Identification of Transforming Growth Factor-β as an Extracellular Signal Required for Axon Specification in Embryonic Brain Development

by

Jason J. Yi

Department of Pharmacology
Duke University

Date: ________________________________

Approved:

_____________________________________
Michael D. Ehlers, Committee Chair

_____________________________________
Robert Wechsler-Reya

_____________________________________
Gerard C. Blobe

_____________________________________
J.H. Pate Skene

_____________________________________
Xiao-Fan Wang

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology in the Graduate School of Duke University 2009
ABSTRACT

Identification of Transforming Growth Factor -β as an Extracellular Signal Required for Axon Specification in Embryonic Brain Development

by

Jason J. Yi

Department of Pharmacology
Duke University

Date: ________________________________

Approved:

_____________________________
Michael D. Ehlers, Committee Chair

_____________________________
Robert Wechsler-Reya

_____________________________
Gerard C. Blobe

_____________________________
J.H. Pate Skene

_____________________________
Xiao-Fan Wang

An abstract of a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology in the Graduate School of Duke University 2009
Abstract

The specification of a single axon and multiple dendrites is the first observable event during neuronal morphogenesis and such structural specialization underlies neural connectivity and nervous system function. Numerous intracellular signaling components that are required for axon specification have been described but how such signaling paradigms are initiated by extracellular factor(s) within the embryonic milieu is poorly understood. Here, I describe how transforming growth factor-β (TGF-β), an embryonic morphogen that directs structural plasticity and growth in various cell types, initiates signaling pathways both in vivo and in vitro to fate naïve neurites into axons. Using conditional knockout strategies, I found that cortical neurons lacking the type II TGF-β receptor (TβR2) fail to initiate axons during development, and interestingly, fail to engage radial migration. In cultured neurons, exogenous TGF-β is sufficient to direct the rapid growth and differentiation of an axon and genetic enhancement of receptor activity promotes the formation of multiple axons. The cellular polarization of receptor activity occurs through the interaction of the type-I TGF-β receptor with Par6, a component of the axon-specifying Par3/Par6 polarity complex. Receptor distribution is restricted to axons, and downstream signaling events required for axon specification are triggered when Par6 is phosphorylated by TβR2. Together, these results indicate that TGF-β is the extrinsic cue for neuronal polarity in vivo and directs neuronal polarity by controlling Par6 activity and cellular migration during axon generation.
Table of Contents

Abstract ......................................................................................................................... iv

List of Figures .............................................................................................................. viii

Acknowledgements ..................................................................................................... x

Chapter 1. Introduction ............................................................................................... 1

Organization of the adult cortex ................................................................................. 2

Development of the embryonic cortex ....................................................................... 2

Radial glia .................................................................................................................... 4

Neuronal morphogenesis and polarity ....................................................................... 5

In vivo and in vitro neuronal development .................................................................. 9

Actin cytoskeleton ....................................................................................................... 10

Rho GTPases .............................................................................................................. 11

Microtubule cytoskeleton .......................................................................................... 14

Microtubule stability in axon specification ................................................................ 16

Polarized trafficking .................................................................................................. 17

The Par polarity proteins ......................................................................................... 20

Par proteins in epithelial polarity ............................................................................ 20

Par proteins in axon specification ............................................................................ 22

Par proteins in cell fate determination ..................................................................... 27

Par proteins in cell migration .................................................................................. 29

The TGF-β superfamily of ligands ........................................................................... 30

TGF-β signaling at the membrane ............................................................................ 33

The structure of type I and type II TGF-β receptors ............................................. 36
List of Figures

Figure 1-1. Stages of neuronal polarization in vivo and in vitro...............................................8
Figure 1-2. The Par proteins in axon specification.................................................................26
Figure 1-3. The TGF-β signaling pathway............................................................................35
Figure 3-1. TGF-β ligand expression is spatially segregated during embryonic development........................................................................................................76
Figure 3-2. Axonal expression of TGF-β receptors in the developing mouse neocortex................................. ........................................................................79
Figure 3-3. The ex vivo electroporation and organotypic slice culture........................................82
Figure 3-4. TGF-β signaling is required for neocortical development in vivo..........................85
Figure 3-5. TGF-β affects migration and morphological dynamics at the axonal pole, but not the dendritic pole......................................................................................................................88
Figure 3-6. Pharmacological inhibition of TGF-β receptors prevents axon Specification............................... ........................................................................91
Figure 3-7. Cell autonomous TGF-β signaling is necessary and sufficient to induce Axons................................................................................................................................................94
Figure 3-8. Restricted TGF-β signaling is required to maintain neuronal Polarity...................................... ........................................................................97
Figure 3-9. Locally applied TGF-β is sufficient to specify axonal differentiation and growth........................................................................................................................................100
Figure 3-10. A model for TGF-β-dependent axon Specification........................................................................106
Figure 4-1. TβR2 is localized to trailing edge axons in neurons engaging Migration........................................................................................................................................118
Figure 4-2. TGF-β receptor polarize to nascent axons........................................................................121
Figure 4-3. TβR1 forms a complex with Par6 during neuronal Development.................................................................124
Figure 4-4. Par6 phosphorylation by TβR2 is required for neuronal polarity in vitro

Figure 4-5. Phosphomimetic Par6-S345E restores axons in TβR2 KO neurons in vivo

Figure 4-6. The modular domains of Par6 and evolutionary conservation of Serine/Threonine 345 in vertebrates

Figure 5-1. Neurons produce TGF-β1 and TGF-β2, but do not secrete ligands at detectable levels

Figure 5-2. Neuronal polarization in single cell cultures, and surface expression of TβR3

Figure 5-3. Synaptic localization of TGF-β receptors and ligands in mature neurons

Figure 5-4. TGF-β treatment of neurons perturbs dendritic spine morphology
Acknowledgements

This work was completed under the guidance of my advisor and mentor Michael Ehlers, who provided me guidance and support throughout my time in his lab. This thesis represents six years of (at times frustrating) work, and it could not have been completed without his vision, encouragement, and willingness to buy a durable lab espresso machine.

My Thesis Committee (Gerard Blobe, Pate Skene, Xiao-Fan Wang, and Rob Wechsler-Reya) has been helpful and supportive throughout, and I thank them for their critical insight and discussion. It has been a pleasure to work with our collaborators Franck Polleux and Paul Barnes from the University of North Carolina – Chapel Hill. I thank all members of the Ehlers lab past and present for their friendship, advice, assistance, and for creating a stimulating work environment. My sincere gratitude goes to Haiwei Zhang, Zhiping Wang, Tingting Wang, Rui Peixoto, Mikyoung Park, Tom Newpher, Yuanyue Mu, Angela Mabb, Sangmi Lim, Ming-Chia Lee, Irina Lebedeva, Marguerita Klein, Matt Kennedy, Hyun-Soo Je, April Horton, Tom Helton, Cyril Hanus, Ian Davison, Kathryn Condon, Tom Blanpied, and Ben Arenkiel.

Finally, I thank my family for their incredible encouragement and support throughout my time at Duke. I am especially grateful to my wife Jennifer for her love, encouragement, and unconditional patience throughout this journey.
Chapter One. Introduction

The polarized architecture of neurons is integral for their capacity to formulate functional neural networks. Typically, neurons are composed of multiple dendrites, which receive synaptic inputs, and a single axon, which transmits action potentials across long distances. This axon/dendrite paradigm facilitates intercellular communication and dictates the directionality of information flow in the nervous system. Proper neuronal function, and ultimately nervous system utility, requires cellular polarization to occur at the appropriate time and at the right location within the embryonic framework. Understanding how such drastic structural divergence occurs within a single cell has remained an enduring challenge in the field of cellular neuroscience.

Much of what we know regarding the molecular events that occur during neuronal polarization have been derived from the primary culture of dissociated rodent neurons. In culture, neurons proceed through stereotyped morphological stages (stages 1-7) in which cellular symmetry is broken when axon specification and growth occurs from the transition of an unpolarized stage 2 to a polarized stage 3 neuron (Dotti et al., 1988). This model system has been particularly advantageous due to its experimental accessibility for cell biological, genetic, and pharmacological methods. Many intracellular signaling pathways required for the establishment and maintenance of neuronal polarity have been elucidated from cultured neurons (Arimura and Kaibuchi, 2007). However, the culture environment is non-isotropic and consists of a diffuse milieu of growth factors, making it a poor system to determine how newborn neurons
communicate with their extracellular environment during development. Therefore, how intracellular signaling pathways are initiated and controlled within the proper context requires further study of cellular behavior within native embryonic tissue.

**Organization of the Adult Cerebral Cortex**

The mammalian cerebral cortex is a large structure that contains centers for higher order cognitive functions such as memory, learning, language, and thought. Based on the work of Korbinian Brodmann in 1909 (Brodmann, 1909), six principle layers are defined within the cortex corresponding to horizontal laminar layers found within the cortical wall, each with distinct morphological and functional identities. These are labeled from I – VI, from the outside-in, respectively, and are constituted by different cell populations. Molecular layer I consists mainly of apical dendrite extensions and horizontal axon projections with a few scattered Cajal-Retzius cells and spiny stellate neurons. The external granular layer II contains small pyramidal neurons and numerous stellate neurons. The external pyramidal layer III is predominantly populated by small to medium size pyramidal neurons, whereas the internal granular layer IV contains various types of stellate and pyramidal neurons and serves as the main target of thalamocortical afferents. The internal pyramidal layer V is comprised of large pyramidal neurons and is the principal source of subcortical efferents. The multiform layer VI contains large pyramidal neurons and many small spindle-like pyramidal and multiform neurons, and projects efferent fibers to the thalamus.
Development of the Embryonic Cortex

A remarkable aspect of brain development is that although neurons are generated from progenitor cells near ventricular zones deep within the brain, newborn neurons migrate long distances to reach their final destinations. This is particularly evident during the process of cortex development in which newborn neurons migrate to the outer surface of the developing cortex. In earlier stages when the cortical anlage is small, the migratory distances are short but as development proceeds, the distances progressively increase, and in the case of the primate brain can reach distances up to 7 mm (Rakic, 2003). Not surprisingly, the migrating neuron encounters a rushing current of extracellular cues that it must correctly perceive, decipher, and ultimately, to which it must properly respond. In reflection of the complexity of this process, various types of molecules have been implicated in disorders of neuronal migration including receptors, adhesion molecules, extracellular matrix proteins, intracellular signaling molecules, and transcription factors (Gleeson and Walsh, 2000; Hatten, 2002; Honda et al., 2003; Ross and Walsh, 2001).

Neuronal migration occurs during embryonic days (E) 11-18 of development in mice and between weeks 10-20 in humans. In both cases, migration occurs in three distinct stages. In the preplate stage, the first postmitotic cortical neurons aggregate in an outside-in sequence to form a transient cell layer termed the preplate (Marin-Padilla, 1971). In the second stage, known as the cortical plate stage, waves of newborn neurons exit the ventricular zone and move in a radial direction toward the preplate to form a new series of layers collectively termed the cortical plate, which consequently splits the preplate into a superficial layer known as the marginal zone, and a deeper layer, termed
the subplate. Between E14-18 in mice and weeks 12-20 in humans, additional swells of migrating neurons arrive at the cortical plate. They by-pass earlier generated neurons to form the cortical layers in an inside-out fashion with deeper layers (VI) being the first to form, and the superficial layers (I) being the last to form (Angevine and Sidman, 1961). As discussed above, the adult mammalian cortex is composed of six distinct layers (I – VI) and the correlation between laminar fate and birth order remains true for all neurons, including excitatory pyramidal neurons that constitute the principle projection neurons, and inhibitory interneurons that modulate local circuits (Fairen et al., 1986; Miller, 1985; Peduzzi, 1988).

Recent studies examining the sites of origin of these two populations of neurons have demonstrated that whereas cortical pyramidal neurons are generated within germinal zones of the dorsal telencephalon (Chan et al., 2001; Gorski et al., 2002; Hatanaka and Murakami, 2002), most, if not all cortical interneurons are born in the ventral telencephalon, which includes the proliferative zones of the ganglionic eminence (Anderson et al., 1997; de Carlos et al., 1996; Lavdas et al., 1999; Tamamaki et al., 1997). Thus, interneurons must travel a circuitous route to their final destinations while cortical pyramidal neurons follow a relatively direct radial path to their laminar position in the developing cortex. Since this thesis focuses on pyramidal neuron development, the following sections will be limited to a discussion of events that occur during the migration of pyramidal neurons.

**Radial Glia**
Radial glial cells are widespread non-neuronal cells in the developing central nervous system of all vertebrates. They are precursor cells to neurons, and are well identified by their characteristic radial morphology. Their somas and apical domains are located within the ventricular zone (VZ), and a long basolateral process extends from its cell body throughout the neural wall and attaches to the basement membrane at the pial surface (Cajal, 1911; Kolliker, 1890; Retzius, 1892). During different phases of the cell cycle, the somata of radial glial cells move to different locations in the VZ. During the M-phase, somata are located apically, and during G1, the somata move basally to the basal most position of the VZ where they undergo S-phase. This interkinetic nuclear migration allows the cells to continually bridge the expanse of the cortical wall.

In addition to its role in proliferation, glial cells assume morphologies and functions that are instrumental for guiding neuronal migration. During early stages of nervous system development, glial cells elaborate processes that span the wall of the neural tube, and these cells provide the substrate for directed cell migration (Rakic, 1972). Intriguingly, there is a synergistic relationship between the glial scaffold and neurons during embryonic development. For example, RF60 a secreted neuronal protein can induce the extension of radial glia processes, ensuring that neurons migrate to a proper distance during cortical development (Hunter and Hatten, 1995). In accordance with this observation, RF60 levels are abundant during mid-gestation when neuronal migration is at its peak, attenuated at later periods when migration subsides, and undetectable in adults (Hunter and Hatten, 1995).
Neuronal Morphogenesis and Polarity

Only recently have advancements in live-tissue imaging techniques allowed the visualization of dynamic events that unfold during early neuronal development in vivo. These studies have demonstrated that neurons generated in cortical proliferative zones journey through distinct morphological stages distinguished by abrupt changes in cell shape, direction of movement, and speed of migration (Noctor et al., 2004; Tabata and Nakajima, 2003). Four distinct phases of migration are now recognized (Noctor et al., 2004) (Figure 1-1A). In the first phase, neurons generated at the ventricular surface move radially away from the ventricle to the sub-ventricular zone (SVZ). In phase two, they pause in the IZ-SVZ for as long as 24 hours and become multipolar cells (Bayer and Altman, 1991; Noctor et al., 2004). Time-lapse imaging of phase two cells has shown that these multipolar cells are incredibly dynamic with rapid extension and retraction of processes, similar to the behavior of stage 2 cultured neurons. Interestingly, many but not all neurons pass through the third phase, during which they extend a process towards the ventricle and often translocate toward the ventricle (Noctor et al., 2004). Upon reaching the ventricle, neurons enter the fourth phase of migration. Here, neurons reverse polarity and extend a pia-oriented leading process, take on the characteristic bipolar morphology of migrating neurons and commence radial migration toward the cortical plate. It is during this migration that axons are specified at the trailing edge of the migrating cell, and extend concomitantly with migration (Noctor et al., 2004) (Figure 1-1A). Once the cell reaches its laminar target within the cortex, axon pruning, dendrite growth and arborization, and synapse formation takes place.
The fact that some cortical neurons by-pass phase three of migration and directly progress from phase two to phase four suggests, that at least in a subset of excitatory cortical neurons, alternate routes of migration and morphogenesis exist (Noctor et al., 2004). Indeed, the timing of axon and dendrite formation varies to a great extent in different types of neurons, and thus, established pathways for neuronal polarity likely do not reflect universal mechanisms for neuronal development. For example, cerebellar granule neuron migration occurs opposite that of pyramidal cells of the cortex and hippocampus. Unlike the ‘inside-out’ pattern of cortical development, cerebellar granule neurons are generated ‘outside-in’ from regions within the proliferative outer granule layer of the cerebellum (Hatten and Heintz, 1995). Newborn granule neurons initially migrate tangentially along the pia matter, and then form axons bilaterally before descending into the inner layer of the cerebellum (Hatten and Heintz, 1995) (Figure 1-1B). Thus, the identification of extracellular cues, plasma membrane proteins, and intracellular signaling molecules during neuronal migration and morphogenesis likely reflect context-specific events governed by the type of neuron generated and the timing and location of development.

**In vivo and In Vitro Neuronal Development**

In addition to cell-type differences in morphogenesis, there are profound dissimilarities between cells developing in vivo and in vitro. The most obvious difference is that whereas cells developing in vivo undergo complex patterns of migration, the soma of neurons in culture are for the most part stationary. Pyramidal neurons in culture do not
Figure 1-1. Stages of Neuronal Polarization *In Vivo* and *In Vitro*

(A) Axon specification in the cortex. Cortical neurons proceed through four stages of development. Stages 1 and 2 consist of a multipolar cell that oscillates between the VZ and SVZ. Stage 3 is marked by the formation of a VZ-oriented process, which subsequently becomes the axon, and translocation of the cell toward the VZ. Stage 4 is characterized by the formation of a CP-oriented leading edge process, and the migration of the neuron toward the CP. Axon growth occurs simultaneously with migration. Abbreviations: VZ, ventricular zone; SVZ, sub-ventricular zone; IZ, intermediate zone; CP, cortical plate. Figure based on (Noctor et al., 2004).

(B) Axon specification in granule neurons of the cerebellum. Granule neurons are derived in the EGL, and conform to a bipolar morphology prior to commencing later migration. At some point, the cells undergo migration through the ML and PCL into the IGL. The bipolar processes subsequently become a bifurcated axon during this descent. Abbreviations: EGL, external granule layer; ML, molecular layer; PCL, purkinje cell layer; IGL, internal granule layer. Figure based on (Hatten and Heintz, 1995).

(C) Axon specification in dissociated hippocampal cultures. After plating, cells proceed through stage 1, in which they are round and form lamellipodia around the soma, to stage 2, in which they sprout several minor processes of roughly equivalent length. In stage 3, one of these processes is specified as the axon and undergoes rapid outgrowth. Dendritic growth and neuronal maturation occur in stage 4 and 5, respectively. Figure based on (Dotti et al., 1988).
Figure 1-1. Stages of Neuronal Polarization *In Vivo* and *In Vitro*

**A**

![Figure A](image)

**B**

![Figure B](image)

**C**

![Figure C](image)
adopt the characteristic bipolar morphology of migrating neurons *in vivo*, but rather, directly transition from a multipolar state consisting of multiple neurites of roughly equivalent length, to a polarized state in which a single neurite is specified as the axon and undergoes rapid growth (Craig and Banker, 1994) (Figure 1-1C). Such discrepancies also can be observed for other neuronal cell types. For example, whereas axons emerge directly from retinal ganglion cells in the absence of immature neurites *in vivo*, retinal ganglion cells in culture possess multiple neurites prior to axon specification (Zolessi et al., 2006). Although the reasons for such differences across various cell types remains poorly understood, the common denominator for all developing neurons *in vivo* and *in vitro* appears to be the initial breaking of symmetry with the compartmentalization of the nascent axon (Craig and Banker, 1994; Noctor et al., 2004; Tabata and Nakajima, 2003). The following sections discuss current theories and molecular pathways involved in this polarity-establishing event in neurons.

**Actin Cytoskeleton**

Whereas the axon shaft is predominantly a microtubule-based structure, the axonal growth cone is an actin-rich structure that exhibits rapid changes in shape, size, and rate of extension. There is broad agreement that local reorganization of the actin cytoskeleton is important for asymmetric growth in polarizing neurons. Classic experiments in cultured rat hippocampal neurons have demonstrated that the future axonal growth cone becomes highly dynamic prior to axon extension and forms and disassembles actin-based structures such as lamellipodia and filopodia (Bradke and Dotti, 1999). Actin filaments
in putative axonal growth cones are more easily extracted with detergents and the global addition of low concentrations of cytochalasin D, which binds actin filaments and prevents their elongation (Brown and Spudich, 1981; Flanagan and Lin, 1980), can induce supernumerary axon formation in developing cells (Bradke and Dotti, 1999). Moreover, local perfusion of a single neurite of an unpolarized stage 2 neuron with cytochalasin D can selectively designate that neurite to become the axon (Bradke and Dotti, 1999).

Despite the robust effects of actin instability during polarization, it is not understood why it is required. One hypothesis is that the state of actin filamentation controls the insertion of microtubules into the leading edge of the axon. In support of this idea, depolymerization of actin filaments in the growth cone of *Aplysia* results in the rapid invasion of microtubules into the peripheral area of the growth cone formerly occupied by actin filaments (Forscher and Smith, 1988), and microtubules extend distally prior to axon extension in embryonic frog neurons (Tanaka et al., 1995). Additionally, a more flexible actin network may promote the fusion of membrane cargo selectively in the designated axon as suggested from studies in neuronal and non-neuronal cells (Muallem et al., 1995; Wang et al., 2008). This may lead to the increased delivery of cargo such as adhesion molecules that could facilitate increased axon extension. Regardless of the biophysical necessity of actin instability, more recent studies on neuronal polarity have focused on genetic components that can impact actin reorganization within the cell. Such studies have centered on the activity, organization, and regulation of the Rho GTPases, which are key molecules that control actin dynamics.
Rho GTPases

The Rho GTPase family members include Rho (RhoA, RhoB, RhoC, RhoD, RhoG, RhoH/TTF, Rho T), Rac (Rac1, Rac2, Rac3), Cdc42, TC10, TCL, Wrch1, Chp/Wrch2, and Rnd (Rnd1, Rnd2, Rnd3/RhoE) (Burridge and Wennerberg, 2004; Van Aelst and D'Souza-Schorey, 1997). They are low molecular weight guanine-nucleotide binding proteins that act as binary molecular switches by cycling between an active GTP-bound state and an inactive GDP-bound state. Of the various Rho GTPase family members, RhoA, Rac1, and Cdc42 have been characterized the most extensively. These molecules are known best for their effects on the actin cytoskeleton, but they also play a role in transcriptional activation, membrane trafficking, and microtubule dynamics (Govek et al., 2005). The activity of Rho GTPases is determined by the stoichiometric ratio of GTP to GDP in the cell, and there are a number of regulatory molecules that influence Rho GTPase behavior. These include Guanine nucleotide exchange factors (GEFs), which activate GTPases by enhancing the exchange of bound GDP for GTP (Schmidt and Hall, 2002); GTPase activating proteins (GAPs), which act as negative regulator of GTPases by enhancing the intrinsic rate of GTP hydrolysis (Bernards and Settleman, 2004); and guanine nucleotide dissociation inhibitors (GDIs), which prevent the exchange of GDP for GTP and inhibit the intrinsic GTPase activity of GTP-bound GTPases (Zalcman et al., 1999).

The initial discovery that Rho GTPases are important for neuronal morphogenesis came from studies conducted in neuronal cell lines. Treatment of rat pheochromocytoma
PC12 cells with nerve growth factor (NGF), or serum starvation of mouse N1E-115 neuroblastoma cells results in the induction of lamellipodia and filopodia and ultimately results in neurite sprouting, a phenomenon dependent of Rac and Cdc42 activity since dominant-negative forms of these molecules inhibits neurite outgrowth (Aoki et al., 2004; Sarner et al., 2000). In contrast, RhoA activation is generally associated with inhibition of neurite initiation and retraction in both PC12 and N1E-115 cells and induces the formation of a thick ring-like structure of cortical actin at the cell periphery (Yamaguchi et al., 2001). Moreover, constitutively-active mutants of RhoA prevent neurite formation and induce neurite retraction (Amano et al., 1998; Hirose et al., 1998; Katoh et al., 1998a; Katoh et al., 1998b; Kranenburg et al., 1997; Sebok et al., 1999), while inhibition of RhoA activity with C3 exoenzyme from *Clostridium botulinum* or dominant-negative forms of RhoA creates lamellipodia and filopodia structures normally induced by Rac1 and Cdc42 activity and promotes neurite formation (Brouns et al., 2001; Fujita et al., 2001; Kozma et al., 1997; Kranenburg et al., 1999; Sebok et al., 1999; Tigyi et al., 1996). The generalization that Rac1 and Cdc42 activity promotes neurite formation and RhoA activity inhibits neurite formation in neuronal cell lines also holds for primary cultures. For example, constitutively-active Rac1 increases, and dominant-negative Rac1 decreases neurite extension in dissociated hippocampal cultures (Schwamborn and Puschel, 2004), and additionally, outgrowth is inhibited by constitutively-active RhoA (Da Silva et al., 2003; Schwamborn and Puschel, 2004), and promoted by the inhibition of RhoA by C3 exoenzyme (Da Silva et al., 2003).

Despite the prevailing antagonistic model of Rho GTPase signaling, it is now
clear that such functional antagonism is not mutually exclusive. Studies using FRET-based probes in PC12 cells have demonstrated that immediately after NGF treatment, Rac1 and Cdc42 activity is transiently and broadly activated throughout the cell with subsequent localized cycling between activity and inactivity at mobile tips of protrusions (Aoki et al., 2004). NGF-induced recruitment of Rac1 to the cell surface sites to form filamentous actin-rich protrusions is associated with a synchronous decrease in RhoA activity (Yamaguchi et al., 2001), suggesting that activation of Rac1 must be accompanied by simultaneous RhoA inactivation. Moreover, the activity of T-cell invasion and metastasis 1 (Tiam1) protein, an important Rac GEF involved in axon specification and growth (Nishimura et al., 2005), can be enhanced by the inactivation of RhoA and overcome by co-expression of constitutively-active RhoA (Leeuwen et al., 1997). These studies demonstrate the synergistic relationship between Rho GTPases and underscore the importance of spatial and temporal organization of these molecules during neuronal morphogenesis. The molecular components involved in Rho GTPase control and organization are discussed in much greater detail in later sections.

**Microtubule Cytoskeleton**

Whereas the growth of an axon requires highly dynamic actin within the leading growth cone, axonal integrity and endurance is dictated by the stability of the microtubule cytoskeleton. Microtubules are rigid filamentous structures 25 nm in diameter and composed of heterodimers of α- and β-tubulins (Hirokawa, 1982, 1998). A microtubule is oriented with plus and minus ends: microtubules polymerize faster at the plus end than
at the minus end, which is less dynamic. Their assembly is GTP-based and in the presence of motors and microtubule-binding proteins, microtubules can self-organize into large-scale cellular structures (Janson et al., 2007; Karsenti et al., 2006; Nedelec et al., 1997).

There are fundamental differences in the ultrastructural organization of microtubules in various cell types. The two main types of microtubule assemblies that are found in cells are asters and parallel or anti-parallel bundles. For example, in epithelial cells, the minus ends of microtubules are oriented towards the apical surface, whereas in fibroblasts, microtubules radiate in various direction from the microtubule-organizing center near the nucleus with their plus ends directed towards the periphery. Such structural variation provides polarity throughout the cell owing to the intrinsic polarity of microtubules and the capacity of motor proteins and various microtubule-associated proteins to distinguish between the two ends. This gives microtubule assemblies the ability to sort proteins and organelles within the cell.

