (Yebra et al., 2003).
Mice homozygous for a targeted mutation in the α3 integrin (Itga3) gene die during the perinatal period with severe defects in the development of the kidneys, lungs, skin, and cerebral cortex (Kreidberg et al., 1996; DiPersio et al., 1997; Hodivala-Dilke et al., 1998; Anton et al., 1999). In the cerebral cortex, disorganized neuronal placement and disrupted laminar organization is evident. BrdU labeling of nascent neurons in normal brains demonstrates that older neurons occupy deeper layers of the cortex, while the youngest neurons occupy the outermost layers adjacent to the marginal zone. In contrast, the location of BrdU-labeled neurons in the α3-deficient cortex bears no relationship to their birthdates, suggesting an impairment in proper neuronal migration (Anton et al., 1999). Furthermore, the loss of α3β1 integrin function induces a gliophilic to neurophilic shift in the adhesive preference of cortical neurons, indicating that α3β1 integrin is required for neuron-glial interactions during glial-guided radial migration (Anton et al., 1999). Live imaging of neuronal migration in Itga3 mutant embryonic cortices indicates a reduced rate of radial and tangential migration (Schmid et al., 2004). In addition, α3 integrin deficient neurons display reduced actin cytoskeletal dynamics, manifested as a decrease in filopodial and lamellipodial activity (Schmid et al., 2004). Altered cytoskeletal dynamics of cortical neurons may compromise ECM and cell-cell recognition during migration, leading to the aberrant cortical organization of the α3 integrin mutant.

**Signaling Ligands of Integrins in the Developing Cortex.**

Integrins respond to a dynamic ligand environment during cortical development, interacting with ECM components such as fibronectin, laminins, tenascin, thrombospondin, glycosaminoglycans, reelin and cell surface receptors including tetraspanins and L1-CAM
Laminin, an integral component of the pial basement membrane, is also expressed in the ventricular zone, subplate and marginal zone of the developing cerebral cortex. At early stages of corticogenesis, during preplate formation, laminin is present throughout the cerebral wall (Hunter et al., 1992). By mid-embryonic stages, during cortical plate formation, laminin is expressed by the subplate and marginal zone, with predominant expression in the subplate (Hunter et al., 1992). In addition, the expression of laminin along radial glial cells suggests that laminin may support an interaction between migrating neurons and radial glial fibers (Liesi, 1985, 1990). Fibronectin is highly expressed in the ventricular zone during preplate formation. This expression pattern indicates that fibronectin may regulate the cell division and cell specification of cortical progenitors (Sheppard et al., 1995). During cortical plate formation, fibronectin is present in the intermediate zone and cortical plate, being expressed by radial glia, migrating neurons and cortical plate neurons. Fibronectin may guide migratory neurons along radial glial fibers to specific cortical regions and subsequently mediate neuron-neuron interactions required for cortical layer formation (Sheppard et al., 1995).

Mice deficient in laminin α5 chain, laminin γ1 nidogen binding site, and perlecan, ECM components of the pial basement membrane, underscore the relevance of integrin ligands in cortical development. Absence of the laminin α5 chain results in a lack of anterior neural tube closure, leading to exencephaly, a cephalic disorder in which the brain is exposed (Miner et al., 1998). Targeted deletion of the laminin γ1 binding site for nidogen, a protein involved in linking basement membrane proteins, produces disruptions in the pial basement membrane which lead to retraction of radial glial endfeet from the pial surface and large
neuronal dysplasias present predominantly in the cortical plate and marginal zone (Halfter et al., 2002). Mice deficient in perlecan, a heparan sulfate proteoglycan, display an intrusion of brain tissue into the ectoderm, resulting in neuronal ectopias and exencephaly (Costell et al., 1999).

Outside of the pial basement membrane, reelin, an extracellular matrix protein secreted by Cajal-Retzius cells of the cortical marginal zone (D’Arcangelo et al., 1995; Ogawa et al., 1995), binds to the α3β1 integrin receptor (Dulabon et al., 2000; Schmid et al., 2005). Absence of reelin in the reeler mouse results in an inability of migrating cortical neurons to split the preplate and migrate past previously generated neurons, giving rise to a roughly inverted cortical plate (Caviness et al., 1972; Caviness and Sidman, 1973; Lambert de Rouvroit and Goffinet, 1998).

Conventionally, reelin binds to two members of the low-density lipoprotein (LDL) family expressed by migrating neurons, including very low-density lipoprotein receptor (VLDLR) and apolipoprotein E receptor type2 (ApoER2) (D’Arcangelo et al., 1999; Hiesberger et al., 1999; Trommsdorff et al., 1999; Kubo et al., 2002; Strasser et al., 2004). The binding of reelin stimulates the tyrosine phosphorylation of Dab1, a cytoplasmic adaptor protein, which associates with the conserved motif NPxY in the cytoplasmic tails of VLDLR and ApoER2 (Trommsdorff et al., 1998; Howell et al., 1999). Dab1 activates and is a substrate of Src and Fyn, Src family nonreceptor tyrosine kinases (Arnaud et al., 2003a, 2003b; Bock and Herz, 2003; Kuo et al., 2005). Mouse mutations in both VLDLR and ApoER2, in both Src and Fyn, or in Dab1, point mutations at five Dab1 tyrosine phosphorylation sites, or natural mutations of Dab1, scrambler and yotari, phenocopies the cortical layering deficits observed in reeler mutants (Sweet et al., 1996; Gonzalez et al., 2005).
1997; Howell et al., 1997; Sheldon et al., 1997; Ware et al., 1997; Yoneshima et al., 1997; Rice et al., 1998; Howell et al., 2000; Benhayon et al., 2003; Kuo et al., 2005).

α3β1 integrin binds to the N-terminal domain of reelin, a region distinct from that shown to interact with VLDLR and ApoER2, and Dab1 associates with the cytoplasmic tail of β1 integrin in a reelin-dependent manner, implicating α3β1 integrin in the reelin signaling pathway (Dulabon et al., 2000; Schmid et al., 2005). Furthermore, the ability of reelin to induce detachment of wildtype cortical neurons from radial glial fibers is abolished in α3β1 integrin-deficient cerebral cortices (Dulabon et al., 2000). Based on these observations, it has been proposed that during radial migration, neuronal α3β1 integrin receptors interact with glial cell surface laminin or fibronectin until neurons arrive at the top of the cortical plate, whereupon the ligand preference of α3β1 integrin receptors shifts from glial laminin or fibronectin to reelin, promoting glial detachment and cortical layer formation (Dulabon et al., 2000). In another model for the role of α3β1 integrin in Dab1-mediated signal transduction, the phosphorylation of Dab1 controls neuron-glial cell adhesion by regulating the expression of α3β1 integrin in migrating cortical neurons (Sanada et al., 2004). Specifically, the phosphorylation of Dab1 residues, Tyr220 and Tyr232, decreases the expression of neuronal α3β1 integrin, thereby mediating the glial detachment of migrating neurons at the top of the cortical plate (Sanada et al., 2004).

**Role of Integrin Signaling in Cortical Differentiation.**

Intrinsic and extracellular factors regulate the ability of cortical progenitor cells in the ventricular zone to produce laminar-specific cortical neurons (McConnell, 1988; McConnell and Kaznowski, 1991; Desai and McConnell, 2000). Extracellular matrix molecules function
as instructive environmental cues, regulating the proliferation and differentiation of embryonic progenitor cells in the central nervous system (Drago et al., 1991). Cellular responses to these extracellular matrix molecules are mediated in large part by integrin receptors (Milner and Campbell, 2002; Clegg et al., 2003). α5β1 integrin and its preferential receptor, fibronectin, are highly expressed in the developing ventricular zone (Yoshida et al., 2003). Laminin α2 chain (a subunit of laminin 2, 4, and 12) and α6β1 integrin, a laminin 2 receptor, are also expressed in the proliferative ventricular zone, their expression levels decreasing from E12.5 until after birth (Campos et al., 2004).

While integrin receptors and their ligands are highly expressed in the cortical ventricular zone, little is known of their role in cell fate specification. In vitro, the absence of β1 integrin in embryonic stem cells promotes their differentiation into cells expressing neuronal markers (Rohwedel et al., 1998). Accordingly, loss of β1 integrin decreases the proliferation and survival of progenitor cells which are nestin-positive, a marker for undifferentiated progenitor cells (Leone et al., 2005). Furthermore, the expression of α5β1 integrin in cortical progenitor cells decreases within the subpopulation that undergoes neuronal differentiation (Yoshida et al., 2003). Therefore, cell-ECM and cell-cell adhesive interactions mediated by β1 integrins may be required for the maintenance of the neural stem cell population within the ventricular zone, whereas a downregulation of β1 integrin receptors may promote the neuronal differentiation of cortical progenitor cells.

Once cortical differentiation is initiated and neuronal migration proceeds, the acquisition of cortical neuronal characteristics, including molecular identity, morphology, and connectivity may require extracellular cues encountered during migration across the cerebral wall (Levitt et al., 1997). α3β1 integrin, which is highly expressed by migrating
cortical neurons, may mediate cell-ECM and cell-cell adhesive interactions that initiate developmental programs necessary for the progressive differentiation of cortical neurons (Anton et al., 1999).

Despite knowledge of the relevance of adhesive interactions during corticogenesis, the mechanisms by which α3β1 integrin affect cerebral cortical development remain poorly understood due to the early lethality of α3 null mice. It is therefore important to determine at a mechanistic level how signals transduced by α3β1 integrin affect the migration of specific classes of neurons and their differentiation during layer formation in the developing cerebral cortex.
CHAPTER 2

NETRIN1-α3β1 INTEGRIN INTERACTIONS REGULATE THE MIGRATION OF GABAERGIC INTERNEURONS THROUGH THE CORTICAL MARGINAL ZONE

The development of the six-layered cerebral cortex, composed predominantly of glutamatergic projection neurons and GABAergic interneurons, depends on the appropriate migration of neurons from ventricular zones of the dorsal and ventral telencephalon to their final position in the emerging cortical plate. Neuronal progenitors from the dorsal telencephalon migrate radially as cohorts along the radial glial scaffold, pass their predecessors in the developing cortical plate, and coalesce into layers, giving rise to glutamatergic projection neurons (Hatten and Mason, 1990; Takahashi et al., 1990; Sidman and Rakic, 1973; Rakic, 1972; Miyata et al., 2001; Nadarajah et al., 2001; Noctor et al., 2001; Rakic, 2003). In contrast, neuronal precursors from the ventral telencephalon, principally the medial ganglionic eminence (MGE), migrate tangentially through the cortical marginal zone (MZ), subplate and lower intermediate/subventricular zones (IZ/SVZ) and enter the cortical plate using the radial glial scaffold, differentiating into GABAergic interneurons (Lavdas et al., 1999; Anderson et al., 2001; Wichterle et al., 2001; Nadarajah and Parnavelas, 2002; Polleux et al., 2002; Marin and Rubenstein, 2003; Yokota et al., 2007).

During neuronal migration, specific adhesive interactions between neurons, glia, and the surrounding extracellular matrix (ECM) are critical for the appropriate migration and placement of cortical neurons. These adhesive interactions are mediated in large part by
integrins, heterodimeric cell surface ECM receptors, which serve as structural links between extracellular ligands and the internal cytoskeleton (Hynes, 2002). The functional significance of integrins to cerebral cortical development is demonstrated by the distinct types of cortical malformations exhibited by mice deficient in αv (Bader et al., 1998; McCarty et al., 2005), α3 (Anton et al., 1999), α6 (Georges-Labouesse et al., 1998), β1 (Graus-Porta et al., 2001), and β4 (Murgia et al., 1998) integrin. However, the selective adhesive interactions needed to coordinate the migration of distinct groups of interneurons and projection neurons into the developing cerebral cortex remains poorly understood.

Recent evidence that α3β1 integrin serves as a receptor for netrin-1 as well as evidence for cross-talk between integrins and classical netrin receptors has opened a new avenue in which to investigate these mechanisms (Yebra et al., 2003; Li et al., 2004; Liu et al., 2004; Ren et al., 2004). Netrins function as short- and long-range diffusible guidance cues, inducing attraction or repulsion of cell migration and axonal outgrowth (Serafini et al., 1994; Wadsworth et al., 1996; Hong et al., 1999; Yee et al., 1999). Deleted in Colorectal Cancer (DCC) act as a netrin-1 receptor to regulate growth cone attraction (Keino-Masu et al., 1996; Serafini et al., 1996), whereas repulsion is mediated by a receptor complex composed of DCC and UNC5A-C receptors (Ackerman et al., 1997; Leonardo et al., 1997; Hong et al., 1999; Keleman and Dickson, 2001). In vitro, Yebra and colleagues (2003) have demonstrated that integrins α6β4 and α3β1 regulate the migration of epithelial cells on netrin-1 through a direct interaction of these integrins with the C-terminal domain of netrin-1, though the significance of these interactions have yet to be determined in vivo. The prominent expression of netrin-1 in the ventricular zone of the ganglionic eminence at E13.5 (Métin et al., 1997), during tangential cortical migration, as well as high expression of α3β1
integrin in migrating neurons (Anton et al., 1999) suggests that netrin1-α3β1 integrin interactions may influence tangential migration of GABAergic interneurons.

