GSK-3 REGULATION OF MIGRATION AND MORPHOGENESIS IN THE DEVELOPING CORTEX

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ABSTRACT

Meghan Morgan-Smith: GSK-3 Regulation of Migration and Morphogenesis in the Developing Cortex
(Under the direction of William D. Snider)

The majority of the neurons in the mammalian cerebral cortex are glutamatergic excitatory neurons that have a specific migration pattern and a well-defined 'pyramidal' morphology. Pyramidal neurons migrate on a radial glia scaffold to reach a layer-specific destination in the cortex and extend two polarized processes: the apical dendrite and the axon. Defining molecular mechanisms of migration and morphogenesis are key to understanding circuit formation in the developing cortex.

Recent studies have demonstrated that GSK-3 has a critical role in controlling neuronal number through regulation of radial progenitor proliferation and intermediate precursor amplification. However, only *in vitro* work has addressed GSK-3 functions related to cortical neuronal morphogenesis. The primary conclusion has been that, downstream of RTK/PI3K pathways, GSK-3 inhibition favors axon specification over dendrite formation. Often, *in vitro* studies cannot be recapitulated *in vivo*. For my thesis, I have generated mouse lines that allow for the *in vivo* deletion of GSK-3 in newly born excitatory neurons of the cerebral cortex.

I have identified a cell autonomous requirement for GSK-3 signaling in neuronal migration. GSK-3 regulation is specific for radial migration as tangential migration is not

affected. Additionally, GSK-3 signaling regulates key aspects of morphogenesis including development of the apical dendrite and orientation of the basal arbor. Interestingly, GSK-3 regulation of migration is not mediated by β-catenin signaling and appears to be independent of the RTK/PI3K pathways. Rather, I find strong reductions in phosphorylation of two microtubule associated proteins: the migration mediator Doublecortin and the semaphorin signaling mediator CRMP-2. Further, the abnormalities in dendritic morphology I describe bear similarities to abnormal semaphorin signaling.

I conclude that GSK-3 signaling is essential for proper circuit formation and connectivity in the developing cerebral cortex via regulation of neuronal migration and polarized morphological elaboration. I further demonstrate that GSK-3 regulation of developing cortical neurons is through a signaling cascade that is distinct from regulation of progenitor homeostasis. My work emphasizes the importance of GSK-3 signaling in multiple aspects of the development of the mammalian cerebral cortex.

To my Family - We are low on sleep but high on love. We can...together!

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Finally, I would have not been able to accomplish this dissertation without the constant support from my husband, Garrett. Each day we support each other with love, laughter and sarcasm. Thank you for your love, support, and for our greatest achievements, Sadie Jaden and Coleson Michael.

'It is not your aptitude, but your attitude that determines your altitude.' - Mr. Bottini, former Social Studies Teacher at Perry Jr. High in New Hartford, NY.

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LIST OF ABBREVIATIONS AND SYMBOLS

APC - adenomatous polyposis coli

aPKC - atypical Protein Kinase C

CDC42 - cell devision cycle 42

CDK5 - cyclin dependent kinase 5

CK1 - casein kinase 1

CK2 - casein kinase 2

CP - cortical plate

CRMP-2 - collapsin response mediator protein 2

DCX - Doublecortin

DISC1 - Disrupted in Schizophrenia 1

DIV - days in vitro

Dvl - Dishevelled

E - embryonic day (post conception)

EGF - Epidermal Growth Factor

ERK - Extracellular signal related kinase

GFP - green fluorescent protein

GSK-3 - Glycogen Synthase Kinase 3

IGF - Insulin Growth Factor

IPC - intermediate progenitor cell

IUE - in utero electroporation

IZ - intermediate zone

JNK - Jun amino-terminal Kinase

LEF - lymphoid enhancer factor

LKB1 - serine threonine kinase 11

LP- leading process

LTD - Long-term depression

LTP - Long-term potentiation

MAP - microtubule associated protein

MGE - medial ganglionic eminence

MZ - marginal zone

NRP1 - neuropilin-1

NPC - neural progenitor cell

P - postnatal day (post birth)

PAR - partitioning-defective proteins

PDGF - Platelet-derived Growth Factor

PI3K - Phosphoinositide-3 Kinase

PKA - Protein Kinase A

PP - preplate or primordial plexiform layer

PTEN - Phosphatase and tensin homolog

RTK - Receptor Tyrosine Kinase

Sema - semaphorin

Ser - Serine

Shh - sonic hedgehog

SP - subplate

STRAD - STE20 Related Kinase

SVZ - subventricular zone

TAB1 - TAK1 binding protein 1

TAB2 - TAK1 binding protein 2

TAK1 - TGF-β Activated Kinase 1

TCF - T-cell Factor

Thr - threonine

TP- trailing process

Tyr - tyrosine

SVZ - subventricular zone

VZ - ventricular zone

α - alpha

β - beta

CHAPTER 1: INTRODUCTION

The proper development of the cerebral cortex underlies the connectivity required for cortical function. I have been fascinated by several aspects of this process including neuronal migration, layer formation and early aspects of neuronal morphogenesis since BBSP interview weekend where Dr. Sabrice Guerrier, then a graduate student of the Polleux Lab, presented a movie of migrating cortical neurons.

Several recent advances have made it particularly exciting to study cortical development. First, mutations in humans and mice that result in layer formation deficits provide clues to the molecular basis of neuronal migration. Second, the development of techniques including mouse mutagenesis and *in utero* electroporation allow for decisive testing of protein function *in vivo*. Finally, the development of cell biological methods now allows for detailed visualization of neurodevelopmental events including the visualization of migrating neurons in real-time.

In both the Polleux and Snider labs I have worked on master kinases that are key regulators of cortical neuronal development. Of particular note, and the main subject of my thesis, is Glycogen Synthase Kinase-3 (GSK-3). GSK-3 signaling has been implicated in the Reelin pathway, probably the best known molecular regulator of migration in the cortex. Further, GSK-3 regulates key microtubule binding proteins like Doublecortin (DCX) and collapsin response mediated protein 2 (CRMP-2), which are known to be important in

migration. Interestingly, cyclin dependent kinase 5 (CDK5), a kinase already known to be important in migration shares many substrates with GSK-3. Finally, GSK-3 is a key mediator in the semaphorin pathway that is known, from Franck Polleux's work, to regulate apical dendrite morphology.

Work from our lab and others have defined a role for GSK-3 in the regulation of neuronal progenitors (Kim, Wang et al. 2009; Fang, Chen et al. 2013). To date there is no mouse genetic work on the functions of GSK-3 in early developing cortical neurons. Defining GSK-3 functions will be very important given that GSK-3 functions in that pathway by which the schizophrenia associated protein, DISC-1, functions in brain development.

As an introduction to this subject matter, Chapter 2 will provide a review of cortical development while Chapter 3 will summarize and GSK-3 signaling. In Chapter 4, I present a study on cortical developmental functions of TAK1, a kinase upstream of LKB1 (my work under the supervision of Franck Polleux). The primary focus of my thesis is Chapter 5, 'GSK-3 regulation of migration and morphogenesis in the developing cortex'. This chapter is formatted as a manuscript to be submitted to eLIFE within the next few weeks. Finally, Chapter 6 is a discussion of future directions that may spring from my thesis work.

CHAPTER 2: Cortical Development

2.1 Overview

The development of the mammalian neocortex is dependent on precisely timed and regulated neurogenesis, cell polarization, migration and morphological development. These processes are the framework for proper circuit connectivity and function. Defining the molecular regulation by GSK-3, or any other protein, requires detailed understanding of the timing and nature of these sequential steps in building the cortex. In this chapter I briefly present a summary of murine cortical development relevant to the experimental findings described in my main paper (Chapter 5). The original literature in this area is vast. I have selectively cited some of the original papers as well as some recent comprehensive reviews. Importantly, a new three-volume series 'Comprehensive Developmental Neuroscience' edited by John Rubinstein and Pasko Rakic contains a volume dedicated to cellular migration and circuit formation with several outstanding chapters that summarize the current state of thinking.

2.2 Rodent and Primate Models

The cerebral cortex is a highly evolved and complex structure that is essential for the more evolved functions of the human brain. Two functional classes of neurons are present in the mammalian cerebral cortex, excitatory pyramidal neurons and inhibitory interneurons. Pyramidal neurons compose approximately 80% of the neurons in the cortex and are

responsible for the excitatory glutamatergic signaling, while interneurons are responsible for inhibitory GABAergic signaling (for review see Rubenstein 2011). If the processes that regulate key aspects of pyramidal neuronal development are disrupted, structural brain malformations and developmental delay can result, as well as more subtle abnormalities that underlie disorders like schizophrenia (Mochida and Walsh 2004; Kumar, Sundaram et al. 2010).

An important point for mouse work is that rodent and primate models exhibit similarities to humans in progenitor regulation, migratory patterns, and neuronal maturation (Molnar, Metin et al. 2006). This similarity makes mouse genetic studies useful for defining the mechanisms underlying cortical development in humans. Importantly, many human symptoms and disease states can be analyzed in genetically modified mouse models.

Though both rodent and primate cortical development have similar patterns, a vast increase in cortical volume is associated with more evolved species (Krubitzer and Kaas 2005). While rodents lack a folded cortex (lissenencephalic), the primate cortex is gyrated due to a massive increase in cell number (Willis 1664). This difference is largely due to the enlarged progenitor area known as the outer subventricular zone, which is vastly enlarged in primates (Smart, Dehay et al. 2002; Hansen, Lui et al. 2010). However, the cell types present and overall process of cortical development are similar between primates and rodents (for review see Molnar 2011). This similarity makes genetic manipulation and elucidation of cortical circuit and function in the mouse model appropriate for our understanding of the development of the cortices of all mammals.

2.3 Progenitor Proliferation and Lamination

The development of the murine neocortex begins around embryonic day 10 (E10) (Angevine 1970; Borrell and Reillo 2012). At this time the neural progenitor cells (NPCs) symmetrically divide to expand the progenitor pool of radial glial cells located in the ventricular zone (VZ) (Haubensak, Attardo et al. 2004; Kriegstein and Alvarez-Buylla 2009). Radial glia are a specialized cell type that have cell bodies located in the VZ with long basal processes reaching the pial surface and shorter apical processes with end-feet near the ventricle (Schmechel and Rakic 1979; Ayala, Shu et al. 2007) (for recent progenitor review see Borrell and Reillo, 2012) **[Figure 2.2]**. Radial glia cells were originally described in small mammals and human fetuses by Giuseppe Magini and Camillo Golgi in the mid-late 1800‘s (see Bentivogolio and Mazzarello, 1999 for a historical review) and were originally thought to provide a scaffold for neuronal migration (Rakic 1971; Rakic 1972). A major advance in the 2000s was the demonstration that in mice, radial glia are the progenitor pool for most of the neurons and glia in the cerebral cortex (Noctor, Flint et al. 2001; Lui, Hansen et al. 2011).

The neurogenic phase of cortical development begins from E10.5 to E12 (Ayala, Shu et al. 2007). During this time the radial glia cells asymmetrically divide to form a daughter radial glial cell and a newly born glutamatergic pyramidal neuron (Angevine and Sidman 1961; Noctor, Flint et al. 2001; Evsyukova, Plestant et al. 2013). Important to my thesis work, radial progenitor proliferation is strongly regulated by GSK-3 (Kim, Wang et al. 2009), see Chapter 3.

Beginning around E14.5 radial glia cells also divide to form intermediate progenitor cells (IPCs) which migrate and populate the subventricular zone (Noctor, Martinez-Cerdeno et al. 2004). These transient progenitors symmetrically divide into daughter IPCs or daughter neurons with intracortical projections (Noctor, Martinez-Cerdeno et al. 2004; Noctor, Martinez-Cerdeno et al. 2008; Kriegstein and Alvarez-Buylla 2009). Both the symmetric divisions of IPCs and the asymmetric divisions of radial glia progenitors are responsible for the rapid expansion of the cortex (Smart, Dehay et al. 2002; Hansen, Lui et al. 2010). A recent study has identified a new role for GSK-3 in IPC amplification (Fang, Chen et al. 2013). This work further solidifies a role for GSK-3 in regulating progenitors and controlling neuronal numbers in the developing cortex.

The neocortex is a highly laminated structure made of six layers. Each layer is identifiable by molecular markers and pyramidal cell morphology (DeFelipe and Farinas 1992; Hevner, Daza et al. 2003; Molyneaux, Arlotta et al. 2007). Importantly, the laminar fate of newly born neurons is dependent on the timing of radial glia cell division. Beginning around E10.5, the first born immature neurons migrate towards the pial surface to form the transient preplate, the beginning of the cortex (Gupta, Tsai et al. 2002; Nadarajah and Parnavelas 2002) **[Figure 2.1]**. Ultimately some these cells will become the most superficial layer of the cortex, layer 1, containing Cajal-Retzius cells that secrete Reelin to regulate migration (see below) (D'Arcangelo, Miao et al. 1995). Recently, a study has suggested that some radial glia cell have specified neuronal fates, arguing that both timing and specific radial glia subtypes are responsible for the lamination of the cortex (Franco, Gil-Sanz et al. 2012).

Newly generated neurons fated to form the laminated cortex are born around E12.5 and migrate to split the preplate and form the beginning of cortical plate (CP) **[Figure 2.1]** (for review see Evsyukova et al, 2013). These first neurons will form the deepest layer of the cortex, layer 6, while the preplate cells transition into the marginal zone and subplate (Marin-Padilla 1978; Gupta, Tsai et al. 2002; Molyneaux, Arlotta et al. 2007) **[Figure 2.1]**. Later born neurons will enter the cortical plate and migrate past previously born neurons to populate the outermost positions in the developing cortex, layers 2-5 **[Figure 2.1]**.

The developmental sequence of birth and migration past existing neurons continues until E18 and gives the cortex an 'inside-out' lamination where the oldest neurons populate the deepest layers and the youngest neurons are located towards the pial surface (Angevine and Sidman 1961; Angevine 1970; Rakic 1974; Marin-Padilla 1978). After E18, radial glia cells retract their processes and differentiate into astrocytes (Mission, Takahashi et al. 1991; Kriegstein and Alvarez-Buylla 2009; Li, Newbern et al. 2012). At this stage the neurons finish migration, extend axons to their targets, and develop dendrite arbors. Ultimately the subplate degenerates and the six-layered cortex remains through adulthood (Luskin and Shatz 1985; Allendoerfer and Shatz 1994).

In addition to the excitatory pyamidal neurons of the neocortex, inhibitory GABAergic interneurons also populate the cortex. These neurons are born outside of the cortex in the medial ganglionic eminence (MGE) and migrate into the cortex beginning at E12.5 (Parnavelas, Barfield et al. 1991; Anderson, Eisenstat et al. 1997; Jimenez, Lopez-Mascaraque et al. 2002). As with excitatory neurons, interneurons also populate all layers of

the cortex. Interestingly, I have found important differences in GSK-3 regulation of excitatory versus inhibitory neurons.

2.4 Neuronal Migration

Migration at the earliest stages of cortical development is characterized by somal translocation. First characterized in the opossum, early born neurons from E10-E12 form the primordial plexiform layer, or preplate, and extend a leading edge to the pial surface (Allendoerfer and Shatz 1994; Nadarajah, Brunstrom et al. 2001; Nadarajah and Parnavelas 2002). The soma then translocates out of the VZ and the neuron retracts the apical process that is extended to the ventricular zone (Morest 1970; Brittis, Meiri et al. 1995; Nadarajah, Brunstrom et al. 2001; Marin and Rubenstein 2003) **[Figure 1.2A]**. This initial form of migration does not require the use of a radial glia scaffold, occurs in a smooth and continuous motion, and is not the primary mode of migration by the majority of neurons that will populate the cortical plate (Nadarajah, Brunstrom et al. 2001; Evsyukova, Plestant et al. 2013). However, somal translocation does occur as the final stage of the second mode of migration, glial-guided migration (Nadarajah, Brunstrom et al. 2001; Marin and Rubenstein 2003).

The second form of migration, first identified in fetal monkey neocortex, is known as glial-guided migration or locomotion (Rakic 1971; Rakic 1972). An immature multipolar neuron derived from a radial glial precursor will migrate to the SVZ, and occasionally back to the VZ, where it extends and retracts neurites (Tabata and Nakajima 2003; Noctor, Martinez-Cerdeno et al. 2004; LoTurco and Bai 2006; Tabata, Kanatani et al. 2009) **[Figure**

2.2A]. These dynamic neurites are thought to sense the local environment for a polarizing cue. The multipolar neuron will then polarize and transition to a bipolar morphology consisting of a short leading process extending towards the pial surface, and a thin trailing process that will become the axon **[Figure 2.2A]** (Brittis, Meiri et al. 1995; Nadarajah, Brunstrom et al. 2001). The bipolar morphology allows for radial glia attachment and guided directional migration (for migration reviews see Marin and Rubenstein, 2003, Gupta et al. 2002, Evsyukova et al, 2013). Neurons migrating in this fashion often move in short bursts, a saltatory migration, with long periods of pause between movement (Nadarajah, Brunstrom et al. 2001). Successive waves of newly generated neurons migrate into the cortical plate using this glial-guided locomotion to form the laminated structure of the cortex.

Inhibitory interneurons use a third form of migration, tangential migration, to populate the cortical plate **[Figure 2.2B]**. These neurons migrate into the cortex in two streams through the marginal zone or subplate (DeDiego, Smith-Fernandez et al. 1994; Anderson, Eisenstat et al. 1997; Lavdas, Grigoriou et al. 1999; Anderson, Marin et al. 2001). These cells migrate tangentially to the radial glia cells in the cortex and do not rely on radial glia interactions to populate the cortical plate (Anderson, Eisenstat et al. 1997; Corbin, Nery et al. 2001; Marin and Rubenstein 2003; Yokota, Gashghaei et al. 2007). Instead, these neurons use a combination of interactions with axons, dendrites and radial glia to reach their final positions (Polleux and Ghosh 2002). Interneurons often change direction and extend and retract leading processes (Kriegstein and Noctor 2004). However, once these cells reach the cortex they invade all layers in a radial fashion, but without proper leading edge orientation along the radial glia scaffold (Polleux, Whitford et al. 2002; Ang, Haydar et al.

2003; Marin and Rubenstein 2003). Importantly, radial and tangential migration are thought to be regulated quite differently (see below).

All neuronal movements require complex and precise regulation of cytoskeletal dynamics. Specifically, the highly dynamic multipolar state, transition to bipolar morphology, and physical movement along radial glia require especially fine control of cytoskeletal dynamics **[Figure 2.3]**. As such, many microtubule associated proteins (MAPS) have been identified as regulators of migration. These include key proteins like Doublecortin (DCX) (des Portes, Pinard et al. 1998; Gleeson, Allen et al. 1998; Friocourt, Liu et al. 2007; Bilimoria, de la Torre-Ubieta et al. 2010), LIS1 (Tsai, Chen et al. 2005), MAP1B (Gonzalez-Billault, Del Rio et al. 2005; Trivedi, Marsh et al. 2005) and CRMP2 (Yoshimura, Kawano et al. 2005; Ip, Shi et al. 2012). Importantly for my thesis work, many of these MAPs are GSK-3 targets and several are also targets of the known migration regulator CDK5 (Tanaka, Serneo et al. 2004; Li, Hawkes et al. 2006; Xie, Samuels et al. 2006)(See Chapter 3 and 5).

In conjunction with cytoskeletal dynamics, adhesive molecules are also required for adherence to the radial glia and migration along its fibers (for adhesion review see Solecki 2012). Adhesion molecules must be inserted in the membrane at the leading edge and also removed from the cell body and trailing process to enable the cell to migrate to the pial surface (Solecki 2012) **[Figure 2.3]**. For this to occur the cell must regulate endocytosis in specific compartments (Shieh, Schaar et al. 2011). Interestingly, GSK-3 has been shown to regulate dynamin phosphorylation (Clayton, Sue et al. 2010) and bulk endocytosis in cultured neurons. Finally, in addition to adhesion, force-generating acto-myosin contractility is required to facilitate migration (Valiente and Marin 2010; Trivedi and Solecki 2011).

To cease migration the neuron senses the appropriate 'stop' signal to detach from the radial glia cell and somally translocate to reach its final position (Nadarajah, Brunstrom et al. 2001) (see Evsyukova et al. 2013 for review). The major signaling molecule associated with ceasing migration is the glycoprotein Reelin. Reelin is secreted from the Cajal-Retzius cells located in the marginal zone and is essential for splitting the preplate and the termination of migration (D'Arcangelo, Miao et al. 1995; Ogawa, Miyata et al. 1995; Franco and Muller 2011; Evsyukova, Plestant et al. 2013). In the absence of Reelin, neurons are unable to split the preplate or migrate past previously born neurons (D'Arcangelo, Miao et al. 1995; Ogawa, Miyata et al. 1995; Evsyukova, Plestant et al. 2013). Again important for my thesis work, GSK-3, CDK5 and the adherence molecule N-cadherin have been implicated downstream of Reelin signaling (Beffert, Morfini et al. 2002; Gonzalez-Billault, Del Rio et al. 2005; Jossin and Cooper 2011).

2.5 Axon and Dendrite Formation

During migration, neurons rapidly develop a polarized morphology that facilitates axonal and dendritic elaboration and targeting. *In vivo* the axon arises from the trailing process during neuronal migration (Rakic 1972; Schwartz, Rakic et al. 1991; Barnes, Solecki et al. 2008). However, much of our knowledge of axon initiation comes from dissociated culture paradigms (Dotti and Banker 1987; Lewis, Courchet et al. 2013). In the favored model of dissociated embryonic hippocampal neurons, multipolar neurons extend and retract their neurites until a single neurite elongates to form an axon (Dotti and Banker 1987; Dotti, Sullivan et al. 1988) **[Figure 2.3]**. This process is regulated by multiple kinases responsible

for regulating motor proteins, membrane trafficking and signal transduction. For example, PI3K (Shi, Jan et al. 2003), LKB1 (Barnes, Lilley et al. 2007; Shelly, Cancedda et al. 2007) and GSK-3 (Shi, Cheng et al. 2004) regulate polarization *in vitro* through signals transduced to downstream substrates. *In vitro* results are now being addressed using *in vivo* models. For example, deletion of LKB1 in progenitors gives rise to neurons that are unable to form an axon *in vivo* (Barnes, Lilley et al. 2007). In contrast, *in vivo* deletion of GSK-3 in cortical progenitors keeps progenitors in a proliferative state (Kim, Wang et al. 2009)(see below). Therefore neuronal morphogenesis was not assessed in the initial study of effects of GSK-3 deletion on neuronal development *in vivo*.

Each layer of the cortex contains pyramidal neurons with a specific morphology and projection pattern. Briefly, deeper layers of the cortex project subcortically while upper layers project intracortically (Gaspard and Vanderhaeghen 2011; Deboer, Kraushar et al. 2013). More specifically, layer VI neurons extend corticothalamic projections while layer V neurons develop mostly corticobulbar, coticospinal and corticotectal projection patterns with a few callosally projecting neurons mixed in (Hallman, Schofield et al. 1988; Molnar and Cheung 2006; Deboer, Kraushar et al. 2013) **[Figure 2.4]**. Layer IV neurons receive sensory input from thalamocortical axons and project short-range axons to cortical targets (Gaspard and Vanderhaeghen 2011). Neurons in the outermost layer II-III, and the focus of my thesis work, project contralaterally through the corpus callosum as well as within the same cortical hemisphere (Molyneaux, Arlotta et al. 2007; Deboer, Kraushar et al. 2013).

Axonal targeting after initial polarization has been studied in multiple model systems and several diffusible cues have been identified. Receptors in the growth cone respond to

chemoattractant and chemorepulsive cues that enable proper targeting. Netrin is an axon chemoattractant and required for the formation of commisures in the spinal cord and the corpus callosum (Lai Wing Sun, Correia et al. 2011; Fothergill, Donahoo et al. 2013). Interestingly, Netrin signaling can act as both a chemoattractant and a chemorepellant (Colamarino and Tessier-Lavigne 1995). In the cortex, semaphorin-neuropilin1 (Luo, Raible et al. 1993; Pasterkamp 2012) ligand receptor signaling acts as a repulsive cue for developing axons. Dual purpose signaling is also evident in semaphorin signaling where semaphorin 3A can act as a chemoattractant for apical dendrites of cortical pyramidal neurons and a repellant for their axons (see below) (Polleux, Morrow et al. 2000).

Morphological development of pyramidal neurons and radial migration are closely associated processes. At the most basic level, during radial-guided migration the leading process will become the apical dendrite and project towards the pial surface (Miller 1981). Branching of the apical dendrites occurs mostly far away from the cell soma in the apical tuft located in the marginal zone. Thinner basal dendritic arbors will develop later and branch robustly in the area near the cell soma (Jan and Jan 2001). Several signaling cascades have been identified as regulators of dendrite formation and arborization. Among these signals, diffusible semaphorins have been identified as key signals required for apical dendrite orientation (Polleux, Morrow et al. 2000; Jan and Jan 2001; Pasterkamp 2012). Semaphorin 3A signaling through its receptor, neuropilin-1 and cofactor soluble guanylate cyclase, attracts the leading edge of a bipolar neuron causing extension towards the pial surface and maturation into an apical process. Remarkably semaphorin signaling acts on the axon to

repel its growth away from the pial surface (Song, Ming et al. 1998; Polleux, Morrow et al. 2000) (see chapter 3).

Importantly for my thesis, GSK-3 has been implicated downstream of semaphorin signaling (see chapter 3).

2.6 Disruptions in Early Cortical Development

In human syndromes, mispositioned neurons in the cerebral cortex can result from changes in progenitor cell cycle or migration failure. In humans the disruption of neuronal migration can result in developmental delays, seizures and death. For example, classic lissencephaly is the result of improper neuronal migration and is characterized by widely separated or absent gyri, decreased cortical lamination and multipolar or inverted neuronal morphology (Kerjan and Gleeson 2007). Groundbreaking genetic studies in humans have identified several genes mutated in migration disorders: LIS1 (Miller-Dieker syndrome, Type I lissencephaly) (Reiner, Carrozzo et al. 1993), doublecortin (X-linked lissencephaly, type I lissencephaly) (des Portes, Pinard et al. 1998; Gleeson, Allen et al. 1998) or tubulin α 1A (TUBA1) (Keays, Tian et al. 2007). These genes have been deleted in mouse models for studies on the mechanisms of migration (Dobyns, Reiner et al. 1993; Forman, Squier et al. 2005; Jaglin and Chelly 2009).

Human Reelin-linked lissencephaly (Norman-Roberts Syndrome) is another form of lissencephaly that is characterized my abnormal lamination (Hong, Shugart et al. 2000). Reelin, a secreted glycoprotein from the Cajal Retzius cells, signals through Dab1 for proper lamination of the cerebral cortex, hippocampus and cerebellum (Rice and Curran 2001;

Lakatosova and Ostatnikova 2012). Mutations in this pathway lead to altered neuronal morphology and cortical lamination and can be studied using the classic mouse model, the Reeler mouse (Tissir and Goffinet 2003; Lakatosova and Ostatnikova 2012).

Importantly for my thesis work, many of the proteins implicated in lissencephaly converge onto the GSK-3 regulatory hub. Reelin has been shown to increase phosphorylation of an inhibitory GSK-3 site, serine 9/21 (Beffert, Morfini et al. 2002) decreasing the phosphorylation of tau. However, reelin also induces the phosphorylation of GSK-3 on an activating tyrosine residue (Tyr216) which correlates with increased phosphorylation of MAP1B (Gonzalez-Billault, Del Rio et al. 2005). These data suggest GSK-3 regulation of cytoskeletal dynamics may be influenced by Reelin signaling in complex ways. Additionally, GSK-3 mediated phosphorylation of DCX is thought to be required for DCX's actions in restricting axon branching (Bilimoria, de la Torre-Ubieta et al. 2010) and migration (Bai, Ramos et al. 2003). Finally, inactivation of GSK-3 by Disrupted In Schizophrenia-1 (DISC1) is essential for progenitor proliferation and cortical neuronal migration. Abnormal regulation of GSK-3 by mutated DISC1 may underly the development of schizophrenia and bipolar disorder (Mao, Ge et al. 2009).