In neurons, microtubule polarity is seen early in the cell with the onset of axon specification. In the axon, all microtubule filaments are uniformly oriented with the plus end facing the growth cone and the minus end facing the soma (Baas et al., 1989) whereas the orientation of microtubule filaments in dendrites is generally random (Baas et al., 1989). Such differences suggest profound deviations in the modes of construction, maintenance, and function between these cellular compartments. Indeed, experiments have demonstrated that microtubule stability is enhanced in nascent axons compared to immature dendrites (Ferreira et al., 1989; Qiang et al., 2006; Yu et al., 2005), the
conversion of dendrites to axons is accompanied by changes in microtubule polarity (Takahashi et al., 2007), and microtubule stabilization with taxol is sufficient to convert dendrites into axons (Gomis-Ruth et al., 2008).

Distinctions are also seen in microtubule binding proteins found in the axon and dendrites, which have been used extensively as sub-cellular biomarkers. The most widely distinguished of these is the axonal protein tau and the somatodendritic protein microtubule-associated protein-2 (MAP-2) (Dotti et al., 1987). Additionally, collapsin response mediator protein 2 (CRMP-2) (Yoshimura et al., 2005), and Adenomatous Polyposis Coli (APC) (Shi et al., 2004) also have been demonstrated to be key regulators of axon formation and stability in hippocampal neurons. Tau, CRMP-2, and APC bind to microtubules during the early phases of neuronal polarity and this binding is thought to enhance microtubule stability, thereby establishing the axon (Jiang et al., 2005; Yoshimura et al., 2005).

Microtubule Stability in Neuronal Polarity

There is now broad agreement that perseverance of microtubule-based structures in developing neurons is largely based on the binding of microtubule-stabilizing factors such as tau, CRMP-2, and APC (Shi et al., 2004; Yoshimura et al., 2005; Zumbrunn et al., 2001). The affinities of these proteins for microtubules are highly dependent on their phosphorylation state, and the pathways that mediate their phosphorylation have received considerable attention (Drechsel et al., 1992; Yoshimura et al., 2005; Zumbrunn et al., 2001). Initial observations regarding tau phosphorylation came from
immunohistochemical studies of postmortem brains of Alzheimer’s Disease patients, where hyperphosphorylated forms of tau are found in paired helical filaments, a classic neuropathological hallmark of disease progression (Grundke-Iqbal et al., 1986; Kosik et al., 1986; Wood et al., 1986). Subsequent studies identified three kinases that phosphorylate tau. These include glycogen synthase kinase 3β (GSK-3β), also known as tau protein kinase I (Ishiguro et al., 1991; Ishiguro et al., 1993); cyclin-dependent kinase 5 (CDK5), also known as tau protein kinase II (Arioka et al., 1993; Kobayashi et al., 1993); and extracellular signal-regulated kinase 2 (ERK2) (Roder et al., 1993). Of these, GSK-3β has been studied extensively for its effects on microtubule stability during axon formation.

GSK-3β is a constitutively active, multifunction kinase that regulates diverse cellular processes including metabolism (Cross et al., 1995), cell proliferation (Diehl et al., 1998), oncogenesis (Harada et al., 1999), and apoptosis (Kauffmann-Zeh et al., 1997). It is highly expressed in the brain and is linked to a variety of nervous system diseases including ischaemic stroke (Sasaki et al., 2001), bipolar disorder (Beaulieu et al., 2008; Klein and Melton, 1996), Huntington’s disease (Carmichael et al., 2002), and Alzheimer’s Disease (Imahori and Uchida, 1997; Pei et al., 1999; Pei et al., 1997; Yamaguchi et al., 1996). In immature neurons, GSK-3β activity is high throughout the cell, including the neurites (Jiang et al., 2005). During axon specification, GSK-3β is deactivated in the nascent axon through phosphorylation at Serine9 (Jiang et al., 2005), an event that depends on local PIP₃ accumulation by PI3-kinase and the activation of the pleckstrin-homology kinase Akt (Cross et al., 1995). Indeed, overexpression of
constitutively active GSK-3β prevents axon formation whereas GSK-3β knockdown by RNA interference (RNAi) or pharmacological inhibition of GSK-3β causes the formation of multiple axons (Jiang et al., 2005; Shi et al., 2004). Altogether, these observations indicate a model in which axon formation occurs when local deactivation of GSK-3β causes the accumulation of unphosphorylated tau, CRMP-2, and APC, thereby stabilizing the microtubule cytoskeleton for axon formation.

**Polarized Trafficking**

A consequence of differential microtubule polarity between axons and dendrites is the mode of trafficking that occurs between the two compartments. In axons, the unipolarity of microtubules dictates that anterograde motor proteins, which drive transport from the cell body to the cell periphery are plus-end directed, whereas retrograde motors are minus-end directed. Conversely, the mixed microtubule arrangement in dendrites dictates a much more complex arrangement. Interestingly, the two representative motors, conventional kinesin and cytoplasmic dynein, which utilize ATP to drive plus-end directed and minus-end direct microtubule movements, respectively, were originally purified from brain lysates (Brady, 1985; Paschal et al., 1987; Vale et al., 1985). A subsequent screen in a mouse brain cDNA library identified a group of 10 molecular motor genes, which were designated as kinesin superfamily proteins (KIFs) (Aizawa et al., 1992).

KIFs are constructed of a conserved globular motor domain that contains a ATP-binding sequence and a microtubule binding sequence (Hirokawa, 1998; Hirokawa et al.,
This globular motor domain, hydrolyzes ATP and generates the chemical energy to drive the KIF along microtubule with intrinsic directionality. Whereas the amino acid sequence of motor domains is well conserved among various KIFs, other regions including the filamentous stalk region and globular tail region are quite variable (Hirokawa, 1998; Hirokawa et al., 1989). In general, motor proteins use their stalk regions to dimerize, although some KIFs possess short coiled-coil regions and exist as monomers, and some form heterodimers among subfamily members (Hirokawa, 1998; Hirokawa et al., 1989). The tail region is used to bind various cargoes (Hirokawa, 1998; Hirokawa et al., 1989). KIFs are broadly grouped into three families based on the position of the motor domain within a molecule. N-kinesins have a motor domain in the N-terminal region, M-kinesins have one in the middle, and C-kinesins have a motor domain at their C-terminus (Hirokawa and Noda, 2008). The intramolecular position in general determines the directionality of the motor with N-kinesins being plus-end directed motors and C-kinesins being minus-end directed motors (Hirokawa and Noda, 2008).

Several KIFs have been demonstrated previously to be important for the delivery of neuronal cargo to proper intracellular compartments. For example, synaptic vesicle precursors are delivered to the axon by KIF1A (Okada et al., 1995), and the glutamate N-methyl-D-aspartate (NMDA) receptor subunit NR2B is transported to dendrites by KIF17 (Setou et al., 2000). More recent studies have highlighted an important and specific role for molecular motors in axon specification. Indeed, live imaging studies in hippocampal neurons using fluorescently-tagged motor domains of KIF1A and KIF5 have shown that
whereas KIF1A accumulates in all neurites, KIF5 selectively accumulates in axons (Jacobson et al., 2006). Several molecules important for axon specification have been identified and these include the polarity protein Par3, which is transported by KIF3A (Nishimura et al., 2004; Shi et al., 2004), and the microtubule binding protein APC and CRMP-2, which are transported by KIF3A (Nishimura et al., 2004; Shi et al., 2004) and KIF5 (Kawano et al., 2005; Kimura et al., 2005), respectively. Moreover, PIP3 is transported to the axon through its interaction with KIF13B (Horiguchi et al., 2006), also known as guanylate kinase-associated kinesin (Hanada et al., 2000). KIF13B interacts with PIP3 binding protein (PIP3BP, also known as centaurin-α) through its forkhead-associated domain (FHA) (Horiguchi et al., 2006). KIF13B and PIP3 accumulate at axonal tips of hippocampal neurons, and both the overexpression of wild-type and dominant-negative forms of KIF13B perturbs axonal establishment (Horiguchi et al., 2006). Collectively, these studies highlight the coincident nature of intracellular signaling, cytoskeletal rearrangement, and trafficking events that must occur properly for axon specification.

**The Par Polarity Proteins**

The six members of the partitioning-defective (Par1-6) gene family were originally identified from forward genetics screens in *C. elegans* (Kemphues et al., 1988). The initial analyses of these mutants revealed that most of these proteins were required for two aspects of cell polarization – asymmetric positioning of the mitotic spindle that facilitates unequal cell division, and polarized positioning of a set of proteins and RNAs
important for assigning cell fate between specific cells (Kemphues et al., 1988). Subsequent cloning and analysis of these genes suggested they act in a novel intracellular signaling pathway. Indeed, Par1 and Par4 encode serine/threonine kinases (Guo and Kemphues, 1995; Watts et al., 2000); Par5 is a member of the 14-3-3 family of proteins, which are recruited to phosphorylated serines and threonines (Morton et al., 2002); Par3 and Par6 are PDZ domain scaffolding proteins (Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999), and Par2 is a RING-finger domain protein that acts as a putative ubiquitin ligase during protein degradation (Levitan et al., 1994; Moore and Boyd, 2004). These proteins also reside in distinct intracellular compartments as initial cell polarization develops in *C. elegans* embryos. Whereas Par3 and Par6 become enriched in the anterior cortex during the one-cell stage, Par1 and Par2 localize to the posterior cortex and Par4 and Par5 remain symmetrically localized throughout the cell (Kemphues et al., 1988).

**Par Proteins in Epithelial Polarity**

The biological significance of Par proteins was appreciated entirely in *C. elegans* until the cloning of the *Drosophila* gene *bazooka* in 1998, which was found to encode a protein closely resembling Par3 (Kuchinke et al., 1998). Subsequent work uncovered additional components of the cellular polarity mechanism, and implicated the Par proteins at the center of a fundamental and ancient pathway that controls cellular polarization in various organisms and cell types (Cowan and Hyman, 2007; Izumi et al., 2006; Joberty et al., 2000; Lin et al., 2000; Tabuse et al., 1998). Epithelial cells are polarized cell types with an apical and basolateral domain and compose the principle boundary between an
organism and its external environment. Sheets of epithelial cells are formed with the construction of cellular junctions between cells, such as adherens junctions and tight junctions. These domains maintain adhesion, regulate tissue permeability and form the physical demarcation between the apical and basolateral domains of individual cells. An important principle in cell biology is that a conserved set of proteins, including the Par proteins, controls cell junction formation and epithelial polarization throughout the metazoa. In *C. elegans*, the anteroposterior segregation of Par proteins changes at the end of the four cell stage in embryos, such that Par3 and Par6 are localized to the apical domain and are essential for the apico-basal asymmetries associated with gastrulation (Nance et al., 2003); Par3 drives apico-basal polarization in *Drosophila* (Kuchinke et al., 1998), and Par3 is localized to the apical/basolateral boundary in mammalian epithelial cells (Izumi et al., 1998) and functions in their assembly (Chen and Macara, 2005). Interestingly, although *Drosophila* epithelial cells possess septate junctions rather than tight junctions, and their position relative to adherens junctions is quite different, Par3 is situated at the apical/basolateral boundary in both cell types, further illustrating the highly conserved role of the Par proteins in cellular polarization.

Although Par3 and Par6 remain the best-studied members of the Par family in epithelial polarity, additional Par proteins have been demonstrated to play key roles in polarity. Par1, also known as microtubule affinity-regulating kinase (MARK) has been demonstrated to determine the organization of the microtubule cytoskeleton, which in turn orients the apical domain of the cell (Cohen et al., 2004). High Par1 activity was shown to convert columnar epithelial cells with a single apical domain and vertically
oriented microtubules into a hepatic-type epithelial cell with horizontally oriented microtubules and multiple apical domains (Cohen et al., 2004). Intriguingly, Par1 known is activated by Par4, which is also known as LKB1 in mammalian cells (Lizcano et al., 2004). LKB1 is a master serine threonine kinase that targets at least 13 other kinases for activation (Lizcano et al., 2004), and is best known as a tumor-suppressor linked to Peutz-Jehgers cancer syndrome (Hemminki et al., 1998). Activated LKB1 exists in a complex with Stradα, a pseudo-kinase that activates LKB1 through an allosteric mechanism (Baas et al., 2003). Remarkably, elevation of LKB1 activity through inducible Stradα expression is sufficient to drive the complete polarization of epithelial cells within 24 hours, even in the absence of cell-cell contacts (Baas et al., 2004). Although the exact mechanism by which LKB1 autonomously drives cell polarity remains poorly understood, it further illustrates the central role of the Par complex during epithelial cell polarity across numerous species.

**Par Proteins in Axon Specification**

More recently, the Par proteins have been studied extensively for their involvement in neuronal development where their activity influences axon specification (Figure 1-2). In stage 2 hippocampal neurons, Par3 and Par6 are present at the tips of all neurites but are selectively retained in the axonal growth and lost from putative dendrites during the transition to stage 3 (Shi et al., 2003). This suggests that the Par3/Par6 complex may serve as a signaling platform in the nascent axon to localize signals required for neuronal polarity. Consistent with this notion, overexpression of Par3 or Par6 disrupts neuronal
polarity, and Par3 overexpression promotes the formation of multiple axons (Shi et al., 2003). In addition, signaling molecules that are regulated by Par3 and Par6, also localize to nascent axons. Some of these include PKCζ (Schwamborn and Puschel, 2004), the Rho GTPase Cdc42 (Schwamborn and Puschel, 2004), Smad ubiquitin regulatory factor 1 and 2 (Smurf1 and Smurf2) (Schwamborn et al., 2007), KIF3A (Shi et al., 2004), Dishevelled (Zhang et al., 2007), and the GEF Tiam1 (Nishimura et al., 2005). Despite the presence of polarity-generating molecules in the axon, our picture of how such developmental asymmetry is achieved with these molecules remains incomplete. An important missing link is the identification of axon-generating molecules that initiate the signaling pathways required for neuronal polarity. In particular, extracellular factors and their cognate receptors that are organized and function within the Par3/Par6 complex have yet to be identified in neurons. Moreover, *Drosophila* neurons generate axons in the absence of Par3 and Par6 (Rolls and Doe, 2004), and studies demonstrating the effect of Par3 and Par6 knockdown in cultured cells or in intact embryonic tissue during axon specification in mammalian neurons are lacking.

Additional members of the Par protein family are also implicated in neuronal polarity. MARK/Par1 has been demonstrated to function downstream of Par3 and Par6 in hippocampal neurons and act as a negative regulator for axon formation. It was previously known that MARK can phosphorylate tau, and this phosphorylation prevents the association of tau with microtubules. Consistent with this observation, inhibition of MARK activity promotes the formation and outgrowth of multiple axons whereas enhanced MARK activity prevents axon formation (Chen et al., 2006b).
LKB1, the activator of Par1, is also a critical mediator of axon formation in neurons. Conditional loss-of-function mouse models have demonstrated elegantly that LKB1 acts specifically during axon generation (Barnes et al., 2007). Conditional mutants were generated by crossing “floxed” LKB1 mice with transgenic mice expressing the Cre recombinase under the control of the Emx1 promoter (Barnes et al., 2007). This allowed the excision of the LKB1 locus from dorsal telencephalic progenitors around E9.5 and the subsequent production of cortical pyramidal neurons lacking LKB1 throughout their lifetime (Barnes et al., 2007; Gorski et al., 2002). Surprisingly, defects in cellular polarity are only seen in neurons and not progenitor cells, and LKB1-null neurons migrate normally and reach their proper migratory targets to form proper cortical layers (Barnes et al., 2007). Neurons lacking LKB1 fail to produce an axon, and the overexpression of LKB1 and STRADα result in the formation of neurons with multiple axons (Barnes et al., 2007; Shelly et al., 2007). The cellular distribution of LKB1 is not polarized (Barnes et al., 2007), but LKB1 is selectively phosphorylated at Serine431, which is required for its activation, only in the axon (Barnes et al., 2007). However, these results remain controversial. Additional observations using shRNA-mediated LKB1 knockdown in the embryonic cortex have demonstrated profound defects in neuronal migration, and the formation of axons and dendrites in opposite orientations, rather than the complete lack of an axon (Asada et al., 2007). Moreover, LKB1 phosphorylation is attributed to c-AMP-dependent protein kinase (PKA) activity, which in turn is activated by extracellular brain-derived neurotrophic factor (BDNF) (Shelly et al., 2007). However, mice lacking BDNF (Ernfors et al., 1994; Jones et al., 1994), or its
Figure 1-2. The Par Proteins in Axon Specification

Member of the Par protein family, with the exception of Par2 and Par5, have been demonstrated to play integral roles during axon specification. The Par proteins can be separated into two classes based on knowledge regarding their signaling pathways. Par4 and Par1 have both been demonstrated to function in neuronal polarity in which the kinase activity of Par4 is required directly to regulate Par1 activity in controlling microtubule dynamics. Par3 and Par6 are scaffolding molecules and they have been studied extensively for their ability to organize Rho GTPase signaling, thereby affecting actin dynamics, during neuronal polarization.
### Figure 1-2. The Par Proteins in Axon Specification

<table>
<thead>
<tr>
<th>Polarity Protein</th>
<th>Role in Axon Specification</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Par1</td>
<td>Kinase also known as MARKs; phosphorylates Tau to prevent microtubule stabilization and polymerization</td>
<td>Chen et al., 2006</td>
</tr>
<tr>
<td>Par2</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Par3</td>
<td>Scaffolding protein that selectively accumulates in the axon; nucleates Rho GTPases</td>
<td>Shi et al., 2003, Nishimura et al., 2005</td>
</tr>
<tr>
<td>Par4</td>
<td>Kinase also known as LKB1; Loss of expression in cortical neurons leads to failed axon formation</td>
<td>Barnes et al., 2008</td>
</tr>
<tr>
<td>Par5</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Par6</td>
<td>Scaffolding protein that selectively accumulates in the axon; nucleates Rho GTPases</td>
<td>Shi et al., 2003</td>
</tr>
</tbody>
</table>
receptor TrkB (Klein et al., 1993) survive until birth and CNS neurons in these animals do not exhibit any defects in axon formation (Ernfors et al., 1994; Jones et al., 1994; Klein et al., 1993). Thus, additional mechanisms must exist to initiate the polarity program during axon specification.

**Par Proteins in Cell Fate Determination**

Further complicating our view of Par protein function in axon generation is their role in regulating asymmetrical cell division during neurogenesis. Asymmetric cell divisions in neuroblasts have been studied extensively in *Drosophila*, and such studies have provided much of our knowledge about the role of Par proteins in cell fate determination (Yu et al., 2006). The creation of distinct daughter cells requires that cell fate determinants be segregated to opposite poles of the cell with the mitotic spindle positioned orthogonal to the axis of this polarization. This ensures that only daughter cells receive the determinants to promote differentiation. Par3 functions to mediate both of these processes by ensuring the basal localization of the fate determinants numb (Uemura et al., 1989) and miranda (Ikeshima-Kataoka et al., 1997), and the proper orientation of the mitotic spindle (Wodarz et al., 1999). Spindle orientation involves a distinct mechanism in which Par3 localizes to the apical surface of neuroblasts during the delamination of the cell from the overlying epithelium (Cai et al., 2003; Schaefer et al., 2000; Yu et al., 2000) and recruits the protein Insecuteable (Insc) (Schober et al., 1999). Insc subsequently recruits Pins, which associates with both G protein αi subunits (Gαi) and the
microtubule-binding protein Mud (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). Loss of function of LKB1 also perturbs asymmetrical cell division in Drosophila neuroblasts, and abrogates proper localization of Par3, Par6, aPKC, and miranda, whereas Pins and Gαi localization, and mitotic spindle rotation is unaffected (Bonaccorsi et al., 2007). Intriguingly, LKB1/Pins double mutants exhibit exacerbated phenotypes of either single mutant, suggesting that LKB1 and Pins act in partially overlapping redundant pathways during asymmetric cell division (Bonaccorsi et al., 2007).

Far less is known about mammalian neuronal differentiation. However, recent experiments using replication-incompetent retroviruses have demonstrated an important role for Par proteins in asymmetrical cell division. Viruses packaged with an shRNA targeted against Par3 in E12 mouse embryos demonstrated perturbations in progenitor cells maintenance (Costa et al., 2008). Whereas the total number of clonal cells decreases, the proportion of neurons within the clonal population increases (Costa et al., 2008), suggesting that neuronal progenitor cells lacking Par3 prematurely exist mitosis. In contrast, overexpression of Par3 or the overexpression of Par6 increases total clonal size, but decreases the number of MAP-2 positive neurons (Costa et al., 2008), suggesting that high levels of Par3 and Par6 maintain the progenitor lineage. These experiments highlight that in addition to roles in neuronal morphogenesis, the control of asymmetrical cell division by Par proteins is also conserved in developing mammalian brains. Thus, more study is needed to distinguish these biological pathways mediated by the Par proteins, and caution is warranted when interpreting data from animal models.
Par Proteins in Cell Migration

Cell migration is an important phenomenon that is critical in development and morphogenesis, wound healing, and cancer. Migration can be directed, or at least in vitro, random; but in either case cells must polarize to possess a leading edge and a trailing edge for proper locomotion. In 1999, the Rho GTPase Cdc42 was shown to be important for generating anterior-posterior polarity in migrating fibroblasts during wound healing (Nobes and Hall, 1999), but the molecular basis of this phenomenon was not known until the discovery in 2000 that Par6 binds Cdc42 through its Cdc42 and Rac1 Interacting/Binding (CRIB) motif (Joberty et al., 2000; Lin et al., 2000). Subsequently, Par6 and PKCζ were demonstrated to dictate migratory directionality, centrosome orientation and microtubule polarity with respect to the nucleus and axis of movement in astrocytes (Etienne-Manneville and Hall, 2001) and fibroblasts (Schlessinger et al., 2007). It is now broadly accepted that Par3 and Par6 polarize to the leading edge of migrating cells (Pegtel et al., 2007; Plant et al., 2003) and a key function of the Par proteins is the spatial coordination of intracellular Rho GTPase activity (Joberty et al., 2000; Lin et al., 2000; Nobes and Hall, 1999; Pegtel et al., 2007; Wang et al., 2003). In migrating cells, the activation of Cdc42 and Rac1 and the inactivation of RhoA activity is observed at the leading edge whereas the inactivation of Cdc42 and Rac1 and the activation of RhoA is seen at the trailing edge (Kraynov et al., 2000; Nalbant et al., 2004; Pertz et al., 2006). This segregation of Rac1/Cdc42 and RhoA activities during cell migration is critical for proper anterior-posterior polarization. For example, Par3 complexes with the Rac GEF Tiam1/2 at the leading edge, which facilitates enhanced
Rac1 activity (Pegtel et al., 2007). Simultaneously, RhoA is targeted for degradation at the leading edge by Smurf1 (Wang et al., 2003), an E3 ubiquitin ligase that binds both Par6 and PKCζ (Ozdamar et al., 2005; Wang et al., 2003). Moreover, Rho-kinase (ROK), a downstream effector of RhoA, phosphorylates Par3 at Ser833, an event that disrupts Par complex formation and prevents anterior-posterior polarity in migrating cells (Nakayama et al., 2008).

Par6 has also been demonstrated to be a key regulator of neuronal migration. In migrating cerebellar granule neurons, Par6 localizes to the neuronal centrosome, which is located proximally in the leading process of the neuron (Gregory et al., 1988; Rakic, 1972). Neuronal migration occurs as a ‘two-stroke’ mechanism with centrosomal movement preceding nuclear translocation (Solecki et al., 2004). shRNA-mediated knockdown of Par6, or overexpression of Par6 disrupts centrosomal positioning and movement, and consequently prevents proper migration of neurons to their anatomical destinations (Solecki et al., 2004). However, the mechanisms by which centrosomal movements are mediated in neurons remains poorly understood.

**The TGF-β Superfamily of Ligands**

Transforming Growth Factor-β (TGF-β) is a morphogen that controls a diverse set of biological processes throughout the metazoa. These include cell proliferation, differentiation, apoptosis, patterning, and specification of developmental fate in embryogenesis as well as in mature tissues (Shi and Massague, 2003). The TGF-β family of cytokines are characterized by six conserved cysteine residues, which are encoded by
42 open reading frames in the human, nine in flies, and six in worms (Lander et al., 2001). It is composed of several subfamilies classified by sequence similarity and the signaling pathways they activate – the TGF-β subfamily composed of TGF-β1, 2, and 3; the bone morphogenetic protein (BMP) subfamily comprised of 20 ligands; the growth and differentiation factor (GDF) subfamily composed of at least 9 members; the activin/inhibin subfamily composed of activin A, AB, B, and inhibin A, B; the glial derived neurotrophic factor (GDNF) subfamily which includes GDNF, artemin, and neuturin; as well as several additional members such as Mullirian inhibiting substance (MIS) and nodal (Knight and GliSter, 2006). The assorted set of TGF-β ligands elicit distinct cellular responses, but they all share common sequence and structural features. The active form of a TGF-β ligand is a dimer stabilized by hydrophobic interactions which are additionally reinforced, in most cases, by an inter-subunit disulfide bridge (Schlunegger and Grutter, 1993). Each monomer comprises several extended β strands interlocked by three conserved disulfide bonds that form a tight structure known as the “cysteine knot” (McDonald and Hendrickson, 1993).

The activation of receptors by ligands is a tightly regulated process that is controlled by two classes of molecules. One class is comprised of a class of membrane-anchored proteins that act as accessory receptors, or co-receptors, to promote ligand binding to serine/threonine kinase receptors. These include the proteoglycan betaglycan, also known as TβR3 (Lopez-Casillas et al., 1991; Wang et al., 1991). TβR3 is known to mediate TGF-β binding to the type II receptor, a role that is particularly critical for TGF-β2 (Cheifetz and Massague, 1991; Lopez-Casillas et al., 1993; Sankar et al., 1995). TβR3
does not bind to activins, but it has been demonstrated to functionally antagonize activin signaling by binding inhibin and facilitating its access to activin receptors, thereby outpacing activin stimulation (Lewis et al., 2000). Moreover, TGF-β can be further refined through the regulated endocytosis of TβR3 (Chen et al., 2003). Additional members include the betaglycan-related protein endoglin, which interestingly binds TGF-β1 and TGF-β3, but not TGF-β2 (Cheifetz et al., 1992); and Cripto, which mediates the binding of Nodal, Vg1, and GDF1 to activin receptors (Cheng et al., 2003; Gray et al., 2006; Gritsman et al., 1999). In addition to positive-regulating membrane factors, the protein BMP and activin receptor membrane bound inhibitor (BAMBI) is a negative regulator of TGF-β signaling (Onichtchouk et al., 1999). BAMBI acts as a decoy receptor and contains an extracellular domain and a short cytoplasmic region with sequence similarities with type I receptors. BAMBI competes with type I receptors for incorporation into ligand-induced receptor complexes, inhibiting receptor activation (Onichtchouk et al., 1999).