Therefore, we have investigated the contribution of netrin1-α3β1 integrin-mediated signal transduction to the migration of GABAergic interneurons from the MGE into the developing cerebral wall in vivo. We have found that netrin-1 and α3β1 integrin are co-expressed along the migratory routes traversed by GABAergic interneurons, with prominent co-localization at the cortical marginal zone. Netin-1 can interact with α3β1 integrins and activate β1 integrins. These interactions promote the oriented migration of cortical interneurons. Furthermore, to examine the role of netrin1-α3β1 integrin interactions on cortical tangential migration in vivo, we generated interneuron-specific α3β1 integrin, netrin-1 deficient mice. Analysis of α3lox/Δlx5/6-Cre, netrin-1−/− double mutant mice provides genetic evidence for netrin1-α3β1 integrin interactions and demonstrates their necessity for regulating interneuronal migration patterns and the number of interneurons migrating through the cortical marginal zone in vivo.

**Netrin-1 and α3β1 Integrin Co-Expression in the Developing Cerebral Cortex.**

To determine the pattern of netrin1 and α3β1 integrin expression in the developing cortex during the tangential migration of GABAergic interneurons, the expression pattern of these proteins was analyzed in the embryonic day 13.5 (E13.5) cerebral cortex. The netrin-1 mutant was originally generated using a gene trap vector to produce a β-galactosidase fusion protein containing a stretch of N-terminal signal sequence of netrin-1 (Skarnes et al., 1995). β-galactosidase expression in these mice faithfully indicates endogenous netrin-1 gene expression. Thus, β-galactosidase and α3 integrin antibodies were applied to coronal brain
sections of netrin-1 heterozygous mutants to examine the expression pattern of netrin-1 and α3β1 integrin in the embryonic cortex. Prominent netrin-1 expression in the ventricular zone of the ganglionic eminence as well as in the marginal zone of the developing cortex was detected (Figure 1 B, E, H). α3 integrin is present in radially and tangentially migrating neurons in the cerebral wall (Figure 1 A, J, L). α3 integrin is prominently co-expressed with netrin-1 in the marginal zone region (Figure 1 C, F, I). Together, these results indicate α3β1 integrin expression in interneurons traversing through netrin-1-rich areas of the developing cerebral cortex during the tangential migration of GABAergic interneurons.

**Secreted Netrin-1 is Localized Along the Migratory Route of Cortical GABAergic Interneurons.**

To examine the expression pattern of functional, secreted netrin-1 in the developing telencephalon during tangential migration, E13.5 cortices were immunolabeled with netrin-1 antibodies. Expression of secreted protein was detected in regions traversed by tangentially migrating interneurons (Figure 1 M). This immunolocalization pattern was not detected in netrin-1 null mice (Figure 1 N, P). The presence of netrin-1 protein in the ventral and dorsal telencephalon was further verified by immunoblotting performed on micro-dissected portions of the telencephalon (Figure 1 P).

**Netrin1-α3 Integrin Interactions.**

To examine the interactions between α3 integrin and netrin-1, we performed co-immunoprecipitations of netrin-1 and α3 integrin. Immunoprecipitation of E13.5 forebrain lysates with anti-netrin-1 antibodies and immunoblotting with anti-α3 integrin antibodies or immunoprecipitation of lysates with anti-α3 integrin antibodies and immunoblotting with
anti-netrin-1 antibodies demonstrates that α3 integrin co-immunoprecipitates with netrin-1 in vivo (Figure 2 A). Previously known ligands for α3 integrin and netrin-1 (i.e., laminin-1 and DCC, respectively) were co-immunoprecipitated in these assays and serve as positive controls (Figure 2 E). Netrin-1 or α3 integrin were not co-immunoprecipitated when α3 integrin deficient or netrin-1 deficient forebrain extracts were used to co-immunoprecipitate netrin-1 or α3 integrin, respectively (Figure 2 D). To further verify an interaction between α3 integrin and netrin-1, we performed co-immunoprecipitations of netrin-1 and α3 integrin using netrin-1-expressing 293 cells transfected with α3 integrin. Immunoprecipitation of these cell lysates with anti-netrin-1 antibodies and immunoblotting with anti-α3 integrin antibodies or immunoprecipitation of lysates with anti-α3 integrin antibodies and immunoblotting with anti-netrin-1 antibodies shows that α3 integrin co-immunoprecipitates with netrin-1 in vitro (Figure 2 B). To establish whether netrin1 and α3β1 integrin associate directly, a mixture of recombinant α3 integrin and netrin-1 proteins was utilized to co-immunoprecipitate netrin-1 and α3 integrin. Immunoprecipitation of these protein complexes with anti-netrin-1 antibodies and immunoblotting with anti-α3 integrin antibodies or immunoprecipitation of protein complexes with anti-α3 integrin antibodies and immunoblotting with anti-netrin-1 antibodies demonstrates that α3 integrin co-immunoprecipitates with netrin-1 in vitro and indicates a direct association between α3 integrin and netrin-1 (Figure 2 C).

**Netrin-1 Activates β1 Integrin Receptors of MGE Progenitor Cells.**

To establish whether the physical interaction between netrin-1 and α3β1 integrin has functional relevance, we examined the effects of netrin-1 on β1 integrin receptor activation.
E14.5 MGE progenitor cells, dissociated from the SVZ/VZ of the MGE, were incubated with netrin-1 containing supernatant, recombinant mouse netrin-1 (50 ng/ml), media alone, or ECM (9.9 mg/ml). Media alone serves as a negative control, while ECM substrate, composed predominantly of laminin, collagen type IV, heparan sulfate proteoglycan and entactin, functions as a positive control for β1 integrin receptor activation. The activation of β1 integrin in netrin-1 treated MGE cells was assessed by application of a β1 integrin antibody (9EG7), which specifically recognizes its active conformation (Galbraith et al., 2007). Supernatants containing secreted netrin-1 or mouse recombinant netrin-1 markedly increased β1 integrin receptor activation of MGE progenitor cells compared to media alone (Figure 2 F), demonstrating a functional, physiologically relevant interaction between netrin-1 and α3β1 integrin.

Netrin1-α3 Integrin Interactions Promote the Migration of MGE Neuroblasts.

The expression pattern and functional association of α3 integrin and netrin-1 in the forebrain at E13.5-E14.5 suggests that netrin-1 may serve to modulate the migration of cortical interneurons from the ventral to dorsal telencephalon through a direct receptor-ligand interaction with α3 integrin. To determine whether netrin-1 can serve as a chemoattractant for MGE neuroblasts through an interaction with α3 integrin, we cocultured netrin-1 expressing HEK 293 cells and E14.5 MGE and examined the patterns of cell migration from the MGE explants towards sources of netrin-1. Migrating neuroblasts were labeled with antibodies to class III β-tubulin. In control experiments, utilizing 293 cell aggregates which do not express netrin-1 and wildtype MGE explants, cells migrate uniformly in all directions from the explant (Figure 3 A, E). However, when a wildtype explant is cultured next to a
netrin-1 expressing 293 cell aggregate, we found a 2.2-fold preference for neuronal migration from the proximal side of the explant towards the netrin-1 source (Figure 3 B, E). When utilizing α3 integrin-deficient explants, we did not observe preferential cell migration toward a netrin-1 source (Figure 3 D, E), but rather uniform cell migration similar to the control experiment was noticed (Figure 3 C, E). These observations suggest that α3 integrin promotes proper netrin-1 mediated attraction of migrating MGE-derived interneurons.

Disruption of Netrin1-α3β1 Integrin Interactions Reduce the Number of Interneurons Migrating Through the Cortical Marginal Zone.

To evaluate the role of netrin1-α3 integrin interactions during tangential neuronal migration in vivo, interneuron-specific conditional α3 integrin, netrin-1 deficient double mutant mice (α3lox−Dlx5/6-Cre, netrin-1−/−) were generated. As Dlx5/6 is expressed throughout the lateral and medial ganglionic eminences at E12.5 (Liu et al., 1997; Stenman et al., 2003), mice with a floxed and null Itga3 allele (α3lox−) in a netrin1 null background, which also contain the Dlx5/6-Cre-IRES-EGFP transgene, allow for specific ablation of α3 integrin in neuronal precursors derived from the ganglionic eminence and permit simultaneous visualization of these cells by EGFP expression. This transgene marks nearly all GABAergic interneurons in the postnatal cerebral cortex (Stühmer et al., 2002). α3 integrin conditional heterozygotes (α3lox+/Dlx5/6-Cre, netrin-1+/−) and α3 integrin conditional, netrin-1 double heterozygotes (α3lox+/Dlx5/6-Cre, netrin-1+/−) are phenotypically normal and were used as controls. We compared the patterns of tangential migration of double mutant mice with α3 integrin conditional (α3lox−Dlx5/6-Cre, netrin-1−/−) and netrin-1 (α3+/Dlx5/6-Cre, netrin-1−/−) single mutant as well as with control mice to establish genetic evidence for netrin1-α3 integrin interactions.
We examined migration patterns at E16.5, at the height of interneuronal migration into the developing cerebral wall. Quantification of interneuron distribution within the cerebral wall indicates that compared to control mice, conditional α3 integrin and netrin-1 single mutant mice exhibited an increase in EGFP$^+$ cells ectopically placed in the ventricular zone (control, 28±1.04%; $a^{\Delta 3}_{\text{lox}}$/Dlx5/6-Cre, netrin-$1^{+/+}$, 32±0.58%; $a^{\Delta 3}_{+}$/Dlx5/6-Cre, netrin-$1^{-/-}$, 34±0.91%; Figure 4 B, D). Netrin-1 single mutants also displayed disorganized interneuronal cell distribution within the marginal zone, in which EGFP$^+$ cells appeared less tightly organized compared to controls (Figure 4 A). In contrast, $a^{\Delta 3}_{\text{lox}}$/Dlx5/6-Cre, netrin-$1^{-/-}$ double mutant mice exhibited a more severe marginal zone deficit, manifested as a substantial reduction in EGFP$^+$ cells migrating along the marginal zone (control, 27±1.06%; $a^{\Delta 3}_{\text{lox}}$/Dlx5/6-Cre, netrin-$1^{-/-}$, 19±0.52%; Figure 4 A, C). Correspondingly, double mutants displayed an increased number of EGFP$^+$ cells ectopically migrating through the ventricular zone (control, 28±1.04%; $a^{\Delta 3}_{\text{lox}}$/Dlx5/6-Cre, netrin-$1^{-/-}$, 37±0.47%; Figure 4 B, D, E). This data suggests that netrin1-α3 integrin interactions modulate distinct patterns of interneuronal movement within the developing cerebral cortex, especially the migration along the outer surface of the developing cerebral cortex.

**Real-Time Assessment of Interneuronal Migration Patterns Through the Cortical Marginal Zone Reveals Abnormal Migration in $a^{\Delta 3}_{\text{lox}}$/Dlx5/6-Cre, Netrin-$1^{-/-}$ Double Mutants.**

To further explore the significance of netrin1-α3 integrin interactions on interneuronal migration patterns *in vivo*, real-time imaging of interneuron migration was performed on control $a^{\Delta 3}_{\text{lox}}$/Dlx5/6-Cre, netrin-$1^{+/+}$ and $a^{\Delta 3}_{\text{lox}}$/Dlx5/6-Cre, netrin-$1^{-/-}$ double mutant E15.5 embryonic cortices. In the cerebral wall, the leading processes of migrating interneurons are
oriented tangentially towards the dorsal telencephalon. We found a significant decrease in the number of interneurons oriented towards the dorsal cortex in α3loox/Dlx5/6-Cre, netrin-1−/− double mutants compared to α3loox/Dlx5/6-Cre, netrin-1+/+ controls (control, 41±2.3%; α3loox/Dlx5/6-Cre, netrin-1−, 34±2.7%; Figure 5 E; Movies S1-S2). Local analysis of migration patterns revealed a 2.7-fold increase in the number of interneurons shifting from tangential migration along the marginal zone to ventricular surface-directed radial migration in double mutant mice compared to controls (Figure 5 A, B, F; Movies S3-S4). The increase in ventricular surface-directed radial migration at the cortical marginal zone provides a mechanism for the reduction of interneurons at the marginal zone of E16.5 α3loox/Dlx5/6-Cre, netrin-1−/− double mutants. In addition, double mutant mice displayed a 5.6-fold increase in the number of interneurons reversing direction in tangential migration in the vicinity of the marginal zone compared to control mice (Figure 5 C, D, G; Movies S5-S6). These results indicate that netrin1-α3 integrin interactions are required for interneurons to maintain directed tangential migration along the cortical marginal zone.

To further investigate netrin1-α3 integrin interactions during interneuronal motility, we tested whether netrin-1 mediated autocrine signaling in migrating interneurons is sufficient for appropriate tangential migration by performing a slice coculture assay. E14.5 MGE explants from control or conditional α3 integrin single mutant mice were placed over the MGE of E14.5 wildtype or netrin-1 mutant coronal brain slices and cultured for 72 hrs. Compared to the control condition, an assessment of α3 integrin deficient explants placed on wildtype cortical slices and control explants placed on netrin-1 mutant cortical slices did not reveal a significant deficit in the number of EGFP+ cells which had migrated to the cerebral cortex (Figure 6 A-C, E-G, I). In contrast, we found a significantly decreased number of
EGFP⁺ interneurons in netrin-1 deficient cortices containing α3 integrin deficient explants compared to controls (62±2.8% and 73±2.5%, respectively, Figure 6 A, D, E, H, I). Additionally, we observed a 25% reduction in the number of EGFP⁺ cells traversing the cortical marginal zone (Figure 6 A, D, E, H, J). Therefore, netrin-1 mediated autocrine signaling in migrating interneurons is not sufficient for proper cortical tangential migration. Instead, netrin-1 must act non-cell-autonomously by long-range and/or paracrine signaling to α3 integrin-expressing interneurons during this migratory process.