2.7 FIGURES AND FIGURE LEGENDS

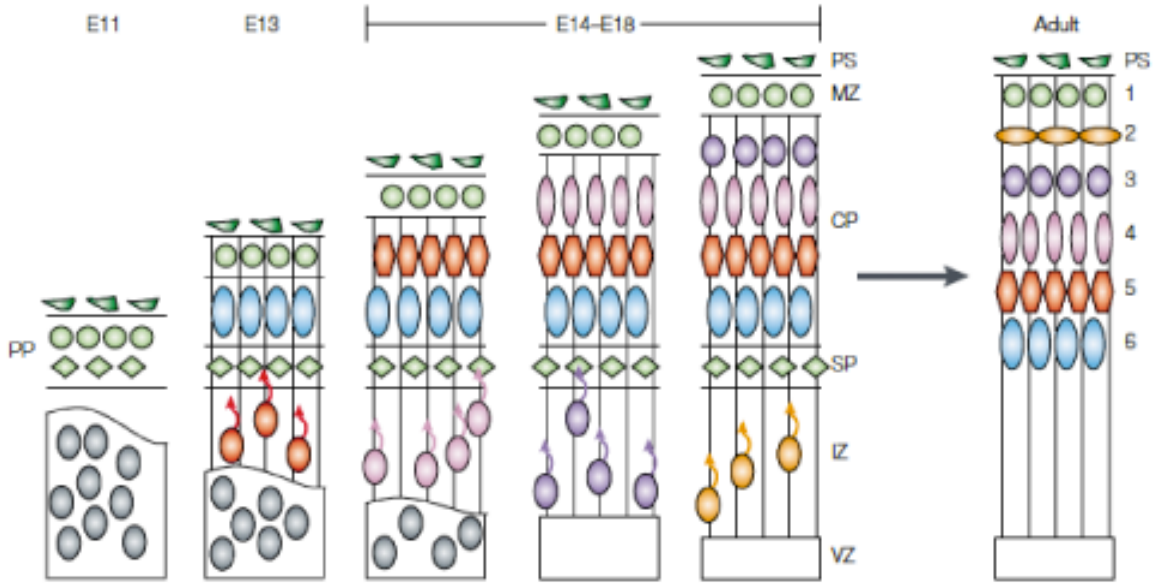


Figure 2.1 Cortical Development.

During the neurogenic phase of cortical development radial glia cells asymmetrically give rise to the first neurons that form the preplate (PP) (green circles and diamonds). The next generation of neurons (blue circles) splits the PP into the marginal zone (MZ) (green circles) and subplate (SP) (green diamonds). The neurons that split the preplate populate the cortical plate and become the deepest layer, layer VI, of the cortex (blue circles). Newly generated neurons migrate past the SP and layer VI to populate the outermost layer of the CP) and become layer V (red). Subsequent neuronal generation by radial glia and intermediate progenitor cells (IPCs) form the remaining layers of the cortex in an inside-out fashion (remaining colored circles). At late embryonic stages, the radial glia cells generate glial cells and SP degenerates to form the final 6-layered cortex observed in the adult. Image adapted from Gupta et al. 2002.

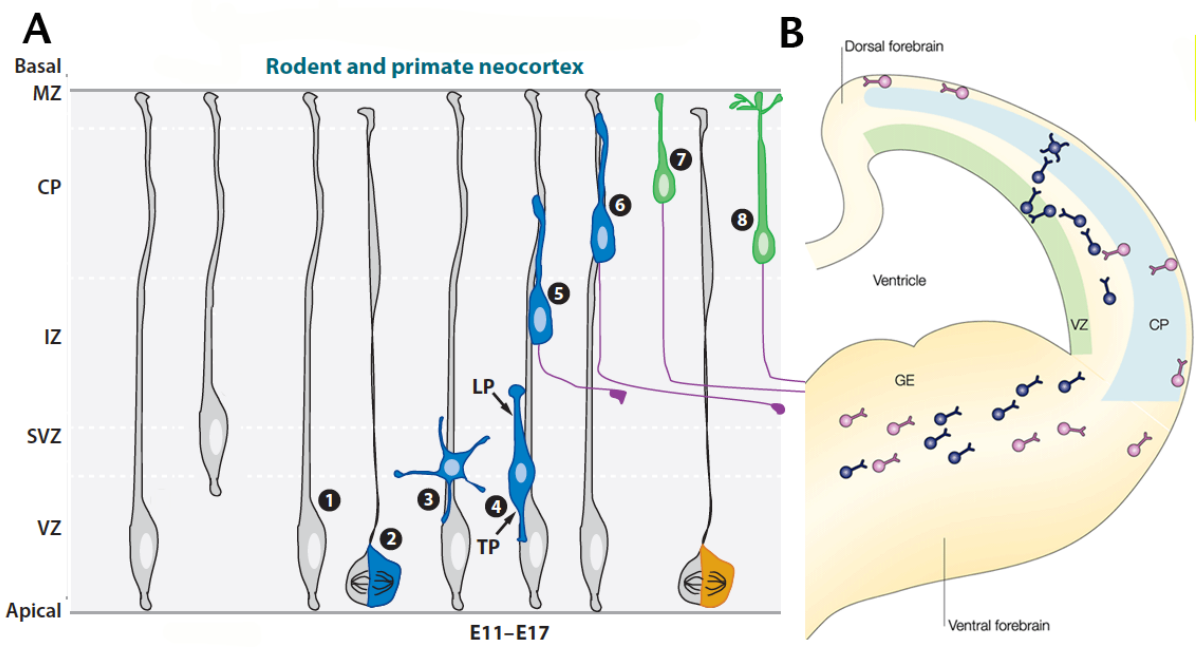


Figure 2.2 Neuronal Migration in the developing cortex

(A) Pyramidal Neuron Migration. In the cortex, radial glia cells retract their apical process to somally translocate towards the pial surface and form the preplate (grey radial glia cells). During the neurogenic phase, radial glia (1) asymmetrically divide (2) to produce a radial glia cell and multipolar daughter neuron (3) that migrates to the subventricular zone (SVZ). The newly born neuron will polarize into a bipolar morphology (4) with a leading process (LP) and trailing process (TP). Upon polarization the neuron will begin glial-guided migration (4-6) until reaching the outermost layer of the cortical plate. During migration the axon extends from the trailing process (purple process). Upon migration termination the neuron leaves the radial glia scaffold, somally translocates to its final position (7) and the leading process becomes the apical dendrite (8) and the axon continues to extent and target.

(B) Interneuron Migration. Interneurons born in the ganglionic emanance (GE) migrate tangentially into the cortex without the glial scaffold. Interneurons migrate in two streams (blue and purple) before diving into the cortical plate to adopt their final positions.
Image adapted from Barnes and Polleux 2009 and Nadarajah and Parnavelas 2002.

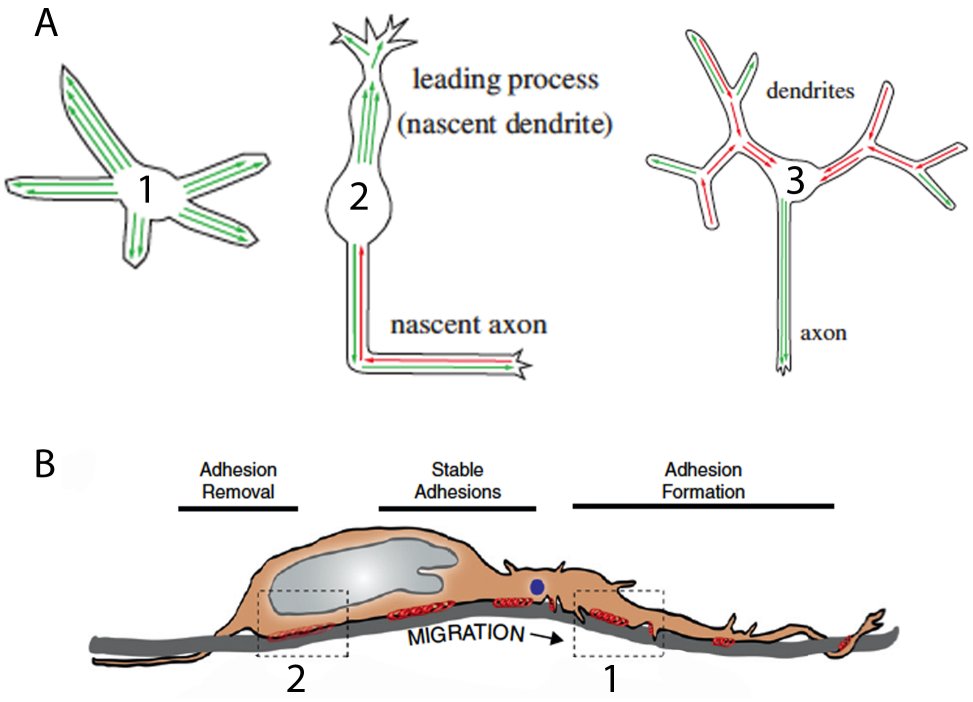


Figure 2.3 Cytoskeletal Organization and Adhesion for Migration

(A) Microtubule rearrangement during migration and maturation. (1) multipolar neurons sense the environment with plus-end microtubules oriented distally (green arrows). (2) Migrating neurons orient plus-end microtubules distally in the leading process (green arrows) and both minus-end distal (red arrows) and plus-end distal (green arrows) microtubules in the trailing process. (3) Developing dendrites increase in minus-end distal (red) microtubules in the dendrites while the axon converts to a plus-end distal microtubule network.

(B) Adhesion requirement for migration. (1) In order for migration to occur adhesion molecules must be inserted into the leading edge for adherence to the radial glia scaffold. (2) Physical movement along the radial scaffold requires removal of adhesions at the rear of the cell soma. Stable adhesions provide stable adherence to the scaffold and are subsequently removed at the rear of the cell after saltatory migration.

Figure Adapted from Sakakibara et al. 2013 and Solecki DJ 2012.

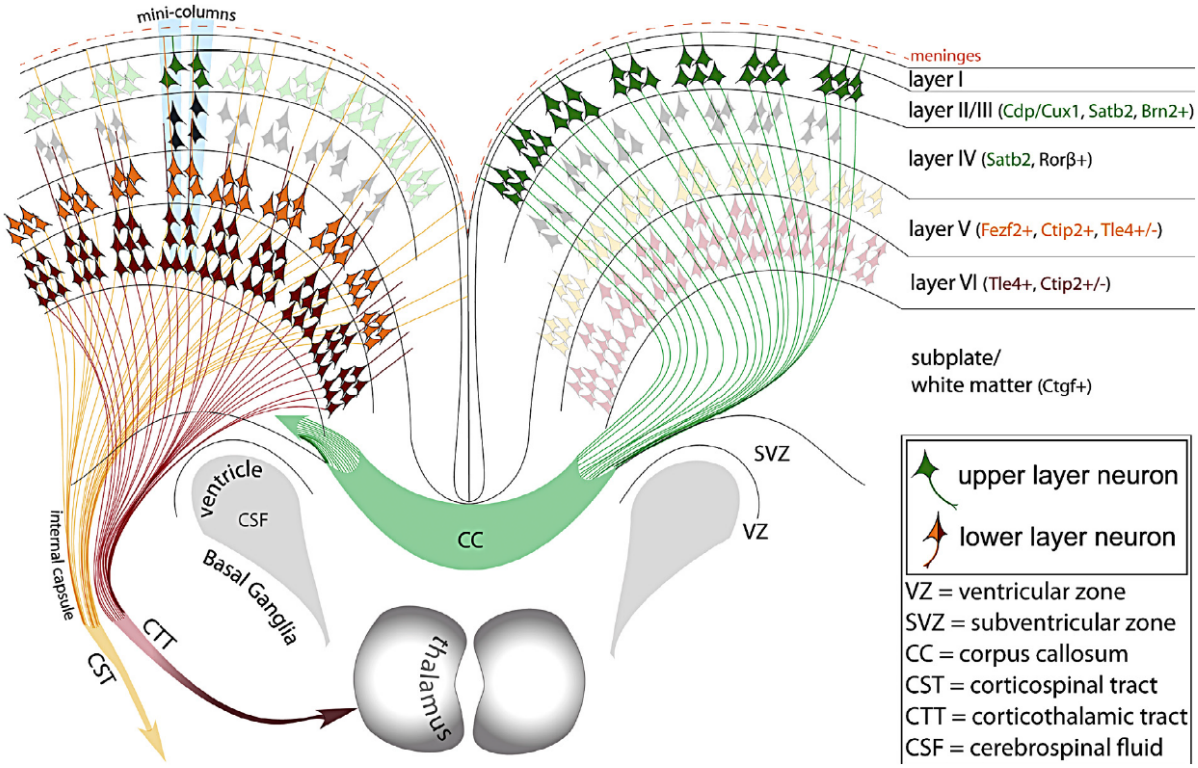


Figure 2.4 Cortical Projections and Laminar Identity

Neurons in each area of the cortex express specific markers and project to specific targets. The deepest layer, layer VI (pink/brown) project to the thalamus while layer V neurons (yellow/orange) project to the spinal cord. Upper layer neurons from layers II and III (green) project intracortically and through the corpus callosum to contralateral cortical targets. Image adapted from DeBoer et al. 2013.

CHAPTER 3: Glycogen Synthase Kinase-3

3.1 Overview

The literature on cortical neuronal migration and morphogenesis suggests that important kinases, like CDK5 and GSK-3, that regulate the functions of microtubule associated proteins might have key roles. Functions of CDK5 related to neuronal migration and layer formation in the cortex have been well studied (Ohshima, Gilmore et al. 1999; Xie, Sanada et al. 2003; Tanaka, Serneo et al. 2004; Xie, Samuels et al. 2006; Ohshima, Hirasawa et al. 2007). I therefore have focused my thesis on functions of GSK-3. This chapter provides an introduction to GSK-3 signaling.

3.2 Glycogen Synthase Kinase-3 Structure and Function

Glycogen Synthase Kinase 3 (GSK-3) is a serine/threonine kinase that was originally identified as a key regulator of glycogen synthase in glycogen metabolism and insulin responses (Woodgett and Cohen 1984). GSK-3 is highly conserved from the *Drosophila* homologue zeste-white3/shaggy (Bourouis, Moore et al. 1990; Siegfried, Perkins et al. 1990) to humans. Despite it's name, GSK-3 is now known to be a multifunctional kinase with a broad range of targets and a key role in several signaling cascades.

GSK-3 α and β are ubiquitously expressed and both are highly expressed in the brain during development (Woodgett 1990) in both neurons and in glia (Ferrer, Barrachina et al. 2002; Kaidanovich-Beilin and Woodgett 2011). In mammals, GSK-3 has two isoforms,

GSK-3α and GSK-3β (Woodgett 1990) that are 85% identical and have a catalytic domain similarity of over 90% (Ali, Hoeflich et al. 2001; Kaidanovich-Beilin and Woodgett 2011). The main differences between isoforms lie outside the catalytic domain in the N-terminus, where the α isoform has an N-terminal extension that may regulate nuclear transport (Azoulay-Alfaguter, Yaffe et al. 2011). Mice and humans have an alternatively spliced variant of GSK-3β of unknown function that contains a 13 amino acid inserted sequence in the kinase domain and is highly expressed in the nervous system (Mukai, Ishiguro et al. 2002).

Differences in isoform function are evident in mouse models where GSK-3α knockout mice are viable (MacAulay, Doble et al. 2007) while GSK-3β knockouts are embryonic lethal (Hoeflich, Luo et al. 2000). These results suggest that in many cell types GSK-3β is the more important family member. However, we have found that one isoform can clearly compensate for the other and that deletion of both isoforms is required to get a full picture of GSK-3 function *in vivo* (Kim, Zhou et al. 2006; Kim, Wang et al. 2009). In the work presented in this thesis both isoforms of GSK-3 have been deleted in neurons of the developing dorsal telencephalon.

3.2.1 GSK-3 Signaling Downstream of RTKs

GSK-3 has high basal activity towards many of its substrates in resting cells and inhibits their function at baseline (Hughes, Nikolakaki et al. 1993; Kaidanovich-Beilin and Woodgett 2011). Unlike most kinases, GSK-3 is inhibited by several upstream signaling pathways, transiently relieving phosphorylation of its substrates and enhancing their function (Hur and

Zhou 2010). GSK-3 is inactivated when phosphorylated on N-terminal Serine 9 (ser9) of GSK-3β (Sutherland, Leighton et al. 1993; Stambolic and Woodgett 1994) and Serine 21 (ser21) of GSK-3α (Sutherland and Cohen 1994). These phosphorylation events are typically mediated by Akt, downstream of receptor tyrosine kinase(RTK)/Phosphoinositide 3-kinase (PI3K) signaling. EGF, PDGF, neurotrophins, IGF and Insulin activate RTKs that, in turn, activate PI3K and Akt culminating in ser9/21 GSK-3 phosphorylation (Cross, Alessi et al. 1995; Kaidanovich-Beilin and Woodgett 2011) **[Figure 3.1]**. Additionally, signals transduced from the RTKs to PAR-6/PAR-3/aPKC can also inactivate GSK-3 kinase activity (Etienne-Manneville and Hall 2003). N-terminal Ser9/21 phosphorylation has been used *in vitro* studies to determine the level of GSK-3 kinase activity in multiple pathways and cell types. It should be noted that these residues may not be a correct readout of kinase activity in all signaling paradigms (see below). Making the situation more complicated, GSK-3 can be activated by the phosphorylation of tyrosine residues, Tyr216 of GSK-3β and Tyr279 of GSK-3α (Hughes, Nikolakaki et al. 1993).

In general, GSK-3 phosphorylates downstream targets with a Serine/Threonine-X-X-X-Serine/Threonine motif. Most of GSK-3 substrates require another phosphorylation event near the GSK-3 site ('priming'), mediated by a different kinase, for maximal GSK-3 activity (Sutherland 2011). Many GSK-3 priming kinases have been identified including, CK1, CK2, JNK, ERK, and PKA (for review see Sutherland C 2011). Though priming is not absolutely required for GSK-3 substrate phosphorylation, priming strongly enhances GSK-3 effect (Thomas, Frame et al. 1999). Of specific interest to this thesis, CDK5 is able to prime and enhance GSK-3 phosphorylation of MAPS and will be a point of discussion in Chapter 7.

3.2.2 GSK-3 Signaling in the Wnt pathway

Importantly, the Drosophila GSK-3 homologue, Shaggy/zeste white 3, was identified in the context of Wnt rather than RTK signaling. In contrast to RTK signaling where GSK-3 is regulated primarily via phosphorylation, GSK-3 regulation via Wnt is based on protein-protein interactions mediated by Disheveled (van Noort, Meeldijk et al. 2002; Doble and Woodgett 2003). Again, GSK-3 is active at baseline, phosphorylating and inactivating β-catenin, and GSK-3 activity towards this substrate is transiently released when Wnt signaling is activated (see below).

The Wnt/β-catenin Signaling Pathway is crucial for embryonic patterning, cell fate determination, polarity and proliferation (Logan and Nusse 2004). Wnts (wingless in *Drosophila)* are secreted glycoproteins that bind to the Frizzled-LRP5/6 receptors and act via Dishevelled (Dvl) and GSK-3 signaling (For review see Logan and Nusse 2004). At basal levels GSK-3 is sequestered by the scaffolding protein Axin (Ikeda, Kishida et al. 1998; Yasuda, Whitmarsh et al. 1999) into a destruction complex that includes the tumor suppressor Adenomatous polyposis coli (APC), the GSK-3 priming kinase, casein kinase 1 (CK1)(Amit, Hatzubai et al. 2002), and β-catenin (Rubinfeld, Souza et al. 1993). In this complex GSK-3 phosphorylates β-catenin on N-terminal residues Thr41, Ser37, and Ser33 (Liu, Li et al. 2002). Phosphorylation of cytoplasmic β-catenin on these residues signal for its degradation via the proteosomal pathway (Liu, Kato et al. 1999) **[Figure 2.1]**.

The exact mechanism for Wnt-induced inactivation of GSK-3 is still under intensive investigation. Upon Wnt stimulation of Frizzled-LRP5/6, Dvl removes GSK-3 from the

destruction complex, probably by protein-protein interaction, and mediates GSK-3 phosphorylation of the accessory Wnt receptor LRP5/6 (Zeng, Tamai et al. 2005; Dale 2006). The disruption of the destruction complex results in the accumulation of non-phosphorylated β-catenin and its subsequent translocation to the nucleus (van Noort, Meeldijk et al. 2002). Nuclear β-catenin binds to T-cell Factor (TCF)/lymphoid enhancer factor (LEF) transcription factors (Behrens, von Kries et al. 1996; van de Wetering, Cavallo et al. 1997) **[Figure 3.1]**. In general, GSK-3 deletion strongly enhances β-catenin accumulation and signaling (Kaidanovich-Beilin and Woodgett 2011), however this predication has not been carefully assessed in all cell types. GSK-3's role in Wnt/β-catenin signaling in developing cortical neurons is addressed in Chapter 5.

GSK-3 activity usually inhibits substrate functions. For example, GSK-3 phosphorylation of β-catenin, cyclin D1 and *c-myc* targets these proteins for degradation, while inhibition of GSK-3 promotes their stabilization and function (Diehl, Cheng et al. 1998; Alt, Cleveland et al. 2000; Sears, Nuckolls et al. 2000). Additionally, phosphorylation of some microtubule associated proteins (MAPs) inhibit tubulin binding **[Figure 3.1]**. For example, GSK-3 phosphorylation of tau decreases its affinity for microtubules (Lindwall and Cole 1984; Drechsel, Hyman et al. 1992; Hanger, Hughes et al. 1992; Brownlees, Irving et al. 1997; Utton, Vandecandelaere et al. 1997). Similarly, GSK3 phosphorylation of APC (Rubinfeld, Albert et al. 1996; Etienne-Manneville and Hall 2003) and CRMP-2 (Inagaki, Chihara et al. 2001; Fukata, Itoh et al. 2002; Yoshimura, Kawano et al. 2005) decrease their association with microtubules. Obviously, changes in microtubule binding properties will alter cytoskeletal dynamics.

However, the situation is complex in that GSK-3 phosphorylation of MAP-1B increases its association with microtubules (Lucas, Goold et al. 1998). Further, GSK-3 activity is required for the activity of some of its substrates, including doublecortin and CRMP-2 downstream of semaphorin signaling. I will address several of these GSK-3 substrates in Chapter 5.

3.2.3 GSK-3 signaling in the semaphorin pathway

Though originally identified as axonal repellants (Luo, Raible et al. 1993), semaphorins are diffusible guidance cues that can be both chemoattractive and repulsive (Pasterkamp 2012). In the cortex, axons are chemorepulsed by the sema signal and, as a result, project their axons away from the sema gradient (Chedotal, Del Rio et al. 1998; Montolio, Messeguer et al. 2009) and limit branching (Dent, Barnes et al. 2004). In addition, sema signaling is also used to mediate axon-axon interaction and by balancing repulsion with adhesion/fasiculation is involved with axon defasiculation and targeting (Vactor, Sink et al. 1993; Pasterkamp 2012). In the opposite direction, semaphorin 3A-neuropilin-1 signaling is a chemoattractive cue that orients the apical dendrite to the pial surface (Polleux, Morrow et al. 2000) **[Figure 3.2]**. Elevating cGMP levels relieves the inhibitory phosphorylation of GSK-3 resulting in increased signaling to substrates and subsequent dendrite orientation.

Increasing evidence suggests that an increase GSK-3 kinase activity is an essential mediator of sema signaling (Eickholt, Walsh et al. 2002; Uchida, Ohshima et al. 2005; Pasterkamp 2012). Indeed a key GSK-3 target, collapsing response mediator protein-2 (CRMP-2) was discovered as downstream of of semaphorin signaling (Uchida, Ohshima et

al. 2005). CRMP-2 is a microtubule associated protein whose phosphorylation by GSK-3 at site Thr514 required for cytoskeletal rearrangements that are an essential to semaphorin biological actions (Yoshimura, Kawano et al. 2005) [**Figure 3.2]**.

Semaphorin signaling via GSK-3 as been implicated in axon and dendrite specification of hippocampal and cortical neurons (Tran, Kolodkin et al. 2007; Shelly, Cancedda et al. 2011) In non-polarized neurons a strong dendrite-specifying semaphorin gradient asymmetrically increases cGMP levels, increasing GSK-3 signaling, and decreasing the phosphorylation of LKB1 on ser431, the residue responsible for specifying the axon (Polleux, Morrow et al. 2000; Shelly, Cancedda et al. 2011; Shelly and Poo 2011; Pasterkamp 2012) [**Figure 3.2]**. This signaling cascade specifies dendrite identity, suppresses axon formation and promotes dendritic growth. In the opposite direction, pser431 LKB1 (Barnes, Lilley et al. 2007; Shelly, Cancedda et al. 2007) and an inactive pool of GSK-3 becomes asymmetrically localized in the neurite being specified as the axon (Jiang, Guo et al. 2005) (see below). After the axon is initiated, semaphorin 3A treatment rapidly activates the GSK-3 pool in the growth cone to facilitate axon repulsion from the semaphorin gradient (Eickholt, Walsh et al. 2002; Montolio, Messeguer et al. 2009).

These actions of GSK-3 in the semaphorin signaling cascade may have strong relevance to GSK-3 regulation of excitatory neuron morphogenesis as outlined in my soon to be submitted manuscript (Chapter 5).

3.3 GSK-3 regulation of polarity; Problems with in Vitro Models

As noted above, after a neuron is born it polarizes to form an axon and a somatodendritic domain and subsequently dendritic processes are elaborated. This process is highly regulated and has been mostly delineated through *in vitro* hippocampal culture models (Dotti, Sullivan et al. 1988; Craig and Banker 1994). Multiple proteins have been implicated as essential components of the polarity cascade (Polleux and Snider 2010). These include PI3K (Menager, Arimura et al. 2004), Akt (Yan, Guo et al. 2006), PTEN (Jiang, Guo et al. 2005), CRMP2 (Inagaki, Chihara et al. 2001; Arimura, Menager et al. 2004), LKB1 (Barnes, Lilley et al. 2007; Shelly and Poo 2011) and GSK-3 (Jiang, Guo et al. 2005; Yoshimura, Kawano et al. 2005; Yoshimura, Arimura et al. 2006).

In vitro experiments suggest a strong role for GSK-3 in regulating neuronal polarity (Kim, Hur et al. 2011). Inactivation of GSK-3 by Ser9 phosphorylation is essential in transitioning the immature neurite into an axon, while pharmacological inhibition of GSK-3 *in vitro* leads to multiple axons per individual neuron (Jiang, Guo et al. 2005). In contrast, expression of a S9A mutant GSK-3 prevents axon formation (Jiang, Guo et al. 2005; Yoshimura, Kawano et al. 2005). Inhibition of GSK-3 and relief of phosphorylation of microtubule associated proteins is thought to enhance microtubule stability, which is important in specifying an axon (Witte, Neukirchen et al. 2008) (for review see Stiess and Bradke 2010).

In an effort to address the role of ser9/21 in an *in vivo* environment, GSK-3 point mutation knock-in mice have been created. Mutant mice expressing GSK-3 with Ser9/21 converted to alanine create, in theory, a ‘constitutively active’ GSK-3. Thus, these mutations

should result in steady basal activation of GSK-3 and excessive and uncontrolled phosphorylation of downstream targets. However, these animals exhibit normal neuronal polarity and develop with no overt abnormalities of neuronal morphology (McManus, Sakamoto et al. 2005; Gartner, Huang et al. 2006). These results raise the possibility that ser9/21 phosphorylation may not be important to the morphological regulation of neurons *in vivo*. The dramatic difference between *in vitro* and *in vivo* GSK-3 phenotypes strongly supports the need to study the role of GSK-3 in a natural *in vivo* environment. My thesis directly addresses the role of GSK-3 in neuronal morphogenesis *in vivo* (Chapter 5).