The other class of molecules is comprised of a diverse group of soluble proteins that act as ligand binding traps, sequestering the ligand and preventing its access to membrane receptors. These include the N-terminal pro-region of the TGF-β precursor, which is cleaved in the secretory pathway (Gentry et al., 1988) and remains non-covalently bound to the bioactive domain as a latency-associated peptide (LAP), which prevents access of the ligand to receptors (Miyazono and Heldin, 1989). It also includes the small proteoglycan decorin (Yamaguchi et al., 1990) and the circulating protein α2-macroblogulin (O'Connor-McCourt and Wakefield, 1987), which both bind to free TGF-
β. Moreover, follistatin binds to Activins (Nakamura et al., 1990) and BMPs (Fainsod et al., 1997), and three distinct protein families, Noggin (Re'em-Kalma et al., 1995), Chordin/SOG (Sasai et al., 1995), and DAN/Cerberus (Piccolo et al., 1999) all bind BMPs.

**TGF-β Signaling at the Membrane**

TGF-β signaling is initiated by distinct serine/threonine kinases on the cell surface, which are brought together through the binding of a ligand molecule. The first example of a TGF-β receptor in the literature can be found from *C. elegans*, in which the orphan receptor Daf-1 was cloned in 1990 (Georgi et al., 1990). The subsequent cloning of an activin receptor in 1991, now known as activin receptor II (ActR2), suggested the existence of an evolutionarily conserved family of serine/threonine kinase receptors required for TGF-β signaling (Mathews and Vale, 1991). The receptor serine/threonine kinase family in the human genome is composed of 12 members – seven type I and five type II receptors, all of which are dedicated for TGF-β signaling (Manning et al., 2002). The only exception to this rule is GDNF, which signals through the receptor tyrosine kinase Ret (Massague, 1996). Type I and II receptors are glycoproteins of approximately 55kDa and 70kDa, respectively, with core polypeptides of 500 – 570 amino acids including the signal sequence (Attisano et al., 1992; Ebner et al., 1993; Lin et al., 1992; Mathews and Vale, 1991; ten Dijke et al., 1993). Both types of receptors are organized into an N-terminal extracellular ligand-binding domain, a transmembrane region, and a C-terminal serine/threonine kinase domain (Shi and Massague, 2003). During signaling,
the constitutively active type II receptor phosphorylates the receptor I kinase domain, which then propagates the signal through various intracellular mechanisms.

The Structure of Type I and Type II TGF-β Receptors

TGF-β receptors are distinguished as either type I or type II based on their structural and functional properties (Massague, 1998). Vertebrate type I receptors are further classified into three groups based on the sequence similarities of their kinase domains and signaling (Wrana et al., 1994a). Structural studies have suggested that the dimeric arrangement of TGF-β ligands requires the formation of a receptor complex composed of two type I and two type II receptors (Hart et al., 2002) (Figure 1-3) activities. In mammals, one group includes TβRI (also known as ALK5), ActR1B, and activin-like kinase (ALK) 7; another includes BMP receptor (BMPR) 1A and 1B; and the third includes ALK1 and ALK2 (Massague, 1998). Type I TGF-β receptors identified in invertebrates include Thick veins (Tkv) and Saxophone (Sax), which act as receptors for Decapentaplegic (Dpp) (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Xie et al., 1994). At the sequence level, Tkv most closely resembles mammalian BMPRI receptors whereas Sax is closer to ALK1 and ALK2 (Massague, 1998). In addition, Baboon (Babo) is a type I receptor that most closely resembles mammalian TβRI, but its endogenous ligand in Drosophila is unknown (Brummel et al., 1999; Wrana et al., 1994b). In C. elegans, Daf-1 has been identified to bind the BMP-like ligand Daf-7 (Georgi et al., 1990).
Figure 1-3. The TGF-β Signaling Pathway

The schematic shows several levels of activation and regulation of TGF-b signals. TGF-b ligands act as dimers and first bind the high-affinity type II TGF-b receptor TbR2). This causes binding of TbR2 to the type I receptor (TbR1), and subsequent activation of TbR1. Activated TbR1 then phosphorylates Smad proteins, which then translocate into the nucleus to affect transcriptional outputs. Various levels of regulation exist including regulation of the bioactivity of the ligand, access of TbR1 to Smads, receptor internalization leading to recycling or degradation, and the intracellular regulation of Smads. Arrows indicate flow of signal. Orange objects represent ligand and receptor activation, gray for Smad and receptor inactivation, green for Smad activation, and blue for nuclear shuttling. Phosphate groups and ubiquitin are represented by green and red circles, respectively. Figure based on (Shi and Massague, 2003).
Figure 1-3. The TGF-β Signaling Pathway
The type II receptor subfamily in vertebrates includes TβR2, BMPR2, and AMHR, which respectively bind TGF-β (Lin et al., 1992), BMPs (Liu et al., 1995; Nohno et al., 1995; Rosenzweig et al., 1995), and MIS (Baarends et al., 1994; di Clemente et al., 1994). ActR2 and ActR2B bind activins when expressed alone or with other activin type I receptors (Attisano et al., 1992; Mathews and Vale, 1991; Mathews et al., 1992), however, ActR2 and ActR2B can bind BMPs 2, 4, and 7 and GDF5 in concert with BMP type I receptors (Hoodless et al., 1996; Nishitoh et al., 1996; Yamashita et al., 1995). Type II receptors identified in invertebrates include Punt in *Drosophila* (Letson et al., 1995; Ruberte et al., 1995) and Daf-4 in *C. elegans* (Estevez et al., 1993).

A unique characteristic of type I receptors is a highly-conserved 30 amino acid region that immediate precedes the protein kinase domain. This region is referred to as the GS domain because of a characteristic SGSGSG sequence found within this region (Wrana et al., 1994a). Ligand-dependent phosphorylation of serines and threonines within the TTSGSGSG sequence of TβRI by the type II TGF-β receptor (TβR2) is required for signaling activation (Souchelnytskyi et al., 1996; Wieser et al., 1995; Wrana et al., 1994a), and the same holds for ActR1B (Attisano et al., 1996). Immediately following the SGSGSG sequence, all type I receptors possess a Leu-Pro motif that serves as a docking site for the immunophilin FKBP12 (Chang et al., 1996; Chen et al., 1997c). FKBP12 binds TβRI and disrupts TGF-β signaling by inhibiting TβRI phosphorylation by TβR2 (Chen et al., 1997c; Wang et al., 1996). Interestingly, FKBP12 binds to TβRI in the basal state and is released upon ligand-induced receptor complex formation (Chen et al., 1997c; Wang et al., 1996), and TβRI mutants defective in FKBP12 binding have
elevated basal activity levels but retain normal signaling in the presence of ligand (Chen et al., 1997c; Wang et al., 1996). Thus, FKBP12 likely guards against spurious activation of TGF-β signaling during ligand-independent encounters between type I and type II receptors. The penultimate residue in the GS domain, which is located at the boundary of the kinase domain, is a threonine or glutamate for all type I receptors. As demonstrated with TβRI (Wieser et al., 1995) and other type I receptors, the substitution of this residue to an aspartate or glutamate endows the receptor with constitutive signaling activity in the cell (Attisano et al., 1996; Hoodless et al., 1996; Kretzschmar et al., 1997b; Wiersdorff et al., 1996; Zou et al., 1997). Thus, the GS domain is a key regulatory region that controls the catalytic activity of the type I receptor.

The kinase domain of both type I and type II receptors conforms with canonical sequences of other serine/threonine kinase domains (Franzen et al., 1993; Mathews and Vale, 1991). Indeed, type I receptors have been shown to phosphorylate their downstream targets the Smad proteins on serine residues (Kretzschmar et al., 1997b; Macias-Silva et al., 1996), and type II receptors phosphorylate themselves and type I receptors on serine and threonine residues, but not on tyrosine residues (Bassing et al., 1994; Lin et al., 1992; Mathews and Vale, 1993; Souchelnytskyi et al., 1996; Wieser et al., 1995; Wrana et al., 1994a). Conserved amino acids that have been demonstrated to coordinate ATP phosphate groups in other protein kinases are also essential for the activity of type I and type II receptor kinases. These include a universally conserved β3-strand lysine (Carcamo et al., 1994; Wrana et al., 1992) and G217 in the glycine loop of TβRI (Weis-Garcia and Massague, 1996). In addition, the regulatory region known as
the T-loop in other protein kinases (Taylor and Radzio-Andzelm, 1994) contains two serine residues in TβR2, and their phosphorylation serves to enhance or diminish the signaling activity of the receptor (Luo and Lodish, 1997). Beyond the kinase domain, type II receptors typically contain a very short C-terminus following the kinase domain, whereas type I receptor have essentially no C-terminal extensions (Massague, 1998).

**Mechanism of Receptor Activation**

At its basal state, TβRI exists as an unphosphorylated receptor (Wrana et al., 1994a) whereas TβR2, ActR2 and ActR2B are phosphorylated (Attisano et al., 1996; Lin et al., 1992; Mathews and Vale, 1993; Schreiber, 1992; Wrana et al., 1994a). The functional significance of this phosphorylation remains poorly understood. It occurs on serine residues, some within the C-terminal tail, and phosphorylation is partially retained in mutant receptors lacking kinase activity (Attisano et al., 1996; Wrana et al., 1994a). Moreover, studies using mutant forms of TβR2 lacking the C-terminal tail had no detectable effect on receptor signaling (Wieser et al., 1993). Phosphorylation also occurs at other sites within TβR2. These include a serine in the juxtamembrane region and serines in the T-loop region of the kinase domain, and both modulate the signaling activity of the receptor (Luo and Lodish, 1997). However, how these phosphorylation events are regulated is unknown.

Studies using epitope-tagged receptors indicate that TβR2 forms ligand-independent homo-oligomers in their basal state (Chen and Derynck, 1994; Henis et al., 1994). These complexes are thought to prime the formation of heteromeric TβRI/ TβR2
receptor complexes in the presence of ligand. Additionally, type I and type II receptors possess an intrinsic affinity for each other, as demonstrated by ligand-independent association in cells overexpressing receptors or in vitro as recombinant proteins (Ventura et al., 1994). These ligand-independent associations are mediated in part by the intracellular portions of these receptors because these regions interact in yeast two-hybrid screens (Chen et al., 1995; Kawabata et al., 1995; Liu et al., 1995; Ventura et al., 1994). Thus, the control of basal receptor activity is critical, and cells likely achieve this through the inhibitory activity of FKBP12 binding to TβR2, as well as tight regulation of receptor quantities. Indeed, it has been demonstrated that in cells expressing moderate levels of TGF-β receptors (Wrana et al., 1994a) or activin receptors (Lebrun and Vale, 1997), the heteromeric receptor complex and phosphorylation of the type I receptor are highly dependent on ligand stimulation.

Ligand binding induces the formation of a stable heteromeric complex of type I and type II receptors (Attisano et al., 1993; Attisano et al., 1996; Carcamo et al., 1994; Franzen et al., 1993; Liu et al., 1995; Rosenzweig et al., 1995; Wrana et al., 1992; Wrana et al., 1994a; Yamashita et al., 1994) that resists dissociation by ionic detergents and chaotropic agents (Wrana et al., 1994a). As mentioned, given the dimeric nature of the ligands, a heterotetrameric receptor composed of two type I and two type II receptors is likely forms. This notion is supported by two-dimensional gel electrophoresis analysis of TGF-β receptor complexes (Yamashita et al., 1994), co-immunoprecipitation of receptors containing distinct epitope tags (Weis-Garcia and Massague, 1996), and genetic complementation between mutant type I receptors (Weis-Garcia and Massague, 1996).
Intriguingly, type II receptor kinase activity, or overall phosphorylation state of TβR2, ActR2, or ActR2B is not increased in the presence of ligand. Thus, such observations imply that type II receptors are constitutively active kinases that only require the ligand to interact with type I receptors (Wrana et al., 1994a).

The formation of the ligand-induced receptor complex leads to the phosphorylation of the type I receptor, which is catalyzed by the kinase activity of the type II receptor (Attisano et al., 1996; Wrana et al., 1994a). TβRI is phosphorylated by TβR2 at serine and threonine residues in the sequence TTSGSGSG of the GS domain (Souchelnytskyi et al., 1996; Wieser et al., 1995; Wrana et al., 1994a) and similar sites are phosphorylated in ActR1B by activin type II receptors (Attisano et al., 1996). In addition to these sites, TβR1 is also phosphorylated at Ser165 in the juxtamembrane region by TβR2, a phosphorylation that may positively or negatively affect TGF-β responses dependent on the biological context (Souchelnytskyi et al., 1996).

**Transcriptional Effects of TGF-β Signaling**

A unique feature of TGF-β signaling is the direct coupling of receptors with Smad transcription factors, which are phosphorylated and activated by type I receptors (Shi and Massague, 2003). Early studies looking at the kinase activity of type I and type II TGF-β receptors found that whereas TβR1 activity is required for cellular responses to TGF-β, TβR1 does not phosphorylate itself or TβR2 (Wrana et al., 1994a). Additionally, constitutively active mutant forms of TβR1 (Wieser et al., 1995), ActR1B (Attisano et al., 1996), BMPR1A and BMPR1B (Hoodless et al., 1996; Kretzschmar et al., 1997b; Zou et
al., 1997), and Tkv (Hoodless et al., 1996; Wiersdorff et al., 1996) were found to generate signaling responses in the absence of ligand. Thus, it is postulated that a bulk of TGF-β signaling must propagate through type I receptors to initiate intracellular signaling cascades to influence distinct physiological endpoints. Subsequent studies that demonstrated that purified BMP type I receptor could phosphorylate Smad1 in vitro (Kretzschmar et al., 1997b), provided clear evidence that TGF-β receptors are directly coupled to downstream transcription factors.

**Structure of Smad Proteins**

The founding member of the Smad family is the product of the *Drosophila* gene *Mad*(mothers against dpp) (Sekelsky et al., 1995). *Mad* was discovered in a genetic screen for mutations that exacerbate the phenotype of weak *dpp* alleles (Raftery et al., 1995), and its discovery led to the identification of many related genes in other model species. Three *Mad* homologues, *sma-2*, -3, and -4, were identified in *C. elegans* and named because of their small body size (Savage et al., 1996). Subsequent homologues were identified in vertebrates and termed Sma/Mad related (Smad). Human, mouse, and from Smads 1-8 were cloned by screening expressed sequence tag (EST) databases or cDNA libraries for Mad homologues (Chen et al., 1996; Eppert et al., 1996; Graff et al., 1996; Hoodless et al., 1996; Imamura et al., 1997; Lechleider et al., 1996; Liu et al., 1996; Nakao et al., 1997; Watanabe et al., 1997; Yingling et al., 1996; Zhang et al., 1996). In addition, Smad2 was independently identified in a screen for inducers of mesoderm formation in *Xenopus* embryos (Baker and Harland, 1996), Smads 6 and 7 were
identified as shear stress-induced genes in endothelial cells (Topper et al., 1997), and Smad4 was initially identified as deleted in pancreatic carcinoma locus 4 (DPC4) in humans (Hahn et al., 1996).

Based on structural and functional criteria, Smads are classified into three families. The first family is comprised of receptor Smads (R-Smads) that are direct substrates of TGF-β family receptor kinases. These include Smad5 and Smad8, which are substrates for BMPR1 (Kretzschmar et al., 1997b) and mediate BMP signaling (Chen et al., 1997b; Graff et al., 1996; Hoodless et al., 1996; Liu et al., 1996; Suzuki et al., 1997; Thomsen, 1996), and Smad2 and Smad3, which are TβR1 substrates and mediators of TGF-β and activin signals (Baker and Harland, 1996; Chen et al., 1996; Eppert et al., 1996; Graff et al., 1996; Lagna et al., 1996). The second group is composed of Smads, known as co-Smads, that participate in signaling by associating with receptor-regulated Smads. The only member of this group is Smad4, which is similar in structure to R-Smads, but does not undergo phosphorylation in response to ligand. Smad4 associates with R-Smads when they are phosphorylated by their corresponding receptors (Hata et al., 1997; Kretzschmar et al., 1997b; Lagna et al., 1996; Zhang et al., 1996). Smad4 function as a co-Smad for all TGF-β pathways and is required TGF-β, activin, and BMP signaling (Lagna et al., 1996; Zhang et al., 1996). The third family of Smads comprises inhibitory Smads (I-Smads) and includes Smads 6 and 7. I-Smads are structurally divergent Smads whose only known activity is to inhibit the signaling of R-Smads. Smad6 preferentially inhibits BMP signaling (Hata et al., 1997; Imamura et al., 1997) and Smad7 inhibits both BMP and TGF-β signaling (Hayashi et al., 1997; Nakao et al.,
Smad proteins contain highly conserved N-terminal MH1 domain and C-terminal MH2 domain joined by an intramolecular linker region that varies in sequence and length (Massague, 1998). The MH1 domain is highly conserved in R-Smads and Smad4, but diverges with I-Smads. The MH1 domain serves to inhibit the transcriptional (Liu et al., 1996) and biological activities (Baker and Harland, 1996) of the MH2 domain in the basal state, likely due to a physical interaction between the two domains (Hata et al., 1997). In addition, the MH1 domain possesses DNA-binding activity in the activated state (Kim et al., 1997; Liu et al., 1997). The linker sequence between the MH1 and MH2 domains are divergent among Smads, and these regions contain multiple phosphorylation sites, which allow crosstalk with other signaling pathways (Kretzschmar et al., 1997a). In addition, the linker region contains a PY motif, which mediates specific interaction with Smurf 1 and 2, which are E3 ubiquitin ligases which target Smads and Smad-associated TGF-β receptors for degradation (Zhang et al., 2001; Zhu et al., 1999). The MH2 domain contains receptor phosphorylation sites in R-Smads (Kretzschmar et al., 1997b; Macias-Silva et al., 1996), has effector function (Baker and Harland, 1996; Liu et al., 1996), and mediates several important protein-protein interactions. These include homo-oligomeric complexes that all Smads form in the basal state (Hata et al., 1998; Hata et al., 1997; Lagna et al., 1996; Shi et al., 1997; Wu et al., 1997), the association of R-Smads with type I receptors (Macias-Silva et al., 1996), the binding of activated R-Smads with Smad4 (Hata et al., 1997), the interaction of Smads with DNA-binding factors (Chen et al., 1997a; Liu et al., 1997), and intriguingly, the association of
Smad3 with the polarity proteins Par3 and Dishevelled (Warner et al., 2003). The MH2 domain also contains a characteristic Ser-X-Ser motif at the extreme C-terminus of R-Smads and phosphorylation of these terminal serine residues by type I receptors drives Smad activation (Abdollah et al., 1997; Souchelnytskyi et al., 1997).

**Smad Signaling and Regulation**

Receptor-mediated phosphorylation of R-Smads leads to their accumulation in the nucleus. In the nucleus, Smad4 and all R-Smads, with the exception of Smad2, bind to DNA in a sequence-specific manner (Shi and Massague, 2003). The minimal Smad binding element (SBE), which was initially identified as the optimal DNA binding sequence for Smad3 and Smad4, is composed of only four base pairs, 5’-AGAC-3’ (Dennler et al., 1998; Yingling et al., 1997; Zawel et al., 1998). Gene expression studies have demonstrated that TGF-β stimulation influences to immediate positive and negative changes in the expression of several hundred genes (Kang et al., 2003), although given the pleiotropic nature of TGF-β signaling, these changes are likely to depend on the physiological context of signaling.

As central mediators of TGF-β signaling, Smads are subject to various types of regulatory mechanisms that integrate and adapt their signaling to the biological status of the cell. In the basal state, R-Smads are predominantly cytoplasmic, whereas the I-Smads are predominantly nuclear, and Smad4 is distributed in both compartments (Shi and Massague, 2003). The nuclear localization of R-Smad/Smad4 complexes during ligand stimulation can be influenced by alternative signaling pathways. For example, in
Xenopus embryos, activin signaling leads to Smad2 nuclear accumulation and induction of mesodermal markers (Grimm and Gurdon, 2002). However, this process is abruptly halted by the phosphorylation of Smad2 within its linker region by MAP kinase (Grimm and Gurdon, 2002). The exact molecular basis for this exclusion is not well-understood, but in addition to MAP kinase, calcium-calmodulin dependent protein kinase II (CaMKII) has also been demonstrated to phosphorylate Smad2 at several residues within its linker region (Wicks et al., 2000). Although these sites are different from the sites of MAP kinase phosphorylation, they also inhibit Smad2 nuclear accumulation (Wicks et al., 2000).

I-Smads can also inhibit Smad-dependent transcription. I-Smads lack a C-terminal Ser-X-Ser motif, and their N-terminal region only contains short segments of MH1 domain homology (Hata et al., 1998; Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997; Tsuneizumi et al., 1997). In fact, Smad6 was originally reported as a truncated Smad only consisting of the MH2 domain (Topper et al., 1997). During BMP signaling, Smad6 does not interfere with receptor-mediated phosphorylation of Smad1, but competes with Smad4 to bind activated Smad1 molecules (Hata et al., 1998). The binding of Smad6 to receptor-phosphorylated Smad1 yields a transcriptionally inert complex, suggesting that Smad6 act as decoy Smads to dampen receptor-mediated transcriptional events during BMP signaling (Hata et al., 1998). Smad7 acts to inhibit TGF-β signaling through an altogether different mechanism. Smad7 resides in the nucleus in the basal state and upon TβR1 or BMP stimulation (Itoh et al., 1998), it forms a complex with the ubiquitin ligases Smurf1 and Smurf2 (Kavsak et al., 2000; Suzuki et
al., 2002). Through their N-terminal C2 domains, Smurf1 and Smurf2 target the Smurf/Smad7 complex to the plasma membrane where Smad7 directly binds to activated TβR1 and inhibits phosphorylation of R-Smads (Kavsak et al., 2000; Suzuki et al., 2002). As a consequence of this membrane localization, Smurf1 also mediates the ubiquitination and turnover of receptors (Ebisawa et al., 2001; Tajima et al., 2003). Interestingly, Smad7 itself undergoes degradation in this process. However, TGF-β and BMPs can transcriptionally activate Smad7 to perpetuate this negative feedback loop (Kang et al., 2003).

**Smad-Independent TGF-β Signaling**

In addition to long-term changes in the transcriptional profile of cells, TGF-β signaling can also have short-term effects, most notably, on the cytoskeleton. However, the extent of Smad-independent signaling in cells is not well understood and remains an area of active research. Previous studies have demonstrated the impact of TGF-β on the activity of the Rho GTPases RhoA, Rac, and Cdc42 (Bakin et al., 2002; Bhowmick et al., 2001; Edlund et al., 2002), which in consequence results in membrane ruffling and lamellipodial formation in response to ligand stimulation (Edlund et al., 2002). TGF-β can also stabilize microtubules (Gundersen et al., 1994), and activate phosphatidylinositol-3-kinase (PI3K), which leads to the phosphorylation and activation of the PI3K effector kinase Akt (Bakin et al., 2000; Vinals and Pouyssegur, 2001). This activation is direct as TβR1 binds PI3K and biochemically-purified TGF-β receptor complexes can catalyze the conversion of PIP2 to PIP3 (Yi et al., 2005).
An interesting facet of TGF-β signaling is that beyond TβR1, TβR2 appears to have few other substrates. However, recent work has demonstrated the phosphorylation of the polarity protein Par6 by TβR2 (Ozdamar et al., 2005), an early event during epithelial-mesenchymal transition (EMT), a form of epithelial cell plasticity import for embryonic development and various cancers (Yang and Weinberg, 2008). The details of this pathway will be explored in detail in following sections, but this study highlights the importance of both Smad-dependent and independent mechanisms during TGF-β signaling.

**Epithelial-Mesenchymal Transition**

The development of organ systems in metazoans begins with a single layer of epithelial cells. During early embryogenesis of most metazoans, mesenchymal cells arise from the primitive epithelium. Unlike epithelial cells that form sheets and layers with apical/basolateral polarity, mesenchymal cells exhibit front/back polarity and rarely establish contacts with neighboring mesenchymal cells. Moreover, mesenchymal cells can invade as individual cells through the extracellular matrix (ECM). Although these distinct cells types have long been recognized in embryos, the direct conversion of epithelial cells into mesenchymal cells was only defined as a distinct cellular program in the 1980s. In a series of experiments (Greenburg and Hay, 1982, 1986, 1988), Greenberg and Hay demonstrated that individual anterior lens epithelial cells from embryos and adults detached from explants when cultured in 3-dimensional collagen gels. These detached cells elongated, and migrated as independent cells. Based on the mesenchymal
morphology of these cells, they concluded that differentiated epithelial cells had the potential to transform into mesenchymal cells through a cellular program they termed epithelial-mesenchymal transition (EMT).

The EMT program is activated at various stages of embryonic development to enable the conversion of epithelial cells into mesenchymal cells. EMT is reversible and thus, the reverse program of mesenchymal-epithelial transition (MET) also occurs during both embryonic development and during several pathological processes (Boyer and Thiery, 1993; Davies, 1996). EMT is defined by three major changes in cellular phenotype (Boyer and Thiery, 1993; Hay, 1995). These include changes in cell morphology from an epithelial type with apical/basal polarity to a spindle-shaped mesenchymal phenotype with migratory protrusions; changes in differentiation markers from cell-cell junction proteins and cytokeratin intermediate filaments to vimentin filaments and fibronectin; and the invasion of motile cells through the ECM.

During development, EMT is observed during various tissue remodeling events. These include mesoderm formation (Viebahn et al., 1995), neural crest development (Duband et al., 1995; Hay, 1995), heart development (Bolender and Markwald, 1979), secondary palate formation (Fitchett and Hay, 1989), male Mullerian duct regression (Trelstad et al., 1982), and tumor progression and metastasis (Thiery, 2002). Of these, early embryonic events such as mesoderm formation and neural crest development are the best studied. The earliest example of EMT occurs during mesoderm formation from the primitive ectoderm, a process that occurs at gastrulation (Kimelman, 2006). The first observable event in mesoderm formation is the invagination of epithelial cells, which is
accompanied by drastic morphological changes in a small population of epithelial cells. These cells undergo narrowing of their apical compartments, the apical redistribution of organelles, and the bulging of the basal compartments (Yang and Weinberg, 2008). When epithelial cells are ready to ingress, the basement membrane is locally breached and ingressing cells lose their cell-cell junctions. Subsequently, these cells undergo mesenchymal differentiation and migrate along the narrow extracellular medium underneath the ectoderm (Yang and Weinberg, 2008).

In neural crest formation, a defining characteristic of vertebrate embryogenesis, the emergence of neural crest cells begins with the presence of a discrete population of cells with round, pleiomorphic shapes, which is in stark contrast to the polarized morphology of nearby neural tube cells (Yang and Weinberg, 2008). The neural crest cells proceed to lose N-cadherin-mediated cell-cell adhesion and become excluded from the neural epithelium (Tucker et al., 1988). Detailed ultrastructural studies have demonstrated that basal lamina disruption occurs at the onset of neural crest migration, and cells in mid-migration upregulate genes required for mesenchymal phenotype and migration (Duband and Thiery, 1987; Nichols, 1981).

