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The Functions of Atypical PKC and the PAR Complex in Axon Growth Inhibition

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Abstract of the Dissertation

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The failure of axon regeneration at injury sites in the central nervous system is due, at least in part, to the formation of a growth-inhibitory environment. The glial scar formed at injury sites contains numerous growth-inhibitory molecules whose intracellular signaling mechanisms remain largely unknown.

Here, I provide evidence that the polarity machinery, consisting of atypical PKC, PAR6 and PAR3, mediates axon growth inhibition induced by NG2, a major chondroitin sulfate proteoglycan in the glial scar.

NG2 activates aPKC. Inhibition of aPKC reduces the inhibitory action of NG2. Phosphomimetic forms of aPKC are sufficient for axon growth inhibition.

NG2 activates Cdc42. This activation is required for aPKC activation and growth inhibition. NG2 increases the association of aPKC with PAR6 and this interaction is also required for NG2-induced inhibition.

The aPKC-binding region of PAR3 contains an aPKC phosphorylation site that is critical for the regulation and function of the PAR complex. NG2 decreases the association of aPKC with PAR3. Blocking aPKC activity prevents this decrease and a non-phosphorylatable mutant form of PAR3 reverses NG2-mediated axon growth inhibition.

NG2 also induces the dislocation of PAR3 to the cell body, suggesting dysfunctional regulation of PAR3.

Rac1 signaling is a downstream event regulated by PAR3. NG2 activates Rac1 in an aPKC- and PAR3-dependent manner. A dominant negative form of Rac1 reverses axon growth inhibition whereas a constitutively active form of Rac1 attenuates axon outgrowth. These together suggest that hyperactivity of Rac1 is involved in the inhibitory effect of NG2.

Ceramide activates aPKC. I therefore evaluated the role of ceramide in axon growth inhibition. Ceramide inhibits axon growth in aPKC-dependent manner. Blocking ceramide signaling reduces the inhibitory effect of NG2. These together suggest that ceramide signaling may be upstream of the PAR complex in a pathway that mediates axon growth inhibition.

In summary, my research characterized the intracellular signaling mechanisms of axon growth inhibition by NG2. I speculate that NG2 triggers ceramide signaling to induce hyperactivity and dislocation of Rac1 through the PAR complex. This dysfunctional regulation of Rac1 may cause axon growth inhibition. These results define a new therapeutic target for axon regeneration after CNS injury.

For Christ and His Kingdom

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List of Abbreviations

aPKC	atypcial PKC
BDNF	Brain-derived neurotrophic factor
CAG	cytomegalovirus (CMV) early enhancer element and chicken beta-actin promoter
CNS	the central nervous system
CRIB	Cdc42/Rac1-interactive binding
CSPG	Chondroitin sulfate proteoglycan
DAG	diacylglycerol
DH domain	DBL homology domain
DMEM	Dulbecco's Modified Eagle Medium
Dvl	Dishevelled
ECM	extracellular matrix
EMT	epithelial mesenchyme transition
F-actin	filamentous actin
FZ	Frizzled
GAG	Glycosaminoglycan
GAP	GTPase-activating protein
GDI	guanine nucleotide dissociation inhibitor
GEF	GTP exchange factor
LIMK	LIM kinase
MAG	myelin associated glycoprotein
NgR	Nogo-66 receptor

OMgp	Oligodendrocytes myelin glycoprotein
OPC	oligodendrocytes precursor cell
OPCA	OR/PC/AID
P domain	the peripheral domain
PB1	Phox and Bem1
PH	Pleckstrin homology domain
PI3K	phosphoinositide 3-kinases
PIP2	phosphatidylinositol (4,5)-bisphosphate
PIP3	phosphatidylinositol (3,4,5)-triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PS	pseudosubstrate
ROCK	Rho-associated protein kinase
RPTPσ	receptor protein tyrosine phosphataseσ
sema4D	semaphorin 4D
Smurf1	Smad ubiquitin regulatory factor 1
T domain	the transitional domain
TGFβ	transforming growth factor β
PAR complex	polarity complex
TNFR	tumor necrosis factor receptor

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Chapter 1 General Introduction

Axon and dendrite structure is fundamental for neuronal function. Axons are in general long and thin processes primarily composed of microtubules and actin-enriched tips. Dendrites are short and wide with branched processes that are uniformly filled with actin. The asymmetric distribution of microtubules and actin within these structures is established by several intracellular signaling mechanisms such as the polarity (PAR) complex and small GTPase signaling.

The majority of dynamic cytoskeletal regulation occurring within developing neurons occurs at the distal tips of axons. This regulation is critical for axonal development. Consistent with this idea, the morphology of axonal tips continues to change during development and local increases in cytoskeleton dynamics enables immature neurites to develop into axons (Bradke and Dotti, 1999). The polarity complex proteins (atypical PKC, PAR6 and PAR3) and some small GTPases are accumulated at axonal tips where they regulate the dynamics of the cytoskeleton (Nishimura et al., 2004; Nishimura et al., 2005; Schwamborn and Puschel, 2004; Shi et al., 2003). There is wide agreement that microtubules function as a major force to push axonal tips whereas actins function to control the motility and direction of microtubules (Bradke and Dotti, 1999, 2000; Lowery and Van Vactor, 2009; Schaefer et al., 2008). In this regard, the polarity machinery is thought to regulate the stability and interaction of these cytoskeletal proteins at the tips of axons for proper axonal development.

How does the PAR complex reorganize the actin-microtubule structure? The PAR complex is closely linked to the Rho family of small GTPases, such as Cdc42, Rac1 and RhoA (Nakayama et al., 2008; Nishimura et al., 2005). These small GTPases function as molecular switches to turn on and off the signaling pathways that regulate actin and microtubule polymerization (Etienne-Manneville and Hall, 2002). Cdc42 and Rac1 activation mainly triggers actin polymerization and branching whereas RhoA activation negatively acts on actin and microtubule polymerization. Microtubule elongation is a major force for axon elongation and actin filaments serve to guide and direct microtubule protrusion into growth cones (Schaefer et al., 2008). Given the cooperative role of microtubules and actin in axon elongation, the GTPases

should also be coordinately regulated for proper axonogenesis. In this regard, the PAR complex mediates the crosstalk among the GTPases to coordinate actin and microtubule dynamics.

The intracellular actions described above are major components in establishing neuronal polarity while several extrinsic cues are additionally required for proper polarization of neurons in the developing brain. This raises the question of whether or not extrinsic cues are linked to the polarity complex. Recently, it has been reported that some stimulatory cues, such as BDNF, TGFβ and WNT, indeed regulate the PAR complex to promote axonal development (Cheng et al., 2011; Yi et al., 2010; Zhang et al., 2007). This work led me to a question; do inhibitory cues regulate the polarity machinery to mediate their inhibitory action on axonal outgrowth?

This question has not only intellectual significance but also clinical implication for successful regeneration of damaged axons. Many inhibitory molecules that are accumulated at the injury site of the central nervous system (CNS) disturb successful regeneration of damaged axons (Yiu and He, 2006). Compared to our knowledge about their pathological role in CNS injury and inhibitory effect on axonal outgrowth, it is largely unknown how exactly these molecules inhibit axonal regrowth. Understanding the intracellular mechanisms of axon growth inhibition is therefore anticipated to provide us with therapeutic targets to improve related pathological conditions of CNS injury.

Axon growth inhibition may be regarded as the delay or reverse process of axonal outgrowth, presumably due to dysfunctional regulation of actin and microtubule dynamics. Consistent with this hypothesis, several inhibitory molecules alter the activity of Rho GTPases (Jin and Strittmatter, 1997; Niederost et al., 2002; Sivasankaran et al., 2004). Furthermore, either genetic or pharmacological manipulation to nullify these alterations attenuates or reverses the inhibition (Jin and Strittmatter, 1997; Monnier et al., 2003). Given the role of the PAR complex in regulating Rho family GTPases, inhibitory cues may thus induce such alteration through the PAR complex to attenuate axon extension.

In my dissertation research, I therefore explored the role of the polarity complex, consisting of atypical PKC, PAR6 and PAR3 in axon growth inhibition induced by NG2, a major inhibitory proteoglycan in the glial scar. In addition, I evaluated the role of ceramide signaling and how it is linked to the polarity proteins in the growth inhibition.

1.1 The functions of the PAR complex in neuronal polarity

In general, differentiated cells, such as epithelia cells, migrating cells and neurons are highly polarized with distinct morphological and functional asymmetry. The establishment of cell polarity is coordinated by a group of the polarity proteins (PAR6, PAR3 and atypical PKC) and Rho family GTPases (Nishimura et al., 2005; Schwamborn and Puschel, 2004; Shi et al., 2003). Since a major role of the PAR complex is to regulate actin and microtubule network through Rho family of GTPases, it is not surprising that the complex is linked to shaping the morphology of cells and the localization of cellular components. In this section, I will discuss each component of the PAR complex, Rho family GTPases and their roles and interactions.

Atypical PKC (aPKC); aPKC is an isoform of the protein kinase C (PKC) family of kinases. The PKC family is categorized into three major classes with different cofactor requirements: conventional (α, β, and γ), novel (δ, ε, η, and θ), and atypical (ζ and λ/ι) (Tanaka and Nishizuka, 1994). The activation of conventional PKCs requires diacylglycerol (DAG) and Ca^{++} that interact with C1 and C2 regulatory region of the kinases, respectively. Novel PKCs, which lack C2 region, do not require Ca^{++} but exhibit their enzymatic activities in the presence of DAG (Ono et al., 1988). DAG or Ca^{++} is independent of the activation of aPKCs, which are deficient in C2 region and contain only a partial C1 region (Ono et al., 1989). Therefore, the activation of aPKC is determined by protein-protein interactions.

aPKC consists of four functional domains and motifs, including a Phox and Bem1 (PB1) domain and a pseudosubstrate (PS) sequence in the N-terminus, and a kinase domain and an ATP-binding site in the C-terminus (Figure 1.1). The PB1 domain recognizes OPR/PC/AID (OPCA) motifs of other proteins, such as PAR-6, ZIP/p62 and MEK5 (Diaz-Meco and Moscat, 2001; Gao et al., 2002; Sanz et al., 1999), allowing aPKC to interact with these adapter proteins. This interaction plays a role in directing the kinase to the signaling events where the adapter proteins are involved, such as cell polarity, inflammation and MAPK kinase pathway signaling.

The PS sequence is homologous to the consensus sequence for aPKC phosphorylation except that the phospho-group acceptor, serine or threonine residue is replaced with a non-phosphorylatable residue, alanine. It presumably stays plugged into the substrate-binding cavity

of the kinase to maintain the enzyme inactive. The kinase domain of atypical PKC has an ATP-binding region. It includes a lysine residue, Lys^{281}, which is essential for its kinase activity. Therefore, a mutant whose Lys^{281} is replaced with other amino acids is used as a kinase-defective form of aPKC. The kinase domain also contains two crucial threonine residues, Thr^{410} and Thr^{560} at its activation loop and turn motif, respectively. They are sequentially phosphorylated by an activation mechanism; the phosphorylation of Thr^{410} releases the pseudosubstrate domain of the kinase, thereby allowing autophosphorylation of Thr^{560} to hold the enzyme at completely active status (Hirai et al., 2003).

Atypical PKC and its diverse roles; Atypical PKC (PKCζ, λ/ι) is involved in multiple physiological processes including cell polarity, mitogenesis, protein synthesis, cell survival and transcriptional regulation. A plausible explanation for such diverse roles of the kinase is its interaction with various molecules, which likely direct the kinase activity to diverse signaling pathways (Hirai and Chida, 2003). For example, ZIP/p62 allows PKCζ to interact with TNFα receptors so that the kinase is able to mediate cytokine signaling. Upon the receptor activation, ZIP/p62 mediates this activation to PKCζ, which then causes NFκB activation for inflammatory cell stimulation. (Sanz et al., 2000; Sanz et al., 1999; Wooten et al., 2001).

PKCλ/ι directly interacts with p70 ribosomal S6 protein kinase (p70S6K) to regulate protein translation (Akimoto et al., 1998). Cooperating with PKB/Akt and mTOR kinase, PKC λ/ι regulates P70S6K activity in response to mitogenic stimuli. The activated p70S6K then enhances the translation of the mRNAs that encodes ribosomal proteins and elongation factors. PKC λ/ι therefore functions as a mediator of mitogen signaling to stimulate protein translation.

PKCζ interacts with a leu-zipper motif–containing protein FEZ1 (fasciculation and elongation protein; zygin/zeta-1) which is a mammalian homologue of *C. elegans* UNC-76. In *C. elegans* unc-76 mutants, axons in fascicles fail to elongate to the full length and to bundle together (Bloom and Horvitz, 1997; McIntire et al., 1992). Therefore, FEZ is necessary for normal axonal elongation and fasciculation in the nematode. In PC12 cells, FEZ is required for NGF-induced differentiation. Coexpression of FEZ and activated form of PKCζ stimulates the neuronal differentiation of PC12 but expression of FEZ alone has no effect. Consistent with this biochemical interaction of these two proteins, FEZ is colocalzed with PKCζ at the membrane. Inhibition of PKCζ tranlocates FEZ from the membrane to the cytoplasm. Together, these data

suggest that FEZ functions as a substrate of PKCζ and directs PKCζ to be involved in axonal outgrowth and neuronal differentiation (Kuroda et al., 1999).

Atypical PKC and the Polarity (PAR) complex; PKCζ interacts with PAR6 and PAR3, through its PB1 domain and catalytic domain, respectively, forming the polarity (PAR) complex. These proteins direct PKCζ to the coordination of Rho GTPases to reorganize actin and microtubule structures in the establishment of cell polarity. In addition, PKCζ directly phosphorylates GSK3β and MARK2 (Arimura and Kaibuchi, 2007; Chen et al., 2006b) to stabilize microtubule structure.

PAR6 consists of three functional domains (Figure 1.2), including a PB1 domain, a PDZ domain and a semi-CRIB domain. The PB1 domain contains an OPR motif, which is recognized by another PB1 domain of PKCζ. It therefore allows the heterodimerization of PKCζ and PAR6. CRIB (Cdc42/Rac1-interactive binding) domain is a short stretch of amino acids that allows PAR6 to interact with active form of Cdc42 (Burbelo et al., 1995). The PDZ domain of PAR6 directly interacts with a PDZ domain of PAR3, letting PAR6 bind with PAR3 (Lin et al., 2000). The semi-CRIB domain of PAR6 lacks two histidine residues that are usually conserved in typical CRIB sequences. Despite the absence of these residues, PAR6 still maintains the ability to recruit the active form of Cdc42 with the assistance of an adjacent PDZ domain (Lin et al., 2000).

The role of PAR6 in the PAR complex is to link Cdc42 to the activation of aPKC. The crystal structure of Cdc42 -PAR6 complex suggests that the binding of active Cdc42 induces a conformational change of PAR6 (Garrard et al., 2003). It is however uncertain whether or not the change is required for the interaction of PAR6 with aPKC and how PAR6 mediates Cdc42 activation to the kinase.

