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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**INVESTGATING BRAIN DEVELOPMENT WITH FORWARD AND REVERSE
GENETICS**

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Biology

by

Stephanie Lee Bielas

Committee in charge:

Professor Richard Firtel, Chair
Professor Joseph Gleeson
Professor Anivan Ghosh
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2007

The dissertation of Stephanie Lee Bielas is approved,
and it is acceptable in quality and form for publication on
microfilm:

Chair

University of California, San Diego

2007

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FIELDS OF STUDY

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ABSTRACT OF THE DISSERTATION
Investigating Brain Development with Forward and Reverse Genetics

by

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Doctor of Philosophy in Biology

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Formation of the mammalian brain requires the coordination of numerous events during embryonic development, the complexity of which is illustrated by the large number of defects that have been identified in humans. Analysis of both human genetic disorders and reverse genetically engineered mice have been invaluable in the endeavor to understand the molecular mechanisms underlying proper cortical development. Likewise, forward genetic screens have the potential of generating novel mouse models for human disorders, which can contribute by identifying novel genes and provide resources to investigate the molecular mechanisms of disease phenotypes. This study demonstrates that a genetic screen of ENU mutagenized mice identified a mouse pedigree that exhibits recessive primary microcephaly. Genetic mapping has identified a novel microcephaly locus for this mutation on chromosome 5qB1. Alternatively,

doublecortin was identified as the gene disrupted in the human female disorder double cortex and the male disorder X-linked lissencephaly. This study identified spinophilin as a novel binding partner for doublecortin and used single and double knockouts of spinophilin and doublecortin to investigate the role of this interaction in cortical neurons. This approach allowed us to propose a novel model for microtubule condensation at the transition zone between the growth cone and neurite shaft, in this way helping to further our understanding of the role doublecortin plays in neuronal migration and axon outgrowth.

I. INTRODUCTION

Mammalian Brain Development

In vertebrates, the anterior neural epithelium of the neural tube undergoes morphological subdivisions to generate vesicle-like structures known as the prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain). The prosencephalon becomes further divided into the telencephalon and diencephalon. The telencephalon gives rise to the cerebral cortex, basal ganglia, and hippocampus, whereas the diencephalon develops into the thalamus, epithalamus, and pretectum in the mature brain. Thus the telencephalon and diencephalon are the embryonic anlagen of higher cognition and integration centers in the brain. Although these two regions of the forebrain are functionally linked, their structural organization is distinct (Rallu et al., 2002). In mammals, the thalamus consists of clusters of neurons organized into nuclei, which are morphological and functional unit, whereas the cerebral cortex is a laminar sheet of neurons where each layer serves specific function (Lim and Golden, 2007). Therefore, one can presume that distinct developmental strategies must be utilized by these two brain regions.

Neural Proliferation

Precise patterns of cell division and migration are crucial to transform the neuroepithelium of the embryonic neural tube into the adult brain. The orientation of the cleavage plane in relation to the apical-basal polarity of the pseudostratified neuroepithelium determines the symmetry of the division with vertical cleavages

resulting in symmetric cell divisions and horizontal cleavages leading to asymmetrical divisions (Chenn and McConnell, 1995). Neurogenesis occurs through a combination of several modes of cell division including symmetrical progenitor cell divisions that expand the pool of neuronal precursor cells, asymmetrical progenitor divisions that give rise to single neurons and symmetrical terminal divisions, where a progenitor cell divides to produce two neurons, thus depleting the pool of proliferative cells (Cai et al., 2002; Gotz and Huttner, 2005; Takahashi et al., 1996). The basal boarder of the neuroepithelium lines the ventricles and this region of proliferation is referred to as the ventricular zone (Parnavelas, 2000). As neurons are generated in the ventricular zone they migrate out of this region to form adult brain structures.

Cerebral Cortical Development

One of the key steps in the development of the cerebral cortex is neuronal migration. The cerebral cortex consists of six layers of neurons that have distinct morphological and functional identities. The development of these neuronal layers involves the migration of neurons in radial and tangential directions to the final laminar position. Neuronal migration to the cerebral cortex occurs during days 11-18 of embryonic development in mouse, and between weeks 10-20 in human and occurs in three basic stages. In the first stage known as the preplate stage, the cortex primordial consists of only two layers: the proliferative zone and the preplate (Rickmann and Wolff, 1981). The preplate layer likely derives from the first wave of migration of neurons out of the ventricualr zone that migrate outward towards the apical surface.

Coincident with this, there is the appearance of a specialized population known as the Cajal-Retzius cell, that may have its origin in the proliferative zone, or may have its origin in a specialized proliferative zone at the CNS midline (Meyer and Wahle, 1999; Monuki et al., 2001). The Cajal-Retzius cells play important roles in subsequent stages of development.

In the second stage, known as the cortical plate stage, waves of postmitotic neurons exit the ventricular zone and move in a radial direction towards the pial surface, establishing the cortical plate within the preplate. This new layer of cells will eventually become the cortical plate and contribute to layers 2-6 of the cortex. The cortical plate neurons split the preplate into a superficial marginal zone and a deeper subplate (Marin-Padilla, 1978). In this way, the second neuronal wave creates a layer between the marginal zone and the subplate, known as the cortical plate. Between E14-E18 in mouse and week 12-20 in human, sequential waves of cortical plate neurons migrate from the proliferative zone into the cortical plate. One of the most distinctive features of this formation is the ‘inside-out’ generation of the cortex, revealed by birthdating studies, which show that early-born neurons constitute the deeper layers, whereas later-born neurons occupy the more superficial layers (Angevine, 1961; Berry, 1965). Therefore, the more superficial layer neurons migrate through the subplate and past all of the previously deposited neurons of the cortical plate to achieve positioning. Additionally, we can infer that each wave of neurons comes into contact with the marginal zone and might encounter some sort of signal to stop migration.

A key observation is that most if not all GABAergic neurons that populate the cerebral cortex are generated from the proliferative zones of the medial and lateral geniculate nuclei. These neurons initially migrate tangentially (parallel to the surface of the neocortex) rather than radially (Anderson et al., 1997). This tangential migration occurs within several different zones in the developing cortex. Strikingly, neurons destined for a particular layer of the neocortex, whether the neuron originated in the neocortical or subcortical proliferative zone of the LGE/MGE, are generated on or around the same date (Valcanis and Tan, 2003). Therefore, before the commencement of long-distance tangential migration, interneurons are already specified with respect to their future layer addresses.

Diencephalic Development

The key feature of diencephalic development is the formation of nuclei along the anterior-posterior and dorsal-ventral axes. In the diencephalons, neural progenitor cells undergo proliferation in the ventral zone of the third ventricle. Once they exit the cell cycle, post mitotic cells migrate to the mantle zone and aggregate into various clusters called nuclei. Differentiated neurons in each nucleus have distinct morphologies, employ specific neural transmitters, and generate neural connection to different regions of the brain (Jones, 1997). In order to build accurate neural circuits, it is essential to form each nucleus in the correct location and at the appropriate time in development. Based on birthdating studies in mammals, it has been hypothesized that the progenitor cells in the ventricular zone are organized as mosaic patches, each giving

rise to neurons in specific nuclei of the diencephalon (Altman and Bayer, 1988). Thus, it has been hypothesized that the initial requirement for the accurate allocation of each nucleus is the precise establishment of the AP and DV identities in the neural progenitor cells (Hashimoto-Torii et al., 2003).

Maturation and Process Outgrowth

Neurons are morphologically characterized by the presence of one or more long, thin neurites that differentiate into dendrites and an axon. Both cytoskeletal networks are thought to fulfill separate, yet coordinated cellular roles in neurite initiation. The highly dynamic actin network is known for its multiple roles in force generation during cell spreading and contraction (Schaefer et al., 2002). The more stable microtubule cytoskeleton is best known for its parallel and antiparallel arrays that might act as compression resistant struts inside the neurite (Ingber, 1993). During neurite initiation cultures neurons extend lamellipodium around the periphery of the cell soma. As neurites form the lamellipodia segments extend away from the cell soma to form a growth cone, concurrent with microtubule advance into the initiation site and formation of an ordered microtubule array. The newly formed protrusion then elongates and microtubules become tightly packed into parallel arrays inside the nascent neurite shaft (Dehmelt and Halpain, 2004). Actin-microtubule interactions are observed in lamellipodium throughout outgrowth as microtubules preferentially grow along actin bundles in filopodia, suggesting a physical link between the structures exist.

The final stage of brain development is maturation, where neurons whose cell bodies have recently reached their adult locations within the brain extend processes and form synaptic connections throughout the CNS. The corpus callosum is the largest interhemispheric commissure. It forms between the 8 and 20 weeks of gestation in humans and between embryonic day 15 and 17 in mice and consists of fascicles of axons from neurons positioned in predominantly in layer 2/3 and 5, but all cortical layers show evidence of projections to the callosum (Yorke and Caviness, 1975). The commissural axons from the cortex are attracted to the midline by chemoattractants of the phylogenetically conserved netrin family and extend through a corridor defined by two midline cell populations, the glial wedge and the indusium griseum (Shu and Richards, 2001; Stein and Tessier-Lavigne, 2001; Tessier-Lavigne and Goodman, 1996). Callosal axons decussate across the midline around embryonic day 16 and do not reach their target until around postnatal day 2 to 3 (Rash and Richards, 2001).

Human Disorders and Mouse Models

The human brain is the seat of consciousness and the means by which we carry out abstract reasoning. Understanding how the brain is assembled during embryonic development will give deeper insights into molecular mechanisms that could provide the basis for therapy and repair. Although a diversity of approaches will be needed to answer all of our questions, an important starting point in studying events in development is often careful analysis of mutant phenotypes. Much of what we know

about brain development has emerged through the study of mutations in mouse and humans.

Defects in Neuroproliferation

Alterations to proliferation and cell death that occur during neurogenesis have been shown to be a major determinant of mammalian brain size. Primary microcephaly is a human disorder that is characterized by a substantial reduction in size of the cerebral cortex and a generalized reduction in size of the remainder of the central nervous system, but with normal cytoarchitecture (Mochida and Walsh, 2001). While there are six loci for autosomal recessive primary microcephaly, four genes have been identified: autosomal recessive primary microcephaly 1 (*MCPH1*), abnormal spindle-like, microcephaly associated (*ASPM*), cyclin-dependent kinase 5 regulatory subunit-associated protein 2 (*CDK5RAP2*) and centromere protein J (*CENPJ*). The essential role of closely regulated neuronal proliferation in this disorder is highlighted by the fact that all four proteins are cell-cycle associated, with three being centrosomal components that apparently have diverse roles that affect mitosis. The *MCPH1/Microcephalin* gene was positionally cloned from two consanguineous Pakistani families and is an infrequent cause of microcephaly (Jackson AP et al. 2002, Roberts E et al 2002, Kumar A et al. 2004). *MCPH1/Microcephalin* has been shown to be a cell cycle regulator through its role in chromosome condensation and DNA (Trimborn et al., 2006) (Xu et al., 2004). The cytogenetic phenotype of prophase-like cells is highly condensed chromosomes within an intact nuclear envelope that is due to premature onset of chromosome condensation during the G2 phase of the cell cycle (Neitzel et al., 2002).

Depletion of MCPH1/microcephalin using small interfering RNA (siRNA) recapitulates this phenotype, indicating that MCPH1/microcephalin is integral to this process (Trimborn et al., 2004). It has been established using patient cells that the decondensation of chromosomes that occurs after completion of mitosis is also delayed. Therefore, MCPH1/microcephalin might be a negative regulator of chromosome condensation (Trimborn et al., 2006).

ASPM, *CDK5RAP2* and *CENPJ* encode centrosomal components, emphasizing the importance of the centrosome in neurogenesis. *ASPM* mutations are the most common cause of microcephaly while mutations in *CDK5RAP2* and *CENPJ* are rare (Badano et al., 2005; Hung et al., 2000; Kumar et al., 2004; Roberts et al., 2002; Trimborn et al., 2005). *ASPM* is predicted to contain one N-terminal microtubule-binding domain that is responsible for mediating its ability to organize microtubules at the spindle pole during mitosis and at the central spindle during cytokinesis (Cox et al., 2006). Both *CDK5RAP2* and *CENPJ* are associated with the γ -tubulin ring complexes within the centrosome, which are responsible for the production of microtubules that form the mitotic spindle (Li and Kaufman, 1996; Megraw et al., 1999; Terada et al., 2003). *CDK5RAP2* is hypothesized to affect neurogenic mitosis by reducing the availability of the microtubules that are needed to build the mitotic spindle and astral microtubule network while *CENPJ* is suggested to inhibit microtubule nucleation and depolymerise microtubules (Cox et al., 2006; Hung et al., 2004). While there is strong human genetic evidence for the role of neuroproliferation in controlling brain size the mechanisms by which this is achieved is still to be determined. It is yet to be

determined if microcephaly genes are modulating expansion of the neural progenitor pool or are involved in the decision to switch from symmetric to asymmetric cell division. Mouse models will be important for further analysis of these issues. Concurrently, it has been demonstrated that increasing the proportion of cycling progenitor cells is sufficient to increase cortical surface area, producing a mouse cerebral cortex with sulci and gyri (Chenn and Walsh, 2002).

Cortical Migration and Lamination

Genetic evidence suggests that proper orientation of the microtubule cytoskeleton and regulation of microtubule associated proteins are necessary for migration of cortical neurons. Few examples accentuate this more profoundly than the human brain developmental disorder Lissencephally. Lissencephally is a cortical migration malformation that is characterized by a lack of cortical folds on the surface of the brain. In lissencephally the cortex is thicker while the normal six layers of the cortex is reduced to four layers consisting of loosely packed cells (Barkovich et al., 1991; Dobyns and Truwit, 1995). Despite a thickening of the cortex the overall brain size tends to be smaller with enlarged ventricles (Allanson et al., 1998). These characteristics are presumed to be the result of a disruption in neuronal migration in which neurons are prevented from reaching their proper orientation in the cortex. Patients with this disorder suffer from severe epilepsy and mental retardation and usually die at a young age (Dobyns et al., 1992). Lissencephally is caused by mutations in Lissencephally 1 (Lis1) (Dobyns et al., 1993; Reiner et al., 1993) and the X-linked gene doublecortin

(Dcx) (des Portes et al., 1998b; Gleeson et al., 1998). Both the LIS1 and DCX are microtubule binding proteins and disruption of their microtubule associated activities is detrimental to cortical development.

LIS1 was the first lissencephaly gene to be cloned (Reiner et al., 1993). LIS1 resembles the β -subunit of the heterotrimeric G-protein and possesses 7 WD (tryptophan/aspartic acid) repeats, domains that mediate protein-protein interactions and are shared by several families of proteins (Garcia-Higuera et al., 1996; Neer et al., 1994). LIS1 was identified as the noncatalytic subunit of platelet-activating factor acetylhydrolase isoform 1b (Hattori et al., 1994), however, the role of LIS1 in cortical development was subsequently characterized by its ability to bind to and regulate microtubule catastrophe (Sapir et al., 1997). Recent evidence of LIS1 regulation of cytoplasmic dynein function has expanded our understanding of the possible functional mechanisms of LIS1 during cortical development (Faulkner et al., 2000; Smith et al., 2000).

Transgenic mouse models elegantly demonstrate the dosage dependent effects of LIS1 on cortical development. Four distinct *Lis1* mutant alleles have been generated, two of which are null alleles and two of which are partial loss-of-function alleles (Cahana et al., 2001; Hirotsune et al., 1998). As the dosage of LIS1 decreases, the severity of the *Lis1* phenotype increases. Mice homozygous for the null alleles die prior to implantation, emphasizing its essential role in viability (Hirotsune et al., 1998). *Lis1* mice heterozygous for the null alleles present with cortical developmental features similar to but slightly milder than those observed in human lissencephaly, with

lamination being disrupted in both the cortex and hippocampus (Cahana et al., 2001; Gambello et al., 2003; Hirotsune et al., 1998). When LIS1 is reduced below 50%, as in compound heterozygotes carrying one null and one loss-of-function allele, cortical development is severely disrupted with abnormal lamination observed in all areas of the brain from the cortex to the cerebellum. The least disruption to cortical development is observed in mice homozygous for the partial loss-of-function allele, which display diffuse hippocampal lamination only (Hirotsune et al., 1998). Cerebellar granule neuron migration assays showed that migration of the cell soma was decreased several fold in mice heterozygous for a null allele compared to wildtype neurons (Gambello et al., 2003; Hirotsune et al., 1998). While these experiments demonstrate the dosage effects of *Lis1*, they also indicate that the *Lis1* phenotype is the result of a decrease in migration during cortical development. These results also suggest that the hippocampus is the brain structure most sensitive to disruptions in migration during development, as the least affected *Lis1* partial loss-of-function homozygote merely exhibits a disruption in hippocampal lamination.

Subcellularly, LIS1 dosage was also found to modulate microtubule dynamics and positively regulate cytoplasmic dynein, characteristics that likely relate to its negative effects on cortical neuron migration (Smith et al., 2000). At the cellular level, LIS1 exhibits punctate staining along microtubules but is most prominently associated with areas of high microtubule density, such as the centrosome and MTOC (Aumais et al., 2001; Smith et al., 2000). LIS1 also colocalizes with components of the dynein and dynactin complexes. When LIS1 is overexpressed in Cos-7 cells there is a perinuclear

shift in the localization of both the p150glued component of dynactin and the intermediate chain of dynein, whereas microtubules disperse to the periphery of the cell. The opposite phenomenon occurs in fibroblasts from *lis1* heterozygous mice. As LIS1 decreases, p150glued and the dynein intermediate chain exhibit a more diffuse perinuclear localization, while microtubules become more concentrated at the cell's center. This suggests that the decrease in migration observed in *Lis1* heterozygous cortical neurons results from a disruption of the microtubule cytoskeleton and aberrant localization of dynein and dynactin complexes.

Some of the preliminary studies characterizing the activities of LIS1 in cortical neurons describe its ability to reduce microtubule catastrophe, resulting in a net increase in microtubule growth (Sapir et al., 1997; Smith et al., 2000). This function of LIS1 is further emphasized by recent finding that LIS1 localizes to the growing plus ends of microtubules together with CLIP-170 (Coquelle et al., 2002). Microtubules are dynamic structures that alternate between periods of growth and shrinkage, a process that allows them to navigate intracellular space (Schuyler and Pellman, 2001). Tethering microtubules to specific subcellular regions of the cell membrane accompanies cell polarization and can be accomplished when proteins localized at the plus end of microtubules are bound by microtubule anchors at the plasma membrane. (Gundersen, 2002). CLIP-170 is the founding member of a family of molecules that bind the plus end of growing microtubules (Akhmanova et al., 2001; Komarova et al., 2002; Perez et al., 1999). CLIP-170 forms short comets at the tips of growing microtubules which are subsequently lost if microtubule growth is arrested (Komarova et al., 2002). While

CLIP-170 caps microtubule tips indiscriminately throughout the cell, CLIP-170 binding partners have been shown to selectively bind only those CLIP-170 capped microtubules oriented towards the direction of migration, using this mechanism to promote a net increase in the growth of microtubules towards the leading edge of the cell (Akhmanova et al., 2001; Schuyler and Pellman, 2001). LIS1 localization with CLIP-170 at microtubule plus end has been shown to promote microtubule bundling in COS-7 cells (Coquelle et al., 2002). It will be important to determine if this interaction enhances the anti-catastrophe capabilities of Lis1 and/or promotes polarization of the microtubule cytoskeleton by increasing growth of microtubules oriented towards the leading process.

Doublecortin was so named for its causative role in the human female disorder double cortex. Males that inherit one affected X chromosome develop the same lissencephalic phenotype associated with mutations in *LIS1*. Females heterozygous for the *DCX* mutation only express the mutation carrying X chromosome in a portion of their cortical neurons, thus presenting with a mosaic phenotype. In this case, neurons expressing wild type *DCX* migrate to the outer cortex, while neurons without *DCX* fail to migrate properly, resulting in a subcortical heterotopic band of neurons in the intermediate zone (Mizuguchi et al., 2002). Therefore, double cortex represents a mosaic phenotype, but whether the migration defect is strictly cell-autonomous still remained unclear. Nevertheless, individuals with lissencephaly typically display severe mental retardation and intractable epilepsy, whereas individuals with double cortex

typically display mild to moderate mental retardation and less severe epilepsy (Berg et al. 1998).

While DCX is not a typical MAP, it does share certain trends with this family of molecules (Feng and Walsh, 2001). Like other MAPs, DCX has two tandem repeats that do not bind individual tubulin dimers but stabilises the tubulin–tubulin lateral contacts in tubulin oligomers and preferentially nucleate 13-protofilament microtubules *in vitro*. Likewise, DCX has been shown to be a potent stabilizer of fully polymerised microtubule lattice against spontaneous catastrophe (Moore et al., 2004; Moore et al., 2006). DCX also has a short serine/proline rich projection domain, suggesting it can promote tight bundling of microtubules, a theory supported by its ability to stabilize microtubules in response to such stresses as cold and microtubule depolymerizing drugs (Chen et al., 1992; Francis et al., 1999; Gleeson et al., 1999a; Horesh et al., 1999). While one repeat is sufficient to bind tubulin, both domains are necessary to bind microtubules and promote polymerization (Taylor et al., 2000). *DCX* patient mutations largely clustered in the microtubule binding domains, suggesting that DCX's microtubule associated activities are an important function for neuronal migration (des Portes et al., 1998a; Gleeson et al., 1999b; Pilz et al., 1998).