**Regulation of Epithelial-Mesenchymal Transition by TGF-β**

Various members of the TGF-β family of ligands are implicated as major induction signals of EMT during virtually all known morphogenetic events (Zavadil and Bottinger, 2005). These include Nodal signaling during mesoderm induction in *Xenopus* (McDowell and Gurdon, 1999), chickens (Shah et al., 1997), and mice (Chen et al.,
induction of neural crest formation by BMPs in *Xenopus* (Marchant et al., 1998), chickens (Liem et al., 1995), and mice (Correia et al., 2007); coordination of cardiac morphogenesis by TGF-β2(Sanford et al., 1997), and the regulation of secondary palatal fusion by TGF-β3 (Proetzel et al., 1995).

More recently, the molecular events that govern TGF-β-dependent EMT have been elucidated. In fluorescence-based mammalian cells screens, the polarity protein Par6 was identified as an intracellular binding protein for TβR1 (Ozdamar et al., 2005). In epithelial cells, Par6 localizes to tight junctions (Joberty et al., 2000) and its interaction TβR1 anchors the receptors to tight junctions (Ozdamar et al., 2005). This interaction is constitutive, and in the presence of ligand, TβR2 is recruited to tight junctions where it phosphorylates Par6 at its penultimate serine, Ser345 (Ozdamar et al., 2005). The phosphorylation of Par6 causes the recruitment of Smurf1, which then targets RhoA for degradation. This localized reduction in RhoA activity subsequently leads to reorganization of the actin cytoskeleton, the loss of tight junctions, and the upregulation of mesenchymal genes (Ozdamar et al., 2005).

**An Extrinsic Factor for Axon Specification**

Various signaling pathways required for neuronal polarization have been identified, most of which have been elucidated from *in vitro* cell culture systems (Arimura and Kaibuchi, 2007). As I have discussed in detail in previous sections, some of these include conserved polarity proteins (Shi et al., 2003), small GTPases and their accessory factors (Bito et al., 2000; Kunda et al., 2001; Nishimura et al., 2005; Schwamborn and Puschel, 2006a).
2004; Watabe-Uchida et al., 2006), the ubiquitin-proteasome system (Schwamborn et al., 2007; Yan et al., 2006), actin reorganization (Bradke and Dotti, 1999), microtubule-associated factors and microtubule motors (Biernat et al., 2002; Jacobson et al., 2006; Mandell and Banker, 1996; Yoshimura et al., 2005), and various kinases and phosphatases (Barnes et al., 2007; Jiang et al., 2005; Kishi et al., 2005). Although various molecular pathways have been implicated in axon specification, the common theme appears to be that most of these signaling components are organized and spatially nucleated by the Par3/Par6 polarity complex.

The existence of extracellular cues required for axon development has been suggested by several studies. These include local actin depolymerization by pharmacological agents (Bradke and Dotti, 1999), laminin (Esch et al., 1999), NgCAM (Esch et al., 1999), IGF (Sosa et al., 2006), and BDNF (Shelly et al., 2007) can trigger axon formation and growth in dissociated cultures. However, the identity of true extrinsic axon specifying factors has been elusive because knockout mice lacking many of these signaling molecular or their receptors show no defect in axon formation (Chen et al., 2008; Ernfors et al., 1994; Jones et al., 1994; Kappeler et al., 2008; Liu et al., 1993; Vicario-Abejon et al., 2004). For example, IGF-1 has been proposed as an extracellular factor required for neuronal polarity in cultured hippocampal neurons (Sosa et al., 2006). In vivo support for this notion comes from mice lacking IGF-1, which have reduced neuropil volume (Liu et al., 1993). However, forebrain cultures from IGF-1 knockout mice have demonstrated significant defects in glial differentiation, suggesting that neuropil reduction results from glial absence rather than axon loss (Liu et al., 1993).
anatomical structure of the hippocampus and cerebellum, regions that express both IGF-1 and IGF receptors, are largely normal in mice lacking IGF-1 (Vicario-Abejon et al., 2004), and although small in size, mice lacking IGF-1 receptors throughout the CNS have a normal lifespan with no apparent nervous system defects (Kappeler et al., 2008). In addition, exogenous brain-derived neurotrophic factor (BDNF) has been shown to activate LKB1 (Baas et al., 2004) through a c-AMP-dependent protein kinase (PKA) pathway leading to axon specification in vitro (Shelly et al., 2007). Curiously, mice lacking BDNF (Ernfors et al., 1994; Jones et al., 1994) or its receptor TrkB (Klein et al., 1993) survive until birth and CNS neurons in these animals show no overt defects in axon formation (Ernfors et al., 1994; Jones et al., 1994; Klein et al., 1993). Thus, other initiating factors must exist to commence neuronal polarization in vivo.

**TGF-β in Central Nervous System Development**

TGF-β signaling components have distinct patterns of expression in the embryonic brain, suggesting their importance in neural development. *In situ* hybridization and immunohistochemical studies have demonstrated that all three TGF-β ligands are expressed throughout mammalian CNS development (Heine et al., 1987; Mecha et al., 2008). Earliest expression is detected in neuroepithelia at E8.5, a time in which neurulation occurs (Mecha et al., 2008). Additionally, TGF-β ligands are expressed in the developing meninges, and within CNS structures during times of active neurogenesis and differentiation (Heine et al., 1987).

In addition to TGF-β ligands, TGF-β receptors are highly expressed in migrating
neurons of the developing cortex (Tomoda et al., 1996), and have been demonstrated to affect neuronal development. For example, in *Drosophila* mushroom body neurons, loss of the *Drosophila* TβR1 receptor punt leads to axon overextension whereas overexpression of constitutively active mutant of punt leads to early axon termination (Ng, 2008). Moreover, TGF-β1 and TGF-β2 ligand have been shown to promote the sprouting and elongation of neurites in dissociated hippocampal cultures (Ishihara et al., 1994), and mutations in TGF-β receptors and signaling components are responsible for a myriad of developmental disorders characterized by mental retardation (Gripp et al., 2000; Loeys et al., 2005), further suggesting their importance in nervous system development. Surprisingly, the role of TGF-β in CNS development has remained largely unexplored.

As already discussed in detail, the TGF-β signaling pathway, as elucidated from other cell types, shares many molecular components with pathways involved in axon specification. For example, TβR1 constitutively binds the polarity protein Par6 (Ozdamar et al., 2005). During EMT, Par6 is a substrate of TβR2 and this event leads to the recruitment of the E3 ubiquitin ligase Smurf1 and localized degradation of RhoA (Ozdamar et al., 2005). This leads to the dissolution of tight junctions, and transforms the cell into a mesenchymal cell capable of migration (Greenburg and Hay, 1982, 1986, 1988). In addition, TGF-β activates PI3-kinase during EMT (Bakin et al., 2000), and Par6, Smurf1, RhoA, and PI3-kinase have each been implicated in axon specification *in vitro* (Bito et al., 2000; Schwamborn et al., 2007; Shi et al., 2003). Moreover, TβR1 binds (Barrios-Rodiles et al., 2005) and influences the activity of p-21 activated kinases.
(Wilkes et al., 2003), which are a class of Cdc42/Rac1 activated enzymes selectively activated in developing axons to control neuronal polarity (Jacobs et al., 2007) and also implicated in cortical neuron migration (Causeret et al., 2008). Altogether, these findings point to the possibility that TGF-β may define an elusive extrinsic cue for axon specification and neuronal polarization in vivo.

**Experimental Rationale and Specific Aims**

The goal of my thesis work was to identify an extrinsic factor that specifies axon formation during neuronal morphogenesis in the developing embryonic brain. To this end, I identified TGF-β as a critical mediator of axon genesis. Although TGF-β signaling has been extensively studied in other cell types, I hypothesized that TGF-β signaling is important for axon specification and development in immature neurons due to the presence of TGF-β signaling components in the embryonic brain, and the similarities between TGF-β signaling pathways with those described for axon specification.

As I have described at length, despite an extensive and increasing understanding of intracellular mechanisms underlying axon specification, the extrinsic cues that direct neuronal polarity in vivo have been obscure. Some of the difficulty in discerning intrinsic versus extrinsic mechanisms rests in the experimental system. The classic dissociated hippocampal neuron culture system utilizes neurons typically derived at embryonic day 18, a period where isolated postmitotic neurons are already in mid-migration in the hippocampus and thus are already inherently polarized (Banker and Cowan, 1977). Moreover, dissociated cultures and the surrounding growth factor-rich growth media are
quasi-isotropic and lack graded or patterned tissue cues that would be normally encountered \textit{in vivo}.

TGF-\(\beta\) signaling has been implicated in neuronal development and dysfunction within TGF-\(\beta\) signaling pathways is responsible for a wide-spectrum of human mental retardation disorders (Loeys et al., 2005; van Steensel et al., 2008). However, despite these insights, the role of TGF-\(\beta\) in immature neurons has remained an unexplored area of study. Based on previous studies that identified the presence of TGF-\(\beta\) signaling components in developing neural tissue, and the similarities between signaling pathways involved in axon specification and TGF-\(\beta\) signaling, the goal of the \textbf{Specific Aims} below was to elucidate the role of TGF-\(\beta\) signaling in neuronal morphogenesis.

\textit{AIM #1: To establish TGF-\(\beta\) as the extrinsic factor required for axon formation in embryonic brain development}

The experiments of \textbf{AIM #1} were directed to test my hypothesis they TGF-\(\beta\) signaling is responsible for axon specification in developing neurons. Several approaches were designed to experimentally test this assertion. First, using immunofluorescence and quantitative image analysis, I found that both T\(\beta\)R1 and T\(\beta\)R2 are highly expressed in the axons of the developing neocortex of mouse embryos, and specifically, that T\(\beta\)R2 is enriched in trailing edge axons of immature neurons. In collaboration with Franck Polleux and Paul Barnes at the University of North Carolina – Chapel Hill, I performed \textit{ex vivo} electroporations and subsequent organotypic slice cultures using a conditional mouse line containing “floxed” alleles (T\(\beta\)R2\textsuperscript{\textit{flox/flox}}) of T\(\beta\)R2. In this manner, I was able
to ablate TβR2 expression in a subset of cortical neurons and visualize cellular morphology within a native embryonic context through the expression of a bicistronic plasmid encoding both GFP and Cre recombinase. I found that whereas neurons expressing GFP alone possessed stereotypical bipolar morphologies consisting for a long leading edge process and a long trailing edge axon, cell lacking TβR2 (TβR2-KO) possessed long leading edge processes but did not elaborate a trailing edge axon. Surprisingly, TβR2-KO cells were mostly mired near the VZ/IZ border, suggesting that in addition to polarity defects, these cells were compromised in their ability to migrate. In live-slice imaging experiments, I found that TβR2-KO neurons do not exhibit any kind of growth in their trailing edge but possess very highly dynamic leading processes that grow, retract, and branch over time. Their defects in migration appeared to arise due to a lack of nuclear translocation. Finally, I confirmed that electroporated cells are not progenitor cells through immunofluorescence for the glial marker, and the observation that TβR2-KO progenitor cells can undergo mitosis to produce neurons that fail to migrate.

AIM #2: To determine whether TGF-β signaling acts in a cell-autonomous manner during axon specification

My experiments in AIM #1 suggested that within intact embryonic brains, loss of TβR2 prevents axon specification in developing neurons. However, because the ex vivo method labels neuronal progenitors within the VZ, our experiments could not address whether TGF-β signaling was required in progenitor cells or in postmitotic neurons. To
distinguish these possibilities, I conducted a set of experiments using primary cultures of dissociated neurons from hippocampi of rats. By overexpressing a kinase-inactive mutant of TβR2, I could successfully prevent axon formation in immature neurons as assessed by immunofluorescence for the axon marker tau-1. In addition, pharmacological inhibition of the kinase activity TβR1 by a small molecule inhibitor also prevented the formation of axons in cultures. I could also generate cells with multiple axons by the overexpression of a wild-type form of TβR2 in immature neurons and in older neurons. Finally, I tested the sufficiency of exogenous TGF-β ligand on axon specification through two methods. First, I generated patterned coverslips containing fluorescent dye-conjugated BSA alone or with TGF-β1, 2, 3. I found that most neurons near TGF-β-containing stripes elaborated their axons toward the stripes, whereas projections near BSA-containing stripes were random. Secondly, streptavidin-conjugated polystyrene beads coated with biotinylated TGF-β1, 2, 3 ligand induced rapid outgrowth of the neurite of contact. Altogether these results suggest that TGF-β is both required and sufficient to induce axon specification in a cell-autonomous manner.

AIM #3: To elucidate the downstream signaling pathways involved in TGF-β-dependent axon specification

An interesting aspect of TGF-β signaling in epithelial cells is that Par6 acts as a scaffold to anchor TβR1 at tight junctions and is a substrate of TβR2 in the presence of ligand. Because Par6 is known to localize to sites of axon formation, I hypothesized that the same mechanisms are preserved in neurons during axon specification. In developing
cortical slices, I found Par6 to localize with TβR1 within periventricular regions of the cortex. Immunoprecipitated complexes from brain lysates probed with an antibody for TβR1 contain both Par6 and Par3, and immunofluorescence for surface TβR1 and intracellular Par6 revealed that both proteins form complexes within filopodia at the tips of neurites. To test whether phosphorylation of Par6 is important for axon specification, I generated point mutants of Par6 that could not be phosphorylated at the TβR2-specific Ser345 site (Par6-S345A) or a phospho-mimetic mutant (Par6-S345E). In dissociated neurons, I found that overexpression of Par6-S345A prevents axon formation, whereas Par6-S345E allows the polarization program to proceed normally. Moreover, in TβR2flox/flox mice, co-electroporation of Cre with either Par6-S345A or Par6-S345E revealed that Par6-S345E could rescue axonal defects whereas Par6-S345A could not. Therefore, these experiments suggest that phosphorylation of Par6 by TβR2 is required for proper axon formation in developing neurons.
Chapter Two. Materials and Methods

In this chapter I will discuss in detail all of the materials and methods I utilized to generate the results shown in future chapters. Ex vivo electroporations in embryonic mice were performed in collaboration with Paul Barnes in the laboratory of Franck Polleux (University of North Carolina, Chapel Hill, NC). All animals were used according to protocols approved by the Institutional Animal Care and Use Committee of the Duke University Medical Center, the University of North Carolina, and in accordance with NIH guidelines.

Reagents

HA-TβR2-WT and Myc-TβR2-K277R (hereafter indicated KR) were gifts from Dr. Gerald Blob (Department of Pharmacology and Cancer Biology, Duke University). pCIG2 and pCIG2-Cre were generated in the laboratory of Franck Polleux (Hand et al., 2005). pEGFP-C2 Par6 was a gift from Dr. Yuh-Nung Jan and Dr. Lily Jan, University of California, San Francisco, CA. pCIG2-Par6-S345A was generated first by introducing a point mutation into Par6 using the sense primer 5’-cgaggttagtttagcggattgcaactgaattaatttgcagtc-3’ and the antisense primer 5’-gactgcagatcagtcgaatcgtgaatccgctaacatcacctcg-3’ in which the highlighted codon indicates the mutation. pCIG2-Par6-S345E was generated as above using the sense primer 5’-cgaggttagtttagcggattgcaactgaattaatttgcagtc-3’ and the antisense primer 5’-gactgcagatcagtcgaatcgtgaatccgctaacatcacctcg-3’ in which the highlighted codon
indicates the mutation. Inserts for both Par6-WT and Par6-S345A Ire PCR amplified and ligated into pCIG2 using SacI and EcoRI sites. All DNA constructs were purified using the Qiagen Maxi endotoxin-free DNA purification kit and verified by sequencing.

**Conditional Allele Mice**

Mice used for my studies contained a “floxed” TβR2 alleles consisting of loxP sites flanking exon 2 of the TβR2 gene in a Black6/C57 genetic background (Chytil et al., 2002). In the presence of Cre recombinase, exon 2 is excised from the genomic DNA resulting in a null allele that fails to express functional protein (Chytil et al., 2002). Mice were obtained from the Mouse Models of Human Cancers Consortium (MMHCC) at the National Cancer Institute. Screening for “floxed” alleles was accomplished using the following primers: 5’ – taacaagttcggagccca – 3’ and 5’ - actctcagaggtcccc – 3’.

**Neuronal and Tissue Culture and Ex Vivo Electroporation**

Primary cultures of hippocampal neurons were obtained from E18 rat embryos from timed pregnancy rats (Charles River Laboratories). In brief, the entire hippocampus was isolated and digested with trypsin at 37°C and dissociated with a fire-polished Pasteur pipette in plating medium containing Neurobasal medium (Sigma) supplemented with B27, Glutamax, 5% fetal bovine serum, and 1 μg/ml gentamycin (all from Invitrogen). Cells were plated at a density of 200 – 300 mm² onto either poly-D-lysine-coated glass coverslips in 12-III plates, or onto 35 mm dishes with glass coverslips attached to the bottom (MatTek). Neurons were cultured at 37°C in 5% CO₂.
**Ex vivo** organotypic neocortical slices were prepared as follows. First, timed-pregnant female mice at E15 were anesthetized with isofluorane and sacrificed by cervical dislocation. Dorsal telencephalic progenitors were labeled by injecting pCIG2 plasmid DNA (0.5 μg/μl) or pCIG2-Cre plasmid DNA (0.5 μg/μl) diluted in a 0.1% fast green solution into the lateral ventricles of decapitated E14.5 Tgfbr2flq/flox mouse heads using a picospritzer II (General Valve). Electric potentials were generated across intact heads using gold-coated electrodes attached to an ECM 830 electroporator using the following parameters: four 100 ms 45V pulses separated by 100 ms intervals. Immediately after electroporation, brains were dissected in Complete MEM (Basal Medium Eagle supplemented with Hank’s Buffered Salt Solution, 50 mM glucose, 2.5 mM HEPES (pH 7.4), 1 mM CaCl2, 1 mM MgSO4, 4 mM NaHCO3, 1x Penicillin-Streptomycin). Dissected brains were embedded in an agarose solution consisting of Complete MEM and 3% low-melting point agarose cooled to 50°C. The brains were coronally Vibratome sectioned at 250 μm slices. Sectioned brain slices were maintained in air-interface cultures on 0.4 μm membrane hanging inserts (Falcon) in 6-well plates. Slices were cultured in Complete MEM supplemented with 5% horse serum. Cultures were maintained at 37°C in 5% CO2.

**Neuronal Culture Transfections**

Neurons were transfected with Lipofectamine 2000 (Invitrogen). They were transfected with appropriate cDNAs after they adhered to coverslips, typically 4-6 hours after plating. For each transfection, 1-1.5 μg of DNA diluted in 50 μl, and 0.8 μl of
Lipofectamine 2000 was mixed and added to coverslips in 12-well plates. When co-transfecting TβR2 constructs with pEGFP-C1 empty vector, a quantity ratio of 2:1 was used, respectively.

**Antibodies**

Primary antibodies used were rat anti-TβR1 and goat anti-TβR2 (both from R&D systems), rabbit anti-TβR1 (Cell Signaling), mouse anti-Tau-1 (Chemicon), mouse anti-Tuj1 (Covance), mouse anti-Nestin (BD Biosciences), mouse anti-TAG1 (Developmental Studies Hybridoma Bank), chicken anti-GFP (Clontech) and goat anti-Par6 (Santa Cruz Biotechnology), TGF-β1/2 (Santa Cruz Biotechnology), VGLUT1 (Synaptic Systems). Immunodetection was accomplished using Alex-488, -568, and -647-conjugated secondary antibodies (Invitrogen).

**Immunofluorescence**

*Dissociated neurons*

The standard protocol for immunochemical labeling of dissociated neurons is as follows: neurons were fixed in a solution of 4% paraformaldehyde/4% sucrose in PBS for 20 minutes. Neurons were washed several times in PBS. For surface receptor labeling, the cells were blocked in a blocking solution of 7% BSA in PBS for one hour at room temperature, then incubated overnight in a humidified chamber at 4°C with appropriate primary antibodies diluted (typically 1:500) in blocking solution. For intracellular labeling, neurons were permeabilized with a solution of 0.1% Triton-X100 in PBS for 10
minutes. Neurons were again rinsed several times in PBS, and then incubated in blocking solution for one hour at room temperature. Neurons were incubated with appropriate primary antibody solutions overnight in a humidified chamber at 4°C. The next day, coverslips were washed several times in PBS and incubated at room temperature in secondary antibody solution (1:500) for one hour in a humidified chamber. Neurons were rinsed several times in PBS and the coverslips were mounted on glass slides using Vectashield (Vector Laboratories).

**Organotypic slice cultures and fixed brain tissue**

Organotypic culture slices were fixed on their culture membranes overnight in 4% paraformaldehyde/4% sucrose with gentle rocking at 4°C. Fixed slices were washed five times for 15 minutes each in PBS at room temperature with gentle rocking. Slices were permeabilized overnight in 0.3% Triton-X100 diluted in blocking buffer consisting of 7% BSA dissolved in PBS at 4°C with gentle rocking. The next day, slices were rinsed several times in PBS and incubated overnight in primary antibody diluted in blocking buffer with 0.1% Triton-X100. The following day, slices were rinsed several times in PBS, and incubated for four hours in secondary antibody diluted in blocking buffer with 0.1% Triton-X100. Slices were washed several times in PBS and the membrane around the slices was cut with a scalpel. The slices were mounted on glass slides in Vectashield mounting medium. Isolated brains from E14.5 and E18 mice were fixed overnight at 4°C in a solution of 4% paraformaldehyde (PFA)/4% sucrose in phosphate buffered saline (PBS). Brains were embedded in a 3% low-melting point agarose solution prepared in
PBS and sectioned at 150 μm on a Vibratome and processed for immunohistochemistry as described.

**Microscopy**

Fluorescent images were acquired on a Zeiss 510 upright confocal scanning microscope (Carl Zeiss, Inc.). Electroporated organotypic slices were imaged after 60 hours in culture. During the acquisition, slices on their intact inserts were placed in a 35 mm glass-bottom dish (MatTek) filled with slice culturing media. The imaging chamber was humidified by fixing a metal mesh on top of the culture inserts and placing a moist Kimwipe on top of the mesh for the duration of imaging. Images were collected on a Nikon Eclipse TE300 microscope equipped with a mechanized and temperature-controlled stage. Confocal images were obtained using a Yokogawa spinning disk confocal (Solamere Technology Group), with excitation from a 50 mW Sapphire 488 LP laser (Coherent) selected and shuttered by an acousto-optical tuning filter (Neos Technologies), and the emission directed by a filter wheel (Sutter Instruments) holding band pass filters (Chroma). Images were acquired using a 20x Nikon Plan-Fluor NA 0.45 extra-long working distance lens and a 12-bit cooled CCD camera (Hamamatsu Inc.). All images in this study were analyzed using Metamorph software (Universal Imaging Corporation).

**Immobilized TGF-β Bead Assay**

Purified recombinant TGF-β1,2,3 (R&D Systems) was biotinylated using the EZ-link
Sulfo-NHS-LC-LC reagent (Pierce) according to the manufacturer’s instructions. 2 μg of each protein was used per biotinylation reaction, and excess biotin was separated from the protein by spin filtration at 10,000 x g for 35 min at 4°C on a 3 kDa Micron size exclusion column (Millipore). The unfiltered phase was removed and incubated with 1 x 10^6 streptavidin-conjugated polystyrene beads (10 μm diameter, Pierce) with gentle shaking at 4°C for 30 min according to the manufacturer’s protocol. The beads were washed in PBS and stored at 4°C until use. For control beads, an equal molar quantity of BSA was used in the preparation.

Experiments were performed on a Nikon Eclipse TE300 inverted microscope as described above. Neurons were imaged in a Ludin chamber (Life Imaging Services) filled with Neurobasal media lacking phenol-red and supplemented with B-27, Glutamax, and buffered at pH 7.3 with 25 mM HEPES (Gibco Invitrogen). Approximately 300 beads were placed into the center of the imaging chamber and images were acquired at a frequency of one image per minute for the duration of the experiment (typically 90 min) using a 20x Nikon Plan-Fluor NA 0.45 extra-long working distance lens.

**Substrate Patterning**

Glass coverslips with a diameter of 18 mm were coated overnight with poly-D-lysine (1 mg/ml) at 37°C. The next day, coated coverslips were washed three times in sterile water and dried. A silicon matrix (J. Jung, Tuebingen, Germany) consisting of 90 μm channels separated by 90 μm intervals was placed over the coverslip. Channels were filled with a 0.1% solution of Alexa 568-conjugated BSA (Invitrogen) with or without 100 ng/ml
TGF-β1, 2, 3 in PBS and incubated for 2 hours at 37°C. Coverslips were washed and cells were plated as described above.

**Image Analysis**

*Ex vivo electroporation*

Computer-based reconstructions of neurons were done using Metamorph and Photoshop (Adobe) software. Organotypic slice cultures were fixed after 5 days of culture using the previously described protocol, and typically stained with anti-nestin antibody to visualize radial glia and anti-GFP antibody to enhance neuronal morphology. Confocal image stacks with a step size of 0.2 μm were acquired. Stacks were further trimmed with respect to planes that captured individual neurons. These stacks were then collapsed into maximum projections and neuronal morphology traces were done in Photoshop.

*Substrate Patterning Assay*

Analysis of axon orientation was done as follows. Cells were cultured for 60 hours and fixed according to standard protocols. Cells were stained with tau-1 and Map-2 antibodies to visualize the axon and dendrites, respectively. For analysis, the 90 μm space between substrate stripes was bisected into 45 μm halves in the plane parallel with the stripes. Tau-1 camera lucida traces of cells seeded next to stripes were mapped onto x-y coordinates where positive y values reflect the perpendicular direction toward the TGF-β stripe and negative y values reflect the perpendicular direction away from the TGF-β stripe oriented. Tau-1 traces were generated using CorelDraw software.
Co-immunoprecipitation and Western Blot

Forebrains from E18 rat embryos were homogenized by pipette in phosphate-buffered saline containing 0.5% Triton X-100, 135 mM NaCl, complete Mini protease tablet (Roche), and PMSF. The homogenate was incubated on ice for 20 min with periodic rocking and cleared by centrifugation at 14,000 x g for 10 min at 4°C. 750 μg of total protein was aliquoted and diluted to a final volume of 800 μl in lysis buffer, and samples were incubated with or without primary antibody overnight at 4°C. For input samples, 37.5 μg of total protein was aliquoted into sample buffer. The following day, GammaBind G Sepharose beads (GE Healthcare) were washed, blocked in a 5 mg/ml solution of BSA with rotation at 4°C for 1 hour, and resuspended in wash buffer (lysis buffer containing 250 mM NaCl). The beads were added to the immunoprecipitation reactions and incubated at 4°C for 1.5 hours. The final complex was washed five times with wash buffer and resuspended in 30 μl of 1x protein sample buffer.

