2017

C-Jun N-Terminal Kinase Signaling Guides the Migration of Interneurons During Cortical Development

Abigail K. Myers

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c-Jun N-terminal kinase signaling guides the migration of interneurons during cortical development

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Dissertation submitted to the School of Medicine
at West Virginia University

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy in
Neuroscience

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Morgantown, WV
2017

Keywords: c-Jun N-Terminal Kinase (JNK), cortical interneurons, developmental cortical disorders, migration

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Abstract

c-Jun N-terminal kinase signaling guides the migration of interneurons during cortical development

Abigail K Myers

The cerebral cortex, responsible for higher order functions, is a layered structure that relies on the precise wiring of two main types of neurons, excitatory neurons and inhibitory interneurons. In order to maintain a critical balance of excitation and inhibition in the cortex, a smaller, yet very diverse, population of cortical interneurons provides inhibition to a much larger cohort of excitatory neurons. This makes interneurons a vulnerable population where the slightest perturbations to their location and wiring with excitatory neurons can contribute to the acquisition of neurological disorders like autism, schizophrenia and epilepsy.

During development, cortical interneurons migrate tangentially from the medial and caudal ganglionic eminences in the ventral forebrain to the cortex. Unlike cortical excitatory neurons that are born in the ventricular zone of the dorsal forebrain and migrate radially on radial glial scaffolding, cortical interneurons are born in the ventral forebrain and migrate tangentially, primarily using molecular guidance cues in the extracellular environment to enter, navigate, and form streams in the nascent cerebral cortex. As development proceeds, interneurons depart from migratory streams on diagonal or radial trajectories in order to populate the cortical plate, where they eventually segregate into layers and make synaptic connections with other cortical neurons. Signaling pathways allow interneurons to express the correct complement of cell surface guidance receptors, transduce signals associated with the activation of those receptors, and facilitate the rearrangement of their cytoskeleton in order to aid in their travel. These processes are essential for the proper entrance of cortical interneurons into the cerebral cortex, their exit from migratory streams, and ultimately their layering in the cortical plate. Yet, how intracellular signaling pathways govern these processes remains largely unknown.

The c-Jun N-terminal kinase (JNK) signaling pathway, primarily known for its role in cell death, plays a large role in cell migration by activating downstream targets that modify the cytoskeleton and cell architecture. However, its importance in cortical interneuron migration has not been established. This work, organized into four main chapters including two main data chapters and an appendix, provides the first evidence that cortical interneurons use JNK signaling to enter and navigate in the developing cerebral cortex. In the first data chapter, we establish that *Jnk* is present within cortical interneurons and *Jnk1* is required for their entry into the cerebral cortex between embryonic day (E) E12.5 and E13.5 of mouse development. The second data chapter provides evidence that JNK signaling is important for maintaining migratory streams and that pharmacological inhibition or genetic ablation of *Jnk* from interneurons incites their exit from migratory streams and subsequent entry into the cortical plate between E14.5 and E15.5. Further, the work provided in the Appendix indicates disruption to the localization of Jnk-phosphorylated doublecortin, a microtubule associating protein important for branching of the leading processes of the migrating interneurons. Together, this work suggests that JNK signaling plays a critical role during cortical interneuron migration and may provide insight into how neurodevelopmental disorders like epilepsy, autism and schizophrenia could arise.

Dedication

To my family, friends, and mentors, thank you for all your love and support throughout all of my education

Acknowledgements

I would like to take a few moments to thank the many people that have helped me throughout my career as a PhD graduate student.

To my advisor, Dr. Eric Tucker, thank you for teaching me everything you know about lab techniques, experiments, and publishing papers, as well as giving me the opportunity to attend conferences and introducing me to colleagues to help me network with others. Thank you for working with me as a team to train other students that have come into and through the lab and for teaching me how to manage and run a lab. Last, but not least thank you for taking me on as your first graduate student. I know this experience hasn't been without its ups and downs, but thank you for sticking with me and working through all the challenging points. You have taught me so much about the field, and I have grown so much from this experience.

To my committee members, Dr. Pete Mathers, Dr. Scott Weed, Dr. Aric Agmon, and Dr. Shuo Wei, thank you for all your guidance, support, and feedback on projects and papers.

To Dr. Dey, thank you for all your support and for providing a listening ear, direction, and feedback when I had a problem or needed advice. I genuinely appreciated our meetings and talks and everything that came out of them.

To Dr. Randy Kulesza and my teaching professors, thank you for giving me the opportunity to teach, for being patient with me while I learned the best strategies to teach, and for teaching me the most up-to-date ways of keeping students engaged.

To my past and current labmates (John, Danielle, Neha, Bree, Emily, Kathryn, Katie, Emily, John, Kelly, Michael, Steven, Jessica, Skye, Catherine), thank you for all your help with the mouse colony and experiments. I am so glad every one of you decided to join the lab. Each one of you has taught me something new, and I am grateful that I have had the opportunity to work with all of you. Thank you for being patient with me during hectic experiment times and defense times, and most of all thank you for your support and friendship. I wish you all the best of luck with your projects and future endeavors.

To Dr. Amanda Ammer and Dr. Karen Martin, thank you for your help with everything confocal. Thank you for including me in training sessions, being my never-ending source of answers when things went wrong with the confocal imaging, and for making my work environment flexible with Imaris. I loved working with you both.

Thank you to my graduate student colleagues and friends (both inside and outside the neuroscience program). I would not have made it this far without the many lunches, dinners, drinks, walks, talks, and hugs. I never thought that I would have grown so much while I was here at WVU, but I had the best friends to help me through everything I faced. I am so grateful for every single one of our friendships.

Thank you to my family and family friends for your guidance, support, and love throughout all of my schooling. Specifically, I would like to thank my parents, Sue and Tom, my brother, Aaron, my sister-in-law, Natalie, my Aunt Donna, Grammie, my best friend, Liz, and my love, Russ, for always being there to celebrate my successes and to encourage me through my struggles. Words cannot describe how much your unwavering love and kindness means to me.

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List of Abbreviations

5HT3aR- 5-Hydroxytryptamine-3a Receptor
ADP- Adenosine Diphosphate
AMPA- α -Amino-3-Hydroxy-5-Methyl-4-Isloxazolepropionic Acid Receptor
Ant1- Adenine Nucleotide Transferase 1
ApoER2- Apolipoprotein-E Receptor 2
Arl13b- ADP-Ribosylation Factor-Like Protein 13B
ATP- Adenosine Triphosphate
CCK- Cholecystokinin
cDKO- Conditional Double Knockout (*Jnk1^{fl/fl}*; *Jnk2^{-/-}*; *Dlx5/6-CIE*)
CGE- Caudal Ganglionic Eminence
CP- Cortical Plate
cTH- Conditional Triple Heterozygous (*Jnk1^{fl/fl}*; *Jnk2^{-/-}*; *Jnk3^{+/-}*; *Dlx5/6-CIE*)
cTKO- Conditional Triple Knockout (*Jnk1^{fl/fl}*; *Jnk2^{-/-}*; *Jnk3^{-/-}*; *Dlx5/6-CIE*)
CXCL12- CXC- Ligand 12
CXCR4/7- CXC-Receptor Motif 4/7
Dab1- Disabled 1
DCX- Doublecortin
Dlx5/6-CIE- Dlx5/6-Cre-Ires-EGFP
DMSO- Dimethyl Sulfoxide
E- Embryonic Day
ErbB4- Erb-B2 Receptor Tyrosine Kinase 4
GABA- Gamma-Aminobutyric Acid
GAD67- Glutamic Acid Decarboxylase 67
GFP- Green Fluorescent Protein
GIN- Green Fluorescent Protein-Expressing Inhibitory Neurons
IFT- Intraflagellar Transport
IZ- Intermediate Zone
JIP- JNK-Interacting Protein

JNK- c-Jun N-Terminal Kinase
LgDel- Large Deletion
LGE- Lateral Ganglionic Eminence
Map2k4/7- Mitogen Protein Kinase Kinase 4/7
Map3k12- Mitogen Protein Kinase Kinase Kinase 12
MAPK- Mitogen Activated Protein Kinase
MARKS- Microtubule Affinity Regulating Kinases
MeCP2- Methyl CpG Binding Protein 2
MGE- Medial Ganglionic Eminence
MKK4/7- Mitogen-Activated Protein Kinase Kinase 4/7
MZ- Marginal Zone
NMDA- N-Methyl-D-Aspartate Receptor
NPY- Neuropeptide-Y
P- Postnatal Day
PBS- Phosphate Buffered Saline
PKA- Protein Kinase A
PTEN- Phosphatase and Tensin Homolog Gene
REM- Rapid Eye Movement
SCG10- Superior Cervical Ganglion 10
SD- Standard Deviation
SDF-1- Stromal Derived Factor-1
SHH- Sonic Hedgehog
SVZ- Subventricular Zone
TAOK2- TAO Kinase 2
VIP- Vaso-Intestinal Peptide
VZ- Ventricular Zone

Chapter 1: Introduction and Literature Review

The Cerebral Cortex

The cerebral cortex, specifically the neocortex, controls our ability to sense and respond to our environment through decision-making, movement, communication, vision, hearing, and sensory perception. It is comprised of, in a broad sense, two mitten shaped hemispheres, each comprising five lobes (frontal, parietal, occipital, temporal, and limbic), organized layers of gray matter, and white matter tracts. Alfred Campbell was one of the first to relate structure to function, identifying neocortical regions important for motor function, vision, audition, and sensation (Campbell, 1905; Loukas et al., 2011). This work was followed by Korbinian Brodmann who performed a detailed histological characterization of the cortex, identifying and describing the cellular composition of numerous areas (Loukas et al., 2011). These well-defined cortical regions are known today as Brodmann's areas (Loukas et al., 2011).

The neocortex is organized into six-layer structure composed of two types of neurons. Excitatory neurons use glutamate as a neurotransmitter and relay neural information over long distances both within and outside of the cerebral cortex and are therefore also known as projection neurons. These neurons are comprised of primarily pyramidal neurons that arise from a diverse group of progenitors in the dorsal (cortical) ventricular zone and subventricular zone (Stancik et al., 2010). They migrate radially to their proper laminar position during cortical development, populating the cortex in an inside-out manner (the earliest born excitatory neurons reside in the deepest layers of the cortex while later born neurons reside in more superficial layers; Rakic, 1972, 1974, 1978). In contrast, inhibitory neurons, also known as cortical interneurons, produce and release the neurotransmitter gamma-aminobutyric acid (GABA) and critically gate the activity of excitatory neurons despite being outnumbered about four to one (Rudy et al., 2011). During development, cortical interneurons are born in the ventral forebrain and migrate tangentially to populate the cortex (Anderson et al., 2001; Wichterle et al., 2001; Xu et al., 2004; Miyoshi et al., 2010). Proper positioning and circuit formation of these two neuronal

types is imperative for normal behavior and cognition (Rakic, 1975; Benes et al., 1991). Perturbations in the development of excitatory neurons and interneurons underlie a large number of human neurodevelopmental disorders. Disrupted positioning of inhibitory interneurons in particular during development may alter their connectivity within the mature cortical circuit and interfere with the normal excitatory-inhibitory balance within the cerebral cortex (Isaacson and Scanziani, 2011). Alterations in excitatory-inhibitory balance within the cerebral cortex are thought to underlie severe neurodevelopmental disorders, including schizophrenia, epilepsy, and autism (Cho et al., 2006; Bozzi et al., 2012; Snijders et al., 2013). Recently, these disorders were termed interneuronopathies to acknowledge the pathological development of cortical interneurons in conjunction with the abnormal development of excitatory neurons (Kato and Dobyns, 2005).

Cortical Inhibitory Interneurons

Cortical interneurons are not a single neuronal type, but an extremely diverse and important population of neurons in the cerebral cortex (Rudy et al., 2011). Together, they prevent hyperexcitability by inhibiting excitatory neurons, and refine and shape the electrical activity of cortical circuits to promote diverse functions, including working memory and attention (Canuet et al., 2011; Murray et al., 2015; Kim et al., 2016b). They account for approximately 20% of all cortical neurons, making them a vulnerable cell population (Rudy et al., 2011). Subtypes of cortical interneurons differ in molecular composition (parvalbumin, somatostatin, 5HT3aR-expressing interneurons constitute three diverse groups), electrophysiological properties (fast-late-, quasi-fast-, non-fast- and irregular-spiking, and intrinsically bursting), synaptic contact area with excitatory neurons (cell body, dendrites, or initial axon segment), and layer location (Flames and Marín, 2005; Rudy et al., 2011; Chu and Anderson, 2015). In recognition of their remarkable heterogeneity, many efforts have been made to classify cortical interneurons based on their morphology, gene expression profile, neurotransmitter or protein expression profile, and

function (Kawaguchi, 1995; Kawaguchi and Kubota, 1997; Ascoli et al., 2008; Rudy et al., 2011; Chu and Anderson, 2015).

The wide diversity of interneurons suggests a unique role for each individual type (Kawaguchi, 1995; Kawaguchi and Kubota, 1997; Ascoli et al., 2008; Rudy et al., 2011). Additionally, studies investigating the underlying pathology in severe neurological disorders have found striking decreases and irregularities in certain populations of cortical interneurons, which further suggests that different subsets could play distinct roles that do not overlap and may account for some of the heterogeneity seen in cortical disorders (Lewis et al., 2012; Snijders et al., 2013; Jacob, 2016). The precise function of individual subsets of cortical interneurons in the normal brain or the ability for each of these populations to compensate or replace one another in disease states is not well understood (Kawaguchi and Kubota, 1993, 1996, 1997; Ascoli et al., 2008).

Parvalbumin-expressing interneurons

Parvalbumin-expressing interneurons are the most abundant subtype in the cortex and have been highly implicated in neurological disease (Schwaller et al., 2004; Gill et al., 2010; Curley et al., 2011; Akakin et al., 2012; Dutton et al., 2012; Lewis et al., 2012; Meechan et al., 2012; Kim et al., 2016a). Parvalbumin interneurons are fast-spiking neurons that reside in all cortical layers except layer 1 (Kawaguchi and Kubota, 1993; Taniguchi et al., 2013). There are actually two major types of parvalbumin-expressing interneurons, each with a distinct morphological and electrophysiological characteristics (Kawaguchi and Kubota, 1997). The first are basket cells, they have a large plexus of axons that extend and target the cell body and proximal dendrites of cortical pyramidal cells (Szentagothai, 1975). In contrast, chandelier cells have distinct morphological features with cartridges of synaptic boutons at the end of each axonal branch that target the initial axon segment of excitatory neurons (Szentagothai, 1975). The

electrophysiological properties of chandelier cells are distinct from basket cells despite having fast-spiking properties like basket cells (Szabadics et al., 2006; Glickfeld et al., 2009; Woodruff et al., 2009). It remains unclear whether chandelier cells depolarize or hyperpolarize excitatory neurons (Glickfeld et al., 2009; Woodruff et al., 2009, 2011). Early studies on chandelier cells have predominately found them to have a depolarizing effect on excitatory neurons, atypical of cortical interneurons (Szabadics et al., 2006; Woodruff et al., 2009). Contrary to these data, another study found that chandelier cells may have a hyperpolarizing, and therefore inhibitory effect, on excitatory neurons (Glickfeld et al., 2009). The latter study indicated that methods used in the previous study to record these neurons were not sufficient to maintain membrane potential or intracellular ion concentrations of excitatory neurons (Glickfeld et al., 2009). Further, studies by Woodruff and colleagues (2011) have further complicated our understanding of these cells, they found chandelier cells may play a dual role at the initial axon segment synapse. Their data suggest chandelier cells may either hyperpolarize or depolarize a postsynaptic excitatory neuron based on the membrane potential of the excitatory neuron (Woodruff et al., 2011). Together, these studies indicate that parvalbumin-expressing interneurons provide a wide variety of inputs to excitatory neurons that thereby intricately shape neural activity in the cortex.

Dysfunction in parvalbumin-expressing interneurons has been implicated in many neurodevelopmental disorders including autism, schizophrenia, and epilepsy (Schwaller et al., 2004; Dutton et al., 2012; Lewis et al., 2012; Sgadò et al., 2013). Studies report many different parvalbumin-expressing neuron pathologies that can underlie these disease states including a decrease in the number, improper laminar position, and incomplete maturity of parvalbumin-expressing neurons in the prefrontal cortex, a location where reasoning, emotion, and decision making is believed to occur (Gill et al., 2010; Curley et al., 2011; Meechan et al., 2012; Hashemi et al., 2016). Specifically, Curley and colleagues (2011) found decreased expression of GAD67 mRNA, an enzyme that converts glutamic acid to GABA, without alterations in the number of

parvalbumin interneurons in patients with schizophrenia. The altered expression of GAD67 was correlated with irregularities in gamma oscillations, brain waves that correlate with focus and attention and often appear during REM sleep (Cho et al., 2006; Curley et al., 2011). Other investigators have reported a loss of GABAergic neurons in epilepsy, citing its loss as a probable cause of hyperexcitability (Schwaller et al., 2004; Gill et al., 2010).

Somatostatin-expressing interneurons

Somatostatin expressing interneurons, located in layers II through VI, have projections to the superficial layers of the cerebral cortex that provide inhibition to the distal apical dendrites of excitatory neurons (Oliva Jr et al., 2000; Ma et al., 2006; Fino and Yuste, 2011). This subtype of interneuron, which is responsible for providing local feedback inhibition to excitatory neurons, demonstrates diverse electrophysiological properties and morphologies (Fino and Yuste, 2011). Somatostatin-expressing cortical interneurons have been characterized using several different mouse lines including GIN, X94, and X98 lines that together account for about 50% of the total population of somatostatin neurons (Ma et al., 2006). The GFP-expressing inhibitory neurons (GIN) mouse line was the first line developed with GFP expression in somatostatin-expressing interneurons residing in the hippocampus and cortex (Oliva Jr et al., 2000). In the somatosensory cortex, the GIN line labeled interneurons residing in layers II, IV, and upper layer V (Oliva Jr et al., 2000). Bipolar neurons were the most prevalent labeled neuron in this line followed by cortical interneurons with a pyramidal shaped soma located primarily in layer II (Oliva Jr et al., 2000).

Further characterization of somatostatin interneurons was done through the X94 and X98 lines created by Ma and colleagues (Ma et al., 2006). The X94 and X98 lines were found to have a similar distribution of somatostatin-expressing neurons to the GIN line, but additionally labeled somatostatin cortical interneuron populations that were sparse or absent in the GIN line (Oliva

Jr et al., 2000; Ma et al., 2006). The X94 mouse line labeled many somatostatin interneurons in layer IV and lower layer V (Ma et al., 2006). The X98 line labeled similarly to the X94, but labeled more somatostatin interneurons residing in layer VI with no labeling in layer IV (Ma et al., 2006).

Somatostatin interneuron morphology reflected their location, targets, and electrophysiological properties. Somatostatin cortical interneurons found in layer IV were a novel finding by Ma and colleagues (2006). These cortical interneurons displayed short range axons and dendrites, and exhibited stuttering and quasi-fast-spiking electrophysiological properties which Ma and colleagues (2006) state may have led to previous mischaracterization of this subtype. A more recent study confirmed these results and found that somatostatin interneurons in layer IV are responsible for inhibiting fast-spiking interneurons and the disinhibition of local thalamocortical circuitry (Xu et al., 2013). Additionally, somatostatin interneurons residing in layer V and VI differ in morphology compared to layer IV interneurons and provide inhibition to surrounding pyramidal cells by extending axons to layer I and contacting their distal apical dendrites (Ma et al., 2006), which is the defining feature of so-called Martinotti cells.

In the absence of somatostatin interneurons, the feedback inhibition is removed and graded signals can continue to increase in intensity causing epilepsy and perhaps sensory overload, a key symptom experienced by patients with autism where sensory stimuli, both sounds or touch, are overwhelming. A reduction in the number of somatostatin interneurons is seen in mice deficient for both *MeCP2* and *PTEN*; patients with mutations in these same genes exhibit autism and epilepsy (Ito-Ishida et al., 2015; Vogt et al., 2015). Ito-Ishida and colleagues (2015) found the *MeCP2*-mutant mice, deficient for somatostatin interneurons, was the basis for their epilepsy whereas deletion of this protein from parvalbumin-expressing interneurons resulted in motor, sensory, memory, and social deficits. These data clearly indicate that somatostatin and parvalbumin interneurons have distinct roles in cortical function.

5HT3a receptor-expressing interneurons

A third heterogeneous population of cortical interneurons express the serotonin receptor 5HT3a, they account for approximately 30% of the total population of interneurons (Morales et al., 1996; Morales and Bloom, 1997; Miyoshi et al., 2010; Vucurovic et al., 2010). They are found in all layers of the cortex, but a large proportion reside in upper layers (Karagiannis et al., 2009; Lee et al., 2010; Miyoshi et al., 2010). Within this population, Miyoshi and colleagues identified nine different subtypes and Lee and colleagues characterized a tenth subtype based on morphology, electrophysiological properties, and chemical markers (Lee et al., 2010; Miyoshi et al., 2010). Early studies suggested and later studies confirmed that the 5HT3aR-expressing populations of cortical interneurons label for chemical markers that include reelin, vaso-intestinal peptide (VIP), cholecystokinin (CCK), calretinin, and neuropeptide Y (NPY; Morales and Bloom, 1997; Lee et al., 2010; Miyoshi et al., 2010). Each of these groups is distinguished by morphological characteristics and electrophysiological properties. The electrophysiological properties of 5HT3aR interneurons are highly diverse, and include late-spiking, burst non-adapting, delayed non-fast spiking, irregular spiking, and intrinsic bursting interneurons (Lee et al., 2010; Miyoshi et al., 2010). Similarly, their morphologies differ ranging from interneurons that are neurogliaform in appearance with small cell bodies and short dendrites, to bipolar and multipolar interneurons with longer reaching dendrites and larger cell bodies (Lee et al., 2010; Miyoshi et al., 2010).

A few studies have linked deficits in these small populations to cortical disorders such as schizophrenia and autism, but their role in developmental neurological disease remains understudied (Hashimoto et al., 2008). Hashimoto and colleagues (2008) identified lower transcript levels of NPY and CCK in the dorsolateral prefrontal cortex in patients with schizophrenia implicating this group in neurological disease. However, further work is required to fully elucidate their role in other developmental disorders.

The Ventral Forebrain and Development of Cortical Interneuron Populations

Each population of cortical interneuron arises from distinct regions of the ventral forebrain during development (Wichterle et al., 1999; Anderson et al., 2001; Nery et al., 2002; Xu et al., 2004; Miyoshi et al., 2010; Ma et al., 2012; Niquille et al., 2013). They migrate using guidance cues to navigate to the correct location in the cortex and to make the appropriate connections, some mentioned above, on excitatory neurons (Polleux et al., 2002; Flames et al., 2004; Zimmer et al., 2008; Bortone and Polleux, 2009; Elias et al., 2010; Rudolph et al., 2010; Sánchez-Alcañiz et al., 2011; Wang et al., 2011; Li et al., 2012; Abe et al., 2014).

The concept that neurons migrated within the developing cortex was first identified through the study of radially migrating neurons (Angevine and Sidman, 1961; Rakic, 1971, 1972). Studies determined that excitatory neurons are born in the ventricular zone from radial glia, migrate up radial glial scaffolding, and drop off in an inside-out manner to organize themselves based on birthdate (Angevine and Sidman, 1961; Rakic, 1974; Bayer et al., 1991; Noctor et al., 2001). Further, there are many important guidance cues and cytoskeletal regulators known to be essential for excitatory neuron migration, such as reelin and doublecortin (Stouffer et al., 2015). Perturbations of excitatory neuron migration result in a spectrum of human malformation syndromes including lissencephaly and the double cortex phenotype (Caviness, 1976; Gleeson et al., 1998, 1999). During subsequent studies of radially migrating excitatory neurons a second cohort of migrating neurons was identified (O'Rourke et al., 1995, 1997). Researchers later solidified that these neurons were GABAergic interneurons and that they migrated from regions in the ventral forebrain instead of the ventricular zone of the dorsal forebrain (Anderson et al., 1997; Wichterle et al., 1999, 2001). GABAergic neurons, including cortical interneurons, are generated in three anatomically distinct compartments within the developing ventral forebrain; the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE), and caudal ganglionic eminence (CGE; Lavdas et al., 1999; Wichterle et al., 1999; Anderson et al., 2001;

Miyoshi et al., 2010). These regions are well-defined morphologically and by their transcription factor expression (Xu et al., 2004, 2008; Cai et al., 2013). Initial studies demonstrated that the MGE but not the LGE produced cortical interneurons that would disperse into adult cortical tissue suggesting that the LGE produced a different population of inhibitory neurons that did not migrate into the cortex (Wichterle et al., 1999). Further studies found that the lateral ganglionic eminence gives rise to inhibitory neurons in the striatum and olfactory bulb (Campbell et al., 1995; Wichterle et al., 1999; Anderson et al., 2001; Stenman et al., 2003; Tucker et al., 2006; Waclaw et al., 2009). Cortical interneurons arise predominately from the MGE and the CGE (Figure 1.1A,B; Wichterle et al., 1999, 2001; Xu et al., 2004; Lee et al., 2010; Miyoshi et al., 2010; Touzot et al., 2016). The MGE gives rise to the largest population of cortical interneurons including the parvalbumin- and somatostatin-expressing interneurons from the ventral and dorsal MGE respectively (Anderson et al., 2001; Xu et al., 2004; Inan et al., 2012). The CGE gives rise to most 5HT3aR expressing interneurons including the VIP, CCK, and a subset NPY interneurons (Nery et al., 2002; Xu et al., 2004; Miyoshi et al., 2010; Touzot et al., 2016). A portion of NPY neurons are somatostatin-positive and are generated in the MGE (Butt et al., 2005).

Cortical Interneuron Migration

Cortical interneurons migrate tangentially from the MGE and CGE to reach the cortical rudiment and infiltrate the cortical plate (Figure 1.1C,E; Xu et al., 2004; Miyoshi et al., 2010; Inan et al., 2012). The birthplace of inhibitory cortical interneurons is therefore distinct from their excitatory neuron counterparts in that excitatory neurons are born in the ventricular zone of the dorsal forebrain and travel radially to enter the cortical plate on glial scaffolding (Rakic, 1972; Noctor et al., 2001). Excitatory neurons then drop off radial glial processes to form layers in the cortical plate and do so based on birthdate in an inside-out manner, with older neurons being deposited in deeper cortical layers (Angevine and Sidman, 1961; Rakic, 1974; Noctor et al., 2001; Greig et

al., 2013). Interneurons migrating tangentially will later distribute together with excitatory neurons in the cortical plate according to birthdate. Early born interneurons from the MGE tend to co-distribute with early born excitatory neurons (Valcanis and Tan, 2003). Conversely, CGE derived inhibitory interneurons laminate primarily in upper layers of the cortex, not by birthdate like MGE derived interneurons (Miyoshi et al., 2010).