PAR3 is a large scaffolding protein, consisting of three PDZ domains, a PKCζ binding region and a coiled-coil domain (Figure 1.2). The first PDZ domain is required for the interaction with PAR6 in the PAR complex. The PKCζ binding region of PAR3 allows the scaffolding protein to interact with the catalytic domain of PKCζ. The PKCζ binding region contains an aPKC phosphorylation site. The phosphorylation of Thr^{827} at this site is known to reduce the affinity of PAR3 to PKCζ (Lin et al., 2000; Nagai-Tamai et al., 2002) and this regulation is

required for the cell-cell contact-induced cell polarization of epithelial MDCK cells (Nagai-Tamai et al., 2002). PAR3 suppresses the activity of PKCζ *in vitro*, presumably due to the binding of PAR3 to the catalytic domain of PKCζ (Lin et al., 2000). Together, this information suggests that PAR3 may function as a scaffold to keep the kinase in a latent state. In this sense, the activation of PKCζ may result in the phosphorylation of PAR3, allowing the active kinase to interact with downstream substrates.

Another functional significance of PAR3 is the activation of Rac1 (Figure 1.2). Rac1 regulates several signaling proteins such as PAKs, LIMK, NWASP and WAVE. Since these proteins are involved in actin polymerization and branching, Rac1 plays a key role in regulating the stability and rigidity of the actin network. The coiled-coil motif of PAR3 with the assistance of the PDZ domains recruits Tiam1 or STEF (Tiam2) and this interaction regulates their activity. Therefore, PAR3 is directly linked to the regulation of Rac1 activity and the PAR complex plays a key role in actin cytoskeleton regulation through PAR3 and Rac1. (Chen and Macara, 2005; Nishimura et al., 2005). In kidney epithelial cells, where PAR3 interacts with Tiam1, PAR3 negatively regulates Tiam1, resulting in Rac1 inactivation. In cultured neurons, where PAR3 interacts with STEF (Tiam2), PAR3 positively regulates STEF, resulting in Rac1 activation. One explanation for these conflicting results may be that these two different GEFs respond in opposite directions upon PAR3 binding.

Since these two GEFs are similar to each other in domain structure, it is however possible that PAR3 regulates their activity in a similar way. Therefore, another explanation may be that Tiam1 and STEF activity may be regulated in a different manner, depending on cell context. In neurons, PAR3 interacts with PKCζ and PAR6 and this interaction is required for the activation of STEF and Rac1. By contrast, in epithelia, where PAR3 negatively regulates Tiam1, this function of the PAR3 is independent of PKCζ and PAR6. Therefore, I speculate that PAR3 may be able to regulate these GEFs in a different way, depending on the interaction with the polarity proteins. In this regard, it is noteworthy that in the trailing edges of migrating cells, where the activity of Rac1 is maintained low, PAR3-Tiam2 complex is dissociated from PKCζ and PAR6 (Nakayama et al., 2008).

The function of PAR3 in cell polarity is also dependent on subcellular localization. Given the function of the complex as a cytoskeleton reorganizer, it needs to be located where actin and

microtubules are actively regulated, such as cellular protrusions and growth cones. The complex is indeed concentrated at the tips of nascent axons in hippocampal neurons and disturbing the PAR complex localization impairs polarity (Shi et al., 2003). What is the mechanism of the localization? Since PAR3 directly binds to KIF3, a plus end-directed microtubule motor protein, other polarity proteins are tethered to PAR3-KIF3 and transported to the axonal tip (Nishimura et al., 2004).

Rho family GTPases; GTPases are molecular switches that control signaling pathways related to significant cellular physiology and behaviors. They cycle between two different configurations; GTP bound (active) state and GDP bound (inactive) state. During the active state, GTPases are able to interact with effector proteins to trigger signaling pathways. Due to the intrinsic enzymatic activity, however, they slowly become GDP-bound state, losing the interaction with the effectors. There are three proteins that regulate GTPases activity. GTP exchange factors (GEFs) catalyze the exchange of GTP and GDP in order to activate GTPases, GTPase-activating proteins (GAPs) promote the enzymatic activity of GTPases to accelerate GTPases inactivation, and guanine nucleotide dissociation inhibitors (GDI) sequester inactive GTPases from the membrane and thereby prevent GTPase activation by GEFs (Etienne-Manneville and Hall, 2002).

The Rho GTPase family includes Rho, Cdc42 and Rac GTPase. Since a major role of Rho GTPases family is the regulation of the actin cytoskeleton, they are able to influence cell polarity, microtubule dynamics, membrane transport pathways and transcription factor activity (Etienne-Manneville and Hall, 2002). Therefore, the alteration of these GTPases often results in dramatic cell shape changes. Due to the complexity of the signaling pathways that they regulate, it has been challenging to interpret their role in cytoskeleton regulation. There is, however, broad agreement regarding the function of these GTPases as follows. Cdc42 activation is related to filopodia formation (Kozma et al., 1995; Nobes and Hall, 1995). Filopodia (microspikes) are spike-shaped, long and thin cytoplasmic projections that are perpendicularly sticking out from lamellipodia. Since these structures are triggered by extensive actin elongation and bundling, the role of Cdc42 is considered to polymerize actin fiber. Rac1 activation promotes lamellipodia formation (Rottner et al., 1999). Lamellipodia are veil-like and wide cytoplasmic expansions. The cytoskeleton structure underlying lamellipodia is the meshwork of filamentous actin that is

formed by multiple Y-shaped actin branching. The role of Rac1 is indeed to trigger the signaling pathway for actin polymerization and branching.

The filamentous actin structures underlying the filopodia and lamellipodia are critical for the regulation of growth cone motility since the F-actin network controls microtubule progression that is a major driving force for growth cone progression (Bradke and Dotti, 1999, 2000; Lowery and Van Vactor, 2009; Schaefer et al., 2008). Therefore, both Cdc42 and Rac1 activities need to be properly coordinated, depending on the activity of each other. In the course of the coordination, the PAR complex functions as a coordinator of Cdc42 and Rac1.

Axon development and cytoskeleton network; Most of what we know about the PAR complex in neuronal polarity comes from studies using cultured embryonic hippocampal neurons (Dotti et al., 1988). In this culture system, mechanically and enzymatically dissociated neurons spontaneously recapitulate the polarization process. Since this process ensues in a reproducible and predictable manner, this system becomes a useful tool to study neuronal polarity (Arimura and Kaibuchi, 2007). After being plated on surfaces, the cells first form several thin filopodia (stage 1). Several hours later, they generate 3-5 short and equal lengths of processes, so-called neurites that undergo repetitive elongation and retraction (stage 2). Later then, this morphological symmetry of neurites breaks. One of them starts growing rapidly while the others remain short (stage 3). The extended process becomes an axon and the other slowly develop into dendrites (stage 4,5) (Dotti et al., 1988).

Among those steps, the transition from stage 2 to 3 appears critical for axon specification. This transitional period is the time for the morphological asymmetry to develop. What then is a major mechanism to break down the symmetry during this transition? It had been suggested that the pulling force generated by filopodia moves the growth cone to elongate the neurite (Lamoureux et al., 1989). However, the pulling force is not sufficient for the movement and growth (Zheng et al., 1994). On the contrary, a pushing force, generated by microtubule elongation, drives the forward movement of the growth cone to elongate the axon shaft (Bradke and Dotti, 1999, 2000; Schaefer et al., 2008). For microtubules to efficiently push the growth cone forward, they need to penetrate into the peripheral domain (P domain), and associate with bundled actin filaments in filopodia (Lowery and Van Vactor, 2009). Highly meshed structures, made of branched actin filaments residing at the transitional domain (T domain) (presumably

enhanced by Rac1 activation), appear to hamper the insertion of microtubules into the P domain (Forscher and Smith, 1988). In this aspect, the actin network at growth cones seems to play a role of permission or prevention for microtubule progression and axonal elongation, depending on its structure or the extent of polymerization (Bradke and Dotti, 2000). Therefore, loosened and unstable structure of actin filament at the growth cone is likely required for microtubule progression and the forward movement of the tip (Bradke and Dotti, 1999).

The morphological asymmetry begins at the growth cone of a neurite that becomes an axon. These prospective axonal growth cones become enlarged and highly dynamic with prominent filopodia and lamellipodia formation (suggesting the instability of actin network) whereas the other growth cones stay relatively small and quiescent (Bradke and Dotti, 1999). Such morphological difference suggests that the cytoskeleton network of axonal growth cones is less stable than those of minor neurite growth cone. Consistent with this idea, the local perfusion of F-actin destabilizing reagents permits a minor neurite to develop into an axon (Bradke and Dotti, 1999; Forscher and Smith, 1988). Bath application of the drug induces the formation of neurons with multiple axons (Bradke and Dotti, 1999). These findings suggest that increasing the instability of actin cytoskeleton at the growth cones is required for the axon formation and extension.

The PAR complex regulates the cytoskeleton network for neuronal polarity; The importance of actin and microtubule dynamics in the growth cone and axon elongation suggests that any molecule impinging on cytoskeleton architecture would play an important role in axon development. The Par complex is associated with microtubule and actin dynamics in several cell polarity models. For example, PKCζ phosphorylates and controls the activities of MARK2 and GSK3ß, which impinge on microtubule dynamics. On the other hand, PAR3 regulates Rac1 activity through STEF/TIAM, a Rac1-specific GEF. Rac1 then regulates actin polymerization through PAK and WAVE.

An important feature of the PAR complex is its spatial distribution during polarization. In neuronal polarization, phosphorylated forms of PKCζ are accumulated at the tip of the neurite that will become the axon (Nishimura et al., 2005; Shi et al., 2003). PAR3 is found in all neurites of unpolarized hippocampal neurons at stage 2. At stage 3, it becomes concentrated on the growth cone of the neurite that becomes the axon. Overexpression of PAR3 causes a neuron to

develop multiple axons. PAR6 is also found in the axonal compartment or at the axonal tip of polarized neurons (Cheng et al., 2011; Shi et al., 2003). Overexpression of PAR6 impairs axonal formation (Shi et al., 2003). Together, these suggest that the PAR complex is involved in the axon formation and its accumulation at the axonal growth cone is required for axon formation. The PAR complex may increase the dynamics of the actin and microtubule network at axonal growth cones. The complex may render actin network more permissive for microtubule progression, allowing microtubules to elongate into filopodia and push forward the growth cone.

1.2 Extrinsic trophic cues and the polarity complex proteins

Most of our knowledge about the PAR complex was obtained from spontaneous polarization models. Given that numbers of extrinsic factors are involved in the development of neurons, these factors likely play a significant role in axonal development. Despite increasing knowledge of the polarity complex, we have just begun to understand how such extrinsic cues modulate the polarity machinery to regulate axonal development. In this section, I will review research regarding the role of extrinsic stimulatory factors in the regulation of the PAR complex and neuronal polarity.

BDNF: BDNF plays an instructive role to determine axon specification in neuronal polarity (Cheng et al., 2011). When hippocampal neurons are grown on BDNF-striped surfaces, the fate of neurites is determined by whether or not they touch BDNF. BDNF signaling appears to locally increase cyclic AMP level and thereby activates protein kinase A (PKA). PKA activation then presumably turns on several signaling cascades.

One of the targets, which is triggered by BDNF-activated PKA, is Smad ubiquitin regulatory factor 1 (Smurf1), an E3 ubiquitin-protein ligase. Smurf1 targets a protein to proteasome degradation by labeling it with poly-ubiquitin. BDNF-induced PKA activation increases the phosphorylation of Smurf1, which changes the substrate preference of the ligase from PAR6 to RhoA. Given the inhibitory effect of RhoA and instructive role of the PAR complex in axon specification, this change works favorably for axon formation. Consistent with this, PKA-phosphorylated form of Smurf-1 is found at axonal tips. Overexpression of RhoA with Smurf1-site mutation prevents BDNF-induced axon formation. In addition, overexpression of Smurf-1 with phosphomimetic mutation at PKA site promotes axon formation. Together, these suggest that BDNF stimulates axon development through PKA signaling and the PAR complex.

TGFβ: TGFβ signaling also plays an instructive role in fating naïve neurites to become axons in the developing brain. When hippocampal neurons are grown on TGFβ-striped surface, the fate of neurites is determined by whether or not they touch TGFβ. The polarity effect of TGFβ was originally revealed in epithelial mesenchyme transition (EMT), where the cytokine induces the phosphorylation of PAR6. This phosphorylation is linked to RhoA degradation, which is required for TGFβ-mediated EMT (Ozdamar et al., 2005). Similar with this model, TGFβ is also required for the proper axon development and migration of neurons in the developing brain. As in the EMT model, TGFβ induces the phosphorylation of PAR6 through a TGFβ receptor, TβR1 in developing neurons. This phosphorylation is required for TGFβ-induced axon specification. It is, however, uncertain how such TGFβ-mediated modification of PAR6 allows axon formation (Yi et al., 2010).

WNT: WNT and its receptor frizzled (FZ) regulate numerous developmental processes, including asymmetric cell division, cell fate determination, planar cell polarity and tissue polarity**.** In developing neurons, WNT functions as an extrinsic stimulatory cue to promote axonal development. How does WNT accomplish this? Recently, it had been reported that the PAR complex is involved in WNT-stimulated neuronal polarity.

Zhang et al, showed that dishevelled (Dvl), an immediate downstream effector of FZ, accumulates at the axonal tips in hippocampal neurons. They also found that suppression of Dvl causes a polarity defect due to the lack of axonal structure whereas its overexpression promotes formation of multiple axons. This suggests that WNT signaling plays a stimulatory role in axon formation. Furthermore, they found that WNT signaling is linked to PKCζ. WNT treatment induces the activation of PKCζ in hippocampal neurons. Consistent with this result, Dvl interacts with PKCζ and its overexpression increases the kinase activity. In addition, dominant negative forms of PKCζ prevent the stimulatory effect of Dvl overexpression on axon formation (Zhang et al., 2007). Together, these results suggest that WNT regulates the PAR complex through frizzled and Dvl pathway to stimulate axonal development.

WNT also functions as a guidance molecule in the developing nervous system. In the developing spinal cord, WNT–Frizzled signaling instructs commissural axons to turn anteriorly after midline crossing (Lyuksyutova et al., 2003). Wolf et al showed that suppression of PKCζ abolishes the WNT-mediated turning and WNT-stimulated outgrowth of commissural axons.

They also found that PAR6 and the phosphorylated form of PKCζ are abundant in the post-crossing axon segment. Together, these results suggest that WNT requires the PAR complex for its functions as an attractive guidance cue (Wolf et al., 2008).

In this section, I have reviewed a few studies regarding the role of extrinsic stimulatory cues in neuronal polarity and axon guidance. These results suggest that these cues have an instructive role in axon specification. In the absence of extrinsic cues, the complex randomly accumulates in one of immature neurites, allowing it to become the axon. The role of extrinsic cues is likely to trigger some signaling pathways to stabilize the PAR complex in the selected neurites but the complex in the other neurites presumably is likely degraded (Cheng et al., 2011; Schwamborn, 2007; Schwamborn and Puschel, 2004; Yi et al., 2010; Zhang et al., 2007).