3.4 GSK-3 Functions *in vivo* in Progenitors

GSK-3 signaling is known to be essential for the regulation of progenitor proliferation in the developing dorsal telencephalon *in vivo*. Kim et al. convincingly showed that deletion of both GSK-3 isoforms in the cortical radial progenitors resulted in an expansion of the progenitor pool and suppressed neuronal differentiation (Kim, Wang et al. 2009) **[Figure 3.3]**. Wnt, Sonic hedgehog (Shh) and Notch signaling were all implicated as enhanced by GSK-3 deletion. Kim and colleagues observed a strong increase in β-catenin levels in the GSK-3 deleted cells demonstrating GSK-3 regulation of β-catenin is essential for progenitor homeostasis. Consistent with these results, Walsh and colleagues have shown that increasing β-catenin levels in progenitors increases the production of neural precursors (Chenn and Walsh 2002). Importantly, the schizophrenia-associated protein, DISC1, is thought to act in part by regulating neural progenitor proliferation via GSK-3 (Mao, Ge et al. 2009). Recently, work from the Ip lab has demonstrated a novel role for GSK-3 IPC amplification via the destruction complex mediator, Axin (Fang, Chen et al. 2013). Axin amplifies IPC conversion

to neurons and was shown to bind GSK-3 as a part of its actions. Taken together, these studies establish critical roles for GSK-3 in the regulation of progenitors in the developing dorsal telencephalon *in vivo* and suggest that GSK-3 signaling importantly controls neuronal number in the developing brain. However, functions of GSK-3 in developing neurons *in vivo* remain elusive and form the subject of my thesis work (Chapter 5).

3.5 GSK-3 Function in Mature Neurons and Human Diseases

Not surprisingly, GSK-3 exhibits important functions in mature neurons. For the most part this regulation has been studied in *in vitro* models. For example, pharmacological inhibition of GSK-3 decreases vesicular transport in axons by reducing tau phosphorylation and binding to the motor protein kinesin-1 (Cuchillo-Ibanez, Seereeram et al. 2008). Additionally, GSK-3 phosphorylates Dynamin 1 (Clayton, Sue et al. 2010) to regulate bulk endocytosis during periods of high activity when clatherin-dependent endocytosis is unable to endocytose large amounts of membrane. Finally, it has been demonstrated that GSK-3 is essential for LTD and over expression blocks LTP (Hooper, Markevich et al. 2007; Peineau, Taghibiglou et al. 2007; Zhu, Wang et al. 2007), possibly through PSD-95 regulation (Nelson, Kim et al. 2013). Though this thesis will not address the role of GSK-3 in mature neurons, this topic will be discussed in Chapter 6.

GSK-3 has important functions in psychiatric and neurologic diseases making further understanding of its role in neural development an urgent priority. For example, abnormal GSK-3 activity may underlie mood disorders. Thus Lithium, a known GSK-3β inhibitor, is the mainstay of treatment for bipolar disorder (Klein and Melton 1996; O'Brien, Harper et al.

2004)(Stambolic, Ruel et al. 1996; De Sarno, Li et al. 2002; Freland and Beaulieu 2012). Additionally, the drug valproate, also sometimes used to treat mood disorders, is also an inhibitor of GSK-3 kinase activity (Chen, Huang et al. 1999; De Sarno, Li et al. 2002).

GSK-3 may also be important in the pathogenesis of schizophrenia (Emamian 2012). Administration of antipsychotics, haloperidol or clozapine inhibits GSK-3 activity via ser9 phosphorylation (Emamian, Hall et al. 2004; Emamian 2012). Additionally, a regulator of GSK-3, Disrupted-in-Schizophrenia 1 (DISC1) has been implicated as a major susceptibility factor for Schizophrenia (De Rienzo, Bishop et al. 2011). DISC1 functions in party by affecting the GSK-3 functions related to progenitor proliferation in both neonates and adults (Mao, Ge et al. 2009).

Finally in Alzheimer's disease, two of the hallmarks of the disease are aggregation of amyloid-β peptides from amyloid precursor protein and the development of neurofibrially tangles composed of hyper-phosphorylated tau (Glenner and Wong 1984). Interestingly GSK-3 inhibition blocks the production of amyloid-β peptides suggesting that GSK-3 is required for normal amyloid precursor protein function (Phiel, Wilson et al. 2003). Additionally, GSK-3 strongly regulates the phosphorylation status of the MAP tau (Ishiguro, Shiratsuchi et al. 1993) (see above). Hyper-phosphorylated tau is found in the neurofibrially tangles in the brains of Alzheimer's patients (Hanger, Hughes et al. 1992; Pei, Braak et al. 1999; Lucas, Hernandez et al. 2001). Hyper-phosphorylated tau also underlies a class of neurodegenerative diseases called tauopathies.

Although my dissertation does not address the functions of GSK-3 in adult neurons, better understanding of GSK-3 regulation during neuronal development, including the regulation of microtubule associated proteins, may inform approaches to inhibiting GSK-3 for therapeutic intervention.

3.6 FIGURES AND FIGURE LEGENDS

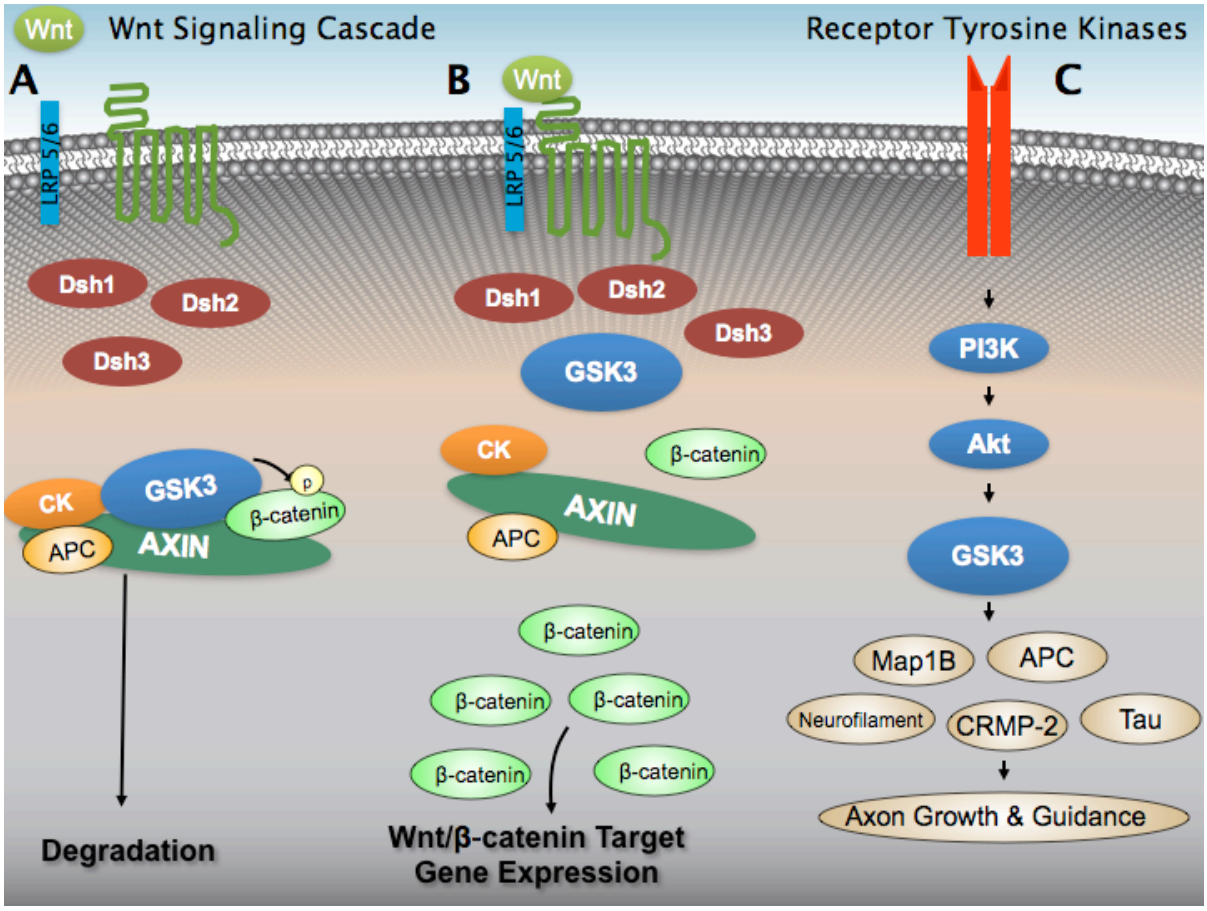


Figure 3.1 GSK-3 Signaling Cascades

(A-B) Canonical Wnt Signaling. (A) At basal levels GSK-3 is associated with casein kinase, APC, Axin and β-catenin in a 'destruction' complex. GSK-3 phosphorylates β-catenin to signal its degradation. (B) Upon Wnt stimulation GSK-3 is sequestered away from the destruction complex leading to the accumulation of β-catenin and altered gene expression. (C) Receptor Tyrosine Kinase Signaling. GSK-3 is downstream of multiple cascades. Signaling through PI3K/AKT leads to ser9 phosphorylation or GSK-3 and GSK-3 inhibition. GSK-3 signals to multiple downstream Microtubule Associated Proteins (MAPS).

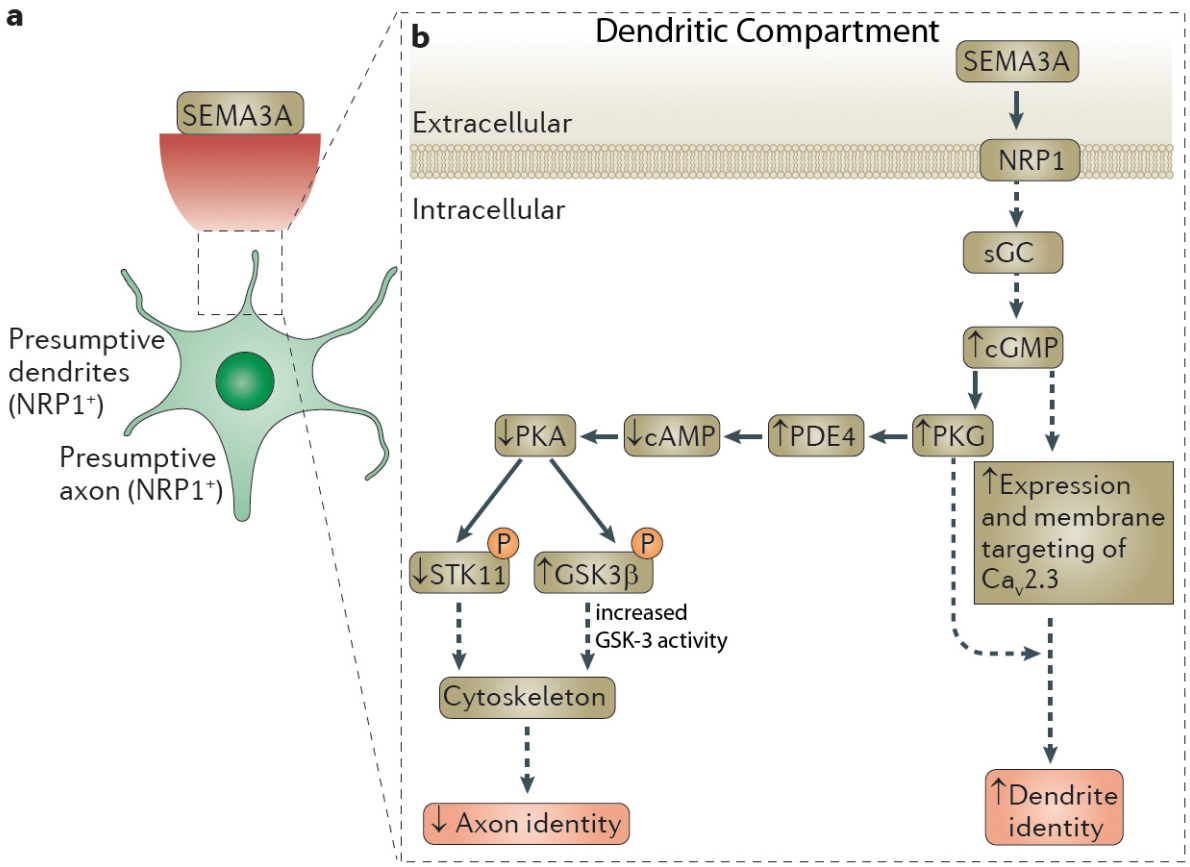


Figure 3.2 Semaphorin signaling in dendrite development

(A) Schematic of semaphorin gradient specifying dendrite formation. (B) A strong semaphorin signal asymmetrically increases cGMP levels to specify dendrite identity. Increased cGMP in the dendrites decreases the phosphorylation of STK11 (LKB1) and GSK-3, increasing GSK-3 signaling in the dendrite. Both Ser431 phosphorylation of LKB1 and GSK-3 inactivation in a single neurite are required initiating an axon. Figure adapted from Pasterkamp 2012.

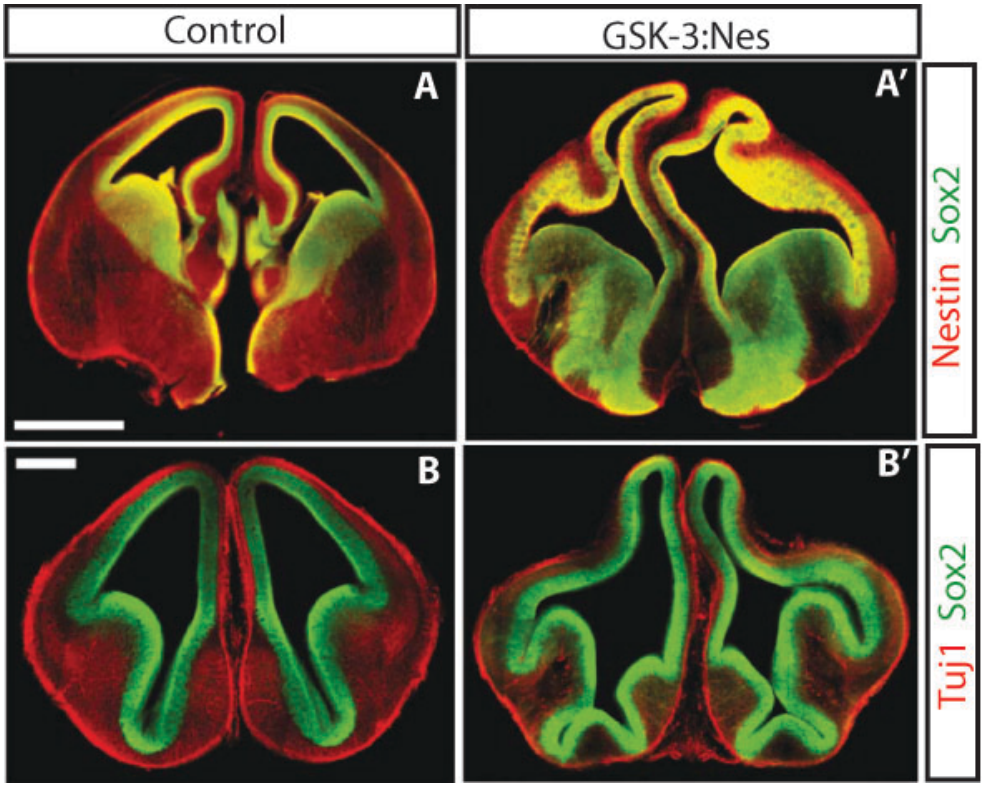


Figure 3.3 GSK-3 regulation of Progenitor Homeostasis

(A-A') Coronal sections of E13.5 control and GSK-3 deleted mice. GSK-3 mutants have an increased progenitor pool (sox2). Nestin labels Radial Glia. Scale 900uM. (B-B') Coronal sections of E13.5 control and GSK- 3 deleted mice. GSK-3 mutants have fewer Tuj1-labeled neurons and increased progenitor pool (Sox2). Scale bar = 500uM. Figure adapted from Kim et al. Nat. Neuroscience 2009

CHAPTER 4: TGF-β Activated Kinase 1

4.1 Overview

Neurons are some of the most highly polarized cells in the body and the polarization of axons and dendrites underlies the proper flow of information in the brain. Currently, the molecular mechanisms responsible for neuronal polarization during brain development *in vivo* are unclear. However, recent work from several labs have started to defined a signaling cascade required for cortical neuron polarization *in vivo.* At the core of this cascade is the phosphorylation of a serine/threonine kinase, LKB1, on its serine residue 431. Here I demonstrate *in vitro* that TGF-β Activated Kinase-1 (TAK1) is capable of associating with LKB1 and phosphorylating LKB1 on serine 431. Additionally, *in vitro* knock-down of TAK1 in dissociated cortical cultures results in delayed polarization, consistent with a role in axon formation. Finally, genetic deletion of TAK1 in progenitors resulted in no overt change in lamination or polarization *in vivo*. Despite promising *in vitro* data, our results indicate that TAK1 is not the major upstream kinase responsible for transducing the polarization cascade *in vivo.*

The work in this chapter was conducted under the direction of Franck Polleux, PhD. Following the completion of the TGF-β Activated Kinase-1 project I generated LKB1:Nex conditional mutants and conducted initial characterization on this mutant line (data not shown). This work was continued by Dr. Polleux at Scripps and was recently published (Courchet, Lewis et al. 2013).

4.2 Introduction

Following cell cycle exit, neurons polarize by undergoing drastic morphological changes to form two distinct domains: a single axon and multiple dendrites. While many proteins have been implicated in the establishment of neuronal polarity and axon specification *in vitro*, such as APC (Shi, Cheng et al. 2004), PI3K (Yoshimura, Arimura et al. 2006), and GSK-3 (Shi, Cheng et al. 2004; Jiang, Guo et al. 2005; Yoshimura, Kawano et al. 2005), few of these genes have been shown to be required for neuronal polarization *in vivo*. However, recent work demonstrated that LKB1, the ortholog of *Caenorhabditis elegans Partitioning-defective* 4 (Par4 or STK11), is critical for the establishment of neuronal polarity *in vivo* (Kishi, Pan et al. 2005; Barnes, Lilley et al. 2007; Shelly, Cancedda et al. 2007; Shelly and Poo 2011).

The vast majority of LKB1 protein is inactive and located in the nucleus with STRADβ (Dorfman and Macara 2008). Upon a polarization cue, LKB1 is shuttled into the cytosol by STRADα and MO25 and this translocation increases the catalytic activity of LKB1 by approximately 10-fold (Baas, Boudeau et al. 2003). In epithelial cells, activation of LKB1 via an inducible STRADα was sufficient to induce polarity (Baas, Kuipers et al. 2004) indicating a critical role for LKB1 in this process. As in epithelial cells, the translocation of LKB1 to the cytoplasm in cortical neurons allows for LKB1 to be activated by phosphorylation of an axon-specifying residue, serine 431 (Barnes, Lilley et al. 2007). This phosphorylation event leads to pLKB1 being sequestered to the a single neurite, phosphorylation of downstream SAD kinases and finally, axon initiation (Barnes, Lilley et al.

2007; Shelly, Cancedda et al. 2007). The kinase upstream of the LKB1 phosphorylation of ser431 is unknown. Recently, the Schneider lab demonstrated that the kinase TAK1 is capable of phosphorylating and inducing LKB1 activity *in vivo* (Xie, Zhang et al. 2006) suggesting that TAK1 could be acting upstream of LKB1 in the polarization cascade.

TGF-β activated kinase 1 (TAK1) is a serine/threonine kinase initially identified as a mitogen-activated protein kinase (Yamaguchi, Shirakabe et al. 1995). While it can be activated by TGF-β, TAK1 has been implicated in both the JNK (Shirakabe, Yamaguchi et al. 1997; Wang, Zhou et al. 1997) and P38 MAPK pathways (Moriguchi, Kuroyanagi et al. 1996) and has critical roles in cell survival, proliferation, stress response and immune regulation (Delaney and Mlodzik 2006). Interestingly, the role of TAK1 has not been assessed in cortical neurons.

TAK1 requires a combination of adaptor proteins, TAK1 Binding proteins 1, 2 and 3 (TAB1, TAB2 and TAB3), for its activation (Shibuya, Yamaguchi et al. 1996; Kishimoto, Matsumoto et al. 2000; Takaesu, Kishida et al. 2000). While TAK1 association with either TAB2 or TAB3 associates the complex to specific receptors, K63-linked ubiquitination along with TAB1 physical association enable TAK1 kinase activity (Shibuya, Yamaguchi et al. 1996; Kishimoto, Matsumoto et al. 2000; Sakurai, Miyoshi et al. 2000; Takaesu, Kishida et al. 2000; Landstrom 2010). Of particular note, TAK1 knockout mice are embryonic lethal (Sato, Sanjo et al. 2005) and therefore the majority of the studies related to TAK1 have been conducted *in vitro*.

Here I have addressed the function of TAK1 in neuronal polarity both *in vitro* and *in vivo* using Emx1-Cre (Gorski, Talley et al. 2002) and a conditional $TAK1^{loxp/loxp}$ mouse (Sato,

Sanjo et al. 2005). I demonstrate *in vitro* that TAK-1 is capable of associating with LKB1 and phosphorylating LKB1 on ser431. The phosphorylation of LKB1 is enhanced by TAB1 co-expression. Additionally, overexpression of TAK1 in newly born neurons results in a delay in axon initiation *in vitro,* consistent with a role for TAK1 in polarization upstream of LKB1. Finally, deletion of TAK1 *in vivo* does not inhibit axon initiation, as seen with LKB1 deletion with Emx1-cre (Barnes, Lilley et al. 2007). We conclude that *in vivo* TAK1 is not a critical regulator of polarity in newly born neurons of the developing dorsal telencephalon.

4.3 Results

TAK1 expression in the developing murine cortex

In vivo deletion of LKB1 in cortical pyramidal neurons results in a loss of axon formation (Barnes, Lilley et al. 2007) **[Figure 4.1]**. Furthermore, re-expression of LKB1 via cortical electroporation of the conditional LKB1 knockout neurons was able to rescue axon formation **[Figure 4.1]**. Interestingly, Barnes et al. identified a residue in LKB1, serine 431, that specifies axon formation. A mutation in LKB1, serine 431 to alanine mutation ($LKB1^{S431A}$), was not able to rescue axon formation **[Figure 4.1]**. These data indicate the *in vivo* importance of LKB1, specifically the phosphorylation of LKB1 on serine 431, in neuronal polarization (Barnes, Lilley et al. 2007).

Recently, TAK1 expression has been shown to regulate LKB1 kinase activity (Xie, Zhang et al. 2006) making it an upstream candidate for LKB1 phosphorylation. However, the role of TAK1 in the developing cortex has never been explored. LKB1 is expressed throughout the cortex but is enriched in the progenitor zone (VZ) of the developing cortex **[Figure 4.2A]**. Similar to LKB1, TAK1 is highly also highly expressed in the ventricular zone of the

developing cortex at E14 **[Figure 4.2A]** suggesting a role in early neuronal regulation. Additionally, the adaptor protein TAB2 also shares the same expression profile **[Figure 4.2A]** suggesting that the TAK1 activating complex is expressed in the VZ of the developing cortex. To date no information on TAB1 or TAB3 expression is currently available. These data suggest that TAK1 is located in at right place and time *in vivo* to regulate neuronal polarization in the developing cortex.

TAK1 phosphorylates LKB1 in HeLa cells

In order to ascertain the role of TAK1-LKB1 signaling in cortical development I sub-cloned TAK1 and its activator, TAB1, and expressed these two proteins in conjunction with LKB1 in HeLa. HeLa cells lack endogenous TAK1, TAB1 and LKB1 expression. Lysates from these transfections were probed with an antibody specifically recognizing phosphorylated serine 431 on LKB1. Interestingly, TAK1 is capable of inducing phosphorylation of LKB1 on serine 431 [**Figure 4.2B]** in the presence or absence of TAB1 transfection. However, the degree of LKB1 phosphorylation is greatly increased with the co-transfection of TAB1, demonstrating the importance of TAB1 for TAK1 function [**Figure 4.2B]**. These results were further supported *in vitro* by the transfection of the $LKB1^{S431A}$ mutant in conjunction with TAK1 and TAB1. This LKB1 mutant is not able to be phosphorylated by the addition of TAK1 and TAB1, providing specificity to the biochemical detection of phosphorylated serine 431 [**Figure 4.2B]**. Finally, transfection of HeLa cells with LKB1, TAB1 and a kinase dead form of TAK1 (KD-TAK1) also failed to phosphorylate LKB1 on serine 431 [**Figure 4.2B]**. Successful expression of TAK1, TAB1 and LKB1 was

verified via western blot analysis **[Supplemental Figure 4.1]**. These data indicate that *in vitro,* TAK1 is capable of phosphorylating LKB1 on the residue responsible for axon formation, serine 431.

TAK1-LKB1 interact *in vitro*

TAK1 is capable of phosphorylating LKB1 *in vitro* suggesting that TAK1 and LKB1 physically associate *in vitro*. To test this hypothesis I transfected HEK293 cells with combinations of LKB1, TAK1 and TAB1 and subjected these lysates to co-immunoprecipitation using an LKB1-specific antibody **[Figure 4.3A, circled area]**. Interestingly, TAK1 and LKB1 associate in every transfection combination that included TAK1 and LKB1 (data not shown). This association occurred regardless of TAB1 transfection, indicating that TAB1 is not responsible for the TAK1-LKB1 interaction and that TAK1 and LKB1 may directly associate.

Interestingly, though LKB1 association with TAK1 was present in all transfections including the two constructs, the amount of TAK1 associated with LKB1 was minimal [**Figure 4.3A, circled area]**. One explanation is that the majority of LKB1 is located in the nucleus, while TAK1 is predominantly located in the cytosol (Baas, Boudeau et al. 2003). To address this issue I conducted a second co-immunoprecipitation experiment and pulled down for TAK1 in the cytosol and then probed for LKB1 association **[Figure 4.3 B].** Indeed, a large amount of cytosolic LKB1 was associated with TAK1, indicating that these two kinases interact *in vitro.*

TAK1 expression in cortical neurons

Phosphorylation of LKB1 on serine 431 enriches LKB1 in the axon (Barnes, Lilley et al. 2007). Therefore we hypothesized that TAK1 may also be preferentially localized to the developing axon. To address the neuronal localization of TAK1, E14.5 cortical neurons were cultured for 3 days and then stained for TAK1 expression. Interestingly, TAK1 is ubiquitously expressed in cortical neurons and appears enriched in the growing axon **[Figure 4.4A].** Given that serine 431 phosphorylation of LKB1 is essential for axon formation, we hypothesized that overexpression of TAK1 would inhibit axon initiation due to mislocalization of TAK1 protein in the cell. Interestingly, overexpression of TAK1 in this paradigm resulted in a multiple short neurites and the absence of a single long axon after three days of culture **[Figure 4.4B]** while control cultures imaged at two and three days *in vitro* depict a normal stage progression in culture **[Figure 4.4C]**. In control neurons at 3DIV approximately sixty percent of control neurons have progressed to a stage 3 (i.e. have a polarized morphology with a single axon). However, only approximately twenty-six percent of TAK1 overexpressing neurons have progressed to stage 3 indicating that TAK1 may regulate neuronal polarization *in vitro.* A similar phenotype is observed by over-activation of LKB1 (Barnes, Lilley et al. 2007; Shelly, Cancedda et al. 2007) suggesting that these two kinases might be in the same pathway.

TAK1 is Not Required for Polarization *in vivo*

To confirm that TAK1 is required for LKB1-mediated neuronal polarization *in vivo,* I generated a cortex-specific conditional knockout for TAK1 by breeding a $TAK1^{loxp/loxp}$ mouse

(Sato, Sanjo et al. 2005) conditional mouse to Emx1-Cre (Gorski, Talley et al. 2002). This conditionally deletes TAK1 in the progenitors of the cortex beginning around E10.5. Similar to LKB1:Emx mutants, the TAK1:Emx mutant mice die at birth due to unknown causes.

Western blot analysis from cortical lysates show a significant reduction in TAK1 protein by E16.5 in TAK1:Emx mutants when compared to littermate controls **[Figure 4.5 E]**. Interestingly, though not unexpected, no change in LKB1 protein was observed in TAK1:Emx mutants. Surprisingly, no change in pser431 LKB1 was observed in the mutants **[Figure 4.5 E]**, suggesting that loss of TAK1 in the developing neocortex does not alter the axon-specific phosphorylation of LKB1 on serine 431. Further *in vivo* analysis of the TAK1:Emx mutant embryos showed no defect in cortical lamination (data not shown).