Cortical interneurons enter the cerebral cortex beginning on embryonic day 12.5 in mice (in humans this begins between 6-8 weeks of embryonic development; Figure 1.1D; Zecevic et al., 2011; Ma et al., 2013). Cortical interneurons mainly travel in two streams, a marginal zone (MZ) stream located just below the pial surface of the cortex, and a subventricular zone stream (SVZ) located above the ventricular zone (Figure 1.1C; Wichterle et al., 2001).

Migrating cortical interneurons travel similarly to radially migrating cells by extending a dynamic leading process (Figure 1.2A) that in conjunction with nuclear kinetic events pulls them forward (Figure 1.2B) in a process called nucleokinesis (Rakic, 1971; Edmondson and Hatten, 1987; Bellion et al., 2005). They differ from excitatory neurons in the frequency in which their leading process branches and the rapidity in which they change directions during migration (Nadarajah et al., 2001; Martini et al., 2009; Lysko et al., 2011), which likely reflects different demands associated with accomplishing guided migration without a radial glial substrate. Each of these elaborate events correlates with the expression of receptors and guidance cues or chemokines (Serrati et al., 2008; Sánchez-Alcañiz et al., 2011; Wang et al., 2011; Baudoin et al., 2012; Lysko et al., 2014).

Migration Guided by Molecular Cues

Guidance cues act as directional maps providing boundaries for migrating neurons within the developing forebrain (Flames et al., 2004; Rudolph et al., 2010; Sánchez-Alcañiz et al., 2011; Wang et al., 2011; Li et al., 2012). Without these maps, migration can go awry causing

devastating cortical dysfunction (Meechan et al., 2012; Volk et al., 2015). Recent studies have found that cortical interneurons can express different receptors based on age, region of the forebrain, and which stream they are migrating (Faux et al., 2010; Antypa et al., 2011). Cortical interneuron guidance cues can be chemoattractive, guiding cortical interneurons through a region, or chemorepulsive, deterring cortical interneurons from an area (Flames et al., 2004; Rudolph et al., 2010; Sánchez-Alcañiz et al., 2011; Wang et al., 2011; Li et al., 2012). Cortical interneurons rely heavily on guidance cues to maintain streams and migrate to the appropriate location within the cerebral cortex (Flames et al., 2004; Rudolph et al., 2010; Sánchez-Alcañiz et al., 2011; Wang et al., 2011; Li et al., 2012).

Early migration in streams

Chemoattractive cues such as stromal derived factor-1 (SDF-1; also known as Cxcl12) provide preferential areas where cortical interneurons expressing the Cxcr4 receptor can migrate and distribute into their two main migratory streams (López-Bendito et al., 2008). The SDF-1 ligand is secreted into the extracellular environment by meningeal cells at the pial surface and transiently-amplifying neural progenitor cells in the SVZ (López-Bendito et al., 2008). The SDF-1 signaling pathway also mediates microtubule dynamics in the leading processes of cortical interneurons and affects the speed of migration and decision making during migration (Lysko et al., 2011). The absence of this cue causes dispersion of cortical interneuron streams and disruption to their placement and wiring with other neurons within the cortex (Sánchez-Alcañiz et al., 2011; Wang et al., 2011). One example where Cxcr4 expression is altered within cortical interneurons is in the DiGeorge Syndrome, or 22q.11 deletion syndrome, which is often associated neurological dysfunction including schizophrenia and autism (Meechan et al., 2012). Meechan and colleagues described the distribution of cortical interneurons in the Large Deletion (LgDel) 22q11.2DS mouse model of DiGeorge Syndrome to be similar to *Cxcr4* mutants. The LgDel model showed a decrease of cortical interneurons traveling within the SVZ stream and an

increase in interneurons in the MZ stream which is comparable to cortical interneuron distributions seen in *Cxcr4* null mice (Wang et al., 2011; Meechan et al., 2012). Moreover, the distribution shifts seen in early cortical development do affect the distribution of parvalbumin interneurons and their circuitry in the adult brain (Meechan et al., 2012, 2015). Dysfunctional wiring of parvalbumin-positive interneurons was associated with cognitive impairment in the LgDel mouse (Meechan et al., 2015). This was assessed with a visual discrimination/reversal task and demonstrated that the LgDel mice had difficulty learning the initial task and further, had difficulty when the task was reversed (Meechan et al., 2015). These data indicate that the proper guidance of cortical interneurons by *Cxcr4* is imperative to for proper function of the adult cortex.

On the other hand, chemorepellents like ephrins repel cortical interneurons from inappropriately invading the ventral forebrain and cortex. Two types of ephrin ligands exist: Type A and Type B, distinguished by their interactions with EphA versus EphB receptors and the fact that the type A's are glycosyl-phosphatidylinositol linked while the type B's are transmembrane (Gale et al., 1996; Flanagan and Vanderhaeghen, 1998). The ephrins thought to guide interneuron migration are mostly ephrin A's (Zimmer et al., 2008; Rudolph et al., 2010). There are several different isoforms of ephrin A's that are expressed in different locations of the developing forebrain. However, studies of ephrin guidance of cortical interneuron migration remain confined to the ventral forebrain. Ephrin A5, expressed in the VZ of both the MGE and LGE, acts to repel cortical interneurons expressing its cognate receptor, EphA4, arising and migrating from the MGE (Zimmer et al., 2008). A stripe assay utilizing cortical interneurons found that interneurons did not enter the stripes of ephrin A5. Additionally, cocultures of MGE derived cortical interneurons and fibroblasts transfected to express ephrin A5 demonstrated that ephrin A5 repels cortical interneurons away from the transfected fibroblasts. Further, ephrin A3 also interacts with EphA4 receptors to guide cortical interneurons within the developing cortex

(Rudolph et al., 2010). The ephrin A3 ligand was found to be expressed in the cortical plate and developing striatum (Rudolph et al., 2010). Slice culture assays inhibiting ephrin A3 made the striatum permissive to cortical interneurons migrating from the MGE and stripe assays demonstrated that ephrin A3 repels cortical interneurons. (Rudolph et al., 2010). However, the involvement of ephrin A3 in the cortex remains unknown. If ephrin A3 signaling acts similarly in the cortical plate to its repellent role in the striatum it is possible that the expression of EphA4 receptors would prevent the entry of cortical interneurons into the cortical plate and promote tangential migration in streams.

There are some guidance cues that affect cortical interneuron migration where it is still unclear whether the guidance cue is chemoattractive or repulsive. Cortical interneurons guided by ErbB4 receptors in response to the neuregulin ligand is one example (Flames et al., 2004; Li et al., 2012). Disruptions to ErbB4 signaling are implicated in cases of schizophrenia (Stefansson et al., 2002). ErbB4 receptors are primarily localized on cortical interneurons migrating from the MGE and in the adult cortex, particularly on parvalbumin- expressing neurons (Yau et al., 2003). ErbB4 is activated specifically by neuregulin 1 and 3 during early brain development (Flames et al., 2004; Li et al., 2012). Li and colleagues (2012) demonstrated that neuregulin 1 and 3 are expressed in different regions of the developing cortex; neuregulin 3 is expressed within the developing cortical plate, and neuregulin 1 is expressed in VZ/SVZ at E14.5. Neuregulin 1 has three distinct isoforms all of which regulate cortical interneuron migration (Flames et al., 2004; Li et al., 2012). The secreted isoforms (neuregulin 1 type I and II) were found to be chemoattractive to migrating cortical interneurons (Flames et al., 2004). The membrane bound isoform (neuregulin 1 type III) created an environment that was permissive for migrating interneurons (Flames et al., 2004). However, it remains uncertain whether neuregulins act as a chemoattractant or a chemorepellent to guide cortical interneuron migration during early development (Flames et al., 2004; Li et al., 2012). Flames and colleagues (2004) suggest that

neuregulins attracts migrating neurons. *In vitro* stripe assays, using dissociated MGE cells and COS cells transfected with neuregulin 1 type III, demonstrated that cortical interneurons migrated towards neuregulin 1 type III expressing COS cells and preferred them over control or nontransfected COS cells (Flames et al., 2004). Similarly, *in vitro* assays utilizing MGE explants grown in culture with neuregulin 1 type I transfected COS cells showed a similar result (Flames et al., 2004). However, a more recent study found neuregulin acts as a chemorepellent and instead of attracting cortical interneurons suggested that neuregulins act to create boundaries through which cortical interneuron streams can travel (Li et al., 2012). *In vitro* MGE explant assays show that when MGE explants are cultured with T293 cells transfected with neuregulin 1 and 3, explants showed polarized growth away from transfected T293 cells (Li et al., 2012). Further, in-utero electroporation of neuregulin 1 within the developing cortex prevents cortical interneuron migration into the area of ectopic expression (Li et al., 2012). Therefore, although neuregulins are important for early cortical interneuron migration their primary role as a guidance factor remains unclear.

Tangential to radial transition

As development advances migrating cortical interneurons need to exit from the cohesive streams they are traveling in to invade the cortical plate and establish residence in the appropriate laminae. This requires tight regulation of chemokine cues and also expression of new cues both in the interneuron as well as in the extracellular environment. Each step in development must proceed without error to establish normally functioning cortex.

Around E15.5 cortical interneurons make a crucial transition from tangential migration to radial migration to infiltrate the developing cortical plate. Premature transitions or failure to make this transition can result in improper positioning of interneurons in the cortex (Sánchez-Alcañiz et al., 2011; Wang et al., 2011; Meechan et al., 2012). Initial studies on this transition suggested that

there is specific regulation of guidance factors which range from regulation of receptors that read chemokine guidance cues (Cxcr4 and Cxcr7) to the expression of adhesion molecules (Connexin43) that mediate the tangential to radial transition (Elias et al., 2010; Sánchez-Alcañiz et al., 2011; Wang et al., 2011; Abe et al., 2014).

The SDF-1 pathway is known for perpetuating a tangential orientation in cortical interneurons through Cxcr4 and Cxcr7 (Sánchez-Alcañiz et al., 2011; Wang et al., 2011; Abe et al., 2014). Elimination of either *Cxcr4* or *7* causes cortical interneurons to disperse from migratory streams and alter positioning of interneurons within the mature cortex, indicating that both receptors are important for spatial and temporal guidance (Sánchez-Alcañiz et al., 2011; Wang et al., 2011). However, studies have shown that these receptors may have distinct roles during cortical interneuron migration, specifically relating to their tangential to radial transition (Lysko et al., 2011, 2014; Sánchez-Alcañiz et al., 2011; Wang et al., 2011; Abe et al., 2014).

Recent studies suggest that Cxcr7 may have both autonomous and non-autonomous roles in cortical interneurons migration and is important for regulating the expression Cxcr4 and SDF-1 (Sánchez-Alcañiz et al., 2011; Wang et al., 2011). During early cortical development at E13.5 in mice, cortical interneurons primarily migrate in two distinct streams highly regulated by Cxcr4 expression and the presence of the SDF-1 ligand (Sánchez-Alcañiz et al., 2011; Wang et al., 2011). Cxcr7 promotes the retention of Cxcr4 at the cell's surface (Sánchez-Alcañiz et al., 2011; Abe et al., 2014). Additionally, at E13.5, Cxcr7 is highly expressed within the cortical plate by excitatory neurons (Sánchez-Alcañiz et al., 2011). *In vitro* studies using somatic cell culture demonstrated that Cxcr7 was expressed on both a cell's surface and internally, whereas Cxcr4 was expressed only on the cell's surface (Boldajipour et al., 2008). Cxcr7 was capable of binding and sequestering SDF-1 internally within the cell (Boldajipour et al., 2008). It is possible that Cxcr7 plays a non-autonomous role during early stages of cortical interneuron migration by acting within excitatory neurons to sequester SDF-1 in the cortical plate to localize cortical

interneuron to streams and prevent cortical interneurons from entering the cortex (Boldajipour et al., 2008; Sánchez-Alcañiz et al., 2011). During later time points the expression of Cxcr7 is down-regulated in the cortical plate, perhaps allowing the cortical plate to be permissive to cortical interneurons potentially through SDF-1 signaling (Sánchez-Alcañiz et al., 2011). Further, the expression of SDF-1 affects the stabilization of microtubule networks within the leading process of cortical interneurons through the phosphorylation of Dcx by protein kinase A (PKA; Lysko et al., 2011, 2014). In the presence of PKA, Dcx binds to and stabilizes microtubules which in turn prevents branching of the leading process (Lysko et al., 2014). In the absence of SDF-1 activation of Cxcr4, microtubule networks destabilize and leading process branching occurs (Lysko et al., 2011). Leading process branching could be one way cortical interneurons turn radially to enter and navigate the cortical plate. This, in conjunction with Cxcr7 regulation of Cxcr4 at the surface of migrating interneurons, could indicate that Cxcr7 is important for organization of cortical interneurons within streams during early development and also their departure to enter the cortical plate during later development.

However, regulation of the SDF-1 pathway is unlikely to be the only regulator of the tangential to radial switch. Recently, studies began to investigate this further and documented differences in the expression of receptors or cues that mediate changes within the migrating interneuron, as well as changes in the expression of environmental cues that occur during this time period that could all work together to promote proper placement in the cortical plate (Bortone and Polleux, 2009; Elias et al., 2010; Baudoin et al., 2012; Lin-Hendel et al., 2016).

Elias and colleagues (2010) were the first to report that both radially migrating excitatory neurons and interneurons use gap junctions to migrate. Connexins are gap junction proteins that create channels to communicate electrical and chemical signals between neurons. During development, excitatory neurons express Connexins and utilize them as cell adhesion molecules, independent of their ability to form pores between cells, to adhere to radial glial cells

during their migration into the cortical plate (Elias et al., 2007). Similarly, they found that expression of Connexin43, a gap junction protein in cortical interneurons, is important for initiating the radial transition (Elias et al., 2010). When Connexin43 was knocked down in interneurons few made a radial transition to travel into the cortical plate (Elias et al., 2010). Electron microscopy showed that radially migrating cortical interneurons can establish connections via Connexin43 with radial glia to migrate into the developing cortical plate in the same way as excitatory neurons (Elias et al., 2010). These studies demonstrate that cortical interneurons may use similar mechanisms as migrating excitatory neurons when contacting and migrating on radial glia. However, it still remains unclear what mechanism provides the on-switch for the expression of these adhesion proteins. Perhaps the expression of adhesion proteins arises following changes to the branching and orientation of the leading process.

Studies have indicated that the primary cilium, a highly specialized system of microtubules, acts to help cortical interneurons read and respond to guidance cues within their environment and orient cortical interneurons as they migrate (Baudoin et al., 2012; Higginbotham et al., 2012). Conditional deletion of the ciliary protein, *Arl13b*, caused defective ciliary dynamics within cortical interneurons which triggered, delayed migration into the developing cortex, and altered the radial distribution of cortical interneurons within the cortical wall (Higginbotham et al., 2012). In *Arl13b* mutants, more cortical interneurons were found in the IZ of the cortical wall, with a marked decrease of interneurons traveling in the MZ and SVZ streams (Higginbotham et al., 2012). Many of the cortical interneurons had increased branching compared to controls, and increased pausing (Higginbotham et al., 2012). Further, *in vitro* cortical interneuron cultures demonstrated that the expression and shuttling of guidance cue receptors were altered in the primary cilium, indicating that the altered distribution of cortical interneurons within the cortical wall may be due to their inability to appropriately process environmental guidance cues (Higginbotham et al., 2012). Additionally, Baudoin and colleagues (2012) found Sonic

Hedgehog (SHH) signaling through the primary cilium orients cortical interneurons during their migration. Depletion of SHH signaling with cyclopamine in *ex-vivo* slices caused cortical interneurons to remain in streams migrating tangentially (Baudoin et al., 2012). However, in the presence of SHH, cortical interneurons departed from migratory streams and entered the cortical plate (Baudoin et al., 2012). Shuttling of the SHH pathway proteins through intraflagellar transport (IFT) was found to be required for cortical interneurons to make the transition to a radial orientation (Baudoin et al., 2012). Disruption to anterograde IFT, by conditionally ablating Kif3a from cortical interneurons, showed that cortical interneurons in *ex vivo* slices could not respond to SHH signaling and were stuck in a persistent tangential orientation (Baudoin et al., 2012). Therefore, appropriate assembly and function of the primary cilium is necessary for cortical interneurons to respond to guidance cues and could play an important role when cortical interneurons make the tangential to radial transition.

Moreover, energy utilization may also assist with the tangential to radial transition during this time. Lin-Hendel and colleagues (2016) demonstrated the need for oxidative phosphorylation for tangentially migrating cortical interneurons that was not required for radial projection neurons migration. Deletion of *Ant1*, a transporter important for transferring ATP across the inner membrane of mitochondria, in mice resulted in cortical interneurons changing directions instead of maintaining their tangential orientation (Lin-Hendel et al., 2016).

In summary, these studies suggest that multiple molecular mechanisms work synergistically to regulate the appropriate timing of cortical interneuron departure from migratory streams. The manner in which these distinct molecular mechanisms are coordinated during interneuron migration is unknown, but intracellular signaling pathways may play a vital role in the process. Signaling pathways are known to modify movement and direction in neurons as well as other migratory cell types through phosphorylation of downstream targets, but their role in early cortical interneuron migration remains incompletely understood.

The c-Jun N-Terminal Kinase Signaling Pathway

The c-Jun N-terminal kinase (JNK) signaling pathway consists of members of the mitogen-activated protein kinase (MAPK) superfamily of protein kinases. Three genes, *JNK1*, *JNK2*, and *JNK3*, encode the terminal kinases of the JNK signaling pathway (Gupta et al., 1996). The family of three *JNK* genes produce a total of ten isoforms through alternative splicing that exist in a short form (46kDa) or a long form (55kDa; Gupta et al., 1996). Each JNK protein is capable of activating different substrates and downstream cascades through phosphorylation of serine and threonine residues. In addition, JNK-interacting proteins (JIPs) assist JNKs by providing a scaffolding that bind JNKs to either increase the probability of being phosphorylated by an upstream effector or to phosphorylate a downstream target (Whitmarsh and Davis, 1998; Whitmarsh et al., 1998).

The JNK pathway has traditionally been associated with its role in apoptosis through its phosphorylation of c-Jun, a downstream activator of the cell death cascade across many cell types (Kuan et al., 1999). However, JNK is important for a multitude of other functions including axon guidance and growth in neurons and cytoskeletal rearrangement. It is important for migration in a variety of cell types including migration of excitatory neurons during brain development (Petrich et al., 2002; Chang et al., 2003; Gdalyahu et al., 2004; Kim and Sharma, 2004; Oliva Jr et al., 2006; Jin et al., 2010; Westerlund et al., 2011).

Brain development and neuronal migration

Early studies implicated JNK signaling in nervous system development and suggest differential roles for each *Jnk* gene in different locations within the brain (Kuan et al., 1999). Kuan and colleagues determined a clear onset of expression of JNK within the developing brain beginning at embryonic day 7 for *Jnk1* and *Jnk2* and embryonic day 11 for *Jnk3* (Kuan et al., 1999). Additionally, they found distinct expression patterns throughout the developing embryo (Kuan et

al., 1999). Initial studies of single deletions did not reveal a neurological phenotype in mice (Kuan et al., 1999). However, combinatorial constitutive deletion of the *Jnk1* and *Jnk2* genes were found to cause embryonic lethality between embryonic day 11 and 12 in mice (Kuan et al., 1999). The *Jnk1/2* knockout mice displayed neural tube closure defects and decreased apoptosis in the hindbrain, in addition to increased apoptosis in the forebrain (Kuan et al., 1999). Combinatorial deletions of *Jnk1/3* and *Jnk2/3* did not show similar anomalies (Kuan et al., 1999).

In adults, JNK proteins from all three JNK genes were found to be highly expressed within the cortex, hippocampus, striatum, and cerebellum of wildtype mice (Brecht et al., 2005). The short isoforms of *Jnk1* and *Jnk3* were predominately expressed over the long isoforms, whereas both short and long isoforms of *Jnk2* were expressed equally. *Jnk1* expression was primarily through the short isoform in all regions (cortex, hippocampus, striatum, and cerebellum; Brecht et al., 2005).

More recent studies demonstrate potential need for all three JNK genes in developing cortex. Single loss of the *Jnk1* or *Jnk2* gene in mice are now known to be sufficient to disrupt excitatory neuron migration in the developing cerebral cortex. *In vivo* studies by Westerlund and colleagues revealed that *Jnk1* is important for excitatory neuron migration and in its absence, causes them to migrate faster into the cortical plate (Westerlund et al., 2011). Additional studies established that excitatory neurons exhibiting a loss of JNK displayed dysfunction during nucleokinesis, and migration defects (Nishimura et al., 2010, 2014). Recently, Zhang and colleagues (2016) demonstrated a need for *Jnk2* in migrating excitatory neurons. The absence of *Jnk2* caused delays in migrating excitatory neurons, suggesting different roles for the *Jnk* genes within migrating neurons (Zhang et al., 2016).

Human mutations in JNK genes are not well documented. Interestingly, the only documented mutations are located in the *JNK3* gene (Shoichet et al., 2006; Kunde et al., 2013). Truncation mutations of the *JNK3* gene cause cognitive deficits and epilepsy (Shoichet et al., 2006; Kunde et al., 2013) suggesting that dysfunctions in the JNK genes could underlie certain cortical disorders. Other more obscure disruptions to the JNK pathway exist and occur through mutations in other genes that either activate the JNK pathway, or function downstream of the JNK pathway, and are a plausible cause of neurological disorders. One such regulator of JNK signaling that is found mutated in autism spectrum disorder is *TAOK2*, which is an upstream activator of JNK function (Calderon de Anda et al., 2012). Although, there is very little evidence to show this interaction, mouse models suggest that these mutations decrease activation of the JNK pathway and subsequently cause defects in developing neurons (Calderon de Anda et al., 2012).

Neuronal motility and movement

Several downstream effectors of JNK signaling regulate cell motility and movement during migration and are known to cause neurological dysfunction when disrupted. Stathmin 10 and MARKS-like protein 1 proteins are important for regulating cytoskeletal elements within excitatory neurons (Westerlund et al., 2011; Björklom et al., 2012). These proteins bind to microtubules and actin filaments, respectively, to regulate branching and neuronal movement. Likewise, doublecortin (*Dcx*), a microtubule-associating protein, is also a phosphorylation target of JNK signaling (Gdalyahu et al., 2004; Jin et al., 2010). In humans, disruption of *DCX* causes severe cortical migration disorders such as double cortex syndrome (Gleeson et al., 1998). Disruption of doublecortin in migrating cortical interneurons decreases branch formation and nucleokinesis events in cortical interneurons making it a likely contributor to other disorders of the cortex (Kappeler et al., 2006). JNK-phosphorylation of doublecortin causes it to localize to growth cones of cultured hippocampal neurons, which perhaps allows the growing axon to

perform different functional movements (Gdalyahu et al., 2004; Lysko et al., 2014; Nishimura et al., 2014). This localization of doublecortin was absent from the growth cone in the presence of a pan-JNK inhibitor (Gdalyahu et al., 2004).

These studies suggest an internal role for JNK in migrating neurons that affect not only the cytoskeletal structure of the neuron, but also its ability to respond to external cues in the environment. It is still not known how JNK signaling activity differs amongst different subsets of neurons.

Objectives and Hypotheses

The JNK signaling pathway plays a crucial role in migrating neurons, specifically excitatory neurons in the developing cortex. Studies from Westerlund and colleagues and Zhang and colleagues show that there are differing, but important roles, for individual *Jnk* genes in migrating excitatory neurons, which can modify the speed that they migrate within the cortex and ultimately affect how they are deposited into distinct layers (Westerlund et al., 2011; Zhang et al., 2016). Truncations of JNK genes in humans suggest that cortical interneurons could be disrupted by altered JNK function, as patients present with epilepsy and intellectual disability; cortical diseases that are associated with deficits in inhibition (Shoichet et al., 2006; Kunde et al., 2013). However, the role that JNK signaling plays in migrating cortical interneurons is not known.

Thus, JNK signaling is likely to play important roles in cortical interneuron development and understanding JNK's role may assist in understanding the pathogenesis of several human neurodevelopmental disorders. Therefore, the primary objective of this research is to characterize the role that JNK signaling plays in early cortical interneuron migration. The first set of experiments in Chapter 2 of my thesis explores the role of JNK signaling during the early entry of cortical interneurons into the cerebral cortex at embryonic day 12.5. The second set of

experiments presented in Chapter 3 of my thesis delves into the role that JNK signaling plays in migratory stream integrity and interneuron laminar positioning in the cortical plate. Our overarching central hypothesis for this work is that JNK signaling plays a crucial role during the early migration of cortical interneurons as they first enter and then find their laminar position in the developing cerebral cortex.

Figure 1.1: Cortical interneuron migration. **A.** Cortical interneurons migrate from distinct regions in the embryonic mouse brain. **B.** A coronal section of an embryonic mouse brain. **C.** Cortical interneurons migrate tangentially from the ventral forebrain in two streams located in the marginal zone (MZ) and subventricular zone (SVZ). **D.** Cortical interneurons arrive at different portions of the cortex at different time points (E=embryonic day of mouse development). **E.** In order to enter the cortical plate cortical interneurons turn to migrate radially (MZ=Marginal zone, CP=Cortical plate, IZ=Intermediate zone, SVZ=Subventricular zone, VZ=Ventricular zone). Figure includes adapted figures from: (Danglot et al., 2006; López-Bendito et al., 2008; Marín et al., 2010)

Figure 1.2: Migrating cortical interneurons. **A.** Migrating cortical interneurons extend a leading process that helps them to navigate their way into the developing cerebral cortex. **B.** Migrating cortical interneurons create a swelling in the leading process that is equipped with the cellular machinery to pull the cell body forward. Figure includes adapted figures from: (Polleux and Anton, 2005; Danglot et al., 2006)

Figure 1.3: The c-Jun-N-terminal kinase (JNK) pathway. JNK is phosphorylated by multiple upstream kinases. JNK is the terminal kinase in this cascade activating multiple downstream targets to regulate many cell growth and migration activities.