For Western blotting, samples were boiled for 5 minutes and spun down to recollect the final volume. Samples were loaded and run on 4 – 15% gradient Tris-HCL polyacrylamide gels (Bio-Rad). Proteins were immobilized by gel transfer to polyvinylidene fluoride (PVDF) membranes (Millipore). The resulting membrane was blocked for 1 hour at room temperature in 5% BSA prepared in Tris-buffered saline containing 0.1% Tween-20. Primary antibodies were diluted in blocking solution (typically at a dilution of 1:1000) and incubated with gentle rocking overnight at 4°C. Signal detection was accomplished using species-specific horse-radish peroxidase (HRP)
conjugated secondary antibodies (GE Healthcare) at a dilution of 1:5000 in 5% milk.

**ELISA**

TGF-β1 and TGF-β2-specific ELISA kits were purchased from R&D Systems and used according to the manufacturer’s protocol. Cells used for analysis were plated in standard plating media consisting of 5% FBS. 24 hours after plating, the plating buffer was switched to serum-free neuronal culture media consisting of Neurobasal supplemented with B27 and Glutamax. For sample collection, 150 μl of media was collected daily and frozen at -80°C until use.

**Statistical Analysis**

All errors bars represent the standard error of the mean. Statistical comparisons were accomplished by using Student’s t-test, where a p < 0.05 was considered statistically significant. All statistical tasks were performed with Origin software.
Chapter Three. TGF-β Signaling is Required for Axon Specification During Embryonic Brain Development

Summary

Morphological polarization is central to cellular function in all tissues. In the mammalian brain, the specification of a single axon and multiple dendrites occurs early in the life of most neuron types, and this polarity choice point determines nearly all aspects of neural connectivity and brain function. Numerous intracellular signaling events occur during axon specification and have been described in detail. However, the identity of the extracellular factor(s) that initiate neuronal polarity in vivo is unknown, and whether extrinsic cues exist at all to direct neuronal polarity in vivo has been uncertain. Here, I demonstrate that transforming growth factor-β (TGF-β), an embryonic morphogen that regulates structural plasticity and growth in various cell types, initiates signaling pathways both in vivo and in vitro to fate naïve neurites into axons. TGF-β ligands are expressed in the developing brain at sites of axon specification, and using conditional knockout strategies, I show that cortical neurons lacking the type II TGF-β receptor (TβR2) fail to initiate axons during development. Exogenous TGF-β is sufficient to direct the rapid growth and differentiation of an axon and genetic enhancement of receptor activity promotes the formation of multiple axons. These results define a long-elusive extrinsic cue for neuronal polarity in vivo that patterns neural circuits in the developing brain.
Introduction

The designation of polarized cellular domains is central to tissue function in all metazoans. Among the most polarized cells, neurons of the mammalian central nervous system (CNS), exhibit asymmetry early in brain development that defines the elaboration and function of all neural circuitry (Kriegstein and Noctor, 2004). In simple formulation, neurons are binary biological units with a distinction between the somatodendritic compartment, which receives and integrates synaptic inputs, and the axon, which transmits action potentials across long distances (Horton and Ehlers, 2003). The specification of such domains occurs early in neuronal development and the molecular events that guide this phenomenon remain the subject of intense research (Arimura and Kaibuchi, 2007).

To date, most of our knowledge regarding axon specification comes from pioneering in vitro studies using dissociated cultures of rodent hippocampal neurons. In this system, dissociated neurons plated on an adherent substrate initially extend several undifferentiated neurites (stage 1-2), then enter a phase of asymmetric growth (stage 3) in which a single neurite undergoes rapid elongation and becomes the axon (Craig and Banker, 1994). Various intracellular signaling pathways important for the transition from an unpolarized to a polarized state have been identified (Arimura and Kaibuchi, 2007). These include conserved polarity proteins (Shi et al., 2003), small GTPases and their accessory factors (Bito et al., 2000; Kunda et al., 2001; Nishimura et al., 2005; Schwamborn and Puschel, 2004; Watabe-Uchida et al., 2006), the ubiquitin-proteasome
system (Schwamborn et al., 2007; Yan et al., 2006), actin reorganization (Bradke and Dotti, 1999), microtubule-associated factors and microtubule motors (Biernat et al., 2002; Jacobson et al., 2006; Mandell and Banker, 1996; Yoshimura et al., 2005), and various kinases and phosphatases (Barnes et al., 2007; Jiang et al., 2005; Kishi et al., 2005). Despite extensive information or intracellular polarity signaling, it is not known how such pathways are initiated during neuronal development.

Several extracellular factors have been proposed to initiate neuronal polarity programs (Esch et al., 1999; Hilliard and Bargmann, 2006; Shelly et al., 2007; Sosa et al., 2006). For example, exogenously applied brain-derived neurotrophic factor (BDNF) can activate the polarity-inducing kinase LKB1 (Baas et al., 2004) through a c-AMP-dependent protein kinase (PKA) pathway leading to axon specification in vitro (Shelly et al., 2007). However, mice lacking BDNF (Ernfors et al., 1994; Jones et al., 1994) or its receptor TrkB (Klein et al., 1993) survive until birth and CNS neurons in these animals do not exhibit any obvious defects in axon formation (Ernfors et al., 1994; Jones et al., 1994; Klein et al., 1993). In addition, IGF-1 has been proposed as an extracellular factor that initiates neuronal polarity in cultured hippocampal neurons in vitro (Sosa et al., 2006). In vivo support for this notion comes from observations that mice lacking IGF-1 have reduced neuropil volume (Liu et al., 1993). However, forebrain cultures from IGF-1 knockout mice show significant defects in glial differentiation, suggesting that neuropil reduction results from glial absence rather than axon loss (Liu et al., 1993). Indeed, the anatomical structure of the hippocampus and cerebellum, regions that express both IGF-1 and IGF receptors, are largely normal in mice lacking IGF-1 (Vicario-Abejon et al.,
Moreover, although growth retarded and microcephalic, mice lacking IGF-1 receptors throughout the CNS have a normal lifespan with apparently intact axon tracts (Kappeler et al., 2008). Thus, other initiating factors must exist to commence neuronal polarization in vivo.

Among diverse extrinsic signals in the developing brain, transforming growth factor-β (TGF-β) is a pleiotropic morphogen that governs a wide variety of cellular processes including cell differentiation, proliferation, apoptosis, and specification of developmental fate (Shi and Massague, 2003). Canonical TGF-β signaling is initiated by the binding of a ligand dimer to receptor serine/threonine kinases at the cell surface. The three closely-related TGF-β ligands (TGF-β1-3) first bind the type II TGF-β receptor (TβR2), which causes its recruitment to the type I TGF-β receptor (TβR1). The formation of this complex allows the phosphorylation of the kinase domain of TβR1 by TβR2, which in turn initiates both immediate and long-term cellular changes through cytoskeletal rearrangements and transcriptional responses, respectively (Shi and Massague, 2003).

TGF-β signaling components have distinct patterns of expression in the embryonic brain, suggesting their involvement in neural development. In situ hybridization and immunohistochemical studies have demonstrated that all three TGF-β ligands are expressed throughout mammalian CNS development (Heine et al., 1987; Mecha et al., 2008). Earliest expression is detected in neuroepithelia at E8.5, a time in which neurulation occurs (Mecha et al., 2008), and TGF-β receptors are highly expressed in migrating neurons of the developing cortex (Tomoda et al., 1996). Moreover, both
TGF-β1 and TGF-β2 ligand have been shown to promote the sprouting and elongation of neurites in dissociated hippocampal cultures (Ishihara et al., 1994), and mutations in TGF-β receptors and signaling components have been attributed to a myriad of developmental disorders characterized by mental retardation (Gripp et al., 2000; Loeys et al., 2005). Despite these insights, the role of TGF-β in CNS development has remained largely unexplored.

Intriguingly, the TGF-β signaling pathway, as elucidated from other cell types, shares many molecular components with pathways involved in axon specification. For example, TβR1 is anchored to tight junctions in epithelial cells through its constitutive interaction with the polarity protein Par6 (Ozdamar et al., 2005). During epithelial-mesenchymal transition (EMT), TβR2 phosphorylates Par6 leading to the recruitment of the E3 ubiquitin ligase Smurf1 and subsequent local degradation of RhoA (Ozdamar et al., 2005). This leads to the dissolution of tight junctions, and transforms the cell into an elongated mesenchymal cell capable of independent migration (Greenburg and Hay, 1982, 1986, 1988). Moreover, TGF-β has been shown to activate the PI3-kinase-Akt signaling axis during EMT (Bakin et al., 2000). Intriguingly, Par6, Smurf1, RhoA, and PI3-kinase have each been implicated in axon specification in vitro (Bito et al., 2000; Schwamborn et al., 2007; Shi et al., 2003). These findings point to the possibility that TGF-β may define an elusive extrinsic cue for axon specification and neuronal polarization in vivo.

Here, I present in vitro and in vivo evidence that TGF-β directs axon establishment in developing neurons. TGF-β receptors are expressed in axons during
embryonic development and receptor kinase activity is required for axon formation and neuronal migration in the developing mouse neocortex. Gain-of-function and loss-of-function experiments show that the level of TGF-β receptor activity in young neurons dictates axon number. Moreover, exogenous TGF-β is sufficient to spatially direct the differentiation and rapid outgrowth of axons. These results demonstrate a crucial role for extrinsic TGF-β signaling in establishing neuronal polarity and axonal identity in the mammalian brain.

Results

TGF-β Ligand Expression is Spatially Restricted to Areas of Neuronal Proliferation and Differentiation

In situ hybridization studies in developing mouse embryos have demonstrated that the localization of different isoforms of TGF-β ligands is highly suggestive of its function during early events in neuronal development. In E14.5 embryos, a time when neurogenesis of layer 5 neurons cortical neurons occurs (Polleux et al., 1997), TGF-β1 ligand is largely absent, or present at very low levels, in the developing CNS, suggesting that its function is not realized until later stages of development (Figure 3-1A). In contrast, TGF-β2 is highly expressed in areas immediately adjacent to the later ventricles (Figure 3-1B). These areas correspond to the VZ and SVZ, which are principal proliferative zones for excitatory pyramidal neuron generation. In addition, time-lapse imaging studies have demonstrated that axon specification in newborn neurons occurs
within the VZ and SVZ at early stages of neuronal migration (Hatanaka and Murakami, 2002; Noctor et al., 2004). Interestingly, TGF-β3 is also highly expressed within the VZ and within medial ganglionic eminence (MGE), which is the principal site for interneuron proliferation (Figure 3-1C). Altogether, these data indicate that TGF-β ligands are present in the brain during embryonic development, and expressed at anatomically known zones of neuronal polarization. Their spatial distribution suggests that whereas TGF-β2 may be involved in pyramidal neuron polarization, TGF-β3 may be involved in interneuron morphogenesis.

**TGF-β Receptors are Expressed in Axons During Neural Development**

If TGF-β is involved in axon specification, I reasoned that TGF-β receptor expression should be evident in axons during embryonic development. I therefore examined TGF-β receptor expression in the developing mouse neocortex using immunohistochemistry, focusing on E14-15 neocortex. Both TβR1 and TβR2 are highly expressed throughout the mouse neocortex, including robust expression in nestin-positive radial glial progenitors (Figure 3-2A). Both receptor types are present at apical domains of radial glia (Figure 3-2B), consistent with previous findings (Falk et al., 2008; Murphy et al., 2004). In addition, TGF-β receptor labeling was present in postmitotic neurons in the cortical plate (CP), as identified by staining with the neuron-specific β-tubulin III marker Tuj1 (Figure 3-2C). Both TβR1 and TβR2 were found in the cell bodies of layer 6 neurons and diffuse TβR1 labeling was found within the intermediate zone (IZ) of the cortical wall (Figure 3-2C). Intriguingly, I observed striking TβR2 labeling along β-
Figure 3-1. TGF-β Ligand Expression is Spatially Segregated During Embryonic Development.

(A) *In situ* hybridization showing the absence of TGF-β1 in an E14.5 mouse embryo. A magnified view of the developing neocortex demarcated by the black box is shown below.

(B) *In situ* hybridization showing the expression of TGF-β2 within peri-ventricular zones in an E14.5 mouse embryo. The expression of TGF-β2 corresponds with anatomical areas where axon specification in pyramidal neurons occurs. A magnified view of the developing neocortex demarcated by the black box is shown below. Arrow indicates the ventricular expression of TGF-β2.

(C) *In situ* hybridization showing the expression of TGF-β3 predominantly in the medial ganglionic eminence (MGE) in an E14.5 mouse embryo. The expression of TGF-β3 corresponds with anatomical areas of inhibitory neuron generation. A magnified view of the developing neocortex demarcated by the black box is shown below. Arrow demarcates the MGE. All images were obtained from the Genepaint database ([http://www.genepaint.org](http://www.genepaint.org)).
Figure 3-1. TGF-β Ligand Expression is Spatially Segregated During Embryonic Development.
tubulin-rich fasciculations within the IZ in E14.5 animals, suggesting the presence of TGF-β signaling machinery in new axons (Figures 3-2C top panels and 3-2D). The presence of both TβR1 and TβR2 in axons became even more prominent at later timepoints, as shown by immunohistochemistry in E18 mouse embryos (Figure 3-2C, bottom panels). To verify this observation, I simultaneously labeled the cortex with an antibody for TβR2 and TAG1, a marker of corticofugal axons (Kawano et al., 1999). TβR2 immunoreactivity showed good coincidence with TAG1 (Figures 3-2E and 3-2F), indicating that TGF-β receptors are present in areas of active axon development in embryonic neurogenesis. These data show that TGF-β signaling components are present in axons during embryonic neurogenesis in the neocortex.

**TGF-β Signaling is Required for Axon Development in Vivo**

To address the role of TGF-β receptor signaling in axon development, I utilized *ex vivo* organotypic cortical slice cultures, which allows the labeling and visualization of neurons in intact developing brain tissue while preserving normal contextual interactions, tissue architecture, neuronal migration, and differentiation (Barnes et al., 2007; Hand et al., 2005; Polleux et al., 1998). During cortical and hippocampal development, neurons are born in the subventricular zone (SVZ) of the lateral ventricles and travel through radial glia-guided migratory routes to laminate the cortex and hippocampus (Kriegstein and Noctor, 2004). Long-term fluorescence imaging studies of newborn neurons in the VZ have shown that axon specification occurs soon after terminal cell division and axon extension occurs during migration (Hatanaka and Murakami, 2002; Noctor et al., 2004).
Figure 3-2. Axonal Expression of TGF-β Receptors in the Developing Mouse Neocortex

(A) Neocortical slices from embryonic day 14.5 (E14.5) embryos processed for immunohistochemistry and triple labeled for TβR1, TβR2, and nestin. Scale bar, 50 μm. MZ, marginal zone; CP, cortical plate; L6, layer 6; SP, subcortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

(B) Magnified panels of area demarcated by white dashed box in (A) showing apical enrichment of receptors in nestin-positive radial glia (arrowheads). Scale bar, 5 μm.

(C) Sections of mouse neocortex at E14.5 (top) or E18.5 (bottom) labeled for TβR1, TβR2 and the neuron-specific β-tubulin III marker Tuj1. Arrow shows tubulin-Tuj1-positive fasciculations in the IZ. Scale bar, 50 μm. Abbreviations as in (A).

(D) Magnified panels of area demarcated by white dashed box in (C) showing the presence of TGF-β receptors in the IZ at E14.5. Arrowhead indicates a single axon positive for both TβR1 and TβR2. Scale bar, 5 μm.

(E) Sections of E14.5 mouse neocortex labeled with the corticofugal axon marker TAG-1, TβR2, and the nuclear stain TOPRO-3 demonstrating the presence of TβR2 in cortical axons at E14.5 (arrows). Scale bar, 50 μm.

(F) Magnified panels of area demarcated by white dashed box in (E) showing strong TβR2 staining in TAG1-positive axons. Scale bar, 5 μm.
Figure 3-2. Axonal Expression of TGF-β Receptors in the Developing Mouse Neocortex
I used a Cre/loxP-based conditional knockout strategy to selectively ablate TβR2 receptors from newborn cortical neurons in vivo. For this, mouse embryos harboring homozygous “floxed” alleles of Tgfbr2 encoding TβR2 (Tgfbr2\textsuperscript{flox/flox}) were subjected to intracranial electroporation to introduce a bicistronic plasmid encoding GFP and Cre recombinase selectively into neuronal precursors in the VZ at E15 (Figure 3-3) (Chytil et al., 2002). Following electroporation, brains were dissected and cortical slices cultured. After three days of culture, neurons begin to migrate toward the cortical plate and after four days, GFP-positive neurons could be seen as layer 5 at the CP. My analysis was conducted after five days of culture, during which time newborn neurons polarize and migrate toward the CP, slices were fixed and the morphogenesis of GFP-positive migrating neurons examined. Immunocytochemical labeling revealed that newborn neurons expressing GFP (and thus Cre) lacked detectable expression of TβR2 (Figures 3-4A), indicating effective conditional ablation of Tgfbr2. In Tgfbr2\textsuperscript{flox/flox} littermate controls electroporated with GFP alone, GFP-positive neurons progressively traveled through the IZ and terminated their migration at the CP, where they began to elaborate dendritic processes. Most migrating neurons possessed stereotypical bipolar morphologies consisting of a leading process and a long trailing edge axon (Figures 3-4B and 3-4C). In contrast, cells expressing Cre and thereby lacking TβR2 failed to establish axons despite the formation of a leading edge process (Figures 3-4B and 3-4D). Whereas 82.3 ± 6.4% of control neurons possessed a morphologically discernable axon, only 30.2 ± 8.4% of Tgfbr2 null cells (TβR2-KO) formed a distinguishable axon (Figure 3-4E). Surprisingly, the dendritic development of neurons at the CP was largely unaffected by
Figure 3-3. The *Ex Vivo* Electroporation and Organotypic Slice Culture

Electroporation in E15 embryos. The lateral ventricles (LV) of embryos were injected and filled with appropriate cDNA solutions visualized with 0.1% fast green labeling. The heads were then electroporated and organotypic slice cultures prepared. After 3 DIV, most GFP-positive neurons are found in mid-migration within the IZ. At 5 DIV, many neurons are seen at their migratory targets at the CP. Magnified panels correspond to areas demarcated by white boxes. Scale bar represents 100 μm for images on the left and 25 μm for magnified panels to the right.
Figure 3-3. The *Ex Vivo* Electroporation and Organotypic Slice Culture
the loss of TβR2, suggesting that TGF-β signaling is critical for axon formation but not dendritic development (Figures 3-4C and 3-4D, bottom panels).

**Loss of TGF-β Signaling in Newborn Neurons Leads to Defects in Migration**

In addition to defects in axon formation, TβR2-KO neurons exhibited impaired migration to the cortical plate. In neocortex from *Tgfbr2*^fl/fl^ mice transfected with GFP and Cre, I observed GFP-positive neurons that migrated normally to the IZ and CP (Figure 3-4C, top panel). However, many TβR2-KO neurons were present within deep cortical layers and failed to migrate (Figure 3-4D, top panel). Cells within this deep layer population were immunonegative for nestin (Figure 3-5A), suggesting that TGF-β signaling affects migration, but not differentiation from radial glial precursors. Interestingly, TβR2-KO neurons in both superficial and deep cortical layers lacked trailing edge axons (Figure 3-4B, 3-4D, and 3-4E), indicating that despite heterogeneous migration defects, TGF-β signaling via TβR2 is required specifically for axon formation in cortical pyramidal neurons. In time-lapse imaging experiments using electroporated brain slices after 60 hours in culture, neurons from *Tgfbr2*^fl/fl^ mice transfected with GFP alone migrated normally through the IZ and elaborated a dynamic leading edge and a trailing edge axon (Figure 3-5A). Similarly, TβR2-KO neurons possessed highly dynamic leading edge processes that extended, retracted and branched over the 20-hour imaging period (Figure 3-5B). However, TβR2-KO neurons lacked nuclear translocation and failed to exhibit any growth within their trailing edge, suggesting that TGF-β signaling affects
Figure 3-4. TGF-β Signaling is Required for Neocortical Development in Vivo

(A) TβR2 immunohistochemistry in neocortical slice cultures of E15 Tgfbr2^flx/flx embryos electroporated with GFP (A) or GFP/Cre (B). Arrows, GFP-positive neurons either exhibiting (A) or lacking (B) TβR2 immunoreactivity. Arrowhead in (A) indicates an axon. Scale bar, 15 μm.

(B) Example images of migrating neurons from Tgfbr2^flx/flx embryos expressing GFP or GFP/Cre. Arrows indicate the presence (left) or absence (right) of axons. Scale bar, 22 μm.

(C) Neocortical slices from control Tgfbr2^flx/flx embryos electroporated with GFP to label newborn neurons. Top panel shows neuronal migration after 5 days. Bottom panels contain camera lucida traces of individual cells showing cells with stereotypical leading edge processes and trailing edge axons (arrows).

(D) Tgfbr2^flx/flx embryo electroporated to express GFP plus Cre in neuronal precursors. Top panel shows defects in neuronal migration after 5 days with many GFP-positive neurons arrested in the SVZ. Bottom panel contains camera lucida traces of individual cells showing cells with leading edge processes but lacking axons (arrows). For (D) and (E), scale bar represents 100 μm for top panels and 20 μm for bottom panels. CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone; DIV, days in vitro.

(F) Quantification of cells containing axons in wildtype (WT, GFP expressing) and TβR2-KO (TβR2-KO, GFP + Cre) neurons. Results were pooled from 3 embryos (n = 88 cells for WT; n= 92 cells for TβR2-KO). *p<0.05, Student’s t-test.
Figure 3-4. TGF-β Signaling is Required for Neocortical Development in Vivo
morphogenetic events at the axonal pole of the cell rather than the dendritic pole (Figure 3-5B). Together, these findings demonstrate an obligatory role for TGF-β signaling in neuronal polarity in developing neocortex.

**Axon Specification by TGF-β Signaling is Cell Autonomous**

Embryonic cortical electroporation initially labels nestin-positive radial glial progenitors in the ventricular zone (VZ) (Hand et al., 2005). Thus, as radial glial cells express TβR2, I could not determine whether the loss axons in TβR2-KO neurons was cell-autonomous or due to effects in neuronal progenitor cells based on my *ex vivo* preparation. To address this issue, I utilized dissociated E18 rat hippocampal neuronal cultures, which consist of terminally-differentiated postmitotic neurons (Banker and Cowan, 1977). In an initial set of experiments, I treated freshly plated postmitotic hippocampal neurons with SB-431542, a potent and selective small-molecule inhibitor of TβR1 (Figure 3-6). Whereas a large majority control cells treated with DMSO alone possessed a single axon (80.3 ± 2.1%; Figure 3-6A and 3-6C), cells grown in the presence of SB-431542 for 72 hours lacked a distinguishable axon (22.9 ± 3.4%; Figure 3-6B and 3-6C). To further determine whether TGF-β signaling acts cell autonomously to specify the axon, I disrupted TGF-β signaling in individual neurons by sparsely co-expressing a kinase inactive mutant form of TβR2 (K277R, hereafter indicated KR) (Wrana et al., 1992) along with GFP. Hippocampal neurons were sparsely transfected after they adhered to coverslips but prior to axon specification (typically 4-6 hours after plating) and fixed 65-
Figure 3-5. TGF-β Affects Migration and Morphological Dynamics at the Axonal Pole, but Not the Dendritic Pole.

(A) Time-lapse imaging of neuronal migration and polarization in E15 Tgfbr2\textsuperscript{flox/flox} embryos electroporated with GFP. The arrowhead marks the soma, and the white arrow marks the leading edge whereas the red arrow marks the axon. See Movie S3. Scale bar represents 20 μm.

(B) Time-lapse imaging of neuronal migration and polarization in E15 Tgfbr2\textsuperscript{flox/flox} embryos electroporated with GFP. The arrowhead marks the soma, and the white arrows mark branches of the leading edge process. Red arrow marks the axon. See Movie S4. Scale bar represents 20 μm.
Figure 3-5. TGF-β Affects Migration and Morphological Dynamics at the Axonal Pole, but Not the Dendritic Pole.
72 hours after transfection at a time when polarity has been established. Whereas cells expressing GFP alone possessed a single, long tau-1 positive axon (Figures 3-7A and 3-7B, top panels), cells expressing TβR2-KR arrested in stage 2 and possessed multiple short neurites of roughly equivalent length that lacked Tau-1 immunoreactivity (Figures 3-7A and 3-7B, bottom panels). Neurons expressing TβR2-KR often produced thin, filopodia-like extensions that suggested disorganization in cytoskeletal arrangement (Figure 3-7B, bottom panels). When quantified, the longest neurite in TβR2-KR expressing cells was on average 166.9 μm shorter than axons of control cells (TβR2-KR, 137.8 ± 45.0 μm; GFP 304.7 ± 14.0 μm) (Figure 3-7C). Moreover, whereas most control neurons expressing GFP possessed a single tau-1 positive axon by 72 hours (84.5% ± 4.4% with one axon; Figure 3-7D), most cells expressing TβR2-KR lacked a tau-1 positive axon (60.8 ± 5.3% with no axon; Figure 3-7D). Together, these results indicate that postmitotic neurons require TGF-β signaling for axon formation in a cell autonomous manner.

To test whether TGF-β signaling is sufficient to specify axons, I enhanced TGF-β signaling by expressing the constitutively active wild-type (WT) form of TβR2 in freshly dissociated neurons 4-6 hours after plating. By 65-72 hours, cells transfected with GFP possessed long single axons whereas cells with increased TGF-β signaling generated multiple long tau-1 positive and MAP-2 negative axons (Figures 3-7E and 3-7F). When quantified, 46.2 ± 4.9% of TβR2-WT expressing cells possessed multiple axons compared to 3.2 ± 2.9% in GFP expressing cells (Figure 3-7G). Together, these results demonstrate that TGF-β receptor kinase activity is necessary and sufficient to
Figure 3-6. Pharmacological Inhibition of TGF-β Receptors Prevents Axon Specification

(A) Dissociated hippocampal neurons from E18 rat embryos treated with DMSO as a vehicle control. Bottom panels show the morphology of a typical DIV 3 neuron consisting of a single long axon (arrow) and multiple short dendrites. Cells were fixed and visualized by staining with Tuj1 antibody. Scale bar, 50 μm top, 5μm bottom panels.

(B) Images showing changes in morphology of DIV 3 neurons grown in the presence of the TβR1 inhibitor SB-431542 (30 μm). Bottom panels show individual neurons with multiple short neurites of roughly equivalent length. Scale bar, 50 μm top, 5μm bottom panels.

(C) Data indicate means ± SEM of the fraction of neurons with a distinguishable axon after treatment with DMSO or 30 μM SB-431542. Data pooled from 3 independent experiments, n=85 cells for GFP; n=112 cells for SB-431542. *p<0.05, Student’s t-test.
Figure 3-6. Pharmacological Inhibition of TGF-β Receptors Prevents Axon Specification
induce axons in developing neurons.