Previous work from the Polleux lab showed that loss of LKB1 resulted in drastically reduced TAG1+ corticofugal axon projections (Barnes, Lilley et al. 2007) **[Figure 4.5 A-B]**. In line with no change in serine 431 phosphorylation of LKB1, TAK1:Emx mutants did not recapitulate this phenotype **[Figure 4.5 C-D]**. Taken together these data indicate that TAK1 is not required upstream of LKB1 for axon formation *in vivo*.

4.4 Discussion

Phosphorylation of LKB1 on serine 431 is critical for cortical neuronal polarization (Barnes, Lilley et al. 2007; Shelly, Cancedda et al. 2007) however the kinase responsible for this phosphorylation event *in vivo* has not been identified. TAK1, a kinase with critical functions in innate and adaptive immune response, was found to phosphorylate LKB1 and induce its kinase activity (Xie, Zhang et al. 2006). I have found that TAK1 phosphorylates

the LKB1 axon-specifying residue ser431 and physically associates LKB1 in HEK293 and Hela cells *in vitro*. Promisingly, overexpression of TAK1 delayed polarization suggesting a role in axon initiation similar to LKB1. However, TAK1 requires TAB1 for kinase activity and therefore overexpression of TAK1 does not directly relate to TAK1 kinase activity towards its substrates. Instead, overexpression could act as a dominant negative due to limited amounts of TAB1 in the cell. Interestingly, Overexpresion of both TAK1 and TAB1 results in rapid cell death by 1 DIV therefore quantitative analysis could not be conducted (data not shown).

Genetic deletion of TAK1 revealed no change in cortical lamination (data not shown) and TAK1:Emx mutants were able to project axons at late embryonic stages. These data suggest that TAK1 is (1) not the major kinase upstream of LKB1 or (2) a compensatory signaling cascade is able to phosphorylate LKB1 to initiate polarization. Recently, TAK1 and TAB1 were shown to phosphorylate SAD kinases downstream of LKB1, further suggesting a role in polarization (Lilley, Pan et al. 2013). However, deletion of TAK1 using Nestin-cre and Islet cre did not alter polarization *in vivo* and double mutants for TAK1 and LKB1 did not enhance any effect of on the downstream SAD kinases *in vivo* (Lilley, Pan et al. 2013).

Future Directions

Though TAK1 was a promising candidate for neuronal polarization *in vitro* no phenotype was observed *in vivo.* Interestingly, TAK1-TAB1 overexpression results in enhanced cell death (data not shown) suggesting a key role for these proteins in neuronal survival. TAK1 is a key kinase in innate and adaptive immunity and has been studied extensively in cell lines. However, the role of TAK1 in neurons has never been addressed. It would be interesting to cross the TAK1 mutant with a Bax knockout to analyze cell death regulation as well as possible changes in glia formation.

Upon completion of the TAK1 project I generated LKB1:Nex mutants to define a role for LKB1 in axon growth and branching. I was able to conduct initial characterization of this genetic deletion prior to Dr. Franck Polleux's departure to The Scripps Research Institute. Though the role of LKB1 in axon branching was recently published (Courchet, Lewis et al. 2013), the role of LKB1 in cortical lamination will be addressed in Chapter 5.

4.5 Experimental Procedures

Mice

Mice were used according to a protocol approved by the Institutional Animal Care and Use Committee and The University of North Carolina at Chapel Hill. Time-pregnant females were obtained by overnight breedings with males with 8am as conception.

Cortical Progenitor Dissociated Cultures

E14.5 dorsal cortices were electroporated and dissected in 4ºC Hank's Balanced Salt Solution (HBSS; Gibco) and dissociated into single cells using Trypsin (Gibco) according to previously described methods (Hand, Bortone et al. 2005). Neurons were plated on glass bottom dishes (MatTek) coated with 0.1mg/mL Poly-D-Lysine (Sigma) and 5µg/mL Laminin (Sigma). Cells were cultured in Neuralbasal-A Medium (Invitrogen), supplemented with 1X B-27 (Gibco), L-glutamine (Gibco), penicillin-streptomycin (Gibco), and N2 (Gemini). Neurons were fixed with 4% PFA and stained for stage progression analysis. All constructs were cloned into the pCIG2 vector (Hand, Bortone et al. 2005). This vector uses a CMV-enhancer/chicken-beta-actin promoter with and IRES-eGFP. The TAK1 kinase dead construct was created by mutating Lysine 63 to a Tryptophan. Images were taken using a LEICA TCS SL confocal microscope. Antibodies Used: Tuj1 (beta-III tubulin)(Sigma), TAK1 (Cell Signaling), MAP2 (Sigma), DRAQ5 (Fisher), Tau (Millipore), GFP (Aves).

HEK293 and HELA Cell Cultures and Transfections

HEK293 and HELA cells were cultured in DMEM with 10% PBS 2mM L-glutamine and penicillin/streptomycin supplements. Cells were transfected using lipofectamine 2000 (4ul) with 2ug of DNA in Opti-mem media and added to cells for 3-12 hours after which media was replaced. Cells were cultured for 24 hours and lysed using RIPA buffer.

Biochemistry

Mouse cortices were dissected from three control (TAK1$^{loxp/+}$:Emx) and mutant (TAK1:Emx) mice from independent litters, collected in RIPA lysis buffer (50mM Tris, pH 7.4, 1% Triton X-100, 0.25% Sodium Deoxycholate, 0.1% SDS, 1mM EDTA, 150mM NaCl) supplemented with protease and phosphatase inhibitors and cleared by centrifugation. Proteins were separated on SDS-PAGE gradient gels, transferred to a PVDF membrane and probed for TAK1 (Cell Signaling), LKB1 (Millipore), pser431 LKB1 (Invitrogen), the internal control Actin (Cell Signaling) and secondary HRP-conjugated antibodies for detection. Blots were washed and detection was performed with a commercially available ECL kit. Co-immunoprecipitation experiments were conducted using cell lines lysed with Co-IP buffer (50mM Tris-Cl, pH 7.4, 15mM EGTA, 100mM NaCl, 0.1% Triton X-100, protease inhibitor, 1mM DTT and 1mM PMSF). 10% of the lysis was collected prior to co-immunoprecipitation for input controls. The remaining lysis was subjected to co-immunoprecipitation using TAK1 C9 (Santa Cruz) or LKB1 D19 (Santa Cruz) and bound to protein A/G beads (Santa Cruz). Samples were washed, centrifuged and the supernatant removed and then boiled in SDS loading buffer to obtain the bound fraction. Samples were run according to western blots (above).

4.6 FIGURES AND FIGURE LEGENDS

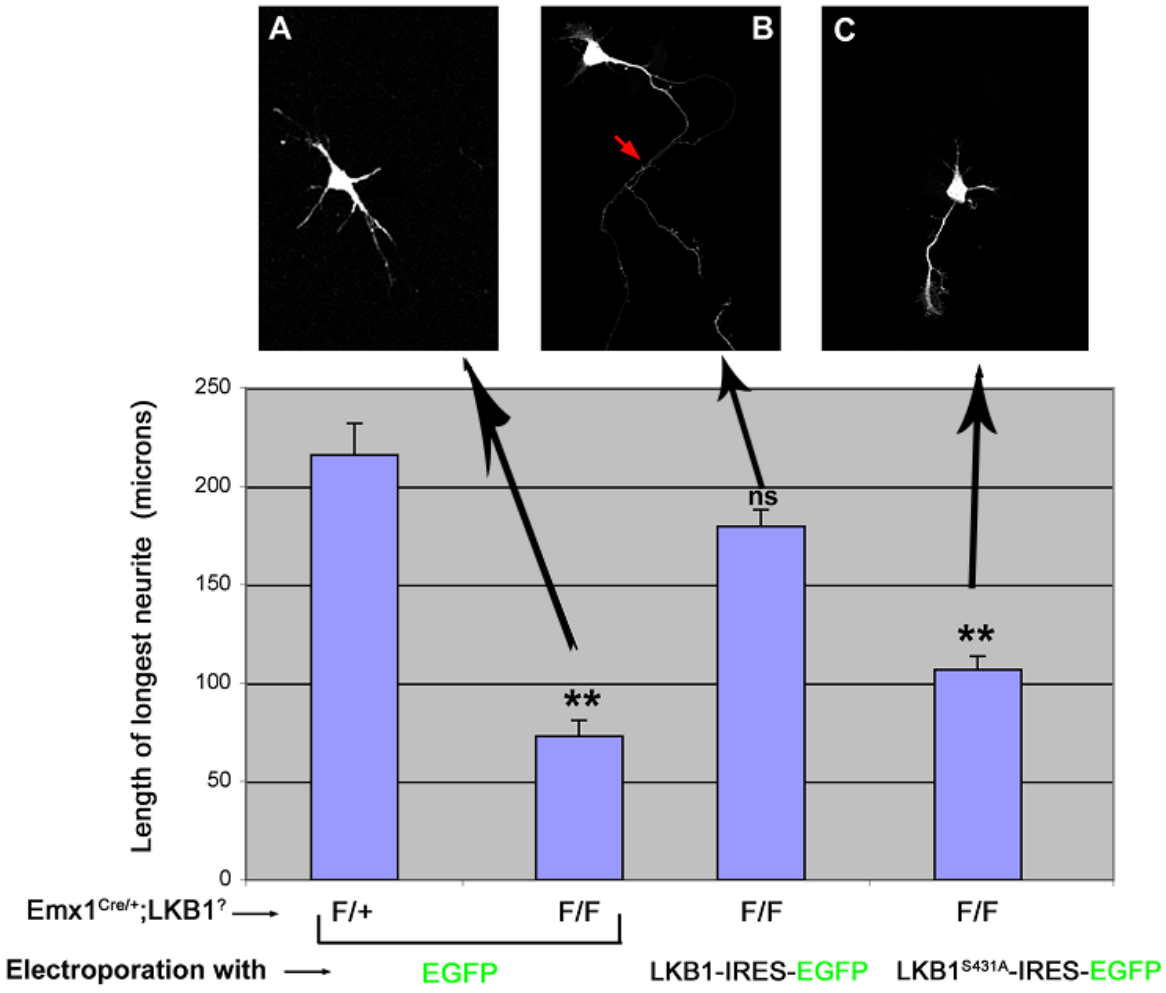


Figure 4.1 Phosphorylation of Ser431 on LKB1 is required for axon formation.

E14.5 LKB1:Emx heterozygous and homozygous embryos were electroporated with GSP, LKB1 or LKB1-S431A. Loss of LKB1 resulted in a decrease in the length of the longest neurite i.e. the axon (A). While this phenotype was fully rescued with wild-type LKB1 electroporation (B) it was not rescued by LKB1-S431A mutant electroporation. (Schema adapted from Barnes et al. 2007, culture data from Polleux and Barnes, Unpublished)

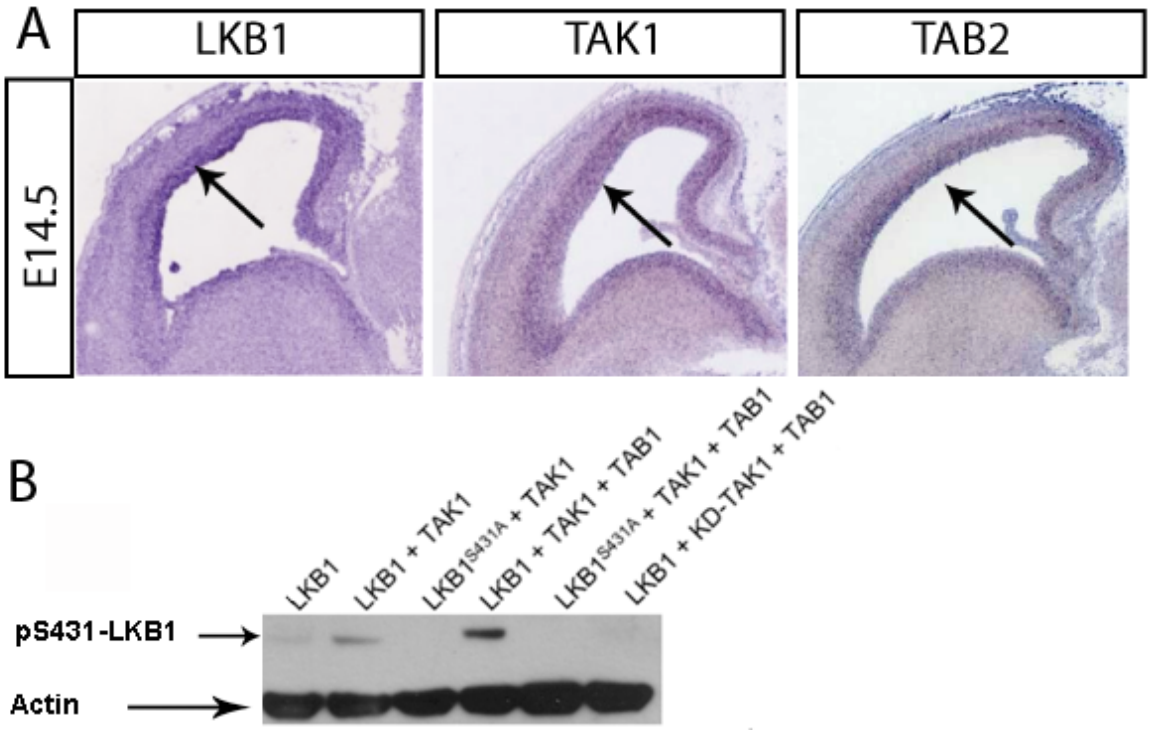


Figure 4.2 Cortical TAK1 Expression and Phosphorylation of LKB1 *in vitro.*

(A) Cortical Expression of LKB1, TAK1 and TAB2 at Embryonic Day 14.5. LKB1 Expression is enriched in the progenitor rich ventricular zone of the developing mouse neocortex (indicated by arrow). Both TAK1 and TAB2 expression are also enriched in the ventricular zone of the neocortex. (Images obtained from genepaint.org)

(B) TAK1 can phosphorylate LKB1 on Ser431 *in vitro.* Hela cells were co-transfected with combinations of TAK1, TAB1, LKB1, LKB1-s431A, and a kinase-dead TAK1 (TAK1-K63W). Cells were harvested 24 hours post transfection and lysed in RIPA buffer containing proteases and phosphatase inhibitors. A) Lysates were subjected to Western Blot analysis and probed for pS431-LKB1 and actin.

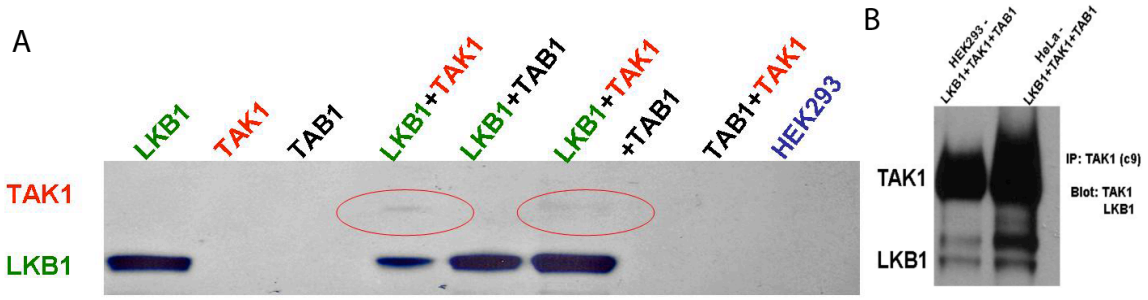


Figure 4.3 TAK1 Physical Association with LKB1 *in vitro.*

(A) TAK1 Physical Association with LKB1 in HEK293 Cells. Co-transfections of plasmids encoding TAK1, TAB1, and LKB1 were co-immuniprecipitated using an LKB1 Antibody and probed for TAK1 association.

(B) HEK293 cells transfected with plasmids encoding TAK1, TAB and LKB1 were co-immunoprecipitated using a TAK1 antibody and probed for LKB1 association.

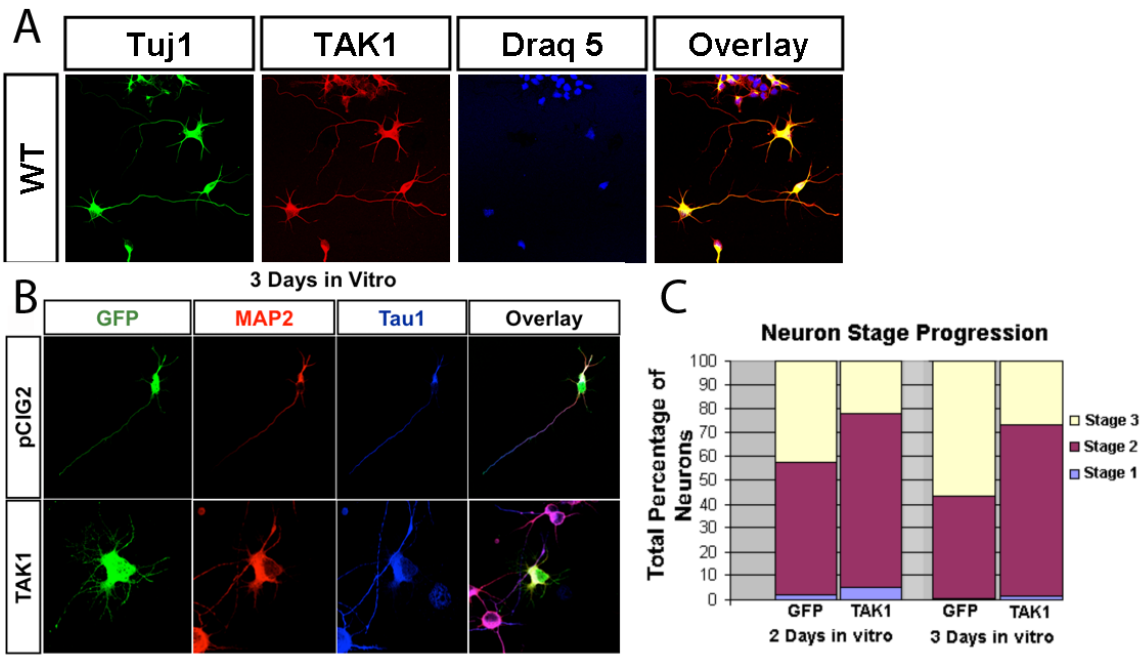


Figure 4.4. TAK1 expression in cortical neurons.

(A) Endogenous TAK1 expression in E14.5 dissociated cortical neurons. Neurons were stained for TAK1, Tuj1 (neuron-specific tubulin isoform), DRAQ5 (nuclear marker).

(B) TAK1 overexpression delays neuronal polarization. TAK1 overexpression at E14.5 followed by imaging 3 days in vitro. TAK1 overexpressing neurons have an increased multipolar stage and delayed transition into a polarized neuron with a developing axon (pCIG2 control). Neurons were stained for GFP, MAP2 (dendritic marker), and Tau1 (axonal marker).

(C). TAK1 overexpression at E14.5 followed by stage progression analysis at 2 and 3 days in vitro. TAK1 overexpression results in an increased multipolar stage at both 2 and 3 days *in vitro* when compared to control.

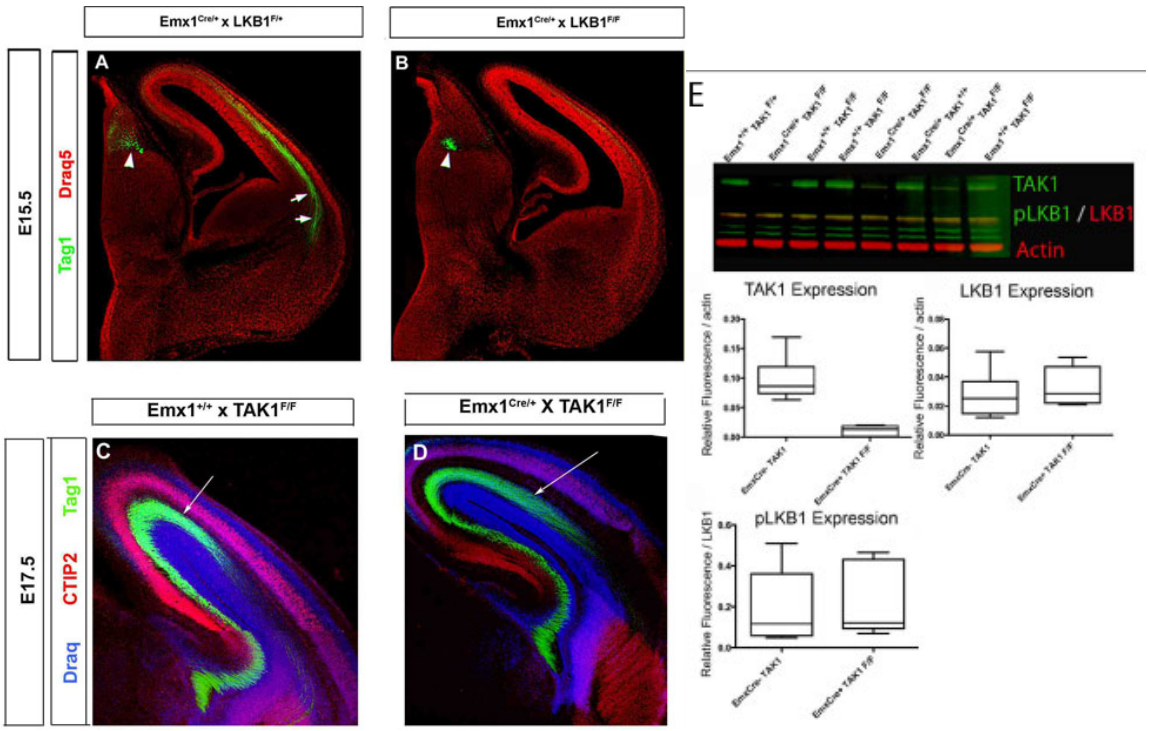


Figure 4.5. TAK1 is not required *in vivo* for axon formation.

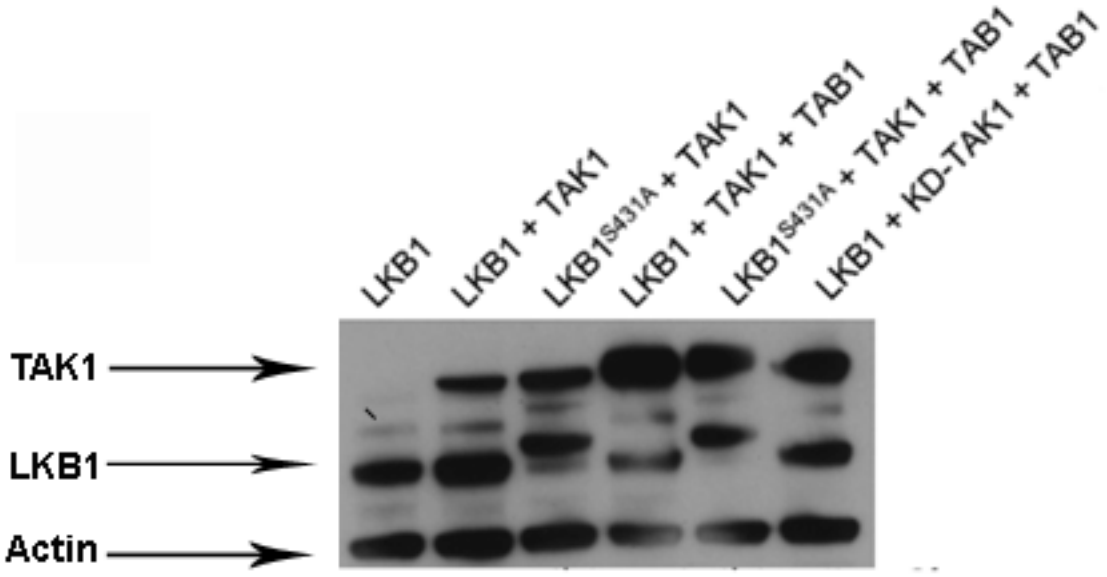
(A-B) Sections from E15.5 LKB1:Emx control and mutant embryos stained for Tag1 (corticofugal axon subset). LKB1:Emx mutants do not produce a Tag1-postive axon.

(C-D) TAK1:Emx control and mutant sections from E15.5 embryos. Sections were stained with DRAQ5 (nuclear marker), CTIP2 (layer IV neurons), and Tag1 (corticofugal axon subset). Both TAK1:Emx control and mutants project axons (arrows).

(E) Western blot analysis of E16.5 TAK1:Emx cortical lysates.. Representative western blot shows visual verification of decreased TAK1 expression in TAK1:Emx mutants when compared to controls. Quantification box plot of TAK1 protein expression in mutants and controls verifies significant decrease in TAK1 protein while no changes were observed in LKB1 and LKB1-s431 expression.

(LKB1 image adapted from Barnes et al. 2007)

4.7 Supplemental Figures and Figure Legends



Supplemental Figure 4.1 Construct Expression in HeLa Cells

HeLa Cells, which lack endogenous TAK1, TAB1 and LKB1 were transfected with combinations of constructs, lysed and probed for expression. LKB1, TAK1 and TAB1 are all expressed in these cells.

CHAPTER 5: Functions of GSK-3 Signaling in Developing Cortical Neurons

5.1 Overview

Glycogen synthase kinase 3 (GSK-3) signaling is known to influence neuronal number in the developing telencephalon via regulation of radial progenitors and intermediate neuronal precursors (INPs). However functions of GSK-3 related to cortical lamination and circuit formation have not been established. In order to assess GSK-3 functions in developing cortical neurons in vivo we created mouse mutants lacking both GSK-3α and GSK-3β in newly born cortical excitatory neurons. GSK-3 deleted neurons expressing upper layer markers exhibited striking failure of radial migration in all areas of the developing cortex. Migration failure was cell autonomous and persisted into postnatal life. In contrast, GSK-3 deletion in interneuron populations did not disrupt tangential movement. Perhaps surprisingly, misplaced GSK-3 deleted neurons extended an axon into the corpus callosum, elaborated an apical process, and developed dendritic arbors. However, the apical dendrite was frequently branched while the basal dendrites exhibited grossly abnormal orientation. In contrast to the regulation of proliferation in progenitors, GSK-3 regulation of migration in neurons appeared to be independent of Wnt/β-catenin signaling. Importantly, phosphorylation of the migration mediator, doublecortin (DCX) at ser327 and phosphorylation of the semaphorin signaling mediator, CRMP-2 at Thr514 were significantly decreased, potentially accounting for the abnormalities observed. Our data demonstrate that

GSK-3 signaling is essential for radial migration and morphogenesis of cortical excitatory neurons in the developing cortex.

5.2 Introduction

GSK-3 α and β are serine/threonine kinases that act as key downstream regulators in multiple signaling pathways, including Wnt/β-catenin, Receptor Tyrosine Kinase (RTK)/ PI3K and Sonic hedgehog (Shh) (Kaidanovich-Beilin, Beaulieu et al. 2012). GSK-3s act via mechanisms that include regulation of transcriptional factors, control of multiple aspects of cellular metabolism, and phosphorylation of cytoskeletal proteins (Hur and Zhou 2010; Kaidanovich-Beilin and Woodgett 2011). Most often, although not invariably, GSK-3s function as negatively acting kinases by inhibiting the functions of substrates at baseline. Inhibition is then relieved via signaling pathways that engage GSK-3s (Doble and Woodgett 2003; Kaidanovich-Beilin and Woodgett 2011). For most GSK-3 substrates, phosphorylation by another kinase near the GSK-3 site ('priming') is required for, or enhances GSK-3 substrate phosphorylation (Cohen and Frame 2001; Doble and Woodgett 2003; Kaidanovich-Beilin and Woodgett 2011). Priming kinases for GSK-3 substrates include cyclin dependent kinase-5 (CDK5), a kinase that is known to regulate important neurodevelopmental events like radial migration (Tanaka, Serneo et al. 2004; Cole, Causeret et al. 2006; Li, Hawkes et al. 2006; Xie, Samuels et al. 2006).

In the nervous system GSK-3β has long been thought of as a target of lithium used in treatment of bipolar disorder (Klein and Melton 1996; O'Brien, Harper et al. 2004). Some of the GSK-3β effects related to lithium actions are due to regulation of signaling downstream of dopamine receptors (Beaulieu, Sotnikova et al. 2004; Beaulieu, Marion et al. 2008; Urs,

Snyder et al. 2012). More recently GSK-3s have been implicated in the pathogenesis of schizophrenia (Emamian, Hall et al. 2004; Mao, Ge et al. 2009; Emamian 2012). Disrupted in Schizophrenia-1 (DISC1), mutated in some familial cases of schizophrenia, is thought to function in part by modulating GSK-3β effects on progenitor proliferation (Mao, Ge et al. 2009; Singh, De Rienzo et al. 2011).