Another significant aspect is that axon guidance shares a common signaling mechanism with neuronal polarity. This idea is more evident in the examples of WNT signaling since this extrinsic cue requires the PAR complex for axon formation and attractive axon guidance. The requirement for the PAR complex in these two distinctive behaviors of neurons is not surprising since dynamic regulation of actin and microtubules is a critical hallmark for both axon guidance and neuronal polarity. Therefore, I speculate that inhibitory cues may also regulate the PAR complex to mediate their inhibitory action.

1.3 Spinal cord injury and extrinsic inhibitory and repulsive cues

The extracellular environment, which consists of extracellular matrix, trophic factors and guidance cues, all constitute information for axonal tracts to find a path for successful targeting. Among extrinsic factors, guidance molecules are relatively well described because of their importance for accurate circuitry formation and the robust behavioral changes of axon growth cones in response to those cues. Depending on the behavioral patterns of the growth cone in response to these cues, they are mainly divided into two categories; attractive and repulsive/inhibitory guidance cues. Since axonal path finding requires complex maneuvering of growth cones such as advancing, pausing and turning, both attractive and repulsive guidance are necessary for such behavior of growth cones. As previously reviewed, a few trophic factors and attractive cues are coupled to the polarity complex in axon development and attractive guidance mechanism. It remains elusive however whether repulsive or inhibitory guidance cues are also linked to the polarity machinery for their inhibitory action on axonal outgrowth.

Many guidance molecules are upregulated during initial nervous system formation and downregulated upon its completion. The inhibitory cues are also highly expressed in the developing brain and downregulated later, suggesting significant roles in nervous system formation. The function of inhibitory cues is, in collaboration with attractive cues, to guide axonal tracts to appropriate targets by regulating the motility and direction of axonal tracts. While the role of attractive cues is to allow the advancement or turning similar to a 'go' traffic signal, conversely the role of inhibitory cues is to prohibit the advancement of growth cones much like a 'stop' signal. Therefore, the inhibitor molecules are equally as important as attractive molecules during brain development (Lowery and Van Vactor, 2009).

Another significance of inhibitory cues is highlighted during pathological conditions of the central nervous system. When axons of the CNS are damaged, their successful regeneration is in general compromised beyond the lesion site. This failure is partially due to the inhibitory molecules associated with myelin debris and glial scar at the injury site. CNS myelin contains numerous inhibitory proteins. Their normal function is presumably to prevent unnecessary branching and sprouting of axons. When myelin fragments upon injury impact, it becomes a major source of inhibition, releasing several inhibitory and repulsive molecules such as Nogo, myelin associated glycoprotein (MAG), OMgp (Oligodendrocytes myelin glycoprotein), semaphorin 4D (Sema4D) and ephrin-B3 (Yiu and He, 2006).

Another source of inhibition is the chondroitin sulfate proteoglycans (CSPGs) that are secreted from reactive astrocytes and oligodendrocytes precursor cells (OPCs). CNS injury, in general, triggers a strong immune response at the injured site, recruiting numerous reactive astrocytes, microglia and OPCs. The CSPGs are secreted by these cells and accumulate in the injured sites, inducing glial scar formation (gliosis) (Pekny and Nilsson, 2005). Gliosis has some beneficial effects to prevent further microbial infection and the spread of cellular damage but due to strong inhibitory and repulsive activity of CSPGs, the scar functions overall as a physical and biochemical barrier to hamper successful axon regeneration (Yiu and He, 2006).

So far, I have reviewed briefly extrinsic inhibitory cues regarding their roles and significance in normal development and pathological conditions. Despite their significance in CNS injury, their intracellular mechanisms are largely unknown. Considering such unmet clinical and biological understanding of CNS injury, my dissertation research focuses on myelin

and glial inhibition. In the following sections, therefore, I will review inhibitory molecules mainly related to myelin and glial inhibition in CNS injury.

Myelin-associated inhibitors and their intracellular signaling; Intact CNS myelin is not inhibitory to axon growth *in vivo* (Davies et al., 1997) but fragmentized myelin is inhibitory both *in vitro* and *in vivo* (Filbin, 2003). Disrupted myelin releases myelin-associated inhibitors such as Nogo, MAG, OMgp, Sema4D and Ephrin-B3.

Nogo is a member of reticulon family of transmembrane proteins (Chen et al., 2000; GrandPre et al., 2000). It includes three isoforms, Nogo A, B and C, which are generated by alternative splicing and different promoter usage. Among these isoforms, Nogo-A has attracted much attention since it is highly expressed in CNS oligodendrocytes. All isoforms share a 66 amino acid loop (Nogo-66), which is necessary for the inhibitory activity (GrandPre et al., 2000). Nogo-A contains a unique region at N-terminal, amino-Nogo, which is not shared by the other isoforms but also shows inhibitory activity. Both Nogo-66 and intact Nogo binds to NgR receptor to mediate axon growth inhibition (Fournier et al., 2001). An antibody targeting Nogo-A or a peptide antagonist of NgR promotes axon regeneration of the corticospinal cord (Cao et al., 2008; Schnell and Schwab, 1990).

The Nogo-66 receptor (NgR), a GPI-linked protein, interacts with the extracellular domain of Nogo-A (Fournier et al., 2001). It also interacts with MAG and OMgp (Domeniconi et al., 2002; Wang et al., 2002b), which are structurally different from Nogo. Due to the lack of an intracellular domain, NgR requires additional co-receptors so that they may transduce the myelin inhibitory signals. Tumor necrosis factor receptor (TNFR) family members, such as p75, TROY and LINGO1 have been so far identified as co-receptors for NgR (Mi et al., 2004; Park et al., 2005; Wang et al., 2002a). Ligand binding appears to induce RhoA activation to cause axon growth inhibition (Park et al., 2005; Shao et al., 2005). For example, MAG binds to NgR, resulting in the dimerization of NgR and p75 or TROY. This dimerization activates the co-receptor in an unknown mechanism. The activated co-receptor sequesters Rho GDI away from RhoA, resulting in the activation of RhoA. The activated GTPase signals through ROCK, LIMK and cofilin to prevent axonal growth (Yamashita and Tohyama, 2003).

MAG (Myelin-associated glycoprotein) is a transmembrane protein expressed in both CNS oligodendrocytes and PNS Schwann cells (Schachner and Bartsch, 2000). It contains five immunoglobulin-like domains in its extracellular domain (Salzer et al., 1987). MAG is released upon myelin disruption and associates with extracellular matrix (ECM) proteins. Interestingly, it promotes neurite outgrowth of embryonic neurons. The activity rapidly turns from promotion to inhibition at the time of birth, suggesting that it acts as a bifunctional cue, depending on developmental status of cells (Turnley and Bartlett, 1998).

OMgp (Oligodendrocyte myelin glycoprotein) is a GPI anchored protein that is enriched in membranes of oligodendroglial-like cells. These cells wrap nodes of Ranvier, which is thought to prevent collateral sprouting of axon (Huang et al., 2005). Less is known about OMgp functions in CNS injury but it prevents collateral sprouting from axons, suggesting its role as an inhibitor upon myelin disruption (Chang et al., 2010; Huang et al., 2005).

EphB3 and Sema4D are the repulsive guidance cues found in CNS myelin (Benson et al., 2005; Moreau-Fauvarque et al., 2003). In general, many guidance molecules are involved in the initial nervous system formation and downregulated when the development is complete. These two molecules are however expressed in myelinating oligodendrocytes during post-natal stage (Yiu and He, 2006). Their role in myelin is unclear but at least seems to play a role of inhibition upon myelin disruption.

Chondroitin sulfate proteoglycans (CSPG) and the glial scar; In addition to myelin inhibitors, CSPGs in glial scars are another inhibitory source to prevent axon regeneration. Injury and myelin disruption cause a glial reaction, recruiting reactive glial cells such as microglia, OPCs, meningeal cells, and astrocytes at the injury site. The recruited glial cells massively secrete inhibitory proteoglycans, resulting in glial scar formation (Rudge and Silver, 1990). This gliosis may have some beneficial effects on axon regeneration because it restricts the inflammation area and cellular degeneration and recruits astrocytes to support axon regrowth (Faulkner et al., 2004). These effects are overridden however by the potent inhibitory extracellular matrix containing CSPGs from reactive glial cells and myelin debris (McKeon et al., 1991; Morgenstern et al., 2002; Yiu and He, 2006).

CSPGs (aggrecan, brevican, neurocan, versican, phosphacan and NG2) are polypeptides with covalently attached highly sulfated glycosaminoglycan (GAG) chain (Morgenstern et al., 2002). Their spatiotemporal expression patterns correlate with glial boundaries in the developing CNS, such as the spinal cord roof plate, optic tectum and dorsal root entry zone (Pindzola et al., 1993; Snow et al., 1990). Therefore, in developing nervous system, CSPGs seem to function as a boundary for axonal tracts. In an injured state, they are upregulated and prevent axon regeneration across the glial scar (Asher et al., 2000; Haylock-Jacobs et al., 2011; Jones et al., 2002).

The intracellular mechanism by which CSPGs mediate inhibition is largely unknown. Signaling pathways such as PKC and RhoA/ROCK signaling have been implicated for CSPG-induced inhibition (Hasegawa et al., 2004; Monnier et al., 2003; Powell et al., 1997; Sivasankaran et al., 2004). Recently, receptor protein tyrosine phosphatase σ (RPTPσ), a protein of LAR family was identified as a receptor for CSPGs through *in vitro* binding assay and transgenic approaches (Shen et al., 2009). It, however, remains unknown whether RPTPσ is linked to PKC or RhoA/ROCK signaling.

NG2; NG2 was first identified as a cell surface antigen of neural tumor-derived cell lines (Wilson 1981). It is a large ~300 kDa integral membrane proteoglycan, containing several sites for GAG chain but only one of these sites, Ser^{999} is attached to GAG chain (Nishiyama et al., 1991; Stallcup and Dahlin-Huppe, 2001). Except the two laminin globular domains in domain 1, the core protein of NG2 is a structurally unique protein with no homology to other protein families (Staub et al., 2002; Tan et al., 2005). The structure of NG2 consists of a large extracellular domain (ECD), a single membrane-domain and a short cytoplasmic tail. The ECD is subdivided into three separate domains based on their structural and functional properties; an N-terminal globular domain (domain1), a central domain (domain 2) containing GAG attachment site and a juxtamembrane globular domain (domain 3) (Ughrin et al., 2003). The ECD has two potential sites for proteolytic cleavage (Asher et al., 2005; Nishiyama et al., 1995). Therefore, NG2 also exists as a soluble and an extracellular matrix-associated form.

NG2 is involved in diverse cellular functions; cell migration (Burg et al., 1998; Burg et al., 1997; Fang et al., 1999), cellular response to growth factors (Goretzki et al., 1999; Grako and Stallcup, 1995) and angiogenesis (Goretzki et al., 2000; Ozerdem et al., 2001; Ozerdem and

Stallcup, 2004). NG2 interacts with various extracellular matrix (ECM) molecules. In particular, it binds directly to collagen VI. This interaction is necessary for the migratory response toward type VI collagen in NG2-expressing glioma cells, suggesting that NG2 is involved in cell migration. NG2 also directly interact with growth factors such as platelet-derived growth factor-AA (PDGF-AA) and basic fibroblast growth factor. Through these molecular interactions, NG2 functions to sequester these growth factors and modulate their cellular response.

NG2 also plays a role as a potent inhibitor of axon growth. NG2 shows typical properties of extrinsic inhibitory molecules; axon growth inhibition, growth cone collapse and repulsive effect (Chen et al., 2002; Dou and Levine, 1994; Ughrin et al., 2003). NG2 or truncated forms of NG2 retard axon outgrowth of CGNs and newborn DRGs (Dou and Levine, 1994). Acute treatment with NG2 induces growth cone collapse in newborn DRGs (Ughrin et al., 2003). In stripe assays, growing axons avoid NG2 stripes. Together, these results suggest that NG2 has typical properties of an inhibitory and repulsive guidance molecule.

NG2 is the major CSPG molecule found in the glial scar, a physical and biochemical barrier to discourage successful regeneration of severed axons. Reactive macrophages, OPCs, meningeal fibroblasts and endothelial cells express NG2. After injury, these cells are recruited to the injury sites and likely shed off the inhibitory proteoglycan to form the glial scar. NG2 expression is upregulated at the injury site within 24 hours, peaks at one week, and remains elevated for another seven weeks (Tang et al., 2003). Coincidently, it is during this time period that most of the severed axons fail to regenerate. Furthermore, neutralizing antibodies of NG2 (targeting the ECD) allow axons of sensory neurons to regrow across the injury site (Tan et al., 2006). These data suggest the significant role of NG2 in the failure of axonal regeneration in the damaged CNS.

NG2 is structurally and biochemically different from other CSPGs. The inhibitory ability of CSPGs mainly relies on their GAG chains since bacterial chondrotinase treatment reduces the inhibitory activity *in vitro* and *in vivo* (Bradbury et al., 2002; Zuo et al., 1998). In contrast, the inhibitory activity of NG2 seems to be a property of its protein core. The extent of GAG chain in NG2 is less than those of other CSPGs (Nishiyama et al., 1991; Stallcup and Dahlin-Huppe, 2001; Tan et al., 2005). NG2 maintains the activity to inhibit axon outgrowth and induce growth cone collapse after GAG chain removal (Dou and Levine, 1994). *In vivo*, neutralizing antibodies

of NG2 (targeting extracellular domain) allow axons of sensory neurons to regrow cross the injury site. These antibodies target domain 1 and domain 3 of NG2 but not GAG chain-attached domain 2, suggesting that the core protein of NG2 but not GAG chain is responsible for the inhibitory action of the proteoglycan.

A recent study, that used an ELISA based-*in vitro* binding assay and transgenic approach, identified receptor protein tyrosine phosphatase σ (RPTPσ), a protein of LAR family as a receptor for CSPGs (Shen et al., 2009). Preliminary binding assays in our lab evaluating the interaction of NG2 with RPTPσ or control CSPGs (a mixture of neurocan, phosphacan, versican, and aggrecan), showed strong CSPG-RPTPσ binding as was reported by Shen et al. in 2009. The binding of NG2 to the receptor was, however, weaker than that of control CSPGs, suggesting the existence of an alternative receptor for NG2. These differences of NG2 from other CPSGs suggest that NG2 may target a unique intracellular signaling mechanism.

Intracellular mechanisms of axon growth inhibition; Considering the numerous ligands and receptors that mediate inhibitory action of fragmentized CNS myelin and the glial scar, targeting individual components may not be the best way to promote regeneration. Therefore, identifying common intracellular pathways shared by the inhibitors would provide greater insight for successful axon regeneration. In this section, I will review briefly a few signaling components that inhibitory molecules trigger and discuss the possibility that the PAR complex is involved in the signaling of these inhibitory cues.