Chapter 2: Cortical Interneurons Require *Jnk1* to Enter and Navigate the Developing Cerebral Cortex

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Published in the Journal of Neuroscience, June 4th, 2014

Abstract

Proper assembly of cortical circuitry relies on the correct migration of cortical interneurons from their place of birth in the ganglionic eminences to their place of terminal differentiation in the cerebral cortex. Although molecular mechanisms mediating cortical interneuron migration have been well studied, intracellular signals directing their migration are largely unknown. Here we illustrate a novel and essential role for c-Jun N-terminal kinase (JNK) signaling in guiding the pioneering population of cortical interneurons into the mouse cerebral cortex. Migrating cortical interneurons express Jnk proteins at the entrance to the cortical rudiment and have enriched expression of *Jnk1* relative to non-interneuronal cortical cells. Pharmacological blockade of JNK signaling in *ex vivo* slice cultures resulted in dose-dependent and highly specific disruption of interneuron migration into the nascent cortex. Time-lapse imaging revealed that JNK-inhibited cortical interneurons advanced slowly and assumed aberrant migratory trajectories while traversing the cortical entry zone. *In vivo* analyses of JNK-deficient embryos supported our *ex vivo* pharmacological data. Deficits in interneuron migration were observed in *Jnk1* but not *Jnk2* single nulls, and those migratory deficits were further exacerbated when homozygous loss of *Jnk1* was combined with heterozygous reduction of *Jnk2*. Finally, genetic ablation of *Jnk1* and *Jnk2* from cortical interneurons significantly perturbed migration *in vivo*, but not *in vitro*, suggesting JNK activity functions to direct their guidance rather than enhance their motility. These data suggest JNK signaling, predominantly mediated by interneuron expressed *Jnk1*, is required for guiding migration of cortical interneurons into and within the developing cerebral cortex.

Introduction

Cortical interneurons play vital roles regulating neurotransmission in the cerebral cortex and their dysfunction is implicated in severe brain disorders including epilepsy and schizophrenia. Mechanisms contributing to the pathological vulnerability of cortical interneurons are unclear, but they may arise during embryonic development when interneurons embark on long distance tangential migration from the medial and caudal ganglionic eminences to the overlying cerebral cortex (Anderson et al., 1997; Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 2001; Nery et al., 2002; Miyoshi et al., 2010). For this reason, molecular mechanisms guiding cortical interneuron migration have been extensively studied (For review, see Faux et al., 2012; Marín, 2013). Many ligands and receptors mediating cortical interneuron dispersion from the ventral forebrain (Powell et al., 2001), motility (Polleux et al., 2002; Pozas and Ibáñez, 2005), chemorepulsion from the striatum (Marín et al., 2001), chemoattraction to the cerebral cortex (Yau et al., 2003; Flames et al., 2004), and formation and maintenance of migratory streams have been identified (Tiveron et al., 2006; Li et al., 2008; López-Bendito et al., 2008; Sánchez-Alcañiz et al., 2011; Wang et al., 2011). However, intracellular signals controlling cortical interneuron migration, particularly those directing migration at critical positions along their trajectories, are largely unknown.

The c-Jun N-terminal protein kinase (JNK) pathway plays obligate roles in mammalian forebrain development (Kuan et al., 1999), and moreover, genetic disruption of JNK function leads to cognitive disorders in humans (Kunde et al., 2013). JNKs are members of the mitogen-activated protein kinase (MAPK) signaling pathway, and are encoded by three related genes in mammals: *Jnk1* (*Mapk8*), *Jnk2* (*Mapk9*), and *Jnk3* (*Mapk10*). All three *Jnk* genes are expressed in the developing mouse brain, but only combinatorial deletion of *Jnk1* and *Jnk2* results in embryonic lethality and profound alterations in

neuronal survival and programmed cell death (Kuan et al., 1999; Sabapathy et al., 1999). Genetic deletions of *Jnk1* or upstream Jnk kinases results in aberrant radial migration of cortical projection neurons (Hirai et al., 2006; Wang et al., 2007; Westerlund et al., 2011; Yamasaki et al., 2011), strongly implicating JNK signaling as a major regulator of neuronal migration in the developing forebrain.

In the current study, we use a combination of *ex vivo* and *in vivo* approaches to demonstrate that cortical interneurons have a cell-intrinsic requirement for JNK signaling—largely mediated by the activity of *Jnk1*—to enter and successfully navigate the developing cerebral cortex. JNK-inhibited cortical interneurons dramatically slow their advancement and take inappropriate trajectories at the entrance to the cortical rudiment. Similarly, cortical interneurons in *Jnk1*-deficient and *Jnk1/2*-deficient embryos exhibit significant delays in their cortical migration. Conditional ablation of *Jnk1* and *Jnk2* from cortical interneurons delays cortical entry, disrupts migratory streams, and perturbs interneuron morphology *in vivo*, but has no effect *in vitro*, suggesting JNK signaling only regulates migration of cortical interneurons in the presence of cortical guidance cues. These findings implicate the JNK pathway, and *Jnk1* in particular, as a key molecular node in the intracellular regulation of cortical interneuron migration *in vivo*.

Materials and Methods

Animals

Animals were housed and cared for by the Office of Laboratory Animal Resources at West Virginia University. Timed-pregnant dams (day of vaginal plug = embryonic day 0.5) were killed by rapid cervical dislocation and mouse embryos were immediately harvested for tissue culture, gene expression, or histological analyses. The following mouse strains were acquired and maintained on a C57BL/6J (The Jackson Laboratory) background: *Dlx5/6 Cre-IRES-EGFP* (*Dlx5/6-CIE*; Stenman et al., 2003), *Mapk8*^{tm1Rjd} floxed (*Jnk1^{fl/fl}*; Das et al., 2007; Kindly provided by Dr. Roger Davis), *Mapk8*^{tm1Flv} knockout (*Jnk1*^{-/-}; Dong et al., 1998; The Jackson Laboratory), and *Mapk9*^{tm1Flv} knockout (*Jnk2*^{-/-}; Yang et al., 1998; The Jackson Laboratory). For slice culture experiments, CF-1 (Charles River) dams were crossed with hemizygous *Dlx5/6-CIE* males. Constitutive mutant embryos were generated by crossing *Jnk1*^{+/-};*Jnk2*^{+/-} females to *Jnk1*^{-/-} or *Jnk2*^{-/-} males. Conditional mutant embryos were generated by crossing *Jnk1*^{fl/fl}, *Jnk2*^{-/-} females to *Dlx5/6-CIE*; *Jnk1*^{fl/+}, *Jnk2*^{-/-} males. All animal procedures were performed in accordance to protocols approved by the Institutional Animal Care and Use Committee at West Virginia University.

Fluorescently Activated Cell Sorting and qRT-PCR

Methods for fluorescently activated cell sorting (FACS), extracting RNA, synthesizing cDNA, and performing quantitative RT-PCR were reported previously (Meechan et al., 2012). Briefly, cortices from *Dlx5/6-CIE*⁺ embryos were individually dissociated with papain (Worthington Biochemical), resuspended in FACS buffer, sorted based on endogenous GFP fluorescence, and *Dlx5/6-CIE*⁽⁺⁾ and *Dlx5/6-CIE*⁽⁻⁾ sorted samples were homogenized separately in Trizol reagent (Ambion). Total RNA was isolated and

cDNA synthesized using the Improm-II RT kit (Promega). qPCR was conducted using EvaGreen (BioRad) reagent and a CFX384 thermal cycler (BioRad). PCR for *Jnk1* (F: AGCAGAAGCAAACGTGACAAC /R: GCTGCACACACTATTCCTTGAG) and *Jnk2* (F: CCAGTAGGATTGCCTGCTTA /R: TGGTCACATGCATACGAGTC) was performed. Gapdh was used as the endogenous reference gene control. Fold-change was determined by the delta-delta CT method (Livak and Schmittgen, 2001).

Organotypic Slice Cultures

Embryos were collected and dissected in ice-cold complete Hank's Balanced Salt Solution (cHBSS; Tucker et al., 2006). Dlx5/6-CIE+ brains were embedded in 3% low melting point agarose (Fisher Scientific), sectioned at 300 μ m on a Leica VT1000 S vibratome, transferred to laminin/poly-D-lysine-coated transwell inserts (BD Falcon; Polleux and Ghosh, 2002) and grown at 37°C with 5% CO₂ in slice culture media (Tucker et al., 2006) for 24 hours. Stock solutions of pharmacological inhibitors [20 mM SP600125 (ENZO Life Sciences), 20 mM AS601245 (ENZO Life Sciences), and 100 mM U0126 (EMD Chemicals, Inc.)] were prepared in DMSO, stored at -20°C, and added to slice culture media immediately prior to use. DMSO was used as a vehicle control at matching solvent concentrations to inhibitors.

Live Imaging Experiments

Live vibratome slices were transferred to Millicell cell culture inserts (Millipore) in FluoroDishes (World Precision Instruments, Inc) filled with slice culture media containing a 1:1000 dilution of DMSO (for controls) or 20 μ M SP600125 JNK inhibitor. FluoroDish preparations were immediately transferred to a Zeiss LSM 510 Meta Confocal Microscope with stable environmental controls maintained at 37°C with 5% humidified

CO₂. Time-lapse z-series were acquired every 10 minutes for 24 hours with a LD Plan-Neofluar 20x/0.4 Korr objective lens.

MGE explant culture and analysis

Brains from embryonic day 12.5 (E12.5) *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* (control) or *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* (conditional double knockout) embryos were removed in ice-cold cHBSS, embedded in 3% low melting point agarose, and sectioned at 300 μ m. Medial ganglionic eminence (MGE) tissue was isolated from appropriate sections and trimmed to generate subventricular zone enriched explants. Each brain was processed individually and yielded approximately two MGE explants plus material for genotyping, which was performed retrospectively. A 1:1 collagen-matrigel mixture was made by preparing a 2 mg/ml working stock of neutralized collagen (BD Life Sciences, Type 1 Rat Tail High Concentration) in phenol-red free neurobasal medium (Invitrogen) and diluting it 1:1 with phenol-red free matrigel (Corning Life Sciences). Ice-cold collagen-matrigel mixture was added to each well of an eight-well Lab-Tek chambered coverglass, and MGE explants were pipetted into matrix-filled wells. MGE-embedded matrices were allowed to solidify for 45 min at 37°C with 5% CO₂, fed with phenol-red free neurobasal medium supplemented with GlutaMAX (Invitrogen), penicillin-streptomycin (Thermo Scientific), and B-27 (Invitrogen), and cultured for two days. Cultured explants were imaged on a Zeiss LSM 510 confocal microscope at a uniform optical thickness. Area measurements were performed on projected montages in ImageJ. Outgrowth area was determined by subtracting the explant area from the total area occupied by both the explant and migrating cells (Figure 8F), and normalized to explant size. Explants were measured blind to genotype and evaluated statistically by a two-tailed unpaired Student's t-test using Prism 6 (GraphPad) software.

Immunostaining of Slice Cultures

Slices were fixed overnight in 4% paraformaldehyde in 1x phosphate buffered saline (PBS) at 4°C. Slices were rinsed from fixative with 1x PBS, and blocked overnight at 4°C in permeability solution (Polleux and Ghosh, 2002) with 5% normal goat serum. Rabbit anti-GFP (Invitrogen; 1:1500) and mouse anti-nestin (BD Transduction Laboratories; 1:1000) primary antibodies were diluted in block, applied to slices, and incubated overnight at 4°C. Slices were thoroughly rinsed in 1x PBS, and incubated overnight at 4°C with secondary antibody solution containing goat anti-rabbit Alexa488 (1:4000; Invitrogen), goat anti-mouse Alexa546 (1:2000; Invitrogen), and the nucleic acid counterstain Draq5 (1:4000; Axxora) diluted in block. Slices were rinsed in 1x PBS and slide mounted in an aqueous mounting medium containing anti-fade reagent.

Cryosectioning and Staining

Embryonic day 13.5 (E13.5) heads were removed and fixed with 4% paraformaldehyde overnight, rinsed in 1x PBS, and progressed through a sucrose series (10, 20, 30%). Cryo-protected heads were embedded with TFM™ Tissue Freezing Medium (Triangle Biomedical) and flash frozen in liquid-nitrogen-cooled 2-methyl butane. Heads were serially sectioned (12 µm) in the coronal plane and slides stored at -20°C prior to use. Slides were rehydrated with 1x PBS for 20 minutes, blocked (as above) for 2 hrs at room temperature, and incubated in primary antibody solution (block with primary antibodies) overnight at 4°C. Primary antibodies included rabbit anti-calbindin (Swant; 1:2000), mouse anti-nestin (BD; 1:1000), and chicken anti-GFP (Abcam; 1:1500). Slides were rinsed with 1x PBS, incubated with secondary antibody and nucleic acid counterstain solution (as above) for 2 hours at room temperature, rinsed in 1x PBS, and coverslipped with an aqueous mounting medium containing anti-fade reagent.

Imaging and Analysis

Immunofluorescently labeled slice cultures and cryosections were imaged on a Zeiss LSM 510 Meta Confocal Microscope with a 10x Fluar or 20x Plan-Apo objective lens. Confocal micrographs were uniformly adjusted for levels, brightness, and contrast in Adobe Photoshop. For quantification of migration, cortical length was measured from the corticostriatal boundary to the cortical arch of each image, and cortices were segmented into 5 equidistant bins (Figures 2.2 and 2.5). Similarly, for quantification of radial distribution (Figure 2.8), a defined region of the lateral cortical wall was cropped from montaged images and equidistantly segmented into six bins from pial to ventricular surfaces. For both studies, the numbers of cells present in each bin were counted and their percentile distributions across all bins were determined for each tissue section. Bin distributions were averaged across sections of the same treatment group or genotype, and statistical significances were determined by two-way ANOVA followed by Fisher's LSD *post-hoc* analyses (GraphPad Prism 6).

4D live imaging movies were analyzed using Imaris (Bitplane). Movies (12 control and 12 inhibitor) were evaluated in the first 12 hours and last 12 hours of each recording. Ten interneurons were tracked in each time segment for all movies. Interneurons were selected for tracking if they could be followed for at least 4 hours. Tracks were discontinued if a cell remained stationary for 40 contiguous minutes, or if the tracked cell could no longer be unambiguously identified. Average velocity, displacement, and straightness values were obtained for each movie (20 cells). Each treatment group contained 120 cells analyzed from 12 movies (n=12). Two-tailed unpaired Student's t-tests (GraphPad Prism 6) were used to determine statistical differences between groups.

Results

Interneurons express Jnk proteins as they first enter the cortical rudiment

We used transgenic mice expressing EGFP under the control of a Dlx5/6 Cre-IRES-EGFP transgene (hereafter referred to as Dlx5/6-CIE) to follow the migration of newly born interneurons into the developing mouse cortex. Dlx5/6-CIE⁺ interneurons first arrive at the entrance to the cortical rudiment on day E12.5 (Figure 2.1A). By E13.5, Dlx5/6-CIE⁺ interneurons have crossed the cortico-striatal boundary and have migrated approximately half the length of the lateral cortical wall (Figures 2.1B, C). As they enter the neocortex, Dlx5/6-CIE⁺ interneurons form two medially oriented migratory streams flanking the emerging cortical plate; an upper marginal zone (MZ) stream and lower sub-ventricular zone/intermediate zone (SVZ/IZ) stream (Figure 2.1C). Thus, the first cohort of Dlx5/6-CIE⁺ cortical interneurons forms streams of migratory cells that rapidly enter the cortical rudiment over a single developmental day.

JNK signaling regulates cell survival (Kuan et al., 1999), radial migration (Westerlund et al., 2011), and axon tract formation (Hirai et al., 2006; Yamasaki et al., 2011) in the developing forebrain. However, the extent to which JNK signaling influences the migration of cortical interneurons is unknown. We examined the expression pattern of total Jnk protein between E12.5 and E13.5 to determine whether JNK signaling could regulate the initial migration of cortical interneurons into the cerebral cortex. Jnk protein is widely expressed in the developing forebrain (Figures 2.1A, B), but appears to be enriched in the nascent cortical plate and mantle region of the ventral forebrain, where newly generated post-mitotic neurons reside. Moreover, Jnk proteins are expressed by tangentially migrating Dlx5/6-CIE⁺ cortical interneurons as they enter the cerebral cortex (Figures 2.1D-F). Quantitative reverse transcriptase PCR analysis of FACS sorted

Dlx5/6-CIE+ and Dlx5/6-CIE(-) cells from dissociated E14.5 cortices indicated that *Jnk1* mRNA is enriched at nearly twofold (average fold change $\pm$ SEM = 1.99 ± 0.22) in Dlx5/6-CIE+ cortical interneurons, while *Jnk2* is uniformly expressed in both populations (Figure 2.1H). Thus, Dlx5/6-CIE+ cortical interneurons express Jnk proteins as they enter the cortical rudiment and, compared to Dlx5/6-CIE(-) cortical cells, selectively express *Jnk1* at higher levels.

Interneuron entry into the cortical rudiment requires intact JNK signaling

To evaluate the initial entry of interneurons into the cerebral cortex under controlled conditions, we prepared live-vibratome slices from E12.5 Dlx5/6-CIE+ brains and cultured them for one day *in vitro* (Figure 2.2A). Initially, very few cortical interneurons were present in the cortical rudiment (Figure 2.2B). Over the next day *in vitro* (DIV), Dlx5/6-CIE+ cortical interneurons rapidly invaded the lateral aspect of the cerebral cortex (Figures 2.2C-D), recapitulating their cortical entry *in vivo* (Figures 2.1A-C).

To determine whether interneuron entry into the cerebral cortex depends on intact JNK signaling, we cultured E12.5 Dlx5/6-CIE+ slices for one DIV in the presence of different concentrations of SP600125, a selective, reversible, pan inhibitor of JNK signaling (Bennett et al., 2001). At 1 μ M, SP600125 had little effect on interneuron entry into the cortex (Figure 2.2E). As the concentration of SP600125 was increased from 5 μ M to 40 μ M, however, a dose dependent inhibition of interneuron migration into the cerebral cortex was observed (Figures 2.2F-H). To quantify interneuron entry into the cortex, we determined the frequency in which Dlx5/6-CIE+ interneurons were found in five equidistant cortical bins (Figure 2.2I). When compared to control sections, interneurons from SP600125-treated sections showed a dose-dependent accumulation in the most lateral cortical bin, and dose-dependent reductions in medial bin locations (Figure 2.2J).

Thus, pharmacological blockade of JNK signaling prevents the initial entry of Dlx5/6-CIE+ cortical interneurons into the cerebral cortex in a dose-dependent fashion.

Blockade of interneuron entry into the cerebral cortex by JNK inhibition is specific and reversible

To determine whether disruption of cortical interneuron migration was specific to JNK inhibition, we evaluated whether pharmacological blockade of the MAPK signal transduction cascade had a similar effect on the initial entry of cortical interneurons into the cortical rudiment. Unlike JNK inhibition, U0126, a potent and selective inhibitor of Mek1/2 (Favata et al., 1998), failed to prevent the entry of Dlx5/6-CIE+ interneurons into the cerebral cortex (Figure 2.3C). Indeed, no differences were found between the advancement of Dlx5/6-CIE+ interneurons in slices treated with 10 μ M U0126 and control slices (Figure 2.3D), confirming previous assessments of U0126 on cortical interneuron migration (Polleux et al., 2002).

To further validate the specificity of JNK inhibition on cortical interneuron migration, we cultured E12.5 Dlx5/6-CIE+ slices for 24 hours in the presence of AS601245, an independent pan-inhibitor of JNK signaling (Carboni et al., 2008). Similar to SP600125 treatment, application of AS601245 significantly impaired entry of Dlx5/6-CIE+ interneurons into the cortical rudiment (Figures 2.3B, D). Indeed, a 5 μ M concentration of AS601245 impaired interneuron entry to an extent approximating a 20 μ M concentration of SP600125 (Figures 2.2G, 2.3B). Thus, application of two independent inhibitors of JNK signaling prevented interneuronal migration into the nascent cortical rudiment, while pharmacological inhibition of MAPK signaling had no effect, suggesting JNK signaling, but not MAPK signaling, is required for the initial migration of cortical interneurons into the cortical rudiment.

We next asked whether blockade of interneuron migration into the cerebral cortex by SP600125 treatment was reversible. E12.5 Dlx5/6-CIE+ slices were exposed to 20 μ M SP600125 for 12 hours, rinsed, and grown for an additional 12 hours in either control medium (“washout” condition; Figure 2.3H) or medium containing freshly prepared 20 μ M SP600125 (“SP600125” condition; Figure 2.3G). When compared to control slices (Figure 2.3F), slices in both washout and SP600125 conditions displayed impaired migration (Figures 2.3G, H). Dlx5/6-CIE+ interneurons showed robust recovery in washout conditions, however, and migrated much further into the cortical rudiment than Dlx5/6-CIE+ interneurons in slices continuously exposed to SP600125 (Figures 2.3E-H). Thus, SP600125 treatment impairs interneuron entry into the cortex, but this effect is largely reversible upon removal of JNK inhibition.

JNK inhibition impairs directed migration of cortical interneurons into the cortical rudiment

To further examine consequences of JNK inhibition on the migration of cortical interneurons, time-lapse recordings were made from E12.5 Dlx5/6-CIE+ slices exposed to control medium or medium containing 20 μ M SP600125. Live images of the cortical entry zone and lateral aspect of the cortical rudiment were acquired every 10 minutes for 24 hours in control and JNK-inhibited conditions, and migratory properties of Dlx5/6-CIE+ cortical interneurons were examined as they entered and navigated the cerebral cortex.

In control slices, Dlx5/6-CIE+ interneurons robustly entered the cortical rudiment and traveled tangentially to fill the lateral cortical wall (Figures 2.4A-D; Movie 2.1, video clip 1). Many Dlx5/6-CIE+ interneurons traversed the cortical entry zone by 12 hours of recording (Figure 2.4C), and by 24 hours, Dlx5/6-CIE+ interneurons had formed MZ and

SVZ/IZ streams on their medial progression through the cortex (Figure 2.4D), often migrating beyond the field of view.

In stark contrast, migration of Dlx5/6-CIE+ interneurons through the cortical entry zone was severely compromised when slices were treated with 20 μ M SP600125 (Figures 2.4E-H; Movie 2.2, video clip 1). Dlx5/6-CIE+ interneurons remained motile, yet failed to advance in medially oriented trajectories. After 12 hours, far fewer Dlx5/6-CIE+ interneurons had entered the cortex (Figure 2.4G) compared to control conditions (Figure 2.4C). By 24 hours, progression of Dlx5/6-CIE+ interneurons into the cortex had improved (Figure 2.4H), but failed to match the extent of interneuron migration seen in controls (Figure 2.4D), as previously quantified (Figure 2.2J). Dlx5/6-CIE+ cells often stacked up in the MZ stream (Figure 2.4H), and remained dispersed in the SVZ/IZ region without forming an organized migratory stream.

To quantify changes in migratory behavior, individual Dlx5/6-CIE+ interneurons were analyzed in control and SP600125-treated slices. Representative cell tracks of cortical interneurons in control (Figures 2.4I-J; Movie 2.1, video clips 2-3) and JNK-inhibited (Figures 2.4K-L) conditions are shown for movies depicted in Figure 2.4. Navigational errors were apparent during the first 12-hours of imaging in SP600125-treated slices, when Dlx5/6-CIE+ interneurons stall their advancement at the entrance to the cortex, and take short, aberrant paths toward the pial or ventricular surfaces (Figure 2.4K; Movie 2.2, video clip 2). Lengths of individual cell tracks (track length) increased during the last 12-hours of imaging in SP600125-treated slices, but Dlx5/6-CIE+ interneurons typically remained misguided (Figure 2.4L; Movie 2.2, video clip 3), and the entire migratory front of cortical interneurons remained less advanced compared to controls.

For statistical comparisons, 240 migratory interneurons from 12 control and 12 SP600125-treated slices were tracked and their trajectories were analyzed. Twenty Dlx5/6-CIE+ interneurons were tracked from each slice: 10 during the first 12 hours, and 10 during the last 12 hours of imaging. Speed of interneuron advancement significantly declined after SP600125 treatment, with greatest deficits occurring during the first 12-hour period (Figure 2.4M). Maximum, mean, minimum, and standard deviation velocities of Dlx5/6-CIE+ interneurons diminished significantly in SP600125-treated slices (values = avg $\pm$ SEM; Control: max = 139.6 ± 4.4 , mean = 59.6 ± 2.0 , min = 7.6 ± 0.5 , SD = 35.1 ± 1.0 $\mu\text{m/hr}$; SP600125: max = 90.3 ± 3.3 , mean = 38.5 ± 1.8 , min = 4.9 ± 0.4 , SD = 21.8 ± 0.9 $\mu\text{m/hr}$). Accordingly, average track lengths from the beginning to ending point of their trajectories (displacement) as well as average cumulative distances traveled (total) were significantly impaired during the first 12-hour period (Control: displacement = 204.6 ± 8.1 , total = 297.2 ± 12 μm ; SP600125: displacement = 98.9 ± 8.3 , total = 208.4 ± 10.7 μm) (Figure 2.4N). Migratory velocities of interneurons in SP600125-treated slices increased during the last 12-hours of imaging (Figure 2.4P), but remained significantly lower than controls (Control: max = 129.6 ± 2.9 , mean = 54.3 ± 1.7 , min = 7.0 ± 0.6 , SD = 32.8 ± 0.8 $\mu\text{m/hr}$; SP600125: max = 107.8 ± 2.7 , mean = 44.5 ± 1.6 , min = 4.9 ± 0.5 , SD = 26.8 ± 0.8 $\mu\text{m/hr}$). As velocity increased during the last 12 hours of imaging, so did distances traveled by individual cortical interneurons. Although both displacement and total track lengths increased, displacement length remained reduced to statistically significant levels in the last 12-hours of imaging (Control: displacement = 197.6 ± 6.4 , total = 270.5 ± 8.4 μm ; SP600125: displacement = 170.6 ± 11.3 , total = 261.7 ± 12.6 μm) (Figure 2.4Q). Directionality of migration as reflected in overall track straightness values, however, was significantly altered in interneurons from SP600125-treated slices during both first (Control: $r = 0.70 \pm 0.01$; SP600125: $r = 0.47 \pm 0.02$) and last 12-hour intervals (Control: $r = 0.74 \pm 0.02$, SP600125: $r = 0.64 \pm 0.02$) (Figures 2.4O, R).