Despite the obvious importance of GSK-3 signaling in pathogenesis and treatment of psychiatric disorders, there are important gaps in information on the role of GSK-3 in the developing brain. It has clearly been established that GSK-3 signaling is a strong regulator of radial progenitor proliferation in the developing cerebral cortex and that these effects are at least partly meditated through β-catenin (Chenn and Walsh 2002; Kim, Wang et al. 2009). Additionally, a recent study demonstrated an important role for GSK-3 in regulating INP amplification, an effect associated with GSK-3 binding to the scaffolding protein Axin (Fang, Chen et al. 2013). Thus, critical roles for GSK-3 signaling in processes that control neuron number in the developing telencephalon have been established.

In contrast, functions of GSK-3 in regulating developing cortical neurons are much less clear. Multiple *in vitro* studies have suggested roles for GSK-3 in regulating neuronal polarity and subsequent axonal and dendritic outgrowth and branching (See Hur and Zhou for review). These functions are thought to be mediated via GSK-3 phosphorylation of microtubule associated proteins (MAPs) including collapsin response protein-2 (CRMP-2) (Yoshimura, Kawano et al. 2005), Adenomatous polypsis coli (APC) (Shi, Cheng et al. 2004; Zhou, Zhou et al. 2004), Tau (Stoothoff and Johnson 2005), microtubule associated protein 1B (MAP1B) (Trivedi, Marsh et al. 2005), Doublecortin (DCX) (Bilimoria, de la Torre-

Ubieta et al. 2010), and subsequent regulation of cytoskeletal dynamics. In general, inhibition of GSK-3β via serine 9 (ser9) and GSK-3α via serine 21 (ser21) phosphorylation and subsequent relief of phosphorylation of downstream targets is thought be required for the formation of the axon and subsequent axon growth (Jiang, Guo et al. 2005; Hur and Zhou 2010). In a similar vein, a study employing *in utero* electroporation of an activating construct suggested that GSK-3 inhibition was essential for radial-guided cortical neuronal migration downstream of LKB1 via a mechanism involving APC (Asada and Sanada 2010). A prediction of this work might be that GSK-3 deletion would enhance axon growth and radial migration. However, to date these effects of GSK-3 on neuronal polarity and migration have not been confirmed with mouse genetic studies. Further, mice with point mutation knockins that prevent ser9/21 phosphorylation are viable and have not been reported to show defects in neuronal morphology or migration (McManus, Sakamoto et al. 2005; Gartner, Huang et al. 2006).

We have now assessed GSK-3 functions in newly born cortical neurons using Nex (NeuroD6)-cre (Goebbels, Bormuth et al. 2006) to mediate recombination in INPs and newly born excitatory neurons in GSK-3 null and conditional alleles. We demonstrate, surprisingly, that GSK-3 activity is essential for radial neuron migration in all areas of the cortex and in the hippocampus. Remarkably, the migration effects appear to be independent of Wnt/β-catenin signaling that mediates GSK-3 functions in neuronal progenitors. At later stages GSK-3 signaling is also essential for dendritic polarization. GSK-3 regulation of migration and morphology is correlated with reduced phosphorylation of DCX on ser327 and CRMP-2

on Thr514. We conclude that GSK-3 is a critical regulator of migration and morphogenesis and that GSK-3 regulation is mediated by phosphorylation of key cytoskeletal proteins.

5.3 Results

GSK-3 signaling is essential for migration of cortical excitatory neurons

To investigate the function of GSK-3 in developing cortical neurons, we generated GSK-3α-/-GSK-3βloxp/loxp: Nex-cre mice (GSK-3:Nex). The Nex-cre line induces recombination in intermediate progenitors of the dorsal telencephalon and early postmitotic neurons beginning at approximately embryonic day 11 (E11) (Goebbels et al., 2006). Prior studies using crosses with reporter lines indicate that recombination occurs in virtually all excitatory pyramidal neurons in the dorsal telencephalon (Goebbels et al., 2006; Monory et al., 2006). Western blot analysis of lysates from the whole cortex, shows a 60% decrease in GSK-3β protein at E19.5 as compared with heterozygous litter mates **[Supplemental Figure 5.1A]**. Remaining GSK-3β protein in the mutants is likely due to lack of recombination in interneurons and developing glia. At E19, GSK-3:Nex brains are roughly the same size as littermate controls. However, GSK-3:Nex mice die shortly after birth (P0-P3) for reasons that have not yet been determined.

To explore neuronal functions of GSK-3, we first assessed cortical lamination at E16, a time of rapid neuronal migration along radial glial processes. Deep layer neurons appeared to be normally positioned **[Supplemental Figure 5.1B]**. Thus staining with Tbr1, a layer 6 marker, revealed a distinctive band in the deeper layers of the cortex in both controls and GSK-3:Nex mutants. In contrast, we noted clear abnormalities in the localization of Cux1

expressing neurons that normally populate Layers 2/3 **[Supplemental Figure 5.1B]**. A clear band of Cux1 positive neurons has formed in controls by E16. In contrast GSK-3:Nex mice exhibit a dispersion of Cux1 cells with fewer neurons reaching the outermost layer, even at this early developmental stage.

Dramatic mislocalization of layer 2/3 neurons was apparent by E19.5. Coronal sections through developing somatosensory cortex showed a large population of Cux1 expressing neurons essentially "stuck" in the intermediate zone and throughout the deeper cortical layers (orange arrows) **[Figure 5.1A-A']**. Indeed some Cux1 expressing neurons in mutants were observed in the ventricular zone (arrowhead). Higher power images showed that although a few Cux1 neurons migrated successfully to layer 2/3 (yellow denoted area), migration of most Cux1 expressing cells failed in the mutants **[Figure 5.1B-B']**.

The migration defect (arrows) in GSK-3:Nex mutants was striking along the entire rostrol/caudal axis at E18.5 as observed in parasaggital sections **[Figure 5.1C-C']**. The migration defect was particularly prominent anteriorly, a developmental profile corresponding to the neurogenic gradient of the developing cortex (Caviness et al., 2009). Images at P3 demonstrated persistent migration failure with almost uniform dispersion of Cux1 neurons across all cortical layers **[Supplemental Figure 5.1C]**. Whether the subset of normally placed neurons did not undergo recombination at an early enough stage for migration to be regulated could not be determined.

We also generated GSK-3αloxp/loxp, GSK-3βloxp/loxp: Nex-cre mice (GSK-3loxp:Nex) using a GSK-3αloxp/loxp mouse line which harbors loxp sites flanking exon 2 of GSK-3α (Doble et al., 2007).. Interestingly, mice with floxed alpha rather than null

alpha alleles survived somewhat longer than the GSK-3:Nex mice but died after the second postnatal week (P15-P17). Western blot analysis of lysates from the whole cortex at P0, shows a 85% decrease in GSK-3α and a 76% decrease in GSK-3β protein when compared with wild type littermate controls **[Supplemental Figure 5.2B-C]**. At P7 GSK-3loxp:Nex mutants exhibited a striking lamination phenotype involving Cux1 neurons **[Supplemental Figure 5.2A]**. This persistent lamination defect demonstrates that GSK-3 deletion results in permanent migration failure and does not simply result in a delay.

Specificity of GSK-3 signaling for radial migration

Importantly, the migration defect in the developing cortex is specific to excitatory pyramidal neurons. In order to monitor the interneuron pool we used the DLX5/6-cre (Monory et al., 2006) line to generate conditional mice lacking GSK-3 differentiating GABAergic interneurons. Interneuron migration was monitored using the AI3 reporter line (Monory et al., 2006) (GSK-3-AI3:DLX). Surprisingly, in both controls and GSK-3 mutants, interneurons exhibited robust migration along the two migratory streams (yellow arrows) from the medial ganglionic eminence (MGE). In GSK-3 mutants, as in controls, interneurons entered all areas of the cortical plate by E19.5 **[Figure 5.2A-A']**.

In order to assess the generality of GSK-3 regulation of radial migration, we assessed migration in developing hippocampus. The hippocampus, like the cortex, is an area where developing neurons migrate along radial glial like processes (Eckenhoff and Rakic, 1984; Nowakowski and Rakic, 1979). Nex-cre expression is evident in developing hippocampal neurons as early as E14 (Goebbels et al., 2006), allowing us to delete GSK-3 in those cells.

Pyramidal neurons generated from the hippocampal primordia undergo migration along radial processes to form CA1/CA3 and the dentate gyrus (DG) (Altman and Bayer, 1990) **[Figure 5.2B]**. The transcription factor CTIP2 marks neurons in the developing CA1 region. GSK-3:Nex mice exhibited a striking hippocampal migration defect. CTIP2 expressing neurons did not migrate properly (yellow arrows) and as a consequence CA1 did not fully develop **[Figure 5.2B-B']**. As a result, CA1-3 and the DG (arrowheads) were disorganized and the hippocampal sulcus was not well defined **[Figure 5.2C-C"]**. These defects were striking in rostrol areas as shown although somewhat less pronounced in caudal sections (data not shown). Interestingly, fimbrial axonal projections (orange arrow) formed in GSK-3:Nex mice demonstrating that even though migration fails, hippocampal neurons were able to polarize and extend appropriately directed axons **[Figure 5.2B]**.

GSK-3 regulation of migration is cell autonomous

To assess whether the migration defect was cell autonomous and to visualize morphology of GSK-3 deleted neurons, we introduced Cre and EGFP into a subpopulation of developing neurons in GSK-3α-/-GSK-3βloxp/loxp mice. NeuroD6 Cre (NeuroD-Cre) and LacZ/EGFP (Z/EG) were injected into the ventricles and co-electroporated at E14-15.5. Electroporation at this age targets radial progenitors that generate mainly upper layer neurons. This co-electroporation technique allowed us to visualize individual GSK-3 deleted neurons in an otherwise control background. Labeled neurons were imaged at late embryonic and postnatal stages.

Deletion of GSK-3 in individual neurons phenocopies the migration delay seen in the GSK-3:Nex mutants. At E19 most control neurons were located in the upper cortical layers as expected **[Figure 5.3A]**. In contrast, most GSK-3 deleted neurons had cell somas that were localized to the deeper layers of the cortex, very few were found in upper layers **[Figure 5.3A']**. Importantly, GSK-3 deleted neurons were clearly able to project axons (orange arrows) suggesting that initial polarization had proceeded in the absence of GSK-3. Further, most GSK-3 null neurons in the cortical plate elaborated a long leading process directed toward the pial surface (arrowheads) **[Figure 5.3A']**. Thus at least the initial stages of dendritic arborization also appeared to proceed in the absence of GSK-3.

The GSK-3 migration defect was strikingly persistent into the postnatal period. In mice electroporated at E14-15.5 and analyzed at P10, GSK-3 deleted neurons remained in the deeper layers and subcortical white matter **[Figure 5.3B-B']**. EGFP expressing neurons co-labeled with Cux1 (red) demonstrating that they were able to acquire the proper laminar markers but were unable to attain the proper position **[Figure 5.3D, arrows]**. Again, long apical processes that reached the pial surface were elaborated by GSK-3 deleted neurons. Arborization was apparent in layer 1 **[Figure 5.3D, yellow arrowheads]**, basal dendrites formed, and axons projecting towards the corpus callosum were evident. Thus GSK-3 regulates some critical aspect of migration but not the early stages of axon and dendrite formation (see below).

In quantifying our results, we found that in control animals the majority of electroporated neurons (73.7%) were found in the upper layers of the cortex (yellow bars) by P10 **[Supplemental Figure 5.3AB]**. In contrast, GSK-3 deleted neurons remained in the

deeper layers of the cortex and only 23% had reached the upper layer 2/3 by P10 ($p=<0.005$) .

GSK-3 deletion in post-mitotic neurons delays multipolar to bipolar transition

A number of in vitro studies have suggested that GSK-3 regulates neuronal polarization. It is plausible that some defect or delay in the polarization might account for migration failure. To test this idea, we electroporated NeuroD-Cre and Z/EG into the GSK-3α-/-GSK-3βloxp/loxp cortex, plated cortical cells in dissociated culture and assessed stage progression at three days in vitro (3DIV). We observed no statistically significant difference in the stage progression between control and GSK-3 deleted neurons **[Supplemental Figure 5.4A]**. Further, GSK-3 deleted neurons that successfully extended an axon remained highly dynamic **[Supplemental Figure 5.4B]** and extended and retracted neurites. Thus at least some processes that require complex cytoskeletal regulation proceed normally in the GSK-3 deleted neurons.

To assess the cell biological mechanisms of GSK-3 regulation of neuronal migration, we co-electroporated NeuroD-Cre;Z/EG or a control pCAG-dsRED construct into the lateral ventricles of control and GSK-3α-/-GSK-3βloxp/loxp mice. This was followed by live imaging of migration ex vivo in a cortical slice preparation (Hand and Polleux, 2011) at 3DIV. In controls, electroporated with pCAG-dsRED, labeled neurons transitioned from multipolar to bipolar morphology and migrated through the cortical plate over a period of 12 hours, as expected **[Figure 5.3E]**. The progress of individual neurons could readily be tracked and is indicated for 3 examples by the progress of the colored arrowheads in the 3

panels. In contrast GSK-3-deleted neurons failed to translocate through the intermediate zone and remained in a multipolar state in the outer subventricular zone (arrowheads) **[Figure 5.3E, and supplemental movie 5.1]**. Further, during the 12 hours of observation most GSK-3 deleted neurons did not transition to a bipolar morphology, a step thought to be required for radial-guided migration.

GSK-3 regulation of axonal and dendritic morphology

GSK-3's ability to regulate multiple cytoskeletal proteins would suggest that GSK-3 deletion would have profound effects on dendrite and axonal arborization. Indeed, GSK-3 inhibition in vitro regulates development of neuronal polarity as well as axon growth and branching (reviewed in Hur and Zhou, 2010). To address GSK-3 regulation of morphology we deleted GSK-3 cell autonomously by in utero electroporation and analyzed dendritic morphology and axon targeting at P15.

As demonstrated above, most GSK-3 deleted neurons failed to migrate and populated the deeper layers of the cortex at P15 **[Figure 5.4A,A';B,B']**. Despite mispositioning, GSK-3 deleted neurons extended axons into the callosum towards the contralateral cortex **[Figure 5.4C]**. In controls we observed axons branching extensively in layers 2/3 and 5 of the contralateral cortex, as previously described (Hand and Polleux, 2011) **[Figure 5.4D]**. In contrast, GSK-3 deleted axons reaching the contralateral cortex very rarely entered the cortical plate and the axons did not arborize in layers 2/3 or 5 **[Figure 5.4C']**. Whether this failure of arborization is a result of deficient axon growth or lack of responsiveness to specific targeting cues remains unclear.

We also analyzed the dendritic arborization in GSK-3 deleted neurons. Many GSK-3 deleted neurons exhibited striking defects in the apical dendrite and misorientation of the basal dendrites **[Figure 5.5]**. Because improper laminar position might affect dendritic arborization we focused analysis on a small subset of GSK-3 deleted neurons that reached layer 2/3. Remarkably, these normally positioned GSK-3 deleted neurons also exhibited abnormally oriented basal dendrites that in many cases, were oriented towards the pial surface rather than towards the deeper cortical layers **[Figure 5.5C]**. The apical dendrites of the GSK-3 deleted neurons properly oriented to the pial surface but often branched and extended apically rather than laterally **[Figure 5.5D]**. Examples of markedly abnormal apical dendrites (orange arrows) and abnormally oriented basal dendrites (blue arrowheads) are shown in Figure 5.5B.

GSK-3 regulation of migration is independent of Wnt/β-catenin signaling

Signaling via β-catenin is an obvious candidate to mediate GSK-3 regulation of migration. In the canonical Wnt cascade, Wnt signaling through the frizzled receptor leads to dishevelled and GSK-3 sequestration, β-catenin accumulation and enhanced transcription (Kaidanovich-Beilin and Woodgett, 2011). In radial progenitors, β-catenin signaling is clearly an important mediator of the effects of GSK-3 deletion (Chenn and Walsh, 2002; Kim et al., 2009).

To determine the role of GSK-3 regulation of β-catenin in the cortical migratory pathway, we utilized a β-catenin mouse that harbors loxP sites flanking exon 3 (βcatEx3:Nex) (Harada et al., 1999). Exon 3 encodes the residues that GSK-3

phosphorylates to signal β-catenin degradation; thus deleting exon 3 renders β-catenin unresponsive to GSK-3 regulation. In contrast to GSK-3:Nex, βcatEx3:Nex mice survive, breed and have no overt behavioral phenotype. They display a rostral midline defect resulting in a lack of the hippocampal commissure **[Figure 5.6A, arrow]**, as seen in other models using stabilized β-catenin (Chenn and Walsh, 2003). However, βcatEx3:Nex mutants exhibit normal cortical lamination and normal distribution of Cux1 neurons in the upper cortical layers **[Figure 5.6B]**. These data suggest that the migration defect associated with GSK-3 deletion may be independent of Wnt/β-catenin signaling.

Further supporting the concept that β-catenin may not be a critical mediator of migration, is that GSK-3loxp:Nex lysates at P0, an age when the migration phenotype is well developed, show little if any change in β-catenin protein levels **[Supplemental Figure 5.2B]**. We cannot rule out changes that would be masked by the dilution effect of non-recombined cells, changes confined to specific cellular compartments, or changes that might be more apparent later in development. For additional assessment of the role of β-catenin signaling we performed global analysis of GSK-3 transcriptional targets at E18 in control and GSK-3:Nex cortical lysates using affymetrix microarray analysis. Consistent with loss of GSK-3 in our conditional mutants, probe level information specific to exon 2 of GSK-3β showed that exon 2 was decreased by an average 2.15 fold [Figure 6C]. However, classic WNT pathway target genes downstream of β-catenin/(TCF)/LEF-1 transcription factors, including CyclinD1, Brachyury, Wisp1, Cdx1, Engrailed2, and Neurogenin 1 were unchanged.

To further assess the role of Wnt/β-catenin signaling in cortical lamination we created a conditional dishevelled 2loxp/loxp (Dvl2) mouse. We then generated a triple mutant by crossing our floxed Dvl2loxp/loxp with existing Dvl1 and Dvl3 nulls and the Nex-Cre line (DVL123:Nex). Deletion of all three DVLs presumably completely abrogates Wnt signaling via the canonical pathway. Remarkably, lamination in the triple allele mutant Dvl123:Nex appears relatively normal at E18 **[Figure 5.6D]**. This result further supports the idea that GSK-3 regulation of migration is not mediated by the WNT/β-catenin cascade.

Taken together, these three lines of evidence suggest that migration defects in GSK-3 deleted neurons are not mediated by changes in β-catenin signaling.

Surprising lack regulation by LKB1, CDC42, PTEN

Recent work utilized RNAi and GSK-3 S9A mutant constructs to conclude that LKB1 inactivation of GSK-3 via ser9 phosphorylation alters neuronal migration (Asada and Sanada, 2010). However, GSK-3 knock-in S9A/S21A GSK-3 'constitutively active' mice develop normally and migration defects have not been reported (Gartner et al., 2006; Jiang et al., 2005). These results indicate that ser9/ser21 phosphorylation status may not correlate with the neurodevelopmental function of GSK-3 in vivo. To further address the issue of GSK-3 inhibition downstream of LKB1, we genetically deleted LKB1 from developing excitatory neurons (LKB1:Nex). LKB1:Nex mutant mice die around P20. Perhaps surprisingly, no lamination abnormalities were observed **[Fig 5.6E]**. These results suggest that LKB1 regulation of GSK-3 activity is not essential for radial-guided neuronal migration in vivo.

In Receptor Tyrosine Kinase (RTK) cascades, GSK-3 lies downstream of PI3K/PIP/AKT. Signals transduced through these cascades inhibit GSK3 by phosphorylation on Ser9/Ser21 (Hur and Zhou, 2010). The phosphatase PTEN suppresses PI3K signaling. To determine if PI3K signaling affects migration we genetically deleted PTEN in neurons using Nex-Cre. Though PTEN:Nex mutants die around birth, no overt lamination defects were observed **[Figure 5.6E]**.

Finally, we deleted a key regulator of Par6-aPKC, CDC42, using Nex-Cre. aPKCs are also known to phosphorylate Ser9/Ser21 of GSK-3 (Etienne-Manneville and Hall, 2003). CDC42:Nex mutants also die shortly after birth, but again we observed no lamination defect in these mutant mice **[Figure 5.6E]**.

These findings taken together suggest that regulation of GSK-3 phosphorylation at ser9/ser21 is not important in the control of radial migration.

Phosphorylation status of GSK-3 substrates

To determine the status of relevant GSK-3 targets we conducted western blot analysis of GSK-3 substrates that have been implicated in migration regulation. As noted above, β-catenin levels were not dramatically changed raising the possibility that β-catenin is regulated differently in post-mitotic neurons versus progenitors. Also we did not detect changes in pSer722 FAK (Bianchi et al., 2005), pSer dynamin-1(Clayton et al., 2010) and pSer129 CREB(Bullock and Habener, 1998; Fiol et al., 1994) **[Figure 5.7B]**. Presumably other kinases contribute to phosphorylation of the putative GSK-3 sites in vivo.

Interestingly, deletion of GSK-3 drastically reduced the phosphorylation of two key proteins previously implicated in migration. GSK-3loxp:Nex mutants showed an 80% decrease in phosphorylated CRMP2 on Thr514 and a 61% decrease in phosphorylated DCX on Ser327 (Gdalyahu et al., 2004) **[Figure 5.7A]**. Changes in the phosphorylation status of these proteins has been implicated in migration, raising the possibility that reduced functions of these proteins toward microtubules mediate GSK-3 effects.

5.4 Discussion

Overview

In this work, we have documented a requirement for cell autonomous GSK-3 signaling in multiple aspects of the development of cortical excitatory neurons. GSK-3 activity is critical for the radial migration of later born, Cux1-expressing neurons in all regions of cortex. Few Cux1 neurons can migrate to upper layers in GSK-3 deleted mice and regulation of migration is clearly cell autonomous. Whether the few neurons that successfully migrate do so in a GSK-3 independent manner or whether the timing of GSK-3 deletion leaves some neurons with residual GSK-3 activity could not be determined.

The GSK-3 requirement appears to be specific for radial migration. Migration of both GSK-3 deleted cortical and hippocampal neurons along radial processes was dramatically impaired. In contrast, tangential migration of neurons deriving from MGE progenitors was not clearly affected. The migration failure was persistent and readily apparent at P15, the latest time point examined.

At later developmental stages, additional abnormalities were noted. Perhaps surprisingly, GSK-3 deleted neurons elaborated appropriately directed axons that elongated for substantial distances. However, after crossing the corpus callosum, axon branching and invasion of target fields in contralateral cortex was compromised. Further, abnormalities of dendritic arborization were readily apparent. Normally, cortical projection neurons in layer 2-3 have an apical process reaching the pial surface, but basal dendrites that are directed laterally and towards the ventricular zone. In striking contrast, basal dendrites of GSK-3 deleted neurons almost invariably pointed upwards towards the pial surface.

GSK-3 regulation of cortical development

Our work builds on a growing body of evidence establishing critical requirements for GSK-3 in cortical development, with different functions characterizing different stages of development. In radial progenitors, GSK-3 signaling is a critical regulator of proliferation. GSK-3 deletion leaves cells "locked" into the proliferative phase due to regulation of proliferation drivers via β-catenin and other signaling mediators. At a later stage, recent work has demonstrated that GSK-3 is a key mediator of the amplification of the INP pool via interactions with the scaffolding protein Axin (Fang et al., 2013). Axin-GSK-3 binding in the cytoplasm is clearly required for expansion of the INP pool, although the mechanism of GSK-3 action was not specified in this study. Interestingly β-catenin regulation of transcription in the nucleus was required for differentiation of INPs into neurons. These effects of GSK-3 signaling on radial progenitors and INPs would clearly strongly influence cortical neuronal number in the developing cortex.

We now find that at later stage, excitatory neuron development is also under important GSK-3 regulation. Thus, Cux1 expressing neurons require GSK-3 activity for migration along radial processes. GSK-3 deletion leads to migration failure of Cux1 positive cells in all regions of the dorsal telencephalon. The behavioral consequences in GSK-3 deleted mice could not be assessed due to early death of the animals. Whether death was due to cortical abnormalities or defects in other cells that underwent recombination with Nex-Cre could not be determined. However, even a very mild form of this type of migration defect would be catastrophic for human brain development.

In contrast to regulation of progenitor proliferation and neural differentiation, GSK-3 regulation of migration appears to be independent of Wnt/β-catenin signaling. Interestingly, the migration defect also appears to be independent from one recently associated with mutants of the schizophrenia associated protein, DISC1. DISC1 regulates progenitor proliferation via interactions with GSK-3, decreased GSK-3 kinase activity and, ultimately, increased β-catenin signaling see (Mao et al., 2009). Interestingly, a recent study demonstrated that DISC1 regulates migration independently of GSK-3 via effects on the centrosome (Ishizuka et al., 2011). However, our work reported here clearly demonstrates that GSK-3 also has a critical role in the regulation of cortical neuronal migration. Radial migration is an enormously complex process requiring progenitor differentiation, dramatic morphological change, intricate mechanisms for cell and nuclear movements and sensing of multiple cues that start and stop the process. Not surprisingly, GSK-3 joins dozens of other molecules implicated in the control of radial migration (see (Ayala et al., 2007) for review). Comparisons with the literature suggest that effects of GSK-3 deletion are

among the most severe that have yet been observed. Interestingly, GSK-3 importantly regulates multiple proteins implicated in migration and is an important mediator in several of the signaling pathways involved. Thus GSK-3 is known to phosphorylate Doublecortin (Bilimoria et al., 2010), FAK (Bianchi et al., 2005), dynamin (Clayton et al., 2010), neurogenin (Li et al., 2012), CRMP-2 (Uchida et al., 2005), and MAP1B (Trivedi et al., 2005). Further GSK-3 mediates Reelin signaling (Beffert et al., 2002), LKB1 effects (Asada and Sanada, 2010), cdc42 effects (Etienne-Manneville and Hall, 2003), integrin signaling (Guo et al., 2007), semaphorin signaling (Eickholt et al., 2002; Uchida et al., 2005) and other pathways that have been implicated in control of radial migration. Finally, GSK-3 shares multiple substrates with CDK5, a kinase that is situated among the most important neuronal intrinsic regulators of migration.

Clearly GSK-3 effects on cortical development extend well beyond the phase of neuronal migration. At stages when axons are projecting to target fields and dendritic arbors are maturing, there continues to be striking regulation by GSK-3. Interestingly the reported effects of deleting GSK-3 in vitro are quite different than the in vivo effects we demonstrate here (see below). From prior studies one might have expected that neurons would elaborate multiple axons, branch extensively, and that dendritic arborization would be suppressed (see Hur and Zhou, 2010 for review). We demonstrate here that GSK-3 deleted neurons can clearly elaborate axons and dendrites both in vitro and in vivo. Differences with prior in vitro studies using inhibitors, silencing RNAs, and activated constructs may relate to the exact timing of full GSK-3 deletion, off-target effects of other techniques, or differences between contextual and dissociated preparations.

Although dendritic arbors form, dendritic arborization abnormalities are striking in GSK-3 deleted neurons. Importantly, abnormalities in apical and basal dendrite orientation have been noted after deletions of guidance molecules and guidance molecule signaling pathways. In particular, interference with semaphorin signaling produces a similar type of interference with apical process development and orientation of basal dendrites (see below).

Mechanisms of GSK-3 regulation

In general GSK-3 acts via two classes of mechanisms: one where GSK-3 activity inhibits substrate function or availability and another where GSK-3 activity is required for substrate function. Therefore, we might expect GSK-3 deletion to enhance processes normally inhibited by GSK-3 activity and to inhibit processes that require GSK-3 activity.