Humans expressing mutations in *DCX* exhibit profound disruptions in cortical development, however, the cytoarchitecture of *Dcx* knockout mice is largely unaffected (Corbo et al., 2002). Detailed analysis has indicated that cortical lamination of hemizygous male mice and female mice homozygous for the *Dcx* null allele is comparable to that of wild type animals. Nevertheless, migration and delamination has

been described for other processes during mouse development (Kappeler et al., 2006; Koizumi et al., 2006a). First, disorganization of the hippocampal lamina is observed in *Dcx* knockouts, a phenotype that is consistent with the role of *Dcx* in migration. The *Lis1* heterozygous phenotype is likewise less severe in mice than in humans, yet the least affected transgenic mouse *Lis1* phenotype (homozygous for the partial loss-of-function allele) is limited to a disruption in hippocampal lamination (Cahana et al., 2001; Hirotsune et al., 1998; Hirotsune et al., 1997). Based on this comparison, it is possible *Dcx* does not cripple migration to the extent that cortical development is disrupted in the mouse, where the migration distances are significantly shorter than in human cortical development. The mild *Dcx* knockout cortical migration phenotype appears to be the result of compensation by other *Dcx* family members such as doublecortin-like kinase 1 (DCLK1), a protein with significant sequence similarity to doublecortin and capable of binding and polymerizing microtubules (Burgess et al., 1999; Burgess and Reiner, 2000; Koizumi et al., 2006b; Lin et al., 2000). While *Dclk1* knockout mice do not present hippocampal delamination, the *Dcx*^{-/-}; *Dclk1*^{-/-} mice have disruptions in cortical as well as hippocampal lamination in a dosage dependent manner, suggesting a redundant role for both *Dcx* and *Dclk1* in proper neuronal migration (Koizumi et al., 2006b; Tanaka et al., 2006).

While cortical migration is unaffected in the *Dcx* knockout, disruptions of neuronal migration have been observed in both interneurons tangentially migrating from the medial ganglionic eminence and olfactory neurons migrating from the subventricular zone through the rostral migratory stream (Kappeler et al., 2006;

Koizumi et al., 2006a). In both cases and excessively branched leading process was observed suggesting a subcellular requirement for Dcx in repression of excessive branching. Unlike the Dcx knockout, RNA interference-mediated depletion of DCX in mouse and rat embryos resulted in a heterotopic band of grey matter within the intermediate zone. However, similar to neuronal disruptions that are observed in the *Dcx* knockout, acute disruption of *Dcx* using RNAi in migrating cortical neurons also resulted excessive branching (Bai et al., 2003). It will be important to determine the cell biological mechanism by which Dcx represses excessive branching.

Recent data has highlighted the important role phosphorylation plays in mediating Dcx affinity for and regulation of the microtubule cytoskeleton. Three kinases have been identified that phosphorylate Dcx and negatively regulate its microtubule interactions (Gdalyahu et al., 2004; Schaar et al., 2004; Tanaka et al., 2004). One kinase responsible for Dcx phosphorylation is cyclin-dependent kinase 5 (Cdk5), a neuron-selective kinase involved in multiple cellular functions. Cdk5 phosphorylation of Dcx at Serine 297 (S297) reduces its affinity for microtubules and ability to polymerize tubulin. Dynamic phosphorylation-dephosphorylation of Dcx at S297 appears to be critical for its function in neuronal migration, because a mutation that cannot be phosphorylated (S297A) or a mutation that simulates phosphorylation at this site (S297D) both blocked Dcx's net effect on migration (Tanaka et al., 2004). These data suggest that dynamic phosphorylation-dephosphorylation of specific residues of DCX may be critical for its function as neuron undergo microtubule alterations and cytoskeleton remodeling during cell movement.

Mutations in a number of signaling molecules, including Cdk5, lead to striking defects in cortical lamination, including an inversion in the cortical layers. Cdk5 was originally identified by homology to other members of the cyclin-dependent kinase family of serine–threonine kinases, which are largely implicated in cell-cycle regulation (Meyerson et al., 1992). While Cdk5 is expressed in many tissues, its kinase activity is surprisingly only detected in developing brain, suggesting that, similar to other such kinases, CDK5 might have a specific activator that regulates its activity. Two activators have subsequently been identified from brain: p35 and p39 (Lew et al., 1994; Tang et al., 1995; Tsai et al., 1994).

Mutations in the *cdk5* gene or in p35/p39 double knockout mice lead to an inversion of the normal inside-out lamination of the cortex but leave the marginal zone intact. The cortices in these mutant mice exhibit distinct phenotypes: In the *cdk5* mutant, the subplate is in the middle of the cortical plate (Gilmore et al., 1998), whereas in the p35 mutant the subplate is positioned beneath the cortical plate with a proportionally larger fraction of cortical neurons present in the cortical plate (Kwon and Tsai, 1998). p35/p39 double knockout mice have a phenotype that is indistinguishable from that of the *cdk5* mutant cortex (Ko et al., 2001). The genetic evidence strongly suggests that p35 and p39 are essential for Cdk5 activity during the development of the nervous system. Thus p35 and p39 are likely to be the principal, if not the only, activators of Cdk5 in brain, and together they regulate migration of neurons past previously deposited neurons in the cortex. Recent analysis of mice support the

A recent time-lapse analysis study has investigated cortical migration in the p35 knockout showing that disruptions in migration were correlated with placement relative to the cortical plate. Post mitotic neurons utilize somal translocation and glial-guided locomotion to achieve their final position in the cortical plate. In the p35 knockout these two modes of migration were largely replaced by a distinct mode of migration termed branched migration (Gupta et al., 2003). The neurons send a leading process that is branched, and neurons moved by propelling their cell soma along the leading process toward a stably fixed branch point. These branched neurons appeared to zigzag through the cortex, eventually splitting the preplate, and once this occurred, their final ascent appeared indistinguishable from somal translocation. This suggests that CDK5 is an important mediator of neuronal migration and that its influence on cortical lamination appears to be independent from its migration associated activities. Such dichotomy is not inconsistent with the many substrates currently known for CDK5 and those yet to be determined, including those substrates that will prove to be important for regulating lamination.

Corpus Callosum Agenesis

Corpus callosum agenesis is associated with several mouse mutants of diverse genes and human neurological syndromes and occurs at an incidence of 2–3 per 100 in the developmentally disabled population (Jeret et al., 1985). The corpus callosum is the largest fiber tract in the brain connecting neurons between the left and right cerebral hemispheres with its principle cognitive function to coordinate and transfer information

between the two hemispheres. Callosal defects occur as a result of the failure of fibers to develop, or cross the midline (Dobyns, 1996). The timing of formation of the corpus callosum coincides and overlaps with the occurrence of complex processes involving neuronal proliferation and migration. Therefore agenesis of the corpus callosum is often part of complex telencephalic malformations, and it frequently appears in various syndromes (Sztriha, 2005).

Several neuronal migration mutants have defective axonal projections, suggesting common mechanisms are involved in both process. However, despite disruption of axon outgrowth, when examined more carefully, these abnormal projections often make their way to the correct target (Royal et al., 2002). Additionally, projection defects that do occur may also be secondary to the mispositioning of neurons rather than a direct effect of the gene on axonal guidance (Fleck et al., 2000; Jossin and Goffinet, 2001). Consistent with these observations, cortical migration appears unaffected in the *Dclk1* knockout mouse, yet they exhibit agenesis of the corpus callosum. In addition, similar to the cortical lamination and hippocampal formation, axon outgrowth exhibits a dosage effect with *Dcx* null alleles as well. While *Dclk1* knockout mice present with agenesis of the corpus callosum, the *Dcx*^{-/y}; *Dckl1*^{-/-} mice have agenesis of all major commissural tracts in a dosage dependent manner consistent with their redundant roles during interhemispheric commissural formation. (Koizumi et al. 2006). This role for *Dcx* in axon outgrowth was observed in one specific genetic background, the Sv129*Pas* strain, where disruption to corpus callosum formation was observed in *Dcx* knockout alone (Kappeler et al., 2007). Likewise, genetic modifiers

may also exist within human populations. A review of CC morphology in a cohort of *DCX* patients has revealed variable corpus callosum defects ranging from agenesis of the corpus callosum to abnormally thick and thin versions (Kappeler et al., 2007).

Genetic Screening Strategies for Brain Development Mutants

Although the mechanisms that regulate development of the brain have begun to emerge, in large part through analysis of reverse genetically engineered mutant mice (Boncinelli et al., 2000; Molnar and Hannan, 2000; Walsh and Goffinet, 2000), many questions remain unanswered. Despite this progress, many aspects of brain development remaining to be explored and would benefit enormously from additional mutant resources. To provide resources for further dissecting brain development, forward genetic screens continue to be performed to identify additional genes involved in this process.

Forward Genetic Screens

Forward genetic approaches in the mouse began to become attractive with and increasingly availability of a dense genetic map and nearly complete genomic sequence. These tools allow the process of gene identification, which was once very cumbersome, to be relatively straightforward. With the initial resurgence of interest in genetic screens, large-scale screens aimed at identifying mutations in broadly defined phenotypic categories were established in a number of different countries (Hrabe de Angelis et al., 2000; Nolan et al., 2000).

In the mouse, random mutagenesis with N-ethyl-N-nitrosourea (ENU) has been used since the 1970s in forward mutagenesis screens. Because spontaneous mutations occur at a low frequency ($\sim 5 \times 10^{-6}$ per locus), mouse geneticists have searched for mutagens to generate new mutations efficiently. Towards this end, X-ray and chlorambucil mutagenesis was explored but often results in a wide variety of chromosomal rearrangements, such as inversion, deletions, and translocations, which usually affect multiple genes (Russell et al., 1989). Conversely, chemical mutagenesis with ENU introduced primarily point mutations into spermatogonial stem cells at a frequency of $\sim 150 \times 10^{-5}$ per locus (Russell et al., 1979). In addition, ENU is easy to administer, and ENU-treated males can be used to generate mutant progeny for many months (Justice et al., 1999).

ENU causes random single-base-pair mutations by direct alkylation of nucleic acids (Justice et al., 1999), so that during DNA replication, these ethylated base pairs cause mistaken identity and introduction of point mutations. Occasionally small deletions can occur (Shibuya and Morimoto, 1993), but in the mouse testis, ENU acts most effectively on spermatogonial stem cells. In mice, the most commonly reported mutations are AT-to-TA transversions or AT-to-GC transitions. In a survey of ENU-induced mutations, Noveroske et al. determined that 63% of mutations were missense mutations, 26% caused abnormal splicing, 10% resulted in nonsense mutations, and $\sim 1\%$ caused "make-sense" mutations, in which a stop codon was converted to an amino-acid-coding codon (Noveroske et al., 2000). Results from subsequent sequence-

based analyses are in line with these original observations (Coghill et al., 2002; Quwailid et al., 2004).

After male mice have been treated with ENU each progeny represents one mutagenized gamete. Because ENU mutagenesis targets spermatogonial stem cells all G1 animals are nonmosaic. On average, the high efficiency of ENU mutagenesis results in identification of a new mutation in any single locus in one out of 500 to 1,500 G1 animals (Justice et al., 1999). Since the mouse is estimated to have ~22,000 to 25,000 genes, each G1 animal is expected to carry ~25 mutations with functional consequences. These mutations result most commonly in hypomorphic alleles. Roughly one out of 10 mutations in a given gene is expected to cause a null allele. In addition, point mutations occasionally result in alleles with hypermorphic (increased), neomorphic (novel), or antimorphic (dominant negative) function (Noveroske et al., 2000). The mutation rate of individual loci can vary between genes. Because ENU preferentially alters AT base pairs, genes with higher GC contents may be affected less frequently than ones with lower GC contents. Furthermore, the sizes of the gene and its functional domains and sensitivity to improper folding all contribute to, but do not act as reliable predictors of, its mutability.

To isolate individual alleles G1 males are bred to uncover either dominant or recessive phenotypes and the offspring of these crosses are then screened for the phenotype of interest. The mouse genome sequence and advances in mapping schemes have accelerated genetic mapping of mutations while identification of strain-specific polymorphisms now allows genetic mapping to occur on most strain combinations

(Waterston et al., 2002). Positional cloning of mutations often provides key entry points into understanding biological pathways. The completion and molecular annotation of the mouse genome sequence, advances in detection of point mutations, and the expansion of genetic techniques facilitate identification of mutated genes.

While ENU mutagenesis screens allow novel genes underlying neural developmental phenotypes to be identified, yeast two-hybrid screens allow new proteins interactions involved in a biological process to be identified through an interaction with a known protein. The yeast two-hybrid system was developed for high-throughput screening during in the early 1990's (Chien et al., 1991; Fields and Song, 1989). According to this screening method, one protein (X) is fused to a DNA-binding domain (BD) from a transcription factor, such as Gal4 or LexA. The BD will bind to upstream sites engineered into reporter genes. The second protein (Y) is fused to a transcription activation domain (AD). If X and Y interact, the AD activates the reporters, leading to the formation of a colony on media where the reporters are required for growth. LacZ is also a common reporter; its activity is detected by blue color on indicator plates. Reporter activation is then selected for and the AD fusion in the surviving clones sequenced. The AD libraries can be generated from cDNA or from pools of AD clones from an array. The sensitivity of this 'library' approach is limited by sampling efficiency for a large library of AD strains and by the number of clones that researchers can practically pick and sequence.

II. A Novel Locus for Murine Autosomal Recessive Microcephaly Maps to 5qB1

Abstract

Primary autosomal recessive microcephaly is a human genetic disorder in which affected subjects are born with a small head circumference. The brain is structurally normal and, apart from intellectual impairment, there are no other significant neurological problems, dysmorphic features, or systematic malformations. Six human loci have been mapped and four genes identified for this disorder. No mouse microcephaly model has been described, but a recent *N*-ethyl-*N*-Nitrosourea (ENU) mutagenesis screen identified line 178TNC that exhibits a 3-5 standard deviation reduction in brain weight. Brain weight was mapped as a quantitative trait locus to chromosome 5qB1, representing the first primary microcephaly locus in mice.

Introduction

Expansion of cortical size in humans has been proposed to underlie the growth in intellectual capacity shown by humans and their ability to execute complex cognitive functions, such as language. Likewise, investigation into human and mouse genetic disorders that result in microcephaly highlight genes that regulate brain growth (Ponting and Jackson, 2005). In humans, microcephaly is the clinical finding of a head circumference measurement more than three standard deviations below the population mean for age and sex (Mochida and Walsh, 2001). Pathological studies of patients with primary autosomal recessive microcephaly indicate a generalized size reduction of the

brain, but with normal overall architecture (McCreary et al., 1996). Four genes have recently been identified that cause autosomal recessive primary microcephaly in humans: microcephalin, abnormal spindle-like microcephaly-associated (ASPM), cyclin-dependent kinase 5 regulatory-associated protein 2 (CDK5RAP2) and centromere associated protein J (CENPJ) (Bond et al., 2005; Jackson et al., 2002; Kumar et al., 2004). All four genes also produce mitotic associated proteins, consistent with the hypothesis that microcephaly is a developmental disorder of neurogenic mitosis (Cox et al., 2006).

The brain is formed through the coordinated processes of neurogenesis/proliferation, migration to proper cortical layer or thalamic nuclei and maturation. Neurogenesis occurs within the ventricular zone (Parnavelas, 2000). Neuroepithelium that lines the lateral ventricles will generate the neurons of the cerebral cortex, basal ganglia, hippocampus, whereas neurogenesis along the third ventricle will give rise to the thalamus, epithalamus and pretectum of the mature brain (Lim and Golden, 2007). Neurogenesis first generates a pool of progenitor cells by symmetric cell division, and neurons are subsequently formed by asymmetric cell division (Huttner and Kosodo, 2005). During cortical development newly generated neurons reach the cortex either by somal translocation, whereby a neuron cell body travels within a pial contacting radial process, or by locomotion, a process by which newborn neurons develop leading and trailing processes and migrate along radial glial fibers (Miyata et al., 2001; Nadarajah et al., 2001; Rakic, 1974). Later born neurons migrate past their predecessors to form the “inside-out” organization of the cortex

(Angevine, 1961). The key feature of diencephalic development is the formation of nuclei. Post mitotic cells migrate from the ventricular to the mantle zone and aggregate into various nuclei (Lim and Golden, 2007). During the final phases of brain development interneurons integrate into local circuits and brain connectivity is established as long distance efferent and afferent projections are generated.

Within the last 20 years, the mouse has been established as an excellent model system for hereditary human diseases. Although no spontaneous or knockout models of mouse microcephaly have been reported, an ENU-induced mutant mouse pedigree 178TNC was identified in the mutagenesis screen performed by Tennessee Mouse Genome Consortium (TMGC) that exhibits a 3-5 standard deviation reduction in brain weight. This is the first description of a murine model of microcephaly and will serve as a valuable resource to study the molecular mechanisms underlying microcephaly.

Results

ENU mutagenesis screen identified a microcephaly pedigree

Brain weight was used as a phenotypic endpoint in an ENU mutagenesis screen performed by the TMGC that identified pedigree 178TNC as a low brain weight family. An average brain weight for 29 178TNC mutant mice was 401mg as compared to 453mg for mice with the similar genetic background and mutation load averaging. This represents a 3-5 standard deviation lower brain weight than wild type controls (Fig. II.1A). Neurohistology analysis was performed to identify deficiencies in the mutants that might account for the reduction in brain weight. One hour intraperitoneal BrdU

injections at 10 weeks postnatal and staining indicate that changes in neuronal proliferation at this stage is unchanged in the mutant. Likewise, no difference was observed between the mutant and wild type for osmium tetroxide staining for myelin, acetylcholinesterase for cholinergic interneurons, anti-GFAP to stain glia, anti-NeuN to stain neurons and anti-ubiquitin to assess neuropathology. While overall brain anatomy does not appear disrupted by cresyl violet staining, the reduced size of the brain is apparent (Fig. II.1B), which is consistent with pathology findings for a human case of primary autosomal recessive microcephaly (McCreary et al., 1996).

Subregion MRI of 178TNC brain

To identify and quantify specific changes in the 178TNC brain we performed MRI to identify any volume differences between mutant and wild type brain regions. To perform this analysis total brain volume was determined by MRI to ensure that brain volumetric differences between the mutant and wild type mice could be detected using this method and that any differences were consistent with the reduction in brain weight previously observed. A significant volumetric reduction of $\sim 4 \times 10^4 \mu\text{m}^3$ was observed for the 178TNC brain in all three orientations scanned: coronal, sagittal and axial (Fig. II.2). To determine any volumetric differences between specific brain regions the brain was subdivided into five separate regions. The white matter subdivision included the corpus callosum, anterior commissure and posterior commissure. The cerebellum/midbrain complex consisted of the pons, medulla, superior colliculus and inferior colliculus. The cortical subdivision included the neocortex and piriform cortex

while the subcortical structures were made up of the hippocampus, hypothalamus, thalamus, striatum and basal ganglia. The olfactory bulb subdivision only covered the olfactory bulbs. The perimeter of each subregion was manually traced in blinded fashion with XVoxtrace software for each 350 μm slice through the brain. Fiducial markers in each orientation were used to identify what region of the brain was being segmented while mouse brain atlases were used for region identification (Fig 3A). The subcortical region was the only region that exhibited statistically significant volumetric reductions in all three orientations. A $\sim 2 \times 10^4 \mu\text{m}^3$ reduction in volume between 178TNC mutant and wildtype mice represents an $\sim 15\%$ decrease in subcortical volume (Fig. II.3B). Statistically insignificant differences were observed between mutant and wild-type volumes for white matter and cerebellum/brain stem/mid-brain complex subregions of 178TNC mutants but surprisingly not for the cerebral cortical region.

Genetic Analysis

To access a candidate interval for the low brain weight mutation 53 affected F2 178TNC mice were genotyped with the MIT microsatellite markers that consists of a panel of polymorphic markers spaced at 10- to 20-cM intervals across the mouse genome. The candidate interval for the 178TNC mutation was mapped to chromosome 5qB1 using quantitative trait loci mapping. The interval was determined using the brain weight phenotype and the locus identified includes the markers from *D5Mit386*, *D5Mit388*, *D5Mit182*, *D5Mit11* and *D5Mit370* with a maximum likelihood ratio statistic score of 58.4 obtained for marker *D5Mit386* (Fig. II.4).

Material and Methods

Animal husbandry

Use of mice in this study was in compliance with the Guiding Principles in the Care and Use of Animals (DHEW Publication NIH80-23), and was approved by the Animal Care and Use review board of the University of Tennessee Health Science Center and Oak Ridge National Laboratories. Mice were maintained at 20-24 °C on a 14/10 h light/dark cycle. Animals were fed Agway Prolab 3000 or Altromin mouse chow and given tap water in glass bottles. Mice were evaluated at 10 weeks of age.

Generation of ENU-induced mutant 178TNC mice

Using an ENU mutagenesis strategy, the 178TNC pedigree was derived from one C57BL/6JRn founder male mouse, treated with 2 doses of 50 mg/kg ENU, was mated with a C3BliA-*Eh*/+ female. A breeding scheme was followed such that genetically homozygous test class mice were generated by the third generation (G₃) of breeding. The fourth and subsequent generations should all carry the abnormal recessive mutation generated by the ENU-mutagenesis event. More details about breeding protocols can be found at the neuromutagenesis domain of the TNGC website. 178TNC mice are available to the scientific community through the TMGC website or through the Neuromice Consortium, a collaborative consortium that maintains and distributed the mutant mice generated through the TMGC ENU-mutagenesis projects.

The C3H pedigree was used as a control throughout this research and was found to be within normal limits in all tests by all domains of the TMGC. This pedigree was identical to the 178TNC in all respects regarding background stain, mutagenesis with ENU, and husbandry.

Brain Weight

Whole brains with olfactory bulbs are removed from decapitated mice and dissected into pre-weighted Petri dishes with sterile 0.9% NaCl.

Histology

Sectioning: Brain tissue is dipped into room temperature isopentane and quick-frozen in pre-cool isopentane on dry ice for 30 minutes. Frozen tissue is incubated on dry ice for 15 minutes and stored at -80°C till tissue is sliced into $20\mu\text{m}$ sections with a disposable cryostat knife at a 20° angle.