Loss of Netrin1-α3β1 Integrin Interactions Decrease the Number and Alter the Positioning of Interneurons in Cortex at P0.

Our in vitro evidence for the significance of netrin1-α3 integrin interactions in interneurons as well as the migration deficits observed in a3lox/Dlx5/6-Cre, netrin-1⁻/⁻ double mutant mice during cortical development in vivo predict a reduction in the number and alteration in the positioning of interneurons in the a3lox/Dlx5/6-Cre, netrin-1⁻/⁻ postnatal cortex, particularly through the cortical marginal zone. Examination of postnatal day 0 (P0) cortices revealed that the number of EGFP⁺ interneurons at the marginal zone in double mutant mice was indeed reduced to 36% of that observed in a3lox/+Dlx5/6-Cre, netrin-1⁺/+ controls (Figure 7 A, D, E). As observed at E16.5, EGFP⁺ interneurons in netrin-1 single mutant mice appeared more dispersed and disorganized within the marginal zone (Figure 7 C). Surprisingly, the total number of EGFP⁺ interneurons in a3lox/Dlx5/6-Cre, netrin-1⁻/⁻ double mutant cortices was also substantially reduced to 45% of that observed in controls (Figure 7 A, D, F).

Similarly, immunolabeling with calbindin antibodies, which label a subpopulation of cortical interneurons, revealed that the number of calbindin⁺ interneurons at the marginal zone and throughout the cerebral cortex were decreased to 42% and 34% in a3lox/Dlx5/6-
Cre, netrin-1−/− brains, respectively, of that observed in controls (Figure 7 G, J, K, L). Conditional α3 integrin single mutant mice displayed a significant 26% reduction in the total number of calbindin+ interneurons in the cerebral cortex compared to controls (Figure 7 G, H, L). In addition, netrin-1 single mutants displayed a disorganization and dispersal of calbindin+ cells within the cortical marginal zone, as observed with EGFP+ interneurons (Figure 7 I). We also noticed an altered distribution of calbindin+ cells within the cortical plate. While calbindin+ cells were found primarily in mid layers of control cortices as reported in wildtype brains (Hof et al., 1999), double mutants displayed a 3.3-fold increase in the number of calbindin+ cells ectopically positioned within deep cortical layers (Figure 7 G, J). This increase in calbindin+ cells within deeper layers, though to a lesser extent, was also observed in conditional α3 integrin and netrin-1 single mutant mice (1.9- and 2.2-fold increases, respectively, compared to controls, Figure 7 G, H, I).

The substantial reduction of interneurons in a3lox−/−Dlx5/6-Cre, netrin-1−/− double mutant cortices may be the result of increased apoptosis or disrupted migration into the cerebral cortex. Therefore, we examined coronal sections of the E16.5 double mutant brain for increased caspase-3 activity. We detected an ~70% increase in cleaved caspase-3+ cells in double mutant cortices compared to controls (Figure S1), providing one explanation for the dramatic reduction of interneurons in a3lox−/−Dlx5/6-Cre, netrin-1−/− double mutant cortices. In addition, we analyzed coronal sections taken across the rostro-caudal axis of the P0 a3lox−/−Dlx5/6-Cre, netrin-1−/− brain to determine whether misrouting of cortical tangential migration may contribute to the deficiency in the number of cortical interneurons in double mutant mice. We found a large ectopic aggregation of EGFP+ interneurons in the ventral telencephalon of double mutants, absent in single mutants and controls (Figure 9 I-L).
Therefore, the misrouting of cortical interneurons may be another possible mechanism for the decrease of interneurons in the $a3^{lox/-}$ Dlx5/6-Cre, netrin-1$^{-/-}$ neocortex. In sum, these data indicate that netrin1-α3 integrin interactions are necessary for the appropriate integration of migratory interneurons within the postnatal cerebral cortex.

**Disorganization and Reduced Numbers of Hippocampal Interneurons in $a3^{lox/-}$ Dlx5/6-Cre, Netrin-1$^{-/-}$ Mutants at P0.**

Nearly all hippocampal GABAergic interneurons derive from the LGE and MGE and migrate tangentially through the cerebral cortex to reach their appropriate destinations within the hippocampus (Pleasure et al., 2000). Since the hippocampus represents the end point of long distance migration from the ventral telencephalon through the cerebral cortex, we anticipated similar hippocampal interneuron and cortical interneuron phenotypes in $a3^{lox/-}$ Dlx5/6-Cre, netrin-1$^{-/-}$ double mutant brains. As predicted, we found a reduction in and dramatic disorganization of EGFP$^+$ interneurons throughout the hippocampus in $a3^{lox/-}$ Dlx5/6-Cre, netrin-1$^{-/-}$ double mutants compared to controls (Figure 8 A, D, E, H). This data suggests that netrin1-α3 integrin interactions may also guide the migration of interneurons to the hippocampus.

**Mechanisms and Relevance of Netrin1-α3β1 Integrin Interactions During Interneuronal Migration Through the Cortical Marginal Zone.**

In this study, we provide genetic evidence for the regulation of tangential migration through the cortical marginal zone by netrin1-α3 integrin interactions. Co-expression data indicate that netrin1-α3 integrin interactions are critical in guiding tangential neuronal migration through the cortical marginal zone. Furthermore, our data show that α3β1 integrin is expressed by cortical interneurons migrating through the marginal zone and lower
intermediate/subventricular zones of the developing cerebral cortex and that netrin-1 is expressed by cells along these migratory routes. *In vitro*, netrin1-α3β1 integrin interactions activate β1 integrin in MGE-derived cells and induce chemotaxis in this cell population via netrin-1 long-range and/or paracrine signaling to α3 integrin-expressing interneurons. *In vivo*, interneuron-specific conditional α3β1 integrin, netrin-1 deficient mice display deficits in patterns of tangential migration and in the number of cortical interneurons migrating along the marginal zone. In the postnatal cortex, aberrant interneuronal positioning and a dramatic reduction in the number of interneurons was observed in the absence of netrin1-α3β1 integrin interactions. Thus, netrin1-α3β1 integrin interactions stimulate and guide the migration of GABAergic interneurons through the cortical marginal zone and, hence, are necessary for the proper integration of cortical interneurons into the postnatal cerebral cortex.

**Materials and Methods**

**Mutant Mouse Strains**

A targeted mutation in mouse α3 integrins was generated as described by Kreidberg et al. (Kreidberg et al., 1996). Genotypes of the mice used were determined by PCR as described earlier (DiPersio et al., 1997). Netrin-1 mutants were generated and genotyped as described by Serafini et al. (Serafini et al., 1996). \(\alpha3^{lox/-}\)Dlx5/6-Cre, netrin-1\(^{-/-}\) double mutant as well as \(\alpha3^{lox/-}\)Dlx5/6-Cre, netrin-1\(^{+/+}\) single mutant and \(\alpha3^{lox/+}\)Dlx5/6-Cre, netrin-1\(^{-/-}\) and \(\alpha3^{lox/+}\)Dlx5/6-Cre, netrin-1\(^{+/+}\) controls were generated by crossing mice with floxed Itga3 alleles in a netrin-1 heterozygous background to mice carrying the Dlx5/6-cre-IRES-EGFP transgene in a netrin-1 and α3 integrin heterozygous background. \(\alpha3^{+/+}\)Dlx5/6-Cre, netrin-1\(^{-/-}\) single mutants were generated by intercrossing mice with the Dlx5/6-cre-IRES-
EGFP transgene in a netrin-1 heterozygous background. In order to distinguish between mice with a floxed and wildtype α3 integrin allele (F/+) and those with a floxed and null α3 integrin allele (F/-), the following primers were used: 5’CCGTCTATGTCTTCATGA ACC-3’, 5’-GGGGAACCTTCCTGACTAG-3’, and 5’-GGAATCCATCCTGGTGATGTC-3’. PCR amplification of floxed and wildtype α3 integrin alleles yield a 130 bp fragment, while amplification of the null α3 integrin allele produces a 285 bp fragment. Genotypes of Dlx5/6-cre-IRES-EGFP mice were assessed by PCR using the following primers: 5’-GCGGT CTGGCAGTAAAAACTATC-3’ and 5’- GTGAAACGCATTGCTGTCACCTT-3’.

**Antibodies and Counterstains**

The following antibodies were used: α3 integrin antibodies (#611045, BD Transduction Labs; 8-4, a gift from Dr. DiPersio, Albany Medical College), β1 integrin (9EG7) monoclonal antibodies (#553715, BD Pharmingen), netrin-1 polyclonal antibodies (#CH23002, Neuromics), laminin-1 polyclonal antibodies (#L9393, Sigma-Aldrich), DCC monoclonal antibodies (#OP45, Calbiochem), calbindin polyclonal antibodies (#AB1778, Chemicon), GFP polyclonal antibodies (#TP401, Torrey Pines Biolabs; #ab13970, Abcam), neuronal class III β–tubulin monoclonal antibodies (#MMS-435P, Covance), and cleaved caspase-3 polyclonal antibodies (#9661S, Cell Signaling Technology). The following counterstains were used: red fluorescent Nissl stain (#N-21482, Molecular Probes) and green fluorescent Nissl stain (#N-21480, Molecular Probes).
Cell Counting

Quantification of EGFP+, calbindin+, and cleaved caspase-3+ cells was performed on coronal sections from at least three animals of each genotype. Cell counts in the E16.5 and P0 cortex were performed in sections corresponding to plates 5-6 of the Chemoarchitectonic Atlas of the Developing Mouse Brain by Jacobowitz and Abbott, 1998. Statistical differences between experimental groups were assessed with the one-way ANOVA and Tukey-Kramer post hoc test using Prism4 software (GraphPad Software Inc.).

Western Blotting for Netrin-1

The ventral telencephalon as well as the lateral and medial dorsal telencephalon was dissected from coronal sections (250 μM) of E14.5 forebrain. Tissue was treated with lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 1% TritonX-100, Roche complete protease inhibitor cocktail and Sigma phosphatase inhibitor cocktail II), centrifuged at 14,000g for 20 min at 4ºC to remove debris and protein concentration was measured using the Bio-Rad Protein Assay reagent. Proteins (50 μg) boiled in reducing Laemmli buffer were resolved on a 7.5% SDS-PAGE gel, transferred to a nitrocellulose membrane, blocked with 5% BSA in 1X PBS, incubated with netrin-1 antibodies, and visualized using peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and a chemiluminescence detection system (Amersham Biosciences).

Immunoprecipitation

E13.5 forebrain tissue was treated with lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, Roche complete protease inhibitor cocktail and Sigma phosphatase
inhibitor cocktail II) and protein extracts were prepared and quantified as described earlier. Proteins were incubated with netrin-1 antibodies or α3 integrin antibodies (8-4) overnight at 4°C. Netrin-1 antibody-protein complexes were incubated with protein G agarose beads (Sigma), while α3 integrin antibody-protein complexes were incubated with protein A agarose beads (Zymed) overnight at 4°C. Protein-antibody-bead complexes were washed several times in 1X PBS. Protein complexes were separated from the beads by boiling in nonreducing Laemmli buffer. Western blotting was performed with netrin-1 antibodies or α3 integrin antibodies (BD Transduction Laboratories).

Netrin-1 expressing 293 cells were transfected with an α3 integrin construct (gift of Dr. Kreidberg, Harvard Medical School) using Effectene reagent according to instructions of the manufacturer (Qiagen). After 48 hours, cells were harvested in lysis buffer (100 mM Tris pH 8.5, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, Roche complete protease inhibitor cocktail and Sigma phosphatase inhibitor cocktail II) and protein immunoprecipitations were performed with netrin-1 or α3 integrin antibodies.

2 μg of purified α3β1 integrin protein (0.2 mg/ml; Chemicon) was incubated with concentrated netrin-1 containing serum free OptiMEM media, isolated from netrin-1 expressing 293 cells and concentrated with a centrifugal filter device (Millipore), and 500 μg/mL of heparin (Fisher Scientific) overnight at 4°C. At the same time, netrin-1 antibodies were incubated with protein G agarose beads (Sigma), while α3 integrin antibodies were incubated with protein A agarose beads (Zymed) overnight at 4°C. The netrin-1 antibody-bead complexes and α3 integrin antibody-bead complexes were washed several times in PBS+ 5% BSA and incubated with α3β1 integrin-netrin1 protein complexes overnight at 4°C. Protein-antibody-bead complexes were washed several times in 1X PBS. Protein complexes
were separated from the beads by boiling in nonreducing Laemmli buffer. Western blotting was performed with netrin-1 antibodies or α3 integrin antibodies (BD Transduction Laboratories).