Multiple studies have suggested that inhibition of GSK-3 kinase activity is important to morphological functions such as establishment of neuronal polarity and cellular migration. For example, several in vitro studies have shown that appropriate regulation of GSK-3β ser9 phosphorylation downstream of Akt is key to establishment of hippocampal neuron polarity. Increasing inhibition results in formation of multiple axons at the expense of dendritogenesis (Jiang et al., 2005; Yoshimura et al., 2005). Effects of GSK-3 inhibition are thought to be mediated by dephosphorylation of CRMP-2, APC and other cytoskeletal mediators with resulting stabilization of microtubules at the tips of axons (Hur and Zhou, 2010; Yoshimura et al., 2005). In a similar vein, astrocyte migration is thought to be mediated in vitro by a CDC42/aPKC mediated ser9 phosphorylation of GSK-3β, dephosphorylation of APC with localization to the microtubule tips, and subsequent stabilization of microtubules at the leading edge (Etienne-Manneville and Hall, 2003). Another recent study employing in utero

electroporation of constructs suggested that an LKB1/GSK-3 pathway resulting in GSK-3β ser9 phosphorylation and APC localization at the leading edge was important in cortical neuronal migration (Asada and Sanada, 2010).

In the studies outlined above, inhibition of GSK-3β kinase activity is indicated by phosphorylation on Ser9, and inhibition of GSK-3α by phosphorylation on Ser21. However, a major unresolved paradox is that mice with GSK-3 α and β point mutation knock-ins that prevent ser9 and ser21 phosphorylation respectively show no widespread morphological abnormalities in vitro or in vivo (Gartner et al., 2006). Similarly, truncation mutants of the Drosophila GSK3- homologue, Shaggy, that lack inhibitory phosphorylation sites are not associated with morphological abnormalities (Papadopoulou et al., 2004). In the context of neuronal migration, although GSK-3 ser9/21 phosphorylation is enhanced downstream of Reelin signaling (Beffert et al., 2002; Gonzalez-Billault et al., 2005), surprisingly GSK-3β kinase activity towards the important substrate MAP1B is increased (Gonzalez-Billault et al., 2005). Finally, our findings reported here do not support critical roles in radial migration for LKB1, cdc42, or the PI3K pathway, all of which are known to regulate GSK-3 ser9/21 phosphorylation. These findings, taken together, raise questions about the importance of negative regulation of GSK-3β via Ser9 phosphorylation on functions of microtubule binding proteins related to migration in vivo.

Our results are more in line with a second type of regulation, GSK-3 activity being required for normal substrate function. Several proteins have been shown to require GSK-3 kinase activity for normal function. These include DCX where GSK-3 mediated phosphorylation is thought to be required for DCX's actions in regulating axon branching

and possibly in migration (Bilimoria et al., 2010). Additional examples possibly relevant to migration are GSK-3 regulation of neurogenin phosphorylation at ser231 (Ma et al., 2008), FAK phosphorylation at ser722 (Bianchi et al., 2005) and dynamin-1 phosphorylation of ser774 (Clayton et al., 2010). GSK-3 phosphorylation regulates functions of these proteins in complex ways, but possibly in ways that may be required for neuronal migration.

A well studied example of a pathway that is associated with upregulation of GSK-3 activity is semaphorin signaling. Semaphorin family members have multiple roles as chemorepellants and chemoattractants for many classes of axons and dendrites (for review see (Pasterkamp, 2012)). Semaphorin signaling activates GSK-3 (Eickholt et al., 2002) resulting in phosphorylation of a key MAP, CRMP-2 (Uchida et al., 2005). In vitro, CRMP-2 phosphorylation is associated with axonal growth cone collapse, whereas CRMP-2 dephoshorylation is associated with formation of multiple axons in hippocampal cultures (Brown et al., 2004). Intriguingly, during neuronal polarization semaphorin signaling is associated with suppression of axons and formation of dendrites (Shelly et al., 2011). Importantly, migration and dendritic abnormalities that we have described here are consistent with cortical neuron phenotypes reported due to manipulation of semaphorin signaling. Thus application of exogenous semaphorin supports apical process formation and dendritic arborization (Polleux et al., 2000). Further interfering with semaphorin, NP1 or Plexin via silencing RNAs and gene knockouts has been reported to interfere with radial migration (Chen et al., 2008; Renaud et al., 2008). Many of the same manipulations have resulted in branched apical dendrites and abnormal dendrite orientation (Morita et al., 2006; Nakamura et al., 2009). Although GSK-3 deletion does not exactly phenocopy these previously

reported semaphorin effects, there are enough similarities to suggest that GSK-3 deletion effects may be due at least in part to abrogation of semaphorin signaling. Finally, in the context of cortical neuronal migration, phosphorylation of CRMP-2 promotes migration. Thus a GSK-3 phosphomimetic CRMP-2 can rescue migration defects associated with knockdown of the semaphorin signaling mediator, α2-chimaerin (Ip et al., 2012).

Conclusion:

In sum we have demonstrated a key cell autonomous role for GSK-3 signaling in regulating radial migration and morphological development of cortical excitatory neurons. Our GSK-3 deletion results are not in line with what might have been predicted form prior studies that have correlated ser9/21 phosphorylation with relief of negative GSK-3 phosphorylation and negative regulation of cytoskeletal associated proteins. In particular we do not find evidence that an LKB1/GSK-3 inhibitory pathway is a key migration regulator. In contrast, our results are consistent with the idea GSK-3 phosphorylation of DCX and CRMP2 are required for appropriate cytoskeletal regulation and migration. Finally, our data are consistent with the idea that GSK-3 activity is essential for many of the effects of semaphorin signaling on cortical development.

5.5 Experimental Procedures

Generation of Conditional Mice

Mice were cared for according to animal protocols approved by the Institutional Animal Care and Use Committees of the University of North Carolina at Chapel Hill.

GSK-3$\alpha^{-/-}$ mice possessing exon 2 deletions and GSK-3α/β loxp flanked exon 2 mice have been previously described (MacAulay, Doble et al. 2007; Patel, Doble et al. 2008). All GSK-3 lines were generously provided by Jim Woodgett. GSK-3:Nex mice were generated by mating GSK-3$\alpha^{-/-}$, GSK-3$^{loxp/loxp}$ with Nex:Cre mice generously provided by Dr. KA Nave (Goebbels, Bormuth et al. 2006). GSK-3 mutant mice and Nex-Cre mice were maintained on a mixed background. Triple allelic male mutants (GSK-3$\alpha^{+/-}\beta^{f/f}$:Nex) fail to survive unto adulthood with death occurring around P25 for reasons not yet determined and GSK-3$\alpha^{-/-}$ males are infertile, as previously reported (Maurin, Lechat et al. 2013).

Ctnnb1 exon 3 floxed (β-catenin constitutively active) mice were previously described (Harada, Tamai et al. 1999). LKB1 floxed mice have been previously described (Bardeesy, Sinha et al. 2002). PTEN floxed mice (Groszer, Erickson et al. 2001) and CDC42 floxed mice were purchased from Jackson Laboratory and have previously been described (Chen, Liao et al. 2006).

Dvl2 floxed allele was generated in the UNC Neuroscience Center Molecular Neuroscience Core using conventional methodology. Dvl1and Dvl3 (Tsang, Lijam et al. 1996) knockout mice were purchased from from Jackson Laboratory and have been previously described. All mice are on a mixed background.

Results shown in all of the figure panels were based on at least three experiments with mice from independent litters, unless otherwise noted.

In Utero Electroporation

Mice were anesthetized using either 2,2,2-Tribromoethanol (4mg/10 g mouse) or isofluorine and embryos were exposed at E14.5 or E15.5. Plasmids (2ug/ul NeuroD-Cre and 1ug/ul Z/EG) mixed with fast green were then microinjected into the lateral ventricle of embryos. Embryos were electroporated with five 50 ms pulses at 30V with a 950 ms interval and returned to the abdominal cavity **[Supplemental Figure 5.5]**. A CF1 foster dam was used to aid postnatal survival studies. Depending on the experiment mice were analyzed at E19.5, P6, P10 or P15.

Ex vivo Electroporation and organotypic cortical slice culture

Cortical progenitor cells were electroporated ex vivo at E15.5 as described previously (Hand et al., 2005). Briefly, E15.5 embryos were decapitated, plasmids were injected into lateral ventricle followed by electroporation with four 30V pulses that were 40ms in duration and separated by a 100ms interval. Following electroporation, brains were dissected and vibratome sectioned at 250μm. Slices were transferred to Poly-D-Lysine and laminin coated culture insert (Millicell, Millipore) in a FluoroDish (World Precision Instruments), then 2ml Basal Medium Eagle with FBS, N2 (Gemini, 400-163), B27 (Gibco, 17054-044), penicillin-streptomycin (Gibco, 15140-122) and L-glutamine (Gibco, 25030-081) supplements were added. Slices were cultured for 3 days at 37ºC and live imaged using an Olympus FV1000 Confocal microscope with stage incubator.

Cortical Progenitor Dissociated Cultures

E14.5-15.5 dorsal cortices were electroporated with NeuroD-Cre; Z/EG, dissected in 4ºC Hank's Balanced Salt Solution (HBSS; Gibco) and dissociated into single cells using Trypsin (Gibco) according to previously described methods (Hand, Bortone et al. 2005). Neurons were plated on glass bottom dishes (MatTek) coated with 0.1mg/mL Poly-D-Lysine (Sigma) and 5μg/mL Laminin (Sigma). Cells were cultured in Neuralbasal-A Medium (Invitrogen), supplemented with 1X B-27 (Gibco), L-glutamine (Gibco), penicillin-streptomycin (Gibco), N2 (Gemini) and FBS. Neurons were fixed with 4% PFA and stained for stage progression analysis. For stage progression analysis heterozygote controls were compared to conditionally deleted neurons (n=3, 1341 cells).

Western Blotting

Mouse cortices were dissected from three control (GSK-3$\alpha^{-/-}\beta^{loxp/+}$:Nex) and mutant (GSK-3:Nex) or three control (GSK-3$\alpha^{loxp/loxp}\beta^{loxp/loxp}$) and three mutant (GSK-3$\alpha^{loxp/loxp}\beta^{loxp/+}$:Nex) mice from independent litters, collected in RIPA lysis buffer supplemented with protease and phosphatase inhibitors and cleared by centrifugation. Proteins were separated on SDS-PAGE gradient gels, transferred to a PVDF membrane and probed for GSK-3 (Invitrogen), the internal control GAPDH (Cell Signaling), and secondary HRP-conjugated antibodies for detection. Blots were washed and detection was performed with a commercially available ECL kit. Image J software (NIH) was used for quantification of band intensity relative to control. Statistical analyses were conductive using Prism software. Differences were considered statistically significant at $p< 0.05$. The following antibodies

were used: GSK-3 (Invitrogen), Actin (Cell Signaling), pFAK ser722 (santa cruz), FAK (Cell Signaling), pCRMP2 Th514 (cell signaling), CRMP2 (cell signaling), pCREB ser129 (santa cruz), CREB (santa cruz), pDCX ser327 (The Reiner lab), pDynamin-1 ser744 (santa cruz), Dynamin-1 (santa cruz), B-catenin (Cell Signaling), GAPDH (Cell Signaling), Cleaved Caspase-3 (Cell Signaling).

Immunohistochemistry

Briefly, 100-350μm free floating vibratome sections of brains were collected in PBS, blocked with 5% normal serum in PBS with 0.1% Triton X-100, and incubated with primary antibodies in blocking solution overnight at 4ºC. For cryosectioned tissue samples were immunostained according to standard procedures. P15 in utero vibratome sections were blocked with 5% normal serum in PBS with 0/1% Triton X-100 and 2% DMSO, and incubated with primary antibodies for 3 days. Slices were rinsed with PBST for 24 hours and incubated with secondary antibodies for 3 days in PBS with 0/1% Triton X-100. GSK-3 staining conducted using Teramide Signal Amplification (PerkinElmer, NEL744001KT).

The following primary antibodies were used in our study: L1 (Chemicon), Cux1 (Santa Cruz), Neuronal Nuclei (Chemicon), GFP (Aves), CTIP2 (Abcam) DRAQ5 (Fisher), TBR1 (1:1000, Abcam), HOECHST (sigma, RFP (Rockland). After rinsing, sections were then incubated with Alexa conjugated secondary antibodies (Invitrogen) overnight at 4ºC, rinsed 3 times in PBS, and mounted with gel/mount (Biomeda).

Quantification of cortical layering

GSK-3α+/-β F/F control mice and GSK-3α-/-β F/F conditionally deleted embryos and postnatal pups were cryosectioned at 200um and used for analysis. The cortical plate was equally divided into five cortical bins **[Supplemental Figure 3]**, cells in each bin were counted using ImageJ software and the percentage of GFP+ neurons in each bin were determined from multiple comparable sections. Using this method, bins four and five correspond to the upper layer 2/3 Cux1 positive layers. A total of 4209 neurons were counted and differences were considered statistically significant at $p =< 0.05$ using an unpaired t-test.

Image Acquisition and Analysis

Images were collected on a Zeiss LSM 710 and/or Zeiss 510 NLO confocal microscope. Z-stack images were collected with 10X or 20X objectives and tiled together to generate high-resolution images of whole brain sections. Axon fluorescence quantification was conducted with ImageJ. Two photon images for P15 neuron tracings were collected using a Zeiss 7MP Multiphoton confocal microscope and cell tracings were conducted using Neurolucida.

Affymetrix Microarray

Dorsal cortices were dissected from three E18 Control (GSK-3α-/-β F/F) and mutant (GSK-3α-/-β F/F;NEX) embryos derived from two independent litters. RNA was prepared using the MiRNAeasy kit (Qiagen) and analyzed using the Affymetrix Mouse Gene 2.0 St array. Following scanning of the array, basic data analysis was carried out using the Partek

Genomics Suite Version 6.12.0712. Transcripts up or down-regulated by 1.5 fold were considered interesting candidates.

Reagents and Collaborations

Dr. Franck Polleux (Columbia University) provided the NeuroD-Cre and Lox-STOP-lox LacZ/EGFP (Z/EG) plasmids and LKB1 mutant mice. CRMP2 plasmids were provided by Nancy Y. IP (The Hong Kong University of Science and Technology, China) and cloned into the NeuroD plasmid. Doublecortin constructs provided by Azad Bonni (Harvard Medical School) and described previously (Bilimoria, de la Torre-Ubieta et al. 2010). The phospho-DCX ser327/Thr321 antibody was provided Dr. Orley Reiner (Weizmann Institute of Science, Israel) and has been previously described (Gdalyahu, Ghosh et al. 2004). Two-Photon images were collected by Vladimir Ghukasyan of the UNC Neuroscience Center Confocal and Multipohoton Imaging Core.

5.6 FIGURES AND FIGURE LEGENDS

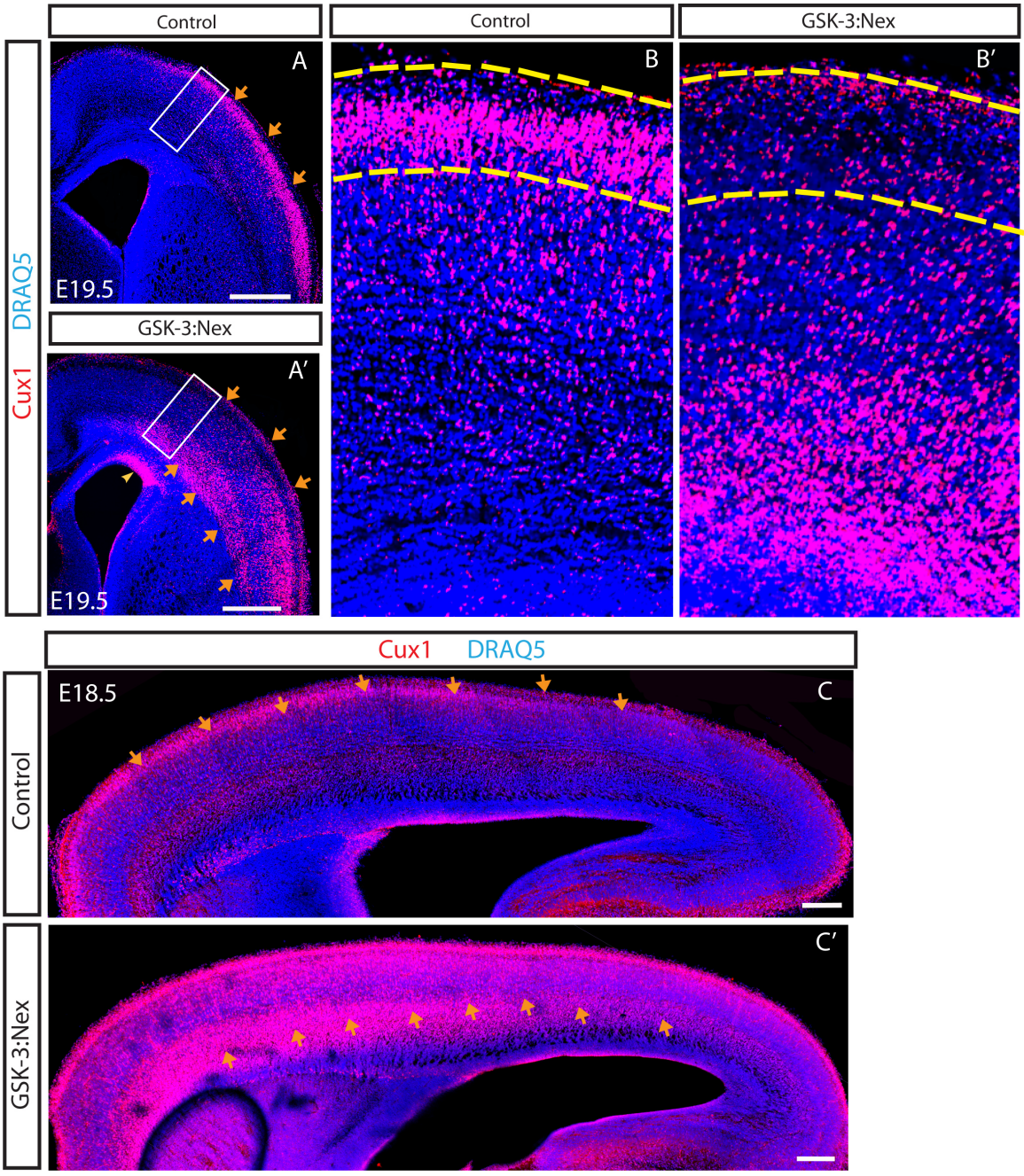


Figure 5.1. GSK-3 signaling is essential for proper lamination of the developing cortex.

(A-A') Cux-1 staining (red) in coronal sections from control and GSK-3:Nex mice at E19.5. Cux-1 neurons are strikingly mislocalized in GSK-3:Nex mutants (arrows) including a small population of neurons that remain in the ventricular zone (arrowhead). Nuclei were counterstained with DRAQ5. Scale bar = 500um. (n=4)

(B-B') Higher magnification of white boxes in (A). The number of Cux-1 expressing neurons reaching layer 2/3 (denoted by yellow dashed lines) is markedly reduced in the GSK-3:Nex mutants.

(C-C') Cux-1 staining in parasagital vibratome sections from control and GSK-3:Nex mutants at E18.5. Cux-1 expressing neurons (arrows) are mislocalized in GSK-3:Nex mutants and populate the deeper layers of the cortex along the entire rostrol/caudal axis. scale bar = 200um.

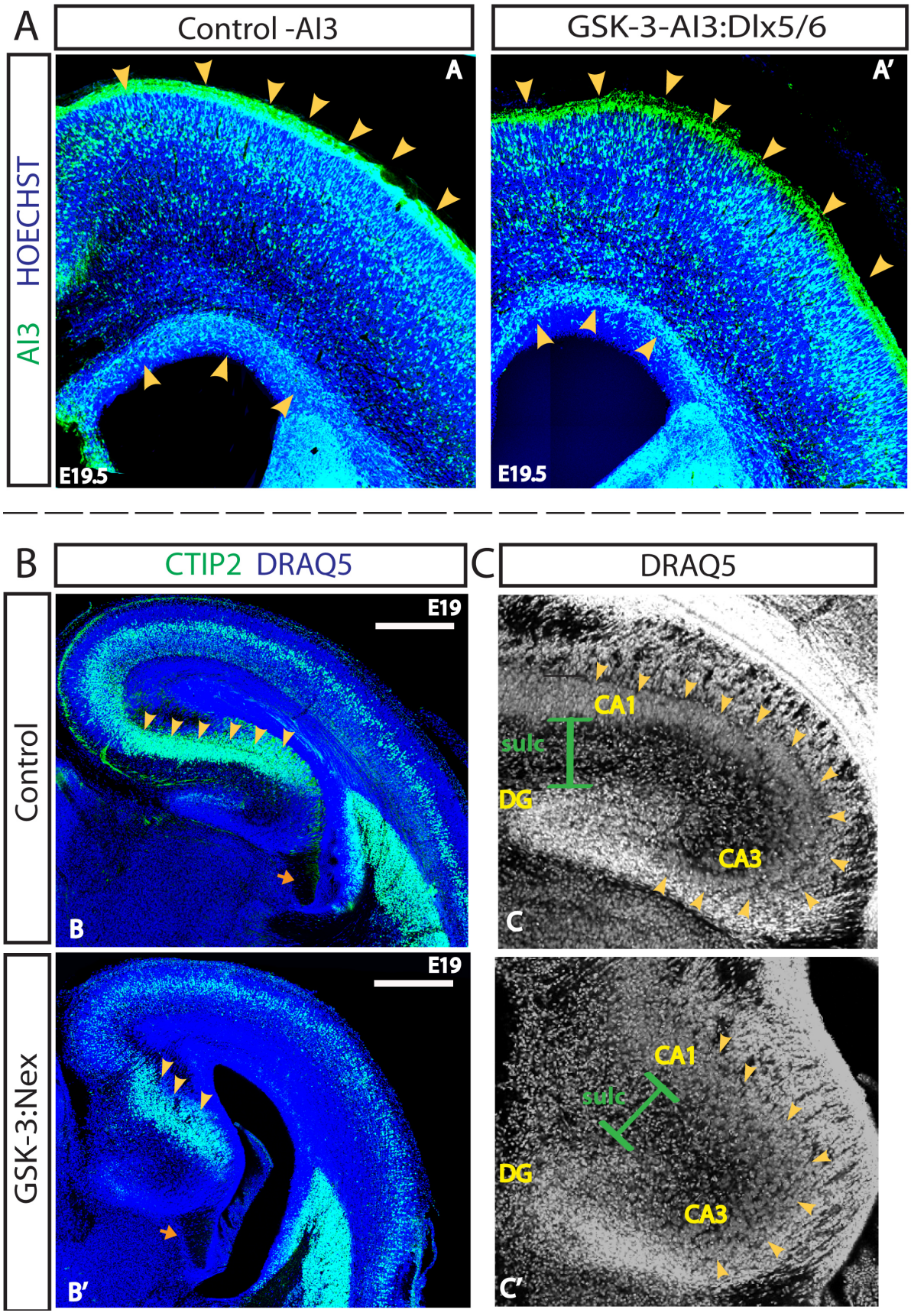
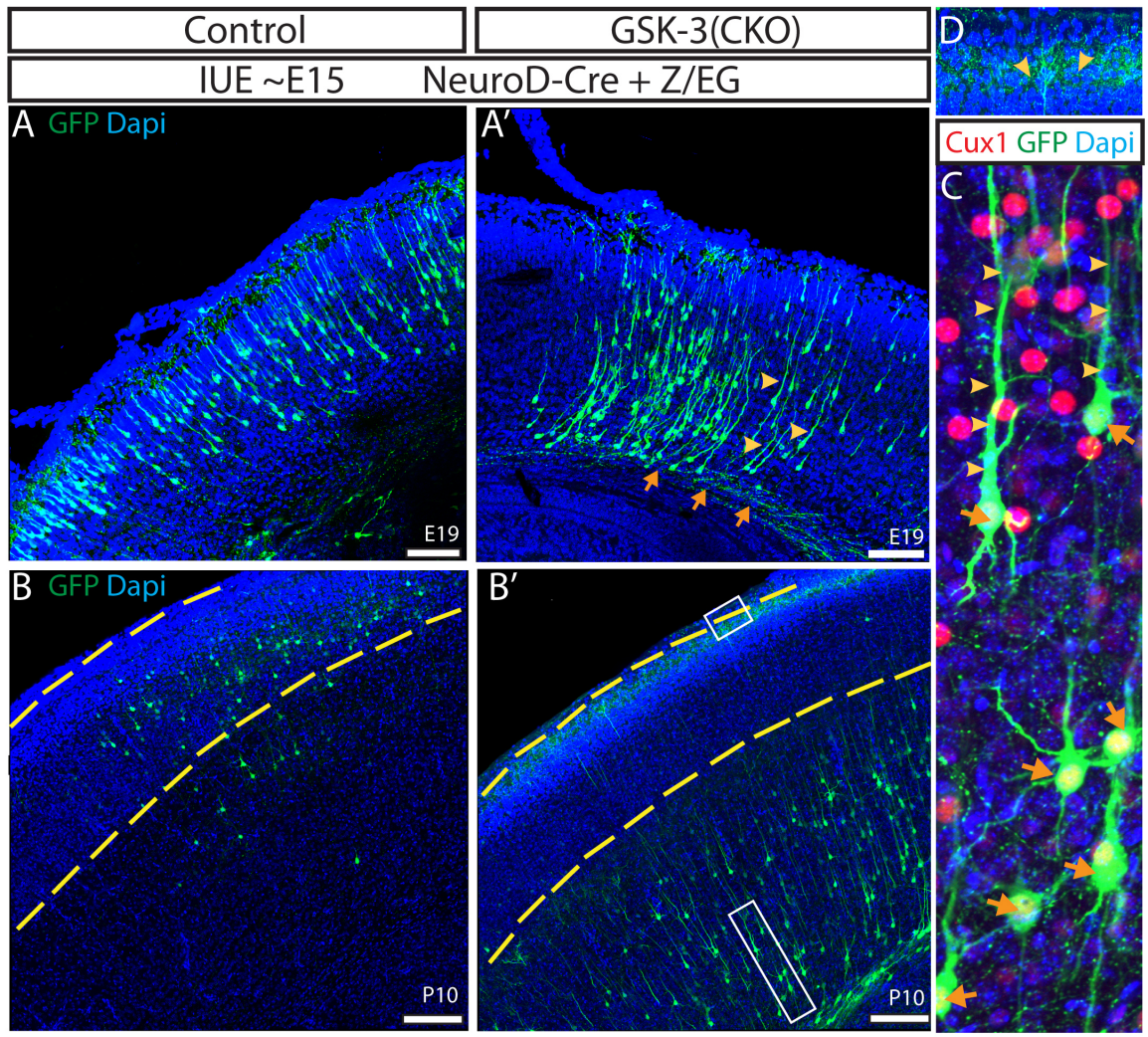

A
Control -AI3
GSK-3-AI3:Dlx5/6
AI3 HOECHST
A
A'
E19.5
E19.5
B
CTIP2 DRAQ5
C
DRAQ5
Control
GSK-3:Nex
E19
E19
B
B'
CA1
sulc
DG
CA3
C
CA1
sulc
DG
CA3
C'

Figure 5.2. GSK-3 signaling is dispensable for tangential migration, but required for radial hippocampal migration.

(A-A') E19.5 coronal sections showing EYFP-expressing interneurons in heterozygous control and GSK-3:DLX5/6 mutants crossed with the Ai3 reporter line. GSK-3 deleted interneurons (green) enter the cortex in two streams as in controls (arrowheads) and showed no overt migration defect. Nuclei were counterstained with Hoechst. (n=3)

(B-B') E19 coronal sections showing CTIP2 (green) expressing neurons in the hippocampus. In the GSK-3:Nex mutants, the pyramidal cell layer (green) does not extend laterally into a compact CA1 region and remains dispersed (arrowheads). Fimbrial axonal projections appear normal in GSK-3:Nex mutants (arrow). Nuclei were counterstained with DRAQ5. Scale bar = 500um. (n=3)

(C-C') Higher magnification of hippocampal area shown in (B). The GSK-3:Nex mutants show disrupted cytoarchitecture. In the mutants, DRAQ5 labeled cells are mislocalized and diffuse (arrowheads) and fail to form clearly defined CA1/CA3 regions of the hippocampus. The GSK-3:Nex mutant mice also lack a clearly defined hippocampal sulcus (green bars) and dentate gyrus (DG).