BrdU injection and staining: mice were weighed, and BrdU injections are given intraperitoneal with a one hour survival time. The dose of BrdU (Sigma) is $0.1\text{cc}/30\text{g}$ body weight using a solution of 7.5mg BrdU ($500\mu\text{l}$ of $0.9\%\text{NaCl}$ and $3.5\mu\text{l}$ 1N NaOH vortexed to mix). After one hour the brain is removed, fixed and mounted. Sections are pretreated for 10 minutes with 0.04% Pepsin (Sigma P-6887) in 1N HCl solution. After 2X washes with DDC at 80°C for 5 minutes, sections were rinsed in series with 70% ethanol at 60°C for 2 minutes, 0.1M PBS at room temperature for 3 minutes and in 0.1M PBS + $1\%\text{H}_2\text{O}_2$ for 3 minutes. Sections were stained with mouse anti-BrdU

(Becton-Dickinson 347580 used at 1-25). Before mounting with Permount sections are counterstained with 0.03% cresyl violet for 20 min, rinsed in dH₂O and dehydrated.

Anti-GFAP staining: 4% PFA fixed sections were pretreated with 1% H₂O₂ in 0.1M PBS for three minutes followed by rinsing with 0.1M PBS for five minutes and stained with rabbit anti-GFAP (Immunon 460740, 6 drops per ml PBS), dehydrated and coverslipped with Permount.

Cytochrome oxidase staining: cytochrome oxidase activity was demonstrated in 4% PFA stained sections by staining with a thoroughly mixed (~30 minutes on a stir plate) and filtered solution of 24mg Cytochrome C (Sigma C-2506), 45mg of Diaminobenzidine (Sigma D-3001), and 3 g sucrose in 60ml 0.01M KPBS (pH 7.40) for 70 minutes in a 35°C water bath (gently shaken and protected from light), rinsed in dH₂O, dehydrated and coverslipped with Permount.

Osmium tetroxide staining: 4% PFA fixed sections were rinsed with 0.1M PBS/ 5% sucrose for 5 minutes then stained with 2% OsO₄/ 5% sucrose for 20 minutes, rinsed in dH₂O, dehydrated and Permount coverslipped.

Acetylcholinesterase staining: 4% PFA fixed sections were AchE stained as follows.

Solution A: add 10ml 0.1M Na-acetate (pH 5.3) to 7ml dH₂O + 0.8ml Cu-glycine (3.75 glycine + 1.6g anhydrous CuSO₄ in 100 ml dH₂O). Solution B: dissolve one 25mg acetylthiocholine iodide (Sigma A-5751) in 1.5ml dH₂O and vortex, then add 0.5ml of 0.1M CuSO₄ followed by immediate vortexing and centrifugation at 2000rpm for 10 minutes. Solution C: dissolve one 25mg tablet of promethazine in 10ml dH₂O and filter into a foil covered vial. Combine solution A with the supernatant from solution B to

make solution D, then add 0.5ml of solution C to 20ml of solution D. Slides are incubate for at least 6 hours in a Coplin jar with light rotation (protected from light), developed in then 1% $(\text{NH}_4)_2\text{S}$ + Aq (Fisher A705-250) for 90 seconds, rinsed in dH_2O and coverslipped with Permount.

NeuN staining: 4% PFA fixed sections were pretreated with 1% H_2O_2 in 0.1M PBS for three minutes followed by rinsing with 0.1M PBS for five minutes. Sections were then stained with mouse anti-NeuN (Chemicon MAB377) used at 1:1000, dehydrated and coverslipped with Permount.

Anti-ubiquitin staining:

Sections were viewed on an Eclipse E800 microscope (Nikon Inc., Tokyo, Japan) equipped with a color camera (Photometrics, Tucson, AZ).

Gene Mapping

Gene mapping was performed at the University of Tennessee, Health Science Center. F1 mice were generated by crossing *178TNC* affected female mice with C3BLiA male mice. F2 mice were produced by brother-sister matings. Mutant mice were identified by low brain weight. MIT microsatellite loci distributed across all autosomes and the X chromosome were used to identify a 100mb interval flanked by markers *D5Mit386* and *D5Mit233* on chromosome 5 that is linked to the low brain weight mutation. Genome-wide significance levels for assessing the confidence of the linkage statistics were estimated by comparing the peak likelihood ratio statistic (LRS) using the MapManager QTX program (version b17). The LRS can be converted to the

conventional base-10 LOD score by dividing it by 4.61 (twice the natural logarithm of 10). The LRS is statistically convenient because its distribution is asymptotically a χ^2 distribution (Knott and Haley, 1992).

Discussion

Primary microcephaly in mice versus humans

Human microcephaly has been traditionally diagnosed based on a small head circumference measurement >3 standard deviations below the age-related mean. In the mouse, we took advantage of the opportunity to measure brain weight directly as an indirect measure of brain volume in an attempt to identify a mouse model of microcephaly. There is evidence however that small head circumference measurements in humans are also consistent with reductions in brain weight and volume. McCreary et al. described the autopsy results of a single case of human primary microcephaly where reduced head circumference was accompanied by an overall reduction in brain size and weight (McCreary et al., 1996). In this case, the size of the cortex and subcortical structures were reduced while the brainstem, cerebellum and spinal cord were unremarkably normal. Likewise, Bond et al. describe MRI scans from microcephaly patient carrying ASPM mutations (Bond et al., 2002). While both cortical and subcortical structures are affected, these human patients exhibit a disproportional reduction in the size of the cortex. These results are slightly different from volumetric results obtained from the 178TNC mutant mice where the cortex was relatively

unaffected while there was a statistically different reduction in volume of the subcortical structures.

Encephalization

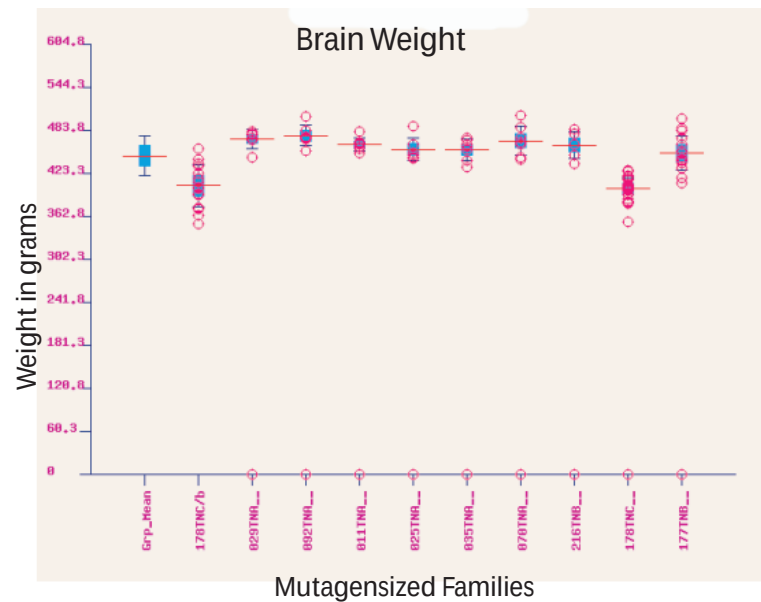
Encephalization is defined as the amount of brain mass exceeding that related to an animal's total body mass. In mammals, >90% of the variation in brain weight can be explained by variation in body weight (Jerison, 1973). Humans are the most extreme outliers from this relationship with a brain size which is ~5-fold larger than that expected based on body size alone (Stephan H, 1988). As microcephaly is a disorder that affects brain size a number of genes identified for this disorder may be found to be differentially regulated in the brain across different mammalian species (Rakic, 1988; Rakic, 1995). Consistent with this hypothesis, the gene mutation underlying the 178TNC phenotype may be differentially regulated between mice and human and explain how in mouse microcephaly mutations can result in a significant reduction in subcortical volume without a significant reduction to cortical volume.

Candidate Genes

All data to date suggest that MCPH is a primary disorder of neurogenic mitosis and not one of neural migration, neural apoptosis, or neural function as all of the known genes for MCPH are expressed in the neuroepithelium and have been shown to play a role in mitosis. Since the timing of symmetric and asymmetric cell division during neural development is crucial to determine the final number of neurons, it has been

proposed that changes in its regulation might be responsible for microcephaly, as well as the brain size differences between species (Rakic, 1995; Woods et al., 2005). There are a number of genes with possible roles in mitotic in the 178TNC candidate interval that would thus be good candidates to screen for mutations: sonic hedgehog, centromere autoantigen A, c-kit, centrosomal protein 135, ephrin receptor A5, centromere autoantigen C1, cyclin-dependent kinase 12 and cell division cycle 7. Identifying the gene mutated in 178TNC mice might make it possible to study whether there is evidence for the adaptive changes in genes between species that might account for the correlated changes in relative brain size during evolution.

A



B

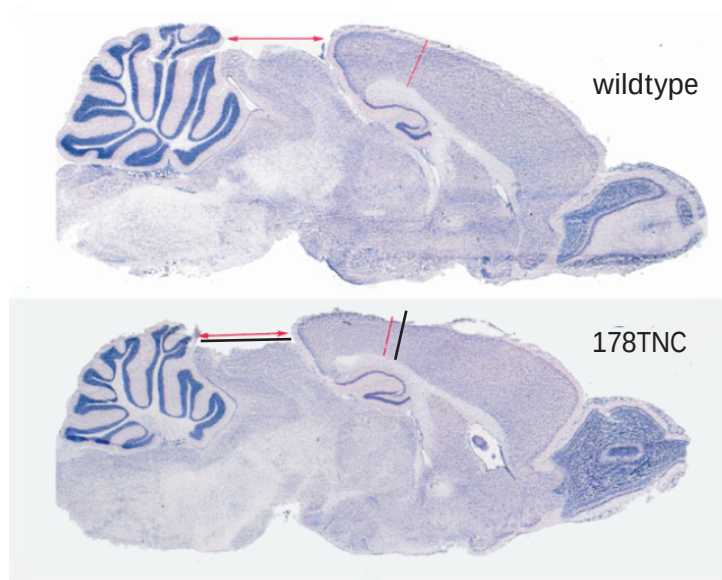
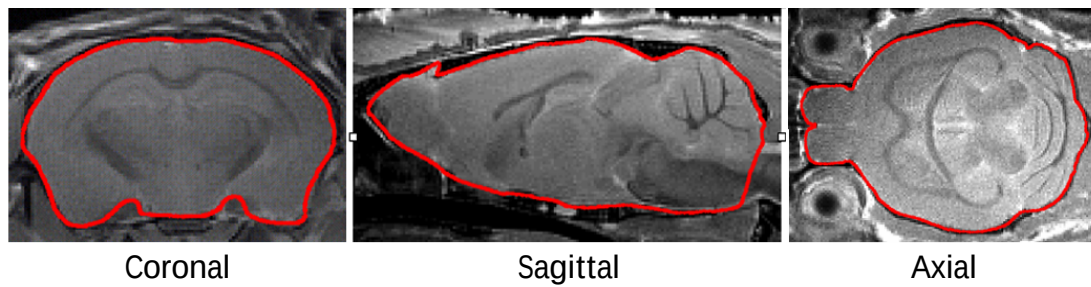


Figure II.1. Reduced brain weight was observed in the 178TNC ENU mutagenized strain. (A) A 3-5 standard deviation reduction in brain weight was observed in the original 178TNC mutagenized strain (178TNC). This reduction in brain weight was recapitulated when the 178TNC mutation was outcrossed into the C57Bl6 strain (178TNC/b). (B) cresyl violet staining of 178TNC and wildtype adult brain slices. The black lines are the same length as the red lines on the wildtype slice.

A



B

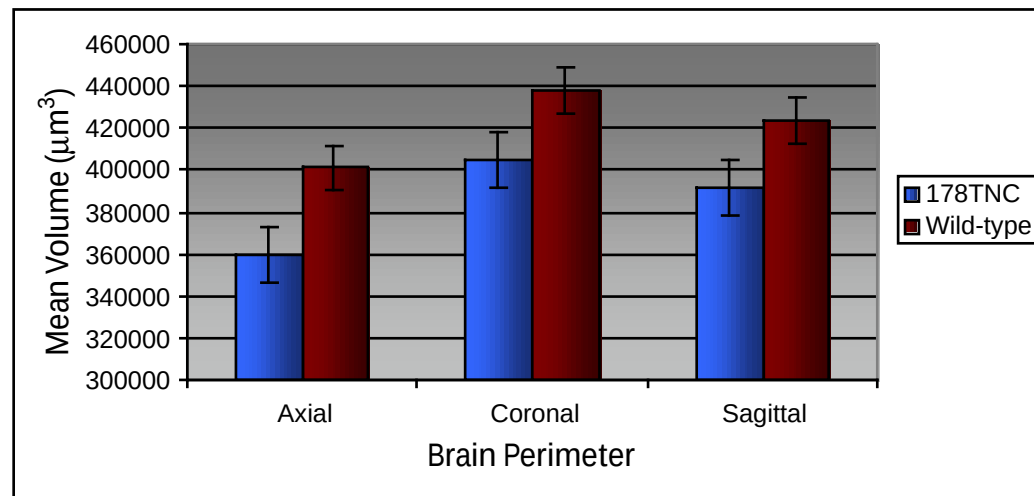


Figure II.2. Total brain volume is reduced in 178TNC mutagenized mice. (A) MRI scans showing coronal, sagittal and axial orientations and parameter tracing of wildtype brain. (B) Total brain volume is significantly reduced in all 3 orientations in the 178TNC strain as compared with wildtype brain volume.

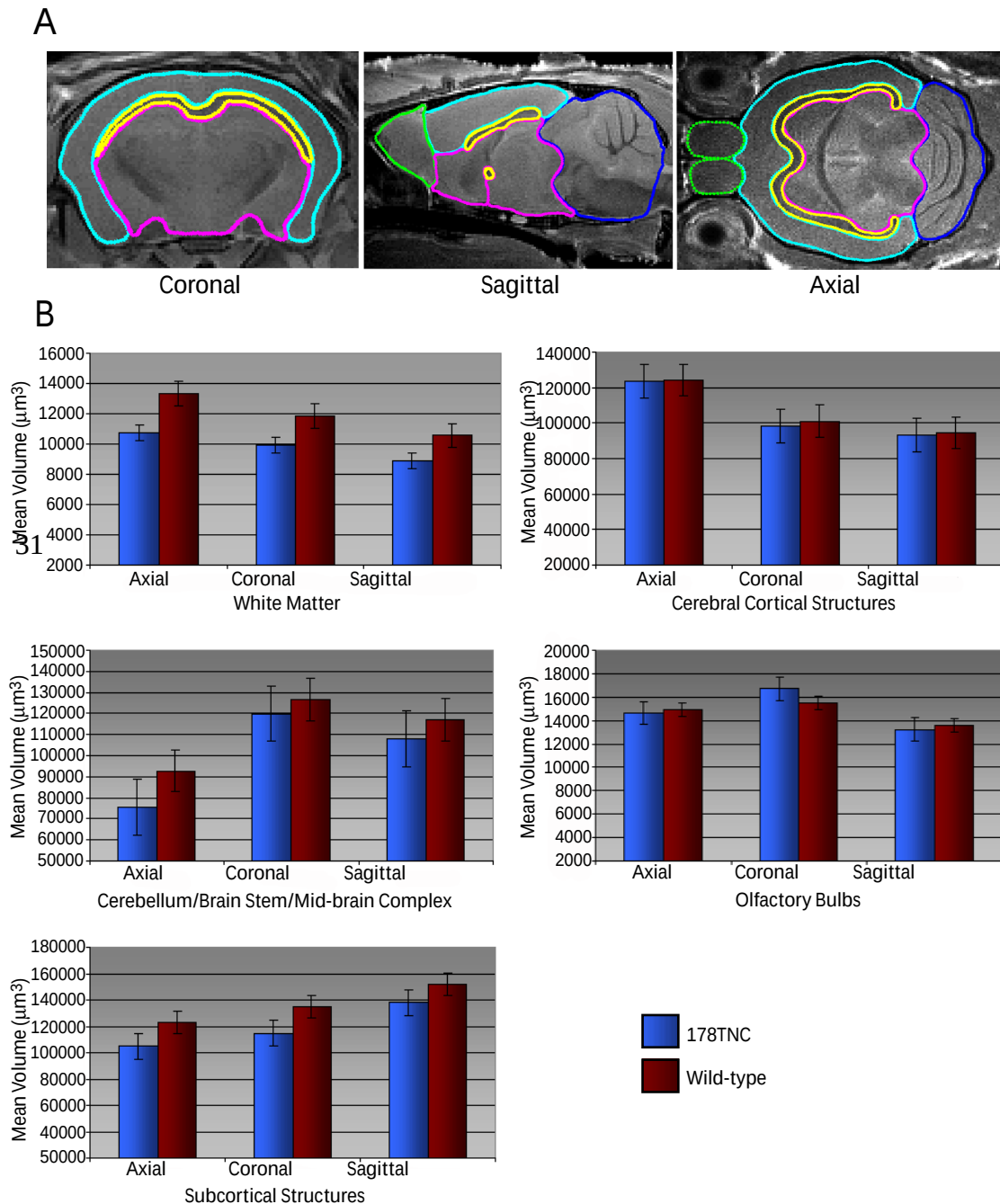


Figure II.3. 178TNC mutant mice show a significant reduction in the volume of subcortical structures as compared to wildtype. (A) The brain was segmented into brain regions which were then compiled to measure regions specific volume differences. The brain was parsed into cortical structures (turquoise), olfactory bulbs (green), white matter (yellow), subcortical structures (purple) and cerebellum/midbrain (blue) with the use of Xvotrace. (B) Volume differences for specific brain regions between wild-type (red) and 178TNC mutant (blue) were compared with a significant reduction in volume measured for subcortical structures in 178TNC mutant mice in all three scanning orientations.

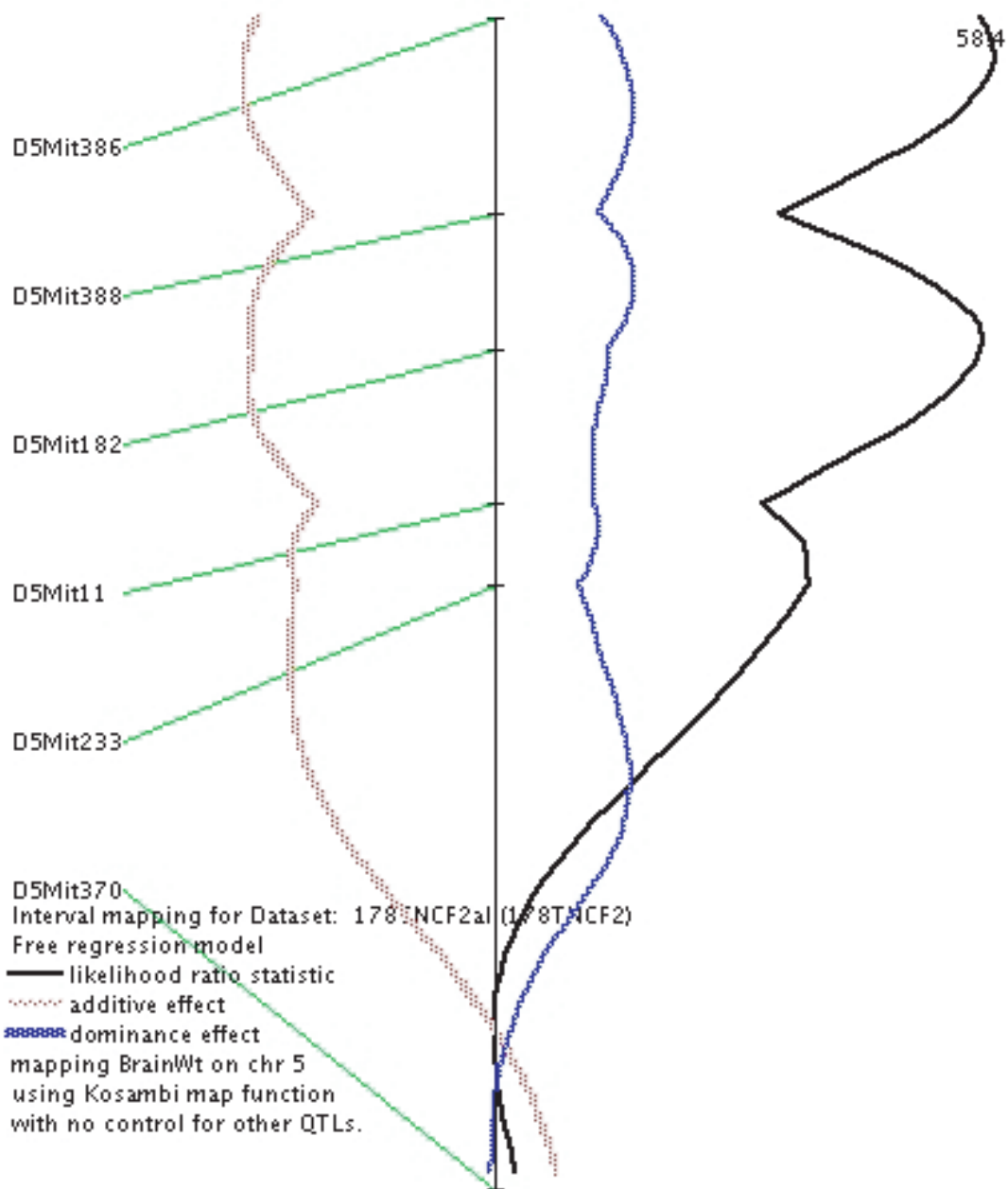


Figure II.4. Quantitative trait locus mapping was performed on 178TNC low brain weight mice to identify a 100mb candidate interval for this mutation on chromosome 5qB1 between markers D5Mit386, D5Mit388, D5Mit182, D5Mit11 and D5Mit370 with a maximum likelihood ratio statistic score of 58.4 which corresponds to a LOD score of 12.7 for marker D5Mit386.

Chapter Acknowledgment

Parts of this chapter were performed in collaboration with Liz Yoder.