**β1 Activation Assay**

The SVZ/VZ of the MGE was dissected from coronal slices (250 μm) of E14.5 wildtype brains, collected in serum free OptiMEM, and dissociated by trituration. 500,000 cells were seeded per well of a 12-well plate coated with poly-L-lysine (0.025 mg/ml; Sigma) or ECM gel (9.9 mg/ml; Sigma). Cells were incubated at 37°C with 5% CO₂ for 4 hrs to allow for adhesion to substrates. To stimulate MGE derived cells with netrin-1, concentrated netrin-1 containing serum free OptiMEM media or recombinant mouse netrin-1 (50 ng/ml; R&D Systems) was added to cells following 2 hrs. of incubation. Cells were washed in 1X PBS, incubated with 9EG7 antibodies for 15 min at 37°C with 5% CO₂, washed twice in 1X PBS, and proteins collected in 50 μl of reducing Laemmli buffer. The proteins were boiled and resolved on a 7.5% SDS-PAGE gel, transferred to a nitrocellulose membrane, blocked with 5% dry nonfat milk in 1X TBST, and visualized using peroxidase conjugated anti-rat IgG secondary antibodies (Jackson ImmunoResearch Laboratories) and a chemiluminescence detection system (Amersham Biosciences).

**Preparation of Hanging Drops**

293 control or netrin-1 expressing cells from 80-100% confluent plates were trypsinized, centrifuged, and resuspended in 100 μl of a rat tail collagen mixture [80% rat tail collagen (3 mg/ml; BD Biosciences), 10% Basal Medium Eagle (GIBCO), and 10% 0.5 M
NaHCO$_3$]. 2 μl aliquots of this cell-collagen mixture were added to the underside of a 10 cm tissue culture dish lid, which was placed over a 10 cm dish containing 10 ml of dH$_2$O. Hanging drops were incubated at 37ºC with 5% CO$_2$ for 2-3 hrs. prior to use.

**Explant Cocultures**

The SVZ/VZ of the MGE was dissected from coronal slices (250 μm) of E14.5 wildtype and littermate α3 integrin mutant brains. At least 2 explants from each brain were placed equidistant from a hanging drop of 293 control or netrin-1 expressing cells embedded in rat tail collagen. Cultures were incubated at 37ºC with 5% CO$_2$ for 24 hrs. and then fixed with 4% paraformaldehyde in 1X PBS (pH 7.4) for 30 min. at room temperature. Cell chemoattraction was quantified with the aid of MetaMorph software (Universal Imaging Corporation) as a ratio of the optical density of MGE cells in a defined area from the explant proximal to the 293 cell aggregate to the optical density of MGE cells in a defined area from the explant distal to the 293 cell aggregate. Statistical differences between experimental groups were analyzed with the Student’s $t$ test using Prism4 software (GraphPad Software Inc.).

**Slice Cocultures**

Slice cocultures were prepared as previously described, with minor alterations (Polleux and Ghosh, 2002). Briefly, cell culture inserts for six-well plates were coated with a laminin and poly-L-lysine solution and placed over wells containing 1.8 ml of slice culture medium. Coronal sections (250 μm) taken at the level of the MGE from E14.5 wildtype and netrin-1 mutant littermates were collected in ice-cold complete HBSS and transferred onto
the coated membrane inserts. E14.5 MGE explants dissected from \( a^3 \text{lox}^+/+, \) Dlx5/6-Cre, \( \text{netrin-1}^{+/+} \) and \( a^3 \text{lox}^{-/}, \) Dlx5/6-Cre, \( \text{netrin-1}^{+/+} \) mice were placed over the MGE of prepared E14.5 wildtype or netrin-1 deficient slices. Slice cocultures were incubated at 37ºC with 5% CO\(_2\) for 72 hrs. prior to fixation with 4% paraformaldehyde in 1X PBS (pH 7.4). Cell migration into the cortex was quantified as described by Cobos et al., 2007. Briefly, four concentric circles were overlaid onto each imaged hemisphere with the center positioned at the dorsal boundary of the MGE explant. Quantification of EGFP\(^+\) cells was performed in the areas of the annuli defined by the concentric circles, boundaries set at the medial and piriform cortex. Using this method, the innermost annulus defines the dorsal LGE, the middle annulus defines the deep migratory route taken by cortical interneurons through the IZ and SVZ, and the outermost annulus defines the superficial migratory route of the MZ. Statistical differences between means were assessed with the Mann-Whitney U test using Prism4 software.

**Real-Time Imaging**

Coronal slices (200 \( \mu \text{m} \)) of E15.5 \( a^3 \text{lox}^{+/+} \) Dlx5/6-Cre, \( \text{netrin-1}^{+/+} \) control and littermate \( a^3 \text{lox}^{-/-} \) Dlx5/6-Cre, \( \text{netrin-1}^{-/-} \) double mutant brains were placed onto nucleopore membrane filters over 35 mm glass bottom dishes containing Minimum Essential Medium (GIBCO) and 10% FBS. Mounted slices were immediately transferred to a 37ºC/5% CO\(_2\) live tissue incubation chamber attached to a Zeiss inverted microscope and a PASCAL confocal laser scanning system and imaged repeatedly every 12 minutes for up to 20 hrs. Real-time interneuronal migration patterns were quantified using Zeiss LSM Image Browser software. Statistical analysis was performed with the Student’s \( t \) test using Prism4 software.
CHAPTER 3

α3β1 INTEGRIN SIGNALING IS NECESSARY FOR THE APPROPRIATE DIFFERENTIATION OF CORTICAL NEURONS

The differentiation of cortical progenitor cells is regulated by intrinsic and extracellular factors. Extracellular matrix molecules serve as instructive guidance cues, regulating the proliferation and differentiation of embryonic progenitor cells (Drago et al., 1991). Integrin receptors recognize and transduce the instructive function of ECM factors into relevant cellular responses.

Integrin receptors and their ECM ligands regulate the cell fate specification of progenitor cells in the cortical ventricular zone. α5β1 and α6β1 integrin receptors as well as fibronectin and laminin, their respective preferential ligands, are highly expressed in the developing cortical ventricular zone (Yoshida et al., 2003; Campos et al., 2004). The absence of β1 integrin in embryonic stem cells promotes their differentiation into cells expressing neuronal markers in vitro (Rohwedel et al., 1998). Likewise, loss of β1 integrin reduces the proliferation and survival of undifferentiated progenitor cells (Leone et al., 2005). Moreover, the expression of α5β1 integrin in cortical progenitor cells decreases within the subpopulation that undergoes neuronal differentiation (Yoshida et al., 2003). Therefore, cell-ECM and cell-cell adhesive interactions mediated by β1 integrins may be required for the maintenance of the neural stem cell population within the ventricular zone, whereas a downregulation of β1 integrin receptors may promote the neuronal differentiation of cortical progenitor cells.
Once cortical differentiation is initiated and neuronal migration proceeds, the acquisition of cortical neuronal characteristics, including molecular identity and cellular morphology, may require extracellular cues encountered during migration across the cerebral wall (Levitt et al., 1997). α3β1 integrin, which is highly expressed by migrating cortical neurons (Anton et al., 1999), may mediate cell-ECM and cell-cell adhesive interactions that initiate developmental programs necessary for the progressive differentiation of cortical neurons.

Here, we show that conditional deletion of α3β1 integrin in migrating cortical neurons and radial glial cells, in which the human glial fibrillary acidic protein promoter is active, results in decreased cortical thickness, and reduced dendritic length and branching of deep layer pyramidal neurons and interneurons. Changes in the positioning of cortical neurons expressing layer-specific markers are also observed. In contrast, the loss of α3β1 integrin in postmigratory interneurons does not affect neuronal differentiation. Therefore, the final differentiation of pyramidal neurons and interneurons is dependent on α3β1 integrin signaling during cortical neuronal migration.

**Characterization of Conditional α3 Integrin Inactivation.**

To determine whether α3 integrin mediated adhesive interactions during neuronal migration are essential for the acquisition and elaboration of different cortical neuronal phenotypes in the mature cerebral cortex, we generated lines of conditional null mice that lack α3 integrin expression in the CNS. We mated mice in which the third exon of the *Itga3* gene is flanked by loxP sites with transgenic mice that express Cre recombinase under the control of the rat nestin promoter and enhancer, the human GFAP (hGFAP) promoter, or the parvalbumin promoter. To maximize the number of cells with a complete loss of α3 integrin
function, we mated mice carrying loxP sites ($\alpha_3^{\text{lox}}$) with mice carrying Cre recombinase in a background that is heterozygous with respect to the null allele of $\alpha_3$ integrin ($\alpha_3^3$). Mating these mice to the nestin-Cre line resulted in transgenic mice deficient in $Itga3$ expression in nestin+ neuronal precursor cells and their derivatives ($\alpha_3^{\text{lox}^/-}$ Nestin-Cre or F/-, Nestin-Cre). The nestin-Cre transgene induces widespread recombination of the reporter genes Rosa26 lacZ$^{\text{lox}}$ and $\beta_1^{\text{lox}}$ in the CNS beginning around E10.5 (Betz et al., 1996; Graus-Porta et al., 2001; Dubois et al., 2006). We also inactivated $\alpha_3$ integrin in cells in which the hGFAP promoter is active. The hGFAP promoter is active in neural progenitors of the dorsal telencephalon as early as E13.5 (Zhuo et al., 2001); therefore, Cre-mediated recombination occurs in radial glial cells and cortical neurons of the developing dorsal telencephalon in these mice ($\alpha_3^{\text{lox}^/-}$ hGFAP-Cre or F/-, hGFAP-Cre). Immunoblotting with $\alpha_3$ integrin specific antibodies indicate a significant downregulation of $Itga3$ expression in the cerebral cortex of postnatal $\alpha_3^{\text{lox}^/-}$ hGFAP-Cre and $\alpha_3^{\text{lox}^/-}$ Nestin-Cre mice (Figure 10 A).

To test whether $\alpha_3$ integrin signaling is required for cortical neuronal differentiation following neuronal migration, we utilized parvalbumin-Cre mediated inactivation of $\alpha_3$ integrin ($\alpha_3^{\text{lox}^/-}$ Parvalbumin-Cre or F/-, Parvalbumin-Cre). The parvalbumin promoter is active in the cerebral cortex beginning at postnatal day 8 (Alcantara et al., 1993; de Lecea et al., 1995). We verified parvalbumin-Cre expression in the mouse cerebral cortex by mating parvalbumin-Cre+ mice with Rosa26 lacZ$^{\text{lox}}$ reporter mice. LacZ expression, as assessed by X-gal staining of parvalbumin-Cre+, Rosa26 lacZ$^{\text{lox}}$ transgenic mouse brains, was present in the cerebral cortex at postnatal day 8 (Figure 10 B). Coronal brain sections labeled with antibodies to $\beta$-galactosidase, an indicator of LacZ expression, also reveal LacZ expression in the cerebral cortex at postnatal day 10 (Figure 10 C). The cellular localization of $\beta$-
galactosidase expression was examined to assess the specificity of β-galactosidase antibodies. As expected, β-galactosidase was localized to the cytoplasm of cortical neurons (Figure 10 D).

The expression of parvalbumin, a calcium-binding protein which marks fast spiking GABAergic interneurons, including basket cells and chandelier cells, correlates with the morphological and functional maturation of this interneuronal population (del Río et al., 1994; de Lecea et al., 1995). Basket cells consist of approximately 50% of interneurons in the juvenile rat somatosensory cortex and parvalbumin immunoreactive cells comprise > 50% of GABAergic interneurons in the rat somatosensory and visual cortex (Ren et al., 1992; Gonchar and Burkhalter, 1997; Markram et al., 2004). Therefore, including chandelier cells, parvalbumin-Cre mediated recombination will likely occur in at least 50% of GABAergic interneurons in the cerebral cortex. An examination of the loss of α3 integrin expression in adult parvalbumin conditional mutants by immunohistochemistry reveals that unlike α3lox/+ Parvalbumin-Cre controls (Figure 10 E-G), parvalbumin+ cells do not express α3 integrin in α3lox/− Parvalbumin-Cre mutants (Figure 10 H-J).

Reduced Cortical Thickness in α3lox/− hGFAP-Cre Mutants.

To determine if α3 integrin plays a role in the emergence of the cerebral cortex, the characteristic laminar organization of adult cerebral cortex of control and Itga3 deficient mice was examined. Coronal sections from α3lox/− hGFAP-Cre and α3lox/+ hGFAP-Cre mouse brains were Nissl stained. In addition to general disorganization of neuronal layering, a 14±3.1% reduction in cortical thickness, measured from the pial surface to the dorsal limit of
the white matter, was detected in α3lox/−hGFAP-Cre mutants compared to α3lox/+hGFAP-Cre controls (Figure 11 A-G).

This significant reduction in cortical width may be due to increased apoptosis, aberrant neurogenesis, or a reduction in neuropil. To assess apoptosis in α3lox/−hGFAP-Cre mutants, we applied a cleaved caspase-3 antibody to brain sections to examine caspase-3 activity and found no change in the levels of activated caspase-3 in α3lox/−hGFAP-Cre mutant mice compared to α3lox/+hGFAP-Cre controls (data not shown). To assess neurogenesis, we BrdU pulsed E15.5 embryos for 1 hr and stained these embryos with BrdU antibodies. We found no change in neurogenesis within the ventricular zone of the dorsal telencephalon in α3lox/−hGFAP-Cre mutants compared to α3lox/+hGFAP-Cre controls (data not shown). In addition, quantification of cell density in Nissl-stained adult α3lox/−hGFAP-Cre mutant and control cortices revealed no significant deficits in Itga3 mutants (data not shown). These results suggest that the reduction in the width of the cortex in α3lox/−hGFAP-Cre mutant mice may be due to a reduction in neuropil.