Improvements observed in migratory speed and track length during the last 12 hours of imaging in SP600125-treated slices likely reflect declining efficacy of the pharmacological inhibitor over the 24-hour imaging period. When interneurons were allowed to grow into the cerebral cortex for 12 hours in control conditions prior to treatment with SP600125, deficits in interneuron migration were comparable to those observed in acutely treated slices. For example, mean interneuron velocity was reduced to $37.2 \pm 1.2 \mu\text{m/hr}$, which was slightly less than the mean velocity observed during the first 12 hours of SP600125 treatment. Furthermore, to evaluate whether the JNK requirement persisted beyond cortical entry at E12.5, we analyzed migratory properties of control and JNK-inhibited cortical interneurons at the leading front of migration in E14.5 cortices. Similar to SP600125 treatment at E12.5, pharmacological inhibition of JNK at E14.5 led to statistically significant reductions in total track length (Control: 267.3 ± 17.4 ; SP600125: $188.1 \pm 8.5 \mu\text{m}$; $p = 0.01^*$), mean velocity (Control: 51.7 ± 0.8 ; SP600125: $32.1 \pm 1.6 \mu\text{m/hr}$; $p = 0.0004^{***}$), and directionality (Control: 0.75 ± 0.05 ; SP600125: 0.56 ± 0.05 ; $p = 0.04^*$). Thus, inhibition of JNK signaling significantly slows the advancement and alters the trajectories of migratory cortical interneurons in the cortical entry zone at E12.5 and at the leading front of migration in E14.5 cortices.

Jnk1 regulates cortical interneuron migration at the cortical entry zone in vivo

In order to determine whether loss of *Jnk* genes compromised migration of cortical interneurons *in vivo*, we evaluated the distribution of calbindin-expressing cortical interneurons in an allelic series of *Jnk1* and *Jnk2* single and combinatorial mutant embryos at E13.5 (Figures 2.5 and 2.6), after the initial cohort of interneurons have entered the cerebral cortex. At this age, calbindin expression labels most tangentially migrating interneurons in the cortical rudiment (Anderson et al., 1997). Five embryos of each genotype were coronally sectioned, and the lateral cortical wall was bilaterally

sampled at four rostro-caudal locations spanning sections containing the rostral MGE to mid caudal ganglionic eminence (CGE; Figure 2.5A). The cortical rudiment was subdivided into 5 equidistant bins spanning the cortico-striatal boundary laterally (Bin1) to cortical arch medially (Bin5; Figure 2.5A), and the percentage of calbindin-positive cortical interneurons found in each cortical bin was determined for each section and averaged across all embryos of the same genotype. Since placement of equidistant bins was done with respect to the length of each cortical hemisphere, our sampling strategy accounts for variation in cortical length that might occur between sections.

In *Jnk1^{+/-}* embryos, calbindin-positive interneurons displayed normal tangential migration into the cortical rudiment at both rostral and caudal probe locations (Figure 2.5B, F). However, calbindin-positive interneurons in both MZ and SVZ/IZ streams of *Jnk1^{-/-}* embryos (Figures 2.5C, G) were less advanced than calbindin-positive interneurons in *Jnk1^{+/-}* embryos (Figures 2.5B, F). Accordingly, the proportion of calbindin-positive interneurons increased laterally (Bin1) and declined medially (Bin3-5; Figures 2.5E, I). In this and subsequently presented data, statistically significant increases in interneuron abundance are seen in Bin1 and statistically significant decreases in interneuron abundance are seen in Bin3 when migratory deficits are present. Bin2 likely remains unchanged due to transitional normalization occurring between Bins 1 and 3, and while reductions of interneuron abundance occur in Bins 4-5, they often do not reach statistical significance due to the relatively small percentages of cells found in those bins at E13.5. Although reductions of interneuron migration were apparent at both rostral and caudal probe locations in *Jnk1^{-/-}* embryos, reductions in medial progression only reached statistical significance at caudal probe locations (Figure 2.5E, 2.5I).

To determine whether reduction of *Jnk2* in combination with loss of *Jnk1* exacerbated migratory phenotypes of cortical interneurons, we analyzed the distribution of calbindin-

positive cortical interneurons in embryos lacking *Jnk1* and a single genomic copy of *Jnk2* (*Jnk1^{-/-};Jnk2^{+/-}*), since *Jnk1/2* double nulls die prior to interneuron migration into the cerebral cortex (Kuan et al., 1999). In *Jnk1^{-/-};Jnk2^{+/-}* embryos, advancement of calbindin-positive interneurons in both MZ and SVZ/IZ streams was significantly diminished relative to *Jnk1^{+/-}* controls at both rostral and caudal probe locations (Figures 2.5D, H). Statistically significant interactions were found between genotype and bin position at rostral and caudal probe locations (Figures 2.5E, I), suggesting loss of *Jnk1* alone and loss of *Jnk1* combined with reduction of *Jnk2* inhibits tangential progression of cortical interneurons *in vivo*. At caudal locations, calbindin-positive interneurons in *Jnk1^{-/-};Jnk2^{+/-}* and *Jnk1^{-/-}* embryos were comparably regressed (Figures 2.5G-I), but at rostral locations, calbindin-positive interneurons in *Jnk1^{-/-};Jnk2^{+/-}* embryos were significantly less advanced than those in *Jnk1^{-/-}* embryos (Figures 2.5C-E). To ensure that potential differences in brain size did not influence interpretation of our results, we measured absolute cortical lengths from every section we analyzed and found no statistically significant differences in cortical length between genotypes by one-way ANOVA (*Jnk1^{+/-}* = 937.8 ± 17.4 , *Jnk1^{-/-}* = 1005.0 ± 21.0 , *Jnk1^{-/-};Jnk2^{+/-}* = 935.4 ± 27.6 μm). Thus, at rostral positions, reducing *Jnk2* in combination with the loss of *Jnk1* has a greater impact on cortical interneuron migration than loss of *Jnk1* alone.

To determine whether *Jnk2* plays a comparable role to *Jnk1* in the initial migration of cortical interneurons *in vivo*, we generated embryos with the reciprocal *Jnk2^{+/-}*, *Jnk2^{-/-}*, and *Jnk2^{-/-};Jnk1^{+/-}* genotypes, and evaluated tangential progression of calbindin-positive cortical interneurons at E13.5. Normal migration of cortical interneurons was observed in *Jnk2^{+/-}* embryos at both rostral (Figures 2.6A, D) and caudal (Figures 2.6E, H) probe locations, and their cortical bin distribution closely matched *Jnk1^{+/-}* embryos (Figures 2.5E, I). Unlike in *Jnk1^{-/-}* embryos, however, tangential progression of calbindin-positive

interneurons was completely unperturbed in *Jnk2*^{-/-} embryos at rostral (Figures 2.6B, D) and caudal probe locations (Figures 2.6F, H). Moreover, removing a genomic copy of *Jnk1* in the context of *Jnk2* deletion did not alter the advancement of calbindin-positive cortical interneurons (Figures 2.6C, D, G, H). Neither MZ nor SVZ streams were regressed in *Jnk2*^{-/-} or *Jnk2*^{-/-};*Jnk1*^{+/-} embryos relative to migratory streams in *Jnk2*^{+/-} controls (Figures 2.6A-G), and the distribution of calbindin-positive cortical interneurons in cortical bins were nearly identical across all three genotypes at rostral and caudal locations (Figures 2.6D, H). As before, cortical rudiment lengths were consistent between genotypes (*Jnk2*^{+/-} = 1032.6 ± 22.0, *Jnk2*^{-/-} = 1033.2 ± 27.6, *Jnk2*^{-/-};*Jnk1*^{+/-} = 1014.5 ± 9.3 μm). Apparently, retaining a single genomic copy of *Jnk1* is sufficient to leave cortical interneuron migration intact and unperturbed in the *Jnk2*^{-/-} background at E13.5.

Cortical interneurons have a cell-intrinsic requirement for Jnk1 in vivo

Genetic ablation of *Jnk1* disrupts the initial migration of interneurons into the cortical rudiment, but since *Jnk1* is expressed in both migratory cortical interneurons and non-interneuronal cells of the developing cortex (Figure 2.1), the requirement for JNK signaling in regulating cortical interneuron migration may not be cell autonomous. Indeed, radial migration of cortical projection neurons is accelerated in *Jnk1*^{-/-} embryos (Westerlund et al., 2011), which could lead to non-autonomous disruptions in the tangential migration of cortical interneurons. To determine whether the initial cohort of cortical interneurons has a cell-intrinsic requirement for JNK signaling to migrate into the cortical rudiment, we used mice expressing the Dlx5/6-CIE transgene to conditionally ablate *Jnk1* within cortical interneurons *in vivo*. Since migration of cortical interneurons was completely unperturbed after constitutive loss of *Jnk2*^{-/-} (Figure 2.6), but reduction of *Jnk2* in *Jnk1* nulls exacerbated interneuron migratory phenotypes (Figure 2.5), we

conditionally ablated *Jnk1* in interneurons of *Jnk2*^{-/-} embryos. This allowed evaluation of interneurons that were completely deficient in both *Jnk1* and *Jnk2*, which would otherwise be impossible due to the mid-gestation embryonic lethality of constitutive *Jnk1/2* double mutation.

We predicted *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* embryos would not have deficiencies in interneuron migration, since interneuron migration was unperturbed in *Jnk2^{-/-};Jnk1^{+/+}* embryos. Indeed, normal tangential migration of cortical interneurons was observed at E13.5 in *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* embryos (Figures 2.7A, E). We analyzed *Dlx5/6-CIE+* cortical interneurons, as well as the calbindin-expressing cohort of cortical interneurons, which constitute most of the *Dlx5/6-CIE+* cells in the cortical rudiment at E13.5. Both populations of labeled cells appeared normally distributed within the cortex of *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* embryos at rostral (Figures 2.7C, D) and caudal probe locations (Figures 2.7G, H). In *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* embryos, however, both *Dlx5/6-CIE+* and calbindin-positive cortical interneurons were significantly regressed in their cortical advancement at rostral (Figures 2.7B-D) and caudal (Figures 2.7F-H) probe locations compared to interneurons in control brains. The distribution of interneurons within *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* cortices were significantly altered from control *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* cortices at rostral (Figures 2.7C, D) and caudal probe locations (Figures 2.7G, H). Similar to *Jnk1^{-/-}* and *Jnk1^{-/-};Jnk2^{+/-}* embryos, cortical interneurons accumulated laterally and diminished medially within *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* cortices, with the largest statistical differences between genotypes occurring in Bin1 and Bin3 (Figures 2.7C, D, G, H). Distributions of cortical interneurons within *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* embryos were comparable to *Jnk1^{-/-};Jnk2^{+/-}* embryos since both rostral and caudal portions of their trajectories were significantly regressed relative to their respective controls (Figures 2.5E, I). This finding is consistent with *Jnk2* loss of function

enhancing a *Jnk1*^{-/-} migratory phenotype at rostral probe locations. Cortical lengths were not significantly different between control and conditional *Jnk1/2* double knockout embryos (*Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* = 949.1 ± 50.1, *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* = 922.7 ± 56.0 μm). Collectively, these data suggest the initial cohort of cortical interneurons has a cell-intrinsic requirement for JNK signaling—largely mediated through the activity of *Jnk1*, but partially compensated for by *Jnk2* when *Jnk1* is lost—in migrating through the cortical entry zone *in vivo*.

Although our results indicate cortical interneurons have a cell-intrinsic requirement for JNK signaling to appropriately enter the cerebral cortex, there are two possible explanations for this requirement: 1) JNK signaling could intrinsically enhance the migratory capacity of cortical interneurons, or 2) JNK signaling could be required by cortical interneurons to sense or respond to environmental cues located in the cortical rudiment. To distinguish between these possibilities, we cultured explants of MGE tissue from E12.5 control or conditional double knockout embryos in a reduced, serum-free *in vitro* environment devoid of cortical cues (Figure 2.8A). MGE explants isolated from either *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* (Figures 2.8B, C) or *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* (Figures 2.8D, E) forebrain slices produced *Dlx5/6-CIE+* migratory interneurons that dispersed in a radial pattern from the explant margins. When migratory outgrowth was measured blind to genotype (Figure 2.8F), no statistical differences in interneuron migration were observed between *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* and *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* explants of MGE tissue (Figure 2.8G). These data strongly suggest that JNK signaling does not act in cortical interneurons to autonomously promote or enhance their motility.

Integrity of cortical migratory streams depends on JNK signaling in vivo

Since we observed statistically significant deficits in the entry of cortical interneurons into the cortical rudiment after conditionally ablating *Jnk1* from interneurons of *Jnk2*-null mutants *in vivo* (Figure 2.7), but failed to see migratory anomalies in the absence of cortical cues *in vitro* (Figure 2.8), we hypothesized that cortical interneurons have a cell intrinsic requirement for JNK signaling to navigate the early cortical rudiment *in vivo*. To determine whether JNK-deficient cortical interneurons are misrouted when first entering the cerebral cortex, we quantified the radial distribution of cortical interneurons within the lateral cortical wall of *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* control and *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* mutant embryos at E13.5 (Figures 2.9A-C). Six equidistant bins were made along the radial axis of the lateral cortex (adjacent to the corticostriatal boundary), and the percentage of *Dlx5/6-CIE+* cortical interneurons appearing in Bins 1 (MZ) through 6 (Ventricular Zone; VZ) were determined for each genotype. In *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* embryos, *Dlx5/6-CIE+* interneurons were predominantly distributed in the MZ (Bin 1) and SVZ/IZ (Bins 3-4) streams (Figures 2.9A, C). In *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* embryos, however, the proportions of *Dlx5/6-CIE+* interneurons located in the MZ stream (Bin 1) and SVZ portion of the SVZ/IZ stream (Bin 4) were diminished compared to control cortices (Figures 2.9B, C). In addition, the percentage of *Dlx5/6-CIE+* interneurons in the IZ (Bins 2-3) was elevated in conditional double mutants compared to controls (Figures 2.9B, C). When compared statistically, distributions of *Dlx5/6-CIE+* migratory interneurons within Bins 1-6 were significantly altered between *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* and *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* genotypes with the largest shifts occurring in Bins 3 and 4 (Figure 2.9C), suggesting JNK-deficient interneurons were dispersing from the SVZ.

Moreover, repositioning of migratory interneurons within the radial axis of the *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* mutant cortex was accompanied by diminished integrity to both MZ

and SVZ/IZ migratory streams. The MZ and SVZ/IZ streams of *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* control embryos (Figures 2.9D, E) were consistently more cohesive than MZ and SVZ/IZ streams of *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* mutant embryos (Figures 2.9 H, I). *Dlx5/6-CIE+* and calbindin-positive cortical interneurons were more loosely organized in *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* cortices, leading to frequent gaps within MZ and SVZ/IZ streams (Figures 2.9H, I). Leading processes of cortical interneurons within the SVZ/IZ stream of *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* cortices were typically straight and medially oriented (Figure 2.9E), while disordered and non-tangentially oriented in the SVZ/IZ stream of *Dlx5/6-CIE; Jnk1^{fl/fl};Jnk2^{-/-}* mutant cortices (Figure 2.9I). In addition, interneuronal processes were often non-contiguous with their cell bodies (Figure 2.9I), suggesting migratory orientations of *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* interneurons were frequently orthogonal to the coronal plane of section. Finally, processes of cortical interneurons in *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* embryos were often more highly branched (Figures 2.9J-K) than those of control interneurons (Figures 2.9F-G). Thus, conditional ablation of *Jnk1* from interneurons of *Jnk2^{-/-}* embryos disrupts the radial distribution of cortical interneurons at the entrance to the cortical rudiment, diminishes the integrity of MZ and SVZ/IZ migratory streams, and leads to morphological alterations in migratory cortical interneurons. Collectively, these data suggest cortical interneurons have an intrinsic requirement for JNK signaling in navigating the cortical entry zone *in vivo*.

Discussion

In the current study, we identified novel and essential roles for JNK signaling in cortical interneuron migration. We established that cortical interneurons express Jnks as they enter the cerebral cortex and, compared to non-interneuronal cortical cells, have enriched expression of *Jnk1*. Using *ex vivo* slice cultures, we demonstrated pharmacological blockade of JNK results in dose-dependent disruption of interneuron migration into the cerebral cortex, and that JNK-inhibited cortical interneurons slow their advancement and take aberrant trajectories as they navigate the cortical entry zone at E12.5. JNK-inhibition at E14.5 similarly perturbed migratory properties of cortical interneurons, indicating their requirement for JNK persists at later embryonic stages. *In vivo* analyses of single and multi-allelic mutations of two *Jnk* genes revealed that loss of *Jnk1* impairs cortical interneuron migration, while loss of *Jnk2* does not, unless *Jnk2* is reduced in *Jnk1* nulls. Finally, we showed conditional deletion of *Jnk1* in interneurons of *Jnk2* nulls recapitulates migratory deficits observed in constitutive *Jnk1^{-/-};Jnk2^{+/-}* embryos, and in addition, disrupts migratory streams and cortical interneuron morphology. Despite this clear intrinsic requirement for JNK activity *in vivo*, JNK-deficient interneurons migrate normally *in vitro*, suggesting JNK regulates cortical interneuron migration in response to cortical guidance cues. Together, our results suggest that cortical interneurons have an intrinsic requirement for *Jnk1* to enter and navigate the developing cerebral cortex, making the JNK signaling pathway a key intracellular mediator of cortical interneuron migration *in vivo*.

JNKs and neuronal migration

JNK signaling orchestrates diverse cellular processes in the developing nervous system including cell survival (Kuan et al., 1999; Sabapathy et al., 1999), axon elongation and

stability (Hirai et al., 2006; Wang et al., 2007; Yamasaki et al., 2011), axon guidance (Qu et al., 2013), and radial migration (Hirai et al., 2006; Wang et al., 2007; Westerlund et al., 2011; Yamasaki et al., 2011). The extent to which individual *Jnk* genes have distinct functions remains to be elucidated (Haeusgen et al., 2009; Yamasaki et al., 2012). Here, we report *Jnk1* plays a greater role than *Jnk2* in cortical interneuron migration. This may either reflect functional differences between *Jnk1* and *Jnk2*, or unequal expression of *Jnk1* and *Jnk2* in migratory cortical interneurons. The contribution of *Jnk3* to cortical interneuron migration is currently unknown.

Radial migration in the developing cortex relies on JNK signaling, yet it is unclear whether *Jnk1*, *Jnk2*, and *Jnk3* play equivalent roles. Deletion of upstream JNK activators including *Map3k12* (Hirai et al., 2006), *Map2k4* (Wang et al., 2007), or *Map2k7* (Yamasaki et al., 2011), inhibits radial migration. Surprisingly, deletion of *Jnk1* has the opposite effect: radial migration into the cortical plate is accelerated in *Jnk1* nulls (Westerlund et al., 2011). These conflicting results could be explained if *Jnk2* and/or *Jnk3* play opposite roles to *Jnk1* in radial migration, since deletion of upstream JNK activators would diminish activity of all available Jnk isoforms, whereas single deletion of *Jnk1* would not. We find pharmacological inhibition of all JNK activity, as well as genetic deletion of *Jnk1* or *Jnk1* and *Jnk2* together, inhibits tangential progression of cortical interneurons. These results indicate that at least *Jnk1* and *Jnk2* act in parallel to promote cortical interneuron migration *in vivo*. Thus, both radial and tangential migration requires *Jnk1*-mediated signaling, but *Jnk1* function exerts opposite effects on radially versus tangentially migrating neuroblasts. Differential expression of downstream JNK targets may underlie the molecular bases of these distinctions. For example, the microtubule regulatory protein SCG10/stathmin-2 is a major downstream effector of *Jnk1* in radially

migrating neuroblasts (Westerlund et al., 2011); cortical interneurons may rely on other JNK substrates, however.

JNK signaling at the “cortical entry zone”

Our data suggest the first cohort of cortical interneurons require JNK activity to enter and navigate the lateral cortical rudiment. We call this segment of their migratory pathway the “cortical entry zone,” which bridges the subcortical telencephalon and neocortex. Extracellular guidance cues located at the cortical entry zone likely direct and concentrate cortical interneurons into newly emerging migratory streams. Indeed, *Cxcl12* is highly expressed within the SVZ/IZ at the cortical entry zone (Stumm et al., 2003, 2007; Tiveron et al., 2006; López-Bendito et al., 2008), and is required for interneuron migration into the cortex via the SVZ/IZ stream (Tiveron et al., 2006; López-Bendito et al., 2008). Thus, cortical interneurons respond to extracellular guidance cues located at the cortical entry zone to correctly navigate the nascent cerebral cortex, and our data suggest JNK signaling critically regulates the spatial and temporal precision for which this occurs.

We provide substantial genetic evidence indicating that JNK signaling regulates interneuron migration at the cortical entry zone *in vivo* (Figure 2.10). Constitutive ablation of *Jnk1* diminishes cortical interneuron migration at E13.5, with significant reductions in migration occurring at caudal cortical locations. Loss of *Jnk2* alone or loss of *Jnk2* with heterozygous reduction of *Jnk1* has no discernable effect, however, suggesting a single genomic copy of *Jnk1* is sufficient to maintain normal migratory properties of cortical interneurons—even in the absence of *Jnk2*. In contrast, heterozygous reduction of *Jnk2* in *Jnk1* nulls impairs cortical interneuron migration more than loss of *Jnk1* alone, since tangential migration into both rostral and caudal portions

of their cortical trajectory are compromised. Thus, compared to *Jnk1*, *Jnk2* plays a relatively minor role in cortical interneuron migration, and its influence is only apparent rostrally when *Jnk1* function is lost. Conditional deletion of *Jnk1* in interneurons of *Jnk2* nulls, which completely eliminates *Jnk1* and *Jnk2* function in cortical interneurons, significantly impairs cortical interneuron migration throughout the rostral-caudal axis of the developing cortex. Not only is cortical entry compromised when *Jnk1* and *Jnk2* function is eliminated from cortical interneurons, but MZ and SVZ/IZ streams are less cohesive, radial positioning of migratory interneurons is altered, and migratory cortical interneurons are more highly branched. In a reduced *in vitro* environment, however, JNK-deficient interneurons migrate normally, suggesting cortical interneurons require JNK to sense or respond to cortical guidance cues rather than promote motility. Taken together, our *in vivo* genetic, *ex vivo* pharmacologic, and *in vitro* migration data suggest cortical interneurons have a cell intrinsic requirement for JNK signaling to guide their migration into and within the developing cerebral cortex.

Two cell biological processes control tangential migration of cortical interneurons: 1) dynamic remodeling of the leading process through branching and elongation, and 2) translocation of the nucleus into the leading process, or nucleokinesis. Both events require precise molecular control of actin and microtubule cytoskeleton (Bellion et al., 2005; Godin et al., 2012), and must be coordinated in a stepwise fashion to orient migration towards chemoattractant guidance cues (Martini et al., 2009). Although cellular and molecular mechanisms underlying the requirement for JNK in migrating cortical interneurons are currently unknown, we hypothesize JNK signaling acts as a molecular bridge between cortically encountered guidance cues and cytoskeletal machinery driving migration. One potential mediator is doublecortin, a microtubule-associated protein regulated by JNK phosphorylation (Gdalyahu et al., 2004; Jin et al., 2010), and required

for proper branching, nucleokinesis, and directed migration of cortical interneurons (Kappeler et al., 2006; Friocourt et al., 2007). The degree to which extracellular guidance cues activate JNK signaling to modulate doublecortin, or other regulators of cytoskeletal dynamics in migrating cortical interneurons, such as Lis1 (Gopal et al., 2010), p27^{Kip1} (Martini et al., 2009), or Rac-GTPases (Tahirovic et al., 2010; Tivodar et al., 2015), remains to be determined.

Relevance to neuropsychiatric disorders

Cortical interneurons are cellular targets for the pathogenesis of several developmental disorders of cortical connectivity, including schizophrenia and autism (Marín, 2012). Recent work in a mouse model of 22q11.2 deletion syndrome (22q11.2DS), which confers significant genetic risk for schizophrenia and autism in humans (Murphy et al., 1999; Fine et al., 2005; Métin et al., 2006; Niklasson et al., 2009), has revealed that embryonic migratory deficits in cortical interneurons lead to laminar repositioning of parvalbumin-positive interneurons in adult cortices (Meechan et al., 2009, 2012). Similar to JNK-deficient interneurons, cortical interneurons in 22q11.2DS brains display delayed cortical entry at E13.5 and irregularities in their migratory streams (Meechan et al., 2009, 2012). Interneuron migratory deficiencies in 22q11.2DS mice are cell autonomous and largely result from reductions in the chemokine receptor Cxcr4 (Meechan et al., 2012). Independent analysis of a similar mouse model confirmed that functional defects in Cxcl12/Cxcr4 signaling underlie interneuron deficiencies in 22q11.2DS mice (Toritsuka et al., 2013), and implicated *Dgcr8* as the mediator. It is currently unknown whether genes within the 22q11.2 locus, including *Dgcr8*, interact with the JNK pathway, but similarities in interneuron migratory deficits are highly suggestive of a connection. One plausible explanation is that Cxcl12-Cxcr4 signaling activates JNK to guide interneuron

migration and promote migratory stream integrity at the cortical entry zone, but this hypothesis remains to be tested.

Uncovering cellular and molecular mechanisms underlying the pathogenesis of cortical connectivity disorders is essential for developing effective strategies for their treatment. Our current work identifies the JNK signaling pathway, and *Jnk1* in particular, as a major regulator of interneuron migration *in vivo*—making the JNK pathway a novel candidate to consider in the etiology of cortical circuit disorders and a target for potential therapeutic interventions.