III. Spinophilin Facilitates PP1-Mediated Dephosphorylation of PSer297

Doublecortin in Microtubule Bundling at the Axonal “Wrist”

Abstract

The axonal shafts of neurons contain bundled microtubules, whereas extending growth cones contain unbundled microtubule filaments, suggesting that localized activation of microtubule-associated proteins (MAP) at this transition zone may bundle these filaments during axonal growth. Dephosphorylation is thought to lead to MAP reactivation, but specific molecular pathways have remained elusive. We find that Spinophilin, a Protein-phosphatase 1 (PP1) targeting protein, recruits the MAP Doublecortin (Dcx) selectively at the “wrist” of growing axons, where it is required for the dephosphorylation of Dcx Ser297, leading to activation. Loss of activity at the “wrist” is evident as an impaired microtubule cytoskeleton along the shaft. These findings suggest that spatially restricted adaptor-specific MAP reactivation through dephosphorylation is important in organization of the neuronal cytoskeleton.

Introduction

Growth cone features that are distinctive from those of the axon shaft were noted first by Cajal (Ramón y Cajal, 1988). The growth cone is the motile end of the axon, is enriched in dynamic actin and microtubule (MT) components, and determines the direction of axonal growth. It has been divided into three regions: the peripheral domain containing a dense meshwork of actin forming lamellipodia and filopodia, the

transition domain where actin filaments anchor into a loose MT network, and the central domain containing splayed MTs and organelles of varying size (Bridgman and Dailey, 1989; Dent and Gertler, 2003). Between the central domain and the axonal shaft is a region surrounded by actin where splayed MTs become bundled into dense parallel arrays (Dehmelt and Halpain, 2004; Rodriguez et al., 2003; Shea, 1999) that we refer to as the neuronal “wrist”. Defects in the “wrist” domain would therefore be predicted to result in defects in the organization of the MT cytoskeleton of the axonal shaft. The molecules that function at these transition zones have not yet been clearly defined.

Among the candidates for mediating MT bundling is Doublecortin (Dcx), a MAP with a role in MT stabilization and bundling that has been localized to the growth cone (Friocourt et al., 2003; Moores et al., 2004). Dcx was initially identified as the causative gene for the human neuronal migration disorder double cortex (des Portes et al., 1998a; Gleeson et al., 1998), and is now widely used as a marker newly postmitotic neurons. It consists of an N-terminal repeated domain (R1 and R2) and a C-terminal serine-proline rich domain. Dcx is localized to the tips of growing neurites (Friocourt et al., 2003), and is incorporated into neuronal MTs during polymerization (Francis et al., 1999). Deletion in mouse results in excessively branched axonal shafts in migrating neurons (Kappeler et al., 2006; Koizumi et al., 2006a), suggesting Dcx may be required for formation of stable MT, possibly at the wrist region.

Recent data has highlighted the important role that Dcx phosphorylation plays in mediating its affinity for and regulation of the MT cytoskeleton (Gdalyahu et al., 2004;

Schaar et al., 2004; Tanaka et al., 2004). One kinase responsible for Dcx phosphorylation is cyclin-dependent kinase 5 (Cdk5), a member of the Cdk family of serine/threonine kinases primarily active in terminally differentiated neurons (Nikolic et al., 1996; Ohshima et al., 1996). Cdk5 phosphorylation of Dcx at serine 297 reduces its affinity for MTs and its ability to polymerize tubulin (Tanaka et al., 2004). The dynamic nature of cytoskeletal reorganization underlying neurite outgrowth and migration predicts that Dcx phosphorylation, like that of other MAPs, is tightly regulated by both kinases and phosphatases (LoTurco, 2004; Schaar et al., 2004). Unlike tyrosine kinases and phosphatases that are represented in the mammalian genome in comparable numbers, the quantity of serine/threonine kinases far exceeds that of serine/threonine phosphatases (Ceulemans and Bollen, 2004). Thus, phosphatase diversity only matches that of the kinases when the number of phosphatase regulators is considered. Much of the data concerning the role of serine/threonine phosphatases for MAPs is based on results of pharmacological studies and thus potential adaptor molecules that may regulate these interactions have not been identified.

Dcx has been shown to interact with a diverse set of proteins including the μ subunit of AP-1 adaptor protein (Friocourt et al., 2001), the cytoplasmic domain of neurofascin (Kizhatil et al., 2002), Lissencephaly-1 (Caspi et al., 2000) and spinophilin (Spn)(Tsukada et al., 2003). Spn is an actin binding protein with an established role in subcellular targeting of protein phosphatase 1 (PP1), a serine/threonine phosphatase that controls many aspects of cellular physiology (Allen et al., 1997; Hsieh-Wilson et al.,

1999; Nakanishi et al., 1997; Satoh et al., 1998). Studies of Spn have focused primarily on its role in dendritic spines where it indirectly regulates excitatory synaptic transmission through modulation of PP1-mediated dephosphorylation of AMPA and NMDA channels (Allen et al., 1997; Feng et al., 2000; Yan et al., 1999). However, through its actin-binding domain Spn is also able to bind and bundle F-actin, which is theorized to be important for regulating spine morphology (Satoh et al., 1998). Consistent with these observations *Spn*^{-/-} mice exhibit abnormal spine number and formation (Feng et al., 2000).

Here we find that that Dcx-Spn-PP1 interaction is critical for the formation of stable microtubules in neurite shafts. Dcx is associated with Spn selectively at the wrist of growing neurites. Deletion of Spn results in mislocalized and excessively phosphorylated (inactivated) Dcx, and deletion of either Spn or Dcx, or knockdown of the catalytic PP1 subunit results in defective MT bundling in neurite shafts. Spn-PP1 promotes Dcx dephosphorylation at Cdk5-phosphorylated Ser297, which restores the MT bundling effects of Dcx. Microtubule bundling at the wrist appears to rely on the association of the Dcx-Spn-PP1 complex, because the cellular phenotype is rescued by *wt* Spn but not by Spn that lacks a PP1-interaction or Dcx-interaction domain. Similarly, the phenotype is rescued by *wt* Dcx but not by Dcx that lacks the phosphorylation site that is acted on by Spn-PP1. We propose that the Spn-PP1-Dcx association may be important for proper MT condensation at the axonal wrist, leading to constriction of the growth cone that results in bundled MT in the neurite shaft.

Results

Identification of the Dcx-Spn interaction using yeast-two-hybrid

Through yeast two-hybrid we identified Spn as a Dcx interacting protein. Spn is a multidomain protein with an N-terminal actin-binding domain, a PSD-95/Dig/ZO-1 homology (PDZ) domain followed by a coiled-coil (CC) domain (Supp Fig. III.1A). Full-length Dcx was used as “bait” to screen a Lex-A library made from E9.5-10.5 whole mouse embryo (Vojtek et al., 1993). Thirteen of the 27 clones identified by the screen carried inserts that encoded portions of the Spn CC domain, a motif that likely mediates protein-protein interactions. When these fragments were aligned, a 47 amino-acid region shared between the fragments highlighted Spn amino acids 650-697 as an important component of the Dcx binding domain on Spn.

Co-affinity purification was used to verify the interaction between full-length Spn and full-length Dcx and to investigate the Spn-binding-domain of Dcx. For this assay, recombinant His-tagged full-length Dcx or fragments bound to nickel beads were incubated with cell lysates of 293T cells transfected with Flag tagged full-length Spn. Interaction with Spn was assessed by immunoblotting supernatant and pellet samples from each assay with an anti-Flag antibody. Full-length Spn was retained on the Dcx-bound beads (Supp. Fig. III.1B). When full-length Spn was incubated together with His-tagged nickel bead-bound Dcx fragments representing the major domains of Dcx [the first repeat (R1), second repeat (R2) and S/P rich tail (S/P) region] the interaction between Spn and Dcx appeared to be mediated mainly by R2. The interaction between Spn and the second Dcx repeat was, however, attenuated with longer Dcx fragments

that contain R2 and either R1 or the S/P. To determine if this was the result of auto-inhibition of Spn/Dcx binding by these domains, R2 was incubated together with R1 or S/P *in trans*. For both combinations of fragments, the interaction between Spn and R2 was diminished (Supp. Fig. III.1C), indicating that R1 and S/P produce an inhibitory effect on Spn binding Dcx R2.

To verify that the association between Dcx and Spn was mediated through the R2 and the CC domains, respectively, a series of deletion constructs was assembled and tested for the ability to co-immunoprecipitate from heterologous cells. In transfected 293T cells, plasmids encoding Flag-tagged full length or DCC (deleted amino acids 649-696) Spn was co-transfected along with Xpress-tagged full length or DR2 (deleted R2) Dcx. Following immunoprecipitation with a-Flag antibody, the supernatants and immunoprecipitants were analyzed by immunoblotting with a-Flag or a-Xpress. We found an association of Spn and Dcx that was not detected with either deletion construct (Supp. Fig. III.1D). Together the data suggests an interaction mediated through the Dcx R2 and Spn CC domains.

Co-immunoprecipitation and co-distribution of Dcx Spn and PP1

We also tested for co-immunoprecipitation of Spn and Dcx from whole brain lysate (WBL). WBL was generated from E16 brains, a developmental stage consistent with peak cortical migration, and incubated with Dcx antisera or control rabbit sera. Spn was immunoprecipitated with Dcx but not with the control serum (Supp. Fig. III.2A), suggesting that Dcx and Spn are in a complex *in vivo*. The blotted

immunoprecipitates were also probed with an antibody specific for PP1. We noted that PP1 was also part of the Dcx-Spn complex *in vivo*. The data suggests that the three proteins may be associated in developing brain.

Co-distribution of Dcx and Spn during neuronal development

Dcx is expressed most prominently during cortical development in postmitotic neurons. To determine the cell populations mostly likely regulated by Dcx/Spn, we compared their protein distribution in embryonic brain from E13, E16 and E18, which represent the neuronal developmental spectrum (Supp. Fig. III.2B). At both E13 and E16 Dcx and Spn immunostaining was pronounced in the intermediate zone, a region of the developing brain where neurons extend axons. Expression became less specific by E18 towards the completion of brain development. Together, these experiments suggest the interaction between Dcx and Spn may be important in neuronal migration or axon outgrowth.

Impaired axon outgrowth in *Dcx* mutant brains

Corpus callosal hypoplasia is apparent in the MRIs of human males with *DCX* mutations (Fig. III.1A). Additionally, published data has that Dcx expression is upregulated in corpus callosal (CC) projection neurons during periods of axonal growth in mice (Arlotta et al., 2005), together suggesting a potential role in axonal extension and/or stabilization. We therefore tested *Dcx* ^{-/-} mice for alterations in axonal outgrowth by labeling projections with DiI at E14.5, 15.5 and 16.5, when axons are

extending in multiple regions of the brain. E14.5 injection into medial subcortical zone labeled the projecting cortico-thalamic axons that extended to the cortico-striatal boundary in *wt* mice, a distance of several millimeters (Fig. III.1B). In *Dcx* ^{-/-} mice, however, we noted that this axonal tract had extended less than 1 mm, and that none of the labeled axons had reached this boundary. By E15.5 in *wt* mice these cortico-thalamic axons had extended into the region of the striatum and thalamus, whereas in *Dcx* ^{-/-} mice only a fraction of these axons had reached the cortico-thalamic boundary, and even fewer had reached the striatum and thalamus (Fig. III.1C-D). By E16.5, these defects became less apparent (Fig. III.1E), while the corpus callosal axonal tract, which typically reaches the medial extent of the telencephalon by this point, showed a defect in axonal length. Although by the time of birth, these developmental delays had not resulted in clean morphological defects, these findings suggest a requirement for *Dcx* in axonal outgrowth. This effect on outgrowth does not appear to be secondary to impaired neuronal migration, because *Dcx* knockout mice do not display discernable defects in the positioning of neurons (Corbo et al., 2002; Kappeler et al., 2006). The data suggests that *Dcx* plays a role in axon outgrowth in both humans and mice.

***Dcx*/Spn/PPI localization at the wrist suggests possible involvement in axonal outgrowth**

To determine if the *Dcx*/Spn interaction may play a role in the *Dcx*^{-/-} axon outgrowth phenotype we assessed the role of this interaction in the cortex by first looked for areas of colocalization in cultured cortical neurons at 1 DIV. At this stage,

neurites have a well-defined shaft capped by a growth cone, but axonal/dendritic differentiation has not yet occurred. Cortical neurons appear more monopolar than hippocampal neurons, which extend multiple short processes concurrently (Dotti et al., 1988). Immunohistochemistry (IHC) results demonstrated that Spn exhibits an unexpected highly restricted distribution at the transition zone between the growth cone and axonal shaft, in the “wrist” region of the cell, while Dcx exhibited its characteristic enrichment along the neurite tip and around the cell body and showed some overlapping distribution with Spn at the wrist. Expression of GFP/RFP-fusion proteins in the developing brain showed nearly identical localization patterns (data not shown). PP1 showed ubiquitous localization in these neurons, but interaction in a complex with Dcx and Spn was confirmed by co-immunoprecipitation (Supp Fig. III.2A). We next assessed the distribution of phospho-serine 297 Dcx (PSer297 Dcx) using a phospho-specific antibody (Tanaka et al., 2004) to this Cdk5 substrate site. PSer297 Dcx showed low but detectable levels in the growth cone but was much reduced in the wrist and the axonal shaft. Thus Spn is enriched at the wrist, and PSer297 Dcx is largely excluded from axonal shafts. This data suggests a model in which Spn/PP1 may mediate dephosphorylation of PSer297 Dcx at the wrist.

We next tested for co-distribution of Spn and Dcx with the MT and actin cytoskeletons in 1DIV cortical neurons. Spn was localized in close association with the actin cytoskeleton, as predicted based on its actin-binding domain, whereas Dcx showed overlapping localization predominantly with MTs (Fig. III.2B). Consistent with the published results for the distribution of actin and MTs in the growth cones,

MTs appeared to be closely associated with actin filaments (Dehmelt and Halpain, 2004). The close association of the MT-bound Dcx and the actin-bound Spn suggest that the Dcx-Spn interaction may serve to coordinate signaling between these cytoskeletal components at the wrist that may in turn be important in axon outgrowth.

Dcx and Spn cooperate to mediate hippocampal lamination and corpus callosum formation

We therefore tested for a shared phenotype between the *Dcx* and *Spn* knockout mice, to determine if the two genes share similar roles in brain development. *Dcx* mutant mice display a delamination of the CA3 region of the hippocampus (Corbo et al., 2002). Previous literature indicates a mild reduction hippocampal size in *Spn* ^{-/-} mice (Feng et al., 2000). To test for a phenotype similar to *Dcx* ^{-/y}, we examined *Spn* ^{-/-} hippocampal anatomy using Cresyl violet stain. Surprisingly, we found that 100% of the mice showed a similar mild delamination of the CA3 region of the hippocampus (Fig. III.2C), which was very similar in appearance to the *Dcx* ^{-/y} mice. This data suggests that the genes may function together in brain development.

We next examined the phenotype of P21 mice deficient for both *Dcx* and *Spn*, in order to determine if there is functional redundancy between these genes during development. To do this, we generated *Spn* ^{-/-}; *Dcx* ^{-/y} double knockout (*DKO*) mice and compared these with the single knockout and *wt* mice. The hippocampal lamination phenotype was slightly more severe in the *DKO* than was observed in either of the single knockouts (Fig. III.2C). Cortical cytoarchitectonics appeared intact in the

DKO, but we noted complete agenesis of the corpus callosum (Conte et al.), which was not observed in either of the single knockout mice. The corpus callosum (CC) represents axonal fibers that originate from cortical projection neurons of layer II/III and V and project to homotypic regions in the opposite hemisphere (Ozaki and Wahlsten, 1998). The ACC was accompanied by Probst bundles, suggesting failed or delayed axonal extension across the midline during embryogenesis. We next sought to determine if this was restricted to the CC, or represented a more general disorder of axonal growth. The anatomy of all four genotypes using Cresyl violet staining was compared, and we noted that the anterior commissure (AC), a major track connecting long distance reciprocal olfactory and orbital-frontal regions one of the other major decussation tracts, was hypoplastic in the *DKO* mouse, whereas it appeared normal in both the single knockout mice (Fig. III.2C). To exclude the possibility that these axonal phenotypes were the result of degeneration rather than failed formation, mice were examined at the P0 timepoint, when these axonal projections have just completed decussation. Pups of each genotype were sacrificed and brain sections stained for L1CAM, an axonal marker that highlights major projections (Chung et al., 1991). We found that these axonal decussations were absent even at this age in the *DKO* mouse, and instead L1CAM-positive fibers terminated prior to midline crossing (Fig. III.2C). We conclude that *Dcx* and *Spn* cooperate to mediate axonal outgrowth of multiple long distance projections within the brain.

In order to be certain that this phenotype was not due to spontaneous ACC observed in some mouse genetic backgrounds, we analyzed P0 offspring from twenty

litters of *Spn* +/-; *Dcx* +/- X *Spn* +/-; *Dcx* +/- matings. From a total of 125 mice, 6 double knockout mice were identified, 5 of which displayed ACC and hypoplastic AC, whereas none of the 119 mice with intermediate genotypes showed these phenotypes in this genetic background ($p < 0.001$, Fig. III.2D). This data suggests that *Dcx* and *Spn* cooperate to mediate long distance axonal growth in the CC and AC during development.

***Spn* and *Dcx* are required for MT bundling in cortical neurons**

Having established partial functional redundancy between *Dcx* and *Spn* in brain development, we next examined for defects in the actin and MT cytoskeletons in cultured cortical neurons from *wt*, *Dcx* *-/-*, *Spn* *-/-* and *DKO* littermates. Neurons from all four genotypes were compared in a blinded fashion following visualization of the cytoskeleton (Fig. III.3A). There were no significant differences in actin staining among the four genotypes in either the growth cone or axonal shaft, and MTs were usually observed in close approximation with actin, extending into the growth cone. However, we noted that both *Dcx* *-/-* and *Spn* *-/-* neurons showed poorly organized axonal MT cytoskeleton with failure to condense MTs into a single shaft. As a result of splayed and unevenly spaced MTs, axonal shafts were widened along their base compared with *wt*. Neurons from *DKO* displayed an even broader leading process with apparently more severe failure of MT bundling. This data suggests that the axonal defect observed in both knockouts may result from impaired MT bundling.

Increased inter-MT distance in the absence of either *Dcx* or *Spn*

To further investigate the ultrastructural basis of this MT phenotype, we performed transmission electron microscopic (EM) analysis of cultured neurons to visualize the cytoskeleton in the growth cone and axonal shaft from stage 1DIV neurons from each of the four genotypes. We noted in *wt* neurons that MTs were nearly always well organized in the axonal shaft and showed uniform orientation and a typical inter-MT spacing of 20-30 μm (Fig. III.3B). The neurons from the *Dcx* $-/-$ and *Spn* $-/-$ mice, however, showed a disordered MT array. These MTs typically veered in oblique directions within the shaft and showed nonuniform spacing. The neurons from the *DKO* mice appeared even more severely disordered, with frequent MT crossing observed, suggesting a failure of bundling. In order to quantitate inter-MT distance (IMD), we collected high-resolution images, traced MTs along their entire visible length and measured nearest neighbor distance at uniform 250 nm intervals along the entire length of the visible neurite from at least 4 neurons of each genotype (for a total of over 300 IMD measurements from each genotype). In *wt* neurons, there were very few locations where MTs crossed paths, (indicated by an $\text{IMD} < 20 \text{ nm}$) whereas these were not infrequently encountered in both single knockouts and even more-so in the double knockout ($p < 0.05$, Fig. III.3C). Additionally, over 40% of the IMD measurements were greater than 50 μm from both single and double knockouts, implying a failure to bundle MTs in the absence of *Dcx* and *Spn*.

Measurements of IMD from ultra-thin EM sections (80 nm) have the disadvantage of frequently sectioning through individual (25 nm) MT fibers. It is also

possible that apparent distances between MTs may be inaccurately determined because the 3D space is projected onto 2D space. To overcome this limitation, we also performed EM tomography from axon shafts of neurons derived from *wt*, *Dcx* *-/-*, *Spn* *-/-* and *DKO* littermates, and traced MTs in a blinded fashion in 3D from 500 nm sections. We noted that in these reconstructions from *wt* mice, MTs were typically closely and uniformly apposed to one another and uniformly oriented along the AP axis of axonal shaft. Reconstructions from single mutant mice appeared less well organized, with greater IMD and shorter MTs, whereas analysis of the *DKO* showed fragmented and poorly aligned MTs (Supplemental movie 1-5). We conclude that the MT defects observed in single knockouts neurons are worsened in *DKO* neurons.

Defective MT bundling is associated with excessively branched neurites at later timepoints in culture

We expected that failure to condense neurite MTs might lead to defects in neuronal morphology at later stages of maturation, as observed for other cytoskeletal factors (Homma et al., 2003). To test this, we analyzed morphology of mutant neurons after 36 hrs in culture, a time when leading neurites have typically organized into stable thin processes, but before final morphology is determined. Obvious defects in morphology were detected in both single *Dcx* *-/-* and *Spn* *-/-* neurons with an increase in branching complexity of the main process (MP) as well as an increase in the number of cells with branches extending from the cell body (i.e. body processes (BP))(Fig. III.3D). Quantification of the number of 2° and 3° branches from the main process as

well as the number of 1° and 2° body processes performed in a blinded fashion demonstrated an excess of such branches. There was an increase in the percentage of neurons with 2° and 3° branches from the MP as well as 1° BP in knockout neurons that were only rarely observed in *wt* neurons at this stage (Fig. III.3E-F). Additionally, the *Dcx*^{-/-}; *Spn*^{-/-} showed a further increase in the number of secondary BP, again which were only rarely observed in either *wt* or *Dcx*^{-/-} or *Spn*^{-/-} neurons. We conclude that, at later stages of maturation, both *Dcx* and *Spn* are required for maintenance of neuronal morphology and suppression of excessive branching that likely result from failure to properly organize the microtubule cytoskeleton, and that suppression of excessive branching of BPs is cooperatively mediated through mechanisms involving *Dcx* and *Spn*.