The Rho small GTPases, such as RhoA, Rac1 and Cdc42 function as regulators of the actin cytoskeleton. RhoA activation is closely linked to growth cone collapse and axon guidance repulsion (Kozma et al., 1997; Liu et al., 2009; Swiercz et al., 2002; Swiercz et al., 2004). RhoA activation is required for the inhibitory action of both myelin and CSPG (Monnier et al., 2003; Niederost et al., 2002). In particular, myelin inhibitors activate RhoA by displacing a Rho-guanine dissociation inhibitor (Rho-GDI) (Yamashita and Tohyama, 2003). Recently, Hsieh et al reported that Nogo signals through RhoA to activate LIM kinase. They claimed that this signaling causes the inactivation of cofilin, an actin depolymerization factor (Hsieh et al., 2006). Therefore, it is speculated that myelin-derived inhibitors may increase the stability and rigidity of actin cytoskeleton through the RhoA signaling described above.

RhoA also negatively regulates microtubule-associated proteins (Arimura and Kaibuchi, 2007; Mimura et al., 2006). MAG and Nogo induce the phosphorylation of CRMP-2 through RhoA/Rho-kinase. This phosphorylation negatively regulates CRMP-2. Since the role of CRMP is to promote microtubule assembly, the phosphorylation results in the down-regulation of microtubule assembly. Consistent with this result, microtubule density is reduced in injured axons (Mimura et al., 2006). In addition, pharmacological stabilization of microtubule allows regeneration of damaged axons *in vivo* (Erturk et al., 2007; Hellal et al., 2011). Together, these results suggest that myelin and glial inhibitors signal through RhoA to modulate actin and microtubule stability for their inhibitory action.

In contrast to RhoA activation, the myelin inhibitors inactivate Rac1 to mediate their inhibition (Kuhn et al., 1999; Niederost et al., 2002). Since Rac1 promotes actin polymerization, the Rac1 inactivation reduces polymerized actin structure at growth cone. Actin has both permissive and non-permissive effects for microtubule progression in growth cones, depending on its polymerized structure (Bradke and Dotti, 2000; Fan et al., 1993; Fritsche et al., 1999). Actin filaments serve to guide microtubule penetration into the growth cones. In this aspect, actin polymerization is required for axon elongation. When the actin cytoskeleton is heavily polymerized and branched, the resulting heavy meshwork of actin filaments likely hampers microtubule motility. Rac1 inactivation, therefore, may result in the shortage of actin filaments to guide microtubules through to the growth cones, attenuating axon growth.

In addition to the Rho GTPases, protein kinase C (PKC) signaling is known to be involved in both CSPG and myelin inhibition (Hasegawa et al., 2004; Powell et al., 2001; Sivasankaran et al., 2004). Myelin and glial inhibitors induce the activation of PKC, presumably due to local elevation of calcium concentration. MAG and Nogo trigger PLC-IP_3 pathway for the calcium elevation (Hasegawa et al., 2004). It remains however elusive how these inhibitors induce PLC activation. Pharmacological suppression of conventional PKC attenuates outgrowth inhibition. Together, these results suggest that myelin inhibitors and CSPGs signal through calcium and PKC to mediate their inhibitory action.

In this section, I have reviewed briefly the signaling mechanisms that myelin and glial inhibitors recruit. In general, these inhibitors antagonistically regulate RhoA and Rac1 to induce alterations in actin and microtubule stability, which causes growth cone collapse and axon

outgrowth inhibition. PKC signaling is also involved but it is still unclear how it is linked to receptor signaling and the GTPases signaling (Hasegawa et al., 2004; Sivasankaran et al., 2004).

In the injured state, severed axons may need to recapitulate the polarization process. The inhibitory environment at the injured site, however, discourages the successful regeneration of damaged axons. Recent findings suggest that myelin and CSPG-based inhibition are related to the alteration in actin and microtubule stability through Rho GTPases and this alteration is required for the inhibition. As reviewed earlier, numerous studies indicate that small GTPases are directly linked to the PAR complex for cytoskeleton reorganization. In developing neurons, the complex links Cdc42 to Rac1 activation (Nishimura et al., 2005). In migrating cells, the PAR complex functions to mediate the antagonistic regulation of RhoA and Rac1 (Nakayama et al., 2008). Therefore, I speculate that inhibitory cues at the lesion site signal through the polarity machinery to regulate the GTPases and thereby discourage axonal regeneration. In my dissertation research, I explored this hypothesis using NG2 as a model for inhibitory cues.

1.4 Specific Aims and Experimental Rationales

My research attempts to elucidate the intracellular signaling pathways of extrinsic growth inhibitory cues. NG2 has typical properties of an inhibitory and repulsive guidance cue: inhibitor of axonal outgrowth and stimulator of growth cone collapse and repulsion. NG2 is, therefore, a proper model to study the intracellular mechanism of axon growth inhibition that is triggered by extrinsic inhibitory cues. In addition, NG2 is an inhibitory proteoglycan most abundantly accumulated at the glial scar. Thus, this research is also expected to provide new insights and therapeutic targets for successful regeneration of damaged CNS.

Aim1: Determine whether PKCζ is required for axon growth inhibition.

Preliminary data suggests that multiple PKC isoforms are involved in axon growth inhibition by NG2. Since PKCζ is a PKC isoform and is associated with the PAR complex, inhibitory cues may regulate the kinase. Thus, Chapter 3 will test whether the kinase activity is altered and required for NG2-mediated inhibition.

Aim2: Identify the mechanism by which NG2 regulates PKCζ and the Par complex to cause axon growth inhibition.

Aim 2.1 Determine whether Cdc42-PAR6 is required for axon growth inhibition; As mentioned in Aim1, NG2 may regulate PKCζ. This regulation requires the alteration of other polarity proteins. Therefore, I will test the following in chapter 4; the activation of Cdc42 and its requirement for PKCζ activation, and the requirement of a Cdc42-PAR6 interaction for NG2 induced-inhibition.

Aim 2.2 Determine whether PAR3-Rac1 is necessary for axon growth inhibition; Cdc42 activation triggers PKCζ and then induces Rac1 activation through PAR3. The balanced activity of these small GTPases is critical for proper stability of actin and microtubule network that is required for axon elongation. Therefore, chapter 5 will examine how the altered PKCζ activity modifies PAR3 and Rac1 and whether these proteins and their modification are required for the inhibition.

Aim2.3 Examine the spatial localization of the Par complex in axon growth inhibition; The polarity proteins are in general concentrated at the tips of axons. This asymmetric distribution is critical for axon formation. Therefore, in chapter 5, I will examine whether NG2 alters the spatial distribution of the PAR complex.

Aim3: Determine whether ceramide signaling is involved in axon growth inhibition.

As other extrinsic molecules, inhibitory cues mediate their activity through certain receptor signaling system. This process is often coupled with the generation of second messenger molecules. Ceramide is linked to the PAR complex in several polarity models and inhibits neurite outgrowth. This suggests that NG2 may trigger the synthesis of ceramide to regulate the PAR complex for axon growth inhibition. Therefore, in chapter 6, I will explore whether ceramide signaling is necessary and sufficient for axon growth inhibition.

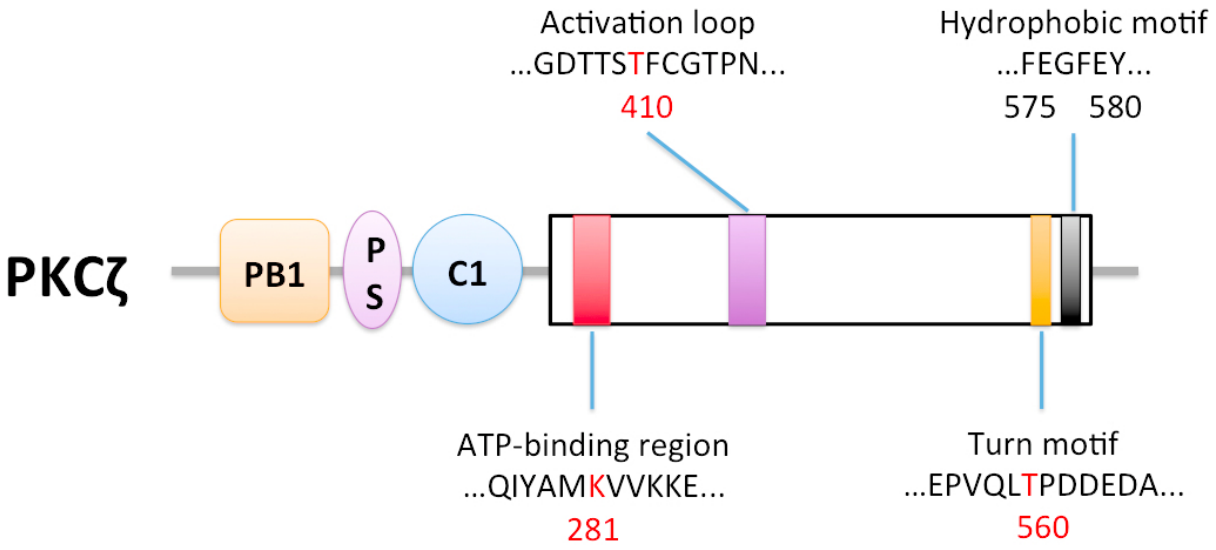


Figure 1.1. Schematic representation of domain structure of PKCζ

The illustration was obtained from a recent review by Hirai and Chida (Hirai and Chida, 2003). PKCζ include a PB1 domain in the N-terminus, a pseudosubstrate (PS), a C1 domain, and a Ser/Thr kinase domain in the C- terminus. The kinase domain contains an ATP-binding region, an activation loop, a turn motif, and a hydrophobic motif. In the ATP-binding region, Lys^{281} is crucial for kinase activity. Thr^{410} in the activation loop is phosphorylated by PDK1 that binds to the hydrophobic motif. Thr^{560} in the turn motif is the autophosphorylation site and its phosphorylation is also essential for the activation (Hirai and Chida, 2003).

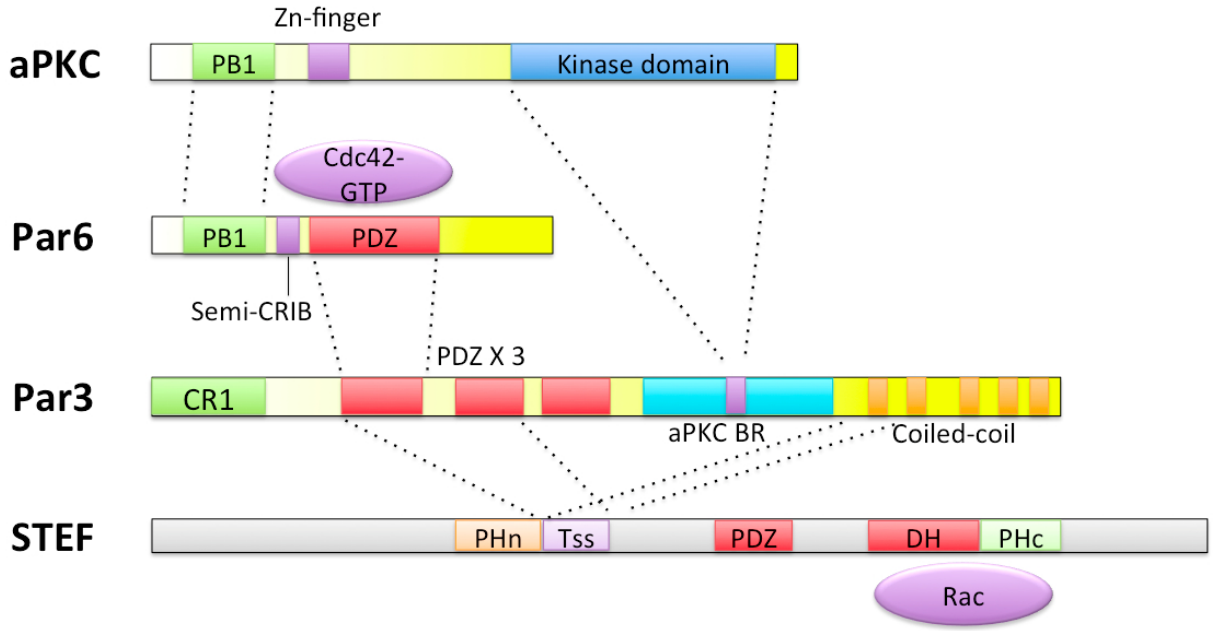


Figure 1.2. Interactions of the PAR complex, STEF and small GTPases

The illustration was obtained from a work of Nishimura et al (Nishimura et al., 2005). Connecting lines indicate regions of the proteins that interact with one another. Details of the domain organization of the PAR complex and STEF are referred to literature (Macara, 2004; Nishimura et al., 2005).

Chapter 2 Materials and Methods

2.1 Materials

Reagents: Laminin, CHAPS, Tween 20, Sodium deoxycholate, Tris base, Poly-$_L$-Lysine, GTPγS, phosphatidylserine and EDTA were purchased from Sigma (St. Louis, MO). ATP, [γ-^{32}P] was purchased form PerkinElmer (Waltham, MA). Agarose, sodium dodecyl sulphate, protein G agarose, glycine, High Fidelity PCR system, Rapid DNA ligation kit, proteinase inhibitor cocktail, and phosphoatase inhibitor cocktail were purchased form Roche (Indianapolis, IN). LB medium, LB agar medium, isopropanol, ammonium persulfate and bisacrylamide were purchased from Fisher (Pittsburg, PA). Acrylamide and PVDF membrane were purchased from Bio-Rad laboratories (Hercules, CA). ECL western blotting detection system and horseradish peroxidase-conjugated secondary antibodies were purchased from GE Healthcare life sciences (Piscataway, NJ). Neurobasal®-A medium, B-27 supplement L-15 medium (Leivobitz), Dulbecco's Modified Eagle's Medium (DMEM), trypsin-EDTA, Dulbecco's Phosphate buffered saline, TEMED, subcloning efficiency DH5α and 1Kb plus DNA ladder, Benchmark Protein ladder and Lipofectamine 2000^{TM} were purchased from Invitrogen (Carlsbad, CA). Wizard® Plus miniprep kit were purchase from Promega (Fitchburg, WI). Endofree Plasmid Maxi kit, Qiagen Gel extraction kit and MiniElute PCR purification kit were purchased from Qiagen (Valencia, CA). Restriction enzymes and DNA modifying enzymes were purchased from Roche or New England BioLabs (Ipswich, MA). PKCζ pseudosubstrate (myristoylated) and Fumonisin B1 were purchased from Enzo Life Sciences (Farmingdale, NY). Chicken Extracellular Chondroitin Sulfate Proteoglycans (CSPG) was purchased from Millipore (Billerica, MA). Trypsin was purchased from Worthington Biochemical (Lakewood, NJ). E18 rat hippocampal tissue was purchased from BrainBits (Springfield, IL). Spargue-Dawely rats were maintained and bred in the university animal facility.