Figures

Figure 2.1: Early arriving cortical interneurons express Jnk proteins. **A-F.** Localization of total Jnk (red) and EGFP (green) expression in Dlx5/6-CIE+ embryonic brain sections. Jnk proteins are widely expressed in the developing forebrain at E12.5 and E13.5, and are particularly enriched in post-mitotic zones. **A.** At E12.5, few Dlx5/6-CIE+ cortical interneurons have entered the cortex (CTX) from subcortical locations. **B, C.** By E13.5, many Dlx5/6-CIE+ cortical interneurons have invaded the cortical rudiment and formed streams of migratory cells in the marginal zone (MZ) and sub-ventricular zone/intermediate zone (SVZ/IZ) of the nascent cortex. **D-F.** Dlx5/6-CIE+ interneuronal cell bodies (asterisks) and processes (arrowheads) co-localize with total Jnk protein in the E13.5 cortex. **G.** Separation of Dlx5/6-CIE(+) and Dlx5/6-CIE(-) cells from a dissociated E14.5 cortex by FACS sorting. Three cortices were independently processed for gene expression analyses by qRT-PCR. **H.** qRT-PCR indicates *Jnk1* transcript is enriched twofold in Dlx5/6-CIE(+) cells compared to Dlx5/6-CIE(-) cells (Student's t-test; $p = 0.02$), whereas *Jnk2* transcript is uniformly expressed in the two populations. CP, Cortical pool.

Figure 2.2: Cortical interneurons require JNK activity to enter the developing cerebral cortex.

A. Schematic representation of slice culture assay. E12.5 Dlx5/6-CIE+ brains are isolated, sectioned, and cultured 24 hours in control or SP600125 (SP) conditions. **B-D.** In control cultures, cortical interneurons robustly migrate into the cortical rudiment during the 24-hour culture period. Migratory front is marked by a flanking arrowhead (MZ region) and arrow (SVZ region) in each image. **E-H.** Entry of cortical interneurons into the cerebral cortex is disrupted by SP600125, a pan JNK inhibitor, in a dose-dependent fashion. **I.** For quantification of interneuron migration in slice cultures, cortices were segmented into 5 equidistant bins and the percentages of cortical interneurons appearing in all cortical bins were determined for each section, and averaged across all sections per treatment group. **J.** Dose-dependent accumulation of cortical interneurons in lateral and decline of interneurons in medial cortical bins following SP600125 treatment. Two-way ANOVA reveals statistically significant interactions between treatment and bin location ($F_{(16, 275)} = 57.5$; $p < 0.0001$). Differences within bins were determined post-hoc by Fisher's LSD tests (**** $p < 0.0001$; *** $p \leq 0.0006$; ** $p < 0.008$).

Figure 2.3: Disruption of cortical interneuron migration by pharmacological inhibition of JNK signaling is specific and reversible. **A-D.** An independent, pan-JNK inhibitor, AS601245 (**B**), diminishes interneuron entry into the cortex compared to control (**A**) cultures, while blockade of Mapk signaling with the Mek1/2 inhibitor U0126 (**C**) does not. **D.** Quantification reveals significant reductions in migration for slices treated with 5 μ M AS601245, but no change in slices treated with 10 μ M U0126 (two-way ANOVA: $F_{(8, 165)} = 15.98$; $p < 0.0001$; Fisher's LSD: **** $p < 0.0001$; *** $p = 0.0002$; ** $p = 0.009$; * $p = 0.049$). **E-H.** Cortical interneuron migration recovers after removal of 20 μ M SP600125. **E.** Quantification reveals that interneuron migration improves when 20 μ M SP600125 is washed out and replaced by control medium half way through the 24-hour culture period (two-way ANOVA: $F_{(8, 165)} = 37.56$; $p < 0.0001$; Fisher's LSD: **** $p < 0.0001$). **F.** Slice cultured in control medium, rinsed at 12 hours, and cultured for an additional 12 hours in control medium. **G.** Slice cultured in 20 μ M SP600125, rinsed at 12 hours, and cultured for an additional 12 hours in fresh 20 μ M SP600125. **H.** Slice cultured in 20 μ M SP600125, rinsed at 12 hours, and cultured for an additional 12 hours in control medium.

Figure 2.4: Migratory properties of cortical interneurons are perturbed following SP600125 treatment at E12.5. **A-D.** In control slices, *Dlx5/6-CIE+* interneurons robustly migrate through the cortical entry zone over a 24-hour imaging period. Cells travel in MZ region (arrowhead in **D**)

and SVZ region (dashed lines in **D**) of the section. **E-H**. In slices treated with 20 μ M SP600125, Dlx5/6-CIE+ cells remain motile, but advance slowly and frequently take aberrant trajectories. Cells pile up in MZ (arrowhead in **H**), failing to advance further. **I, J**. Tracks from ten individual cells in the first 12 (**I**) and last 12 (**J**) hours of a control movie. Individual cell tracks are long, straight and medially directed. **K, L**. Tracks from 10 individual cells in the first 12 (**K**) and last 12 (**L**) hours of an inhibitor movie. Cell tracks are short, crooked, and less medially oriented in SP600125-treated slices, particularly in the first 12 hours of imaging. Tracks are pseudo-colored by time (bars in **I-L**). **M-O**. Comparison of interneuron migration in SP600125-treated and control slices during the first 12 hours of recordings. SP600125 treatment leads to statistically significant reductions in migratory velocity (**M**), track length (**N**), and track straightness (**O**) corresponding to directionality. **P-R**. Comparison of interneuron migration in SP600125-treated and control slices in the last 12 hours of recordings. Interneuron migratory velocity (**P**) increases in SP600125-treated slices during the second 12 hours of recording due to waning inhibitor efficacy, but remains significantly reduced from controls. Track length (**Q**) of JNK-inhibited interneurons is also increased during the last 12 hours, but displacement values remain significantly altered. Directionality (**R**) of interneurons from SP600125-treated slices remains significantly altered in the last 12 hours. Significance levels after performing two-tailed unpaired Student's t-tests are denoted as: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Figure 2.5: Genetic ablation of *Jnk1* impairs migration of cortical interneurons *in vivo*. **A.**

Sampling strategy for mutant embryo analyses (Figures 2.5-2.7). Five E13.5 brains were serially sectioned and bilaterally sampled at four rostral-caudal locations per genotype (Cx, cortex; L, LGE; M, MGE; C, CGE; S, septum; T, thalamus). Cortices were divided into 5 equidistant bins, and the percentages of calbindin-positive cells appearing in cortical bins were determined. Data from rostral (slices 1 and 2) and caudal (slices 3 and 4) positions were analyzed separately. **B-E.** Distribution of calbindin-positive cells in *Jnk1*^{+/+} (**B**), *Jnk1*^{-/-} (**C**), and *Jnk1*^{-/-};*Jnk2*^{+/+} (**D**) cortices

at rostral positions. Furthest extent of interneuron progression is marked by closed (MZ) and open (SVZ/IZ) arrowheads in each image. **E.** Statistically significant interactions were found between genotype and bin location (two-way ANOVA: $F_{(8, 60)} = 2.742$; $p = 0.0120$). Compared to *Jnk1*^{+/-} controls, migration of calbindin-positive cortical interneurons was significantly impaired in *Jnk1*^{-/-};*Jnk2*^{+/-} embryos (Fisher's LSD: *** $p = 0.0005$; * $p = 0.019$ in Bin 1, 0.042 in Bin 3). **F-I.** Distribution of calbindin-positive cells in the caudal cortex of *Jnk1*^{+/-} (**F**), *Jnk1*^{-/-} (**G**), and *Jnk1*^{-/-};*Jnk2*^{+/-} embryonic brains (**H**). **I.** Statistically significant interactions were observed between genotype and bin location (two-way ANOVA: $F_{(8, 60)} = 2.463$; $p = 0.0223$). Migration of calbindin-positive cortical interneurons was significantly impaired when *Jnk1*^{-/-} (Fisher's LSD: ** $p = 0.005$; * $p < 0.049$) and *Jnk1*^{-/-};*Jnk2*^{+/-} cortices (Fisher's LSD: ** $p = 0.003$; * $p = 0.017$) were compared to *Jnk1*^{+/-} controls.

Figure 2.6: Unlike *Jnk1*, genetic ablation of *Jnk2* does not disrupt migration of cortical interneurons *in vivo*. At both rostral (A-D) and caudal (E-H) levels, calbindin-positive cortical interneurons are equally distributed in *Jnk2*^{+/-} (A, E), *Jnk2*^{-/-} (B, F), and *Jnk2*^{-/-}; *Jnk1*^{+/-} (C, G) embryos. No statistically significant interactions were found between genotype and bin location at either rostral (two-way ANOVA: $F_{(8, 60)} = 0.131$; $p = 0.998$; D) or caudal positions (two-way ANOVA $F_{(8, 60)} = 0.135$; $p = 0.997$; H).

Figure 2.7: Cortical interneurons have a cell-intrinsic requirement for JNK signaling to migrate through the cortical entry zone *in vivo*. *Jnk1* was conditionally ablated in *Dlx5/6-CIE*+ cells of *Jnk2*^{-/-} embryos. **A-D.** Distribution of *Dlx5/6-CIE*+ (green) and calbindin-positive (red/yellow) cortical interneurons in the rostral cortex of *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* (**A**) and *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* embryonic brains (**B**). **C, D.** Statistically significant interactions were found between genotype and bin location for *Dlx5/6-CIE*+ (two-way ANOVA $F_{(4, 40)} = 11.55$; $p < 0.0001$; **C**) and calbindin-positive cortical interneurons (two-way ANOVA $F_{(4, 40)} = 12.42$; $p < 0.0001$; **D**). Migratory advancement of interneurons was significantly impaired in *Dlx5/6-*

CIE;Jnk1^{fl/fl};Jnk2^{-/-} cortices (Fisher's LSD: *Dlx5/6-CIE*: **** $p < 0.0001$, *** $p = 0.0003$; calbindin: **** $p < 0.0001$, ** $p = 0.002$). **E-H.** Distribution of *Dlx5/6-CIE*+ and calbindin-positive cortical interneurons in the caudal cortex of *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* (**E**) and *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* embryonic brains (**F**). **G, H.** Statistically significant interactions were found between genotype and bin location for both *Dlx5/6-CIE*+ (two-way ANOVA: $F_{(4, 40)} = 8.336$; $p < 0.0001$) (**G**) and calbindin-positive (**H**) cortical interneurons (two-way ANOVA: $F_{(4, 40)} = 11.98$; $p < 0.0001$). Similar to rostral levels, migratory advancement of interneurons was significantly impaired at caudal levels in *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* cortices (Fisher's LSD: *Dlx5/6-CIE*: *** $p = 0.0002$ in bin1, 0.0007 in bin 3; calbindin: **** $p < 0.0001$, *** $p = 0.0007$, * $p = 0.029$).

Figure 2.8: *In vitro* migration of cortical interneurons from explants of MGE tissue does not rely on JNK signaling. **A.** Illustration of experimental design (see Materials and Methods). **B-C.** Explant of MGE tissue isolated from an E12.5 *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* embryonic brain section and cultured for 2 days in a collagen:Matrigel matrix. Interneurons have migrated from explant borders in a radial pattern. **D-E.** MGE explant from a E12.5 *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* brain grown under identical conditions, and exhibiting a similar pattern of interneuron outgrowth. **F.** Areas measured from explant shown in **B** and **C**, illustrating that outgrowth area (dark blue) was determined by subtracting the explant area (light blue) from the total area occupied by both explant and migrating cells. **G.** Plot representing mean values of normalized outgrowth area (outgrowth area/explant area) for 10 explants from each genotype. DIC images (**B, D**) were used to identify explant boundaries, while perimeters of migrating cells were traced from images containing the EGFP channel alone (**C, E**).

Figure 2.9: Cortical interneurons lacking *Jnk1* and *Jnk2* disperse from migratory streams and adopt branched morphologies *in vivo*. **A-C.** Radial distribution of *Dlx5/6-CIE*⁺ cells (green) in the lateral cortex of E13.5 *Dlx5/6-CIE; Jnk1^{fl/+}; Jnk2^{-/-}* (**A**) and *Dlx5/6-CIE; Jnk1^{fl/fl}; Jnk2^{-/-}* embryos

(**B**). Equivalently sized cortical probes were equidistantly binned along the radial axis (Bin 1 = MZ - Bin 6 = VZ) and the percentage of Dlx5/6-CIE+ cells appearing in each bin was determined (n = 5 embryos/genotype). **C**. Radial distribution of Dlx5/6-CIE+ cells is altered in *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* cortices (two-way ANOVA: $F_{(5, 48)} = 13.26$; $p < 0.0001$). Proportionately fewer Dlx5/6-CIE+ cells are located in bin 4 (SVZ region; Fisher's LSD = **** $p < 0.0001$), while more are located in bin 3 (IZ region; Fisher's LSD = **** $p < 0.0001$). **D-G**. Dlx5/6-CIE+ (green) and calbindin-positive cells (red/yellow) from E13.5 *Dlx5/6-CIE;Jnk1^{fl/+};Jnk2^{-/-}* cortices. **D**. Cortical interneurons within the MZ travel in a coherent stream with few gaps (open arrowhead). **E**. Interneurons within the cortical SVZ/IZ stream bare medially directed (image right) leading processes. **F-G**. Examples of isolated Dlx5/6-CIE+ cells with simple, unbranched leading processes. **H-K**. Dlx5/6-CIE+ (green) and calbindin-positive cells (red/yellow) from E13.5 *Dlx5/6-CIE;Jnk1^{fl/fl};Jnk2^{-/-}* cortices. **H**. Mutant MZ streams are patchy, containing many gaps (open arrowheads). **I**. Cortical interneurons are loosely organized within the mutant SVZ. Many interneuronal cell bodies (arrows) have no apparent leading processes, and many interneuronal processes (arrowheads) have no apparent cell bodies, suggesting many interneurons are orthogonally positioned to the plane of section. Interneurons with attached processes (asterisks) are often non-medially directed. **J-K**. Dlx5/6-CIE+ cells from mutant cortices often have branched morphologies. Also note aberrant branching of interneurons in **B** and **I** compared to **A** and **E**.

Figure 2.10: Migration of cortical interneurons through the cortical entry zone *in vivo* requires intact JNK signaling, largely mediated by interneuron expressed *Jnk1*. Schematic drawings of the cortical rudiment at E13.5; ventro-lateral is left, dorso-medial is right. Red shading represents JNK-dependent cortical “entry zone”. LOF, loss of function. Control *Jnk1*^{+/+}, *Jnk2*^{+/+}, *Dlx5/6-CIE;Jnk1*^{fl/+}; *Jnk2*^{-/-} genotypes, as well as *Jnk2*^{-/-} and *Jnk2*^{-/-}; *Jnk1*^{+/+} genotypes, display normal tangential migration at rostral (top left) and caudal (top right) cortical locations at E13.5. Cortical interneuron migration within *Jnk1*^{-/-} embryos is diminished to statistically significant levels (red interneurons) at caudal (middle right), but not rostral locations (middle left). *Jnk1*^{-/-}; *Jnk2*^{+/+} constitutive mutants and *Dlx5/6-CIE;Jnk1*^{fl/m}; *Jnk2*^{-/-} conditional mutants have significantly diminished migration (red interneurons) at both rostral (bottom left) and caudal locations (bottom right). Additionally, interneurons completely devoid of both *Jnk1* and *Jnk2* are abnormal positioned in the radial axis of the cortex, disrupting the integrity of MZ and SVZ/IZ streams, and exhibit branching anomalies.

Movie 2.1. Live imaging of cortical interneuron migration through the cortical entry zone at E12.5 in a control slice (see Figure 2.4). **Video clip 1.** Ventro-lateral, bottom left; Dorso-medial, upper right. Dlx5/6-CIE+ cells organize into migratory streams and rapidly invade the nascent cortical rudiment over the 24-hour imaging period. **Video clip 2.** Cell track 1 (purple spot and tail) represents a Dlx5/6-CIE+ cell tracked during the first 12-hour imaging period. Cell 1 maintains a medially directed trajectory, but shifts from SVZ to VZ regions of the slice during the tracking period. **Video clip 3.** Cell track 2 (red spot and tail) represents a Dlx5/6-CIE+ cell tracked during the last 12-hour imaging period. Cell 2 maintains a long, medially directed trajectory, but shifts positions within the SVZ/IZ stream during the tracking period. Spots mark tracked cells, and tails represent where the cell traveled over the last hour of time.

Movie 2.2. Live imaging of cortical interneuron migration through the cortical entry zone at E12.5 in an SP600125-treated slice (see Figure 2.4). **Video clip 1.** Ventro-lateral, bottom left; dorso-medial, upper right. Dlx5/6-CIE+ cells tentatively migrate into the cortical entry zone during the first 12 hours of recording, often migrating slowly into the cortex only to reverse directions and leave. Migration improves during the last 12 hours of recording, but many cells pile up in the MZ stream or spread out in the SVZ/IZ region without advancing past the cortical entry zone. **Video clip 2.** Cell track 1 (purple spot and tail) represents a Dlx5/6-CIE+ cell tracked during the first 12-hour imaging period. Cell 1 migrates a short distance medially, traveling from the SVZ/IZ to the MZ regions of the slice, only to stall, turn, and migrate back towards the cortico-striatal border. **Video clip 3.** Cell track 2 (red spot and tail) represents a Dlx5/6-CIE+ cell tracked during the last 12-hour imaging period. Cell 2 migrates in a radial trajectory, traveling from the VZ to the MZ region of the slice, only to turn and migrate laterally, like Cell 1. Spots mark tracked cells, and tails represent where the cell traveled over the last hour of time.

Chapter 3: JNK Signaling Preserves Cortical Interneuron Stream Integrity and Radial Organization of the Cortical Plate

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Abstract

The precise migration of cortical interneurons from the ganglionic eminences to the cerebral cortex is essential for cortical circuit formation and prevention of severe neurodevelopmental disorders like autism, schizophrenia, and epilepsy. Cortical interneurons navigate the developing cerebral cortex utilizing well-known molecular guidance cues, however the intracellular signaling pathways that coordinate their directed migration are not well understood. Recently, we identified the c-Jun N-terminal kinase (JNK) pathway as an important regulator of cortical interneuron migration, where it mediates their advancement into the cortical entry zone and proper assembly of migratory streams during early stages of corticogenesis. In the current study, we demonstrate a vital role for JNK signaling as cortical interneurons transition from tangential to radial migration. Pharmacological inhibition of JNK signaling in *ex-vivo* slice cultures caused cortical interneurons to evacuate from migratory streams and precociously enter the cortical plate. Cortical interneurons partially reassemble into migratory streams after removal of the pharmacological blockade, suggesting that migratory stream integrity depends on intact JNK signaling. Live imaging of JNK-inhibited *ex-vivo* slices demonstrated increased diagonal and radial trajectories of interneurons evacuating from the subventricular zone stream and increased time spent in the cortical plate upon arrival. Similarly, complete genetic loss of JNK signaling from cortical interneurons in *ex-vivo* slice cultures leads to dispersion of cortical interneurons from the SVZ stream and subsequent filling in of the cortical plate. Evaluation of *Jnk1^{fl/fl}; Jnk2^{-/-}; Jnk3^{-/-}; Dlx-CIE* cortices at embryonic day (E) 15.5 revealed interneuron clusters within the entry zone of the cortex and less unified subplate and SVZ streams. Time-lapse imaging at E14.5 of *Jnk1^{fl/fl}; Jnk2^{-/-}; Jnk3^{-/-}; Dlx-CIE* cortices demonstrated that cortical interneurons escaping the MZ stream trigger the formation of interneuron clusters in the cortical plate. Further, evaluation of underlying radial glial scaffolding suggest that the clusters of cortical interneurons may form in conjunction with disruption and detachment of radial glial cell

processes from the pial surface. These data suggest that JNK is important for mediating the tangential to radial transition of migrating cortical interneurons and could be vital for the development of the underlying cortical architecture.

Introduction

Proper formation of inhibitory interneuron circuitry within the developing cortex is imperative for normal cortical function, including the prevention of over-excitation. Even minor disruptions to interneuron distribution and wiring in the cerebral cortex are suspected to cause common neurological disorders like autism, schizophrenia, and epilepsy (Meencke and Veith, 1992; Meechan et al., 2012; Volk et al., 2015). Cortical circuit malfunction can result from a variety of causes; however, early migratory deficits can have a profound impact on cortical circuitry by impairing the final placement and function of cortical interneurons.

Post-mitotic cortical interneurons travel tangentially from the medial and caudal ganglionic eminences in the ventral forebrain by deciphering molecular cues to form and migrate in streams (Nery et al., 2002; Xu et al., 2004; Miyoshi et al., 2010). As development proceeds, cortical interneurons transition from tangential to radial migration to enter the cortical plate where they will layer with excitatory neurons to form the layers of the mature cerebral cortex (Miyoshi and Fishell, 2011). The mechanism by which cortical interneurons mediate this tangential to radial switch appears to be multi-factoral (Chédotal and Rijli, 2009; Elias et al., 2010; Baudoin et al., 2012). Recent studies have attributed the tangential to radial switch to the expression of connexin43 (Elias et al., 2010), the chemokine SDF-1/Cxcl12 and its receptors Cxcr4/Cxcr7 (Sánchez-Alcañiz et al., 2011; Wang et al., 2011), the morphogen SHH (Baudoin et al., 2012)), and regulation of cellular machinery through the primary cilium (Baudoin et al., 2012; Higginbotham et al., 2012). To date, the intracellular signaling pathways regulating the tangential progression of cortical interneurons in migratory streams and the timing of migratory stream exit are largely unknown.

The c-Jun N-terminal kinase pathway includes upstream activators (MKK4 and MKK7) and downstream targets (DCX, Map1B) (Gdalyahu et al., 2004; Kawauchi et al., 2005; Jin et al., 2010; Coffey, 2014). The terminal kinase is made up of 3 *Jnk* genes: *Jnk1*, *Jnk2*, and *Jnk3* and

known to regulate migration through the dynamic remodeling of cell cyto-architecture (Gupta et al., 1996; Gdalyahu et al., 2004; Westerlund et al., 2011; Björklom et al., 2012). JNK signaling activates regulatory proteins such as doublecortin (Gdalyahu et al., 2004), Map1b (Chang et al., 2003), connexin43 (Barker and Gourdie, 2002) to influence cellular dynamics. Additionally, it mediates guided migration through chemokine receptors such as CXCR4 (Serrati et al., 2008; Zhe et al., 2012)) within different cell types to affect migration (Friocourt et al., 2007; Elias et al., 2010; Sánchez-Alcañiz et al., 2011; Wang et al., 2011). Therefore, deficits in JNK signaling could have implications in perturbing cortical interneuron orientation in migratory streams and their transitions from tangential to radial modes of migration (López-Bendito et al., 2008; Elias et al., 2010; Lysko et al., 2011, 2014; Sánchez-Alcañiz et al., 2011). Since our previous research showed that cortical interneurons require JNK signaling for their initial entry into the cerebral cortex and their organization within migratory streams between E12.5 and E13.5 of mouse development (Myers et al., 2014), we next considered whether JNK signaling was also required at later stages of cortical interneuron migration.

In the current study, we use pharmacological inhibition of JNK signaling in *ex vivo* slice cultures and *in vivo* deletion of the *Jnk1*, *Jnk2*, and *Jnk3* genes from interneurons to investigate the role of JNK signaling in the tangential progression of cortical interneurons in migratory streams and the timing of migratory stream departure. Our findings suggest that JNK signaling is required for the maintenance of migratory streams and that inhibition of JNK signaling may facilitate the switch from tangential to radial modes of migration in order to promote the infiltration of cortical interneurons into the nascent cortical plate.

Materials and Methods

Mice

All mouse procedures were completed in accordance to the West Virginia Animal Care and Use Committee. CF-1 dams were mated to *Dlx5/6-Cre-IRES-EGFP* (Dlx5/6-CIE) males to achieve time pregnancies at embryonic day 14.5 (E14.5) for our slice culture experiments. C57 *Jnk1^{fl/fl}; Jnk2^{-/-}* dams were mated with *Jnk1^{fl/+}; Jnk2^{-/-}; Dlx5/6-CIE* males and *Jnk1^{fl/fl}; Jnk2^{-/-}; Jnk3^{-/-}* dams were mated *Jnk1^{fl/+}; Jnk2^{-/-}; Jnk3^{+/-}; Dlx5/6-CIE* males with to achieve time pregnancies at E15.5. The day of vaginal plug was embryonic day 0.5.

Slice Culture for Dosage Experiment and Washout Experiments

The day prior to collecting the embryos, a six well plate of transwell membranes were coated with a solution of poly-L-lysine and laminin. On the experimental day, the transwell membranes were rinsed with sterile water and left to dry. Dlx5/6-CIE positive embryos were collected at embryonic day 14.5 (E14.5). The embryonic brain was removed from the embryo. All brains were embedded in 3% agarose and sectioned into 300 μ M slices on a Leica vibratome. Slice culture media containing either Dimethyl Sulfoxide (DMSO), or a 20 μ M or 40 μ M concentration of the SP600125 inhibitor was added beneath the transwell membranes. Slices were placed on a transwell membrane and cultured in an incubator maintained at 37°C with 5% CO₂. All slices were fixed with 4% paraformaldehyde.

Frozen Embedding of Cultured Sections

Post fixation with 4% paraformaldehyde, slices were transferred through a sucrose gradient (10%, 20%, and 30%) over three days by switching to the next consecutive percentage after a day's time. Slices were embedded in agar sucrose to help free them from the transwell membrane. The agar sucrose embedded slices were transferred to a silicon mold and embedded in Tissue Freezing Medium. The slice embedded in the silicon mold was frozen by

plunging it into 2-Methylbutane chilled by liquid nitrogen. The frozen slices were then stored at -80 degrees.

Cryosectioning and Immunocytochemistry

Frozen slices were sectioned at 12µm on a Leica cryostat. Sections were laid down on to Superfrost Plus slides. Slides were painted with a hydrophobic barrier pen. 1x phosphate buffer saline (1xPBS) was added to each slide to rehydrate the processed sections. Permeabilization solution with 5% normal goat serum was added to the slides to block the sections. Rabbit anti Tbr1 (1:250) and Chicken anti GFP (1:1500; Abcam) primary antibodies labeled the developing cortical plate and cortical interneurons respectively. Secondary antibodies, goat anti-rabbit 546 and goat anti-chicken 488, were used to fluorescently label the primary antibodies. A nuclear counterstain was added to help identify cortical interneurons and assess cellular health.

Live Imaging

Live embryonic brain slices were processed the same as for the fixed preparations above. Slices were cultured on a transwell membrane in a glass bottom dish in either control (DMSO) or 20 µM concentration of the SP600125 inhibitor. Slices were incubated at 37°C at 5% CO₂ and imaged on a Zeiss 710 confocal microscope with a 20x neofluar objective for 12 hours.