***Spn* necessary for *Dcx* localization to wrist region**

The localization of *Spn* during axonal outgrowth suggests a role at the “wrist”, perhaps in recruitment of *Dcx* to this location to aid in MT organization. To test this, we cultured neurons from *Spn*^{-/-} and *wt* littermates and stained for tubulin and *Dcx*. We found that in *wt* neurons, *Dcx* and tubulin showed closely overlapping localization. Using this method, *Dcx*-positive MT structures were uniformly visualized extending from the soma into the growth cone, and these MTs were always positive for *Dcx* along the entire length of the filaments (Fig. III.4A). The intensity of *Dcx* staining appeared to increase near the growth cone, as described (Friocourt et al., 2003). In *Spn*^{-/-} neurons, however, *Dcx* failed to localize to the growing axon. In these neurons, *Dcx* was

observed in the cell soma in comparable levels to *wt*, where localization overlapped with MTs based on tubulin staining. In the growth cone and emerging axon shaft, though, the MT cytoskeleton was largely devoid of Dcx immunoreactivity. This observation was quantified by comparing the relative fluorescence intensity for Dcx along the axon shaft in a series of *Spn* *+/+* and *-/-* neurons. We observed that Dcx signal intensity gradually increased in more distal regions of the axon shaft and was highest in the proximal growth cone/wrist region in *Spn* *+/+* neurons (Fig. III.4B). In *Spn* *-/-* neurons, signal intensity gradually decreased in more distal regions, and did not show a maximum in the distal axon or neurite tip.

To confirm these results using a different method, we electroporated primary cortical neurons with a construct encoding red fluorescent protein (RFP)-labeled Dcx (Tanaka et al., 2004), and compared localization in *Spn* *+/+* and *-/-* neurons. After image acquisition, neurons were scored by a blinded observer as to whether Dcx-RFP was localized to the tip of the neuron or localized more diffusely (Fig. III.4C). We found that in *Spn* *+/+* neurons, the vast majority showed Dcx-RFP localization preferentially in the proximal growth cone/wrist region, whereas *Spn* *-/-* neurons showed Dcx more diffusely localized with MTs throughout the cell ($p < 0.01$, Fig. III.4D). We additionally compared the relative fluorescent intensity along the axonal shaft and found that in *Spn* *+/+* the signal typically increased in more distal regions, whereas in *Spn* *-/-* neurons the signal gradually decreased in more distal regions (Fig. III.4E). No defects in *Spn* localization were found in *Dcx* *-/-* neurons, and neither protein appeared aberrantly distributed in low-power histological brain sections from

the corresponding knockout (data not shown). The data suggest that Spn is required for proper localization of Dcx to axonal tips in cortical neurons.

Spn and Dcx interaction is sufficient to link actin and MTs

The factors that coordinate the integration of the actin and MT cytoskeletons in neurons are not well known. Recombinant Spn has been shown to mediate actin polymerization and Dcx has been shown to mediate MT polymerization (Horesh et al., 1999; Satoh et al., 1998). To determine if Spn and Dcx binding are sufficient to mediate this integration, we tested the ability of purified Dcx and Spn to co-recruit these cytoskeletons in a cell-free assay. Recombinant Spn was added to actin previously polymerized with Alexa 488-labeled phalloidin, resulting in the formation of notable F-actin filaments (Fig. III.4F). Similarly, rhodamine-conjugated brain-derived MAP-depleted tubulin (previously stabilized with a low dose of taxol) was treated with recombinant Dcx, which led to the formation of MT aster-like structures from which MT bundles emanated. The combination of labeled actin and tubulin showed no particular affinity for one another, and neither did the addition of labeled tubulin to Spn-stabilized actin or did the addition of labeled actin to Dcx-stabilized MTs. However, when Spn-stabilized actin and Dcx-stabilized MTs were combined, there was significant overlap of the two labeled cytoskeletal components. This effect was not merely due to clumping of the two cytoskeletons, because taxol/phalloidin stabilization of these cytoskeletons did not lead to overlap. This data suggests that the Dcx-Spn interaction is sufficient to mediate linking of the actin and MT cytoskeletons *in vitro*.

PP1 is capable of mediating dephosphorylation of PSer297 Dcx

Previous work has established that the kinase Cdk5 is in a complex with the phosphatase PP1 and Spn in brain (Agarwal-Mawal and Paudel, 2001; Futter et al., 2005), indicating that this complex is well poised to mediate dynamic phosphorylation/dephosphorylation of substrates. We therefore tested whether PP1 was capable of dephosphorylating the Cdk5-mediated phosphorylation of Dcx Ser297. In this experiment, recombinant Dcx was added to an activated recombinant Cdk5/p25 mixture, resulting in robust phosphorylation of Dcx at Ser297 based both on analysis by autoradiogram and with a phospho-specific antibody to the Ser297 site following SDS-PAGE analysis (Fig. III.5A). The addition of the non-specific calf intestinal phosphatase (CIP) led to a notable reduction in both the autoradiogram signal and the reactivity with the PSer297 Dcx antibody. Increasing concentrations of recombinant PP1 were then compared with CIP for the ability to dephosphorylate this site. We noted a dose-dependent decrease in autoradiogram signal and PSer297 Dcx antibody reactivity following PP1 treatment. We conclude that PP1 is capable of mediating the dephosphorylation of PSer297 Dcx.

Spn enhancement of PP1-mediated dephosphorylation of PSer297 Dcx

Because Spn targets PP1 to phosphoproteins, we next tested whether Spn was capable of enhancing the PP1-mediated dephosphorylation of PSer297 Dcx. The addition of even high concentrations of Spn in the absence of PP1 had no effect on the

state of PSer297 Dcx (Fig. III.5B). For this experiment, we reduced the PP1 concentration to a level where PP1 alone had no effect on the phosphorylation state of Ser297 Dcx (Fig. III.5C). We then added increasing concentrations of Spn to this mixture while maintaining a constant concentration of PP1. We noted a dose-dependent dephosphorylation of PSer297 Dcx that was mediated by Spn. This effect was observed both using autoradiogram and the PSer297 Dcx specific antibody. We conclude that Spn is capable of enhancing the PP1-mediated dephosphorylation of PSer297 Dcx.

Modulation of PSer297-specific phosphorylation by Cdk5 and PP1

The previous data suggests that the Spn-PP1 complex is sufficient to mediate dephosphorylation of PSer297 Dcx, but it raises the question of whether it is necessary to mediate this effect. To address this, we applied membrane-permeable roscovitine, a specific Cdk5 inhibitor (IC₅₀ 200 nM, vs. > 500 nM for cell cycle-related Cdk5 tested (Meijer et al., 1997) or tautomycin, a selective PP1 inhibitor (IC₅₀ 1nM vs. > 10 nM for PP2A and other phosphatases (MacKintosh and Klumpp, 1990) to cultured neurons. Primary cortical neurons from E14 were incubated in increasing concentrations of roscovitine or tautomycin for two hrs. Subsequently, cells were lysed and analyzed by Western using a pan-Dcx antibody, a PSer297 Dcx-specific antibody and a PThr321 Dcx-specific antibody, the latter which recognizes a Jun kinase phosphorylation site (Gdalyahu et al., 2004). As roscovitine concentration was increased, there was progressively less reactivity of the PSer297 Dcx antibody, without significant change in

either the total Dcx or PThr321 Dcx reactivity, suggesting that Cdk5 activity is required for Ser297 Dcx phosphorylation (Fig. III.5D). Application of tautomycin had the opposite effect, as expected, where increasing concentrations led a significant increase in PSer297 Dcx reactivity, without significant change in either the total Dcx or PThr321 Dcx reactivity. We conclude that inhibition of PP1 leads to an increased level of phosphorylation of PSer297 Dcx. Because it was previously shown that phosphorylation of this site results in relative inactivation of MAP activities of Dcx, the Spn-PP1 complex can thus serve to reactivate Dcx.

PSer297 Dcx is excessively phosphorylated in the absence of Spn

The data indicates that PP1/Spn are sufficient and PP1 activity is necessary for PSer297 Dcx dephosphorylation. In order to determine if Spn is necessary for proper dephosphorylation of PSer297 Dcx, we examined *Spn* *-/-*, *+/-* and *+/+* littermates for PSer297 reactivity. Whole brain lysates from E16 littermates were prepared, and CIP was added to half of each as a non-specific phosphatase. The samples were then analyzed by Western for Spn, Dcx, and PSer297 Dcx. We found no significant alteration in Dcx levels in any of the genotypes. However, there was gene dosage-dependent excessive phosphorylation of PSer297 Dcx in the *Spn* *+/-* and *-/-* brain lysates (Fig. III.5E). Reactivity was nearly two-fold higher in the *+/-* brains and a four-fold higher in the *-/-* than *+/+* brains based on quantitative luminometry (Fig. III.6F). The data together suggests that Spn-PP1 is necessary and sufficient for PSer297 Dcx dephosphorylation.

PP1 function required for MT bundling during neurite growth

The identification of PP1 as part of the Dcx-Spn complex (Suppl. Fig. III.2A) prompted us to test PP1's role in MT bundling during neurite outgrowth using genetic knockdown. PP1 consists of two regulatory subunits and a catalytic subunit (PP1 γ), the subunit previously shown to directly associate with Spn (MacMillan et al., 1999). We first tested protein levels of PP1 γ using a previously validated PP1 γ siRNA vs. control (Santa Cruz Biotechnology) in N2A cells. Three days after transfection, cells were lysed and analyzed by Western for PP1 γ 1 (brain-expressed isoform of PP1 γ) vs. tubulin control (Fig. III.6A). We found a 6-fold reduction in expression levels compared with untransfected or control siRNA transfected cells, suggesting that this siRNA mediates robust knockdown of PP1 γ expression. To verify this produced a decrease in protein levels in cortical neurons, cells were *in utero* electroporated with siRNA and an EGFP reporter plasmid to label transfected cells, then subsequently fixed and stained for PP1 γ 1. As expected, cells that were positive for the EGFP reporter showed notably less PP1 γ 1 signal compared with untransfected cells or cells transfected with a control siRNA (Fig. III.6B). Cortical neurons were also fixed and stained to visualize the MT and actin cytoskeleton. We found that MTs failed to bundle in the majority of PP1 γ siRNA expressing cells (Fig. III.6C). This was quantitated by evaluating the percentage of cells with the phenotype of splayed MT in the primary neurite shaft. We found that approximately 80% of PP1 γ electroporated cells showed this phenotype compared with

approximately 15% of controls ($p < 0.01$, Fig. III.6D). We conclude that PP1 catalytic activity is required for bundling of MTs within the leading neurite.

Functional mutants do not rescue MT bundling defect in knockout neurons

Because of the shared MT bundling phenotype observed in *Spn* and *Dcx* knockout neurons, we hypothesized that the interaction between these two proteins is critical for proper MT bundling in neurites. Because we found that Spn aa L649-Q696 (part of the coiled-coil domain) constituted the consensus Dcx binding domain (Suppl. Fig. III.1A), we deleted these residues from Spn (Spn Δ CC), and, as expected, found that this construct failed to co-immunoprecipitate wt Dcx in co-transfected 293T cells (Suppl. Fig. III.1D). Therefore, we tested whether either EGFP-Spn or EGFP-Spn Δ CC was capable of rescuing the MT bundling defect that we had detected in *Spn*^{-/-} neurons. E14 *Spn*^{-/-} neurons were electroporated with constructs encoding either plasmid until DIV 1, then stained for MTs and EGFP, and scored for the bundling defect. We noted that the majority of *Spn*^{-/-} neurons with wt Spn showed a normalization of the MT array, indicating rescue of the leading process phenotype. However, most neurons electroporated with the Spn Δ CC showed persistent MT bundling defects (Fig. III.7A). Quantification of the number of cells with either bundled or splayed MTs in the leading neurite showed rescue in 78% vs. 25% with wt or mutant constructs, respectively (Fig. III.7B, $p < 0.01$). We conclude that the association between Spn and Dcx is required for microtubule bundling. Similarly, we tested the EGFP-Spn4A (residues 457-460 KIKF) mutant construct, which was

previously found to lack binding with PP1 (Hsieh-Wilson et al., 1999; Oliver et al., 2002), to determine if it was capable of restoring the microtubule array in *Spn*^{-/-} neurons. We again noted that most neurons electroporated with EGFP-Spn4A showed persistent MT bundling defects (Fig. III.7A), and quantification showed only the majority of cells with this defect (Fig. III.7B, $p < 0.01$). We conclude that the interaction between Spn, Dcx and PP1 is important component of the molecular mechanisms for MT bundling in neurites.

Having demonstrated that the Spn/PP1 complex serves to dephosphorylate PSer297Dcx, we next tested whether phosphorylation mutants at that site are capable of rescuing the MT cytoskeletal defect in *Dcx*^{-/-} neurons. We therefore tested Dcx297A, which is incapable of phosphorylation at this site, and Dcx297D, which mimics phosphorylation at this site. We found that *wt* but neither mutant showed a rescue of the splayed MT phenotype (Fig. III.7C). Quantification of the percentage of cells with either bundled or splayed MTs in the leading neurite showed rescue in 82% vs. 19% and 14% with *wt*, 297A or 297D mutations, respectively (Fig. III.7D, $p < 0.01$). Together, the data suggest that the interaction of Spn and Dcx as well as the dynamic regulation of the S297 phosphorylation state of Dcx is necessary for maintenance of the MT cytoskeleton during neurite outgrowth.

Spn-PP1 mediated Dcx dephosphorylation recovers MT activity

PSer297 Dcx phosphorylation by Cdk5 results in a 60% decrease in MT polymerization ability (Tanaka et al., 2004), and the data indicates that the Spn-PP1

complex is necessary and sufficient for dephosphorylation at this site. In order to test whether this dephosphorylation results in reactivation of the MAP activity of Dcx, we employed the turbidimetric MT polymerization assay (Gaskin et al., 1974). In this assay, application of recombinant Dcx to MAP-depleted brain-derived tubulin results in robust polymerization during the 1000-second incubation period (Horesh et al., 1999) (Fig. III.8A). We then added activated recombinant Cdk5/p25 to the reaction, which should result in phosphorylation of the Ser297 Dcx site, and re-zeroed the absorbance reading. There was a subsequent decrease in the turbidity over the following 2500 seconds, suggesting that phosphorylation of Dcx by Cdk5 results in depolymerization of MTs that had been previously polymerized with Dcx. We then added roscovitine to inactivate Cdk5, together with recombinant Spn-PP1, which should result in dephosphorylation of the P-Ser297 Dcx, and re-zeroed the absorbance reading a second time. An increase in the turbidity was once again observed over the following 2000 seconds, suggesting MT re-polymerization.

We performed two controls for this experiment. In the first, we concurrently added roscovitine together with Cdk5/p25 to the reaction at the beginning of the second incubation to block the kinase activity of Cdk5. This resulted in little change in the overall turbidity of the reaction (Fig. III.8B), suggesting the kinase activity of Cdk5 acting on Dcx is necessary for depolymerization. We then added tautomycin concurrently with the Spn-PP1 at the beginning of the third incubation to block the phosphatase activity of PP1. Likewise, this resulted in little change in turbidity. We noted that roscovitine or tautomycin alone resulted in little change in turbidity (Fig.

III.8C). In these experiments, turbidity is affected by both MT polymerization and by bundling/cross linking, and thus it was not possible to determine whether these Dcx regulators were altering the organization of MT or leading to depolymerization. To address this, we also performed co-sedimentation experiments, to determine the weight of the resultant MT pellet at the end of these experiments. We found that the pellet weight (which is correlated with the mass of MTs) showed changes that mirrored turbidity (Fig. III.8D). The data suggests that dynamic phosphorylation and dephosphorylation can influence the effect of Dcx on MT polymerization.

Discussion

Here we present molecular and genetic data that supports a novel model of how MT condensation occurs at the wrist region during neurite extension. In this model, actin-associated Spn at the neurite wrist enhances the PP1-mediated dephosphorylation of Dcx, which reinstates the MT bundling effects of Dcx (Fig. III.8E). The model is supported by data indicating disordered MT bundling along the neurite shaft in *Spn*^{-/-} and *Dcx*^{-/-} cultured neurons, which was more severe in the *DKO*. In addition, similar MT disorganization is observed along neurite processes in PP1 siRNA treated cortical neurons. A requirement for Spn in mediating Dcx dephosphorylation at S297 was evident in the increased levels of P^{Ser297} Dcx in the *Spn*^{-/-} brain. MT bundling appeared to require the interaction of the Spn/PP1/Dcx complex, as Spn and Dcx forced expression were capable of rescuing this splayed MT phenotype in respective knockouts, but protein mutants lacking the ability to form this complex showed failure

to rescue the phenotype. This data suggests that Spn is an important adapter molecule to modulate PP1 specificity for Dcx dephosphorylation during cortical development.

Genetic requirements for corpus callosal development

We show delay in axon outgrowth in *Dcx*^{-/y} brains, yet these fibers still eventually appear to project to their correct location, suggesting a time-dependent defect in axons outgrowth. This might make the projection of these neurons more susceptible to further genetic perturbations, such as we demonstrate in the *DKO*, and has been previously demonstrated for dosage-dependent interaction with *Dclk1* (Koizumi et al., 2006a) and a strain dependent effect (Kappeler et al., 2007) of *Dcx* on CC development. While *Dclk1*^{-/-} mice present with ACC, the *Dcx*^{-/y}; *Dclk1*^{-/-} mice have disruptions in cortical lamination as well as agenesis of all major commissural tracts. Spn has been shown to interact with both Dcx and Dclk1 (Tsukada et al., 2003), therefore it is possible that the ACC observed in the *Spn*^{-/-}; *Dcx*^{-/y} is an additive effect of the absence of either Dclk1 or Dcx to interact with Spn. However, since *Spn*^{-/-} alone does not display ACC, it is likely that Dcx has Spn-independent effects on MT regulation in CC development, so that these axons are delayed but reach the midline within the permissive window for decussation (Wahlsten et al., 2006). The factors may play in the variable expressivity of the ACC phenotype in humans with *Dcx* mutations (Kappeler et al., 2007).

The role of *Dcx* in regulation of branching

Previous data has suggested a requirement for *Dcx* in repressing excessively branched neurites during migration in both subventricular zone and medial ganglionic eminence neurons (Kappeler et al., 2006; Koizumi et al., 2006a; Koizumi et al., 2006b), although the mechanisms had not been explored. Similarly, acute disruption of *Dcx* using RNAi in migrating cortical neurons showed excessive branching (Bai et al., 2003). Since *Dcx* and *Spn* have both been shown to be expressed in migrating as well as postmigratory projection neurons, this data suggests that the *Dcx/Spn/PP1* complex may be important in maintenance of the MT cytoskeleton during neuronal migration as well. With disruptions in the *Dcx/Spn/PP1* complex, MT at the wrist fail to bundle, leading to splayed MT along the process that may underlie aberrant branching. Branching is typically initiated at the growth cone and along the shaft when bundled MT splay apart, allowing longer MT to breakdown and the shorter MT to invade the nascent branches (Kalil et al., 2000; Szebenyi et al., 1998). The excessive branching observed in *Dcx*-/*y* neurons is thus consistent with the splayed MT exhibited along the length of the process. Likewise, the even more disarrayed MT we identified in the DKO is consistent with the increase in the frequency of branching observed in this genotype compared with either single knockout.

Integrators of the actin and MT cytoskeleton

Much of the understanding of neurite outgrowth has focused on the role of the actin or MT cytoskeleton independently, and only recently has data emerged to suggest

how these two major cytoskeletal components may be coordinated in this process (Dehmelt and Halpain, 2004; Rothenberg et al., 2003). This integration is presumably mediated by either single molecules that contain both an actin- and MT-binding domain, or by pairs or complexes of molecules that together contain these domains. Several cellular factors including ACF7, MAP2, and Pod-1 contain modular actin and MT binding domains and are themselves capable of crosslinking or integrating the two cytoskeletons (Bernier et al., 2000; Karakesisoglou et al., 2000; Roger et al., 2004; Rothenberg et al., 2003). Other factors have been reported that can integrate intermediate filaments and these two cytoskeletons (Yang et al., 1999). However, there are few examples of molecular complexes that can *bridge* between these cytoskeletal components. One such example is the IQGAP1/CLIP-170 interaction, in which activated Rac/CDC42 recruits IQGAP1, an actin-binding protein, with CLIP-170, a MT plus-end binding protein, to form a tripartite complex for cellular polarization (Fukata et al., 2002).

The current data suggest that the Spn-Dcx interaction may also mediate cross-talk between the actin and MT cytoskeletons. Spn and Dcx display maximal overlap in distribution in the proximal part of the growing neurite tip, at the site of constriction of the neurite that follows the broad growth cone. Dcx has a well-characterized role in MT modulation (Moore et al., 2004; Sapir et al., 2000; Taylor et al., 2000) and Spn not only targets PP1 but is also capable of cross-linking F-actin into bundles (Nakanishi et al., 1997; Satoh et al., 1998).

In vitro studies have shown Dcx can bridge the actin and MT cytoskeletons independent of Spn (Tsukada et al., 2005). We have also observed that high concentrations of purified Dcx can stabilize and bundle phalloidin labeled actin filaments, however low concentrations of Dcx do not exhibit this effect, but can still crosslink the actin and MT cytoskeletons in combination with Spn. However, it remains a possibility that Dcx may have Spn-independent interactions with actin.

Genetic specificity of MAP reactivation through dephosphorylation

Axonal extension appears to consist of three basic events occurring at the growth cone: protrusion, engorgement, and consolidation (Dent and Gertler, 2003). In this model, protrusion consists of extension of actin-based lamellipodia and filopodia, which then serve as substrates for the extension of MTs. Engorgement consists of movement of vesicles and organelles, likely directed by MT-based transport. Consolidation occurs as the proximal part of the growth cone assumes a cylindrical shape, which probably relies on the bundling of loosely associated MTs. However, little data has been provided to highlight the molecular mechanisms underlying the process. The data presented here suggests that the Dcx/Spn/PP1 interaction may play a role in MT bundling during the consolidation step. In this model, actin-bound Spn recruits MT-bound phosphorylated Dcx to the “wrist” region of growing neurites, where PP1 mediates its activation through dephosphorylation. We hypothesize that this effect is part of the molecular machinery involved in the transition from splayed to bundled MTs.