**Altered Layer-Specific Marker Expression in α3lox/−hGFAP-Cre Mutants.**

To determine whether α3β1 integrin is necessary for determining the final layer-specific phenotype of cortical neurons, the expression pattern of layer-specific markers in α3lox/−hGFAP-Cre mutants was evaluated. Coronal sections of the neocortex were labeled with anti-reelin, anti-Brn-1, anti-Er81, and anti-Tbr-1 antibodies at postnatal day 4 (P4) to label neurons of layer I, II-IV, V, and VI, respectively. At early postnatal ages, reelin, a large extracellular protein, is highly expressed by Cajal-Retzius cells and GABAergic interneurons in layer I (Alcantara et al., 1998). A 30±4.7% increase in the number of reelin-expressing
cells within this layer was detected in $\alpha_3^{\text{lox/-}}$hGFAP-Cre mutants (Figure 12 A, B). Brn-1, a class III POU domain transcription factor, is highly expressed by layer II-IV cortical neurons (McEvilly et al., 2002) while Er81, an ETS transcription factor, is expressed by layer V cortical neurons (Gray et al., 2004), and Tbr-1, a transcription factor of the T box family, is a marker for cortical layer VI (Hevner et al., 2001). Measurement of the width of Brn-1, Er81, or Tbr-1 labeled distribution indicates that there is an 18±2.3% and a 12±2.1% increase in the width of Brn-1+ and Er81+ cell distribution, respectively, in $\alpha_3^{\text{lox/-}}$hGFAP-Cre mutant mice compared to $\alpha_3^{\text{lox/+}}$hGFAP-Cre controls (Figure 12 C-F). However, only a small, but not significant change in Tbr-1+ cell distribution is evident in $\alpha_3^{\text{lox/-}}$hGFAP-Cre mutants compared to $\alpha_3^{\text{lox/+}}$hGFAP-Cre controls (Figure 12 G, H). This may be due to formation of layer VI neurons (at ~E12.5) prior to the onset of Cre-mediated recombination and Itga3 inactivation, which occurs at E13.5. Together, these results suggest that $\alpha_3$$\beta_1$ integrin may be required for determining the layer-specific positioning and phenotype of cortical neurons.

**Neuronal Differentiation is Compromised in $\alpha_3^{\text{lox/-}}$hGFAP-Cre Mutants.**

To directly assess whether $\alpha_3$$\beta_1$ integrin is necessary for specifying the final differentiation of cortical neurons, the morphology of deep layer pyramidal neurons was examined in adult $\alpha_3^{\text{lox/-}}$hGFAP-Cre mutants. Since neuronal activity and neurotrophins released by pyramidal neurons regulate the differentiation of GABAergic interneurons (Marty et al., 1997; Marty et al., 2000), we also analyzed the morphology of interneurons to examine any indirect consequences that conditional inactivation of $\alpha_3$ integrin may have on their differentiation. Adult $\alpha_3^{\text{lox/-}}$hGFAP-Cre and $\alpha_3^{\text{lox/+}}$hGFAP-Cre mouse brains were Golgi stained and the total neuritic length, number of primary neurites, total number of
branch points, and number of primary, secondary, tertiary, and quaternary branch points of deep layer pyramidal neurons and interneurons was measured. For pyramidal neurons, the apical and basal dendritic lengths were also measured. The primary apical dendritic length and number of primary neurites of pyramidal neurons appear not to be affected in $\alpha_3^{\text{lox/-}}$ hGFAP-Cre mutant mice (Figure 13 A-C, E, F). However, the total neuritic length, total basal dendritic length, total number of branch points, number of primary branch points, and number of secondary branch points was reduced in pyramidal neurons of $\alpha_3^{\text{lox/-}}$ hGFAP-Cre mutants by 32.4±6.6%, 36.1±7.5, 35.6±6.0%, 31.7±5.7%, and 52.2±10.4%, respectively, compared to $\alpha_3^{\text{lox/+}}$ hGFAP-Cre controls (Figure 13 A-C, E, F). Similarly, in interneurons of $\alpha_3^{\text{lox/-}}$ hGFAP-Cre mice, the total neuritic length, number of primary neurites, total number of branch points, number of primary branch points, and number of secondary branch points was decreased in $\alpha_3^{\text{lox/-}}$ hGFAP-Cre mutants by 62±4.6%, 18±4.2%, 53±8.1%, 51±7.7%, and 69±15.3%, respectively, compared to controls (Figure 13 D-F). Thus, $\alpha_3\beta_1$ integrin is required during migration for the appropriate final differentiation of cortical pyramidal neurons and interneurons.

**Differentiation of Interneurons and Pyramidal Neurons is Unaffected in $\alpha_3^{\text{lox/-}}$ Parvalbumin-Cre Mutants.**

To determine whether $\alpha_3\beta_1$ integrin signaling is necessary for cortical neuronal differentiation following neuronal migration, we examined the morphology of deep layer interneurons in adult $\alpha_3^{\text{lox/-}}$ Parvalbumin-Cre mutants as described above. We also examined the morphology of deep layer pyramidal neurons in order to assess any indirect effects that conditional inactivation of $\alpha_3$ integrin may have on neuronal differentiation. We found that total neuritic length, the total number of neurites emanating from the cell soma, the total
number of branch points, and branching complexity was unaltered in interneurons of conditional mutants compared to controls (Figure 14 A, B, D-F). The morphology of pyramidal neurons was, likewise, unaffected (Figure 14 C, E, F). Therefore $\alpha_3\beta_1$ integrin signaling is likely not required for cortical interneuron differentiation following neuronal migration.

**Mechanisms of $\alpha_3$ Integrin Mediated Cortical Differentiation.**

In this study, we demonstrate a requirement for $\alpha_3\beta_1$ integrin signaling in cortical differentiation. Inactivation of $\alpha_3\beta_1$ integrin in migrating cortical neurons and radial glial cells results in reduced cortical thickness and altered positioning of layer-specific cortical neurons. Furthermore, we observed a striking reduction in dendritic length and branching of deep layer pyramidal neurons and interneurons. In contrast, the inactivation of $\alpha_3\beta_1$ integrin in postmigratory interneurons does not modify neuronal morphology. Thus, $\alpha_3\beta_1$ integrin mediated adhesive interactions during cortical neuronal migration regulate the final differentiation of pyramidal neurons and interneurons.

**Materials and Methods**

**Mutant Mouse Strains**

A targeted mutation in mouse $\alpha_3$ integrins was generated as described by Kreidberg et al. (Kreidberg et al., 1996). Genotypes of the mice used were determined by PCR as described earlier (DiPersio et al., 1997). To generate $\alpha_3^{\text{lox/}}$Nestin-Cre, $\alpha_3^{\text{lox/}}$hGFAP, or $\alpha_3^{\text{lox/}}$Parvalbumin-Cre mutants and controls, we crossed mice carrying loxP sites ($\alpha_3^{\text{lox}}$) with mice carrying the nestin-Cre, hGFAP-Cre, or parvalbumin-Cre transgene in an $\alpha_3$ integrin...
heterozygous background. In order to distinguish between mice with a floxed and wildtype α3 integrin allele (F/+) and those with a floxed and null α3 integrin allele (F/-), the following primers were used: 5’-CCGTCTATGTCTTCATGAACC-3’, 5’-GGGAACCTTCCTGACTA-3’, and 5’-GGAATCCATCCTGGTTGATGTC-3’. PCR amplification of floxed and wildtype α3 integrin alleles yield a 130 bp fragment, while amplification of the null α3 integrin allele produces a 285 bp fragment. Genotypes of Nestin-Cre, hGFAP-Cre, and Parvalbumin-Cre mice were assessed by PCR using the following primers: 5’-CGGGTCTGGCAGTAAAAACTATC-3’ and 5’-GTGAAACGCATTGCTGTCACTT-3’, 5’-ACTCCTCATAAAGCCCTCG-3’ and 5’-ATCACTCGTTGCATCGACC-3’, and 5’-TGTCCTATTACTGACGTAC-3’ and 5’-AGTGCCTTCAACGCTAGAG-3’, respectively.

**Antibodies and Counterstains**

The following antibodies were used: α3 integrin antibodies (BD Transduction Labs), parvalbumin antibodies (Chemicon), β-galactosidase antibodies (MP Biomedicals, Inc.), cleaved caspase-3 antibodies (Cell Signaling Technology), BrdU antibodies (Becton Dickinson), reelin antibodies (a gift from Dr. A. Goffinet), Brn-1 antibodies (a gift from Dr. A. Ryan), Er81 antibodies (Developmental Studies Hybridoma Bank), and Tbr-1 antibodies (a gift from R. Hevner). The following stains were used: X-gal (Roche).

**Western Blotting for α3 Integrin**

Cortices were dissected from 3 week old α3lox/¬Nestin-Cre or α3lox/¬hGFAP-Cre mutant and control brains. Tissue was treated with lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 1% TritonX-100, Roche complete protease inhibitor cocktail and Sigma...
phosphatase inhibitor cocktail II), centrifuged at 14,000g for 20 min at 4°C to remove debris and protein concentration was measured using the Bio-Rad Protein Assay reagent. Proteins (40 μg) boiled in reducing Laemmli buffer were resolved on a 7.5% SDS-PAGE gel, transferred to a nitrocellulose membrane, blocked with 5% BSA in 1X PBS, incubated with α3 integrin antibodies, and visualized using peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) and a chemiluminescence detection system (Amersham Biosciences).

**Nissl Staining and Golgi Impregnation.**

For Nissl staining, adult coronal sections were placed in 1:1 chloroform/100% ethanol for 1 hr, rehydrated through an ethanol series, and incubated in 0.05% thionin solution for 2-2.5 min. These sections were then differentiated through an ethanol series, cleared in xylene substitute (Fisher) and mounted in Permount (Fisher). Nissl+ cortical cell density was quantified in a 100,000 μm² area in layers II and III of the adult motor and somatosensory cortex. For Golgi impregnation, freshly dissected adult brain hemispheres were fixed in a 4:1 volume ratio of 3.5% K₂Cr₂O₇ and 10% formalin for 2 days followed by incubation in 3.5% K₂Cr₂O₇ alone for 1 day. Brain hemispheres were briefly treated in a series of 0.75% AgNO₃ baths and incubated in fresh AgNO₃ for 2 days. Brains were sectioned with a vibratome at 150 μm and collected in 70% ethanol. Sections were dehydrated through an ethanol series, cleared in xylene substitute (Fisher) and mounted in Permount (Fisher). The morphology of Golgi stained cortical neurons was assessed in cells of layers IV and V of the adult motor and somatosensory cortex.
Quantification and Statistical Analysis

All quantification was performed on coronal sections corresponding to the motor and somatosensory cortex from at least three animals of each genotype. The width of the adult cerebral cortex was measured from the pial surface to the dorsal limit of the white matter. BrdU\(^+\) cells were quantified in a 5,000 µm\(^2\) area within the ventricular zone of the E15.5 dorsal telencephalon. Cleaved caspase-3\(^+\) cells were quantified in a 400,000 µm\(^2\) area spanning the entire width of the P4 neocortex. BrdU\(^+\) and cleaved caspase-3\(^+\) cells were normalized to total cell number. The number of reelin\(^+\) cells was assessed in a 10,000 µm\(^2\) area of the cortical marginal zone. Measurement of the width of Brn-1, Er81, or Tbr-1 labeled distribution was normalized to the entire width of the postnatal day 4 cortex. Statistical differences between experimental groups were assessed with the Student’s \(t\) test using Prism4 software (GraphPad Software Inc.)
CHAPTER 4
DISCUSSION

Receptor-Ligand Interactions which Regulate the Tangential Migration of Cortical GABAergic Interneurons.

Extracellular factors, including motogens, directional guidance cues, and stop signals, variously regulate the tangential migration of GABAergic interneurons into the developing cerebral wall. Motogenic factors promote the motility of interneurons; directional guidance cues steer interneurons into appropriate regions of the telencephalon; and stop signals halt their migration in the cerebral cortex.