Quantification for Dosage and Washout Experiments

Images were cropped to 600x1500. The cropped images were binned into 10 equidistant bins. Cortical interneurons were counted using the count tool in Adobe Photoshop. The angles of the leading processes were determined by adding a protractor layer onto the image in Adobe Photoshop. Angle 0 degrees was aligned to the closest bin line.

Quantification for Live Imaging Experiments

Movies were analyzed using the Imaris software (Bitplane). Cortical interneurons were tracked from a lateral, middle, and medial region of the subventricular zone (SVZ) stream. One box was created in the cortical plate region to assess the movement of cells in this region overtime.

Cortical interneurons were tracked for at least 2 hours using the Spots feature. Cell velocity was exported from the software. The leading process of the cortical interneurons was used to assess direction. Cell direction was noted as either tangential, diagonal, or radial. A numerical score was given to each direction (0, 0.5, or 1 respectively) and the number of transitions for each of the cells was noted. An average of 10 cells were tracked per movie 5 movies were tracked from each condition.

Results

JNK signaling maintains the integrity of cortical interneuron migratory streams

Cortical interneurons initially travel from lateral to medial locations within the developing cerebral cortex in tangentially oriented migratory streams located in the subventricular/intermediate zone (SVZ/IZ), subplate, and marginal zone (MZ), but as development proceeds, interneurons switch from tangential to diagonal or radial modes of migration in order to enter the nascent cortical plate (Wichterle et al., 2001). Although extracellular cues underlying migratory stream maintenance (Wang et al., 2011; Baudoin et al., 2012; Li et al., 2012) and cell-cell adhesive cues mediating the tangential to radial switch (Elias et al., 2010) have been identified, intracellular signaling pathways operating downstream of guidance receptors to orchestrate this complex change in migratory behavior are poorly understood. Since we previously identified a critical role for c-Jun N-terminal kinase (JNK) signaling in promoting the initial entry of cortical interneurons into the cortical rudiment and preserving their tangential orientation within migratory streams (Myers et al., 2014), we

hypothesized that JNK may facilitate later stages of cortical interneuron migration as well. In order to determine if JNK signaling is required to maintain the integrity of pre-formed streams of migratory cortical interneurons, we cultured live-vibratome sections from embryonic day 14.5 (E14.5) Dlx5/6-Cre-Ires-EGFP positive (Dlx-CIE+) brains in either control or JNK inhibited conditions for 12 hours. JNK inhibition was attained by using SP600125 (Bennett et al., 2001), a pan-JNK inhibitor, at 20 μ M or 40 μ M; concentrations that delayed or prevented the entry of interneurons into the cerebral cortex between E12.5 and E13.5 of mouse development (Myers et al., 2014). Cortical interneuron distribution was analyzed in two regions of the cerebral cortex: the cortical entry zone and the leading edge of interneuron migratory streams (Figure 1B). Stream dispersion was measured by dividing each region into 10 equidistant bins from the pial to ventricular surfaces and assessing the distribution of cortical interneurons across the radial axis of the cortical wall.

At E14.5, interneurons in the cortical rudiment are predominantly found traveling in robust, organized streams within the marginal zone (MZ) and subventricular zone (SVZ). As expected, MZ and SVZ streams were preserved in slices that were cultured for 12 hours in control conditions at both the entry zone and the leading edge of cortical interneuron migration (Figures 3.1A-C). JNK-inhibited slices, however, showed disbanding of cortical interneurons from migratory streams and precocious filling in of the cortical wall (arrows) after treatment with 20 μ M (Figures 3.1D-F) and 40 μ M SP600125 (Figures 3.1G-I). Moreover, interneuron stream disbanding was most prominent in medial cortical regions (Figures 3.1C, F, I). Quantification of interneuron distribution at the laterally positioned entry zone showed a trend for interneuron dispersion from the SVZ and MZ streams (Figure 3.1J, upper graph), but this trend did not reach statistical significance. When interneuron distribution was quantified at the medially positioned leading edge, however, robust differences were found between control and SP600125-treated slices. Disruption to the marginal zone and subventricular zone streams in JNK-inhibited slices

resulted in significant alterations in the radial distribution of cortical interneurons in the cortical wall (Two-way ANOVA $F_{(18,120)} = 3.582$; $p < 0.0001$). Post-hoc analyses showed statistically significant decreases of cortical interneurons traveling in the marginal zone (Fisher's LSD test: 20 μ M, Bin 1 $p = 0.0344$; 40 μ M, Bin 1 $p = 0.0002$), intermediate zone (Fisher's LSD test: 20 μ M, Bin 4 $p = 0.0005$), and subventricular zone (Fisher's LSD test: 20 μ M, Bin 5 $p = 0.0039$; 40 μ M, Bin 5 $p = 0.0445$), and a significant increase of cortical interneurons in the ventricular zone (Fisher's LSD test: 20 μ M, Bin 8 $p = 0.0217$; 40 μ M, Bin 9 $p = 0.0312$; Figure 3.1J, lower graph). More interneurons were also found in the nascent cortical plate of JNK inhibited slices, further distinguishing them from controls.

In addition to radial displacement, we quantified the orientations of interneuronal leading processes to assess the trajectory of cortical interneurons after 12 hours of JNK inhibition. Angles of leading processes were measured using a digital protractor that was aligned tangentially to the cortical wall with zero degrees always pointing towards the dorsal-medially positioned cortical arch. Interneurons with clearly distinguishable leading processes were sampled throughout the entire medial-lateral extent of the cortical rudiment. Cortical interneurons in control slices maintained a tangential orientation with leading processes angled between 0-30 degrees (Figure 3.1K, green traces) in the cortical plate (99/200 cells), subventricular zone (121/254 cells), and ventricular zone (7/16 cells). Analysis of leading processes of cortical interneurons in the SP600125-treated slices revealed a dose dependent decrease in tangentially oriented cortical interneurons in the cortical plate (20 μ M: 57/178 cells; 40 μ M: 82/256 cells) subventricular zone (20 μ M 80/250 cells; 40 μ M 57/237 cells), and ventricular zone (20 μ M: 11/41 cells; 40 μ M: 5/55 cells; Figure 3.1K, blue traces-20 μ M and red traces-40 μ M). The decreased frequencies of tangentially oriented (0-30 degrees) cortical interneurons in SP600125-treated slices were statistically significant in both the subventricular zone (Chi Square Analysis: 20 μ M: $p = 0.0004$; 40 μ M: $p < 0.0001$) and cortical plate (Chi Square

Analysis: 20 μ M: $p=0.0008$; 40 μ M: $p=0.0002$). Many cortical interneurons in SP600125-treated conditions displayed a wider array of angles, with numerous cortical interneurons angled opposite of their correct trajectory, and others angled toward the cortical plate and ventricular zone regions. Again, increased frequencies of cortical interneurons with aberrant orientations in SP600125-treated slices were statistically significant in the cortical plate regions (Figure 3.1K)

Collectively, our data suggest that JNK inhibition leads to the departure of cortical interneurons from migratory streams, and that migratory stream integrity might differ in its requirement for JNK signaling along the medial-lateral axis. Interneuron streams in the medial cortex appear more sensitive to pharmacological inhibition of JNK signaling than in the lateral cortex.

Stream integrity can be partially restored after disruption of JNK signaling

To determine if the stream integrity that was lost in the SP600125 pharmacologically treated tissue could be recovered through restoration of JNK signaling, we performed a washout experiment on Dlx-CIE+ slices. Slices were cultured *in-vitro* for 12 hours in control media, or media treated with 20 μ M or 40 μ M SP600125. After rinsing with control media at 12 hours, half of the cultures initially treated with SP600125 were replaced with media containing the same concentration of SP600125, while the other half of SP600125-treated cultures were replaced with control media. All slice cultures were grown for an additional 12 hours.

Slices treated with 20 μ M or 40 μ M SP600125 for 24 hours contained cortical interneurons that dispersed from the subventricular zone stream at both the entry zone and leading edge.

Exaggerated loss of interneurons from migratory streams and dispersion throughout the cortical wall, including in the laterally positioned entry zone, were likely due to the extended duration of SP600125 treatment (24 hours of treatment in Figure 3.2 and 3.3 versus 12 hours of treatment in Figure 3.1). Following 24 hours of JNK inhibition, cortical interneurons accumulated in the cortical plate and the ventricular zone regions resulting in statistically significant changes in their

radial distribution at the entry zone (two-way ANOVA: $F_{(18,120)}=2.579$; 20 μM $p=0.0012$; $F_{(18,120)}=3.292$; 40 μM $p<0.0001$) and leading edge (two-way ANOVA: $F_{(18,120)}=2.785$; 20 μM $p=0.0005$; $F_{(18,120)}=8.786$; 40 μM $p<0.0001$). As before, the subventricular zone showed a significant decrease in the abundance of cortical interneurons indicating that JNK inhibition led to evacuation of the SVZ/IZ stream (Figure 3.2J and 3.3J). Additionally, stream spreading and inconsistencies/gaps were often observed in the MZ, and rounded cortical interneurons without migratory processes were found in the cortical plate, especially at the leading edge (Figure 3.2F and 3.3F).

Washout slices showed evidence for recovery in some regions of the radial axis (Figure 3.2J, 3.3J). In slices that were washed out from treatment with 20 μM SP600125, recovery of interneuron distribution was documented in the cortical plate and ventricular zone at the entry zone (Figure 3.2). At the leading edge of the same slices, recovery of cortical interneuron distribution was found in the lower subventricular zone stream and lower portion of the cortical plate, where the subplate is located (Figure 3.2). A similar result was seen in slices that were washed out from treatment with 40 μM SP600125 (Figure 3.3B). These slices showed recovery in the cortical plate with trends toward recovery in the ventricular zone (Figure 3.3D), however, recovery following washout of 40 μM SP600125 was less complete than recovery following washout of 20 μM SP600125.

Our results indicate that restoration of JNK signaling can partially recover radial distribution of interneurons in the cortical wall and lead to the reinvestment of migratory streams. This suggests that acute JNK inhibition misroutes cortical interneurons, but their ability to respond to environmental cues located in the radial axis of the cortical wall remains intact, since interneurons are apparently able to reorient and reengage in a tangential migratory program when JNK signaling is restored. This ability to recover may be dose-dependent, however, as

extreme displacement from migratory streams brought about by severe loss of JNK signaling appears largely irreversible.

Pharmacological loss of JNK signaling causes cortical interneurons to leave the subventricular zone stream and spend more time in the cortical plate

To determine the dynamic behavior of cortical interneurons in the absence of JNK signaling, we live-imaged E14.5 slice cultures from *Dlx-CIE+* embryos in control and SP600125 treated conditions. Cortical interneurons were tracked in the subventricular zone to assess the orientation of their migratory trajectory, distance traveled, and velocity.

Cortical interneurons in slices treated with 20 μ M SP600125 showed dramatic evacuation from the subventricular zone stream when compared to controls that were imaged over the same 12-hour period (Figures 3.4A, B). Indeed, in SP600125 treated slices, only 30.56% (44/144 interneurons) of the cortical interneurons remained in the SVZ stream, whereas 69.44% (100/144 interneurons) of the interneurons departed. When compared to control slices, where 70.14% (101/144 interneurons) of interneurons remained in the SVZ and only 29.86% (43/144 interneurons) departed, highly statistically significant differences were found (T-test $p < 0.0001$; Figure 3.4C top). When the migratory orientations of interneurons traveling in or departing from the SVZ stream were quantified (144 interneurons and 12 movies for control and inhibitor), we found that JNK inhibited cortical interneurons spent more time in diagonal (35.36%; 2282/6468 frames) and radial (22%; 1460/6486 frames) orientations and less time in tangential orientations (42.64%; 2744/6486 frames) compared to controls, which spent 23.07% (929/3985 frames) of their time traveling diagonally, 7.36% (310/3985 frames) of the time traveling radially, and 69.56% (2746/3985 frames) of the time traveling tangentially. When compared, highly significant differences were found (T-test: Diagonal: $p = 0.002$; Radial: $p < 0.0001$; Tangential: $p < 0.0001$; Figure 3.4C middle). Cortical interneuron velocity (T-test: Max, Mean Min, St. Dev: $p < 0.0001$; Figure 3.4C bottom), displacement (T-test: $p < 0.0001$, Figure 3.5A) and track straightness (T-

test: $p < 0.0001$, Figure 3.5B) were significantly decreased in the pharmacologically treated slices.

Cortical interneuron behavior in the cortical plate was also measured by tracking interneurons that entered a standardized box fit to the margins of the cortical plate. In SP600125-treated conditions, cortical interneurons that entered the cortical plate were less likely to have a tangential orientation, and more likely to have a diagonal orientation (Figure 3.4D, left). The length of time that interneurons spent in the cortical plate was statistically longer in SP600125-treated slices compared to controls (T-test: $p < 0.0001$; Figure 3.4D, middle), and accordingly, interneurons in SP600125-treated slices displayed decreased migratory velocity in the cortical plate region (Figure 3.4D, right).

Together, our results indicate that JNK signaling is required to maintain the tangential orientation and migratory velocity of cortical interneurons traveling in the SVZ stream. The robust infiltration of the cortical plate upon JNK inhibition suggests that downregulation of JNK signaling could mediate the timing of cortical interneuron departure from migratory streams and subsequent entry into the cortical plate.

Genetic loss of JNK signaling causes cortical interneuron stream dispersion and cortical plate accumulation

In order to determine if genetic loss of JNK function disrupts the integrity of migratory streams, *ex-vivo* slices from an allelic series of JNK mutants were analyzed. Since *Jnk1*;*Jnk2* double mutants are early embryonic lethal (Kuan et al., 1999), and combinatorial loss of *Jnk1* and *Jnk2* has a greater effect on interneuron migration than loss of *Jnk1* alone (Myers et al., 2014), we conditionally deleted *Jnk1* from interneurons of *Jnk2* null embryos, and then further examined the contribution of *Jnk3* by conditionally deleting *Jnk1* from interneurons of *Jnk2* null;*Jnk3* heterozygotes or *Jnk2*;*Jnk3* double knockouts to completely eliminate JNK signaling in cortical interneurons. Organotypic slices from the allelic series of JNK mutant brains were prepared at

E14.5 and imaged live for 18 hours. Cortical interneurons in both conditional triple mutant mice showed a shift in radial distribution from the subventricular zone to the cortical plate region (Figure 3.5). At first glance, the SVZ stream in the conditional triple mutant mice disperses and cortical interneurons accumulate within the cortical plate compared to the *Jnk1*; *Jnk2* mutant controls.

In order to evaluate whether genetic removal of JNK function altered the distribution of interneurons in the cortical wall, still movie frames at 0, 6, 12, and 18 hours from *Jnk1^{fl/fl}*; *Jnk2^{-/-}*; *Dlx-CIE* (cDKO), *Jnk1^{fl/fl}*; *Jnk2^{-/-}*; *Jnk3^{+/-}*; *Dlx-CIE* (cTH), and *Jnk1^{fl/fl}*; *Jnk2^{-/-}*; *Jnk3^{-/-}*; *Dlx-CIE* (cTKO) slices were quantified. At 0 hour, cortices from all four genotypes showed equivalent radial distribution of cortical interneurons (Figure 3.6, two-way ANOVA: $F_{(18,120)}=1.035$; $p=0.4274$), suggesting that cortical interneurons are not significantly displaced in the E14.5 brain. By 6 hours, the distribution of cortical interneurons in the cortical wall begins to shift (Figure 3.6, two-way ANOVA: $F_{(18,120)}=2.383$ $p=0.0028$). Cortical interneurons in cTH and cTKO cortices show a statistically significant loss of cortical interneurons from the SVZ (Figure 3.6, Fisher's LSD test: Bin 5: cDKO vs cTH: $p=0.0069$; cDKO vs cTKO: $p=0.019$; Bin 6: cDKO vs cTH: $p=0.0131$; cDKO vs cTKO: $p=0.0355$), statistically significant increases in the MZ (Figure 3.6, Fisher's LSD test: Bin 1: cDKO vs cTH: $p=0.0069$; cDKO vs cTKO: $p=0.0029$), and trends toward significant increases in the upper cortical plate (Figure 3.6, Fisher's LSD test: Bin 2: cDKO vs cTH: $p=0.0627$; cDKO vs cTKO: $p=0.0613$). At 12 hours, the MZ stream and upper cortical plate region in the cTH and cTKO cortices display statistically significant increases cortical interneuron distribution while the SVZ stream is further compromised (Figure 3.6, two-way ANOVA: $F_{(18,120)}=4.037$; $p<0.0001$; Fisher's LSD test: Bin 6: cDKO vs cTH: $p=0.0014$; cDKO vs cTKO: $p=0.0395$; Bin 7: cDKO vs cTH: $p=0.0146$; cDKO vs cTKO: $p=0.0004$; Bin 8: cDKO vs cTKO: $p=0.0174$). By 18 hours, the distribution of cortical interneurons has drastically changed throughout the radial axis. The SVZ stream counts reveal dramatic loss of cortical

interneurons (Figure 3.6, two-way ANOVA: $F_{(18,120)}=4.069$; $p<0.0001$; Fisher's LSD test: Bin 6: cDKO vs cTH: $p=0.0356$; Bin 7: cDKO vs cTH: $p=0.0156$; cDKO vs cTKO: $p=0.003$; Bin 8: cDKO vs cTH: $p=0.0055$; cDKO vs cTKO: $p=0.014$), while cortical interneurons subsequently fill the MZ stream (Figure 3.5, Fisher's LSD test: Bin 1: cDKO vs cTH: $p=0.0054$), IZ (Figure 6, Fisher's LSD test: Bin 4: cDKO vs cTH: $p=0.0054$), and cortical plate region (Fisher's LSD test: Bin 2: cDKO vs cTH: $p=0.0102$; cDKO vs cTKO: $p=0.0011$; Bin 3: cDKO vs cTKO: $p=0.0063$).

These results indicate that genetic loss of JNK signaling leads to dispersion of cortical interneurons from the SVZ stream and premature entry into the cortical plate. The incomplete evacuation of interneurons from the SVZ stream of conditional triple knockouts compared to the pharmacologically treated slices may suggest that complete loss of all JNK signaling increases interneuron dispersion through non-autonomous mechanisms.

Genetic loss of JNK signaling causes interneuron clustering in the cortical plate and intermediate zone regions

In order to assess cortical interneuron stream integrity *in-vivo*, we evaluated cortices from conditional double and triple knockout embryos at E15.5. Compared to cDKO cortices, the subplate stream was sparsely populated at more dorsal-medial regions of the cortex and almost unrecognizable laterally in cTH, and cTKO (Figure 3.7A-C). Moreover, clusters of cortical interneurons were found in the cortical plate and intermediate zone at the entry region of the cortex in cTH, and cTKO (Figure 3.7B2, 3.7C1-3), which varied in size, number, and location within the cortical plate to intermediate zone region. The severity of interneuron clustering increased with genetic deletion of *Jnk3*: absent in cDKO cortices, sparse in cTH cortices, and prevalent in cTKO cortices. Cortical interneurons residing in clustered areas appeared non-migratory as leading processes were small or absent in coronal sections (Figure 3.7B2, 3.7C1-3). Despite having rounded cell bodies and little to no migratory processes, interneurons within clusters did not label for cleaved caspase 3, a marker of apoptotic cell death, or prematurely

express markers of differentiation, including parvalbumin or calretinin (data not shown).

Disruptions to the MZ (open arrows, Figure 3.7), subplate (solid arrows, Figure 3.7), and SVZ stream (asterisks, Figure 3.7) were often present near clustered regions, indicating that interneurons likely evacuate streams adjacent to where clusters are forming (Figure 3.7B, C).

These data suggest that cortical interneuron clusters are associated with the loss of *Jnk3* from *Jnk1; Jnk2* conditional double knockout mice. This could be due to the evacuation of the MZ, subplate, and SVZ streams, which appear broken and devoid of interneurons in regions where clusters exist. The ablation of all JNK signaling from cortical interneurons may affect the polarity of the cortical interneurons after they disengage from migratory streams and enter the cortical plate, since they appear rounded in shape with no prominent leading processes, but show no sign of apoptotic cell death or post-migratory differentiation.

Cortical interneuron clusters form from the marginal zone stream in JNK deficient cortices

To determine the origins of cortical interneuron clusters, we further assessed our live imaging movies from E14.5 JNK deficient mutants to analyze the behavior of cortical interneurons that accumulated in the cortical plate. Cortical interneurons in cTKO cortices developed clusters formed by cells escaping the MZ stream and invading the cortical plate during the first 6 hours of the movies (Figure 3.8). Interneurons from the subplate and SVZ region of the cTKO slices infiltrated the cortical plate, as well. These aberrant migratory behaviors were not observed in cDKO cortices (Figure 3.8). However, similar but less prominent defects were seen in movies obtained from the cTH cortices (data not shown), matching our *in vivo* findings. The accumulation of cortical interneurons within the cortical plate was quantified by counting the number of cortical interneurons (10 movies from 5 slices/genotype) within a 100X50 μm box. The cTKO cortices accumulated the most interneurons in the cortical plate at 0, 3, 6, 9, and 12 hours (Figure 3.9: two-way ANOVA: $F_{(8,60)} = 3.562$; $p = 0.0019$).

Our data suggest that the loss of *Jnk3* from *Jnk1* and *Jnk2* deficient interneurons promotes departure from migratory streams and entry into the cortical plate. The newly invading cortical interneurons then promptly recruit nearby interneurons from the subplate and SVZ migratory streams. These alterations in interneuron migration could be explained by an autonomous change, such as in the expression of cell surface guidance receptors or downstream signaling, or due to non-autonomous changes in environmental guidance cues that promote entry into the cortical plate and intermingling with other stream exiting interneurons.

Cortical interneuron clusters originate in regions of radial glial disruption

In order to determine if the cytoarchitecture of the developing cortical plate was disrupted at sites of interneuron clusters, we investigated the integrity of radial glial scaffolding in cDKO and cTKO embryos using Nestin labeling. Radial glial cells in the cortex of cTKO were found to be disrupted in areas of interneuron clusters compared to cDKO cortices (Figure 3.10). Radial glial scaffolding was not well organized in cTKO cortices (Figure 3.10). Endfoot detachment from the pial surface was seen in some, but not all embryos (Figure 3.10). Prevalence of radial glial scaffolding seemed sparse and the structure was disrupted with missing or spindly projections (Figure 3.10). In addition, Hoechst staining revealed potential disruptions to migrating excitatory neurons (Figure 3.10). Together, our data suggest that cortical interneuron clustering occurs where there are disruptions in radial glial scaffolding and/or potential disturbances in the cellular architecture of the cortical plate.

Discussion

In present study, we found that the tangential progression of cortical interneurons in migratory streams depends on the JNK signaling pathway. Pharmacological inhibition of JNK signaling using *ex-vivo* slice cultures revealed a dose-dependent dispersion of cortical interneurons from migratory streams. Furthermore, displaced interneurons were able to repopulate migratory

streams upon removal of the pharmacological blockade, indicating that migratory stream dispersion is partially recoverable. Live imaging of *ex-vivo* slice cultures showed that acute JNK inhibition led to rapid stream dispersion, as JNK-inhibited cortical interneurons switched from tangential to radial modes of migration, exited the SVZ stream, and infiltrated the cortical plate region. Similar to acute pharmacological inhibition, deletion of all three *Jnk* genes from cortical interneurons resulted in dispersion from migratory streams in *ex vivo* slices. *In vivo*, we found that complete loss of JNK signaling from interneurons led to the accumulation of cortical interneurons in clusters within the cortical plate and intermediate zone region of the developing cortex. Live imaging revealed that clusters of cortical interneurons were formed from pockets of MZ-derived interneurons that dipped into the cortical plate and intermingled with other interneurons that evacuated from the SVZ stream. Additionally, the normal columnar architecture created by radial glial scaffolds was perturbed in regions of the cortical plate that were populated by clusters of cortical interneurons. Together, these results suggest that JNK signaling is required to maintain the integrity of cortical interneuron migratory streams and that JNK signaling levels may gate the entry of interneurons into the cortical plate.

JNK signaling and cortical interneuron migration

JNK signaling is known to direct numerous aspects of cortical development including the migration of radially and tangentially migrating neurons (Westerlund et al., 2011; Myers et al., 2014), and leading process branching (Gdalyahu et al., 2004), and axonogenesis (Oliva Jr et al., 2006). Additionally, mutations in JNK genes, specifically JNK3, are implicated in neurodevelopmental disorders including intellectual disability and epilepsy (Shoichet et al., 2006; Kunde et al., 2013), which might arise from the dysfunction of cortical interneurons. Our previously published work demonstrated that JNK signaling, specifically *Jnk1*, plays an important role during early cortical interneuron migration. Pharmacological blockade of JNK signaling delayed the initial entry of cortical interneurons into the cerebral cortex (Myers et al.,

2014). Furthermore, genetic ablation of *Jnk1*, or combinatorial deletion of *Jnk1* and *Jnk2* within cortical interneurons, also delayed their entry into the cortex (Myers et al., 2014). Additionally, we found that the orientation of cortical interneurons in migratory streams and their radial positioning in the cortical wall was disrupted, suggesting that JNK signaling might also be important for the initial assembly of cortical interneurons into organized streams (Myers et al., 2014). The current study is the first to suggest that JNK signaling plays a role in migratory stream maintenance and mediating the tangential to radial transition that interneurons make as they leave migratory streams in order to populate the nascent cortical plate. Indeed, both pharmacological inhibition and genetic ablation of JNK signaling causes interneurons to switch from tangential to radial migration, depart from streams, and accumulate in the cortical plate. Although it is currently unclear how JNK signaling regulates the tangential progression of cortical interneurons in migratory streams or how the withdraw of JNK activity facilitates the tangential to radial switch, the JNK signaling pathway may intersect with other molecular pathways known to facilitate this key transition of migratory behavior

Stromal derived factor-1 (SDF-1)/Cxcl12 is a chemokine that serves as a chemoattractant to cortical interneurons that express the Cxcr4 and Cxcr7 chemokine receptors (Liapi et al., 2008; López-Bendito et al., 2008; Wang et al., 2011). In the absence of SDF-1 or the Cxcr4 or 7 receptors, cortical interneurons disperse from migratory streams, leading to premature accumulation in the cortical plate during pharmacological inhibition and *in-vivo* genetic ablation (Sánchez-Alcañiz et al., 2011; Wang et al., 2011; Abe et al., 2014). Specifically, cortical interneurons in Cxcr4, Cxcr7, and SDF-1 null mice transition to a radial orientation and migrate from the MZ and SVZ streams to deposit in the cortical plate (López-Bendito et al., 2008; Sánchez-Alcañiz et al., 2011; Wang et al., 2011; Abe et al., 2014), a phenotype that is very reminiscent of JNK deficient cortical interneurons. Early deposition of interneurons in the cortical plate is also seen in a mouse model of 22q11.2 deletion syndrome—a developmental disorder

linked to schizophrenia and autism—and these migratory anomalies are at least partially caused by diminished expression of Cxcr4 in cortical interneurons (Meechan et al., 2012). Although JNK and chemokine signaling have not yet been linked in cortical interneurons, Cxcr4 is known to activate JNK signaling in breast cancer cells to promote invasion (Serrati et al., 2008). It is tempting to speculate, therefore, that JNK signaling acts downstream of Cxcr4 activation to facilitate tangential progression of cortical interneurons in migratory streams.