Control
GSK-3(CKO)
IUE ~E15
NeuroD-Cre + Z/EG
A GFP Dapi
A′
E19
E19
B GFP Dapi
B′
P10
P10
D
Cux1 GFP Dapi
C

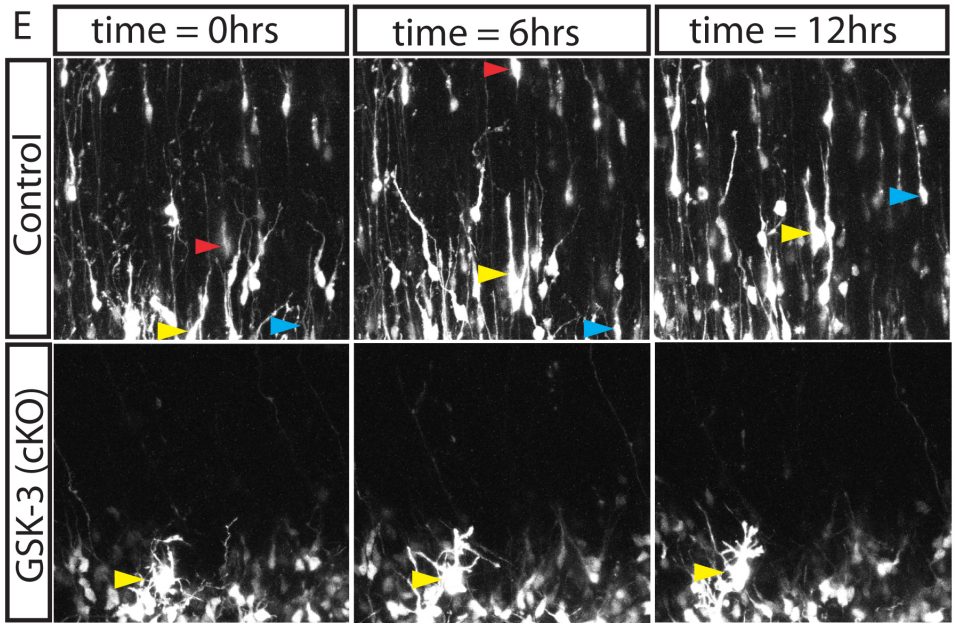

E
time = 0hrs
time = 6hrs
time = 12hrs
Control
GSK-3 (cKO)

Figure 5.3 GSK-3 regulation of cortical lamination is cell autonomous and persistent.

(A-A') E19 coronal sections after *in utero* electroporation at E14.5 with NeuroD-Cre and Z/EG plasmids. Electroporated cells were visualized with anti- EGFP (green) and nuclei were stained with DAPI (blue). GSK-3 deleted neurons remain in the deeper layers of the cortex but elaborate a long pial-directed process (yellow arrows). scale bar = 200um (n=5, two independent litters)

(B-B') Coronal section at P10 after E14.5 electroporation, as in A. GSK-3 deleted neurons remain in the deeper layers of the cortical plate and fail to reach layer 2/3 (denoted with yellow bars). scale bar = 200um (n=3, two independent litters)

(C) Higher magnification of GSK-3 deleted neurons in B' (large box). GSK-3 deleted neurons in deeper layers co-label with Cux (red) (orange arrows), extend axons towards the corpus callosum and elaborate apical dendrites (yellow arrowheads).

(D) Higher magnification of GSK-3 deleted neurons dendritic arbors in layer 1 (arrowheads) of GSK-3 deleted neuron in B' (small box).

(E) GSK-3 deletion delays multipolar to bipolar transition. Still images from time-lapse imaging of organotypic slice cultures at 3DIV. pCAG-dsRED or NeuroD-Cre;Z/EG were injected into the ventricles of GSK-3$\alpha^{-/-}$GSK-3$\beta^{F/F}$ embryos and electroporated at E15. Representative images were taken at time 0, 6, and 12 hours. Control dsRed neurons migrate through the cortical plate (yellow, red and blue arrows show individual neurons at the different time points.) (n=2 controls). GSK-3 deleted neurons fail to migrate through the cortical plate and exhibit persistent multi-polar morphology (yellow arrowheads). (n=2 control, n = 4 mutants)

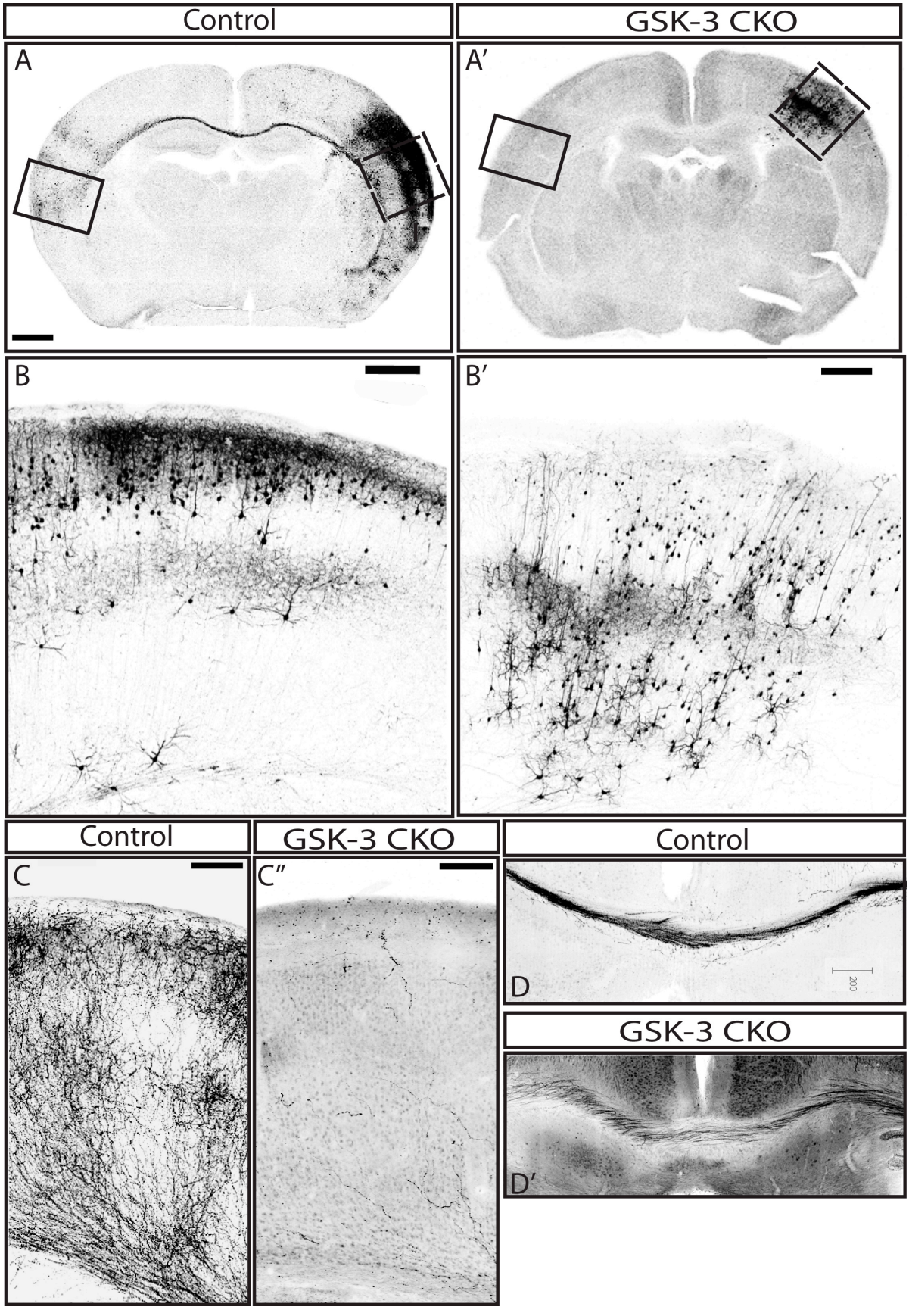

Control
GSK-3 CKO
A
A′
B
B′
Control
GSK-3 CKO
Control
C
C″
D
GSK-3 CKO
D′

Figure 5.4. GSK-3 regulation of axon projections

(A) Control and GSK-3 deleted neurons at P15 immunostained with antibodies against eGFP (black). Electroporation was performed with the same methods and constructs as in Figure 3A using GSK3$\alpha^{+/+}\beta^{loxp/loxp}$ controls and either GSK-3$\alpha^{-/-}$GSK-3$\beta^{loxp/loxp}$ or GSK-3^{loxp} mice for GSK-3 deletion. n=3 from three litters.

(B) Higher magnification of electroporated neuron region in A,A' (dashed lines). Control neurons populate the outer layers of the cortex while GSK-3 deleted neurons remain dispersed throughout all layers of the cortical plate. scale bar 200um

(C) High magnification of axonal arbors in A, A' (box). Control axons enter the cortical plate and densely arborize in layers 2/3 and 5 in the contralateral cortex. GSK-3 deleted neurons extend axons that extend in the corpus callosum, but axonal arborization in contralateral cortex is sparse. scale bar 200um

(D) High magnification of axons in the corpus callosum from control and GSK-3 deleted neurons

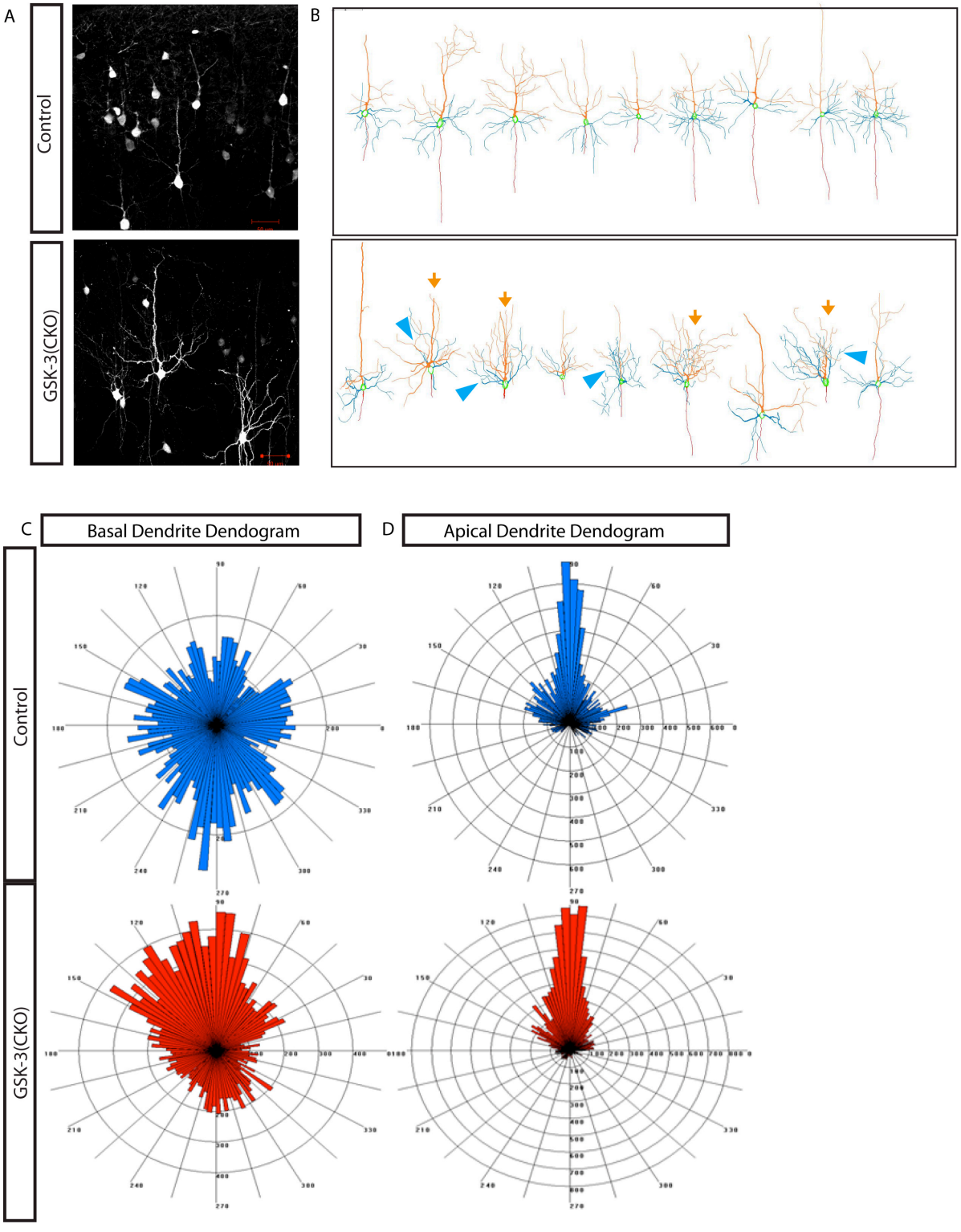
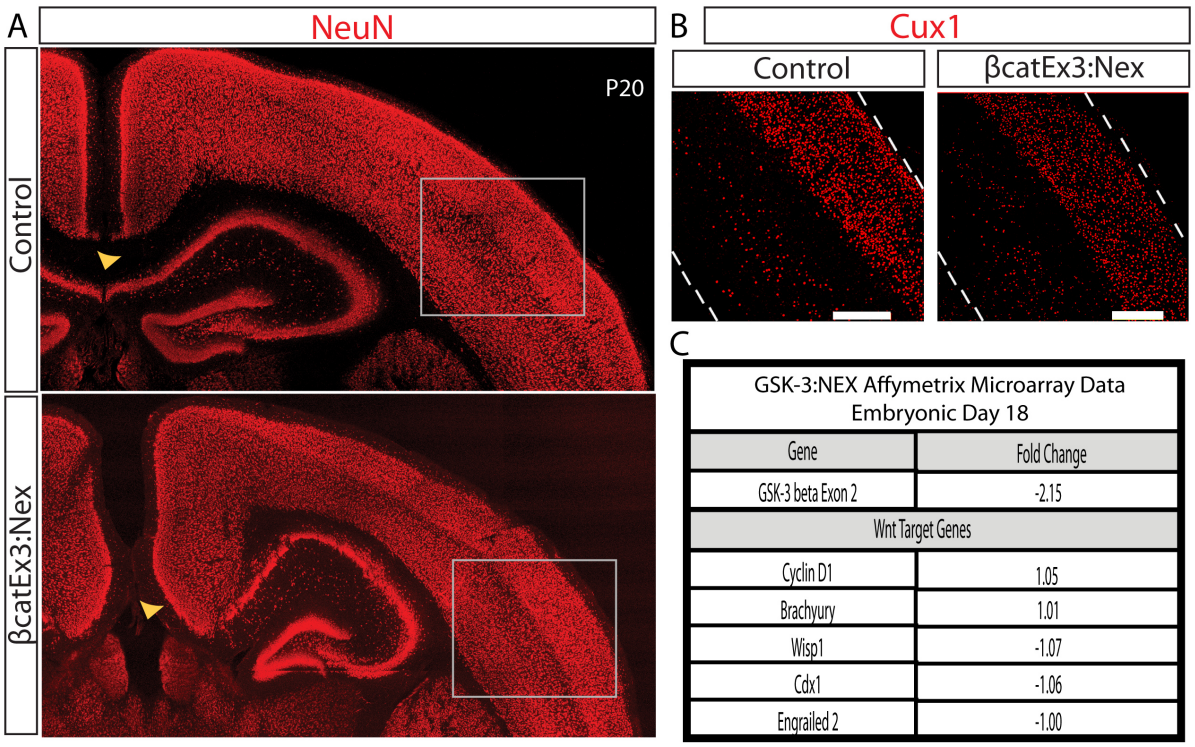

A
Control
GSK-3(CKO)
B
C
Basal Dendrite Dendogram
D
Apical Dendrite Dendogram
Control
GSK-3(CKO)

Figure 5.5 GSK-3 regulation of dendrite polarization.

(A) Control and GSK-3 deleted neurons in the upper layers at P15, immunostained with antibodies against eGFP (black) using same methods as Figure 4. GSK-3 deleted neurons have abnormally polarized arbors.

(B) Reconstructions of control and GSK-3 deleted neurons in the upper layers of the cortex. The axon (red) projects towards the ventricle in control and GSK-3 deleted neurons. Apical dendrites (orange) and basal dendrites (blue) are more branched (arrows) and basal dendrites (blue) are mispolarized (arrowheads) in GSK-3 deleted neurons.

(C-D) Basal dendrite and apical dendrite quantification. Dendrogram shows that basal dendrites (C) frequently project towards the pial surface in GSK-3 deleted neurons. (D) Apical dendrites project properly in GSK-3 deleted neurons but have abnormal morphology (see B). (n=3, 15 control and 16 mutants quantified).



C

GSK-3:NEX Affymetrix Microarray Data Embryonic Day 18	
Gene	Fold Change
GSK-3 beta Exon 2	-2.15
Wnt Target Genes	
Cyclin D1	1.05
Brachyury	1.01
Wisp1	-1.07
Cdx1	-1.06
Engrailed 2	-1.00

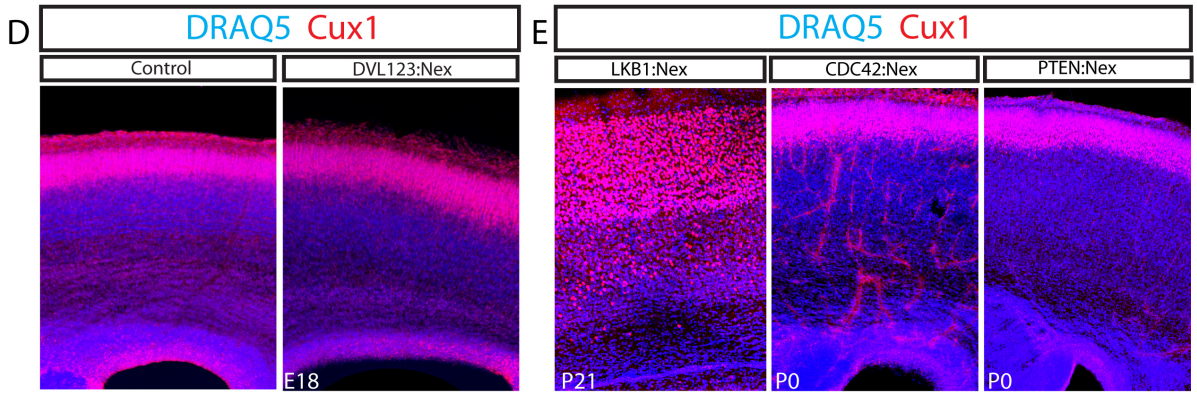


Figure 5.6. GSK-3 regulation of migration is independent of Wnt/B-catenin and RTK signaling.

(A) Coronal sections of somatosensory cortex at P20 stained with antibodies against NeuN. βcatEx3:Nex mutants exhibit altered hippocampal cytoarchitecture with a rostral midline defect (arrow). (n=3 from 3 independent litters).

(B) High magnification of boxes in A. Cux-1 neurons (red) are localized to layer 2/3 in both controls and βcatEx3:Nex mutants.

(C) Coronal sections of cortex showing DRAQ5 (blue) and Cux-1 (red) expressing neurons in Dvl123:Nex at E18. Cux-1 neurons reach layer 2/3 in both controls and Dvl123:Nex triple mutants. (n=3 from 2 independent litters).

(D) Wnt target gene expression in GSK-3:Nex cortex. Cortical lysates were collected at E18 for affymetrix microarray analysis. GSK-3B exon expression was reduced as expected. Classic WNT target genes of (TCF)/LEF-1 transcription factors were unchanged.

(E) Coronal sections of somatosensory cortex stained for Cux-1 (red) and Draq5 (blue). LKB1:Nex mutants: Cux-1 expressing neurons at P21 are localized in layer 2/3 (n=3). CDC42:Nex and PTEN:Nex mutants: Cux-1 expressing neurons at P0 are normally localized (n=3).

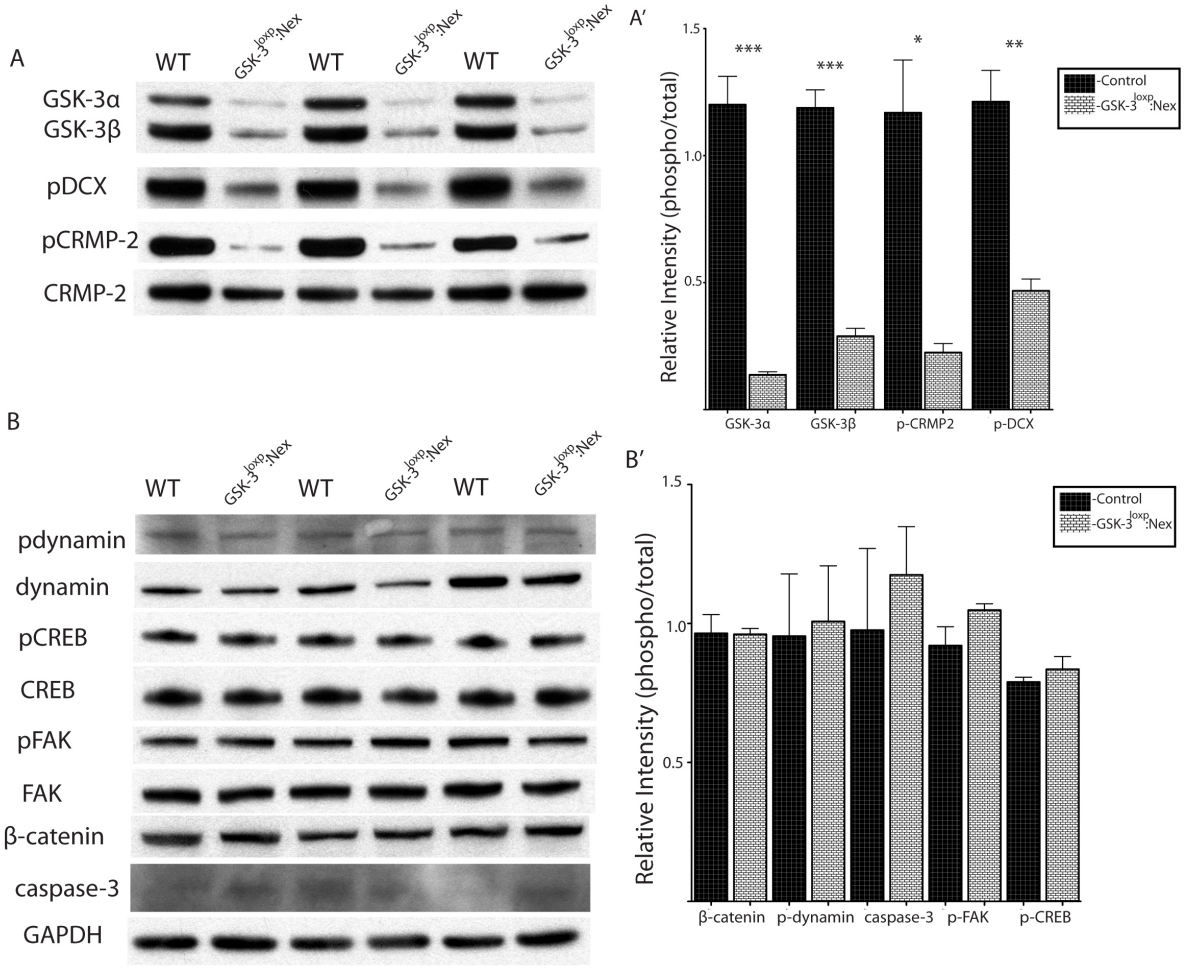
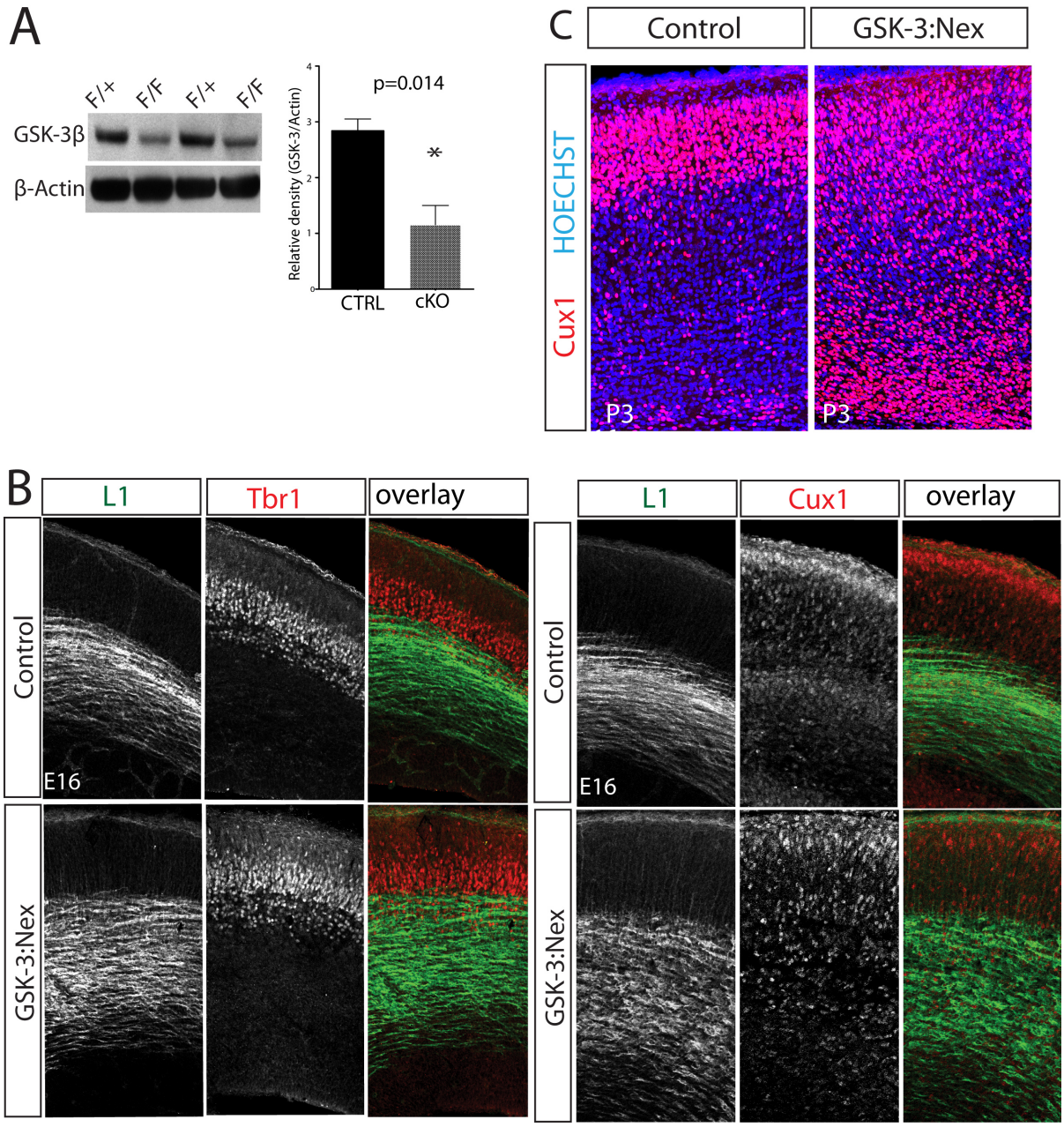

A
WT
GSK-3Joxp:Nex
WT
GSK-3Joxp:Nex
WT
GSK-3Joxp:Nex
GSK-3α
GSK-3β
pDCX
pCRMP-2
CRMP-2
A′
Relative Intensity (phospho/total)
1.5
1.0
0.5
***
***
*
**
Control
GSK-3Joxp:Nex
GSK-3α
GSK-3β
p-CRMP2
p-DCX
B
WT
GSK-3Joxp:Nex
WT
GSK-3Joxp:Nex
WT
GSK-3Joxp:Nex
pdynamin
dynamin
pCREB
CREB
pFAK
FAK
β-catenin
caspase-3
GAPDH
B′
Relative Intensity (phospho/total)
1.5
1.0
0.5
Control
GSK-3Joxp:Nex
β-catenin
p-dynamin
caspase-3
p-FAK
p-CREB