Antibodies: Anti-PKCζ antibody (sc-216), anti-Rac1 antibody (sc-217) and anti-Myc antibody (sc-40) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Phospho-PKCζ/λ (9378) antibody was purchased from Cell Signaling Technology (Danvers, MA). Anti-PAR3 antibody (07-330) and anti-Tau (MAB361) was purchased Millipore (Billerica, MA). Monoclonal anti-FLAG®M2 antibody (F3165), anti-β-actin antibody (A5316) were purchased from Sigma (St. Louis, MO). Living Colors® DsRed Polyclonal Antibody (632496) was

purchased from Clontech (Mountain View, CA). Neuronal Class III β-Tubulin (Tuj1) monoclonal antibody was purchased from Covance (Princeton, NJ).

siRNAs: Scrambled control siRNA was kindly provided by Dr. Holly Colognato, Stony Brook University. To target the endogenous PKCζ in primary cells, ON-TARGETplus SMARTpool PRCKZ, the mixture of the following different sequences of siRNA targeting rat PKCζ (NM 022507) was purchased from Dharmacon (Lafayette, CO); 5`-CCACGACGAUGAGGATATC-3` (864-882), 5`-TCGGAAACATGACAATATC-3` (654-672), 5`-TCACACGTCTTGAAAGGAT-3` (1456-1474) and 5`-CGATGCCGATGGACACATT-3` (1152-1170).

Plasmids:

1) pCAG-EGFP : A CMV early enhancer/chicken β actin promoter (CAG) plasmid was constructed to express exogenous genes in primary neurons. pCIG2 (a kind gift from Dr. Yimin Zou, University of California at San Diego) was linearized with MscI and the resulting 5` overhang sequences were Klenow-filled. The cassette containing multiple cloning sites, IRES, and EGFP sequence was removed by Xho I digestion. A fragment containing the multiple cloning sites and EGFP ORF was obtained from pEGFP-N1 plasmid (Clontech, Mountain View, CA) by XhoI and NotI (blunted by Klenow) digestion and ligated into the linearized pCIG2.

2) pCAG-PKCζ-EGFP : The ORF of rat PKCζ was amplified by PCR from pHACE-PKCζ (a kind gift from Dr Jaewon So, Inha University, South Korea) with the primers 5`-CCGCTCGAGCT GCCACCATGCCCAGCAGGACCGACCCC-3` and 5`-CGGAATTCTGCACGGA CTCCTCAGCAGACAG-3`, and cloned into the XhoI and EcoRI sites of pCAG-EGFP.

3) pCAGJC : The expression cassette of EGFP in pCAG-EGFP was excised by AgeI and BsrGI digestion. The resulting overhang sequences were filled in with Klenow and self-ligated. This vector was used to clone small epitope-tagged expression cassettes.

4) pCAGJC-myc-PAR6C : Myc-tagged human PAR6C expression cassettes was PCR-amplified from pk-myc-PAR6C (Joberty et al., 2000)Addgene plasmid 15474) with the primers 5`-GCGAGCTCCTGCCACCATGGCCCGGCCGCAGAGGACT-3` and 5`-CGGAATTCTCAGAGGCTGAAGCCACTACCATC-3` and cloned into the SacI and EcoRI sites of pCAGJC.

5) pCAGJC-myc-PAR6ΔCRIB : semi-CRIB domain sequence of Myc-tagged PAR6C expression cassette was deleted by using PCR as the following; First, the 5` fragment of semi-CRIB domain were PCR-amplified with the primers 5`-GCGAGCTCCTGCCACCATGGCCCGGCCGCAGAGGACT-3` and 5`-CCGTCGGTGGGTCTCAGGCAGTGCCACTGGCCGCAGCAAGAG-3` and 3` fragment with primers 5`-CTCTTGCTGCGGCCAGTGGCACTGCCTGAGACCCACCGACGG-3`and 5`-CGGAATTCTCAGAGGCTGAAGCCACTACCATC-3`. These two fragments are then adjoined by PCR reaction with the following primers; 5′-GCGAGCTCCTGCCACCATGGCCCGGCCGCAGAGGACT-3` and 5`-CGGAATTCTCAGAGGCTGAAGCCACTACCATC-3` and the resulting fragment was cloned into the SacI and EcoRI sites of pCAGJC.

6) pCAGJC-myc-PAR6Δ135P: 135 proline residue of Myc-tagged PAR6C expression cassette was deleted by using PCR. First, the 5` fragment of semi-CRIB domain were PCR-amplified with the primers 5`-GCGAGCTCCTGCCACCATGGCCCGGCCGCAGAGGACT-3` and 5`-GGAAACCTGGCGGAAATCTTGCAGGCTGATTAGCAAGGGTGG -3` and 3` fragment with primers 5`- CCACCCTTGCTAATCAGCCTGCAAGATTTCCGCCAGGTTTCC -3`and 5`-CGGAATTCTCAGAGGCTGAAGCCACTACCATC-3`. These two fragments are adjoined by PCR with the primers 5′-GCGAGCTCCTGCCACCATGGCCCGGCCGCAGAGGACT-3` and 5`-CGGAATTCTCAGAGGCTGAAGCCACTACCATC-3` and the resulting fragment was cloned into the SacI and EcoRI sites of pCAGJC.

7) pCAGJC-myc-PAR3b

The expression cassette of Myc-tagged PAR3b was cut from pk-myc-PAR3b (Joberty et al., 2000) with HindIII and EcoRI and cloned into the same sites of pCAGJC.

8) pCAGJC-myc-PAR3b S824A

To mutate PKCζ-phosphorylation site of PAR3b, I used *in vitro* site-directed mutagenesis kit (Quick Change™) from Agilent technologies (Santa Clara, CA). According to the manufacturer's manual, pCAGJC-myc-PAR3b was PCR-amplified with the primers 5`-GAAGGATTTGGACGTCAGGCTATGTCAGAAAAACGCACA-3` and 5`-

TGTGCGTTTTTCTGACATAGCCTGACGTCCAAATCCTTC-3`. The PCR reaction mixture was treated with DpnI to remove the template strands and used for transformation. Several colonies were grown for plasmid preparation. The mutation of each plasmid was confirmed by sequencing analysis.

9) pCAGJC-dTomato: pC1-dTomato (a kind gift from Dr. Natsuko Kumamoto, Osaka University, Japan),containing a dTomato version of pC1-EGFP (Clontech) was digested by NheI and the resulting sticky ends were blunted by Klenow. The fragment including dTomate expression cassette and multiple cloning sites excised with XmaI and was cloned into the XhoI (Klenow-filled in) and XmaI sites of pCAGJC.

10) pCAGJC-dTom-Cdc42 WT and pCAGJC-dTom-Cdc42 DN (T17N): Cdc42 expression cassette of pcDNA3-EGFP-Cdc42 wild type (WT) or dominant negative (DN) was excised by BsrGI and XhoI and then cloned into the same sites of pCAGJC-dTomtato.

11) pCAGJC-dTom-Cdc42 CA (Q61L): To mutate wild type of Cdc42 into constitutively active (CA) form, I used *in vitro* site-directed mutagenesis kit (Quick Change™) from Agilent technologies (Santa Clara, CA). According to the manufacturer's manual, pCAGJC-dTom-Cdc42 WT was PCR-amplified with the primers 5`-TTTTTGATACTGCAGGGCTAGAGGATTATGACAGATT-3` and 5`-AATCTGTCATAATCCTCTAGCCCTGCAGTATCAAAAA-3`. The PCR reaction mixture was treated with DpnI to remove the template strands and transformed. Several colonies were grown for plasmid preparation. The mutation of each plasmid was confirmed by sequencing analysis.

12) pCAGJC-dTom-Rac1 WT, pCAGJC-dTom-Rac1 DN(T17N) and pCAGJC-dTom-Rac1 CA(G12V): Expression cassettes of pCMV-HA-Rac1 wild type, dominant negative and constitutively active Rac1 (kind gifts from Dr. Simon Halegoua at Stony Brook University) were amplified by PCR with the primers 5`-CCGCTCGAGCTATGCAGGCCATCAAGTGTGTGG-3` and 5`-CGGAATTCTTACAACAGCAGGCATTTTCTC-3` and cloned into the XhoI and EcoRI sites of pCAGJC-dTomato

2.2 Methods

The purification of NG2 extracellular domain (NG2 ECD); To obtain the pure form of

NG2 ECD, NG2 ECD-expressing stable cell line (Clone 6.11) was cultured as previously described (Tillet et al., 1997; Ughrin et al., 2003). To obtain NG2 ECD-containing supernatant, the stable cell line was cultured in Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS), until the culture became confluent. Cells were then washed with serum-free DMEM twice and fed with a half of regular volume of serum-free DMEM. After 2~3 days later, the culture supernatant was collected and filtered to remove cells and debris.

To purify NG2 ECD, I used ÄKTA prime™ FPLC system (GE healthcare life sciences, Piscataway, NJ). 100~200 ml of the culture supernatant was loaded onto a DEAE ion exchange column (HiTrap, DEAE Sepharose Fast Flow, 5 ml, GE healthcare life sciences) pre-equilibrated with buffer A (50 mM Tris pH 8.0, 0.2 M NaCl). After all of supernatant was flowed through, the column was washed with additional 100 ml of buffer A at the pressure of 0.5 MPa and 2 ml/minute of flow rate. The binding fraction was eluted with 100 ml of 0.2 M to 0.8 M NaCl linear gradients. The resulting fractions were analyzed using SDS-PAGE followed by silver staining and immunoblotting with anti-NG2 antibody (Levine and Stallcup, 1987). The fractions containing pure NG2 were pooled, dialyzed in phosphate buffered saline, pH 7.4 (PBS) overnight. The dialyzed protein was then concentrated by using Centricon® (100kDa, Millipore), aliquoted and stored at -80 °C.

Primary culture of cerebellar granule neurons; Cultures of dissociated cerebellar granule neurons (CGNs) were prepared as described previously (Dou and Levine, 1994). Briefly, cerebella were dissected from the brains of P7~9 rats. After the pial layers were removed, the tissues were treated with 0.025 % trypsin PBS for 20 minutes at 37 °C and dissociated into single-cell suspension by pipetting in DMEM containing 10 % FBS. Cells were washed twice with L-15 medium and suspended in DMEM containing 10 % FBS. Neurons were plated onto either a glass coverslip coated with 50 μg/ml of poly-L-lysine (PLL) and protein substrates or a culture dish coated with 25 μg/ml of PLL.

Primary culture of hippocampal neurons; For primary culture of hippocampal neurons, embryonic day 18 Sprague/Dawley hippocampi were purchased from BrainBits (Springfield, IL). Neurons were isolated and dissociated from the tissues by following the manufacturer's protocol. In brief, hippocampal tissues were incubated with 2 mg/ml papain (Worthington) in Hibernate® E-Calcium (Invitrogen) at 30 ºC for 30 minutes, followed by

trituration in Hibernate/B-27 media. Removing undispersed pieces by gravity, cells were resuspended in plating medium (B-27/Neurobasal + 0.5 mM Glutamax® +25 μM glutamate). Neurons were plated at a density of 5~10 X 10^3 cells/cm^2 onto the glass coverslips coated with 50 μg/ml of poly-L-Lysine (Sigma) or 50 μg/ml of poly-L-Lysine + 10 μg/ml NG2 and cultured at 37 °C and 5 % CO2 for 24~48 hours. Cells were then fixed with 4 % paraformaldehyde/4 % sucrose in 0.1 M phosphate buffer pH 7.4 and stained with Alexa Fluor® 488-conjugated phalloidin and anti-tau antibody.

Analysis of neuronal polarity; To quantify the polarization of cells, five to ten fields per coverslip were randomly selected and imaged with fluorescence microscopy (Axio imager system, Carl Zeiss). Axon and dendrite were determined by immunostaining; a tau-positive process extending more than twice a cell diameter was defined as an axon. Phalloidin-positive neurites were defined as dendrites. More than 130 cells for each group were examined from three independent experiments.

Immunocytochemistry; Cultures were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4, blocked and permeabilized with blocking solution (2 % donkey serum, 0.02 % Sodium-deoxycholate, 0.01 % NP-40 in phosphate buffered saline, pH 7.4) for 30 minutes. Cells were then incubated with primary antibodies diluted in blocking solution for 1 hour. The primary antibodies and their dilution ratios were as follows; anti-PKCζ (1:200) (Santa Cruz biotechnologies), anti-PAR3 (1:100) (Upstate Biotechnologies), monoclonal anti-Tau (1:100) (Millipore) and Neuronal Class III β-Tubulin (Tuj1) monoclonal antibody (1:500) (Covance). After washing 3 times with blocking solution, Alexa Fluor® 488 or 594-conjugated donkey anti-mouse or donkey anti-rabbit secondary antibodies (diluted in 1:1000, 30 minutes incubation, Molecular Probes) were used to detect the primary antibodies. After washing 3 times with blocking solution and twice with PBS, the cover slips were mounted on slides in Fluoremount G™ (Southern Biotech, Birmingham, AL).

Neurite outgrowth assay; Neurite outgrowth assay were performed as described previously (Dou and Levine, 1997). Briefly, CGNs were plated at the density of 1 X 10^4 cells onto the 15 mm glass coverslips treated either with 50 μg/ml of PLL and 2 μg/ml of laminin alone (control substrate) or PLL, 2 μg/ml of laminin along with 10 μg/ml of NG2 ECD (NG2 substrate). Cells were cultured in DMEM containing 10% FBS for 20~24 hours, fixed with 4%

paraformaldehyde in 100 mM phosphate buffer and stained with a neuron-specific marker. To quantify the average neurite length per cell, five to ten fields per coverslip were randomly selected and imaged with fluorescence microscopy (Axio imager system, Carl Zeiss). A process extending more than a cell diameter was defined as a neurite. Length was measured from the axon initial segment to the tip of a neurite by using line measurement tool in Metamorph™ (Molecular Device). For cells that had more than one neurite, only the longest one was measured. In genetic modification experiments, only transfected cells marked by dTomato expression were counted for analysis. Neurite length data were then logged onto Excel™ spreadsheet (Microsoft), and mean neurite length per cell was calculated. Each experiment was repeated at least 3 times unless otherwise noted and comprised duplicate coverslips for each condition. A minimum of 300 cells was measured for each experimental condition.

Adult mouse DRG culture and neurite measurement; DRGs were dissected from six weeks old mice and treated with dispase® II (Roche) and collagenase (Sigma) and titurated. Approximately 1000 cells were plated onto a 15 mm glass coverslip treated either with 50 μg/ml of PLL and 2 μg/ml of laminin alone (control substrate) or PLL, 2 μg/ml of laminin along with 10 μg/ml of NG2 ECD (NG2 substrate). Cells were cultured in Neurobasal® A- supplemented with B-27® for 48 hours, fixed with 4 % paraformaldehyde in 100 mM phosphate buffer and stained with anti-Tuj antibody (1:1000). Cells were visualized by using Alexa Fluor® 488 or 594-conjugated donkey anti-rabbit secondary antibodies (1:1000).