It has been hypothesized that certain factors must account for the reactivation of MAPs by dephosphorylation, but current studies have relied on pharmacological manipulation of phosphatases. The current data provide both genetic and biochemical evidence that the Spn-Dcx interaction leads to PP1 mediated dephosphorylation of Dcx. PP1 is a ubiquitously distributed serine/threonine phosphatase involved in numerous cellular processes with many molecular targets, making it difficult to identify specific targets and mechanisms (Ceulemans and Bollen, 2004). Consistent with this, we found broad distribution of PP1 in young cortical neurons and that PP1 alone is capable of dephosphorylating Cdk5 phosphorylated PSer297 Dcx. However, subcellular targeting factors such as Spn bind to the canonical PP1-binding sequence RvxF, and can restrict PP1 activity by directing the enzyme to discrete subcellular compartments and, in some cases, by modulating its activity toward particular substrates (Terrak et al., 2004; Terry-Lorenzo et al., 2002).

This is the first demonstration to our knowledge of specific adaptor molecules that are required for MAP reactivation. This may be a general mechanism of reactivation of MAPs. Both genetic and pharmacologic evidence support a role for Spn in the PP1-mediated dephosphorylation of Dcx. Spn is capable of enhancing the PP1-mediated dephosphorylation of the PSer297 site of Dcx. Spn ^{-/-} mice had dramatically increased levels of PSer297 Dcx in developing brain. Furthermore, treatment of wt brain lysates with the specific PP1 inhibitor, tautomycin led to significantly increased levels of PSer297 Dcx. The phosphatases mediating dephosphorylation of Dcx at other sites are not specifically known, although evidence suggests that PP2A may serve to

regulate the phosphorylation state of Ser47 (Schaar et al., 2004). Further identification of the specific phosphatases and targeting subunits for Dcx and other proteins will require both biochemical and genetic evidence.

MATERIALS AND METHODS

Yeast Two-Hybrid Screen

The yeast two-hybrid screen was performed as previously described (Vojtek et al., 1993), using a full-length human Dcx fused in frame to LexA (LexA-Dcx) as the bait. A L40 yeast strain was transformed with LexA-Dcx, and then transformed with a mouse E10.5 total embryonic cDNA library (a gift from Dr. Stan Hollenberg). An estimated 1×10^6 transformants were plated for histidine selection, and approximately 27 transformants were recovered after 3-5 days at 30°C. Histidine-positive colonies were collected and screened for β -galactosidase activity using a filter assay and were confirmed as “true positive” clones by plasmid isolation (Hoffman and Winston, 1987) and co-transformation in the L40 strain.

Affinity Purification

293T cells were transfected with the Flag-tagged Spn pcDNA3 (Hsieh-Wilson et al., 1999) using SuperFect (Qiagen) according to the manufacturer’s recommendations. Each of the pET-28a(+) constructs were used to express recombinant HIS₆-tagged protein in BL21 DE3 *Escherichia coli* (Novagen) with HIS resin according to

manufacturer's recommendations. Recombinant Spn was prepared as HIS₆-tagged proteins as described (Hsieh-Wilson et al., 1999).

Kinase/Phosphatase Assay

Proteins were concentrated to 2 mg/ml, and diluted to equimolar concentrations as described (Taylor et al., 2000). Recombinant GST-fusion Cdk5/p25 proteins were used at 1mg/ml in a kinase reaction as previously described (Niethammer et al., 2000). Spn, PP1 (Upstate), and Dcx kinase reaction were incubated for 45 min at 37 °C in PP1 buffer. Samples were boiled then analyzed by Western.

Animals

Spn animals were maintained in a mixed SvJ/129 background. *Dcx* animals were maintained on a mixed 129/BISwiss background, and were extensively intercrossed to generate mice of desired genotype. *WT* time-pregnant C57Bl6 pregnant mice were obtained from Harlan Labs (Indianapolis, IN). Animal work was performed on littermates, and was carried out in compliance with Institutional Animal Care and Use Committee approved protocols.

Cortical Cultures

Cortical neurons were isolated and cultured as described (Zaman et al., 1999). After 24 hrs of culture in 37 °C, 5% CO₂ incubator, the cells were fixed and immunostained. Tautomycin (Calbiochem) and Roscovitine (Calbiochem) were

dissolved in DMSO and increasing concentrations for each drug were incubated with cortical neurons in cell suspension for 2 hrs at 37°C, 5% CO₂.

Electroporation

Isolated cortical neurons were electroporated with a Nucleofector device performed according to Mouse Neuron Nucleofector™ Kit for primary mouse hippocampal neurons specification (Amaxa Inc.). Neurons from littermates of various genotypes were electroporated with full-length pcDNA3 encoding Spn-GFP or SpnΔCC (ΔL649-Q696), or pcDNA3.1 encoding Dcx-RFP with specific mutations (Tanaka et al., 2004).

Immunofluorescence Microscopy

Cortical cultures were fixed at either 18-24hrs (short term cultures) or 36 hrs (for branching cultures) in culture with 4% paraformaldehyde (PFA) and permeabilized with either saponin or Triton-X blocking solution. Whole brains were immersion-fixed in 4% PFA at 4 °C overnight and cryoprotected in sucrose/PBS. Brains were sectioned either by cryostat to 10 μm thickness or to 50μm thickness with a sliding microtome. The antibodies used were rabbit anti-Spn (Upstate Biotechnology, Lake Placid, NY) (1:200), guinea pig anti-Spn (1:100), mouse anti-PP1 (Santa Cruz Biotechnology, Santa Cruz, CA) (1:50), rabbit anti-Dcx (Gleeson et al., 1999b) (1: 200), goat anti-Dcx (1:200) (C-18, Santa Cruz Biotechnology), rabbit anti-PSer297 Dcx antibody (1:50)(Tanaka et al., 2004) and rat anti-L1 antibody (1:200) (Chemicon). Sections were

counterstained with Hoechst (1:10,000) (Molecular Probes) or Nissl. Cultured neurons were examined using 100x oil-immersion lens on a DeltaVision system captured using 0.2 μm Z-steps. Quick projections of each series was generated using DeltaVision Softworks version 3.3.6. Fluorescence intensity along the neurite shaft for the line profiles were measured using the arbitrary line profile tool of Softworks. Fluorescent intensity and neurite lengths were normalized between 5 separate neurons for each condition. Brightfield microscopy was used to score the Spn/Dcx brain sections for corpus callosum and anterior commissure anatomy. A branched process was defined as a process that was longer than the diameter of the cell body and was more than one cell-body distance away from the tip of the main process (to differentiate these from branched growth cone).

Immunoprecipitation and Western Blot Analysis

Cells or mouse brains were lysed with modified RIPA buffer with protease inhibitors (AEBSF, PMSF, aprotinin, leupeptin), treated with 1-4 μl CIP (10 U/ μl) and incubated at 37°C for 1hr. For immunoprecipitation, mouse telencephalon was homogenized in modified RIPA buffer and incubated with Protein A Sepharose (Pharmacia) beads pretreated with anti-Dcx antibody or control rabbit serum for 1 hr. Beads were washed with PBS four times and analyzed by Western blot. Antibodies used include goat anti-Dcx (1:800) (C-18, Santa Cruz Biotechnology, Santa Cruz, CA), guinea pig anti-Spn (1:2000), mouse anti-PP1 (1:1000) (Santa Cruz), mouse anti-flag

(1:300)(Sigma), phospho-specific rabbit anti-Dcx P_{Ser297} (1:100) (Tanaka et al., 2004), phospho-specific rabbit anti-Dcx P₃₂₁ (1:2000) (Gdalyahu et al., 2004).

Spn Δ CC (Δ L649-Q696) and XDcx Δ R2 (Δ D175-F257) were generated through serial site directed mutagenesis and restriction enzyme removal of indicated fragments. To determine binding affinity for X-Dcx and F-Spn respectively, constructs were transfected into 293T cells with Superfectamine (Qiagen) reagent according to manufacturer's specifications. Immunoprecipitation was performed as described above with rabbit anti-Flag (Sigma). Western analysis was probed with mouse anti-Flag (Sigma, 1:1000) and mouse anti-Xpress (Invitrogen, 1:200).

Electron Microscopy

Neurons were acutely dissociated and cultured according to standard protocol. Cortical neurons were fixed with 2% glutaraldehyde in cacodylate solution, postfixed in 1% osmium tetroxide, stained with 2% uranyl acetate, dehydrated and mounted in Durcupan as described (Yu and Baas, 1994). Mounted samples were sectioned at 80 nm on a Leica EMUC6 microtome, floated on coated copper grids and stained with Sato's lead solution. Microscopy was performed with a JEM-4000EX IV microscope. Microscopy films were projected and MT wall-to-wall distances between neighboring MTs across the process were measured manually every 250nm along the process. MTs were differentiated from other filamentous structures based on their uniform 25 nm width. Contingency table analysis was performed on the data to determine the significance of the bin distributions between the four genotypes.

Three-dimensional tomographic analysis was performed on 0.5 μ m sections stained with uranyl acetate and lead salts, and coated with carbon (for stability in the electron beam) and colloidal gold (Sigma, 20nm, for fiducial marking). The specimens were irradiated before initiating a tilt series in order to limit anisotropic specimen thinning during image collection. During the image collection, the illumination was held to near parallel beam conditions and optical density maintained constant by varying the exposure time. Electron tomographic volumes were computed with the IMOD package from 5 tilt series collected at 4000x on a JEOL 4000EX operated at 400kV, sampled at 2° increments from -60° to +60°, using an established protocol. Negatives (Kodak 4489 film) were scanned on a Nikon Coolpix 9000 scanner at 1200 dpi. The pixel resolution was 5.3 nm. The three-dimensional reconstructions were visualized using the software tool Analyze (Mayo Foundation, Rochester, MN). MTs were segmented in longitudinal sections of the volume with the computer program Xvoxtrace. The segmented MTs were rendered with the utility Synu (National Center for Microscopy and Imaging Research, San Diego, CA). These programs allow one to step through slices of the reconstruction in any orientation and to track and model features of interest in three dimensions. From the rendered volumes, the anaglyphic stereo pairs and movie segments were generated using Amira (Mercury Computer Systems, San Diego, CA).

PP1 γ 1 siRNA

Lyophilized control and mouse PP1 γ siRNA duplex (Santa Cruz Biotechnology) were transfected into N2A cells with Lipofectamine (Invitrogen) according to manufacturer's instruction for siRNA transfection. After 3 days in culture cells were lysed with modified RIPA buffer containing protease inhibitors (AEBSF, PMSF, aprotinin, leupeptin). PP1 γ knockdown was determined by western analysis probed with PP1 γ (N-19) (Santa Cruz Biotechnology) and chemiluminescence quantified by Gene Gnome bio imager (Syngene). E13 intraventricular injection of control and PP1 γ siRNA (20uM, Santa Cruz Biotechnology, 1ug/ul pGE2hrGFP (Stratagene, electroporation marker) 0.01% Fast Green (USB, injection marker)) was performed via mouth pipette. *In utero* electroporation was performed as previously described (Tabata and Nakajima, 2001) using 7mm tweezertrods (Harvard Apparatus). Cortical cultures were generated 48hr post electroporation.

Actin/MT Crosslinking Assay

Lyophilized actin was resuspended in A-buffer (5mM Tris HCl [pH 8.0], 0.2 mM CaCl₂, 0.2 mM Na₂ATP, 0.5 mM DTT) for 1 hr on ice and diluted to a working concentration of 0.4 mg/ml in 10X KMEI buffer (500 mM KCl, 10mM MgCl₂, 10mM EGTA, 100mM Imidazole, pH 7.0). Actin filaments were polymerized by incubating monomeric actin with 60nM Alexa 488-phalloidin for 30 min at room temperature. Rhodamine-labeled tubulin (Cytoskeleton) was mixed 1:10 with PCP tubulin (cytoskeleton) and diluted to a final concentration of 1.0 mg/ml in BRB80 buffer (80 mM PIPES [pH 6.8], 1 mM MgCl₂, 1 mM EGTA). MTs were prepared by

polymerizing rhodaminated tubulin for 10 min at 37°C with 0.5 μ M taxol. Crosslinking assays were done by combining Alexa 488-phalloidin stabilized actin, taxol stabilized rhodaminated MTs, and protein (0.75 μ g BSA, 0.75 μ g recombinant Dcx, 0.8 μ g recombinant Spn) for 10 min, fixing for 2 min in 0.1% glutaraldehyde/20% glycerol. Samples were spotted on a slide and data captured on a DeltaVision imaging system.

Turbidity Assay

MT polymerization assays were performed as previously described (Gleeson et al., 1999a; Taylor et al., 2000). Recombinant Dcx (7 μ g) was incubated with 100 μ g of tubulin in 100 μ l reaction containing 1X G-PEM (80 mM Na Pipes, 0.5 mM MgCl_2 , 1.0 mM EGTA, 1 mM GTP pH 6.8) and kinase buffer minus ATP. Tubulin polymerization was recorded as turbidity of reaction as measured by the diffraction of 345nm wavelength. After 10 min 3 μ g of recombinant Cdk5/p25 and 50 μ M ATP were added to the Dcx reaction and diffraction was zeroed. The reaction was allowed to proceed for 40 min at which time 100 μ M roscovitine, recombinant Spn (0.1 μ g) and 0.001 units of PP1 were added. Diffraction was zeroed and the turbidity was recorded for an additional 20 min. 100 μ M roscovitine and 10 nM tautomycin were used to inactivate Cdk5 and PP1 respectively.

Co-sedimentation

PCP-purified tubulin (100 μ g) was incubated with recombinant Dcx (10 μ g) that was pretreated with 5 μ g active or inactive (100 μ M roscovitine) Cdk5/p25 (30 min at

37°C) followed by 0.1 μ g/0.001units active or inactive (10 nM tautomycin) Spn/PP1 (20 min 37°C). The 100 μ l reaction containing 1X G-PEM (80 mM Na Pipes, 0.5 mM MgCl_2 , 1.0 mM EGTA, 1mM GTP pH 6.8) and kinase buffer were pelleted at 37,000 rpm for 20 min at 37°C. The pellets were dried at room temperature for 5 min before weighting three times and averaging the measurements.

Figure III.1. Delayed axonal extension in *Dcx* ^{-/-} brains. A. Midline sagittal T1-weighted brain MRI from normal and a boy with a deletion of *DCX* exon 7-8. Normal shows a well-formed corpus callosum, whereas the patient shows a severe corpus callosal hypoplasia (red arrows). Optic nerve (arrowhead), cerebellum (*), cortex (**). B. DiI injected medial subcortical region at E14.5 shows extension of fibers to the cortico-striatal boundary (double arrows) and into striatal-thalamus region (triple arrows). Mutant shows minimal axonal extension from the injection site at this timepoint. C. E15.5 DiI injection shows robust labeling of corticothalamic axons past the cortico-striatal boundary (box 1) and into the striatal thalamus region (box 2), whereas mutant shows diminished extension through these regions. High power view (box 1 and 1') shows few labeled axons in mutant, whereas box 2 and 2' shows fewer labeled axons have reached their target by this timepoint. E. E16.5 DiI injection shows diminished axon extension of the corpus callosal tract (arrowhead) in mutant compared with *wt*, whereas there has been some catch-up growth of corticothalamic tract by this age (arrows).

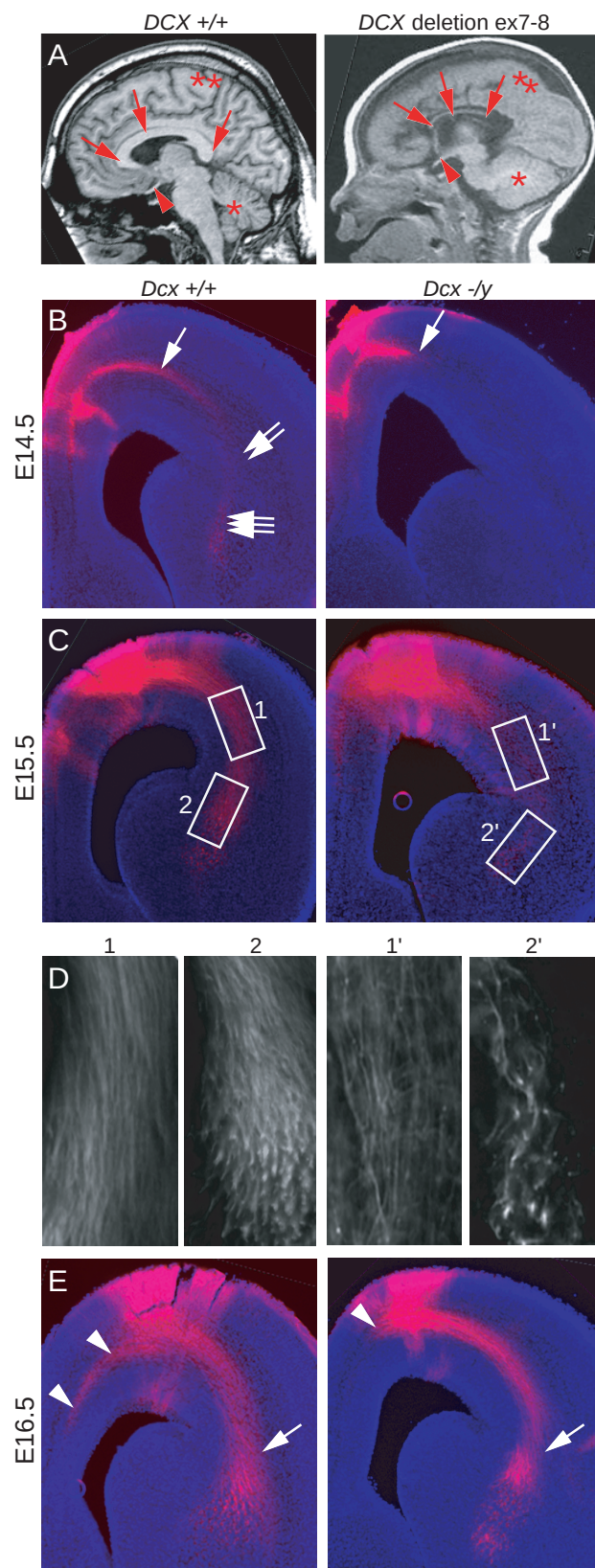


Figure III.2. Spn and Dcx share protein distribution and co-function during brain development. (A) Overlapping localization of Spn, PP1, Dcx and PSer297 Dcx. Dcx exhibited characteristic enrichment along MTs just central to the wrist (double arrows), the axonal shaft (arrowhead) and cell body (double arrowheads), PP1 was distributed diffusely, Spn was enriched in the wrist of the growth cone (double arrowheads), while PSer297 Dcx localized predominantly to the distal wrist region. Note in Merge that PSer297 Dcx was largely excluded from regions of Spn localization (scale bar 10 μ m). (B) Dcx predominantly co-localized with MTs, whereas Spn localizes predominately to the wrist region. (C) *Dcx* and *Spn* cooperate to mediate axonal outgrowth of multiple long distance projections within the brain. Both *Spn*^{-/-} and *Dcx*^{-/y} hippocampus at P21 showed defective lamination of the CA3 region of the hippocampus (arrow), whereas *Spn*^{-/-}; *Dcx*^{-/y} (*DKO*) showed possibly worsened defect compared with either single knockout. The granule cell layer appeared unaffected (arrowhead). Mol = dentate gyrus molecular layer, SLM = stratum lacunosum moleculare, Spy = stratum pyramidale, SR = stratum radiatum. The corpus callosum (CC) was evident in all but the *DKO*, where it was replaced by Probst bundles (PB). The anterior commissure (dashes) showed normal full appearance in all but the *DKO*. Midline indicated by arrowhead. At P0, callosal fibers (stained with L1CAM) were seen to decussate (arrows) in all but the *DKO*, where they terminated in Probst bundles (arrowheads). (D) Expressivity of absent corpus callosum and hypoplastic anterior commissure among offspring from 20 litters of double heterozygous matings. Genotypes are listed along the top. Number of mice with each phenotype of the total number of mice of each genotype are listed. Note that none of the mice except the *Spn*^{-/-}; *Dcx*^{-/-} showed the absent corpus callosum and hypoplastic anterior commissure phenotype. *Dcx*^{-/-} entries include both ^{-/-} females and ^{-/y} male null mice. * = $p < 0.01$, Chi squared test.