Intracellular signaling events that initiate dynamic changes in the cytoskeleton of leading processes are not well understood, but their precise spatial-temporal control is critical for orienting cortical interneurons during their migration. Interestingly, doublecortin (Dcx), a microtubule binding protein known to regulate leading process branching and migration of cortical interneurons (Friocourt et al., 2007), is a downstream target of JNK signaling (Gdalyahu et al., 2004; Kawauchi et al., 2005; Jin et al., 2010). Recent studies suggest that SDF-1 can modulate the branching of the leading process through DCX (Lysko et al., 2014). Therefore, a potential link exists between SDF-1 and JNK signaling in migrating cortical interneurons.

Previous research by Lysko and colleagues indicate a role for protein kinase A (PKA) phosphorylation of DCX in the migration of cortical interneurons. They established that in the presence of high levels of SDF-1, PKA phosphorylation of DCX is low allowing DCX to bind and stabilize microtubules and decrease branching compared to controls which could subsequently maintain the tangential trajectory seen during their early migration in streams.

Downregulation/low expression of SDF-1 or CXCR4 increased PKA phosphorylation of DCX initiating branching (Lysko et al., 2014), which could potentially mediate the tangential to radial transition during cortical interneuron migration. Consequently, it is possible that during low PKA phosphorylation of DCX, JNK-phosphorylation of DCX could mediate the binding of DCX to microtubules when SDF-1 is high to maintain cortical interneuron migratory streams, and when

SDF-1 and JNK activity is low, PKA phosphorylation of DCX initiates new branch formation to mediate the tangential to radial transition. However, this still remains to be determined.

The primary cilium, a structure within the leading process of cortical interneurons, further assists in guiding their trajectory (Baudoin et al., 2012; Higginbotham et al., 2012). The primary cilium, comprised of microtubules, is assembled by intraflagellar transport (IFT) and extended to determine the direction of travel by sensing guidance cues from the surrounding environment. It contains receptors for many different guidance pathways including Neuregulin-1, Slit, and Cxcr4, a known regulator of JNK signaling (Serrati et al., 2008; Baudoin et al., 2012; Higginbotham et al., 2012; Métin and Pedraza, 2014). Mutations in the ciliary protein, Arl13b or disruptions to sonic hedgehog (SHH) pathway are known to disrupt the assembly of the primary cilium and the localization of the aforementioned receptors which in turn could affect their migration by influencing the downstream activation of JNK signaling (Baudoin et al., 2012; Higginbotham et al., 2012). As previously discussed, expression of Cxcr4 in the presence of SDF-1 distinctly promotes tangential travel of cortical interneurons and could do so through activation of JNK signaling and further DCX (Gdalyahu et al., 2004; Lysko et al., 2014). However, upregulation of other signaling pathways could initiate other roles for JNK in migration. Baudoin and colleagues (2012) found that expression of SHH promotes the tangential to radial transition in cortical interneurons through the assembly of the primary cilium. In the presence of SHH, patched releases smoothened to move into the cilium along with anterograde transport of IFT proteins to further elongate the cilium (Baudoin et al., 2012). Kif motor proteins are involved in binding cargo for anterograde transport. JNK interacting proteins (JIP) bind cargo to Kifs indicating that JNK signaling may be involved in the anterograde movement of proteins to promote the growth of the primary cilium (Bowman et al., 2000; Verhey et al., 2001). Further, Baudoin and colleagues (2012) suggest that functional IFT could mediate interactions between cortical interneurons and cortical interneurons and other cells within the

cortex. Therefore, having functional IFT could promote the expression of the adhesion molecules required for cortical interneurons to interact with other cell types, such as radial glia which might also require JNK signaling to help promote the tangential to radial transition (Petrich et al., 2002; Elias et al., 2010).

Another important regulator of the tangential to radial transition in cortical interneurons is the gap junction protein connexin43 (Petrich et al., 2002; Elias et al., 2010). Elias and colleagues (2010) found that overexpression of connexin43 promoted (and knockdown inhibited) the switch from tangential migration to radial migration in cortical interneurons by promoting adhesion between cortical interneurons and radial glial cells. Interestingly, studies in cardiomyocytes demonstrated that activation of JNK signaling can influence the expression of connexin43 (Petrich et al., 2002). Specifically, JNK activation was associated with downregulation of connexin43 (Petrich et al., 2002). Therefore, it is possible that low levels of JNK signaling in interneurons could regulate the timing of migratory stream exit by upregulating connexin43 expression, which would promote the tangential to radial transition.

JNK and radial migration

Our results suggest that in addition to migratory stream disruption, total genetic ablation of JNK from cortical interneurons causes disruptions to the columnar structure of radial glial cells and the disorganization of cells in the nascent cortical plate. In our triple knockout model, *Jnk1* was conditionally deleted from the interneurons of *Jnk2/Jnk3* double knockout mice. Therefore, all cells were deficient for *Jnk2* and *Jnk3*, while only cortical interneurons (and other cells of the Dlx5/6 lineage) were deficient for all three *Jnk* genes. Previous studies have demonstrated unique roles for individual *Jnk* genes during radial migration into the developing cortical plate. Westerlund and colleagues (2011) showed that genetic ablation of *Jnk1* enhances the migration of excitatory neurons into the cortical plate at E15.5, and disruptions to the subplate and layer VI at E17.5. More recently, Zhang and colleagues (2016) found that knockdown of *Jnk2* decreased

migration into the cortical plate, but knockdown of *Jnk3* did not perturb radially migrating neurons. This suggests that loss of *Jnk2* could disrupt the laminar position of radially migrating excitatory neurons, however this remains to be determined *in-vivo*. The consequences for combinatorial loss of two or more *Jnk* genes on cortical development have not been evaluated prior to our studies, but it is possible that deletion of *Jnk2* and *Jnk3* could alter the migration or differentiation of excitatory cortical neurons, even if *Jnk1* remains intact. Therefore, disruptions in radial glial architecture and the disorganization of the nascent cortical plate seen in our triple knockout mice may be due to constitutive loss of *Jnk2* and *Jnk3*. It is also possible that complete loss of *Jnk* in cortical interneurons non-autonomously disrupts radial glia and neurons of the cortical plate in our triple mutants. Conversely, signals from disrupted radial glia could cause interneurons to cluster in regions where radial glial endfeet have detached from the pial surface. Cortical interneurons interact with radial glial cells when migrating in the developing cortex, and when probed by radial glial endfeet in the marginal zone, some interneurons respond by turning towards the cortical plate (Yokota et al., 2007). Moreover, disruption to radial glia perturbs interneuron migration in ferrets (Poluch and Juliano, 2007), again suggesting that cell-cell interactions between interneurons and radial glia are essential for normal aspects of cortical development. Molecular mechanisms underlying communication between cortical interneurons, radial glia, and potentially, radially migrating projection neurons, remain unknown, but it is possible that JNK signaling coordinates important features of this intercellular communication. Considerable work remains in order to untangle autonomous from non-autonomous functions of JNK during cortical development, as well as uncovering the unique roles for each of the *Jnk* genes in the process.

JNK3

Expression of the *Jnk3* gene is localized to namely the central nervous system and therefore, may play a large role in neuronal migration and development (Kuan et al., 1999). Mutations in

the *JNK3* gene in humans have resulted in neurological impairment and disease (Shoichet et al., 2006; Kunde et al., 2013), however knockdown of *Jnk3* in radially migrating neurons did not reveal a migration phenotype (Zhang et al., 2016). Therefore, investigating the role *Jnk3* plays during cortical interneuron migration and development is pertinent. Our studies indicate a genetic dosage requirement for JNK signaling in cortical interneurons. Loss of *Jnk3* in combination with loss of *Jnk1* and 2 leads to departure of interneurons from migratory streams and clustering of interneurons in the cortical plate, as well as defects in the columnar structure of the radial glial cells and radially migrating projection neurons.

Conclusions

It remains clear that JNK signaling is intricately involved in the early migration of both cortical interneurons and radially migrating projection neurons. However, the precise temporal and spatial activity of JNK, as well as the autonomous versus non-autonomous functions of JNK, remains unknown. Therefore, elucidating upstream activators and downstream effectors of JNK signaling in cortical interneurons will be essential to determine intrinsic functions of JNK in cortical interneuron development. Indeed, deciphering mechanisms of JNK function is imperative for better understanding and ultimately treating neurodevelopmental and psychiatric disorders that impinge upon cortical circuit function.

Figures

Figure 3.1: JNK-inhibition disrupts migratory stream integrity. Dlx5/6-CIE+ cortical interneurons travel tangentially in migratory streams under control (A-C) conditions. SP600125-treatment leads to dose-dependent dispersion from both SVZ and MZ streams at 20 μ M (D-F) and 40 μ M (G-I). Intermediate zone (arrows) fills with Dlx5/6-CIE+ interneurons (compare D, G to A; J) and interneuron orientation shifts from tangential (B, C) to diagonal or radial (E-F, H-I) after JNK

inhibition. Differences between bins were determined post hoc by Fisher's LSD tests (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). JNK inhibition leads to a shift in migratory trajectories as measured by angular divergence from the tangential plane in the cortical plate/intermediate zone region, but not in the subventricular zone. Differences were determined by Chi squared analysis: cortical plate (150-180 degrees, 20 μM : $p = 0.0003$; 40 μM : $p = 0.0052$; 180-210 degrees, 20 μM : $p = 0.031$; 40 μM : $p = 0.0483$); subventricular zone (90-120 degrees; 20 μM : $p = 0.0001$; 40 μM : $p = 0.0045$, 120-150 degrees, 40 μM : $p = 0.0088$; 150-180 degrees, 20 μM : $p = 0.0006$; 40 μM : $p = 0.0026$; 180-210 degrees, 40 μM : $p = 0.0419$ and 240-270 degrees, 20 μM : $p = 0.0598$; 40 μM : $p = 0.0028$; 270-300 degrees; 20 μM : $p = 0.0863$; 40 μM : $p < 0.0001$; and 330-360 degrees, 40 μM : $p = 0.0109$) (**K**)

Figure 3.2: Removal of the JNK inhibitor improves migratory streams integrity. Cortical interneurons disperse from migratory streams during 24 hours of 20 μM SP600125 (**D-F**) compared to controls (**A-C**). Washout of the SP600125 at 12 hours promotes recovery in the subventricular zone stream (**G-I, J**) after initial disruption. In addition, the cortical plate and intermediate zone regions show decreased accumulation (**J**) Differences between bins were determined post hoc by Fisher's LSD tests (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

Figure 3.3: Cortical interneuron streams show trends of recovery in the ventricular zone (VZ) after JNK inhibition at 40 μ M concentration. Cortical interneurons disperse from migratory streams during 24 hours of 40 μ M SP600125 (**D-F**) compared to controls (**A-C**). Washout of the SP600125 at 12 hours promotes recovery in the VZ region (**G-I, J**) after initial disruption. Differences between bins were determined post hoc by Fisher's LSD tests (**** p <0.0001, *** p <0.001, ** p <0.01, * p <0.05).

Figure 3.4: Tangential progression in migratory streams requires JNK activity. **A.** Dlx5/6-CIE+ interneurons travel in tangentially oriented migratory streams in control slices. **B.** Interneurons disperse from streams and vacate the SVZ within 12 hours of 20 μ M SP600125 exposure. **C.** SVZ-positioned interneurons in SP600125-treated slices travel slower and in a diagonal or radial trajectory compared to controls. **D.** Cortical interneurons spend more time and exhibit slower migration speeds in the cortical plate of JNK inhibited slices.

Figure 3.5: Cortical interneurons migrating within the SVZ of pharmacologically treated slices have decreased displacement and less straight tracks when compared to controls.

Figure 3.6: Genetic loss of JNK signaling from cortical interneurons disrupts migratory stream integrity. Cortical interneurons leave migratory streams during a period of 18 hours of *ex vivo* slice culture from embryonic day 14.5 in conditional triple knockouts compared to conditional double knockouts. Complete genetic loss of *Jnk* from interneurons leads to dispersion of cortical interneurons from the subventricular zone stream and accumulation in the cortical plate. Differences between bins were determined post hoc by Fisher's LSD tests (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

Figure 3.7: Cortical interneurons form clusters *in vivo* in *Jnk1^{fl/fl}; Jnk2^{-/-}; Jnk3^{-/-}; Dlx5/6-CIE* cortices at E15.5. Increasing allelic loss of *Jnk3* in addition to loss of *Jnk1* and *Jnk2* causes cortical interneurons to cluster in the cortical plate and intermediate zone streams (**A, B, C, dotted lines**). **A.** *Jnk1^{fl/fl}; Jnk2^{-/-}; Dlx5/6-CIE* embryos display intact and well infiltrated MZ, SVZ, and subplate streams. **B, C.** Clustered patches of cortical interneurons disrupt the MZ (**B2, C1-3, open arrows**) subplate stream (**B, C, solid arrows**) and the SVZ (**B, C; Astericks**) in the entry region of the cortex in *Jnk1^{fl/fl}; Jnk2^{-/-}; Jnk3^{+/-}; Dlx5/6-CIE* and *Jnk1^{fl/fl}; Jnk2^{-/-}; Jnk3^{-/-}; Dlx5/6-CIE*. Patches include rounded interneurons that appear non-migratory (**B2, C1-3**).

Figure 3.8: Cortical interneurons form clusters originating from the marginal zone stream. **Top row:** *Jnk1^{fl/fl}; Jnk2^{-/-}; Dlx5/6-CIE* cortices maintain an organized thin MZ stream with minimal accumulation of cortical interneurons within the cortical plate. **Bottom row:** *Jnk1^{fl/fl}; Jnk2^{-/-}; Jnk3^{-/-}; Dlx5/6-CIE* cortices accumulate patches of cortical interneurons in the MZ stream that continue to protrude down into the cortical plate as time elapses and recruit cortical interneurons migrating within the SVZ stream

Figure 3.9: Cortical interneurons in cTKO cortices accumulated faster and more abundantly than cortical interneurons in cTH and cDKO cortices over 12 hours. Significance levels after performing two-way ANOVA are denoted as follows: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

Figure 3.10: Cortical interneuron clustering in *Jnk1^{fl/fl}; Jnk2^{-/-}; Jnk3^{-/-}; Dlx5/6-CIE* cortices coincides with disruption to radial glia. Nestin labeling (**red**) reveals pial surface detachment of radial glial endfeet and incomplete columnar structure (**B, inset, solid arrows**) in *Jnk1^{fl/fl}; Jnk2^{-/-}; Jnk3^{-/-}; Dlx5/6-CIE* compared to *Jnk1^{fl/fl}; Jnk2^{-/-}; Dlx5/6-CIE* embryos (**A, inset, open arrows**). Neurons labeled with Hoechst in *Jnk1^{fl/fl}; Jnk2^{-/-}; Dlx5/6-CIE* are arranged in even columns (**A, inset, white solid lines**) unlike *Jnk1^{fl/fl}; Jnk2^{-/-}; Jnk3^{-/-}; Dlx5/6-CIE* which have lost their organization (**B, inset**).

Chapter 4: Summary and Future Directions

Summary

This work revealed a new and vital role for JNK signaling during early cortical interneuron migration. In chapter 2, we tested whether cortical interneurons migrating into the cortical rudiment required JNK signaling to enter and navigate the developing cerebral cortex. We used *ex vivo* slice pharmacology and *in vivo* genetic deletion to demonstrate that the loss of JNK signaling delayed the migration of cortical interneurons into the nascent cortical rudiment between E12.5 and E13.5. We established that this early delay is primarily due to elimination of *Jnk1*. Constitutive knockouts of *Jnk1* showed delayed migration of cortical interneurons in caudal sections of the embryonic cortex, and the loss of one allele of *Jnk2* in combination with *Jnk1* delayed interneuron migration in both rostral and caudal sections. Elimination of only *Jnk2* did not disrupt cortical interneuron migration, and loss of one allele of *Jnk1* in combination with deletion of *Jnk2* did not delay migration. Since dual constitutive deletion of *Jnk1* and *Jnk2* is embryonic lethal, we conditionally deleted *Jnk1* in combination with constitutive deletion of *Jnk2* using a *Dlx5/6*-CRE-IRES-EGFP (*Dlx5/6*-CIE) transgene to delete both *Jnk1* and *Jnk2* from cortical interneurons. Conditional double knockout (*Jnk1^{fl/fl}*; *Jnk2^{-/-}*; *Dlx5/6*-CIE) cortices had delayed migration compared to controls (*Jnk1^{fl/+}*; *Jnk2^{-/-}*; *Dlx5/6*-CIE). However, this delay was not determined to be solely autonomous, since in the absence of cortical guidance cues, JNK-deficient cortical interneurons migrated comparably to controls. Additionally, we found cortical interneuron streams to be disrupted. Cortical interneurons were not oriented tangentially within the SVZ stream and both the MZ and SVZ stream lacked integrity.

In chapter 3, we tested if JNK signaling was involved in the maintenance of cortical interneuron migratory streams. This study was completed between E14.5 and E15.5 when cortical interneuron streams are well-formed. Pharmacological blockade of JNK signaling in *ex-vivo* slice cultures at E14.5 showed evacuation of the SVZ stream with successive filling of the cortical plate, which was partially reversible when JNK inhibition was removed. Dynamic

evaluation of cortical interneurons through time-lapse imaging showed that JNK-inhibited cortical interneurons transitioned from tangential migration to diagonal or radial migration and exited the SVZ stream at a higher frequency than controls. Further, when JNK-inhibited cortical interneurons reached the cortical plate they spent more time there than control interneurons. Next we sought to investigate the consequences of *in-vivo* loss of JNK signaling on cortical interneuron stream spreading and found no differences between cDKO's and *Jnk1^{fl/+}; Jnk2^{-/-}*; *Dlx5/6-CIE* control cortices in E14.5 + 18 hours of growth slices. However, deletion of *Jnk3* in combination with *Jnk1* and *Jnk2* from cortical interneurons showed SVZ disruption and filling of the cortical plate that was similar to the SVZ dispersion and cortical plate filling seen in *ex vivo* slices during pharmacological inhibition using a pan-JNK inhibitor. In fact, the radial distribution of cortical interneurons lacking all three *Jnk* genes was almost identical to the distribution seen during pan-JNK inhibition. *In-vivo* analysis of E15.5 cortices lacking *Jnk3* in combination with *Jnk1* and *Jnk2* identified clusters of cortical interneurons within the cortical plate in the entry region of the cortex with subsequent disruption to the subplate stream. Further, exploration of cortical plate filling in cTKO cortices revealed disruption and aggregation of cortical interneurons within the MZ stream. Live imaging of *ex vivo* slices revealed that cortical interneurons from the MZ stream invaded the cortical plate and aggregated into clusters with cortical interneurons from the SVZ stream. This prompted us to look at the underlying architecture of the radial glia and the laminar allocation of projection neurons in the early cortical plate. Radial glial cells were disrupted along their radial axis. Decreased nestin labeling indicated loss of radial glial processes and disruptions to endfeet at the pial surface. The columnar organization of cells within the nascent cortical plate was also disrupted when compared to cDKO cortices.

This body of work suggests that JNK signaling plays an important role during the migration of cortical interneurons as they first enter the cerebral cortex and progress medially in streams of migratory cells. JNK mediates the integrity of migratory streams within the developing cortex,

potentially by orienting cortical interneurons. However, it still remains unknown which JNK genes are responsible for this to occur and how these genes work in conjunction with each other to produce progressive movement in streams.

We know from this work that *Jnk1* plays a dominant role in the progress of streams early on in migration, but this dominant role may change as the advancement of cortical interneuron streams in cDKO cortices catch up to *Jnk1^{fl/+}; Jnk2^{-/-}; Dlx5/6-CIE* controls by E15.5 (Appendix Figure A1). At E13.5, cortical interneurons in cDKO cortices showed delays in migration and disruption to their radial distribution whereas *Jnk1^{fl/+}; Jnk2^{-/-}; Dlx5/6-CIE* cortices did not show this delay or disruption. Therefore, at early time points, one copy of *Jnk1* is sufficient for normal migration. Two days later, at E15.5, the distribution and advancement of cortical interneurons is similar between cDKO and *Jnk1^{fl/+}; Jnk2^{-/-}; Dlx5/6-CIE*, which may indicate that the role of *Jnk1* in cortical interneuron migration might change over time. It is unclear if *Jnk1* could still play a role in the radial distribution of cortical interneurons within the developing cerebral cortex and if this influences the overall distribution of cortical interneurons in the mature cortex. The distribution of cortical interneurons in cDKOs and *Jnk1^{fl/+}; Jnk2^{-/-}; Dlx5/6-CIE* cortices remain similar into adulthood at postnatal day 21 (Appendix Figure A2). However, it is still not known if the laminar position of cortical interneurons in either the adult cDKOs and *Jnk1^{fl/+}; Jnk2^{-/-}; Dlx5/6-CIE* cortex differs from a wildtype control brain. If the layer distribution of cortical interneurons is disrupted, *Jnk1* and/or *Jnk2* may become increasingly important during migration. Loss of just one allele of *Jnk1* could be detrimental to interneuron migration and further laminar position within the cerebral cortex. If cortical interneurons are distributed normally in the adult cDKO, this could mean a switch in roles for these *Jnk* genes. Further, it is still unclear how and if *Jnk3* is involved in cortical interneuron migration and if it could be compensating for *Jnk1* or *Jnk2* at these timepoints.

To elucidate the role of *Jnk3* in interneuron migration, and to untangle the differential contributions of each of the *Jnk* genes, we began a series of studies on mice with *Jnk3* deletions. Preliminary results from constitutive genetic deletion of *Jnk3* at E13.5 show no differences between the advancement of cortical interneuron migratory streams in *Jnk3*^{+/-} and *Jnk3*^{-/-}. Therefore, *Jnk3* may not play a significant role in the early entry of cortical interneurons into the cerebral cortex when deleted independently of other *Jnk* genes (Appendix Figure A3). However, by E15.5 loss of *Jnk3* in combination with loss of *Jnk1* and *Jnk2* creates clustering of cortical interneurons and has detrimental effects on radial glia and the structure of the developing cortical plate. Additionally, we do not know if *Jnk3* compensates for the loss of *Jnk1* or the combinatorial loss of *Jnk1* and *Jnk2* at E13.5 or E15.5. Pharmacological blockade of JNK signaling at E12.5 prevented a large majority of cortical interneurons from entering the cerebral cortex. Therefore, the delayed entry and loose organization of cortical interneuron streams that is found in *Jnk1* and *Jnk2* deficient cortices could be due to compensation by *Jnk3*.

Future directions

While the current work breaks down the involvement of *Jnk1* and *Jnk2* during early migration and the additive effect of loss of *Jnk3* to *Jnk1* and *Jnk2* mutants in migratory stream integrity, further work is needed to elucidate the involvement of each individual *Jnk* gene throughout cortical development. It is clear from our work that the role of *Jnk1* and *Jnk2* changes throughout cortical interneuron migration, specifically between E13.5, when interneurons first enter the cerebral cortex, and E15.5, when the leading edge of cortical interneuron migration reaches the medial most aspect of the cortical rudiment (Chapter 2, Appendix Figure A1). Therefore, future experiments should focus on examining the cell type and developmental expression of each *Jnk* gene. This will help us further understand the role of each gene during the course of both tangential migration of cortical interneurons and radial migration of excitatory neurons during cortical development.

Experiments:

- In order to evaluate the expression of *Jnk* transcripts in cortical interneurons at different age points, flow sorting and/ or in-situ hybridization could be utilized.
 - o A time course using cell sorted cortical interneurons and respective cortices to investigate the expression levels of *Jnk* genes within cortical interneurons and the surround cortex to further understand the temporal changes of each *Jnk* gene during the cortical development using qRt-PCR.
 - o In-situ hybridization could be utilized to visualize the expression of each *Jnk* gene in single, double, and triple *Jnk* mutants. This method would help us to understand where each *Jnk* is expressed during each time point and which *Jnk* genes could be compensating for others in *Jnk* mutants.

Further, it still remains unclear how JNK signaling affects the temporal distribution of neurons within the cortex. Previous research has focused on the importance of JNK signaling in radial migration and development. Westerlund and colleagues (2011) demonstrated that constitutive loss of *Jnk1* enhances the migration of excitatory neurons into the cortical plate. Contrary to the function of *Jnk1*, Zhang and colleagues (2016) found that knockdown of *Jnk2* in migrating excitatory neurons slows migration, however found no effect was found when *Jnk3* was depleted. This effect may be due to where the Jnks are localized and function within the excitatory neurons. Previous research by Westerlund and colleagues showed that the localization of Jnk, cytosolic or nuclear, could have an effect on their migration as well. They found that knockdown of cytoplasmic JNK enhanced and knockdown of nuclear migration slowed the migration of excitatory neurons (Westerlund et al., 2011). Therefore, *Jnk1* may function to decrease migration and *Jnk2* might enhance migration during normal cortical development and there by mediate temporal aspects of cortical plate development. Cortical interneurons use cues from their environment, which includes other migratory neuronal

populations, in order to successfully navigate the cortical rudiment. Genetic ablation of *Jnk1* or *Jnk2* could not only disrupt radial migration, but also alter temporal aspects of cortical interneuron migration. Additionally, our work suggests that combinatorial loss of *Jnk2* and *Jnk3* from either radially migrating neurons or from interneurons could affect either population (Chapter 3). Future work should focus on not only the loss of *Jnk* from interneurons, but also combinatorial loss of *Jnk* from excitatory neurons. It is unclear whether the loss of *Jnk2* and *Jnk3* in our mutant cortices at E15.5 contributes to the disruption in columnar architecture and also the radial glial structure or if the loss of *Jnk1* from interneurons in combination with the constitutive loss of *Jnk2* and *Jnk3* causes the disruption to migrating excitatory neurons and their scaffolding.