To quantify the average neurite length per cell, five to ten fields per coverslip were randomly selected and imaged with fluorescence microscopy (Axio imager system, Carl Zeiss) for each group. The images of DRGs were binarized and skeletonized with black (background) and white (cell body and neurites) at Metamorph® software. The total white pixel numbers of each cell were counted, logged onto Excel™ spreadsheet (Microsoft) and mean of pixel number per cell was calculated.

Imaging analysis of the subcellular localization of PKCζ and PAR3; CGNs were prepared from P5~P7 rat as previously described. 1 X 10^4 CGNs were plated onto a 15 mm glass coverslip treated either with 50 μg/ml of PLL and 2 μg/ml of laminin alone (control substrate) or PLL, 2 μg/ml of laminin along with 10 μg/ml of NG2 ECD (NG2 substrate). Cells were cultured in DMEM containing 10 % FBS for 20~24 hours, fixed with 4 % paraformaldehyde in 100 mM

phosphate buffer and stained with either anti-PKCζ antibody (1:1000) or anti-PAR3 antibody (1:100). Cells were visualized by using Alexa Fluor® 488 or 594-conjugated donkey anti-mouse or donkey anti-rabbit secondary antibodies (1:1000). An Olympus FluoView® 1000 confocal laser-scanning microscope was used to imaged the immunostained cells. FluoView® 1000 viewer and Metamorph® software were used for the analysis of each image. 10~15 of multiple Z stack images per a cell were acquired within the planes that include the cell body.

For PKCζ imaging, in each acquired image file, a plane containing the middle part of the cell body was selected and exported for the analysis at Metamorph® software. A pixel intensity distribution was measured across the selected cell body image with linescan function of Metamorph® software. The distribution data were exported to and the histogram was plotted in Excel spreadsheet™.

For PAR3 imaging, in each acquired image file, a plane containing the brightest PAR3 signal at the axonal compartment was selected and exported to Metamorph® software. To measure PAR3 signal of axon, a line was traced along the axon of each cell and its average pixel intensity was measured with linescan function of Metamorph® software. For PAR3 signal of each cell body, a circle was traced along the edge of each cell and average pixel intensity of the area was measured with area measurement tool of Metamorph® software. To calculate PAR3 signal ratio between soma and axon of each cell, mean average pixel intensity of PAR3 at cell body was divided by that of axon.

Cell culture and transient transfections; Amaxa nucleofection system (Lonza AG) was used to deliver plasmids or siRNAs into primary neurons. For each nucleofection, $5X10^6$ of dissociated CGNs and either 0.75~2.5 μg of plasmid DNA or 10~200 pmole of siRNA (Dharmacon) was suspended in 100 μl of nucleofection solution and nucleofected with G-013 program. The cell suspension was immediately diluted with 1 ml of DMEM containing 10% FBS in the electroporation cuvette and 50~100 μl of cell suspension was plated onto a round glass coverslip with 15 mm diameter coated with PLL and protein substrates. For biochemical analysis, 2~3 X 10^7 cells were plated onto PLL-coated 60 mm culture dishes.

Lipofectamine 2000 (Invitrogen) was used for plasmid or siRNA transfection of HT22 cell, an immortalized mouse hippocampal cell line (Davis and Maher, 1994). 4 μg of plasmid

DNA and 10 μl of Lipofectamin 2000 were respectively diluted in each 250 μl of Opti-MEM I reduced serum medium (Invitrogen). After 5 minutes, diluted DNA and reagent were combined and incubated for 20 minutes at room temperature. Then, the transfection complexes were directly added to HT22 cell culture with 90~95% confluence in 35 mm culture dish. The cultures were incubated for 24 hours and subjected to treatments and the following biochemical analysis.

NG2 Treatment, western blot and immunoprecipitation; For biochemical analysis, either 5 X 10^6 non-transfected CGNs or 10 X 10^6 transfected CGNs were plated onto each 35 mm culture dish coated with 25 μg/ml of PLL and cultured in Neurobasal®-A with B-27 supplement for overnight. 3 X 10^5 HT22 cells were plated onto 35 mm culture dish and cultured in DMEM containing 10% FBS overnight.

Primary cells were starved in Neurobasal®-A without B-27 for 3~5 hours in the presence or absence of relevant compounds**.** HT22 cells were starved either in serum-free DMEM for 3~5 hours or in Neurobasal®-A with B-27 overnight followed by Neurobasal®-A without B-27 for 3~5 hours before treatment.

For acute treatment, purified NG2 were applied to the culture at the final concentration of 10~30 μg/ml for the indicated time. Cells were immediately washed twice with cold PBS and lysed in either 0.3 % or 1.0 % CHAPS lysis buffer (20 mM phosphate buffer pH 7.4, 150 mM NaCl, 1 mM EDTA and either 0.3 % or 1.0 % CHAPS) supplemented with proteinase and phosphatase inhibitor cocktail (Roche). Insoluble fractions were removed by centrifugation at 16000 g for 10 minutes and soluble fractions were subjected to immunoblotting or immunoprecipitation.

For immunoprecipitation, 1~2 μg of primary antibodies and 20 μl of protein G bead (Roche) were added to 100~500 μg of cell lysate and incubated under constant agitation for 2~4 hours or overnight at 4 °C. The resulting immunocomplex were then washed 4 times with the lysis buffer, resuspended in 25~50 μl of 2X protein sample buffer containing DTT and boiled for the following SDS-PAGE and immunoblotting.

Either 10~50 μg of cell lysate protein or 10~100% of immunoprecipitated complex per lane was loaded and separated on 8~12% SDS-PAGE gel. The proteins were then transferred onto PVDF membrane at 100 V for 60 minutes or 90 V for 3 hours using Mini trans blot system

(Bio-Rad). The blots were briefly washed twice with Tris-buffered saline with tween 20 (TBST, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05 % Tween20) incubated with primary antibodies diluted in TBST with 5% milk for overnight at 4 °C.

The primary antibodies and their dilution ratios were as follows; Anti-PKCζ antibody (1:1000), anti-Rac1 antibody (1:1000) and anti-Myc antibody (1:1000), Anti-Phospho-PKCζ/λ antibody (1:1000), Anti-PAR3 antibody (1:2000), Monoclonal anti-FLAG®M2 antibody (1:2000), anti-β-actin antibody (1:50000), and Living Colors® DsRed Polyclonal Antibody (1:2000).

The blots were then washed three times for 5 minutes each with TBST and incubated horseradish peroxidase-conjugated secondary antibodies (1:2000; GE healthcare life science) diluted in TBST with 5% milk for 30 minutes to 2 hours. Then, the blots were washed three times with TBST for each 5 minutes, developed with Amersham ECL kit (GE healthcare life science) and exposed to HyblotCL autoradiograph film (Denville Scientific) to detect specific chemoluminescence.

Cdc42 activation assay and *in vitro* GDP and GTP loading: HT22 cells were transfected with dTomato-tagged Cdc42 (dTom-Cdc42) and Myc-tagged PAR6 (Myc-PAR6). For in vitro loading, EDTA was added to cell lysates to 20 mM final concentration. The lysates were then incubated with either 1 mM GDP or 100 μM GTPγS with constant agitation at 30 °C for 30 minutes. The reactions were stopped by adding $MgCl_2$ (to 60 mM, final concentration) and used for the subsequent immunoprecipitation procedure.

***In vitro* kinase assay;** The activity of PKCζ was assayed by measuring ^{32}P-incorporation to PKCζ-specific peptide substrate (Kazanietz et al., 1993; Nishikawa et al., 1997). Recombinant plasmid of FLAG PKCζ (pICG2-FLAG PKCζ) was transfected into CGNs and expressed for overnight. The cells were serum-starved for 3~5 hours and treated with 10 μg/ml of NG2 ECD for 10~30 minutes. After treatment, cells were immediately washed with cold PBS and lysed in 0.3 % CHAPS lysis buffer (20 mM phosphate buffer pH 7.4, 150 mM NaCl, 1 mM EDTA and 0.3 % CHAPS) supplemented with proteinase and phosphatase inhibitor cocktail (Roche). The insoluble fraction was removed from the lysates by centrifugation at 16000 g for 10 minutes. 20~100 μg of each lysate was immunoprecipitated with 1 μg of anti-FLAG antibody and 20 μl of

Protein G bead for 2 hours under constant agitation. The beads and immunocomplex were centrifuged and washed in 0.3 % CHAPS lysis buffer. After repeating the washing 3 times, the bead and immunocomplex were then washed once with and resuspended in 40 µl of Kinase buffer (8 mM MOPS, pH 7.0, 1mM EDTA) and 2~8 µl of resuspension was diluted in the reaction mixture containing 50 µM peptide substrate and 20 µg/ml phosphatidylserine. The assay mixtures were preincubated at 30 °C for 10 minutes. The reaction was started by adding 5 µCi of [γ^{32}P]ATP. After 20 minutes, 20 µl of assay mixture was spotted onto P81 paper (Upstate) to stop the reaction. The paper was washed 3 times with 0.75% phosphoric acid for 5 minutes each, once with acetone for 5 minutes, air-dried and transferred into a scintillation vial. Each 10 ml of scintillation cocktail was added to the each vial and the radiation activity of each reaction was measured in the scintillating counter LS 6500 (Beckman).

Statistics; Statistical analysis was performed using SigmaStat software (Systat Software, San Jose, CA), and either student's t-test or Mann–Whitney rank sum test were used. For comparison of multiple data sets with normal distribution, one-way ANOVA test was used followed by post-hoc Tukey's test. For data sets that failed to pass normality test, ANOVA on ranks was used followed by Dunn's test. Data are presented as mean ± standard error (SE). $p < 0.05$ is considered significant.

Chapter 3 PKCζ is required for axon growth inhibition

3.1 Introduction

Conventional PKCs (PKCα and PKCβ) are known to be involved in axon growth inhibition by myelin-associated inhibitors and CSPGs. It is known that PKC is involved in biochemical events at the growth cone. For instance, PKC phosphorylates GAP43 and this phosphorylation is related to maintaining growth cone stability (He et al., 1997). Inspired by this observation, Powell et al for the first time showed PKC mediates repulsive neurite guidance at an astrocyte boundary. In this work, they found that pharmacological down-regulation and inhibition of PKC signaling allows neurites to cross the boundary between permissive and non-permissive astrocytes. In addition, they showed that an anti-NG2 antibody increases the percentage of cells that cross the boundary (Powell et al., 2001). Together, these results suggest that some inhibitory guidance cues attached to non-permissive astrocytes, probably NG2, signal through PKC to induce the repulsive or inhibitory activity.

Recently, Hasegawa et al showed that myelin inhibitors signal through calcium and PKC to induce axon growth inhibition and growth cone collapse (Hasegawa et al., 2004). In their study, they showed that MAG and Nogo elevate intracellular Ca^{2+} by triggering Gi, a heterotrimeric GTP-binding protein and phospholipase C (PLC). This Ca^{2+} elevation is required for the inhibitory action of these inhibitors since inhibition of calcium signaling attenuates the growth inhibition. These myelin inhibitors induce the activation of conventional PKCs such as PKCα and PKCβ. Inhibition of conventional PKC signaling attenuates the outgrowth inhibition and growth cone collapse induced by MAG and Nogo. In addition, inhibition of calcium signaling prevents the activation of PKC and inhibitory action of MAG and Nogo peptide, suggesting that these two signaling components are linked to each other in myelin inhibition.

Another research group also observed the requirement for PKC in myelin- and CSPG-mediated inhibition (Sivasankaran et al., 2004). In their work, they showed that myelin inhibitors and CSPGs induce the activation of PKC and inhibition of PKC attenuates the inhibitory activity of myelin and CSPG inhibitor. Furthermore, they showed that infusion of PKC inhibitor stimulates dorsal column axon regeneration *in vivo*.

Collectively, the previous data indicate that the inhibitory effect of myelin inhibitors and inhibitory proteoglycans is mediated through conventional PKC signaling. How does PKC signaling cause outgrowth inhibition? Sivasankaran et al showed that PKC might regulate cytoskeleton through RhoA signaling (Sivasankaran et al., 2004) , which is linked to cytoskeleton stability. This connection between PKC and RhoA remains controversial. Hasegawa et al found that myelin inhibitors induce the activation of both PKC and RhoA but these events seem to be independent of each other. (Hasegawa et al., 2004). Therefore, it remains elusive how conventional PKC mediates the inhibitory action of myelin and glial inhibitors.

Suppression of conventional PKC partially attenuates the inhibitory activity of myelin and glial inhibitors, suggesting that other signaling components may also contribute to myelin and glial inhibition. In chapter 1, I claimed that the polarity complex might be involved in the inhibitory activity of extrinsic inhibitory cues with several lines of circumstantial evidence. PKCζ is a component of the PAR complex. Among PKC isoforms, PKCζ is the most relevant, to cell shape change since it regulates the dynamics of actin and microtubule through the PAR complex and microtubule-associated proteins. This led me to a hypothesis that extrinsic inhibitory cues may signal through the kinase to mediate their inhibitory action. Here, using NG2, an inhibitory proteoglycan as a model for extrinsic inhibitory cues, I address the question of whether PKCζ is required for axon growth inhibition.

3.2 Results

3.2.1 PKCζ is required for axon growth inhibition

From preliminary pharmacological experiments, it was found that broad range PKC inhibitors such as H7 completely reversed NG2-induced axon growth inhibition whereas inhibitors of conventional PKCs (Gö6976) or both conventional and novel PKCs (calphostin C) only partially restored normal neurite length (Some of the data are shown in Figure 3.1). A cell-permeable PKCζ-specific pseudosubstrate peptide inhibitor (referred herein as ZIP) more robustly reversed the inhibition by NG2 than other isoform-specific inhibitors of PKC (Figure 3.1). NG2 also inhibited the growth of dorsal root ganglia (DRG) neurons while the zip peptide restored normal neurite length to adult mouse DRGs grown on NG2-coated surfaces (Figure 3.2). These observations suggest that NG2 requires an alternative signaling mechanism involving

PKCζ, whereas myelin-associated inhibitors and chick embryo CSPGs (a mixture of neurocan, phosphacan, versican, and aggrecan) require conventional PKC signaling (PKCα and PKCβ) (Elizabeth M. Powell, 2001; Sivasankaran et al., 2004).

To further examine the role of PKCζ in axon growth inhibition, I used siRNA to suppress PKCζ expression (Fire et al., 1998). To determine the ability of PKCζ-specific siRNA to suppress PKCζ expression, various amount of PKCζ siRNA along with HA-PKCζ was transfected into HT22 cells. 24 hours after transfection, PKCζ levels of each cell lysate were measured by immunoblotting with an anti-PKCζ antibody. Transfection of a siRNA pool directed against PKCζ effectively but not transfection of a scrambled siRNA reduced PKCζ level of HT22 cells (Figure 3.3a). A similar examination was conducted in primary cells. Rat cerebellar granule neurons were electroporated with various amount of either scrambled or PKCζ-specific siRNA. 24 hours after the transfection, PKCζ level of each cell lysate was measured by immunoblotting. Transfection of a siRNA pool directed against PKCζ but not a non-targeting pool effectively reduced PKCζ level of CGNs (Figure 3.3b). To investigate the effects of PKCζ suppression in axon growth inhibition, siRNA-transfected granule neurons were grown on control substrate or NG2 substrate. Transfection of CGNs with scrambled siRNA did not affect axonal growth on control substrate or NG2 substrate. The transfection of CGNs with PKCζ siRNA allowed the neurons to extend significantly long axons on NG2 substrate (Figure 3.4), suggesting a requirement of PKCζ for axon growth inhibition.