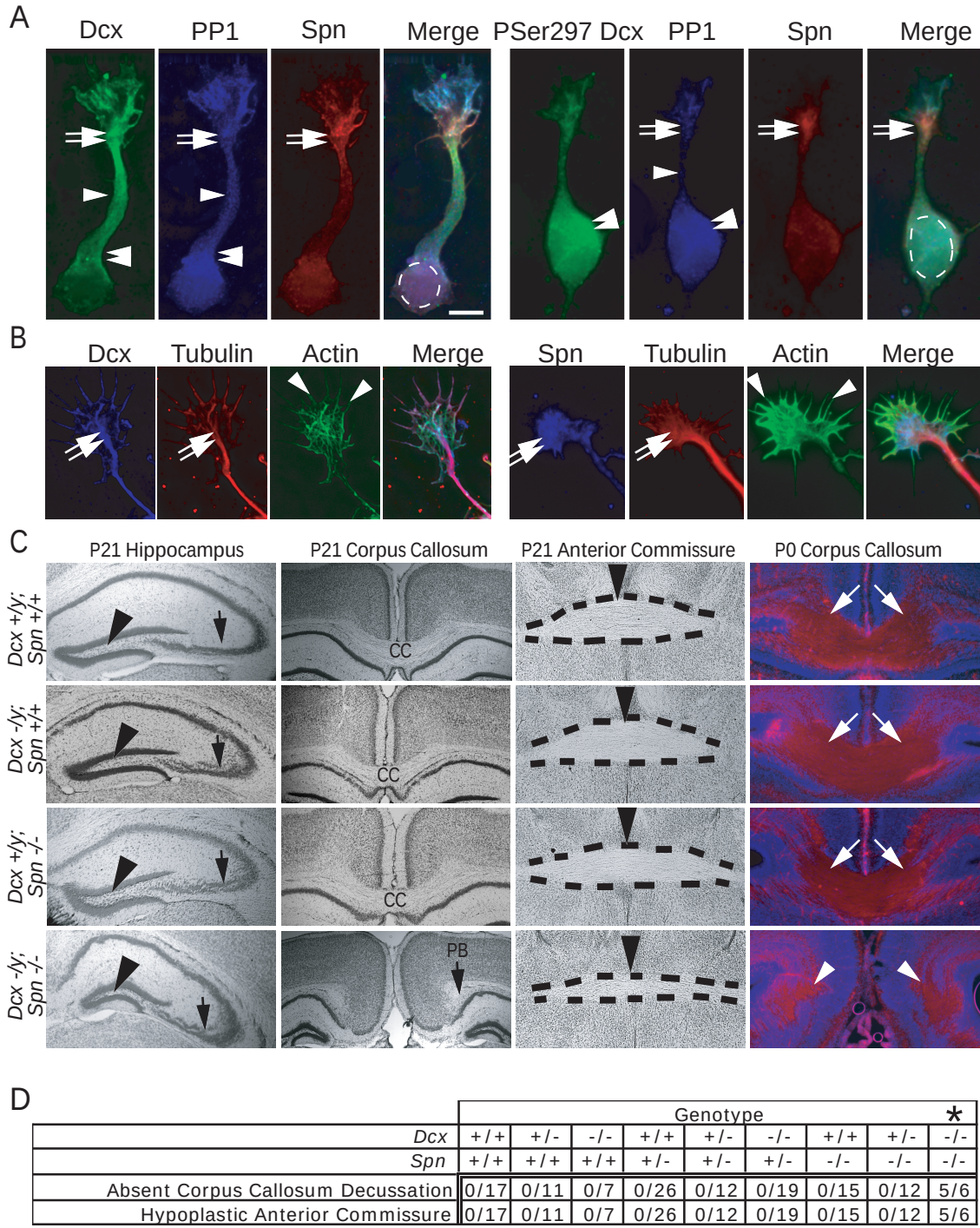


Figure III.3. Disrupted neurite shaft MT cytoskeleton in *Dcx*, *Spn* and *DKO* neurons results in excessively branched neurite phenotype. (A) In *wt* neurons, there were condensed MTs in the neurite shaft (arrowhead). Wrist (double arrow) and growth cone (arrow) are well-delineated. In *Dcx* $-/-$ or *Spn* $-/-$ neurons, MTs failed to form a condensed structure. Instead MTs were splayed (arrowhead), and as a result, the neurite width was increased. (B) MT spacing and orientation is disrupted in axonal shafts in single and double knockout neurons observed with transmission electron microscopy. MTs typically ran in parallel and maintain relatively consistent spacing along the neurite shafts in cultured cortical neurons in *wt* littermates, The parallel array was disrupted in both single knockouts, and instead showed excessive crossing and more random spacing, and is more severe in the *DKO*. Bottom half (dashes) of each shows line tracings depicting MTs. 8000X. (C) Binned inter-MT distance between nearest neighbor is significantly greater in single knockouts and slightly more severe in *DKO* neurons. $N = 1499$ total measurements, from 9 *wt*, 5 *Dcx* $-/-$, 4 *Spn* $-/-$ and 5 *DKO* neuronal shafts from two separate culture experiments. Error bar = SEM. $* = p < 0.05$, Chi squared contingency table. (D) Excessively branched processes occur in the absence of *Dcx* or *Spn*. In *wt* neurons, cells typically display a single monopolar main process (arrow) with occasional 2° branches (double arrow) after 36 hrs in culture. Both *Dcx* $-/-$ and *Spn* $-/-$ neurons exhibit excessive numbers of 2° (double arrows) and 3° (triple arrows) branches from the primary neurite, as well as an increased number of processes extending from the cell soma (arrowhead). This was most striking in *DKO*. (E) Method of quantification of neurite branching. Branches from the main process (MP) are termed 2° MP, and branches from 2° MP are termed 3° MP. Neurites extending from the soma are termed body processes (BP), and branches from the BP are termed 2° BP. (F) Analysis of branching morphology in neurons among different genotypes. *WT* cells typically had a single MP, with average number of 2° MP per cell less than 0.5. The *Dcx* $-/-$, *Spn* $-/-$ and *DKO* neurons all showed 2-3 times this number as well as notable frequency of 3° MP. Average number of Bps per cell was less than 0.05 in *wt* but 4 times greater in mutant cells. Furthermore, 2° BP were only noted with any frequency in *DKO* neurons. $* = p < 0.05$, pairwise comparison, Student *t*-test.

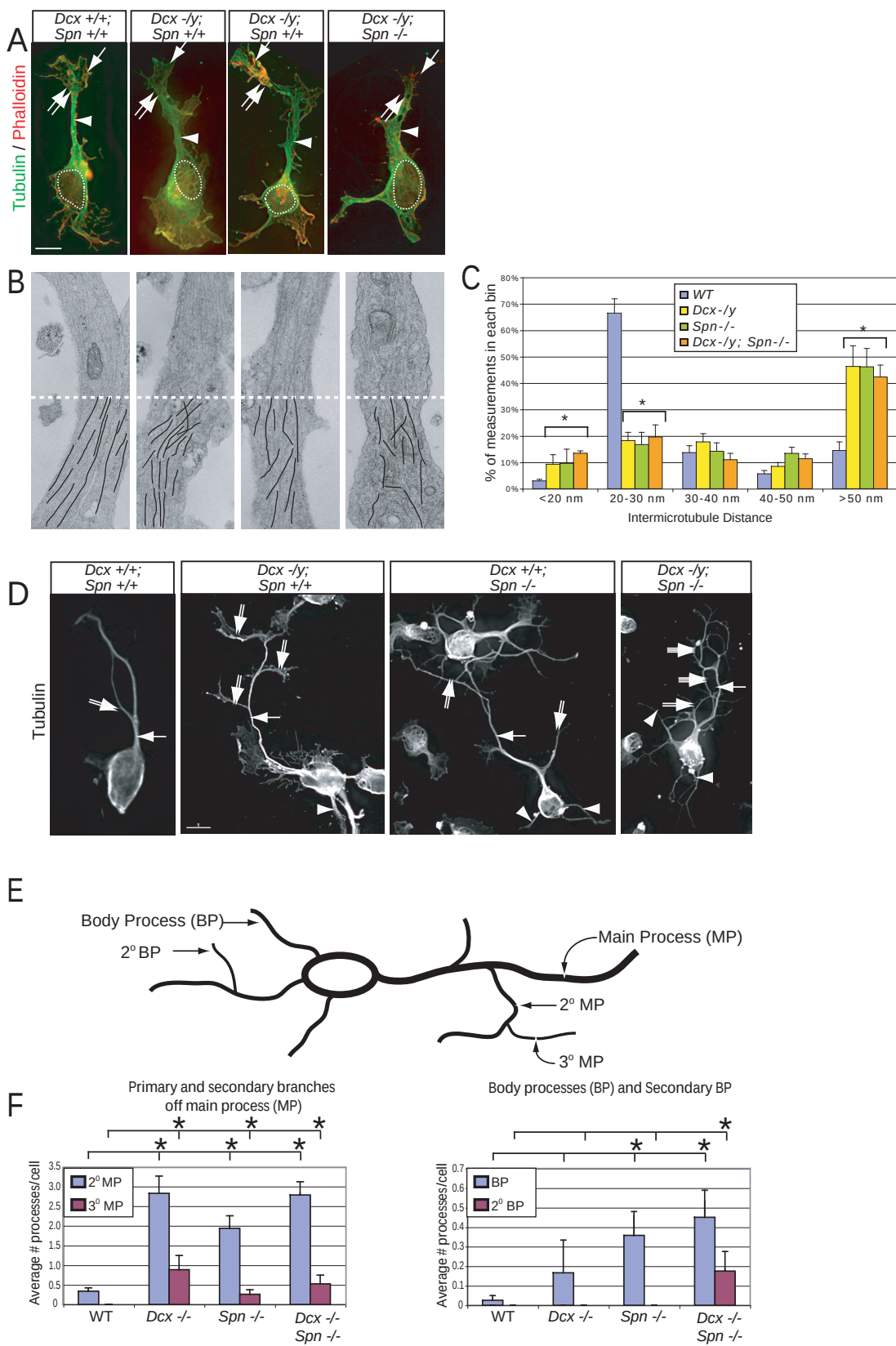


Figure III.4. Spn required for Dcx enrichment at neurite tips, and Dcx/Spn interaction sufficient to co-recruit actin and MT cytoskeletons. (A) E14 *Spn*^{-/-} and *wt* littermate cultured cortical neuron for Dcx and β -tubulin. Enrichment of endogenous Dcx at neurite tips (arrow) is disrupted in *Spn*^{-/-} neurons. Note disarrayed MTs (arrowhead) in knockout. Nucleus indicated by dashes. Scale bar = 10 μ m. (B) Line profiles of Dcx immunofluorescence intensity along the neurite (as depicted by the red line in the schematic) of cultured cortical neurons, shows absence of enrichment of Dcx at distal axonal segment in *Spn*^{-/-} versus littermates. (C) E14 cultured cortical neurons transfected with Dcx-RFP, which fails to enrich in neurite tips in *Spn*^{-/-} (arrows). (D) Quantification of Dcx-RFP localization at neurite tips versus throughout the cell. Error bar = SEM. *N* = number of cells in each group presented to blinded investigator. (E) Line profiles of overexpressed Dcx-RFP intensity along the neurite of cortical neurons shows absence of enrichment at distal segment. (B) and (E), representative of 5 neurons each condition, and neurite length and fluorescent intensity were normalized. (F) Purified Spn and Dcx sufficient to link phalloidin-stabilized actin and taxol-stabilized MTs. (a) Spn added to actin leads to production of filaments. (b) Dcx added to tubulin leads to asters of MTs. (c) Cytoskeletons alone show no co-recruitment, and neither is tubulin recruited to Spn-stabilized actin (d) or actin recruited to Dcx-stabilized tubulin (e). However, Spn-stabilized actin and Dcx-stabilized MTs show significant co-recruitment of the two cytoskeletons (f). Repeated in triplicate.

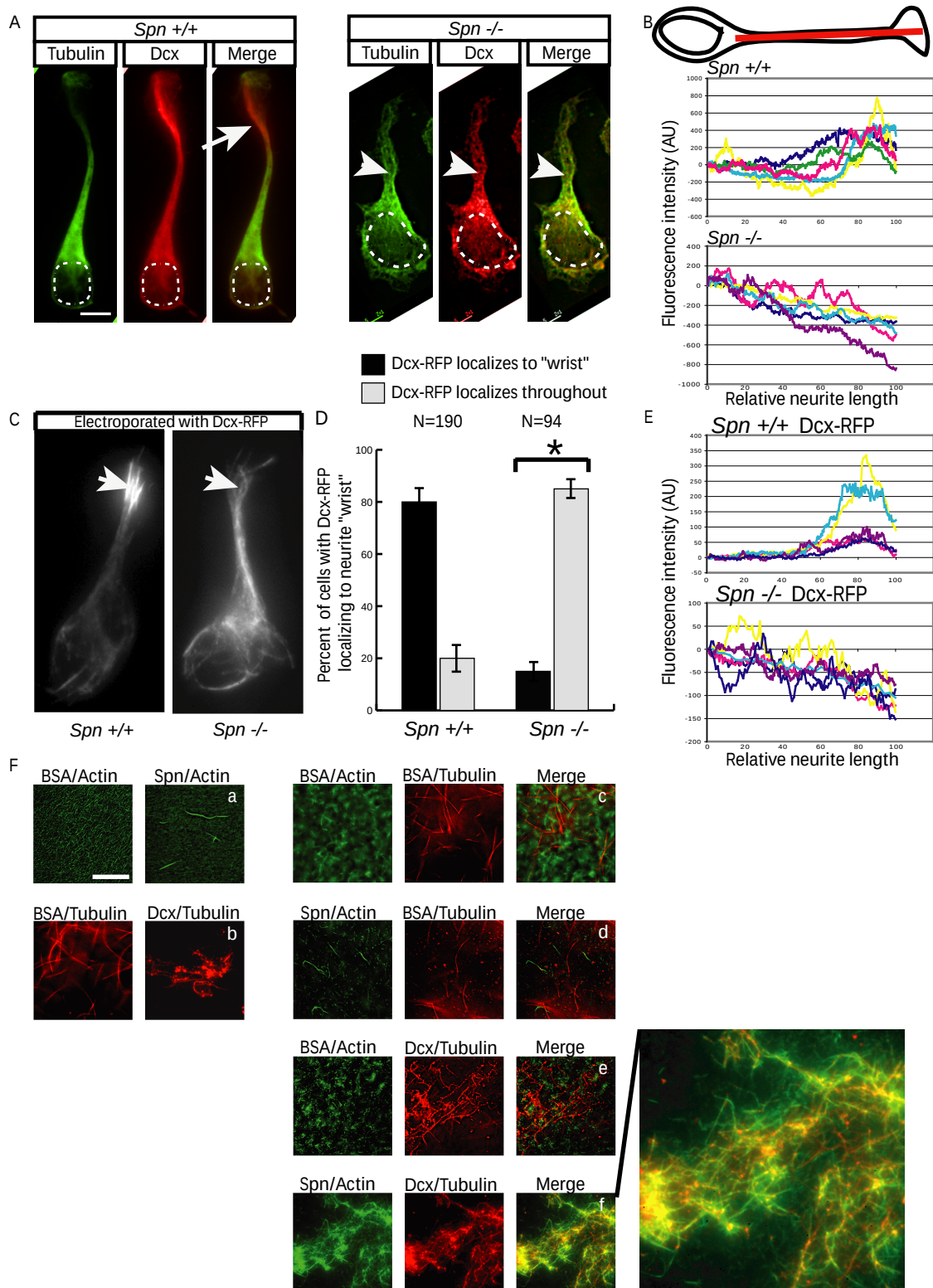


Figure III.5. Spn required for PP1-mediated dephosphorylation of PSer297 Dcx. (A) PP1 at high unit concentrations is capable of dephosphorylating Dcx at PSer297, based on autoradiogram or immunoreactivity with α PSer297 following [32 P] incorporation. PP1 is a more specific phosphatase for the PSer297 site than CIP, resulting in nearly complete dephosphorylation at all concentrations tested. (B) Spn alone has no effect on α PSer297 reactivity or [32 P] retention. (C) Spn promotes PP1 dephosphorylation of Dcx at Ser297. Low levels of PP1 (5X lower than used in A) in the absence of Spn have no effect on [32 P] retention or α PSer297 reactivity but increasing amounts of Spn promotes dephosphorylation of Dcx by PP1. (D) PP1 and Cdk5 reciprocally phosphorylate Ser297 of Dcx in cortical neurons. Disassociated E14 cortical neurons were treated for 2 hrs with increasing concentrations of roscovitine (inhibits Cdk5) or tautomycin (inhibits PP1). Western analysis using antisera to Dcx and two different phospho-specific antibodies, PSer297 and PThr321 shows roscovitine blocked PSer297 reactivity but not PThr321, whereas tautomycin had the opposite effect. (E) Dcx Ser297 phosphorylation increased in *Spn*^{-/-} WBL. Brain lysates from E16 littermates was split and half was CIP treated. PSer297 reactivity increased as *Spn* dosage was decreased. (F) Quantification of PSer297 Dcx band intensity standardized to control shows a four-fold increase in reactivity in *Spn*^{-/-} versus +/+.

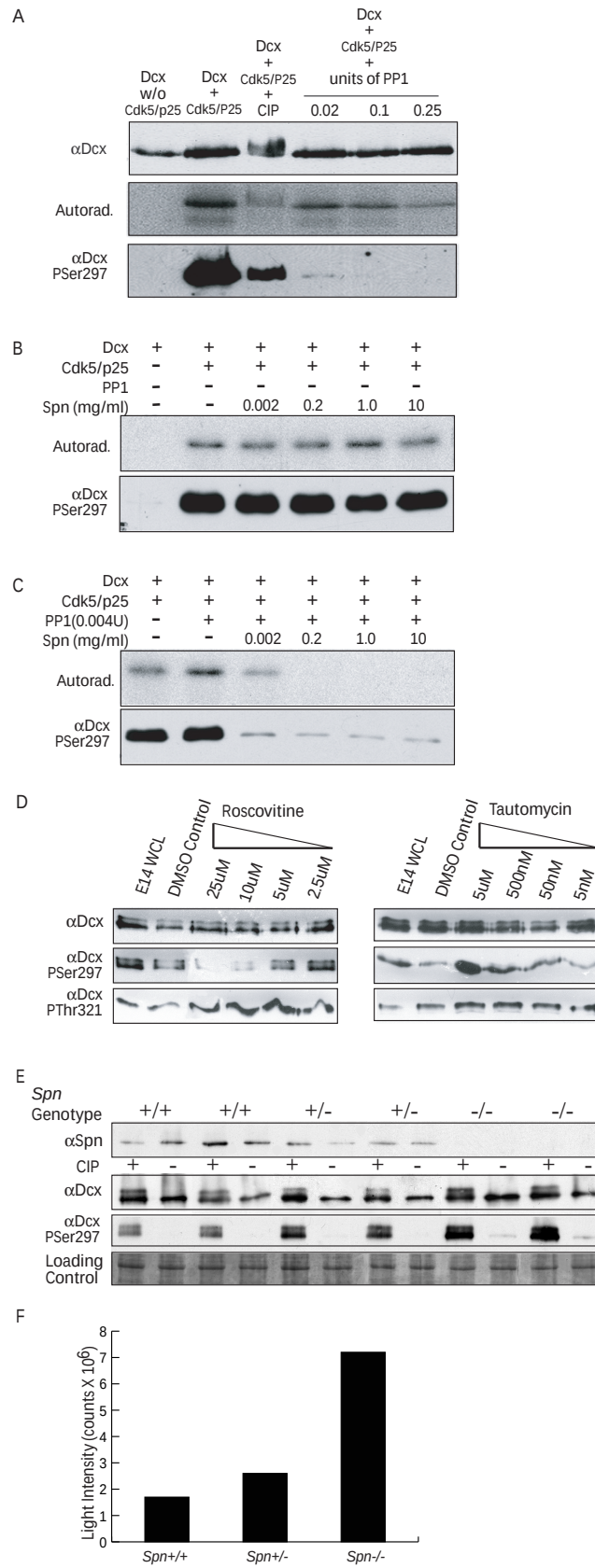
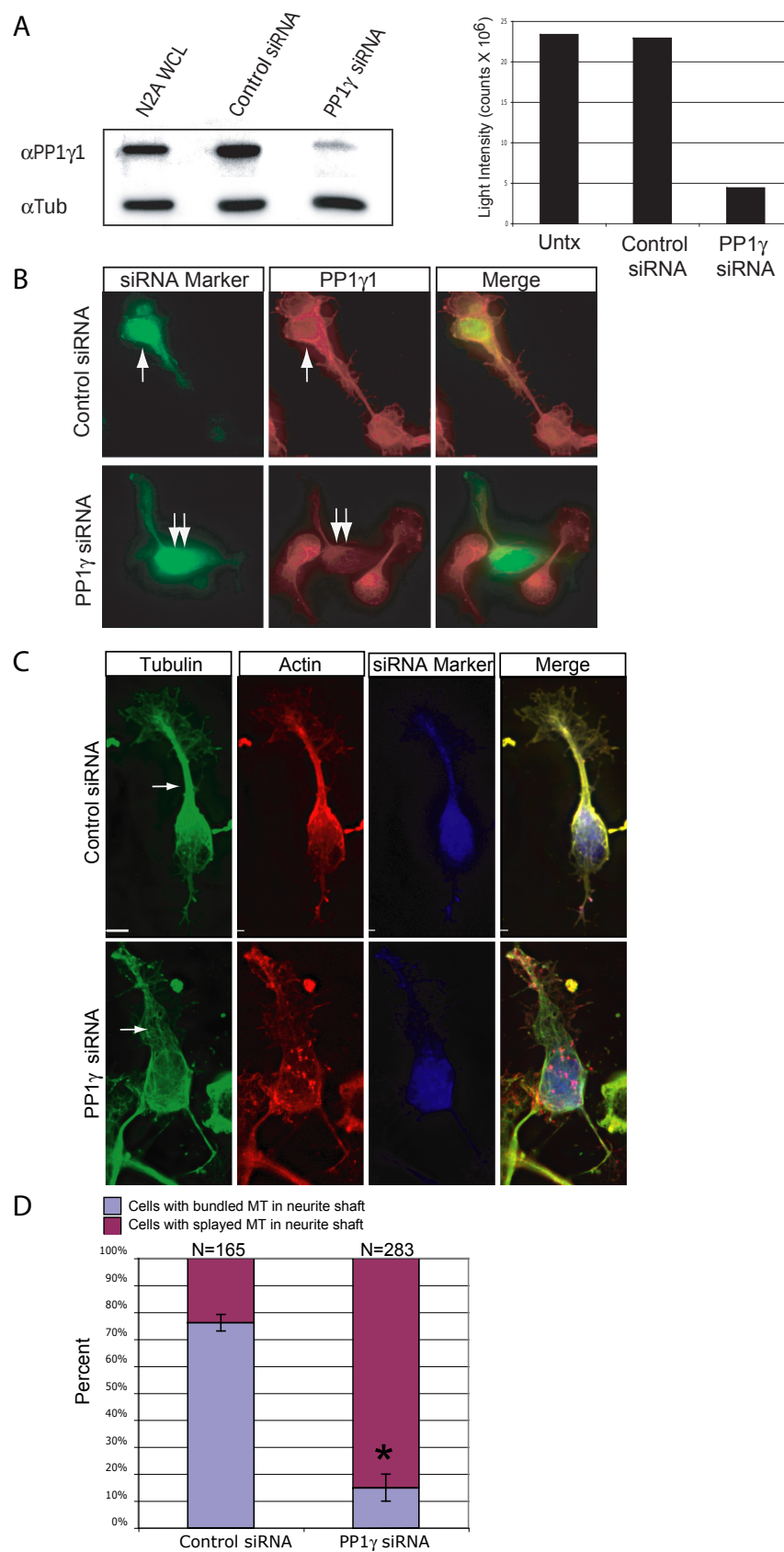