Reelin is a glycoprotein secreted by Cajal-Retzius cells that prevents the entrance of migrating neurons into the MZ region and promotes the inside-out organization of excitatory neurons in the cerebral cortex (D'Arcangelo et al., 1995, 1997). Defects in the reelin pathway are known to cause epilepsy and lissencephaly among other severe neurological disorders (D'Arcangelo et al., 1995; Trommsdorff et al., 1999). The reelin pathway is activated by several extracellular receptors including ApoER2 and carried out by intracellular proteins such as Dab1 (Trommsdorff et al., 1999). ApoER2 recruits JNK interacting proteins (JIPs) to form a complex with JNK and with the intracellular proteins, Dab1 (Stockinger et al., 2000). Therefore, reelin could potentially activate JNK within migrating excitatory neurons in the developing cortex. Further, it is unknown whether the JNK-deficient mice express normal levels of reelin, but even if they do, the loss or disruption to JNK signaling in the JNK-deficient mutant mice could impair reelin signaling in the cTKO cortices, which could explain why we see disruption to the architecture of the cortical plate.

Forthcoming experiments should examine whether JNK signaling is required autonomously or non-autonomously for both interneurons and excitatory neuron migration. Further investigation

into the role of JNK signaling within migrating excitatory neurons might hint at an autonomous role for JNK within migrating excitatory neurons. If JNK signaling is required for excitatory neuron migration and is disrupted, this could potentially disrupt cortical interneuron migration in a non-autonomous manner.

Experiments:

Conditional triple knockout mice

- To determine the autonomous requirements for JNK for either cortical interneurons or excitatory neurons and the impact that eliminating JNK might subsequently have on the migration of the other cell population, we can create a conditional single, double, and triple mutant mice for *Jnk1*, *Jnk2* and *Jnk3* and breed in different cre lines to eliminate JNK in cortical inhibitory neurons (Dlx5/6-CIE, Somatostatin-cre, 5HT3aR-cre) and excitatory neurons (Emx1-cre). This would be important for understanding their role within cortical interneurons and allow us to resolve how the loss of JNK signaling within a certain type of neuron impacts the surrounding environment.

Slice culture transplant assays

- In order to test whether JNK-deficient interneurons can modify their environment, we can place JNK-deficient interneurons on to wildtype cortices to examine whether JNK signaling is required cell autonomously for cortical interneuron migration and whether JNK-deficient interneurons can alter the radial scaffolding in the host tissue, non-autonomously altering radial migration.
- Further, the reverse transplant experiment can be performed to determine if there are non-autonomous requirements for JNK signaling that influence cortical interneuron migration. Dlx5/6-CIE wildtype cortical interneurons will be added to JNK-deficient

cortices to determine if a JNK-deficient environment influences cortical interneuron stream formation and orientation.

- To determine the non-autonomous threshold for JNK signaling in both JNK-deficient interneurons and excitatory neurons, we will introduce JNK-deficient interneurons to JNK-deficient cortices. We can investigate the *Jnk* genes that are required by both interneurons and excitatory neurons non-autonomously to migrate properly within the developing cortex. If normal migration cannot be attained, we can document the phenotypes that are associated with loss of different *Jnk* genes in interneurons and excitatory neurons.

Electroporation to label radial glia

- In order to visualize and characterize radial glial morphology in our JNK-deficient cortices, we can electroporate a construct to label brain lipid binding protein (BLBP) in single, double, and triple mutants (Fen et al., 1994; Guo and Anton, 2014). Based on the morphology of radial glial cells labeled with an antibody for Nestin in our cTKO cortices, it is possible that we will see detached radial glial cell endfeet from the basement membrane or radial glial endfeet that do not reach the pial surface, and abnormal radial glial architecture and branching. Further, we could use the BLBP construct to label radial glia in the slice culture transplant assay (mentioned above) to visualize the behavior of the radial glia interacting with cTKO cortical interneurons in wild-type tissue.

Immunocytochemistry

- Antibody labeling for other cortical interneuron markers at E15.5 such as somatostatin
 - o Somatostatin neurons are primarily known to migrate within the MZ stream during development and are part of the first cohort to enter the cerebral cortex.

Therefore, it would be beneficial to see if somatostatin positive neurons are present in cortical interneuron clusters.

- Immunolabeling for markers of reelin in JNK-deficient tissue to look for ectopic or absent expression at E15.5
- Examine markers for radial glial endfeet and basal lamina at the pial surface such as BLBP, laminin, or cadherin at E15.5
- Expand time series to later time points (postnatal) to look at the consequences of the disruptions to radial glia and cortical plate
 - o Look for ectopic interneurons using antibodies for parvalbumin, somatostatin, VIP
 - o Immuno-label for layer markers to see if layers include the correct cell types

Breed new cre driver lines into triple knockout line

- In order to test if different cohorts of cortical interneurons require *Jnk1* in the same manner and behave the same when deleted in combination with other *Jnk* genes, we will use cre driver lines to knockout *Jnk1* and visualize different subsets of cortical interneurons. We can continue to examine E15.5 using different cre driver lines that will knockout *Jnk1* in different subsets of cortical interneurons such as somatostatin or 5HT3R to understand if the same phenotype as the *Dlx5/6-CIE* emerges in the triple knockouts. Using different cre driver lines will help us characterize the behavior of the ectopic cortical interneurons that aggregate in the cortical plate because the labeled cohorts will label fewer cortical interneurons.

Western blot for expression of reelin pathway proteins

- In order to determine if JNK-deficient mice express normal amounts of reelin and if the downstream intracellular proteins within the reelin pathway like Dab1 are expressed at normal levels we could run a western blot using single, double, and triple *Dlx5/6-CIE*

positive *Jnk* mutant cortical tissue. We could use cell sorting to separate Dlx5/6-CIE expressing cells (interneurons) and Dlx5/6-CIE non-expressing cells (all other cortical cells) and examine the level of the protein components of the reelin pathway in each cohort.

Previous studies suggest an autonomous requirement for JNK signaling through the CXCR4 pathway. The CXCR4/SDF-1 pathway is known to guide cortical interneurons within their migratory streams (Liapi et al., 2008; López-Bendito et al., 2008; Wang et al., 2011; Meechan et al., 2012; Abe et al., 2014). In the absence of this pathway cortical interneurons are known to disperse from migratory streams and layer in the cortical plate (Wang et al., 2011). CXCR4 can activate JNK signaling in breast cancer cells to promote invasion in response to SDF-1 (Serrati et al., 2008). However, there is currently no link between JNK signaling and *Cxcr4* in cortical interneurons. SDF-1 has been shown to modify the cytoskeleton through changes in *Dcx* (Lysko et al., 2014). Increased SDF-1 stabilizes microtubules and promotes tangential migration for cortical interneurons. In the absence of SDF-1, microtubules destabilize, which leads to an increase in branching to promote the exit of cortical interneurons from their migratory streams (Lysko et al., 2014). JNK signaling is known to modify changes within the cytoskeleton through *Dcx* to increase or decrease branching in PC12, N2A, and primary cerebellar neuron cultures (Gdalyahu et al., 2004). Our cDKOs of *Jnk1* and *Jnk2* show changes in the distribution of JNK-phosphorylated *Dcx* from the leading process to the soma, where it might destabilize microtubules and lead to increased branching (Appendix Figure A4). At E13.5, we found that cortical interneurons in the cDKOs showed increased branching and spreading of streams. Future studies should focus on *Cxcr4*'s activation of JNK and downstream regulation of *Dcx* as this may underlie the stream dispersion we see during pharmacological inhibition and *in-vivo* genetic deletion of JNK. JNK signaling levels have not been studied in CXCR4 mutant cortices. Therefore, future studies should look at *Cxcr4* expression in *Jnk* mutants and *Jnk* expression in

Cxcr4 mutants to investigate if there is a connection between JNK and Cxcr4 that regulates stream integrity through downstream activation by JNK.

Experiments:

Evaluate JNK expression in Cxcr4 mutants

- In order to test if JNK signaling is regulated by Cxcr4, we can breed Cxcr4 mutant mice with Dlx5/6-CIE and sort cortical interneurons from respective cortices to look at JNK expression in the absence of Cxcr4 signaling
- Additionally, we can look at the distribution of downstream targets within cortical interneurons especially Dcx and Jnk-phosphorylated Dcx using antibody labeling as well as examine the expression levels of Jnk-phosphorylated Dcx through cell sorting.

Evaluate expression of Cxcr4, SDF-1, and Dcx in *Jnk* mutants

- In order to evaluate whether the expression of Cxcr4 is altered in JNK-deficient cortical interneurons, we can antibody label for Cxcr4 in single, double, and triple mutant cortices to look for up or down regulation of Cxcr4 and to look for distribution changes within the cortical interneurons. Additionally, we can sort Dlx5/6-CIE cortical interneurons and look at changes in expression of Cxcr4.
- In-situ hybridization to look at expression of SDF-1 in single, double, and triple mutant cortices
- Antibody labeling for Dcx and Jnk-phosphorylated Dcx in single, double, and triple mutant cortices and cell sorting comparing the expression of Dcx and Jnk-phosphorylated Dcx between Dlx5/6-CIE positive cortical interneurons and Dlx5/6-CIE negative tissue.

Electroporation to label Dcx

- Create a pCIG3-mCherry-Dcx fusion plasmid that will be electroporated into Dlx5/6 or JNK-deficient slices at embryonic day 13.5. This will allow us to simultaneously label interneurons with cytoplasmic GFP and visualize the subcellular localization of red-fluorescent Dcx within migrating interneurons. In our single, double, and triple *Jnk* mutants we will look to see if the localization of Jnk-phosphorylated Dcx has shifted from the leading process to another location within cortical interneurons such as the cell body like we have found in our cDKOs.

Last of all, calcium signaling in migrating neurons is extremely important for nucleokinesis and might modulate portions of the cytoskeleton (Moya and Valdeolmillos, 2004). Although calcium signaling has not been extensively examined in our *Jnk* mutants, there is a decrease in calbindin labeling in cortical interneurons during early development in cTKO cortices (Appendix Figure A5). Calbindin labeling is confined primarily to the cell body of labeled cortical interneurons, and absent from axonal fibers extending from the developing cortical plate (Appendix Figure A5). Calbindin acts within cortical interneurons as a calcium buffer by sequestering free calcium (Baimbridge et al., 1992). Lower expression of calbindin could indicate that JNK signaling could influence the availability of calcium within cortical interneurons. Intracellular calcium is important for nucleokinesis (Moya and Valdeolmillos, 2004). Increases in calcium signaling near the proximal end of the leading process was shown to co-localize with tubulin and suggested to interact with Dcx through activation of calcium/calmodulin kinases (Moya and Valdeolmillos, 2004). Previous research indicates that calcium/calmodulin kinases can activate the MAPK pathway specifically the JNK pathway suggesting that increased calcium could signal through JNK to influence cytoskeletal architecture within the leading process (Enslen et al., 1996; Kim and Sharma, 2004). Further, calcium transient modulation within cortical interneurons has been linked to the expression of the potassium-chloride transporter, KCC2, and GABA_A and AMPA/NMDA receptor mediated depolarization (Bortone and Polleux, 2009). Migrating cortical interneurons were found to exhibit frequent calcium transients during

knockdown of KCC2 in 2D interneuron cultures (Bortone and Polleux, 2009). Moreover, these calcium transients were further mediated by the expression of GABA_A and AMPA/NMDA receptors through L-type voltage sensitive calcium channels (Bortone and Polleux, 2009). GABA_A and AMPA/NMDA receptor depolarization and knockdown of KCC2 lead to increased cortical interneuron motility and migration (Bortone and Polleux, 2009). Conversely, increased expression of KCC2 and decreased GABA_A and AMPA/NMDA receptor depolarization lead to decreased cortical interneuron movement (Bortone and Polleux, 2009). If the cortical interneurons in cTKO cortices fail to appropriately utilize calcium, these studies could explain why we see cortical interneurons aggregating within the cortical plate. This research suggests that JNK could influence cortical interneuron migration (motility and speed) through calcium signaling and could do so by influencing the expression of KCC2, GABA_A, and AMPA/NMDA receptors and regulating the cytoskeleton through activation by calcium/ calmodulin kinases.

Experiments:

Live imaging of MGE explants with a calcium sensor

- In order to understand calcium dynamics in the JNK-deficient cortical interneurons, MGE explants can be grown in collagen and/or Matrigel. Dlx5/6-CIE wild-type cortical interneuron MGE explant cultures will be used as controls to compare the calcium dynamics in cDKO and cTKO explants. Fura-2, a calcium sensing dye, will be added to the explants to visualize movement of calcium in cortical interneurons migrating away from the explant. Explants will be imaged live on a Zeiss 710 confocal microscope and cortical interneuron calcium dynamics, motility, and branching dynamics will be assessed via Bitplane's Imaris software.

Calcium modulation of the cytoskeleton in the absence of JNK

- Use photo-activatable JNK inhibitors to remove JNK signaling from cortical interneurons migrating from MGE explants in conjunction with the addition of Fura-2 to see if there is a change in calcium distribution within migrating cortical interneurons while imaging the explants live on a confocal. When imaging is complete immuno-label for cytoskeletal components, like Dcx and tubulin, which might have been altered with the removal of JNK.

Expression of KCC2, GABAA, and AMPA/NMDA receptors

- In order to determine if the expression levels of KCC2 transporter, GABA_A, and AMPA/NMDA receptors could be the reason we see aggregation of cortical interneurons within the cortical plate, we will use cell sorting to assess the transporter and receptor expression levels in Dlx5/6-CIE, cDKO, and cTKO in cortical interneurons by comparing the Dlx5/6-CIE positive fractions.

Final Thoughts

A wide variety of neurodevelopmental disorders impacting cortical circuitry have been ascribed to deficits in GABAergic interneurons. The recognition of disorders affecting the assembly and function of inhibitory GABAergic interneuron circuitry has increased in the past decade. It is still unclear how cortical interneurons use and process guidance cues to migrate, organize into laminae, and make connections with surrounding neurons. Therefore, it is imperative that researchers continue to investigate the underlying pathologies beginning with early embryonic development. In this work, we have identified a neurodevelopmental role for the JNK signaling pathway in regulating and mediating cortical interneuron entry into the cerebral cortex, as well as maintaining stream integrity. Although downstream mechanisms are yet to be determined, future work will have major impacts on how we understand the pathology of each of these

diseases and will build on and connect previous research together linking pathways that are already implicated in causing neurological disease. Pinpointing the exact role of JNK signaling in cortical interneuron migration will give researchers and medical professionals a better understanding of the underlying causes of cortical disorders like autism, schizophrenia, and epilepsy, as well as create avenues for development of further treatment of these disorders.

Appendix

Figure A1: *Jnk1^{fl/+}; Jnk2^{-/-}; Dlx5/6-CIE* and *Jnk1^{fl/fl}; Jnk2^{-/-}; Dlx5/6-CIE* cortices display similar advancement and radial distribution at E15.5. Although, interneuron delays were previously seen between *Jnk1^{fl/+}; Jnk2^{-/-}; Dlx5/6-CIE* and *Jnk1^{fl/fl}; Jnk2^{-/-}; Dlx5/6-CIE* cortices at E13.5, at E15.5, the advancement of cortical interneurons seems to even out (**A solid arrow; B, solid arrow**). Further, the radial distribution of cortical interneurons in *Jnk1^{fl/+}; Jnk2^{-/-}; Dlx5/6-CIE* (**C**) and *Jnk1^{fl/fl}; Jnk2^{-/-}; Dlx5/6-CIE* (**D**) cortices is not different. Quantification of the radial distribution of cortical interneurons revealed no statistically significant differences (**E**).

Figure A2: Young adult *Jnk1^{fl/+}; Jnk2^{-/-}; Dlx5/6-CIE* and *Jnk1^{fl/fl}; Jnk2^{-/-}; Dlx5/6-CIE* cortices share similar distributions of cortical interneurons. Sections from three **(A,1-3)** rostro-caudal levels were stained, imaged, and quantified from postnatal day (P)21 brains. Cortices were divided into a medial, central, and lateral partition **(B)**. Each partition was equidistantly binned from the pial surface to the corpus callosum **(B)**. The distribution of Dlx5/6-CIE positive cortical interneurons did not differ between *Jnk1^{fl/+}; Jnk2^{-/-}; Dlx5/6-CIE* and *Jnk1^{fl/fl}; Jnk2^{-/-}; Dlx5/6-CIE* cortices in medial, central, or lateral partitions **(C1, D1, E1)**. Parvalbumin cortical interneurons expressing Dlx5/6-CIE showed a similar distribution to the Dlx5/6-CIE positive population of cortical interneurons and no differences were found in the medial, central, and lateral partitions **(C2, D2, E2)**.

Figure A3: Similar to *Jnk2*, genetic ablation of *Jnk3* at E13.5 does not disrupt cortical interneuron migration. The advancement of calbindin labeled cortical interneurons did not differ between the *Jnk3*^{+/-} (A, D) and the *Jnk3*^{-/-} (B, E) at the rostral or caudal locations. Quantification of the advancement of calbindin positive cortical interneurons showed no statistical differences between the *Jnk3*^{+/-} and *Jnk3*^{-/-} in the rostral (C) and caudal locations (F).

Figure A4: Jnk-phosphorylated Dcx labeling is shifted to the cell body in cortical interneurons in *Jnk1^{fl/fl}; Jnk2^{-/-}; Dlx5/6-CIE* cortices at E13.5. Cortical interneurons migrating within the SVZ stream primarily express Jnk-phosphorylated Dcx in their leading process (**middle left**). However, in the absence of *Jnk1* and 2, Jnk-phosphorylated Dcx heavily labels the cell bodies of SVZ stream cortical interneurons (**middle right**).

Figure A5: Calbindin labeling is reduced in cortical interneurons and absent in axon projections at E15.5 in *Jnk1^{fl/fl}; Jnk2^{-/-}; Jnk3^{-/-}; Dlx5/6-CIE* cortices. Axon projections labeled in *Jnk1^{fl/fl}; Jnk2^{-/-}; Dlx5/6-CIE* (A, solid arrows; B, dotted lines) are absent in *Jnk1^{fl/fl}; Jnk2^{-/-}; Jnk3^{-/-}; Dlx5/6-CIE* cortices (C, solid arrows; D, dotted lines). Calbindin labeling in *Jnk1^{fl/fl}; Jnk2^{-/-}; Jnk3^{-/-}; Dlx5/6-CIE* cortical interneurons is limited to the cell body with sparse labeling of leading processes (D, open arrows) unlike *Jnk1^{fl/fl}; Jnk2^{-/-}; Dlx5/6-CIE* cortices where the majority of cortical interneuron leading processes are labeled (B, open arrows).

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Curriculum Vitae

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Education

Doctor of Philosophy in Neuroscience, May 2017
West Virginia University, Morgantown, WV
Mentor: Eric S. Tucker, PhD

Certificate in University Teaching, May 2014
West Virginia University, Morgantown, WV

Masters of Science in Biomedical Sciences, May 2010
Lake Erie College of Osteopathic Medicine, Erie, PA
Mentor: Randy J. Kulesza, PhD

Post Baccalaureate Program, April 2009
Lake Erie College of Osteopathic Medicine, Erie, PA

Bachelor of Science in Kinesiology, August 2006
Pennsylvania State University, University Park, PA

Published Papers

Cortical interneurons require *Jnk1* to enter and navigate the developing cerebral cortex

Myers, A.K., Meechan, D.W., Adney, D.R., Tucker, E.S.
J. Neurosci. 2014 Jun 4;34(23):7787-801.

- Selected for cover art

Prenatal diagnosis of congenital femoral deficiency and fibular hemimelia

Radler C., **Myers A.K.**, Hunter, R., Arrabal P.P., Herzenberg J.E., Grill F.
Prenat. Diagn. 2014 Oct; 34(10): 940-945.

Neonatal conductive hearing loss disrupts the development of the Cat-315 epitope on perineuronal nets in the rat superior olivary complex.

Myers A.K., Ray J., Kulesza R.J. Jr.
Brain Res. 2012 Jul 17;1465:34-47.

Maternal attitudes towards prenatal diagnosis of idiopathic clubfoot.

Radler C., **Myers A.K.**, Burghardt R.D., Arrabal P.P., Herzenberg J.E., Grill F.
Ultrasound Obstet Gynecol. 2011 Jun;37(6):658-62.

Accepted Abstracts

JNK Signaling Maintains the Integrity of Cortical Interneuron Migratory Streams During Corticogenesis

Myers, A.K., Baker, K., Snow, J.P., Hickling, S.E., Smith, C.A., Tucker, E.S

- Society for Neuroscience, Chicago, IL
 - 2015 Poster Presentation

Cortical Interneurons Require JNK Signaling to Preserve Migratory Streams During Corticogenesis

Myers, A.K., Baker, K., Snow, J.P., Smith, C.A., Tucker, E.S.

- Van Liere Research Day, West Virginia University, Morgantown, WV
 - 2015 Oral Presentation, awarded first place

Cortical Interneurons Require *Jnk1* to Enter and Navigate the Developing Cerebral Cortex

Myers, A.K., Meechan, D.W., Adney, D.R., Tucker, E.S.*

- Gordon Research Conference, Neural Development, Salve Regina University, Newport, RI
 - 2014 Poster Presentation

Cortical Interneurons Require JNK Signaling to Preserve Migratory Streams During Corticogenesis

Myers, A.K., Snow, J.P., Tucker, E.S.

- Van Liere Research Day, West Virginia University, Morgantown, WV
 - 2014 Poster Presentation, awarded second place

Cortical Interneurons Require C-Jun N-Terminal Kinase Signaling to Enter and Navigate the Developing Cerebral Cortex

Myers, A.K., Reilly, E.C., Meechan, D.W., PhD, Adney, D.R., LaMantia, A-S., Tucker, E.S.*

- Gordon Research Conference, Neural Development, Salve Regina University, Newport, RI
 - 2012 Poster Presentation

Cortical Interneurons Require C-Jun N-Terminal Kinase Signaling to Enter and Navigate the Developing Cerebral Cortex

Myers, A.K., Reilly, E.C., Meechan, D.W., Adney, D.R., LaMantia, A-S., Tucker, E.S.

- Appalachian Regional Cell Conference, Charleston, WV
 - 2012 Poster Presentation, awarded Best in Group Poster Presentation

Cortical Interneurons Require *Jnk1* and *Jnk2* to Enter and Navigate the Cerebral Cortex

Myers, A.K.[†], Tucker, E.S.*[‡], Meechan, D.W., Adney, DR, LaMantia, A-S

- Society for Neuroscience, Washington, DC and San Diego, CA
 - 2011*, 2013[†]. 2014[†] Poster Presentation

Jun Kinase Signaling is Required for Interneuron Migration into the Cerebral Cortex

Myers, A.K., Tucker, E.S., Meechan, D.W., Adney, D.R., LaMantia, A-S.

- Van Liere Research Day, West Virginia University, Morgantown, WV
 - 2012 Poster Presentation

Development of Perineuronal Nets in the Rat Auditory Brainstem

Myers, A.K., Kulesza, R.J. Jr.

- Lake Erie College of Osteopathic Medicine (LECOM) Research Day, Erie, PA
 - 2009 Poster Presentation

Relapse of Idiopathic Clubfoot After the Ponseti Method

Myers, A.K., Burghardt, R., Sivaramalingam, S., Lamm, B.M.*, Herzenberg, J.E.

- American College of Foot and Ankle Surgeons Annual Meeting
 - 2009 Oral Presentation, * Honorable Mention Award

Maternal Experience with the Prenatal Diagnosis of Idiopathic Clubfoot

Radler, C., **Myers, A.K.**, Burghardt, R., Arrabal, P.P., Herzenberg, J.E.*, Grill, F.

- Pediatric Orthopedic Society of North America (POSNA) Annual Meeting
 - 2009 Oral Presentation*
- American Orthopedic Association (AOA) Annual Meeting
 - 2009 Poster Presentation*

Invited Lectures

Cortical Interneurons Require c-Jun N-Terminal Kinase (JNK) Signaling to Preserve Migratory Stream Integrity during Corticogenesis

November 21, 2014

Lake Erie College of Osteopathic Medicine, Erie, PA

The Role of JNK Signaling in the Developing Cortex

March 10, 2014

Lake Erie College of Osteopathic Medicine, Erie, PA

Teaching Experience

Graduate Medical Neuroanatomy, NBAN 793B

Spring 2015

Neurobiology and Anatomy, West Virginia University, Morgantown, WV

- Instructor
 - Assisted with gross brain labs
 - Lectures: Autonomic Nervous System, Connectopathy, Emotion

Cellular Methods, BMS 706

Fall 2012-Fall 2015

Biomedical Sciences. West Virginia University, Morgantown, WV

- Instructor, Model Systems (2012-2014)
 - Created a lesson, homework, and review clicker questions
- Instructor, Immunohistochemistry and Microscopy (2014)
- Journal club facilitator (2015)

Anatomy and Physiology 2, NBAN 207

Fall 2012

Neurobiology and Anatomy, West Virginia University, Morgantown, WV

- Laboratory Teaching Assistant
 - Assisted students at the cadavers by helping them locate structures and understand functions of identified organs and tissues

Neuroanatomy

Spring 2014, 2015

Anatomy, Lake Erie College of Osteopathic Medicine, Erie, PA

- Instructor, Basic Neurohistology
 - Created a lesson and exam questions

Honors & Awards

WVU Foundation Distinguished Doctoral Scholarship

2016 West Virginia University, Morgantown, WV

First Place in Oral Presentation

2015 Van Liere Research Day, West Virginia University, Morgantown, WV

Second Place in Poster Presentation

2014 Neuroscience Retreat, Oglebay Resort, Wheeling, WV

Second Place in Poster Presentation

2014 Van Liere Research Day, West Virginia University, Morgantown, WV

Best in Group Poster Presentation

2012 Appalachian Regional Cell Conference, Charleston, WV