Figure 5.7. Phosphorylation of GSK-3 Targets

A-A') Western blots of P0 cortical lysates from GSK-3^{loxp}:Nex mutants and GSK-3 $\alpha^{loxp/loxp}$ $\beta^{loxp/loxp}$ controls. Levels of GSK-3 proteins and phospho-target proteins are shown. Strong reductions in phosphorylation of doublecortin on ser327/Thr321 and CRMP2 on Thr514 are evident. A' Quantification of relative densities from A. (n=3)

B-B') Western blots of cortical lysates at P0 showing levels of other GSK-3 targets. No changes were observed in phosphorylation of dynamin, pCREB, pFAK, β-catenin. No change was observed in cleaved caspase-3. GAPDH was used as a loading control. B' Quantification of relative densities (n=3)

5.7 SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

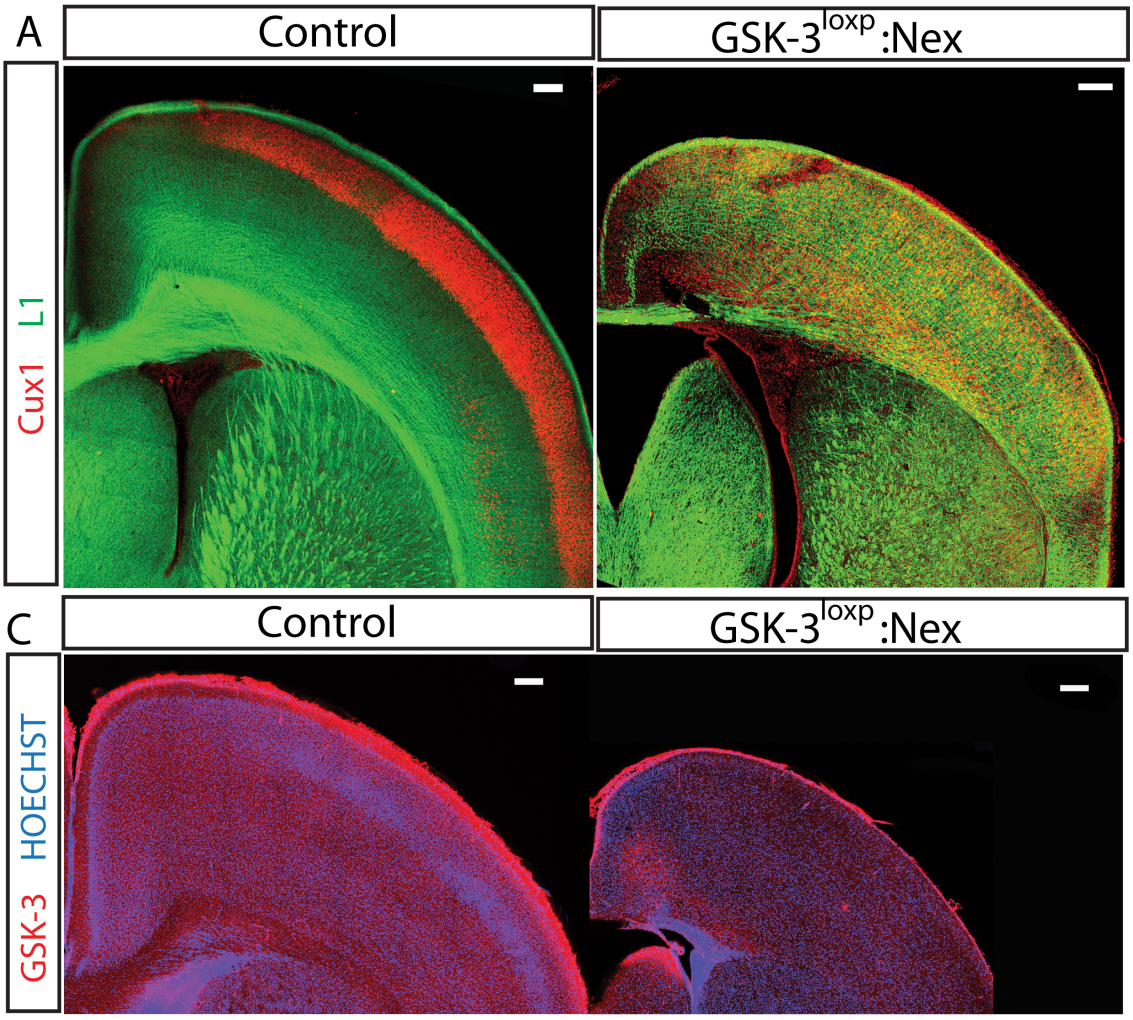


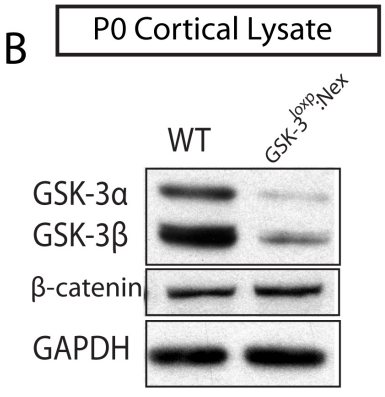
Supplemental Figure 5.1. Deletion of GSK-3 in the developing cortex resorts in lamination defects at multiple time points throughout development.

(A) Representative western blot confirms strongly reduced GSK-3β protein levels in the E19 GSK-3:Nex cortex compared to heterozygous control (n=3). Duplicate protein samples shown. Relative Density *p value < 0.05, unpaired t-test.

(B) Coronal cryostat sections at E16. Layer 6 TBR1 neurons populate appropriate layers in control and GSK-3:NEX mutants(n=1). Cux-1 (red) expressing neurons, marker for upper layer 2/3 neurons, are mislocalized in GSK-3:Nex mutant coronal sections (n=2).

(C) Coronal vibratome sections showing Cux-1 expressing neurons at P3. Neurons are mislocalized in GSK-3:Nex mutants (n=1). Nuclei were counterstained with Hoechst.


A
Control
GSK-3loxp:Nex
Cux1 L1
C
Control
GSK-3loxp:Nex
GSK-3 HOECHST

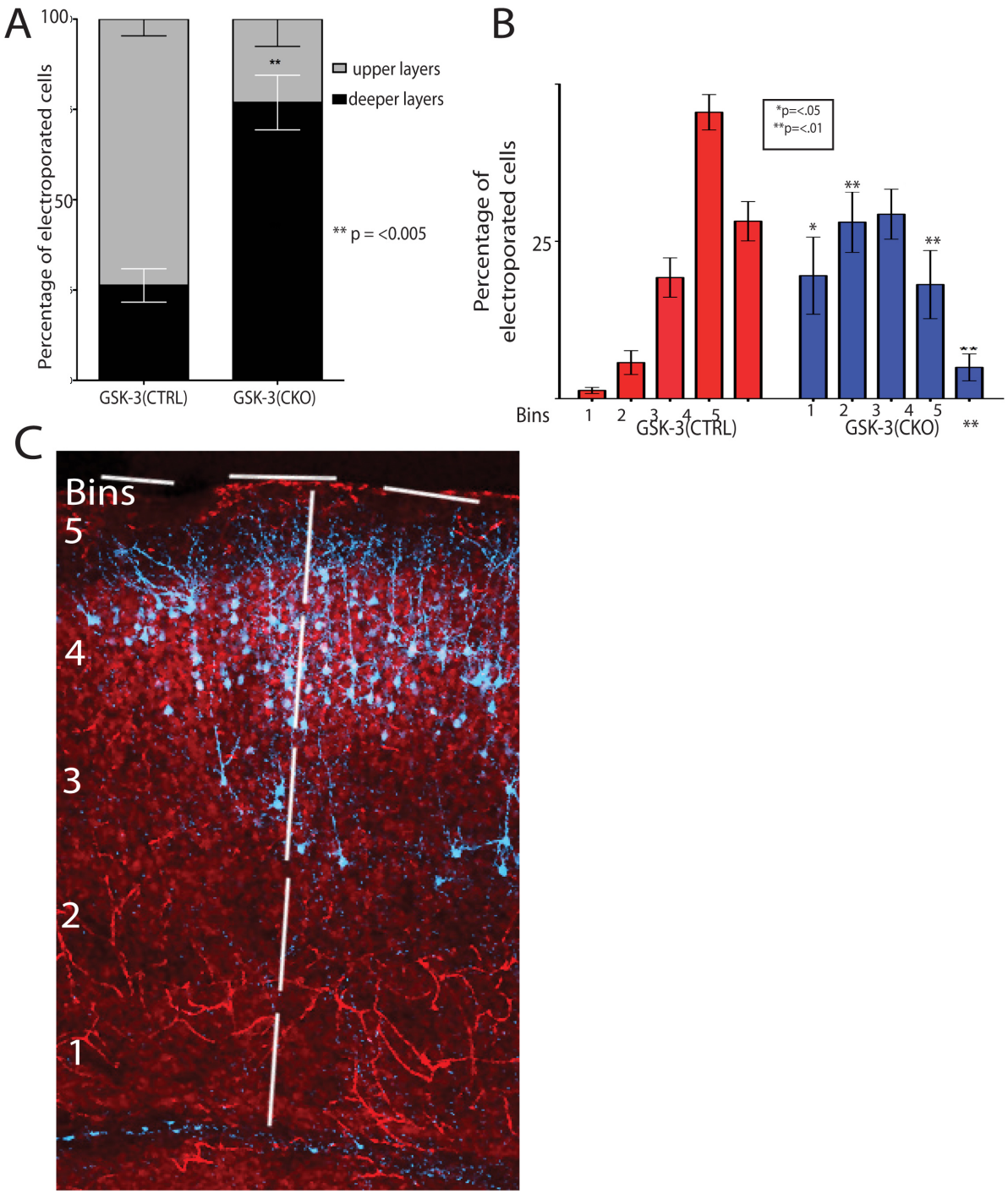

B
P0 Cortical Lysate
WT
GSK-3loxp:Nex
GSK-3α
GSK-3β
β-catenin
GAPDH

Supplemental Figure 5.2. GSK-3^{loxp}:Nex mice display altered lamination at P7.

(A) P7 GSK-3^{loxp}:Nex mutants stained with Cux1 (red) show altered lamination at with Cux1 expressing neurons spread throughout all layers of the cortex. Littermate controls show normal Cux1 lamination in layer 2/3. Scale bar = 200um

(B) Western blot of in P0 cortical lysates confirms strongly reduced total GSK-3 GSK-3α and GSK-3β proteins compared to GSK-3 $\alpha^{loxp/loxp}$ $\beta^{loxp/loxp}$ controls. No change in β-catenin levels was observed. GAPDH was probed as a loading control (n=3).

(C) P7 GSK-3^{loxp}:Nex mice stained with Hoechst (blue) and GSK-3 (Red). Control mice have GSK-3 spread throughout the cortical plate. Littermate GSK-3^{loxp}:Nex mutants show decreased GSK-3 staining throughout the cortical plate and high expression in layer 1. Scale bar = 200um

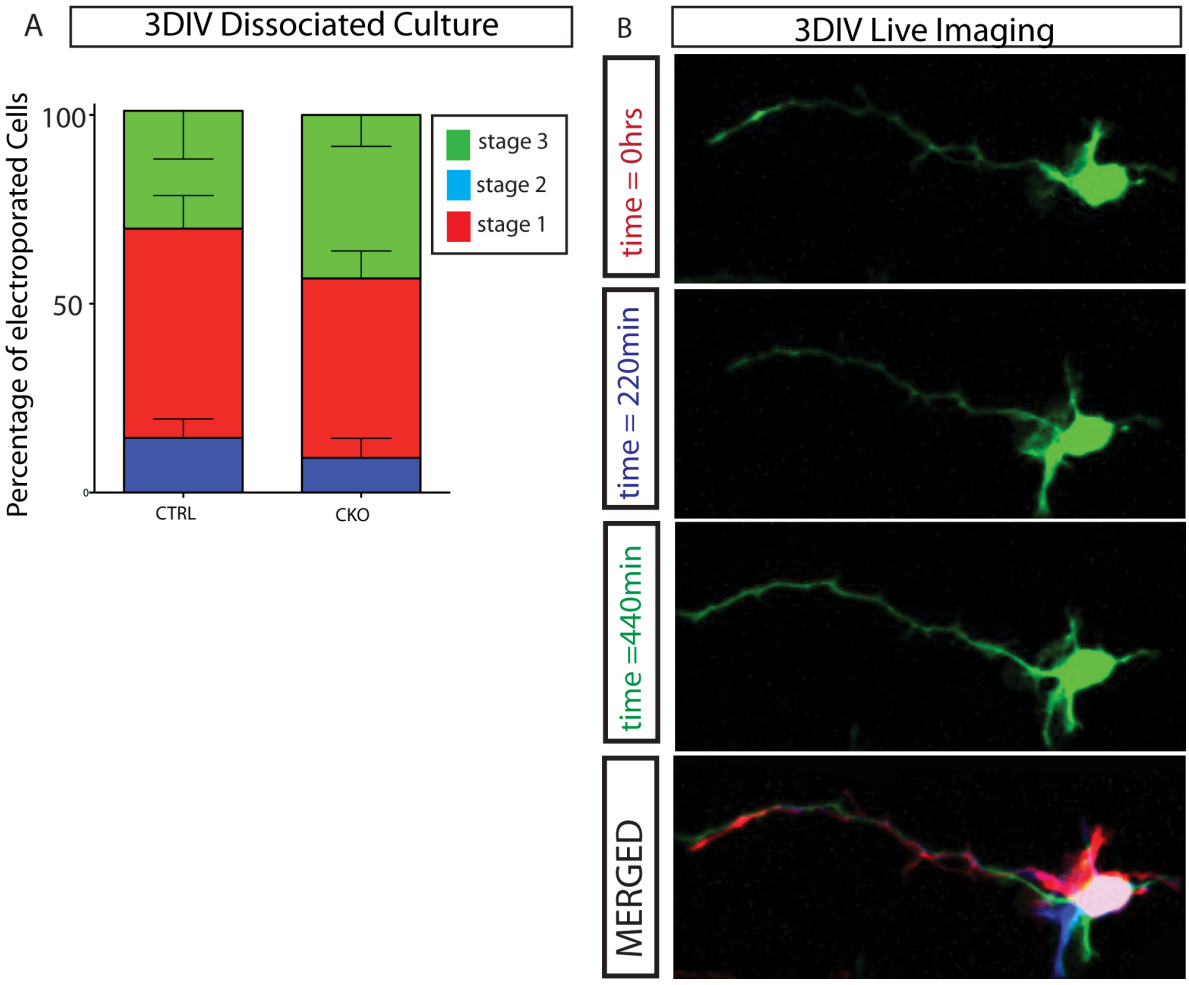

A
100
50
Percentage of electroporated cells
**
upper layers
deeper layers
** p = <0.005
GSK-3(CTRL)
GSK-3(CKO)
B
*p=<.05
**p=<.01
Percentage of electroporated cells
25
Bins
1
2
3
4
5
GSK-3(CTRL)
GSK-3(CKO)
C
Bins
5
4
3
2
1

Supplemental Figure 5.3. P10 Quantification of lamination in electroporated pups.

(A) Quantification of electroporated neurons in at P10 in upper versus lower layers of the cortex. (n=3, 4209 neurons counted) ** p value = 0.003, unpaired t-test.

(B) Postnatal day 10 quantification of electroporated neurons in each cortical bin across the cortical plate. Significant changes were found in deeper layer bins 1-2 and upper layer bins 4-5, further indicating a failure of migration in the mutants. (n=3, 4209 neurons). unpaired t-test * p = < 0.05, ** p=<0.01

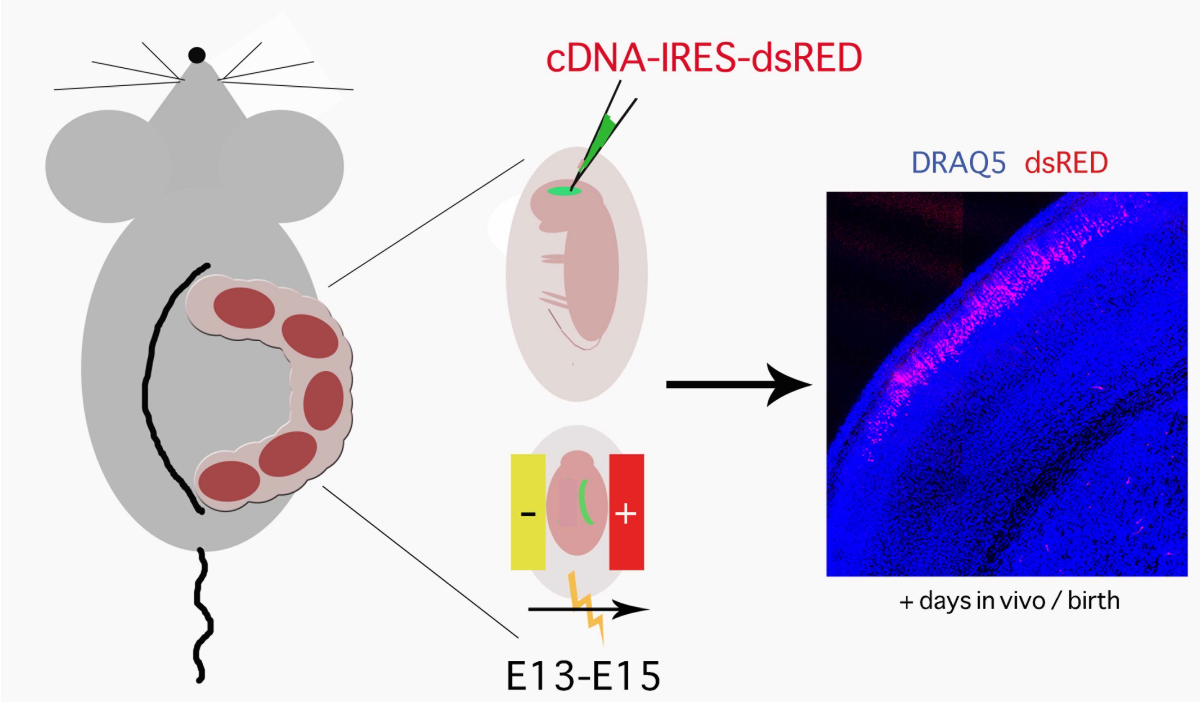
(C) Representative coronal section from control electroporated mouse (blue) co-labeled with MAP2 (red). The cortex was divided into 5 cortical bins and the neurons populating each bin were counted using ImageJ



Supplemental Figure 5.4. GSK-3 deleted neurons polarize and are highly dynamic.

(A) Stage progression analysis of dissociated control and GSK-3 deleted neurons reveals no significant delay in polarization (n=3, 1341 neurons).

(B) Live imaging of dissociated GSK-3 deleted neurons reveal dynamic neurites. Images were taken every 11 minutes. Representative images at time 0 (red), 220 minutes (blue), and 440 minutes (green). Merge Image is pseudo colored.



Supplemental Figure 5.5. ***In utero*** **Experimental Procedure.**

A pregnant E13-E15.5 mouse is anesthetized and uterine horns exposed. cDNA is injected into the lateral ventricles of the developing embryo and current is applied to direct the construct into the neuronal progenitors in the ventricular zone of the developing cortex. The uterine horns are replaced internally for normal embryonic development to continue. Injected pups can be taken embryonically or postnatally for analysis. Image above is a coronal vibratome section stained for dsRED and imaged at P3 to show normal lamination.

CHAPTER 6: Discussion and Future Directions

6.1 Summary of Findings

The most striking feature of my findings is the almost complete failure of radial migration in most GSK-3 deleted newly born cortical neurons. Migration failure occurred even though initial stages of axon extension and dendritic arborization were not affected. This finding suggests very specific cytoskeletal regulation by GSK-3 rather disruption of all microtubule mediated processes.

Of course many molecules are required for radial migration. However our preliminary findings with GSK-3 gain of function (data not shown, see below) suggest that GSK-3 may join a small group that are not only required, but are able to regulate the process. Further, GSK-3 appears to be important regulatory hub, being downstream of reelin and upstream of FAK and dynamin in potential regulation of adhesive properties. More importantly, GSK-3 is implicated as working to together with CDK5 in the regulation key MAPs that are essential to migration. The role of DCX is well established, but GSK-3 also strongly regulates CRMP-2. The role of CRMP family members in migration has only recently been appreciated.

GSK-3 also has important functions in aspects of neuronal morphogenesis, particularly dendritic orientation. My work emphasizes the importance of GSK-3 kinase activity at several stages of cortical development from progenitor homeostasis (Kim et al., 2009) to cortical lamination and morphogenesis.

6.2 Discussion

Cortical migration is an extraordinarily complex process that requires proper sensing of migratory cues and rapid cytoskeletal rearrangement. Both the classic Reelin cascade and other migration signaling cascades converge onto dynamic cytoskeletal regulation (Ayala, Shu et al. 2007; Barnes and Polleux 2009). Multiple MAPs (Dehmelt and Halpain 2004), MAP1B (Del Rio, Gonzalez-Billault et al. 2004; Gonzalez-Billault, Del Rio et al. 2005), Lis1 (Tsai, Chen et al. 2005), and DCX (Bai, Ramos et al. 2003; Tanaka, Serneo et al. 2004; Koizumi, Tanaka et al. 2006; Bilimoria, de la Torre-Ubieta et al. 2010) have all been identified and essential for proper lamination. However signaling cascades upstream of these migration associated MAPs have not been fully defined.

Importantly for my findings, CDK5 is well known to regulate cortical neuronal migration (Dhavan and Tsai 2001; Xie, Sanada et al. 2003; Tanaka, Serneo et al. 2004; Xie, Samuels et al. 2006). Further, CDK5 is known as a 'priming' kinase for GSK-3 (Sengupta, Wu et al. 1997; Noble, Olm et al. 2003; Li, Hawkes et al. 2006). Cdk5 phosphorylation of a substrate enhances GSK-3 function while *in vitro* inhibition of both GSK-3 and Cdk5 ablates the phosphorylation of downstream substrates (Gonzalez-Billault, Del Rio et al. 2005). Cdk5 has also been identified as a regulator of neuronal morphology in response to semaphorin signaling (Sasaki, Cheng et al. 2002). Now, I have shown GSK-3 is a key regulator of neuronal migration and morphology, in a manner that may involve DCX and semaphorin signaling. My data suggests that CDK5 priming and subsequent GSK-3 phosphorylation of DCX and CRMP-2 regulate radial migration. My results provide a first step in elucidating a

model in which converging migratory cues siphon into one pathway reliant on GSK-3 signaling and regulation of cytoskeletal elements.

Interestingly, CDK5 is implicated in radial migration through via FAK, CRMP2 and DCX phosphorylation (Xie, Sanada et al. 2003; Tanaka, Serneo et al. 2004; Uchida, Ohshima et al. 2005). Our GSK-3 mutants have a similar phenotype to the CDK5 mutants, an elongated multipolar stage followed by failed migration to the proper lamina and dendrite morphological abnormalities, though GSK-3 mutants appear to have a more severe defect. Although we saw no change in FAK phosphorylation, we do have significant changes in phosphorylation at GSK-3 sites of both DCX and CRMP-2. These results may suggest that GSK-3 regulation of migration is cytoskeletal as opposed to adhesive.

A key validation of my idea would be a rescue of the GSK-3 deletion phenotype. Currently, we are cloning phosphomimetic versions of both DCX and CRMP-2 in the pNeuroD vector to perform these rescue experiments. We hypothesize that phosphomimetic DCX expression could rescue the migration defect observed in the GSK-3 deleted neurons. We also hypothesize that phosphorylation of CRMP-2 may rescue the dendrite polarity, and possibly the axonal defects seen in GSK-3 deleted neurons. Additionally, we will attempt a double expression of phosphomimetic versions of DCX and CRMP-2 in an attempt to rescue both the migration and morphology defects in the GSK-3 deleted neurons.

Another key validation experiment is that over-expression of GSK-3 should enhance migration. Preliminary *in utero* over-expression experiments appear to show neurons with increased migration. Overexpression analysis at E19.5 shows neurons in the outermost layer of the cortex and they appear more densely compacted in the boundary between layer II and

layer I (data not shown). Obviously, this preliminary data must be replicated and quantified. However, it is promising that overexpression of GSK-3 would have the direct opposite effect from GSK-3 deletion. Future experiments may include later time points to assess GSK-3s overexpression on morphology and axon targeting *in vivo* as well as assessment of CRMP-2 and DCX via immunohistochemistry.

We have shown that the defect in neuronal migration is independent of Wnt signaling. Interestingly, we also see no evidence that ser9 phoshorylation of GSK-3 is important for this phenotype. We have found that PTEN, LKB1 and CDC42 regulation of GSK-3 are not required *in vivo.* Interestingly, the GSK-3 deletion phenotype is similar to the migration and morphological phenotypes in mice were sema3A and NRP1 have been knocked down by siRNA. Indeed, GSK-3 is an important mediator of semaphorin signaling (see chapter 3) suggesting that semaphorin signaling through GSK-3 may be partially responsible for the migration and morphological abnormalities we see in GSK-3 - deleted mice.

6.2 Future Directions

Semaphorins and spines

Though our work has not addressed the issue of spine development in GSK-3 deleted neurons, it is likely that GSK-3 regulation of microtubules could alter spine morphology and circuit function. Importantly, disrupted semaphorin signaling increases dendritic spine numbers and alters morphology (Tran, Rubio et al. 2009). As previously stated, semaphorin signaling activates GSK-3 therefore we would hypothesize that GSK-3 deleted mice would recapitulate dendritic spine morphology seen with disrupted sema signaling *in vivo*.

Long-term Depression

Interestingly, GSK-3 is essential for LTD while GSK-3 over expression has been shown to block LTP (Hooper, Markevich et al. 2007; Peineau, Taghibiglou et al. 2007; Zhu, Wang et al. 2007), possibly through PSD-95 regulation (Nelson, Kim et al. 2013). GSK-3 has also been implicated *in vivo* to regulate endocytosis during periods of high activity (Clayton, Sue et al. 2010). Therefore, we would expect changes in LTD and possibly LTP in neurons lacking GSK-3 expression. Electrophysiological recordings to assess the function of GSK-3 deleted neurons could uncover another *in vivo* role for GSK-3 in the circuitry and signaling of mature neurons.

Long-term Survival

We have seen no increase or decrease in cell death markers in embryonic and postnatal stages when migration is occurring. However, for animals that survive for substantial times postnatally we begin to see appearance of apoptotic markers in the second postnatal week and evidence of neurodegeneration by P21. This is very surprising as one would hypothesized, based on the literature, that GSK-3 inactivation would promote cell survical. We do not yet know whether this is a cell autonomous effect. This is an important area to pursue both for implications for the use of Lithium and because it goes completely against conventional wisdom as GSK-3 inhibition has previously been associated with increased survival rather than increased cell death.

Analog sensitive allele.

GSK-3 deletion in progenitors results in embryonic death while deletion in newly born neurons results in early postnatal death. Therefore, in order to assess the role of GSK-3 signaling in later stages of cortical function, a reversible GSK-3 inhibition will be crucial. An analog sensitive allele for GSK-3β recently became available from Taconic and has been used to study GSK-3 activity related axon degeneration *in vitro* **(Chen, Maloney et al. 2012)**. This line help with uncovering novel roles for GSK-3 signaling in mature neurons *in vivo,* and would also allow for behavioral studies that are impossible due to the early death of our genetically deleted lines.

Lithium and GSK-3 inhibitors in humans

Finally, GSK-3 is already known to be an interesting target for therapeutic intervention. Lithium, which targets GSK-3 in the CNS, has been used for years to treat bipolar disorder (Klein and Melton 1996; O'Brien, Harper et al. 2004)(Stambolic, Ruel et al. 1996; De Sarno, Li et al. 2002; Freland and Beaulieu 2012). GSK-3 is expressed in all tissues and, in the developing cortex, and as we have shown, is a critical regulator of almost every facet of neuronal development. While lithium affects dopamine receptor signaling: our work suggests that it may also affect neural proliferation in adulthood (Kim, Wang et al. 2009). Our future work will shortly sort out GSK-3 regulation of spines, LTD and survival. Known effects and potential effects related to structural change would suggest increased caution in using the drug.

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