To further explore the requirement of PKCζ in axon growth inhibition, I used a dominant-negative form of the kinase to suppress PKCζ activity and examined its effect on NG2-induced inhibition (Wolf et al., 2008). CGNs were transfected with 1~2 μg of kinase-deficient PKCζ ($PKC\zeta^{K281W}$), grown on either control or NG2 substrate for 24 hours and fixed for imaging and measurement of the neurite length. As shown in Figure 3.5, the transfection of CGNs with $PKC\zeta^{K281W}$ but not wild-type PKCζ allowed the cells to extend significantly long axons on NG2 substrate but not that of wild type PKCζ. $PKC\zeta^{K281W}$ was less effective in restoring axon growth on NG2 substrate than PKCζ siRNA, presumablely due to its inhibitory effect on basal growth of axon or insufficient expression level (Figure 5.2).

3.2.2 NG2 activates PKCζ

NG2 increases the phosphorylation of PKCζ; The requirement of PKCζ for axon growth inhibition suggests that NG2 may activate the kinase. The activation of PKCζ consists of two steps; the phosphorylation of threonine 410 (Thr^{410}) releases the pseudosubstrate domain of the kinase, thereby allowing autophosphorylation of threonine 560 residue (Thr^{560}) to render the enzyme completely active (Hirai et al., 2003). Therefore, I measured the phosphorylation of Thr^{410} as an indication of the activation of PKCζ. Rat CGNs were grown overnight, treated with soluble NG2 and lysed. PKCζ and the phosphorylated form of PKCζ were detected by immunoblotting with anti-PKCζ and anti-phospho-PKCζ antibodies, respectively. The signals of phosphorylated PKCζ were normalized to those of total PKCζ and the resulting ratios (p-PKCζ/PKCζ) were compared to the ratio of control treatment for relative comparison. As shown in Figure 3.6, the treatment with NG2 led to the rapid phosphorylation of PKCζ at Thr^{410}. This increase was sustained in the presence of NG2, suggesting that NG2 CSPG indeed induces the activation of PKCζ.

NG2 increases the activity of PKCζ; Since measuring phosphorylation is not a direct measurement of kinase activity, I developed an *in vitro* kinase assay for the direct measurement of PKCζ activity. Multiple isoforms of PKC appear to be involved in the inhibition by NG2 CSPG as mentioned earlier. Most anti-PKCζ antibodies target the C-terminus of the protein whose amino acid sequences are highly homologous among PKC isoforms. Therefore, immunoprecipitating endogenous proteins by using these antibodies may cause non-specificity in the assay. To resolve these problems, I took advantage of epitope-tagged recombinant PKCζ (FLAG-PKCζ). FLAG-PKCζ was overexpressed in HT22 cells (Davis and Maher, 1994), a neuronal cell line which responds to short-term treatment with NG2 CSPG with an increase in Thr^{410} phosphorylation (Figure 3.7). The recombinant kinase in the cell lysates was then immunoprecipitated by anti-FLAG antibodies. The immunoprecipitates were washed with detergent-containing lysis buffer three times, equilibrated with and resuspended in small volume of detergent-free kinase assay buffer. They were then incubated with [γ^{32}P]ATP and PKC isoform-specific peptide substrates in the absence or presence of isoform-specific PKC inhibitors to measure the kinase activity and specificity of immunoprecipitates. As shown in Figure 3.8a, the immunoprecipitates efficiently phosphorylated the peptide substrates for atypical and novel

PKC but not the one for conventional PKC as previously reported (Kazanietz et al., 1993; Nishikawa et al., 1997). Furthermore, a PKCζ-specific inhibitor significantly reduced ^{32}P-incorporation to atypical and novel PKC peptide substrate, whereas Gö6976, a conventional PKC inhibitor was ineffective.

Since these results suggest that this assay measures PKCζ activity with specificity and sensitivity, I performed this assay in NG2-treated HT22 cells. The lysates of cells were immunoprecipitated and ^{32}P-incorporation was measured as described above. As shown in Figure 3.8b, short-term treatment with NG2 increases ^{32}P-incorporation to the substrate in HT22 cells. Similar to this result, in CGNs, NG2 CSPG induced a significant increase of PKCζ activity in CGNs (Figure 3.9).

NG2 induces PKCζ translocation to the plasma membrane; When activated, PKC isoforms are translocated from a cytoplasmic compartment to the plasma membrane (Kazi and Soh, 2007; Petit et al., 2005). Consistent with these results, there was a significant increase in the amount of PKCζ associated with the plasma membrane and a decrease in the cytoplasmic pool of PKCζ after growth on NG2 (Figure 3.10). Short-term treatment of the cultures with phorbal 12-myristate 13-acetate (PMA) had no effect on the compartmentalization of PKCζ whereas the addition of the zip peptide (2μM) 3 hr before lysis prevented this increase in membrane association (not shown).

To further explore the translocation of PKCζ by NG2, CGNs were grown either on control substrate or NG2 substrate for 24 hours, fixed and stained with a PKCζ-specific antibody. The distribution of immunofluorescence was analyzed by using confocal microscopy and imaging software. PKCζ signal was evenly distributed in the cytoplasm of the cells grown on control substrate whereas the signal was concentrated at the plasma membrane of cells grown on NG2 substrate. (Figure 3.11). To confirm this result, CGNs were transfected to express a green fluorescence protein (GFP)-tagged PKCζ (PKCζ–EGFP), grown on control substrate or NG2 substrate, fixed and the distribution of GFP signal was analyzed. As shown in the bottom panel of Figure 3.11, there was an increase in the association of GFP signal with the plasma membrane of the cells grown on NG2 substrate. These observations suggest that substrate form of NG2 CSPG induces the translocation of PKCζ to the membrane.

3.2.3 PKCζ is sufficient for axon growth inhibition

Both the requirement for and the activation of PKCζ suggest that upregulation of the kinase activity alone is sufficient to inhibit axon growth. To explore this, I created two phosphomimetic forms of PKCζ (PKCζ^{T410E} or PKCζ^{T410,560E}) by converting either Thr^{410} or both Thr^{410} and Thr^{560} to glutamic acid, a phosphomimetic residue of threonine (Hirai et al., 2003). To examine the effect of phosphomimetic forms of PKCζ in axon growth, rat CGNs were transfected with 1~2μg of these mutant forms of PKCζ. They were then grown for 22~24 hours on control substrate, and fixed for imaging and measurement of neurite length. As shown in Figure 3.12, expression of either PKCζ^{T410E} or PKCζ^{T410,560E} significantly reduced axon lengths on control substrate, which is an effect comparable to NG2-mediated inhibition.

3.2.4 NG2 promotes polarization and axon formation of hippocampal neurons

Atypical PKC plays an important role in the establishment of cell polarity. In hippocampal neuronal polarity, suppression of PKCζ inhibits axonal development from immature neurites (Chen et al., 2006a; Schwamborn and Puschel, 2004; Shi et al., 2003). Therefore, enhancement of PKCζ activity may either accelerate the polarization or promote axon formation in this type of neuron. To evaluate this idea, dissociated hippocampal neurons were grown either on poly-L-lysine-coated surfaces (control substrate) or NG2-coated surfaces (NG2 substrate) in the absence or presence of zip peptide. Cells were then fixed at day *in vitro* (DIV) 1 or DIV 2, stained with fluorescent phalloidin and immunostained with an anti-tau antibody and imaged by fluorescence microscopy. The imaged cells were quantified and scored by their developmental stages (Dotti et al., 1988) and the number of axons per cell. As shown in Figure 3.13, most of the cells grown on control substrate extended either a single axon or no axon. There was, however, a significant increase in the number of neurons with either a single or multiple axons when the cells were grown on NG2 substrate. These results suggest that NG2 functionally activates PKCζ, promoting axon formation in hippocampal neurons.

3.3 Discussion

Here I showed that PKCζ is required for the outgrowth inhibition by NG2. This inhibitory proteoglycan induces the activation of PKCζ, judging from NG2-induced increase in the phosphorylation, kinase activity and membrane translocation of PKCζ. In addition, I showed

that PKCζ is sufficient for axon growth inhibition. Together, these results suggest that NG2 signals through PKCζ to mediate its inhibitory activity.

This finding is unique, compared to the previous reports where conventional PKC (PKCα and PKCβ) signaling is required for axon growth inhibition (Hasegawa et al., 2004; Powell et al., 2001; Sivasankaran et al., 2004). Since inhibition of conventional PKC attenuates significantly, but partially, the outgrowth inhibition, it is possible that additional but unidentified signaling components contribute to axon growth inhibition. Consistent with this idea, in preliminary pharmacological experiments, broad range PKC inhibitors such as H7 completely reversed NG2-induced axon growth inhibition (data not shown) whereas inhibitors of conventional PKCs (Gö6976) only partially restored normal neurite length (Figure 3.1). Together, these data suggest that extrinsic inhibitory molecules may recruit multiple PKC isoforms to mediate their inhibitory activity and PKCζ may function as a major signaling component.

Another explanation is that NG2 signals mainly through PKCζ whereas myelin inhibitors and other inhibitory proteoglycans recruit PKCα and PKCβ to mediate their inhibitory effect. It is noteworthy that the chick embryo CSPG in the previous studies is a crude extract whose major components are neurocan, phosphacan, versican, and aggrecan (Hasegawa et al., 2004; Sivasankaran et al., 2004). Consistent with this idea, pharmacological inhibition of PKCζ failed to reverse the inhibition by the CSPG mixture (Figure 3.14), suggesting that PKCζ is a unique signaling mechanism for the inhibitory action of NG2.

Recently, RPTPσ was identified as a receptor for CSPG. In this report, the authors showed that neurocan binds specifically to RPTPσ and removal of GAG chains reduces their interaction. They also showed that RPTPσ deficiency reduces inhibitory effect of aggrecan on neurite outgrowth and allows sensory neurons to extend further their axons in a spinal cord lesion (Shen et al., 2009). Several results, however, suggest that NG2 is a unique proteoglycan from other CSPGs. For example, compared to chick embryo CSPG, NG2 binds weakly to the receptor. (Given the stickiness of NG2, this binding might not represent specific interaction.) In general, CSPGs, such as neurocan and aggrecan, rely mainly on their GAG chains for the interaction with the receptor and inhibitory activity. The inhibitory activity of NG2 is, however, a property of protein core but not its GAG chains, as reviewed earlier. Altogether, these arguments suggest that, NG2 activates unidentified receptor systems and recruits PKCζ to

mediate its inhibitory activity whereas other inhibitory proteoglycans signal through RPTPσ and conventional PKC signaling

The requirement for and activation of PKCζ suggest that the activation of PKCζ is a mechanism by which NG2 inhibits axon growth. How does the activation of PKCζ cause axon growth inhibition? Given that overexpression of phosphomimetic forms of PKCζ phenotypically copies the inhibitory action of NG2, it is reasoned that dysfunctional upregulation of the kinase may cause axon growth inhibition. In this sense, it is noteworthy that balanced small GTPases (Cdc42/ Rac1) activity is critical for proper axon outgrowth (Kaufmann et al., 1998; Luo et al., 1994). These studies showed that either increasing or decreasing the activity of these small GTPases prevents axon outgrowth *in vivo*. Consistent with this result, Nishimura *et al.* showed that expression of a constitutively active form of Cdc42 or Rac1 attenuates axonal growth in cultured hippocampal neurons (Nishimura et al., 2005). Furthermore, collapsin (semaphorin 3A), a repulsive guidance cue, requires the activation of Rac1 for its inhibitory activity (Jin and Strittmatter, 1997). Since PKCζ is linked to the regulation of Cdc42 and Rac1 through the PAR complex (Nishimura et al., 2005), balanced or a certain range of PKCζ activity is likely critical for balanced activity of the GTPases. Out of such optimal range, the kinase would cause hyper or hypo-activity of these small GTPases, that is likely unfavorable for axon elongation. Together, these suggest that NG2 may induce hyperactivity of PKCζ to collapse the balanced activity of the small GTPases, which presumably attenuates axon outgrowth.

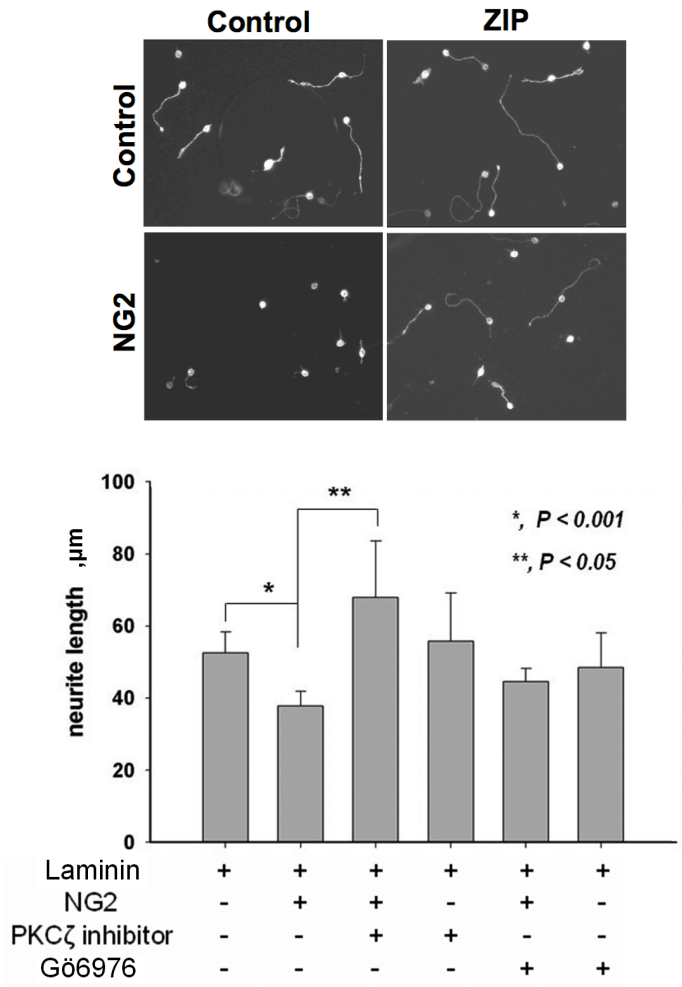


Figure 3.1. PKC signaling is required for axon growth inhibition.