**Regulated of TGF-β Signaling is Required for Polarity Maintenance**

To test whether careful regimentation of TGF-β signaling is required through neuronal development, I expressed TβR2-WT in fully polarized DIV5 neurons to augment TGF-β signaling after polarity establishment. When analyzed two days later (DIV7), nearly half of TβR2-WT expressing cells elaborated multiple long tau-1 positive axons (49.0 ± 2.8%, n = 43, Figure 3-8A, top panels; and 3-8B), a phenotype seldom observed for control cells (3.3 ± 2.7% neurons with ≥ 2 axon, n = 38; Figures 3-8A, bottom panels; and 3-8B). Together, these data show that, even following polarization, TGF-β signaling retains the ability to induce axon generation in neurons. Thus, this result implies that global TGF-β must be downregulated in the cell in order to maintain proper polarity. The cell may achieve this through two mechanisms. First, it may actively limit TGF-β signaling by limiting the expression of signaling components, or by increasing the expression of signaling inhibitors. Second, signaling may be polarized so that only parts of the cell, namely the axon, receive TGF-β innervation while the dendrites remain unstimulated by TGF-β. The distinction between these two mechanisms will be made in the following chapter.
Figure 3-7. Cell Autonomous TGF-β Signaling is Necessary and Sufficient to Induce Axons

(A) Dissociated hippocampal neurons from E18 rat embryos expressing GFP or GFP + TβR2-KR were fixed and stained for the axonal marker tau-1. Cells were transfected 4-6 hours after plating and fixed 65-72 hours later. Arrow indicates tau-1 positive axons (arrowheads) that are absent from cells expressing TβR2-KR. Scale bar, 50 μm.

(B) Camera lucida traces of neurons expressing GFP (top) or GFP + TβR2-KR (bottom). Arrows indicate axon. Scale bar, 20 μm.

(C) Average lengths of the longest neurite in control cells and cells expressing TβR2-KR. n = 12, *p<0.05, Student’s t-test.

(D) Quantification of axon number in cells expressing GFP alone or GFP plus TβR2-KR. Data pooled from at least 3 independent experiments. GFP, n = 42; TβR2-KR, n = 53; *p<0.05, Student’s t-test.

(E) Camera lucida traces of neurons expressing TβR2-WT. Arrows indicate axons. Scale bar, 20 μm.

(F) Neurons expressing GFP or GFP + TβR2-WT showing multiple tau-1 positive axons emerging from cells expressing TβR2-WT (arrows). Scale bar, 20 μm.

(G) Quantification of axon numbers in cells expressing GFP alone or GFP plus TβR2-WT. GFP, n = 41; TβR2-WT, n = 44; *p<0.05, Student’s t-test.
Figure 3-7. Cell Autonomous TGF-β Signaling is Necessary and Sufficient to Induce Axons
Exogenous TGF-β is Sufficient to Spatially Direct Neurite Outgrowth and Axon Specification

Signaling molecules required for axon specification become spatially restricted to nascent axon tips (Arimura and Kaibuchi, 2007). To address whether local TGF-β signaling drives axon specification, I adhered biotinylated TGF-β ligand onto streptavidin-conjugated polystyrene beads and examined cellular responses upon local bead contact. Strikingly, when TGF-β beads were placed in contact with single neurites of unpolarized neurons, I observed rapid growth of the contacted neurite within minutes characteristic of the rapid outgrowth that occurs during axon specification in vitro (Figures 3-9A and 3-9B). Stimulated neurites doubled in length over the course an hour while the lengths of unstimulated neurites remained unchanged (Figure 3-9C). The observed effect of local TGF-β was not due to mechanical contact as polystyrene beads conjugated with bovine serum albumin (BSA) had no effect on neurite growth (Figure 3-9D). To examine whether rapid neurite outgrowth induced by local TGF-β coincided with molecular differentiation of an axon, I cultured hippocampal neurons on coverslips with a striped pattern of TGF-β substrate (Walter et al., 1987). I placed a silicon matrix molded with 90 μm wide channels each separated by 90 μm onto coverslips coated with poly-D-lysine. Channels were filled with either Alexa 568-conjugated BSA alone or together with a mixture of all three TGF-β isoforms, TGF-β1, 2, and 3, which then adhered to the substrate. In this manner, I could generate and visualize spatially-restricted zones of elevated TGF-β ligand and assess whether locally enhanced extracellular TGF-β affects axonal differentiation. After 72 hours, neurons were fixed and dendrites and axons
Figure 3-8. Restricted TGF-β Signaling is Required to Maintain Neuronal Polarity

(A) Augmented TGF-β signaling induces axons after polarity establishment. Cells were transfected with GFP plus TβR2-WT at DIV5 and fixed 48 hours later. Arrows indicate tau-1 positive MAP-2 negative axons. Scale bar, 20 μm.

(B) Quantification of axon numbers in DIV5 cells expressing GFP alone or GFP plus TβR2-WT. GFP, n = 38; TβR2-WT, n = 43; *p<0.05, Student’s t-test.
Figure 3-8. Restricted TGF-β Signaling is Required to Maintain Neuronal Polarity

A

<table>
<thead>
<tr>
<th></th>
<th>GFP</th>
<th>Tau-1</th>
<th>MAP-2</th>
<th>Merged</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td><img src="image1" alt="GFP" /></td>
<td><img src="image2" alt="Tau-1" /></td>
<td><img src="image3" alt="MAP-2" /></td>
<td><img src="image4" alt="Merged" /></td>
</tr>
<tr>
<td>GFP + TβR2-WT</td>
<td><img src="image5" alt="GFP + TβR2-WT" /></td>
<td><img src="image6" alt="GFP + TβR2-WT" /></td>
<td><img src="image7" alt="GFP + TβR2-WT" /></td>
<td><img src="image8" alt="GFP + TβR2-WT" /></td>
</tr>
</tbody>
</table>

B

![Bar graph](image9)

- GFP
- GFP + TβR2-WT

% (%)

1 Axon  ≥2 Axons

<table>
<thead>
<tr>
<th>1 Axon</th>
<th>≥2 Axons</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>75</td>
</tr>
</tbody>
</table>

![Scale bar](image10)
visualized by MAP-2 and tau-1 immunoreactivity, respectively. In cases where neurons adhered near the border of a TGF-β stripe, the nascent axon invariably arose from the neurite that contacted the stripe and the tau-1 positive axon projected into the stripe (Figure 3-9E, top panels). Interestingly, neurons seeded directly on a TGF-β stripe formed multiple tau-1 positive axons (Figure 3-9E, bottom panels).

To quantify the effect of local TGF-β on axon induction, I compared the directional projection of axons on a cell-by-cell basis using tau-1 immunoreactivity. I divided the uncoated area between the coated stripes into 45 μm halves and cells with somas completely contained in either half were imaged for analysis. Camera lucida traces of tau-1 images were overlaid onto a grid as a compilation such that cells projecting axons towards the closest stripe were oriented upwards and projections away from the stripe were oriented downwards on the grid (Figure 3-9F). Using this method, I found that 85.7 ± 7.3% of neurons had axons that arose from neurites which contacted zones of TGF-β (Figure 3-9G). On the other hand, axons were randomly directed in cells grown on striped coverslips containing BSA alone (Figure 3-9H). Together these findings demonstrate that local TGF-β is sufficient to rapidly induce neurite growth and axon differentiation in a spatially directed manner.
Figure 3-9. Locally Applied TGF-β is Sufficient to Specify Axonal Differentiation and Growth

(A) Shown is an E18 + DIV 1 hippocampal neuron undergoing selective neurite extension. The blue circle labels a TGF-β-conjugated bead. Black arrowheads indicate neurites untouched by beads. The red arrow indicates a neurite initially in contact with the bead. Times indicated in hours:minutes. Scale bar, 25 μm. See Movie S1.

(B) Camera lucida traces of TGF-β induced neurite growth. Neurites initially contacting TGF-β beads are indicated in red. Scale bar, 25 μm.

(C) Fold increase in the length of neurites either contacting (red) or not contacting (black) TGF-β beads. Data represent means ± SEM. n = 12.

(D) Fold increase in the length of neurites either contacting (red) or not contacting (black) BSA beads. Data represent means ± SEM. n = 14.

(E) Localized TGF-β spatially orients axon specification. Cells were seeded on coverslips patterned with stripes of TGF-β. After 48 hours, cells were fixed and stained for tau-1 and MAP-2 to visualize the axon (arrow) and dendrites (arrowheads), respectively. White dashed lines indicate the borders of the TGF-β stripe. Scale bar, 50 μm.

(F) For analysis, the 90 μm space between stripes was bisected. Tau-1 camera lucida traces of cells seeded next to stripes were mapped onto x-y coordinates where positive y values reflect the perpendicular direction toward the TGF-β stripe and negative y values reflect the perpendicular direction away from the TGF-β stripe oriented. Scale bar, 50 μm.

(G) Multiple overlaid camera lucida traces of tau-1 staining from neurons grown on TGF-β (G) or BSA (H) striped coverslips. Individual cell traces shown in black. The summed average is shown in red. n = 45, 41. Scale bar, 25 μm.
Figure 3-9. Locally Applied TGF-β is Sufficient to Specify Axonal Differentiation and Growth
Discussion

In this study, I showed that the extrinsic morphogen TGF-β mediates axon specification in the developing brain. The kinase activity of TGF-β receptors is necessary for axon generation both in vitro and in vivo, and enhanced kinase activity is sufficient to drive the formation of multiple axons in dissociated neurons. Moreover, local application of TGF-β ligand induces axon differentiation.

Identification of an Extrinsic Signal for Axon Specification

Despite an extensive and increasing understanding of intracellular mechanisms underlying axon specification (Arimura and Kaibuchi, 2007), the extrinsic cues that direct neuronal polarity in vivo have been obscure. Some of the difficulty in discerning intrinsic versus extrinsic mechanisms rests in the experimental system. The classic dissociated hippocampal neuron culture system utilizes neurons typically derived at embryonic day 18, a period where isolated postmitotic neurons are already in mid-migration in the hippocampus and thus are already inherently polarized (Banker and Cowan, 1977). Moreover, dissociated cultures and the surrounding growth factor-rich growth media are quasi-isotropic and lack graded or patterned tissue cues that would be normally encountered in vivo.

The existence of extrinsic cues for neuronal polarity has been suggested by the fact that a number of external manipulations including local actin depolymerization (Bradke and Dotti, 1999), laminin (Esch et al., 1999), NgCAM (Esch et al., 1999), IGF
(Sosa et al., 2006), and BDNF (Shelly et al., 2007) can trigger axon formation and growth in dissociated cultures. However, whether such factors play a role in neuronal polarity in vivo is contested since knockout mice lacking many of these signaling molecular or their receptors show no defect in axon formation (Chen et al., 2008; Ernfors et al., 1994; Jones et al., 1994; Kappeler et al., 2008; Liu et al., 1993; Vicario-Abejon et al., 2004). There may be several explanations for such discrepancies. The first is the possibility of redundant pathways involved in axon formation. The polarization of axon and dendritic domains is at the heart of basic neuronal function, and this construction defines the utility of a neuron. Therefore, it is highly likely that higher order animals have evolved multiple mechanisms of axon generation to compensate for defects during development. Secondly, establishment is a complex process, one that is likely oversimplified by our current model. For example, the cell biological lines separating overall neuronal health, axon specification, growth, and guidance remain murky, and future work to distinguish these events will likely clarify the current model of neuronal polarization.

Additional factors have complicated the identification of extrinsic cues for axon specification in vivo. First, many secreted factors and their cognate receptors are required at early periods of embryonic development prior to neuronal migration and polarization. Indeed, TβR2-null mice exhibit embryonic mortality around E10.5 due to defects in yolk sac hematopoiesis and vasculogenesis (Oshima et al., 1996). Secondly, many secreted factors act through redundant signaling pathways. For example, all three TGF-β isoforms signal through identical type I and type II receptors (Shi and Massague, 2003). The genetic loss of one TGF-β isoform induces the upregulation of other isoforms in the CNS
(Knuckey et al., 1996; Mecha et al., 2008), and the expression of TGF-β1 from the TGF-β3 genomic locus is partially sufficient to rescue the cleft palate phenotype in TGF-β3-deficient mice (Yang and Kaartinen, 2007). Thus, the temporal precision of genetic manipulation is critical for assessing the stage- and cell-type-specific nature of TGF-β signaling components in embryonic development. I solved this problem through acute and selective disruption of the Tgfbr2 locus by expressing Cre recombinase in neuronal progenitors of the neocortex in E15 Tgfbr2^floX/floX embryos. This approach allowed me to generate Tgfbr2 null alleles immediately prior to terminal neuronal differentiation in vivo. Using this method, I was able to observe previously unknown effects of TGF-β signaling on axon specification in a native embryonic environment.

**TGF-β in Brain Development**

The mRNAs of the three TGF-β isoforms show different distributions in the developing mouse brain. At E15, TGF-β1 expression is expressed at nearly undetectable levels while TGF-β2 is expressed periventricular to the lateral ventricles where cortical neurogenesis occurs, and TGF-β3 expression is primarily limited to the medial ganglionic eminence, the site of GABAergic neuron differentiation (Figure 3-1; see [http://www.genepaint.org](http://www.genepaint.org)) (Lavdas et al., 1999). The localization pattern of TGF-β2 in the embryonic brain suggests that TGF-β2 is the TGF-β ligand that mediates axon specification in cortical neurons. Indeed, the expression pattern of TGF-β2 coincides with the anatomical orientation of neocortical axons since principal neurons arising from the lateral ventricle all project their axons toward the ventricular zone. Thus, newborn
neurons in the ventricular zone will be exposed to a gradient of TGF-β2 ligand with highest levels present near the ventricular surface, a scenario conducive to ventricular zone-oriented axon generation. Consistent with this idea, I found that TβR2-KO neurons fail to form axons and exogenous TGF-β is sufficient to spatially direct the differentiation and growth of axons. Altogether, these observations suggest a model in which a gradient of TGF-β2 directionally activates TGF-β receptors on the ventricular pole of newborn neurons, thereby giving a uniform vector to axon specification (Figure 3-10).

**TGF-β-dependent Signaling Events in Axon Specification**

The pleiotropic nature of TGF-β ligands is ideal for initiating broad signaling events that have been demonstrated to occur in axon specification. In particular, it is well established that TGF-β initiates the activation of PI3-kinase (Yi et al., 2005), a major effector of the axon-specification signaling pathway. Previous experiments have demonstrated that pharmacological inhibition of PI3-kinase or the overexpression of PTEN, which converts PI(3, 4, 5)P into PI(4, 5)P, both inhibit the formation and maintenance of axons in developing neurons (Jiang et al., 2005; Shi et al., 2003). Moreover, TGF-β is known to activate Smurf1 and Smurf2, which are both E3 ubiquitin ligases required for axon specification (Schwamborn et al., 2007).

A question that will be addressed in the following chapter is how TGF-β receptors are organized in the plasma membrane. In epithelial cells, TβR1 and TβR2 are highly polarized at the cell surface with TβR1 positioned at tight junctions and TβR2 positioned throughout the apical domain (Ozdamar et al., 2005). Interestingly, the positioning of
TβR1 is highly dependent on the polarity protein Par6, a key mediator of neuronal polarity in developing neurons (Ozdamar et al., 2005). Moreover, Par6 is a substrate of TβR2, an event that is required for the progression of EMT. The mechanisms of the interaction between TβR1 and Par6, and how TGF-β-dependent signaling occurs through Par6 during neuronal development are explored experimentally in greater detail in the following chapter.

**TGFβ-mediated Transcriptional Events in Neuronal Development**

Axon specification is an enduring structural change, one that likely requires permanent alterations to the cellular transcriptional program. However, little is known about how such genetic programs are altered or regulated during stages of neuronal development. TGF-β signaling is well studied for its transcriptional effects in which the transcriptional factors Smad2 and Smad3 are directly activated by TβR1, causing nuclear translocation and modulation of a vast range of transcriptional responses (Shi and Massague, 2003). Thus, it is tempting to speculate that direct coupling of TGF-β receptor activity to gene expression allows neurons to consolidate changes in cellular structure and gene expression to a single event. Indeed, we found that both TβR1 and TβR2 kinase activity is required for axon specification in neurons, suggesting that transcriptional events initiated by TβR1 are important for neuronal polarity.

Consistent with this notion, several reports point to the importance of Smad activity during development. For example, Smad2 is required for axonal morphogenesis in cerebellar granule neurons (Stegmüller et al., 2008) and yeast two-hybrid screening has
Figure 3-10. A Model for TGF-β–dependent Axon Specification

A gradient of TGF-β in the VZ biases axon development toward the VZ. The detection of TGF-β gradients is mediated by TβR1 and TβR2, which then initiate intracellular signaling cascades through Par6, leading to directed axon specification.
Figure 3-10. A Model for TGF-β–dependent Axon Specification
identified Par3 and Dishevelled, which are both important mediators of axon specification (Shi et al., 2003; Zhang et al., 2007), as binding partners for Smad3 (Warner et al., 2003). Additional experiments in cerebellar granule neurons have demonstrated a role for Smad2 and TGF-β in axonal morphogenesis. Indeed, whereas pharmacological inhibition of TβR1 with SB-431542 or shRNA-mediated knockdown of Smad2 expression promotes axon elongation, overexpression of Smad2 prevents axon elongation (Stegmuller et al., 2008). In the nucleus, Smad2 acts as a scaffold to bring the transcriptional regulator SnoN to the Cdh1-Anaphase Promoting Complex, where it is targeted for proteasomal degradation (Stegmuller et al., 2006). Thus, the authors conclude that Smad2 activation and subsequent nuclear translocation is important for its scaffolding ability in axon morphogenesis (Stegmuller et al., 2008). However, whether the transcriptional activity of Smad2 is required remains unclear.

Interestingly, mutations in genes involved in TβR1 signaling have been attributed to human developmental disorders, which are all characterized by mental retardation. These include Loeys-Dietz syndrome, which arises due to mutations in TβR1 and TβR2 (Loeys et al., 2005), and Shprintzen-Goldberg syndrome, which is attributed to mutations in TβR2 (van Steensel et al., 2008). Moreover, TALE homeobox protein TG-interacting factor (TGIF) is a repressor of Smad2-dependent transcription (Wotton et al., 1999) that is mutated in individuals with holoprosencephaly (Gripp et al., 2000), further illustrating the importance of TGF-β-dependent transcriptional events in neural development (Gripp et al., 2000). Although the mechanisms remain to be investigated, these observations raise the possibility that mutations to transcriptional responses of TGF-β signaling alter
CNS development, leading to profound defects in CNS maturation and function.

**Control of Polarity and Migration by TGF-β**

In addition to axonal defects, the loss of TGF-β signaling also resulted in unexpected defects in neuronal migration. Indeed, previous work in mice conditionally lacking the LKB1 kinase demonstrated that whereas cortical neurons in these animals lacked axons, their ability to migrate remains largely intact (Barnes et al., 2007). Interestingly, a luminescence-based mammalian interactome-mapping (LUMIER) screen identified three binding partners in the mammalian genome for TβR1 (Barrios-Rodiles et al., 2005). These include the polarity protein Par6, which is further investigated in the following chapter; occludin, a member of the tetraspanin family of transmembrane molecules, and a vital structural component of epithelial tight junctions (Furuse et al., 1993); and p-21 activated kinase 1 (PAK1), a member of the PAK family of Rac/Cdc42-activated kinases involved in TGF-β signaling, cell migration, actin reorganization, and neuronal development (Barrios-Rodiles et al., 2005; Bokoch, 2003; Nikolic, 2008; Wilkes et al., 2003). Intriguingly, previous work has demonstrated that in addition to its role in axon specification (Shi et al., 2003), Par6 is required for centrosome positioning and glia-guided migration in cerebellar granule neurons (Solecki et al., 2004). In addition, the loss of PAK1 activity by shRNA-mediated knockdown has been shown to result in defective neuronal migration and morphogenesis in intact cortical slices (Causeret et al., 2008).

Much like the localized accumulation of Par6 in the axon, PAK1 is also involved in axon specification, and experiments in dissociated cultures have revealed that PAK1 is
locally activated in the axon (Jacobs et al., 2007). Thus, such observations raise a few questions about the nature of TGF-β signaling during neuronal development. What could account for such varying phenotypic effects? One possibility is that TβR1 signals activated various independent pathways to impact distinct aspects of cell behavior. Indeed, TGF-β is well known for its effects on a plethora of cellular pathways that affect various behaviors. These include modulation of the activity of Rho GTPases (Zhang, 2009), PI-3 kinase (Yi et al., 2005), GSK-3β (Yi et al., 2005), PAK kinases (Murphy et al., 2004), and various transcriptional effects mediated by Smad proteins (Massague, 1998). An additional possibility is the existence of a feedback mechanism between the axon and soma to coordinate cellular movements with morphogenesis. Indeed, work in non-neuronal cell types during migration has revealed that migration is a coordinated process in which molecular events at the leading edge of the cell, such as actin turnover mediated by Cdc42 and Rac1, must occur in concert with trailing edge events that contract the cell body toward the vector of motion (Van Aelst and D'Souza-Schorey, 1997). The exact mechanisms of this phenomenon wait further investigation, but altogether, these observations suggest that TGF-β receptors exist at the origin point of multiple signaling pathways that control both neuronal migration and morphogenesis during embryonic brain development.
Chapter Four. Phosphorylation of Par6 by TβR2 is required for axon specification

Summary

The specification of a single axon and multiple dendrites occurs early in neuronal differentiation and such morphological polarization is central to neural connectivity and brain function. Transforming growth factor-β (TGF-β), an embryonic morphogen that regulates structural plasticity and growth in various cell types, has been demonstrated to act specifically to specify axons in developing pyramidal neurons in vivo and in vitro. However, the mechanisms by which TGF-β signaling is confined to axons remains unknown. The polarity protein Par6 is a PDZ-domain scaffolding protein that acts as a molecular hub for various signaling proteins, and has been demonstrated to be important for recruiting and nucleating signaling molecules important for axon specification. In epithelial cells, Par6 is localized to tight junctions where it anchors the type I TGF-β receptor, TβR1, at the plasma membrane. In this study, I find that the association between Par6 and TβR1 is preserved in developing neurons, and signaling by TGF-β receptors requires phosphorylation of Par6. Using a combination of in vivo and in vitro genetic methods I find that Par6 phosphorylation at Serine 345 by TβR2 is required for proper axon specification, and this event is sufficient to rescue axonal defects in TβR2-null cells within intact brain tissue.

Introduction
The designation of polarized cellular domains defines the division of labor critical for proper neuronal function and neural connectivity. From a general morphological perspective, neurons are binary biological units with a distinction between the somatodendritic compartment, which receives and integrates information, and the axon, which transmits signals across long distances. The specification of such domains occurs early in the life of a neuron and the molecular events that guide this phenomenon remain the subject of intense research in developmental neuroscience.

Consistent with localized changes in the architecture of the cell, the signaling events underlying neuronal polarity are highly polarized. For example, actin instability in a single neurite fates that neurite to become the axon (Bradke and Dotti, 1999), molecules involved in axon growth an specification locally accumulate in the axon (Horiguchi et al., 2006; Nishimura et al., 2004; Schwamborn et al., 2007; Schwamborn and Puschel, 2004; Shi et al., 2003), and signaling molecules undergo local activation (Barnes et al., 2007; Jacobs et al., 2007) and inactivation (Jiang et al., 2005; Yoshimura et al., 2005) within nascent axons. However, how such signaling events are activated and maintained within such restricted cellular domains is poorly understood.

An interesting aspect of axon specification is the involvement of the conserved Par polarity complex. The six members of the partitioning-defective (Par1-6) gene family were originally identified in *C. elegans* (Kemphues et al., 1988). It is now clear that this conserved ensemble of Par proteins defines an ancient signaling pathway required for asymmetric cell division and morphological polarity. Indeed, whereas Par1 and Par4
encode serine/threonine kinases (Guo and Kemphues, 1995; Watts et al., 2000); Par5 is a member of the 14-3-3 family of proteins, which are recruited to phosphorylated serines and threonines (Morton et al., 2002); Par3 and Par6 are PDZ domain scaffolding proteins (Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999), and Par2 is a RING-finger domain protein that acts as a putative ubiquitin ligase during protein degradation (Levitan et al., 1994; Moore and Boyd, 2004).

Recent studies have shown extensively that Par proteins are integral during neuronal development and mark sites of axon specification. For example, in stage 2 hippocampal neurons, Par3 and Par6 are present at the tips of all neurites but are selectively retained in the axonal growth and lost from putative dendrites during the transition to stage 3 (Shi et al., 2003). Based on such observations, and the scaffolding nature of the Par3/Par6 complex, this has led to a model in which Par3/Par6 acts as a signaling platform in the nascent axon to localize signals required for neuronal polarity. Consistent with this notion, overexpression of Par3 or Par6 disrupts neuronal polarity, and Par3 overexpression promotes the formation of multiple axons (Shi et al., 2003). Further supporting this model is the observation that signaling molecules that are regulated by Par3 and Par6, also localize to nascent axons. Some of these include PKCζ (Schwamborn and Puschel, 2004), the Rho GTPase Cdc42 (Schwamborn and Puschel, 2004), Smad ubiquitin regulatory factor 1 and 2 (Smurf1 and Smurf2) (Schwamborn et al., 2007), KIF3A (Shi et al., 2004), Dishevelled (Zhang et al., 2007), and the GEF Tiam1 (Nishimura et al., 2005). Moreover, mice lacking brain-specific expression of the kinase LKB1, which is also known as Par4, possess normally migrating neurons that lack axons.
(Barnes et al., 2007). Altogether, these observations suggest that various components of the polarity machinery controls profound aspects of axon development.

Despite the presence of polarity-generating molecules in the axon, our picture of how such developmental asymmetry is achieved with these molecules remains grossly incomplete. Perhaps the biggest missing link is the identification of extracellular axon-generating molecules that initiate the signaling pathways required for neuronal polarity. More specifically, how extracellular factors and their cognate receptors are organized and control the Par3/Par6 complex have yet to be identified in neurons. For example, BDNF and IGF-1 have both been proposed as extrinsic axon-specifying molecules in neurons (Shelly et al., 2007; Sosa et al., 2006). In addition to discrepancies in functional data regarding in vivo mouse models lacking BDNF and IGF-1 (Jones et al., 1994; Kappeler et al., 2008; Klein et al., 1993; Liu et al., 1993), how these receptors are organized within the framework of the Par complex remains unknown.

Intriguingly, the TGF-β signaling pathway, as elucidated from other cell types, shares many molecular components with pathways involved in axon specification. For example, TβR1 is anchored to tight junctions in epithelial cells through its constitutive interaction with the polarity protein Par6 (Ozdamar et al., 2005). During EMT, TβR2 phosphorylates Par6 leading to the recruitment of the E3 ubiquitin ligase Smurf1 and subsequent local degradation of RhoA (Ozdamar et al., 2005). This leads to the dissolution of tight junctions, and transforms the cell into an elongated mesenchymal cell capable of independent migration (Greenburg and Hay, 1982, 1986, 1988). Moreover, TGF-β has been shown to activate the PI3-kinase-Akt signaling axis during EMT (Bakin
et al., 2000). Par6, Smurf1, RhoA, and PI3-kinase have each been implicated in axon specification in vitro (Bito et al., 2000; Schwamborn et al., 2007; Shi et al., 2003). These findings point to the possibility that TGF-β may define an elusive extrinsic cue for axon specification and neuronal polarization in vivo.