Figure III.6. PP1 catalytic activity is required for proper MT bundling during neurite outgrowth. (A) N2A neural cells were electroporated with control or PP1 γ siRNA and then lysates analyzed by Western. Tubulin shows roughly equal intensity, whereas there was a 6-fold reduction in PP1 γ 1 intensity following siRNA. (B) Reduced PP1 γ 1 expression following PP1 γ siRNA transfection in primary neurons. Cells were electroporated with EGFP and scrambled siRNA as control. Note reduced expression of PP1 γ 1 following specific (arrows) but not control (arrow) siRNA treatment. (C) PP1 γ knockdown associated with failure of MT bundling and broadened primary neurite. Arrow highlights typical appearance of neurite and bundled MTs in primary neurite in control siRNA treated cells. PP1 γ siRNA treated cells show a disrupted MT cytoskeleton (arrow). (D) Quantification showing percentage of cells with splayed MT phenotype in primary neurite shaft significantly increased following PP1 γ siRNA treatment. $N = 165$ and 283 for control and PP1 γ siRNA, results averaged from two experiments. $* = p < 0.05$, Student t -test. Scale bar $5 \mu\text{m}$



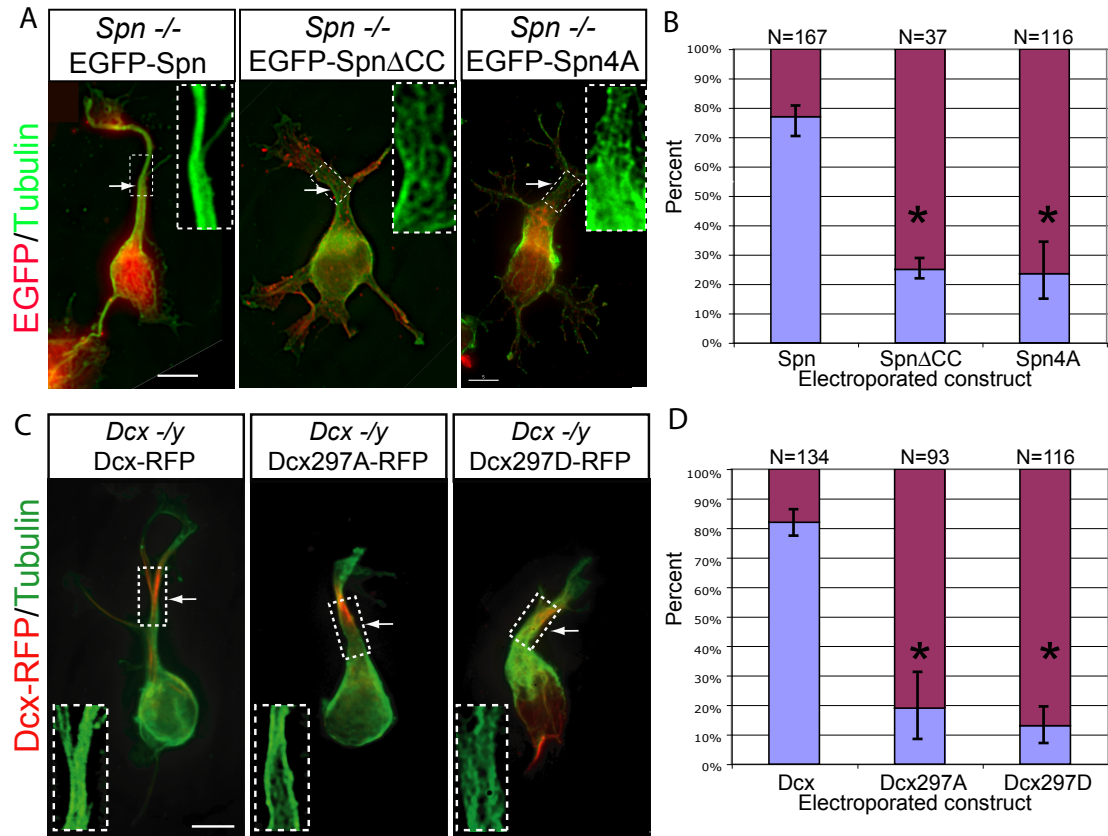
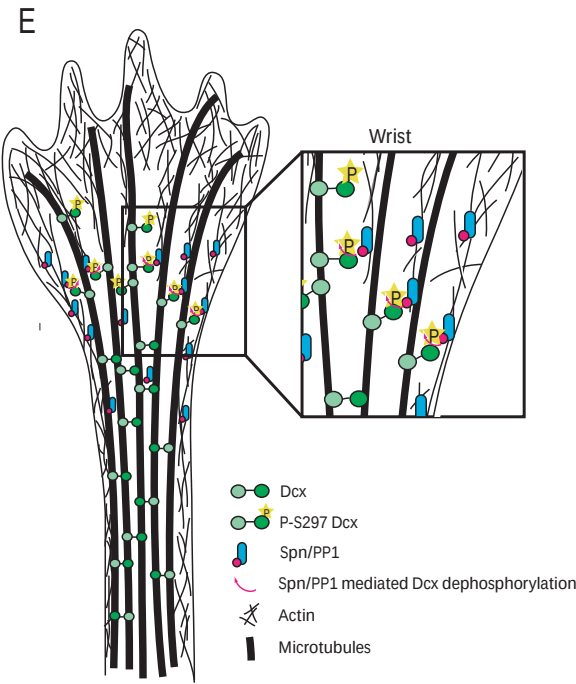
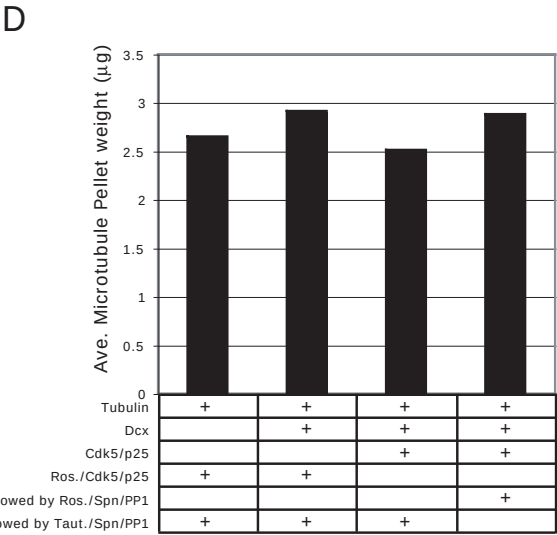
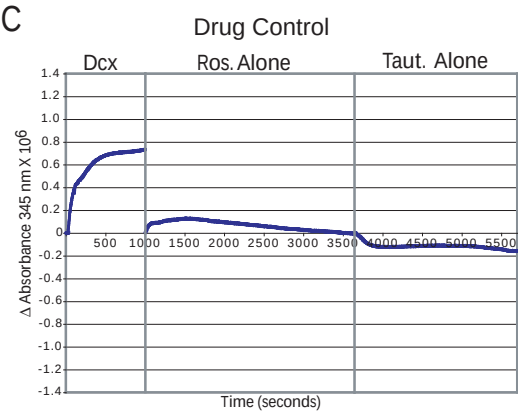
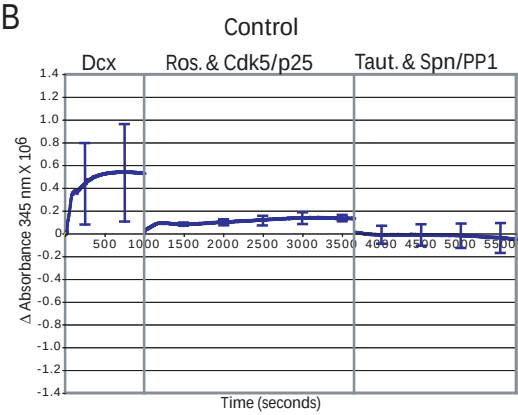
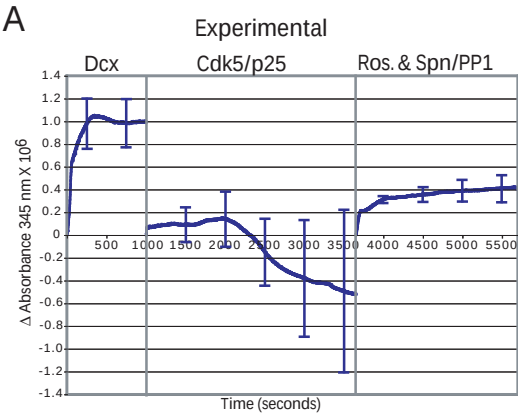


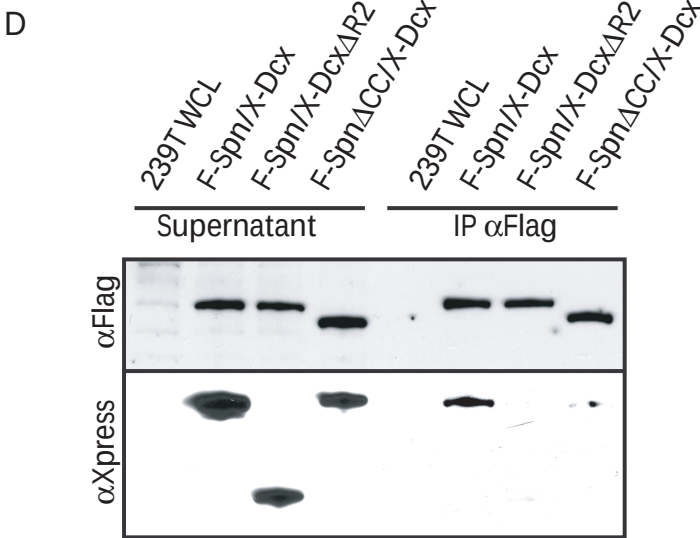
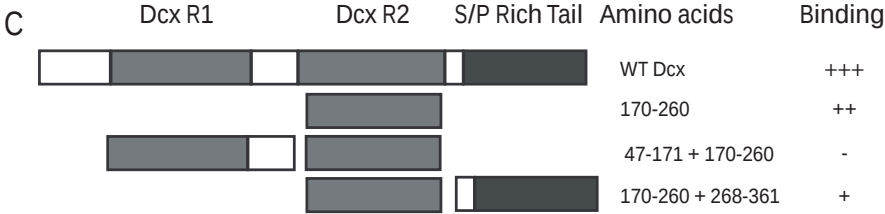
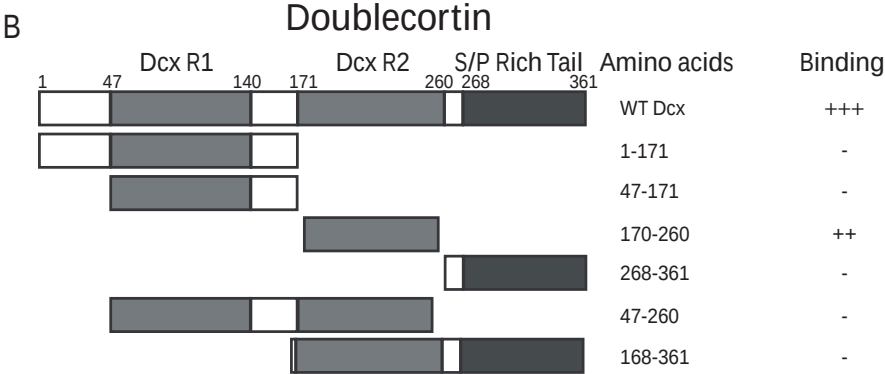
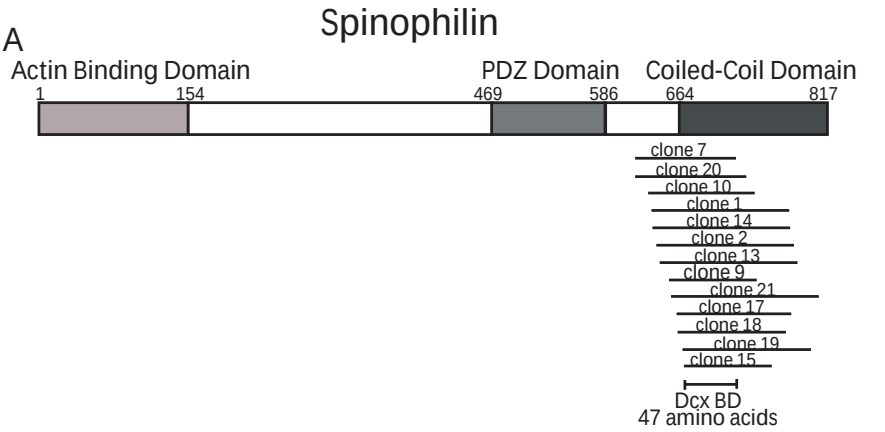
Figure III.7. Association between Spn, PP1 and Dcx is required for MT bundling in neurites. (A) SpnDCC does not rescue the MT bundling defect in *Spn*^{-/-} neurons. Acutely dissociated *Spn*^{-/-} neurons were electroporated with EGFP-Spn or EGFP-SpnDCC, cultured until stage 1-2 neurites were visible, then fixed and stained for EGFP and MTs. *Spn*^{-/-} neuron with Spn showed normalization of the leading process morphology, with a single narrow MT-filled shaft, whereas those electroporated with SpnDCC showed persistence of the defect in the MT cytoskeleton. Inset shows higher power of MT bundling defect used for scoring. (B) Neurons scored for the MT bundling defect shown were a N = 110 for Spn and 37 for SpnDCC, * $p < 0.01$ Student t-test. (C) DcxS297A and S297D do not rescue the MT bundling defect in *Dcx*^{-/-} neurons. Acutely dissociated *Dcx*^{-/-} neurons were electroporated with Dcx-RFP or corresponding mutations, cultured until stage 1-2 neurites were visible then fixed and stained for MTs. (D) *Dcx*^{-/-} neuron with Dcx showed normalization of the leading process morphological defect, with a single narrow MT-filled shaft, whereas both mutations showed a widened neurite shaft and poorly condensed MT. Inset shows higher power of MT bundling defect in neurite shaft. Scale bar = 5 μ m.

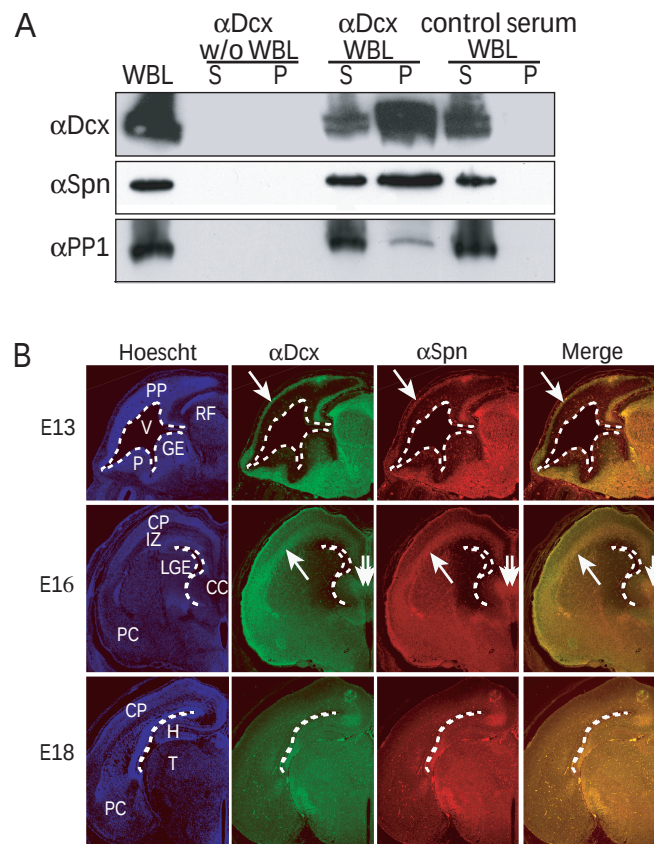
Figure III.8. Spn-PP1-mediated dephosphorylation reinstates the tubulin polymerization effect of Dcx tubulin. Turbidimetric assays with phosphocellulose-purified tubulin incubated with recombinant Dcx serially treated with Cdk5 and Spn-PP1 modulate Dcx tubulin polymerization capabilities. (A) Dcx and tubulin co-incubated for 1000 sec shows robust increase in turbidity. Subsequently, activated Cdk5/p25 was added to phosphorylate Ser297, which resulted in a net decrease in turbidity over the next 2500 sec. Subsequent addition of roscovitine (to block Cdk5 activity) and Spn-PP1 (to dephosphorylate P_{Ser297} Dcx) were added, which resulted in another net increase in turbidity over the next 2500 sec. (B) Cdk5/p25 that was pretreated with roscovitine, or Spn-PP1 that was pretreated with tautomycin had no net effect on turbidity. (C) Neither roscovitine nor tautomycin alone had any net effect on turbidity in this assay. Error bars = SEM from three trials. (D) Co-sedimentation analysis of dynamic regulation of Dcx phosphorylation on MT polymerization. Four separate trial conditions were assessed. Lane 1 was purified tubulin treated with inactive (roscovitine-preincubated) Cdk5/p25 then inactive (tautomycin-preincubated) Spn/PP1. Lane 2 was the same except that Dcx was added prior to other constituents, leading to increased MT pellet weight. In lane 3, active Cdk5/p25 was added after Dcx, followed by inactive Spn/PP1, leading to a return to baseline weight. In lane 4, active Cdk5/p25 was added, followed by roscovitine and active Spn/PP1. Thus, the dephosphorylation of previously phosphorylated sites could occur. This was associated with an increased MT pellet weight. Averaged from two experiments. (E) Model for the role of Dcx and Spn in neurite development. In this model Dcx is localized to MTs throughout the neurite, and P₂₉₇Ser Dcx is localized predominantly to the distal “wrist” region of the growth cone. Spn is restricted in localization to the “wrist” region, where it is complexed with PP1 to mediate dephosphorylation of P₂₉₇Ser Dcx. This leads to reactivation of Dcx, with subsequent MT crosslinking activity that is necessary for MT bundling in the neurite shaft.



Supplemental Figure Legends

Supplemental Figure III.1. Dcx-Spn interaction detected by yeast two-hybrid, and verified by co-affinity and co-immunoprecipitation to be dependent upon the Spn CC domain and Ecx R2 domain. (A) Yeast two-hybrid screen using Dcx as “bait” identified fragments of Spn in over half of the clones. A minimal 47 amino acid fragment of the CC domain was shared by all of the clones identified. Spn is a multidomain actin binding protein. (B) Co-affinity fragment analysis indicated that the R2 domain of Dcx bound Spn. Strength of affinity indicated by “+”. (C) The Dcx R1 and the S/P domain inhibited the Dcx R2 interaction with Spn *in trans*. (D) 293T cells transfected with plasmids encoding either Flag (F)-tagged full-length Spn or Spn Δ CC, and Xpress (X)-tagged full-length Dcx or Dcx Δ R2, immunoprecipitated with α -Flag, then analyzed by Western. Untransfected cells were included to control for antibody specificity, and supernatants contained roughly equal amounts of protein. Full-length but not deleted Dcx and Spn were co-immunoprecipitated in this assay.





Supplemental Figure III.2. In vivo co-immunoprecipitation of Spn and Dcx is Co-immunoprecipitation from E16 mouse brain lysates (WBL). Dcx specific antisera co-immunoprecipitated Spn and PP1, versus control sera or absent WBL. (E) Co-distribution of Dcx (green) and Spn (Waterston et al.) throughout development, especially in regions of major axonal tracts. E13 (sagittal, arrow = preplate/early cortical plate), E16 (coronal, arrow = intermediate zone containing axonal tracts and migrating neurons, double arrow = developing corpus callosum), and E18 (coronal). CC = corpus callosum, CP = cortical plate, Dashed lines, V = ventricles, GE = ganglionic eminence, H = hippocampus, IZ = intermediate zone, LGE = lateral ganglionic eminence, P = pallium, PP = preplate, RF = rhombic flexure, PC = paleocortex, T = thalamus.

Supplemental Movie III.1-5.

Movies can be accessed at:

(http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6WSN-4NMMB5G-

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3D tomographs through axonal shafts of cultured cortical neurons derived from *wt*, *Dcx* *-/-*, *Spn* *-/-*, or *Dcx* *-/-*; *Spn* *-/-* mice. White lines represent individual MTs traced along their entire visible length. Red represents cellular membrane. Note that in both *wt* neurons, the inter-MT distance is relatively uniform and MTs are uniformly oriented along the axonal shaft. In single knockout neurons, inter-MT distance is greater, and the shaft is of greater width. In *DKO* neurons the MTs are fragmented and poorly bundled.

Chapter Acknowledements

Parts of this chapter have been published and were taken from the following article, of which the dissertation author was the primary author:

Bielas SL, Serneo FF, Chechlac M, Deerinck TJ, Perkins GA, Allen PB, Ellisman MH, Gleeson JG. Spinophilin facilitates dephosphorylation of doublecortin by PP1 to mediate microtubule bundling at the axonal wrist. *Cell*. 2007 May 4;129(3):579-91.

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