Hepatocyte growth factor and neurotrophins, brain-derived neurotrophic factor and neurotrophin-4, serve as motogenic cues for cortical GABAergic interneurons. Hepatocyte growth factor, a multi-functional secreted molecule, is expressed in the ventricular zone of the ganglionic eminence and cortex as well as through the cortical plate. HGF binds to the MET receptor expressed by migrating interneurons, promoting movement from the ventricular zone of the GE and between regions of highest HGF expression (Powell et al., 2001). HGF stimulates the movement of cells in cultured forebrain slices, while the addition of HGF antibodies reduces cell motility. In addition, exogenous application of HGF to organotypic forebrain slices significantly disrupts the migration of GABAergic interneurons from the GE to the cortex. Furthermore, mice deficient in urokinase-type plasminogen activator receptor (u-PAR), critical in activating HGF by cleaving the inactive pro-form of HGF, display reduced numbers of calbindin-positive interneurons in the P0 cortex (Powell et
Of the neurotrophins, BDNF is expressed in the marginal zone, subplate, and ventricular zone of the cerebral cortex, while NT-4 is expressed in the ventricular zone of the lateral and medial ganglionic eminence and marginal zone and cortical plate of the dorsal telencephalon (Maisonpierre et al., 1990; Timmusk et al., 1993; Friedman et al., 1998; Fukumitsu et al., 1998; Polleux et al., 2002). TrkB, their high affinity tyrosine kinase receptor, is expressed in cortical GABAergic interneurons (Klein et al., 1990; Gorba and Wahle, 1999). Exogenous BDNF and NT-4 promote the migration of MGE-derived cells into the cerebral cortex, whereas application of an inhibitor of the Trk family receptor tyrosine kinases results in a decrease in the migration of interneurons into the cortex (Polleux et al., 2002). Furthermore, mice deficient in the TrkB receptor exhibit a reduction in the number of calbindin-positive migrating into the cortex (Polleux et al., 2002).

Although motogenic factors induce cell motility, the movement imparted is undirected. Semaphorin 3A and semaphorin 3F, neuregulin-1, and glial cell line-derived neurotrophic factor, guidance cues for migrating cortical interneurons, provide directionality to cell movement. Sema3A and Sema3F are expressed in the developing striatum of the ventral telencephalon. Neuropilin-1 (Npn-1) and neuropilin-2 (Npn-2), transmembrane receptors expressed by tangentially migrating interneurons, bind and mediate a chemorepulsive response to Sema3A and Sema3F, respectively (Marin et al., 2001). This chemorepulsive activity directs migrating cortical interneurons away from the striatum as demonstrated by neuropilin-1 or neuropilin-2 loss of function experiments, which result in a dramatic increase in the number of interneurons invading the striatum and a concomitant decrease in the number of interneurons entering the cortex (Marin et al., 2001).
Membrane bound and diffusible isoforms of NRG-1, a neuregulin family member, are present during cortical tangential migration, whereas ErbB4, the high affinity NRG-1 receptor, is expressed by migrating interneurons (Yau et al., 2003; Flames et al., 2004). In particular, NRG1-CRD, membrane bound isoforms of NRG-1, are expressed in the LGE, whereas NRG1-Ig, secreted isoforms of NRG-1, are expressed in the developing cortex (Flames et al., 2004). NRG1-CRD serves as a permissive corridor in the LGE for the migration of cortical interneurons, while NRG1-Ig is a chemoattractant for GABAergic interneurons (Flames et al., 2004). Loss of ErbB4 or NRG-1 function in vivo, leads to a decrease in the number of interneurons in the postnatal cerebral cortex (Flames et al., 2004). Therefore, NRG-1 isoforms function as short- and long-range attractants for migrating interneurons.

GDNF, a neurotrophic factor, is expressed in the marginal zone, cortical plate, subventricular zone, and ventricular zone of the dorsal telencephalon. Expression of GFRα1, a receptor for GDNF, occurs in the subventricular zone and mantle of the MGE as well as the cortical marginal zone, corresponding to a subpopulation of GABAergic interneurons (Pozas and Ibanez, 2005). In vitro, GDNF promotes MGE cell motility and functions as a chemoattractant for MGE-derived cells. Furthermore, mice deficient in GDNF or GFRα1 exhibit a reduction in the number of GABAergic interneurons present in the cerebral cortex (Pozas and Ibanez, 2005).

Following directed migration into the developing cerebral wall, GABAergic interneurons enter the cortical plate and settle in specific layers according to their birthdate, following an inside-out sequence of positioning (Fairen et al., 1986). Stop signals which inhibit cell migration are necessary for the subsequent layer-specific positioning of cortical
interneurons. Neurotransmitters, glutamate and GABA, may serve as stop signals to arrest the migration of cortical interneurons. Glutamate is highly expressed in the cortical plate (Behar et al., 1999) and released by corticofugal axon growth cones in the intermediate zone (Metin et al., 2000), while GABA is released by tangentially migrating interneurons in the intermediate zone (Poluch and Konig, 2002). Glutamate binds to AMPA receptors, the activation of which results in neurite retraction, sufficient to inhibit the migration of cortical interneurons in slice culture (Poluch et al., 2001). GABA-mediated activation of GABA_A receptors also inhibits cortical neuronal motility (Behar et al., 1998).

Netrin1-α3β1 Integrin Signaling in the Tangential Migration of Cortical GABAergic Interneurons.

Cortical GABAergic interneurons, which originate in the ganglionic eminences, take distinct tangential migratory trajectories to the developing cerebral cortex. Here, we show that migrating GABAergic interneurons express the adhesion receptor, α3β1 integrin, and that netrin-1, a diffusible guidance cue, is expressed along the superficial and deep migratory routes traversed by GABAergic interneurons toward the cerebral cortex, with strongest co-expression at the cortical marginal zone. We provide functional, in vitro evidence for netrin1-α3β1 interactions and demonstrate that these interactions stimulate chemotaxis in cortical interneurons and are achieved by netrin-1 long-range and/or paracrine signaling to α3 integrin-expressing interneurons. In addition, in vivo analysis of interneuron-specific conditional α3β1 integrin, netrin-1 mutant mice reveals deficits in the extent of migration, in the maintenance of directed tangential migration patterns, and in the number of interneurons migrating along the marginal zone. Following tangential migration, aberrant interneuronal positioning and a substantial decrease in the number of interneurons was observed.
throughout the postnatal cortex. Together, these data demonstrate that netrin1-α3β1 integrin interactions are required to promote and maintain the migration of GABAergic interneurons through the cortical marginal zone and consequently for the appropriate integration of cortical interneurons into the postnatal cerebral cortex.

Conventionally, α3β1 integrin and netrin-1 regulate neuronal migration independently. α3β1 integrin, a preferential laminin receptor, is highly expressed by migrating neurons in the developing cortex, where it is required to regulate actin cytoskeletal dynamics in response to migration modulating cues (Schmid et al., 2004). In contrast, netrin-1 binds to DCC receptors to guide the migration of striatal matrix neurons into the striatal primordium and modulate the ventral tangential migration of neurons in the lateral olfactory tract (Hamasaki et al., 2001; Kawasaki et al., 2006). Therefore, it is plausible that netrin1 and α3β1 integrin also control independent signaling pathways involved in regulating cortical tangential migration. This is supported by our observations that tangential migration deficits in α3lox−/−Dlx5/6-Cre, netrin-1+/+ and α3+/−Dlx5/6-Cre, netrin-1−/− single mutant mice are, in general, much less severe than those displayed by double mutants.

Tangentially migrating interneurons take strictly defined, superficial and deep, routes to the developing cortex. In a superficial route to the striatum, interneurons migrate into the marginal zone or subplate, whereas a deep route to the striatum guides migrating interneurons into the lower intermediate/subventricular zones. These migratory trajectories are governed by directional guidance cues. GDNF and different isoforms of NRG-1 function as attractants to guide interneurons into the cortex (Pozas and Ibanez, 2005; Flames et al., 2004), while chemorepellent activity from the preoptic area impedes the migration of interneurons in a ventral direction and factors, including semphorins 3A and 3F, restrict
migration into the developing striatum (Marin et al., 2001). These factors, however, have not been shown to specify which migratory routes are taken by GABAergic interneurons into the cortex in vivo.

We present genetic evidence of interactions within the central nervous system which preferentially guide GABAergic interneurons into the cortex through the cortical marginal zone. Real-time imaging analysis of tangential migration in E15.5 $a^{lox/-}_{3}$Dlx5/6-Cre, netrin-1$^{-/-}$ double mutant mice reveals, at the marginal zone, an increase in tangential to radial transitions directed toward the ventricular surface as well as an increase in tangentially disoriented migratory behavior. By E16.5, the migration of GABAergic interneurons through the marginal zone is significantly reduced in $a^{lox/-}_{3}$Dlx5/6-Cre, netrin-1$^{-/-}$ mutants, underscoring the relevance of netrin1-α3β1 integrin interactions for tangential migration in this region.

Of note, the expression of classical netrin-1 receptors, DCC and neogenin, which regulate attraction, spatially and temporally coincides with cortical tangential migration. At E13.5, neogenin is expressed in the cortical marginal zone, whereas DCC is expressed in both the marginal zone and intermediate zone of the developing cerebral wall (Gad et al., 1997; Shu et al., 2000). Therefore, it is possible that netrin1-DCC mediated signaling may act in concert with netrin1-α3β1 integrin interactions to regulate the migration of GABAergic interneurons.

Adhesive Interactions Found to Control Cortical Neuronal Differentiation.

The role of adhesive interactions in cortical differentiation, from cell fate specification of neural progenitor cells to the final phenotypic differentiation of cortical...
neurons, is only beginning to be uncovered. Current work has revealed adhesive functions for integrin receptors and adherens junctions in modulating cortical neuronal differentiation. As described previously, cell-ECM and cell-cell adhesive interactions mediated by β₁ integrins in the cortical ventricular zone may be necessary to sustain the neural stem cell population, whereas a downregulation of β₁ integrins contribute to the neuronal differentiation of cortical progenitor cells (Rohwedel et al., 1998; Yoshida et al., 2003; Leone et al., 2005).

Recently, conditional ablation of β₁ integrin in cortical neurons and radial glia using nestin promoter-driven Cre recombinase reveals dramatic deficits in the morphological differentiation of neurons and glia in dissociated cortical cultures (Belvindrah et al., 2007). These deficits are attributed to a deficiency of β₁ integrin in radial glial cells, since conditional deletion of β₁ integrin at E11.5 in cortical neurons alone utilizing NEX promoter-driven Cre recombinase (NEX-Cre) has no significant effect on the differentiation of neurons or glia (Belvindrah et al., 2007). However, NEX-Cre is expressed in a subpopulation of progenitor cells within the SVZ that give rise to a subset of glutamatergic pyramidal neurons in upper cortical layers (Wu et al., 2005). As NEX-Cre is expressed in a subpopulation of cortical neurons, it would be difficult to discern differentiation deficits in NEX-Cre targeted cortical neurons within heterogeneous dissociated cortical cultures and, therefore, draw conclusions regarding the role of β₁ integrin in cortical neuronal differentiation.

Adherens junctions, cell-cell adhesion structures, composed primarily of cadherins, α-catenins, and β-catenins, regulate neural progenitor cell proliferation. The extracellular domain of cadherins, single transmembrane proteins, mediates cell-cell adhesion through homophilic interactions. The cytoplasmic domain associates with β-catenin, which interacts
with α-catenin. α-catenin connects cadherin-catenin complexes with the actin cytoskeleton (Hirano et al., 1992; Yamada et al., 2005; Kobielak et al., 2004). It is postulated that the proportion of the cell surface occupied by adherens junctions serves as an indicator of cell density in the ventricular zone of the dorsal telencephalon. Increases in the cell surface area occupied by adherens junctions result in a downregulation of the Hedgehog signaling pathway, initiating a negative feedback loop which reduces the proliferation of neural progenitor cells (Lien et al., 2006). Cortical progenitor cells express αE (epithelial)-catenin, which links adherens junctions with the Hedgehog signaling pathway. Conditional deletion of αE-catenin at E10.5 in CNS stem/neural progenitors utilizing nestin promoter-driven Cre recombinase triggers inappropriate activation of the Hedgehog signaling pathway, leading to a shortening of the cell cycle, decreased apoptosis, and subsequent cortical hyperplasia (Lien et al., 2006).

**α3 Integrin Signaling in Cortical Differentiation.**

*In vitro*, conditional deletion of β₁ integrin in cortical neurons and radial glial cells using nestin promoter-driven Cre recombinase results in profound deficits in the morphological differentiation of neurons and glia (Belvindrah et al., 2007). Our work demonstrates that of the β₁ integrin receptors, α3β1 integrin mediated signaling during cortical neuronal migration modulates the differentiation of pyramidal neurons and interneurons *in vivo*. However, it is possible that the morphological changes observed in α3lox⁻/⁻hGFAP-Cre mutants are secondary to migration deficits and a lack of appropriate network connectivity or that the aberrant cortical neuronal differentiation in α3lox⁻/⁻hGFAP-Cre mutants is secondary to deficits in the radial glial scaffold. Conditional ablation of α3β1
integrin specifically in migrating cortical neurons will determine whether neuronal α3β1 integrin is necessary and sufficient for appropriate cortical neuronal differentiation.

The expression of parvalbumin defines a large postmigratory subpopulation of interneurons and correlates with their morphological and functional maturation (del Rio et al., 1994; de Lecea et al., 1995). Unlike α3lox/-hGFAP-Cre mutants, inactivation of α3 integrin by Parvalbumin promoter-driven Cre recombinase does not alter neuronal morphology. However, this data does not provide conclusive evidence that α3 integrin-mediated interactions are not required for the final differentiation of cortical neurons following neuronal migration, since α3 integrin inactivation by Parvalbumin-Cre is restricted to a subset of cortical interneurons. Yet, these results are consistent with α3β1 integrin expression during cortical development, which is highest during neuronal migration and layer formation. Specifically, α3 integrin expression is high in migrating cortical neurons and significantly downregulated in postmigratory cortical plate neurons (Anton et al., 1999; Sanada et al., 2004).