My previous work demonstrated that TGF-β signaling is required for axon establishment both in vivo and in vitro. I showed that the kinase activity of TGF-β receptors is required for proper axon formation, and the loss of TGF-β signaling in migrating neurons results in growth defects at the axonal pole of the cell, rather than the dendritic pole. Moreover, enhancement of TGF-β signaling by overexpressing TβR2 is sufficient to drive the formation of multiple axons. However, the downstream signaling pathways of TGF-β signaling, particularly how polarized TGF-β signaling is achieved in developing neurons remains unknown. Here, I present evidence that TGF-β receptors are organized at sites of axon specification through its interaction with Par6. Using biochemical methods, I show that TβR1 binds to Par6 in embryonic brain tissue and this complex resides at the tips of neurites of developing neurites, often marking filopodia protrusions. Moreover, phosphorylation of Par6 at Serine 345 by TβR2 is critical for proper axon development as mutation at this residue to an alanine (Par6-S345A) to block phosphorylation prevents axon formation. Finally, I show that in ex vivo organotypic cultures lacking TβR2, overexpression of a Par6 mutant carrying a phosphomimetic glutamate mutation at position 345 (Par6-S345E) is sufficient to rescue axon defects, whereas overexpression of Par6-S345A cannot rescue this defect. These results link secreted cues with the activity of the Par3/Par6 polarity complex during axon
specification, and demonstrate a crucial role for extrinsic TGF-β signaling in establishing neuronal polarity and axonal identity in the mammalian brain.

Results

TGF-β Receptor Distribution is Polarized during Neuronal Development

How is spatially localized TGF-β signaling achieved? As shown in the previous chapter, the expression of TGF-β2 ligand is highly restricted to the VZ and SVZ of embryonic neocortex (Figure 3-1B), precisely the location of axon initiation (Hatanaka and Murakami, 2002; Noctor et al., 2004). Further spatial restriction could arise by localization of TGF-β receptors. Indeed, in epithelial cells TβR1 is confined to tight junctions and TβR2 localizes to the apical domain (Falk et al., 2008; Murphy et al., 2004; Ozdamar et al., 2005). As loss of TβR2 prevents axon but not dendrite formation (Figure 2), I tested whether the axon-specific effect of TGF-β signaling results from subcellular confinement of receptor distribution. I first examined immature E14.5 neurons exiting the SVZ of the lateral ventricle and found that TβR2 immunoreactivity is concentrated within trailing edge processes (Figures 4-1A), which become the axon (Noctor et al., 2004). When I quantified TβR2 immunoreactivity in these cells I found elevated expression of TβR2 throughout trailing-edge axons, but not in leading edge processes (Figure 4-1B). Intriguingly, I did not observe any overt enrichment of TβR1 expression in neurons at this stage, suggesting that TβR1 is expressed at low levels, or highly compartmentalized in immature neurons.
To elaborate further on this observation, I visualized TGF-β receptor distribution in dissociated rat hippocampal neurons, a well-established model system for studying the polarized distribution of signaling molecules during neuronal development (Dotti et al., 1988). I cultured neurons from E18 rat hippocampi and visualized the surface distribution of TβR1 and TβR2 during axon specification by immunolabeling. In stage 2 neurons lacking axons (DIV 2), TβR1 and TβR2 were present diffusely over the cell surface with a few receptor clusters evident as discrete foci at the tips of all neurites (Figures 4-2A, top panels; and 4-2B). In contrast, by stage 3 (DIV 4), TβR1 and TβR2 were concentrated in the axon and largely absent from dendrites (Figure 4-2A, bottom panels; and 4-2C). Quantitative analysis revealed that axonal growth cones had 54.5 ± 0.1% more TβR1 and 72.3 ± 0.1% more TβR2 than dendritic growth cones on the same neurons (Figure 4-2D). These results indicate that TGF-β receptors are retained in the nascent axon and lost from dendrites as neurons polarize, suggesting a basis for axon-specific TGF-β signaling.
Figure 4-1. TβR2 is Localized to Trailing Edge Axons in Neurons Engaging Migration

(A) Tuj1-positive migrating cortical neurons from an E14.5 mouse embryo showing enrichment of TβR2 in trailing-edge axons (arrows) of neurons migrating away from the ventricular zone (VZ) toward the cortical plate (CP). Scale bar, 20 μm.

(B) Linescans of TβR2 showing enrichment of TGF-β receptors within the axon. Signal intensity was normalized to the first pixel value of the linescan within the soma. Distance was normalized to reflect the fractional distance of total process length away from the soma, n=12.
Figure 4-1. TβR2 is Localized to Trailing Edge Axons in Neurons Engaging Migration
TβR1 Forms a Complex with Par6 in Neurons

How does TGF-β signaling induce axon formation within developing neurons? In epithelial cells, TGF-β receptor activity is localized to tight junctions within the plasma membrane through an interaction between TβR1 and Par6, a member of the conserved Par3/Par6/PKCζ polarity complex (Hung and Kemphues, 1999). Interestingly, Par6 is required for multiple aspects of neuronal development including axon specification (Shi et al., 2003), cell division (Costa et al., 2008), neuronal migration (Solecki et al., 2004) and dendritic spine morphogenesis (Zhang and Macara, 2008). Moreover, in epithelial cells, Par6 is directly coupled to TβR1 at tight junctions where in the presence of ligand TGF-β signals are transduced through Par6 (Ozdamar et al., 2005). Thus, I hypothesized that TGF-β signaling may induce axon formation in vivo by directly coupling to Par6. I simultaneously labeled brain slices from E17 mouse embryos with antibodies against Par6 and TβR1 and found that both are enriched within apical domains of radial glia in the VZ (Figure 4-3A and 4-3B).

I next tested whether TβR1 and Par3/Par6 form a biochemical complex in developing brain. To this end, I performed co-immunoprecipitations from rat forebrain lysates prepared from E17 embryos. In lysates probed with a TβR1 antibody, both Par6 and Par3 were found in immunoprecipitates (Figure 4-3C), suggesting that TβR1 exists as a complex within embryonic brains. However, I could not rule out the possibility that the bulk of this interaction is either indirect, or exists in cell-types other than neurons. To distinguish these possibilities, I utilized immunofluorescence to resolve
Figure 4-2. TGF-β Receptors Polarize to Nascent Axons

(A) Surface-labeled TβR1 and TβR2 in dissociated E18 rat hippocampal neurons form discrete clusters at the tips of all neurites (arrowheads) in stage 2 cells prior to polarization (top panels). A few hours later during axon specification, receptor distribution becomes polarized to the axon in stage 3 neurons (arrows) while receptor staining is reduced in dendrites (arrowheads). Scale bar, 20 μm.

(B) Magnified panels demarcated by white boxes in the top right panel of (C) showing sparse surface TβR1 and TβR2 staining at tips of undifferentiated stage 2 neurites. Scale bar, 3 μm.

(C) Magnified panels demarcated by white boxes in the bottom right panel of (C) showing enriched TβR1 and TβR2 within the growth cone of the axon and loss of surface receptor expression from the growth cone of a dendrite. Scale bar, 4 μm.

(D) Integrated intensities of TβR1 (left) and TβR2 (right) immunofluorescence at the growth cones of axons and dendrites of stage 3 neurons. Values were normalized to the shortest neurite in each cell. n = 12 cells. * p<0.05, Student’s t-test.
Figure 4-2. TGF-β Receptors Polarize to Nascent Axons
the localization of these two proteins in neurons within the context of active neuronal development.

In dissociated hippocampal neurons, previous studies have demonstrated that Par6 is present within all neurites prior to neuronal polarization. Upon axon specification, Par6 undergoes selective accumulation in the axon, suggesting that Par6 accumulation within a single neurite fates that neurite to become the axon (Shi et al., 2003). Thus, based on the selective accumulation of TβR1 in axons (Figure 4-2), and based on our biochemical data, we hypothesized that Par6 and TβR1 must segregate into the axon together. Indeed, in unpolarized stage 2 neurons, we found that surface TβR1 and Par6 colocalize in discrete punctate clusters at the tips of all undifferentiated neurites (Figure 4-3D, top panels), particularly within filopodial protrusions (Figure 4-3E). In stage 3 neurons, both surface TβR1 and Par6 were selectively found within the axon (Figure 4-3D, bottom panels). Altogether, these observations are consistent with the notion that TGF-β signals locally to Par6 during neuronal development.

**TGF-β Signaling is Mediated Through Par6 Phosphorylation**

In EMT, TβR1 stimulation causes the recruitment of TβR2 to the TβR1/Par6 complex at tight junctions, which subsequently results in Par6 phosphorylation of Par6 by TβR2. This occurs at the penultimate serine residue in Par6, Serine 345 (Ser345), and this phosphorylation event leads to the recruitment of the E3 ubiquitin ligase Smurf1, and the subsequent localized degradation of RhoA (Ozdamar et al., 2005). Interestingly, RhoA has been studied extensively for its role in axon specification, and previous studies have
Figure 4-3. TβR1 Forms a Complex with Par6 During Neuronal Development

(A) Co-localization of TβR1 and Par6 at the VZ of E17 mouse neocortex. Shown is immunohistochemical triple labeling for TβR1, Par6, and the radial glial marker nestin. Scale bar, 50 μm.

(B) Magnified image of the boxed region in (A) showing enriched expression of TβR1 and Par6 in the VZ. Scale bar, 5 μm.

(C) Par6 and Par3 co-immunoprecipitate with TβR1. E18 rat forebrain lysate were subjected to immunoprecipitation with a TβR1 antibody, and co-precipitating proteins were immunoblotted for TβR1, Par6, and Par3. Input lane represents 5% of the total protein quantity used for immunoprecipitation.

(D) Par6 and surface TβR1 polarize into the axon. Immunocytochemistry for Par6 and surface TβR1 in E18 dissociated rat hippocampal neurons. A stage 2 cell is shown on top panels and a stage 3 cells is shown in the bottom panels. Scale bar, 20 μm.

(D) Magnification of box in (A) showing co-localization of TβR1 clusters and Par6 in undifferentiated neurite tips. Scale bar, 2 μm.
Figure 4-3. TβR1 Forms a Complex with Par6 During Neuronal Development

A

B

C

IP: TβR1 Ab
IB:

TβR1
Par6
Par3

TβR1 Ab IP
Lysate
Control

D

E
demonstrated that whereas constitutively-active RhoA prevents axon establishment (Da Silva et al., 2003; Schwamborn and Puschel, 2004), inhibition of RhoA by C3 exoenzyme promotes axon formation (Da Silva et al., 2003). Thus, I next test whether Par6 phosphorylation by TβR2 is critical for axon specification.

I generated Par6 mutants to prevent phosphorylation by generating a point mutant at Ser345 into an alanine (Par6-S345A) or to mimic phosphorylation by mutating Ser345 into a glutamate (Par6-S345E). I overexpressed both mutants into freshly plated primary dissociated hippocampal neurons. Whereas neurons expressing GFP alone produced long, single axons (Figure 4-4A, top panels), cells expressing Par6-S345A (Figure 4-4A, middle panels) lacked axons similar to the effect previously observed upon inhibition of TGF-β receptor signaling. In contrast, neurons expressing Par6-S345E resembled GFP-expressing controls and had single long axons (Figure 4-5A, bottom panels). Indeed, whereas neurons expressing Par6-S345E formed axons (% cells with axon: GFP, 82.5 ± 7.2%; Par6-S345E, 72.4 ± 10.1%), expression of Par6-S345A markedly inhibited axon formation (27.9 ± 5.6% cells with axons; Figure 4-4B). Thus, these results support a crucial role for Par6 phosphorylation at serine 345 in axon specification.

**TGF-β Signaling Mediates Axon Formation In Vivo through Phosphorylation of Par6**

Thus far, I have demonstrated using biochemical and immunofluorescence methods that TβR1 and Par6 form a stable complex in embryonic brains, simultaneously redistribute
Figure 4-4. Par6 Phosphorylation by TβR2 is Required for Neuronal Polarity in Vitro

(A) Par6 phosphorylation is required for axon differentiation. Hippocampal neurons were transfected with GFP, GFP-Par6-S345A, or GFP-Par6-S345E. Cells were fixed and analyzed for polarized growth. Whereas the majority of GFP and Par6-S345E expressing neurons possessed axons (arrow), neurons expressing Par6-S345A did not form distinguishable axons. Arrowhead marks the soma. Scale bar, 50 μm.

(B) Data represent means ± SEM of the percent neurons with an axon. GFP, n = 38; Par6-S345A, n = 34; Par6-S345E, n = 44. *p<0.05, Student’s t-test.
Figure 4-4. Par6 Phosphorylation by TβR2 is Required for Neuronal Polarity in Vitro
into axons upon neuronal polarization in vitro, and phosphorylation of Par6 by TβR2 is critical for axon specification. To address whether Par6 phosphorylation by TGF-β receptors determines neuronal polarity in vivo, I performed genetic rescue experiments examining axon initiation and polarized migration of neocortical neurons expressing mutant forms of Par6 that either occlude (Par6-S345A) or mimic (Par6-S345E) phosphorylation by TGF-β receptor signaling by TβR2.

Canonical TGF-β signaling was genetically ablated in neocortical progenitors by intracranial electroporation of Cre in E15 Tgfbr2^{flox/flox} mice. Par6 constructs were co-expressed along with Cre. Neocortical slices were then prepared and cultured for five days to allow for neuronal migration and polarization to proceed. TβR2-KO neurons expressing Par6-S345A were similar in morphology to TβR2-KO neurons and failed to initiate axon growth (Figures 4-5A and 4-5C). In contrast, TβR2-KO neurons expressing Par6-S345E reliably produced axons (Figures 4-5B and 4-5D), suggesting that phosphorylation of Par6 can rescue axon specification in the absence of TGF-β receptor signaling. Quantitative analysis revealed that TβR2-KO neurons expressing Par6-S345E possessed axons at nearly the same frequency as WT neurons (70.3 ± 6.8% with axons, compared to 85.2 ± 6.2% for WT neurons expressing GFP; Figures 4-5E and 4-5G). On the other hand, Par6-S345A failed to rescue axon formation in TβR2 KO neurons (36.6 ± 9.2% of cells with axons compared to 31.6 ± 7.2% for TβR2-KO alone; Figure 4-5E). In other words, phosphomimetic Par6 bypasses the requirement for TGF-β signaling and introduction of a negative charge at a single residue in Par6, serine 345, is sufficient to
Figure 4-5. Phosphomimetic Par6-S345E Restores Axons in TβR2 KO Neurons In Vivo

(A) Neuronal progenitors in E15 Tgfbr2<sup>flox/flox</sup> embryos were electroporated with Cre plus Par6-S345A and examined five days later. Bottom panel shows representative traces of neurons in the IZ and CP. Migrating neocortical neurons possessed leading edge processes but lacked trailing edge axons and had impaired migration, phenotypes similar to TβR2 KO neurons (see Figure 2).

(B) A phosphomimetic mutant of Par6 rescues axon specification. Neuronal progenitors E15 Tgfbr2<sup>flox/flox</sup> embryos were electroporated with Cre plus Par6-S345E. The bottom panel shows representative traces of neurons in the IZ and CP. Note that expression of Par6-S345E rescued axon formation (arrows) in TβR2 KO neurons. For (A) and (B), scale bars are 50 μm for top panels and 20 μm for bottom panels. CP, cortical plate; IZ, intermediate zone.

(C) Morphology of migrating TβR2-KO neurons expressing Par6-S345A, showing the absence of an axon (arrows). Scale bar, 22 μm

(D) Morphology of a migrating TβR2-KO neuron expressing Par6-S345E showing the presence of a trailing axon. Scale bar, 22 μm.

(E) Data represent means ± SEM of the percent neurons with an axon. Experiments were averages from at least 3 embryos. Par6-S345A, n = 89; Par6-S345E, n = 84; *p<0.05, Student’s t-test.
Figure 4-5. Phosphomimetic Par6-S345E Restores Axons in TβR2 KO Neurons *In Vivo*
render axons. Together, these results demonstrate that axon formation \textit{in vivo} requires TGF-β signaling via phosphorylation of Par6. Thus, spatially restricted signaling by the extrinsic morphogen TGF-β defines a necessary and sufficient extracellular signal for axon specification \textit{in vivo}.

\textbf{Discussion}

In this study, I have shown that TGF-β-mediated axon specification requires signaling through the polarity protein Par6. Par6 and TβR1 form a stable biochemical complex in developing embryonic brains, Par6 and TβR1 co-localize within filopodial protrusions at the tips of neurites in developing neurons, and upon axon specification, both proteins accumulate into the nascent axon. Moreover, Par6 phosphorylation by TβR2 is critical for axon formation as Par6 mutants that inhibit phosphorylation by TβR2 prevent axon establishment and mutants that mimic phosphorylation are sufficient to rescue axonal defects caused by genetic TβR2 ablation \textit{in vivo}. These results define a long-elusive extrinsic signal for neuronal polarity \textit{in vivo} and links extracellular signaling by TGF-β to the conserved Par3/Par6/PKCζ polarity complex during early stages of neuronal polarization.

\textbf{Axon Specification Through Extracellular Control of the Par3/Par6 Complex}

I have shown that TGF-β signaling for neuronal polarity depends on phosphorylation of Par6 – an evolutionarily conserved multimodular scaffolding protein originally identified as a regulator of asymmetric zygote division in \textit{C. elegans} (Watts et al., 1996). Mass
spectrometric analyses of Par6 interactions have shown that it is a central hub for various signaling pathways including Rho GTPases (Brajenovic et al., 2004). Par6 is a highly modular protein. From its N-terminus to its C-terminus, Par6 interacts with TβR1 through its N-terminus; contains a PB1 domain, which is important for its interaction with PKCζ; a Cdc42-Rac1 interaction-binding (CRIB) motif, which allows Par6 to direct Cdc42/Rac1 activity; and a single PDZ which allows it to interact with other PDZ molecules such as Par3 (Figure 4-6A). On the other hand, in the presence of TGF-β, Par6 phosphorylation by TβR2 recruits the ubiquitin ligase Smurf-1 which in turn promotes the proteasomal degradation of RhoA (Ozdamar et al., 2005). In this manner, the local stoichiometry of Rho GTPases can be modified at the site of TGF-β signaling to alter local actin organization. Notably, local actin reorganization is a well-documented hallmark of axon specification (Bito et al., 2000; Bradke and Dotti, 1999; Schwamborn et al., 2007). Our findings support the notion that TGF-β provides the extrinsic signal in vivo to initiate these conserved signaling pathways for axon differentiation.

A prevalent theory in neuronal polarity is that cellular symmetry is broken through a positive feedback loop that amplifies signaling cascades within the nascent axon (Andersen and Bi, 2000; Turing, 1952). How could TGF-β signaling achieve this? One possibility is that TGF-β signaling amplifies events leading to axon specification by stabilizing the Par3/Par6/PKCζ complex. For example, the RhoA effector Rho-kinase/ROCK/ROK antagonizes Par complex formation by phosphorylating Par3 at threonine 833 leading to dissociation of Par3 from Par6 and PKCζ (Nakayama et al., 2008). At the cellular level, this post-translational modification prevents the
Par3/Par6/PKCζ complex from nucleating in migrating cells, resulting in the loss of front-rear polarity (Nakayama et al., 2008). Engagement of TGFβ receptor signaling coupled with Par6 phosphorylation, local RhoA degradation, and reduced Rho kinase activity could produce the reverse effect leading to positive feedback stabilization of the Par3/Par6/PKCζ complex. Although precise details and a thorough analysis of these signaling pathways in intact tissue awaits future study, such observations imply a model in which TGF-β can facilitate the local accumulation and feedback stabilization of polarity-generating molecules for axon specification.

**Evolutionary Divergence of Neuronal Polarity Programs**
Although polarity proteins involved in neuronal development are highly conserved, their roles in neuronal polarization have diverged. For example, whereas Par3, Par6, and PKCζ regulate polarity in mammalian neurons (Barnes et al., 2008; Shi et al., 2003), *Drosophila* mushroom body neurons lacking or overexpressing Par3, Par6, or aPKC exhibit normal axon and dendritic morphologies (Rolls and Doe, 2004). This has been a confounding problem in the field, one that remains to be fully addressed. The TβR1/Par6 interaction was originally discovered within the epithelial tight junction, a specialized cell-cell junctional complex that demarcates the apical and basolateral domains (Ozdamar et al., 2005). Interestingly, tight junctions are vertebrate-specific structures and
Figure 4-6. The Modular Domains of Par6 and Evolutionary Conservation of Serine/Threonine 345 in Vertebrates.

(A) A schematic representation of the modular structure of Par6. Par6 contains three conserved motifs: a PB1 domain which it uses to bind PKCζ, a CRIB domain to bind GTP-bound forms of Cdc42 and Rac1, and a PDZ domain through which it associates with Par3. In addition, the N-terminus of Par6 has been demonstrated to be important for its binding to TβR1. The TβR2 phosphorylated site, Serine 345, is the penultimate amino acid residue in Par6.

(B) The TβR2 phosphorylation site in Par6 is a vertebrate-specific feature. The sequence of the last eight amino acids for Par6 proteins from C. elegans, Drosophila, Xenopus, Zebrafish, Platypus, Mouse, Rat, and Humans are shown in alignment. The penultimate residue of vertebrate forms of Par6 are conserved as a serine or threonine, as shown in red.
Figure 4-6. The Modular Domains of Par6 and Evolutionary Conservation of Serine/Threonine 345 in Vertebrates.

A

B

<table>
<thead>
<tr>
<th>Species</th>
<th>Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. elegans Par6</td>
<td>N D S D S G E D</td>
</tr>
<tr>
<td>Drosophila Par6</td>
<td>V K D G V L H L</td>
</tr>
<tr>
<td>Xenopus Par6</td>
<td>E D G T V L T L</td>
</tr>
<tr>
<td>Zebrafish Par6</td>
<td>E D G T V I T L</td>
</tr>
<tr>
<td>Platypus Par6</td>
<td>E D G T G L T L</td>
</tr>
<tr>
<td>Mouse Par6C</td>
<td>G D V S G F S L</td>
</tr>
<tr>
<td>Rat Par6C</td>
<td>G D V S G F S L</td>
</tr>
<tr>
<td>Human Par6C</td>
<td>G D G S G F S L</td>
</tr>
</tbody>
</table>
the TβR2 phosphorylation site on Par6 is present as a serine or threonine in all vertebrate isoforms of Par6, but this site is not conserved in invertebrates such as *Drosophila* and *C. elegans* (Bose and Wrana, 2006) (Figure 4-6B). Such divergence suggests that regulation of the Par3/Par6/PKCζ complex acquired divergent mechanisms throughout evolution and neuronal polarity programs in vertebrates became altered as a result. Moreover, in the present study, we have shown that phosphorylation of Par6 at the TβR2 phosphorylation site is downstream of TGF-β dependent axon specification in the mammalian brain. The absence of such Par6 phosphorylation in invertebrates may contribute to differential tissue organization, and possibly contribute to size differences in vertebrate and invertebrate nervous systems.

**Par6 as a Master Organizer of the Polarity Complex**

The current study demonstrates the integral function of Par6 as a scaffolding protein that organizes various events that occur during the early stages of neuronal polarity. Par6 has undergone gene duplication in mammals and there are three mammalian isoforms of Par6 (Par6A, B, C), which all share the same protein interaction domains. Whereas Par6A is primarily localized to tight junctions in epithelial cells, Par6B is localized to the cytosol, and Par6C is found both in the cytosol and tight junctions (Gao and Macara, 2004). Such differences in localization may reflect distinct functions in cellular processes. In addition to TβR1, Par6 is known to function as a molecular hub within the polarity network and physically interacts with a vast number of signaling molecules (Barrios-Rodiles et al., 2005; Bose and Wrana, 2006). These include several molecules well-established to
function in neuronal polarity and include GSK3β (Jiang et al., 2005; Shi et al., 2004; Yoshimura et al., 2005), Adenomatous polyposis coli (Shi et al., 2004), Cdc42 (Schwamborn and Puschel, 2004), PKCζ (Shi et al., 2003), Par3 (Nishimura et al., 2004; Shi et al., 2004; Shi et al., 2003), Rac1 (Schwamborn and Puschel, 2004), and Smurf1 (Schwamborn and Puschel, 2004).

The same luminescence-based mammalian interactome-mapping (LUMIER) screen used to identify Par6 as a binding partner of TβR1 also identified two additional binding partners for TβR1 (Barrios-Rodiles et al., 2005). These include occludin, a member of the tetraspanin family of transmembrane molecules, and a vital structural component of epithelial tight junctions (Furuse et al., 1993); and p-21 activated kinase 1 (PAK1), a member of the PAK family of Rac/Cdc42-activated kinases involved in TGF-β signaling, cell migration, actin reorganization, and neuronal development (Barrios-Rodiles et al., 2005; Bokoch, 2003; Nikolic, 2008; Wilkes et al., 2003). Intriguingly, previous work demonstrated that PAK1 is locally activated in nascent axons (Jacobs et al., 2007), required for axon formation (Jacobs et al., 2007), and the loss of PAK1 activity results in defective neuronal migration and morphogenesis in intact cortical slices (Causeret et al., 2008).

Although the link between TGF-β signaling and PAK family kinases in neuronal development remains to be explored, these observations suggest that TβRI receptors exist at the origin point of multiple signaling pathways that control both neuronal migration and morphogenesis during embryonic brain development. In my experiments, I observed that overexpression of Par6-S345E, which mimics TβR2-dependent phosphorylation of
Par6, could rescue defects in axon generation, but could only partially rescue migrational defects of TβR2 ablation. This suggested that additional TGF-β-dependent pathways, perhaps through a PAK1-dependent mechanism, account for the observed migrational defects.

We propose that by providing a spatially delimited extrinsic signal present at the time of neurogenesis, TGF-β acts to initiate a geometrically constrained program that polarizes newborn neurons. A gradient of TGF-β together with a polarized distribution of TGF-β receptors ensures the differentiation of a trailing process axon, and along with directed migration to the cortical plate, ensures anatomical fidelity conserved across the mammalian forebrain. More broadly, such a mechanism may provide a general paradigm in diverse tissues for extrinsic control of tissue polarity.
Chapter Five. Conclusions and Future Directions

In the preceding chapters, I identified TGF-β as the principal extracellular factor that specifies axons in cortical and hippocampal neurons during embryonic brain development. Disrupting TGF-β signaling through genetic ablation of TβR2 results in cells neurons that specifically lack growth in the axonal pole of the cell while dendritic growth remains highly dynamic. Curiously, the loss of TGF-β signaling in vivo also resulted in profound migrational defects, suggesting that TGF-β controls multiple facets of neuronal development. These results were confirmed in vitro with experiments using dissociated hippocampal neuronal cultures. Moreover, augmentation of TGF-β signaling through the overexpression of TβR2 was sufficient to induce supernumerary axon formation, and the localized delivery of extracellular TGF-β ligand was also sufficient to directionally orient axon growth and differentiation. The transduction of TGF-β signaling required the phosphorylation of the polarity protein Par6 by TβR2 at its penultimate Ser345 residue. Indeed, mutation of the phosphorylation site in Par6 prevented neuronal polarization whereas a phosphomimetic mutant of Par6 was sufficient to rescue the phenotype of TβR2 loss in developing cortical slices.

The Source of TGF-β

In situ hybridization studies have shown that TβR2 ligands are highly spatially localized with TβR2 being the principal ligand present within the VZ and SVZ of lateral ventricles, which is the site of axon determination during pyramidal neuron development. However,
the source of TGF-β within these zones remains unknown. TGF-β is produced by various cell types within an organism and within the developing neocortex, and immunohistochemical studies have confirmed that TGF-β is produced by both neurons and radial glia (Unsicker et al., 1996). Confocal microscopy studies in epithelial cells have demonstrated that TGF-β containing vesicles are localized to the apical domain of cells (Murphy et al., 2004), suggesting that TGF-β ligands are selectively secreted from the apical domain of epithelial cells. Interestingly, radial glial cell organization is similar to classical epithelial cells. Radial glial cells are attached to the cortex such that their apical domains are oriented at the VZ and their basolateral endfeet are oriented at the CP. Thus, based on evidence from epithelial cells, it is tempting to speculate that high TGF-β concentrations at the VZ is achieved through secretion at apical domains of radial glial cells, which would result in high secretion levels at the VZ.

The localization of TGF-β1 and -2 is striking in neurons. In dissociated hippocampal neurons stained with a TGF-β antibody that recognizes both TGF-β1 and TGF-β2, I found labeling at filopodial protrusions throughout the cells, with selective localization within axons in polarized neurons (Figure 5-1A). Thus, this raises the possibility that TGF-β-dependent axon specification occurs through an autocrine mechanism, at least in vitro. I measured the quantity of TGF-β produced by neurons grown in serum-free media using an ELISA assay for both TGF-β1 and TGF-β2 (Figure 5-1B). After plating in standard plating media containing 5% fetal bovine serum (FBS), cells were cultured for 24 hours then switched to serum-free Neurobasal Media containing B27 supplement and Glutamax. Media was then collected over the next 5-7
days. Intriguingly, I could not detect any TGF-β in the media, even when cells were seeded at very high densities (Figure 5-1B). As controls, I used media collected from HEK-293T cells and neuronal culture media supplemented with FBS, and was able to detect the presence of TGF-β in all of these preparations, demonstrating the sensitivity of my ELISA assay (Figure 5-1B).

In addition to the above-mentioned experiment, the use of a TGF-β antibody, which binds extracellular forms of TGF-β1, 2, and 3 had no effect on axon formation in dissociated cultures. This was in stark contrast to the effects of the TβR1 kinase inhibitor, SB-431542, and suggested that perhaps antibody treatment is not sufficient to block autocrine signaling mechanisms. I took this line of thinking one step further. Using standard culturing conditions, I serially diluted cells such that only five cells were plated on each 18 mm coverslip in serum-free plating media. The cells were cultured in standard conditions for five days and carefully screened on a low-power phase contrast microscope to identify coverslips with a single plated neuron. I reasoned that because TGF-β is required for axon specification, if cells could polarize in isolated conditions, TGF-β signaling must function through an autocrine mechanism, at least in vitro. In these cultures, I found that cells that survived as single cells on coverslips did not exhibit any overt morphological polarity (Figure 5-2A). In other words, these cells did not possess a single long process that could be distinguished as an axon upon initial observation. However, and quite surprisingly, immunofluorescence for tau-1 and Tuj1 revealed that all of these cells possessed a single neurite that was positive for tau-1 (Figure 5-2A).
Figure 5-1. Neurons Produce TGF-β1 and TGF-β2, but Do Not Secret Ligands at Detectable Levels

(A) Hippocampal neurons stained with an antibody that recognizes both TGF-β1 and TGF-β2 isoforms. Staining was seen in filopodia at tips of neurites in both stage 2 (top panels) and stage 3 (bottom panels) neurons. In stage 3 neurons, immunoreactivity was enriched in the axon. Scale bar represents 10 μm for the stage 2 neuron and 20 μm for the stage 3 neuron. Insets showed magnified images demarcated by white boxes. Scale bar represents 5 μm.

(B) ELISA performed to detect TGF-β1 and TGF-β1 secretion by neurons. Hippocampal neurons were cultured in the absence of serum and culture media collected at the indicated times. Neither ligand was detected. However, ligands were detected when exogenously added to media, in the presence of serum, or in media from HEK 293T cells. Numbers represent means ± SEM, and reflect averages from two independent experiments with three samples per experiment.
Figure 5-1. Neurons Produce TGF-β1 and TGF-β2, but Do Not Secret Ligands at Detectable Levels

<table>
<thead>
<tr>
<th></th>
<th>TGF-β1 Concentration (pg/ml)</th>
<th>TGF-β2 Concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIV1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DIV2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DIV3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DIV5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DIV7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DIV2*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DIV3*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DIV5*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Plating Media (N27)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N27 + 400pg TGF-β1</td>
<td>487.89 + 15.9</td>
<td>387.353 + 6.8</td>
</tr>
<tr>
<td>N27 + 5% FBS</td>
<td>683.48 + 10.8</td>
<td>N/A</td>
</tr>
<tr>
<td>HEK 293T Media</td>
<td>2643.16 + 84.2</td>
<td>896.45 + 45.0</td>
</tr>
<tr>
<td>N27 + 20% HEK 293T Media</td>
<td>497.18 + 1.8</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Thus, this suggests that although axonal outgrowth is affected in these cells, their ability to polarize remains intact, suggesting that TGF-β may act through an autocrine mechanism within dissociated neurons. Although such observations regarding the intrinsic nature of neuronal polarity are intriguing, they most likely highlight the differences between *in vitro* and *in vivo* neuronal development. Whereas the plane of axon specification in neurons developing *in vivo* is uniform toward the VZ, axon specification in cultures is random in orientation. Long-term imaging experiments have demonstrated that developing neurons *in vivo* are born without any overt morphological polarity and initially resemble stage 2 neurons in culture with several minor neurites of equivalent length. However, virtually all neurons generated near the lateral ventricles specify their axons toward the VZ, suggesting that this uniform vector of axon specification likely results from an existing gradient of TGF-β prior to neurogenesis. Together, these observations suggest that whereas neurons possess an intrinsic ability to polarize, axon specification *in vivo* is likely influenced by the secretion and organization of TGF-β by structures that exist in the brain prior to neurogenesis. I suspect that future work focusing on the role of radial glia in the maintenance of the TβR2 gradient within peri-ventricular zones should produce fruitful insight into the regulation of axon specification.
Figure 5-2. Neuronal Polarization in Single Cell Cultures, and Surface Expression of TβR3.

(A) A hippocampal neuron cultured as a single unit in the absence of serum. Cell was cultured for 5 days and fixed and stained for Tuj1 and the axonal marker Tau-1. Although the cell did not exhibit any overt morphological polarity, it retained its ability to segregate tau into a single neurite (arrow). Scale bar, 20 μm.

(B) TGF-β1 is expressed at the surface of neurons. A hippocampal neuron simultaneously surface stained for TβR3 and Tuj1. Receptor labeling was prominent throughout the cell and at the tips of neurites. Scale bar represents 3 μm.
Figure 5-2. Neuronal Polarization in Single Cell Cultures, and Surface Expression of TβR3.
The Mechanism of Axon Growth and Maintenance

Experiments regarding axon growth in dissociated hippocampal neurons have focused on the distal growth cone. For example, the accumulation of Par3/Par6 (Shi et al., 2003), kinesins (Jacobson et al., 2006), and the inactivation of GSK-3β (Jiang et al., 2005) have all been characterized at the tips of neurites and at the tip of the nascent axon upon polarization. However, axon growth appears to occur by a different mechanism in migrating neurons during embryonic brain development. As cells migrate toward the CP, axonal membrane is secreted at the proximal axon segment, rather than at the distal growth cone. This suggests that in contrast to dissociated neurons, axon growth in vivo occurs within the proximal axon. Interestingly, the proximal axon segment, also known as the axon hillock, is a well-characterized cellular structure, most frequently identified by a patch of Ankyrin G protein (Jenkins and Bennett, 2001). In mature neurons, the axon hillock is enriched with voltage-gated sodium channels and serves as an integration center for signal inputs to inform the neuron whether to depolarize (Wollner and Catterall, 1986), and in younger neurons, it has been demonstrated to serve as a diffusional barrier for membrane proteins (Winckler et al., 1999).

In different formulation, the general structure of a polarized neuron can be viewed to be similar in layout as an epithelial cell. Like epithelial cells, neurons have an analogous apical domain (axon), a basolateral domain (somatodendritic compartment), and a tight junction-like diffusional barrier that separates the two compartments (axon hillock). Interestingly, proteins that have been demonstrated to function in neuronal polarity are often found within tight junctions in epithelial cells. These include, TβR1
(Ozdamar et al., 2005), Par3 (Izumi et al., 1998), Par6 (Suzuki et al., 2001), PKCζ (Izumi et al., 1998), and LKB1 (Baas et al., 2004). Therefore, do polarity proteins that function in axon specification function to establish the proximal axon segment? Future experiments addressing the contribution of the axon hillock to neuronal polarity, and to what extent known regulator of neuronal polarity affect hillock formation will likely yield useful information. Moreover, how membrane addition occurs at the proximal axon segment and how membrane is trafficked and targeted to that domain in migrating neurons will also yield interesting results.

**Negative Regulation of Axon Growth**

Most of the research effort in the field has been focused on the molecular events that positively regulate axon specification, but there is little known about events that negatively regulate axon formation. Throughout my studies, I have observed that the formation of cells with multiple axons is very rare, suggesting the presence of strong negative regulators of axon specification after one neurite is selected as the axon. How can a cell accomplish this? The answer in part comes from my observations that polarity-initiating components such as TGF-β receptors, are selectively polarized into nascent axons. However, the mechanisms by which TGF-β receptors are targeted to axons remain unknown.

Work in this and other labs have demonstrated that neurons possess a highly complex network of secretory compartments to ensure proper targeting of transmembrane molecules to correct destinations. For example, during long-term potentiation (LTP), a
well-known cellular model of learning and memory, AMPA receptors are supplied to activated synapses by the actin-binding motor myosin Vb in a calcium-dependent manner (Wang et al., 2008). Moreover, AMPA receptor content is maintained within synapses by local capture and recycling of receptors by endocytic zones, which are positioned laterally to dendritic spines (Lu et al., 2007; Park et al., 2004). Do such mechanisms exist in early axons? A recent genome-wide RNA-mediated interference screen in *C. elegans* identified various molecules that regulate endocytosis, among which were the anterior polarity proteins Par3, Par6, aPKC, and Cdc42, but not the posterior polarity proteins Par1 or Par2 (Balklava et al., 2007). Using antibody-feeding assays, the authors found that Par proteins regulate different modes of membrane trafficking in HeLa cells expressing a truncated form of Par6 missing the first 101 amino acids. Whereas the uptake of MHCI, a molecule that undergoes clathrin-independent endocytosis was unaffected, its recycling was reduced two to three fold. In contrast, the recycling of transferrin receptor, a molecule that undergoes clathrin-dependent endocytosis, was not affected but its uptake was inhibited by 30% (Balklava et al., 2007). Interestingly, TGF-β receptors are constitutively endocytosed and can undergo recycling and degradation dependent on clathrin-dependent and independent endocytosis, respectively (Di Guglielmo et al., 2003). Thus, it is tempting to speculate that Par6 can increase the surface dwelling time of TβR1 during clathrin-dependent events, and facilitate increased receptor recycling during clathrin-independent endocytosis.

In addition to intracellular mechanisms, axon specification also may be mediated by soluble and insoluble classes of TGF-β signaling regulators. Of these, the
proteoglycan betaglycan, also known as TβR3 (Lopez-Casillas et al., 1991; Wang et al., 1991), may be an important regulator. TβR3 is known to mediate TGF-β binding to the type II receptor, a role that is particularly critical for TGF-β2 (Cheifetz and Massague, 1991; Lopez-Casillas et al., 1993; Sankar et al., 1995). TβR3 is expressed at the surface of developing neurons where it also forms clusters at neurite tips, but whether it polarizes into the axon like TβR1 and TβR2 awaits further investigation. It also includes the small proteoglycan decorin (Yamaguchi et al., 1990) and the circulating protein α2-macroblogulin (O'Connor-McCourt and Wakefield, 1987), which both bind to free TGF-β. Although much work remains to elucidate such mechanisms, in situ hybridization studies in P1 mice have suggested that decorin is expressed as a gradient with highest expression within young neurons and low expression in older neurons and at the VZ (Kappler et al., 1998).

**Neurodevelopmental Disorders Arising from Defects in TGF-β Signaling**

Despite early insights suggesting a role for TGF-β signaling in neurodevelopment, the biology of TGF-β signaling in CNS development has remained a largely unexplored area of study (Ishihara et al., 1994; Tomoda et al., 1996). However, recent discoveries that mutations in TGF-β signaling components give rise to a wide spectrum of human neurodevelopmental disorders has further suggested the importance of TGF-β signaling in CNS development and function (Gripp et al., 2000; Loeys et al., 2005; van Steensel et al., 2008; Verschueren et al., 1999). Axon specification is an enduring neuronal change, one that suggests long-term changes in the cellular transcriptional program. An
intriguing aspect of TGF-β signaling in axon specification is that TGF-β receptors are directly coupled to Smad transcription factors, which sets up a direct signaling circuit between events at the cell membrane and transcriptional changes in the nucleus. Indeed, previous studies in cerebellar granule neurons have demonstrated a link between TGF-β-specific Smad2 activation and axon morphogenesis (Stegmuller et al., 2008).

Neurodevelopmental disorders in humans arising from dysfunctional Smad2 or Smad3 have yet to be reported, likely representing the integral role of these proteins in very basic events of neurodevelopment. However, proteins involved in transcriptional regulation of Smad proteins have been reported to cause mental retardation in humans. These include TALE homeobox protein TG-interacting factor (TGIF), a repressor of Smad2-dependent transcription (Wotton et al., 1999) that is mutated in individuals with holoprosencephaly (Gripp et al., 2000). It also includes, Smad-interacting protein 1 (SIP1), another repressor of Smad2 (Verschueren et al., 1999) implicated in Hirschsprung disease, a congenital disease characterized by mental retardation, delayed motor development, epilepsy, and microcephaly (Wakamatsu et al., 2001). Although the mechanisms remain to be investigated, these observations raise the possibility that mutations to transcriptional responses of TGF-β signaling alter CNS development, leading to profound defects in CNS maturation and function. It remains to be seen if such detrimental defects arise solely from defects in axon formation, or if other developmental processes, such as dendrite growth and synaptogenesis are affected. In addition, future experiments that address genetic targets of Smad2 signaling in neurons, and the biological context in which Smad2 is selectively activated for transcription
remain to be seen.

There are additional links between TGF-β signaling in neurodevelopmental disorders. As stated in the previous chapter, occludin, Par6, and PAK1 kinase (Barrios-Rodiles et al., 2005) were identified from a screen in epithelial cells to physically associate with TβR1. These include. Of these, PAK1 is known to play a role in cortical neuron migration (Causeret et al., 2008) and axon specification (Jacobs et al., 2007). PAK1 belongs to a family of Cdc42/Rac1 activated kinases of which PAK3 is implicated in X-linked mental retardation in humans (Allen et al., 1998). PAK1 and PAK3 share ~80% homology, and experiments in hippocampal slice cultures have revealed that PAK1 can rescue dendritic spine morphology defects induced by the loss of PAK3 activity (Boda et al., 2008).

**TGF-β in Synaptogenesis**

After the establishment of polarity, components of the axon specification machinery can be found throughout the cell during later phases of development. For example, during active periods of synaptogenesis, both Par3 and Par6 localize to dendritic spines and influence spine morphogenesis (Zhang and Macara, 2006, 2008). Indeed, whereas shRNA-mediated knockdown of Par6 leads to a ~80% reduction in spine density in cultured hippocampal neurons, overexpression of full length Par6 nearly doubles spine density as compared to controls (Zhang and Macara, 2008). Intriguingly, the effect of Par6 overexpression on spine density was dependent on an intact N-terminal domain, which contains TβR1 interacting domain (Zhang and Macara, 2008). Thus, this raises the
question as to whether TGF-β receptors, and in turn TGF-β signaling is also found in dendritic spines.

As a preliminary investigation into whether TGF-β receptors localize to sites of synaptic contacts, I constructed a TβR1-fusion protein in which GFP was inserted in frame directly after the signal sequence (GFP:TβR1). Thus, this chimeric molecule could progress through the secretory pathway without disturbing any interactions within its intracellular domain. When expressed in DIV 25 cells, GFP:TβR1 displays a strikingly synaptic localization. Receptors were seen at the tips of dendritic spines and intriguingly, receptors were found within endosomal structures at the base of spines and within dendritic shafts, suggesting that local recycling of TβR1 may occur within dendritic spines (Lu et al., 2007; Park et al., 2004; Wang et al., 2008) (Figure 5-3A). To confirm this observation, I performed surface immunofluorescence for TβR1 and found endogenous TβR1 within spines, suggesting that TGF-β signaling may play a role in synaptogenesis or synaptic function (Figure 5-3B). In addition to TβR1, I performed double immunofluorescence for TβR1/2 ligand in mature cells along with the excitatory synapse marker VGLUT1, which marks presynaptic boutons of excitatory synapses within neurons. Intriguingly, pools of TGF-β ligands
Figure 5-3. Synaptic Localization of TGF-β Receptors and Ligands in Mature Neurons.

(A) DIV 21 culture hippocampal neurons were co-transfected with a GFP:TβR1 fusion construct and an mCherry cell fill and fixed after 48 hours. Receptors were distributed as punctate clusters throughout dendrites. *Insets:* Upon closer examination, receptors were found in dendritic spines (arrows) and within endosomal structures at the base of spines (arrowheads). Scale bars represent 20 μm, and 5 μm for insets, respectively.

(B) Endogenous TβR1 surface labeling in DIV 30 hippocampal neurons. Surface receptor immunoreactivity was seen in punctate clusters off the shaft of the dendrite. These puncta resembled labeling at dendritic spines. Scale bar, 20 μm.

(C) Staining for TGF-β1 and 2 ligand in DIV 18 neurons reveals the presence of TGF-β pools in excitatory synapses. Both synapses without spines (C1) and synapses at spines (C2) possessed pools that directly apposed VGLUT1 puncta. Scale bar represents 20 μm. Scale bar for C1 represents 4 μm and 6 μm for C2.
Figure 5-3. Synaptic Localization of TGF-β Receptors and Ligands in Mature Neurons.
existed immediately apposed to VGLUT puncta, indicating that TβR1 ligand secretion could occur at sites of synaptic contacts (Figure 5-3C). What is the function of TGF-β signaling at dendritic spines? Dendritic spines are actin-rich structures, and thus, I tested whether TGF-β signaling could impact spine morphology. To this end, I performed live cell imaging experiments on DIV 21 neurons transfected with GFP to visualize a cell fill. Spines at this stage of development were highly dynamic and motile in their basal state protrusions (Figure 5-4A). Upon TGF-β stimulation, spines became even more highly dynamic and lost their smooth mushroom shape, often producing multiple long protrusions from the head of the spine (Figure 5-4B). Although these experiments are preliminary, such rapid changes in morphology suggest that TGF-β retains the ability to reorganize the actin cytoskeleton and subsequently, impact morphology in dendritic spines. In addition to short-term morphological changes in synapse development, TGF-β may induce long-term changes at synapses to influence synaptic plasticity and transmission. Early studies in *Aplysia* have demonstrated that the application of TGF-β to *Aplysia* sensorimotor synapses is sufficient to induce long-term facilitation (Zhang et al., 1997), an effect that is independent of serotonin (Zhang et al., 1997), but dependent on the MAP kinase cascade (Chin et al., 2006). Moreover, TGF-β-dependent facilitation occurs long term (24 – 48 hours) and not short term (5 – 15 minutes) in these neurons (Zhang et al., 1997). In pyramidal neurons in the mouse hippocampus, kainic acid treatment in intact hippocampal slices leads to increased Smad2 phosphorylation and reporter gene activation, which peaks 24 hours after kainic acid treatment, further suggesting
Figure 5-4. TGF-β Treatment of Neurons Perturbs Dendritic Spine Morphology.

(A) Live cell imaging was performed on a DIV 21 neuron with images collected every 20 seconds. Under basal conditions, spines were highly dynamic but maintained their morphological integrity.

(B) The same spine imaged after the addition of 20 ng/ml TGF-β1, 2, and 3. Spines underwent dramatic morphological changes, forming multiple protrusions over the duration of the imaging session. Scale bar, 4 μm.
Figure 5-4. TGF-β Treatment of Neurons Perturbs Dendritic Spine Morphology.

A

B + TGF-β1, 2, 3
role for TGF-β in later phases of neuronal potentiation (Luo et al., 2006). Thus, TGF-β likely influences synapse function by short-term changes to the actin cytoskeleton and by long-term changes, which are likely to be mediated by transcriptional responses. It will be interesting to see if the ability of TGF-β to reorganize the actin cytoskeleton has immediate effects on early synapse potentiating events such as myosin Vb-dependent cargo delivery to synapses (Wang et al., 2008), as well as long-term changes to cell content.

**TGF-β in Neurodegeneration**

An intriguing observation regarding TGF-β mouse models is the recent discovery that TGF-β signaling mediates neurodegeneration. Although mice completely lacking TGF-β1 die during embryonic development, certain mouse strains have been demonstrate to tolerate, at least to some extent, the loss of TGF-β1 innervation during development (Brionne et al., 2003). Interestingly, Tgfb1−/− mice are born below expected Mendelian frequencies, and typically live a few days to weeks and subsequently succumb to a wasting syndrome. Interestingly, postnatal day 1 (P1) animals have a compact and thinner neocortex with aberrant lamination, and further analysis by dUTP nick end labeling (TUNEL) and caspase-3 staining has revealed increased cell death in homozygous null animals (Brionne et al., 2003). Consistent with the notion of increased neuronal susceptibility, 5 to 6 month old Tgfb1+/− animals injected with kainic acid display a 17-fold increase in activated caspase-positive cells compared to wild-type controls, suggesting that TGF-β1 acts as a neuroprotective factor (Brionne et al., 2003).
Additional lines of evidence have pointed to TGF-β1 in alleviating neurodegeneration. Alzheimer’s Disease (AD) is a progressive neurodegenerative disorder characterized pathologically by the accumulation of Aβ peptide in amyloid peptide plaques within the brain. Transgenic mouse models simultaneously expressing human APP (hAPP), which is the precursor protein for Aβ, and TGF-β1 display a three-fold reduction in the number of amyloid plaque deposits and a 50% reduction in the overall Aβ load within the cortex and the hippocampus, as compared to hAPP mice (Wyss-Coray et al., 2001). In line with these observations, TGF-β1 transgenic mice also possessed fewer dystrophic neurites as determined by hyperphosphorylated forms of tau and neuritic morphology (Wyss-Coray et al., 2001). Much more work is needed to understand the various effects of TGF-β signaling in neurodegeneration. In particular, it will be important to understand the cell types involved in these various paradigms of neuroprotection mediated by TGF-β1.


Boyer, B., and Thiery, J.P. (1993). Epithelium-mesenchyme interconversion as example
of epithelial plasticity. APMIS 101, 257-268.


Chen, C., Ware, S.M., Sato, A., Houston-Hawkins, D.E., Habas, R., Matzuk, M.M., Shen,


outgrowth and neuronal migration. Dev Biol.


Hayashi, H., Abdollah, S., Qiu, Y., Cai, J., Xu, Y.Y., Grinnell, B.W., Richardson, M.A.,
Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta

Heine, U., Munoz, E.F., Flanders, K.C., Ellingsworth, L.R., Lam, H.Y., Thompson, N.L.,

Hemminki, A., Markie, D., Tomlinson, I., Avizienyte, E., Roth, S., Loukola, A., Bignell,

transforming growth factor-beta receptors form homo-oligomers. J Cell Biol 126, 139-
154.


Hirokawa, N. (1982). Cross-linker system between neurofilaments, microtubules, and
membranous organelles in frog axons revealed by the quick-freeze, deep-etching method.

organelle transport. Science 279, 519-526.

proteins, KIFs: structure, function, and dynamics. Physiol Rev 88, 1089-1118.

Hirokawa, N., Pfister, K.K., Yorifuji, H., Wagner, M.C., Brady, S.T., and Bloom, G.S.
(1989). Submolecular domains of bovine brain kinesin identified by electron microscopy
and monoclonal antibody decoration. Cell 56, 867-878.

Hirose, M., Ishizaki, T., Watanabe, N., Uehata, M., Kranenburg, O., Moolenaar, W.H.,
the Rho-associated protein kinase (p160ROCK)-regulated neurite remodeling in

179


signalling through PI(3)K and PKB. Nature 385, 544-548.


Lebrun, J.J., and Vale, W.W. (1997). Activin and inhibin have antagonistic effects on ligand-dependent heteromerization of the type I and type II activin receptors and human
erythroid differentiation. Mol Cell Biol 17, 1682-1691.


Paschal, B.M., Shpetner, H.S., and Vallee, R.B. (1987). MAP 1C is a microtubule-activated ATPase which translocates microtubules in vitro and has dynein-like properties.
J Cell Biol 105, 1273-1282.


receptor for bone morphogenetic proteins. Proc Natl Acad Sci U S A 92, 7632-7636.


cellular responses. EMBO J 15, 6231-6240.


Trelstad, R.L., Hayashi, A., Hayashi, K., and Donahoe, P.K. (1982). The epithelial-


endosomes and AMPA receptors for postsynaptic plasticity. Cell 135, 535-548.


201


rescues the cleft palate phenotype of Tgfb3 null mutants. Dev Biol 312, 384-395.


Mol Cell 1, 611-617.


Zumbrunn, J., Kinoshita, K., Hyman, A.A., and Nathke, I.S. (2001). Binding of the adenomatous polyposis coli protein to microtubules increases microtubule stability and is
Biography

Jason J. Yi

Born: September 16, 1978 in Seoul, South Korea

Education

Ph.D. Pharmacology, Duke University, 03/09
B.S. Biochemistry and Molecular Biology, Dickinson College, Carlisle, PA

Research Experience

Graduate Student, Duke University 08/03 – 03/09
Research Associate, Cellular Genomics Inc., Branford, CT, 06/01 – 07/03
Research Assistant, Dickinson College, 12/00 – 5/01
Research Assistant, Fox Chase Cancer Center, Philadelphia, PA, 06/00 – 08/00

Publications

Jason J Yi, A Paul Barnes, Franck Polleux, and Michael D Ehlers (2009). TGF-β Signaling Specifies Axons During Brain Development. (Submitted)