We have established that cell-ECM and/or cell-cell adhesive interactions mediated by α3β1 integrin during neuronal migration are necessary for the appropriate differentiation of cortical neurons. In the future, it will be important to determine the contribution of α3β1 integrin cell surface binding partners and ECM ligands to the aberrant neuronal differentiation phenotypes exhibited by α3 integrin mutants. This will allow us to elucidate the mechanisms of α3 integrin mediated cortical neuronal differentiation.
Future Directions.

Specific Aim 1. Determine whether netrin1-α3β1 integrin interactions are induced by HGF during cortical tangential migration.

Our results show that netrin1-α3β1 integrin interactions regulate the tangential migration of GABAergic interneurons into the developing cerebral wall. Recent data suggests that association of α3β1 integrin and netrin-1 is essential for hepatocyte growth factor-induced migration of epithelial cells on netrin-1 (Yebra et al., 2003). In the developing cortex, HGF is a motogen for tangentially migrating GABAergic interneurons. Therefore, we will test the hypothesis that the tangential migration of interneurons may be regulated by netrin1-α3β1 integrin interactions in an HGF-dependent manner.

To examine the role of hepatocyte growth factor in tangential migration dependent on netrin1-α3β1 integrin interactions, we will use Dlx5/6-cre-IRES-EGFP mice to specifically ablate α3 integrin in neuronal precursors derived from the ganglionic eminence. Dlx5 and Dlx6 are expressed throughout the lateral and medial ganglionic eminences at E12.5 and the Dlx5/6-cre-IRES-EGFP transgene marks nearly all GABAergic interneurons in the postnatal cerebral cortex (Liu et al., 1997; Stühmer et al., 2002; Stenman et al., 2003). Utilizing mice with a floxed and null Itga3 allele in a netrin1 null background, which also contain the Dlx5/6-cre-IRES-EGFP transgene (a3lox/Dlx5/6-Cre, netrin-1−/−) will allow us to inhibit α3 integrin expression specifically in Dlx5/6-expressing interneurons and permit simultaneous visualization of these cells by EGFP expression. α3 integrin conditional heterozygotes (a3lox/+Dlx5/6-Cre, netrin-1+/−) are phenotypically normal and will serve as controls.

The ability of HGF to function as a motogen in the a3lox/Dlx5/6-Cre, netrin-1−/− cortex will be tested. MGE or cortical explants from E13.5 a3lox/Dlx5/6-Cre, netrin-1−/− double mutant or control mice will be cultured with COS cell aggregates transiently transfected with...
HGF. The migration of cells derived from control explants is expected to scatter away from the COS cell source of HGF. If netrin1-α3β1 integrin interactions are promoted by hepatocyte growth factor, the migration of cells in explants derived from a3lox−/−Dlx5/6-Cre, netrin−1−/− double mutants will be compromised. Migration will be assessed by quantifying the distances around the explant traversed by migrating cells (Pozas and Ibanez et al., 2005).

The effect of HGF on cell migration mediated by netrin1-α3 integrin interactions will also be tested using organotypic slice cultures. Agarose beads coated with HGF (Snow Brand, Tochigi, Japan) will be positioned along the migratory route of GABAergic interneurons in the ventral telencephalon and cerebral wall of E13.5 coronal slices. BSA soaked beads will serve as a control. Migrating interneurons in slices from control mice are expected to scatter away from HGF coated beads. If the results of this experiment are consistent with the proposed hypothesis, the migration of GABAergic interneurons in slices from a3lox−/−Dlx5/6-Cre, netrin−1−/− double mutants will be less responsive to beads soaked with HGF. The effects of HGF on migrating interneurons will be determined by quantifying the number of GABAergic interneurons at various positions along their migratory route in relation to the coated agarose beads. Also, live imaging of GFP labeled interneurons will be used to evaluate the rate of migration, orientation, and process dynamics of control and a3lox−/−Dlx5/6-Cre, netrin−1−/− interneurons in response to HGF.

Specific Aim 2. Determine whether α3β1 integrin signaling in migrating neurons is necessary for the appropriate final differentiation of distinct classes of cortical neurons.

Our data show that conditional deletion of α3β1 integrin in migrating neurons and radial glial cells, in which the human glial fibrillary acidic protein promoter is active (α3lox−/−hGFAP-Cre), results in decreased cortical thickness, and reduced dendritic length and
branching of deep layer pyramidal neurons and interneurons. Changes in the positioning of cortical neurons expressing layer-specific markers are also observed. In contrast, the differentiation of interneurons and pyramidal neurons is unaffected in α3lox/− Parvalbumin-Cre mutants, in which α3β1 integrin is ablated in postmigratory interneurons. Yet, a specific role for neuronal α3β1 integrin in modulating cortical differentiation during migration remains to be established. Thus, we will test the hypothesis that the final layer-specific phenotype of cortical neurons is dependent on adhesive interactions mediated by neuronal α3β1 integrin during migration.

The hGFAP promoter is active in neural progenitors of the dorsal telencephalon as early as E13.5, resulting in inactivation of α3 integrin in radial glial cells and migrating neurons. Therefore, the deficits observed in the cortical neuronal differentiation of α3lox/− hGFAP-Cre mutants may be secondary to deficits in the radial glial scaffold. To specifically ablate α3 integrin expression in migrating cortical neurons, α3lox/Dlx5/6-Cre mutants will be utilized. The Dlx5/6 promoter will permit inactivation of the Itga3 gene throughout the lateral and medial ganglionic eminences at E12.5, targeting the majority of migrating GABAergic interneurons (Liu et al., 1997; Stühmer et al., 2002; Stenman et al., 2003). Immunoblotting with α3 integrin antibodies demonstrates a significant downregulation of Itga3 expression in the cerebral cortex of postnatal α3lox/− Dlx5/6-Cre mice (Figure 10 A).

To determine whether adhesive interactions mediated by α3β1 integrin in migrating cortical neurons are necessary for specifying the final differentiation of cortical neurons, the morphology of deep layer interneurons and pyramidal neurons will be examined in α3lox/− Dlx5/6-Cre mutant mice. Adult α3lox/− Dlx5/6-Cre and α3lox+/Dlx5/6-Cre mouse brains will be Golgi stained and the total neuritic length, number of primary neurites, total number of

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branch points, and number of primary, secondary, tertiary, and quaternary branch points of deep layer interneurons and pyramidal neurons will be measured. Apical and basal dendritic lengths will also be assessed in pyramidal neurons.

The differentiation of interneurons and pyramidal neurons is unaltered in $\alpha_3^{\text{lox/}}$ Parvalbumin-Cre mutants compared to controls, suggesting that $\alpha_3\beta_1$ integrin does not modulate cortical neuronal differentiation following neuronal migration. However, the number of neurons targeted in $\alpha_3^{\text{lox/}}$ parvalbumin-Cre mutant mice may be too limited for a comprehensive assessment of $\alpha_3$ integrin function in cortical neuronal differentiation, therefore, $\alpha_3^{\text{lox/}}$ synapsin I-Cre mice will be generated. Cre expression from the synapsin I promoter occurs as early as embryonic day 12.5 in differentiated neurons throughout the CNS, but is inactive in neuronal progenitors and glial cells (Zhu et al., 2001). Analysis of these mouse mutants will be performed as described for the $\alpha_3^{\text{lox/}}$ parvalbumin-Cre mice.

To evaluate the mechanisms of $\alpha_3$ integrin mediated neuronal differentiation, the role of metalloproteinases in this process will be examined. Metalloproteinases are zinc-dependent enzymes of the Metzincin superfamily, whose subfamilies include matrix metalloproteinases (MMPs) and A Disintegrin and Metalloproteinases (ADAMs). The MMP family contains at least 23 members, most of which are secreted proteins. MMPs alter ligand availability for integrin receptors by remodeling the extracellular matrix and function as ectodomain sheddases, inducing proteolytic release of the ectodomains of membrane-bound cell-surface proteins. The proteolytic activity of the metalloproteinase domain has been shown to be required for cranial neural crest cell migration (Alfandari et al., 2001). In contrast, the ADAM family, which consists of at least 30 members that are largely transmembrane proteins, contain a disintegrin as well as a metalloproteinase domain. The
disintegrin domain can interact with integrins, thereby modulating integrin-mediated cell
migration (Huang et al., 2005). In C. elegans, unc-71, an ADAM protein, acts with integrins
and netrin to promote cell migration (Huang et al., 2003). Gene chip analysis of wildtype
and α3 mutant cortices as well as our real time PCR results indicate that ADAM5 mRNA
expression is downregulated twofold, while MMP3 mRNA expression is upregulated
sixteen-fold in the α3 integrin deficient cortex at E14. This suggests that α3 integrin
mediated signal transduction cascades induce ADAM5 expression and repress MMP3
expression in the wildtype cortex during cortical neuronal migration.

To test whether ADAM5 and MMP3 contribute to the aberrant neuronal
differentiation phenotypes exhibited by α3 integrin mutants, we will first use MMP3 and
ADAM5 specific in situ hybridization probes to confirm and map the differential expression
of these metalloproteinases in α3 mutant and wildtype embryonic cortices (E16). If
differential expression is noticed, we will then evaluate the expression patterns of substrates
for these metalloproteinases in wildtype and α3 mutant cortices, focusing on substrates that
are known to be expressed in the embryonic cortex and are relevant to cortical neuronal
differentiation (e.g., reelin, laminin, fibronectin). To test the function of these
metalloproteinases in α3 integrin mediated differentiation of cortical neurons, shRNA
constructs for MMP3 and ADAM5 will be generated by inserting annealed oligonucleotide
sequences specific to MMP3 (5´-GATCTCAAAACAATCCATGGAGCCAGTTCAAGAGAC
TGGCTCCATGGATTGTTTTTTTGGAAAC-3´ and 5´-TCGAGTTCCAAAAAAACAA
TCCATGGAGCCAGTCTCTTTGAACGCTCCATGGATTGTTTTGA-3´) and ADAM5
(5´-ATCTCGCGACAGCTCTACAACAGAATTCAAGAGATTCTGTTGTAGAGCTGTC
GTTTTTGAAAC-3´ and 5´-GAGTTCCAAAAACGACAGCTCTACAACAGAAATCTCTT
GAATTCTGTTGTAGAGCTGTCGCGA-3') into the pCYLH vector. The efficiency of shRNA mediated α3 integrin knock down will be determined by testing the effect of α3 integrin targeting constructs on the expression of α3 integrin protein in primary embryonic cortical neurons. Targeting constructs will be transfected into E13.5 primary cortical cells, followed by immunoblotting cell extracts with an anti-α3 integrin antibody to confirm that α3 integrin protein expression is diminished in shRNA expressing cells. Also, MMP3 or ADAM5 expression plasmids using the chicken β-actin promoter to drive gene expression will be generated. Wildtype and α3 mutant embryonic cortical neurons (E16) will be transfected with either metalloproteinase expression vectors or metalloproteinase shRNA constructs and seeded onto reelin, laminin, or fibronectin coated substrates. Neuronal differentiation will be evaluated by labeling neurons in these cultures with Tuj-1 or neuronal cell type specific antibodies (i.e, anti-GABA for interneurons, anti-glutamate for pyramidal neurons) to evaluate the size of neuronal soma, neurite length, and the number of branches. If either of these metalloproteinases play a role in α3 mediated cortical neuronal differentiation, one would expect the expression of ADAM5 and downregulation of MMP3 to rescue the deficits in α3 mutant cortical neurons.

In summary, the proposed experiments in specific aim 1 and 2 will allow us to further examine the role of α3β1 integrin in distinct aspects of cortical neuronal migration and differentiation, processes instrumental to neuronal laminar organization in cerebral cortex. In particular, specific aim 1 will determine the involvement of hepatocyte growth factor in tangential migration dependent on netrin1-α3β1 integrin interactions and specific aim 2 will identify whether neuronal α3β1 integrin signaling is necessary for determining the final layer-specific phenotype of cortical neurons. Together, these aims will enable us to establish
whether neuronal migration and differentiation are linked through mechanisms that involve
the engagement of adhesive interactions by cortical neurons during neuronal translocation in
the developing cerebral cortex.
Figure 1. Expression of netrin-1 and α3β1 integrin in the developing cortex. (A-C) Coronal sections of the forebrain at embryonic day 13.5 labeled with α3 integrin (A) and β-galactosidase (B) antibodies demonstrate that tangentially migrating interneurons express α3β1 integrin (arrowheads in A) and indicate that netrin-1 is present at the cortical marginal zone (arrows in B) and ventricular zone of the ganglionic eminence (asterisk in B). Panel C is a merge of A and B. (D-F) High magnification of the developing cerebral wall boxed in (C) shows colocalization (arrow in F) of netrin-1 (E) and α3β1 integrin (D) at the marginal zone. (G-I) Tangentially migrating interneurons at the marginal zone express α3β1 integrin (arrowheads in G and I) and netrin-1 (arrowheads in H and I). (J-L) Radially migrating neurons in the intermediate zone express α3 integrin (arrowheads in J and L), but not netrin-1 (K and arrowheads in L). The scale bar represents 15µm. (M) Coronal section of the forebrain at embryonic day 13.5 labeled with netrin-1 antibodies demonstrate that secreted netrin-1 is expressed in the developing forebrain during tangential neuronal migration. (N) Netrin-1 expression was not detected in the forebrain of E13.5 netrin-1 null mice by immunohistochemistry. (O-P) Immunoblot of netrin-1 in the ganglionic eminence, lateral pallium, and dorsal pallium of E14.5 forebrain (P) indicate that netrin-1 is expressed in each of these regions (schematized in O).
Figure 2. Netrin1-α3β1 integrin interactions. To verify netrin1-α3β1 integrin interactions, E13.5 forebrain extracts (A), netrin-1 expressing 293 cell lysates (B), or a mixture of netrin-1 and α3β1 integrin recombinant proteins (C) were immunoprecipitated (IP) with α3 integrin or netrin-1 antibodies and western blotted (WB) with these antibodies. (A-C) In each assay, netrin-1 coimmunoprecipitated with α3 integrin and vice versa. (D) As negative co-immunoprecipitation controls, α3 integrin does not associate with immunoprecipitated netrin-1 when using E13.5 α3 integrin deficient forebrain extracts and, likewise, netrin-1 does not interact with immunoprecipitated α3 integrin when utilizing E13.5 netrin-1 deficient forebrain extracts. (E) The co-immunoprecipitation of α3 integrin and laminin-1 as well as netrin-1 and DCC from E14.5 forebrain lysates serve as positive controls. (F) Robust β1 integrin receptor activation is observed following concentrated supernatant netrin-1 (sup), recombinant mouse netrin-1 (rm), and ECM stimulation of MGE progenitor cells compared to control.
Figure 3. Netrin1-α3β1 integrin interactions promote the migration of MGE neuroblasts. (A and B) MGE explants from E14.5 wildtype mice cocultured with control (A) or netrin-1 expressing (B) 293 cell aggregates show preferential migration toward a netrin-1 source. The scale bar represents 300µm. (C and D) MGE explants from E14.5 α3 -/- mice cocultured with control (C) or netrin-1 expressing (D) 293 cell aggregates display uniform cell migration. Quantification of MGE cell chemoattraction is expressed as a ratio of the optical density of MGE cells proximal to the 293 cell aggregate to the optical density of MGE cells distal to the 293 cell aggregate. All error bars = SEM. * = p < 0.05, Student’s t test (data from 3 independent experiments).
Figure 4. Disruption of Netrin1-α3β1 Integrin Interactions Reduce the Number of Interneurons Migrating Through the Cortical Marginal Zone. (A) Cortical tangential migration through the marginal zone at E16.5 is significantly reduced in α3lox/lox Dlx5/6-Cre, netrin-1−/− double mutants compared to single mutant and control mice. α3+/+Dlx5/6-Cre, netrin-1−/− mutants exhibited a disorganization of cell distribution within the marginal zone. The scale bar represents 100µm. (B) A corresponding increase in EGFP+ cells ectopically migrating through the ventricular zone is evident in α3lox/lox Dlx5/6-Cre, netrin-1−/− double mutants. α3lox/lox Dlx5/6-Cre, netrin-1+/− and α3+/+Dlx5/6-Cre, netrin-1−/− single mutant mice also exhibited a significant, though smaller, increase in EGFP+ cells ectopically placed in the ventricular zone compared to controls. (C) Quantification of cell density at the marginal zone. (D) Quantification of cell density at the ventricular zone. (E) Quantification of marginal zone cell density adjusted for the corresponding increase in ventricular zone cell density. All error bars = SEM. * = p < 0.01, one-way ANOVA, Tukey-Kramer post hoc.
Figure 5. Real-Time Assessment of Interneuronal Migration Patterns Through the Cortical Marginal Zone Reveals Abnormal Migration in α3lox−/Dlx5/6-Cre, Netrin-1−/− Double Mutants. (A-D). Live imaging of E15.5 cortices reveals an increase in the number of interneurons transitioning from tangential migration along the marginal zone to ventricular surface-directed radial migration in α3lox−/−Dlx5/6-Cre, netrin-1−/− double mutants (B, cells highlighted in blue) compared to α3lox+/+Dlx5/6-Cre, netrin-1+/− controls (A, cells highlighted in red). The number of interneurons reversing their tangential trajectories in the vicinity of the marginal zone is increased in α3lox−/−Dlx5/6-Cre, netrin-1−/− double mutant cortices (D, cells highlighted in blue) compared to controls (C, cells highlighted in red). The scale bar represents 50µm. (E) Quantification of the number of interneurons migrating towards the dorsal telencephalon in controls and double mutant mice. (F-G). Quantification of the number of interneurons transitioning from tangential to radial migration (F) and reversing tangential trajectories (G) along the marginal zone. Time elapsed since the onset of observations is indicated in minutes. All error bars = SEM. * = p < 0.05, Student’s t test. ps, pial surface.
Figure 6. Migration of Interneurons into the Cortex and Through the Marginal Zone Depends on Non-Cell-Autonomous Netrin-1 and Cell-Autonomous α3 Integrin Functions. (A-H). Slice coculture assay of α3lox/+, Dlx5/6-Cre, netrin-1+/+ control and α3lox−/−, Dlx5/6-Cre, netrin-1−/− E14.5 MGE explants positioned over the MGE of coronal sections from E14.5 wildtype and netrin-1−/− mice. α3 integrin deficient explants placed on wildtype cortical slices (B, F) and control explants placed on netrin-1 mutant slices (C, G) did not reveal a significant deficit in the number of EGFP+ cells which had migrated to the cerebral cortex or through the marginal zone, whereas the number of EGFP+ cells in the cortex and marginal zone of netrin-1 deficient slices containing α3 integrin deficient explants (D, H) is significantly reduced compared to the control condition (A, E). The scale bar represents 200µm. (I-J). Quantification of cell migration to the cortex (I) and through the marginal zone (J). All error bars = SEM. * = p < 0.05, Mann-Whitney U test (n = 10 slice cocultures per experimental group). mz, marginal zone; cp, cortical plate; iz, intermediate zone; svz, subventricular zone; vz, ventricular zone.
Figure 7. Loss of Netrin1-α3β1 Integrin Interactions Decrease the Number and Alter the Positioning of Interneurons in Cortex at P0. (A-D). $\alpha^3_{lox/-}$ Dlx5/6-Cre, netrin-$1^{-/-}$ double mutant mice (D), unlike $\alpha^3_{lox/-}$ Dlx5/6-Cre, netrin-$1^{+/+}$ (B) and $\alpha^{+/+}$ Dlx5/6-Cre, netrin-$1^{-/-}$ (C) single mutants, display a dramatic reduction in the number of EGFP$^+$ cells at the marginal zone and throughout the P0 cortex compared to controls (A). The scale bar represents 200µm. (E-F). Quantification of the number of EGFP$^+$ cells in the marginal zone (E) and cortex (F). (G-J). The number of calbindin$^+$ interneurons in the marginal zone and cortex of $\alpha^3_{lox/-}$
dlx5/6-Cre, netrin-$1^{+/-}$ double mutants (J) is substantially diminished. A reduction in the number of cells in the cortex is also observed in $\alpha^3_{lox/-}$ Dlx5/6-Cre, netrin-$1^{+/-}$ single mutant mice (H), but not in $\alpha^{+/-}$ Dlx5/6-Cre, netrin-$1^{-/-}$ single mutants (I). Both single mutant (H, I) and double mutant mice (J) display an increase in ectopic calbindin$^+$ cells positioned in the lower half of the cortical plate compared to controls (G). The scale bar represents 200µm. (K-L). Quantification of the number of calbindin$^+$ cells in the marginal zone (K) and cortex (L). All error bars = SEM. * = p < 0.01, one-way ANOVA, Tukey-Kramer post hoc test. mz, marginal zone; cp, cortical plate; iz, intermediate zone; svz, subventricular zone.
Figure 8. Increased Cell Death in α3lox/Dlx5/6-Cre, Netrin-1−/− Double Mutants (A-B). Active caspase-3 antibodies (red) applied to E16.5 cortices demonstrate significantly increased cell death in α3lox/Dlx5/6-Cre, netrin-1−/− double mutant cortices (B) compared to α3lox/+Dlx5/6-Cre, netrin-1+/+ controls (A). The scale bar represents 200µm. (C). Quantification of caspase-3 activity. * = p < 0.01, Student’s t test.
Figure 9. Misrouted Cell Migration and Disorganization and Reduced Numbers of Hippocampal Interneurons in $\alpha_3^{\text{lox/}}$ Dlx5/6-Cre, netrin-1$^{-/-}$ Mutants at P0. (A, D, E, H). A reduction in and dramatic disorganization of EGFP$^+$ interneurons throughout the hippocampal subfields of $\alpha_3^{\text{lox/}}$ Dlx5/6-Cre, netrin-1$^{-/-}$ double mutants is evident (D, H) compared to controls (A, E). The scale bar represents 500μm. (A-D, I-L). An ectopic aggregation of cells observed in $\alpha_3^{\text{lox/}}$ Dlx5/6-Cre, netrin-1$^{-/-}$ double mutant mice (D, L), is not observed in single mutant (B, C, J, K) and control (A, I) mice.
Figure 10. (A).
Characterization of Conditional α3 Integrin Inactivation.
Immunoblots of α3 integrin in control and F/−, hGFAP-Cre+, F/−, Nestin-Cre+, or F/−, Dlx5/6-Cre+ postnatal cortices indicate that α3 integrin expression is significantly reduced in F/−, hGFAP-Cre+, F/−, Nestin-Cre+, and F/−, Dlx5/6-Cre+ mutants. (B-D). LacZ expression, indicated by X-gal staining, in coronal sections of the parvalbumin-cre+, Rosa26lacZlox transgenic cerebral cortex occurs at postnatal day 8 (B). Application of β-galactosidase antibodies to postnatal day 10 sections (C) indicates a similar expression pattern. The scale bar represents 200µm. High magnification of the cerebral cortex (D) reveals cytoplasmic β-galactosidase expression. The scale bar represents 20µm. (E-J).
Immunohistochemistry for α3 integrin (E, G, H, J) and parvalbumin (F, G, I, J) in adult parvalbumin conditional mutants (H-J) shows that unlike controls (E-G), parvalbumin+ cells do not express α3 integrin (white arrows). The scale bar represents 20µm.
Figure 11. (A-D). Reduced Cortical Thickness in α3<sup>lox/lox</sup>/hGFAP-Cre Mutants. Nissl stain of coronal sections of adult cortices reveals a reduction in the cerebral cortical width of α3 F/-, hGFAP-Cre mutants (B and D) compared to F/+, hGFAP-Cre controls (A and C). The scale bar represents 100µm. (E, F). Nissl stain of sagittal sections of adult brains indicates a pronounced decrease in the width of the caudal cerebral cortex of α3 F/-, hGFAP-Cre mutants (F) compared to F/+, hGFAP-Cre controls (E). The scale bar represents 200µm. (G). Quantification of cortical thickness in control and F/-, hGFAP-Cre mice. All error bars = SEM. * = p < 0.01, Student’s t test.
Figure 12. (A, B). Altered Layer Specific Marker Expression in $\alpha3^{lox/-}$ hGFAP-Cre Mutants. Reelin immunohistochemistry reveals, compared to controls (A), an increase in reelin-expressing cells of layer I in $\alpha3$ mutants (B). The scale bar represents 50µm. (C, D). Brn-1 labeling indicates ectopic distribution of Brn-1 in $\alpha3$ mutant mice (D) compared to controls (C). The scale bar represents 100µm. (E, F). Er81 immunohistochemistry also reveals ectopic distribution of Er81 in $\alpha3$ mutants (F) compared to controls (E). (G, H). Tbr-1 labeling indicates that the distribution of Tbr-1 in the $\alpha3$ mutant cortex (F) is mostly unperturbed compared to control cortex (E). Quantification of layer-specific marker expression is indicated adjacent to the corresponding images. All error bars = SEM. * = p < 0.001, Student’s t test.
Figure 13. Defective Cortical Differentiation in α3lox/hGFAP-Cre Mutants. (A, B). Representative Golgi stained pyramidal neurons of cerebral cortex in control [F/+, hGFAP-Cre; A] and mutant [F/−, hGFAP-Cre; B] mice. The scale bars represent 20µm (C, D). Quantification of neurite length, apical and basal dendrite length, number of primary neurites, total branch points, and branching complexity of deep layer pyramidal neurons (C) and interneurons (D). (E, F). Representative camera lucida images of Golgi stained deep layer pyramidal neurons (top) and interneurons (bottom) of control (E) and Itga3 mutant (F) mice. All error bars = SEM. * = p < 0.01, Student’s t test.
Figure 14. Differentiation of Cortical Neurons is Unaffected in $\alpha^3\text{lox/-}$ Parvalbumin-Cre Mutants. (A, B). Representative Golgi stained interneurons of cerebral cortex in control [F/+, Parvalbumin-Cre; A] and mutant [F/-, Parvalbumin-Cre; B] mice. The scale bars represent 20µm. (C, D). Quantification of neurite length, apical and basal dendrite length, number of primary neurites, total branch points, and branching complexity of deep layer pyramidal neurons (C) and interneurons (D). (E, F). Representative camera lucida images of Golgi stained deep layer pyramidal neurons (top) and interneurons (bottom) of control (E) and Itga3 mutant (F) mice.
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