Dissociated cerebellar granule neurons (CGNs) from postnatal rats were grown either on control substrate (laminin) or on NG2 substrate (NG2) in the absence or presence of the indicated PKC inhibitors. A cell-permeable peptide inhibitor of PKCζ potently reversed NG2-induced growth inhibition whereas the conventional PKC inhibitor, Gö6976, had only partial effects. The upper panel shows typical cell fields and the histogram plots mean neurite lengths ± SEM. Control denotes coating coverslips with laminin. NG2 denotes substrates coated first with 2 µg/ml of laminin and subsequently with 10 µg/ml of NG2.

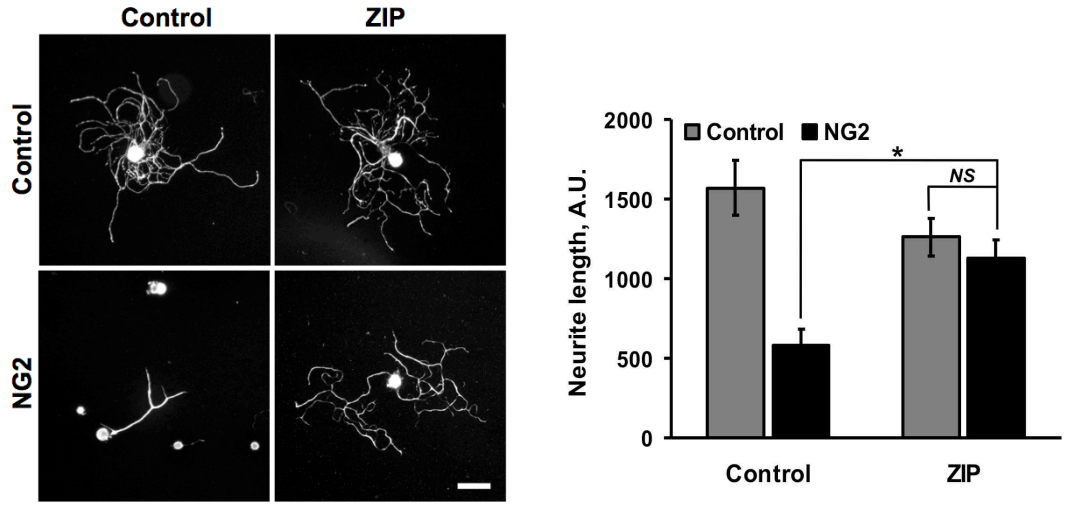


Figure 3.2. NG2 inhibits neurite outgrowth of DRG neurons.

Dissociated DRG neurons from adult mice were grown either on control substrate or on NG2 substrate in the absence or presence of a cell-permeable peptide inhibitor of PKCζ, ZIP. The pharmacological suppression of PKCζ allowed the neurite outgrowth of DRG neurons on NG2 substrate. The left panel shows typical cell fields (Scale bar 50 μm) and the histogram to the right plots mean neurite lengths ± SEM. Control denotes coating coverslips with laminin. NG2 denotes substrates coated first with 2 μg/ml of laminin and subsequently with 10 μg/ml of NG2 (n=1, > 36 cells were counted and scored in each condition; *$p < 0.001$; Dunn's post hoc test).

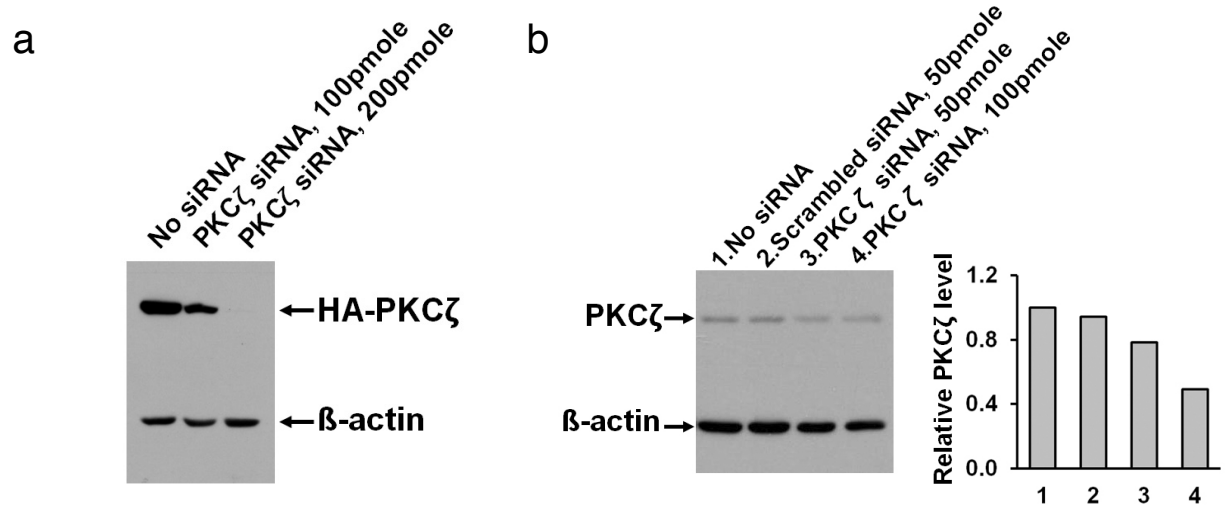


Figure 3.3. RNAi-mediated suppression of PKCζ

a) RNAi-mediated suppression of recombinant PKCζ. HT22 cells were transfected with HA-PKCζ and the indicated amount of siRNAs. The levels of HA-PKCζ in lysates were detected by immunoblotting with anti-HA antibody. The transfection of PKCζ efficiently reduced the level of recombinant PKCζ in dose-dependent manner. b) RNAi-mediated suppression of endogenous PKCζ. Rat CGNs were transfected with indicated amount of either scrambled siRNA or PKCζ-targeting siRNA. Cell lysates were analyzed by SDS-PAGE and immunoblotting with anti-PKCζ antibody. The histogram shows densitometric quantitation of the PKCζ-knockdown.

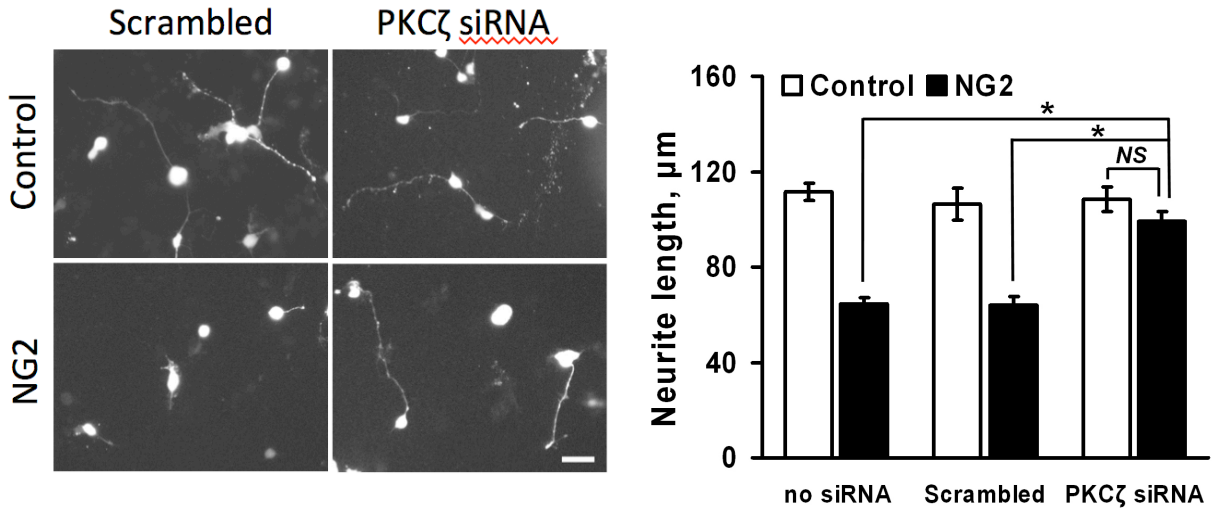


Figure 3.4. PKCζ is required for axon growth inhibition.

The effect of PKCζ siRNA on axon growth inhibition. 30 pmole of scrambled control or PKCζ-targeted siRNA along with 1.0 µg of dTomato plasmid (pCAGJC-dTomato) was transfected into CGNs. The left panel shows typical cell fields (Scale bar 50 µm) and the histogram to the right plots mean neurite lengths ± SEM (n=1 in scrambled siRNA experiment, n=3 in control and PKCζ siRNA experiment, * $p < 0.001$; Dunn's post-hoc test).

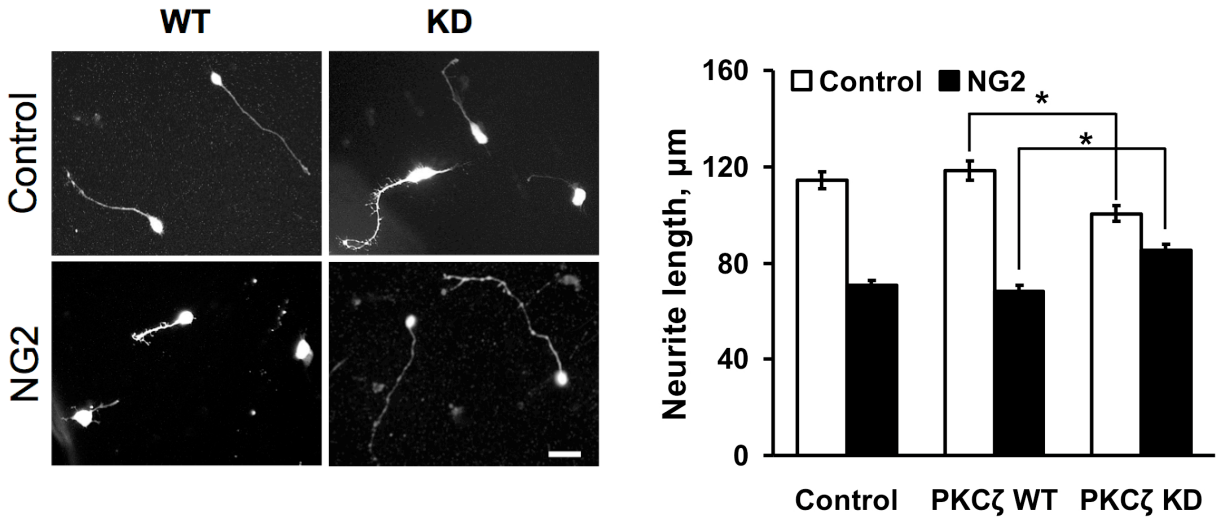


Figure 3.5. Kinase-dead (KD) PKCζ reverses axon growth inhibition.

The effect of kinase-deficient (KD) form of PKCζ in axon growth inhibition. 1~2 µg of indicated PKCζ expression plasmids along with 1 µg of dTomato plasmid was transfected into CGNs. (Scale bar 50 µm). The left panel shows typical cell fields and the histogram to the right plots mean neurite lengths ± SEM (n=3, * $p < 0.005$; Dunn's post-hoc test).

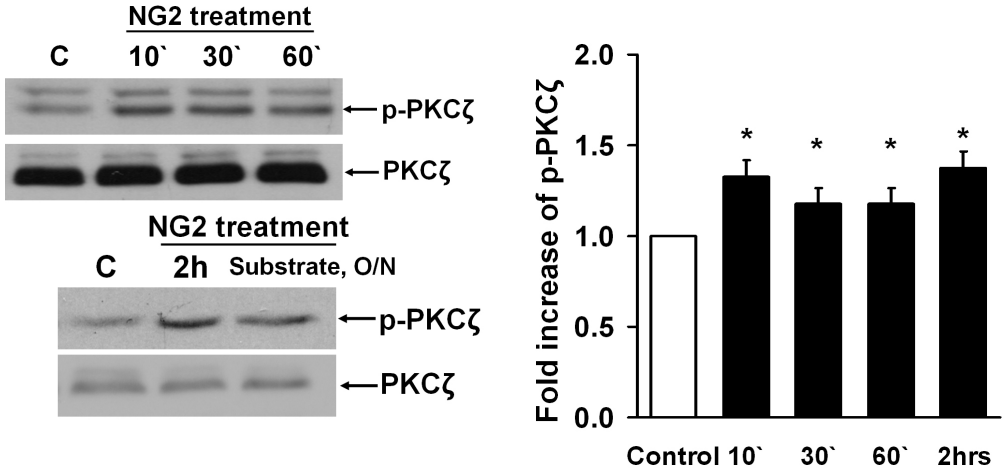


Figure 3.6. NG2 increases the phosphorylation of PKCζ.

PKCζ phosphorylated at T410 (p-PKCζ) was detected by immunoblotting with specific anti-phospho-PKCζ antibody. Bath application of soluble NG2 (top) or growth on NG2 coated substrates (bottom panel) induced the phosphorylation of PKCζ in CGNs. The histogram to the right shows the fold increase in phospho-PKCζ ± SEM relative to control cells after normalization to total PKCζ (n=3-6, * $P < 0.05$, Student's t-test).

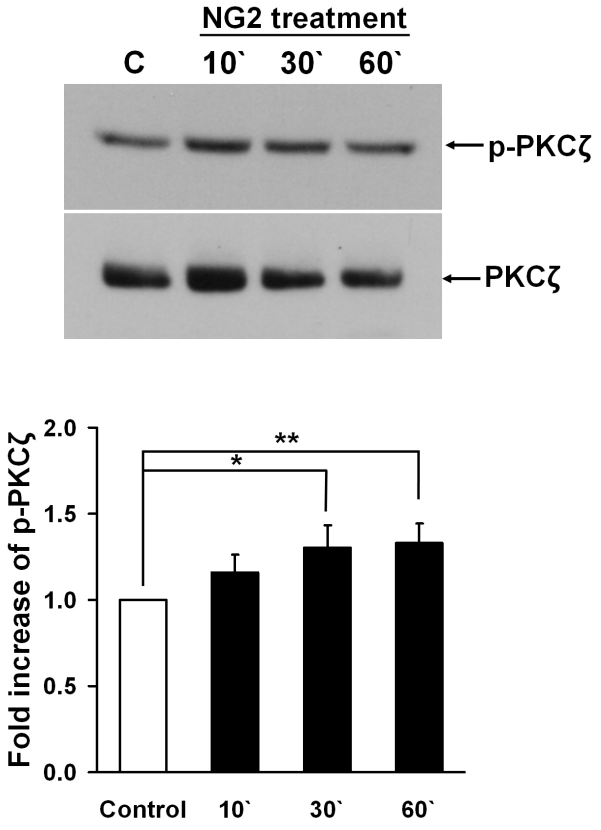


Figure 3.7. NG2 induces the phosphorylation and activation of PKCζ in HT22 cells.

PKCζ phosphorylated at Thr^{410} (PKCζ-P-Thr^{410}) was detected by immunoblotting with specific anti-phospho-PKCζ antibody. Bath application of soluble NG2 (10 μg/ml) induced the phosphorylation of PKCζ in HT22 cells. The histogram shows the average ratio of phospho-PKCζ/total PKCζ ± SEM (n=3, normalized to control, * P= 0.68, ** P < 0.05, Students' t-test).

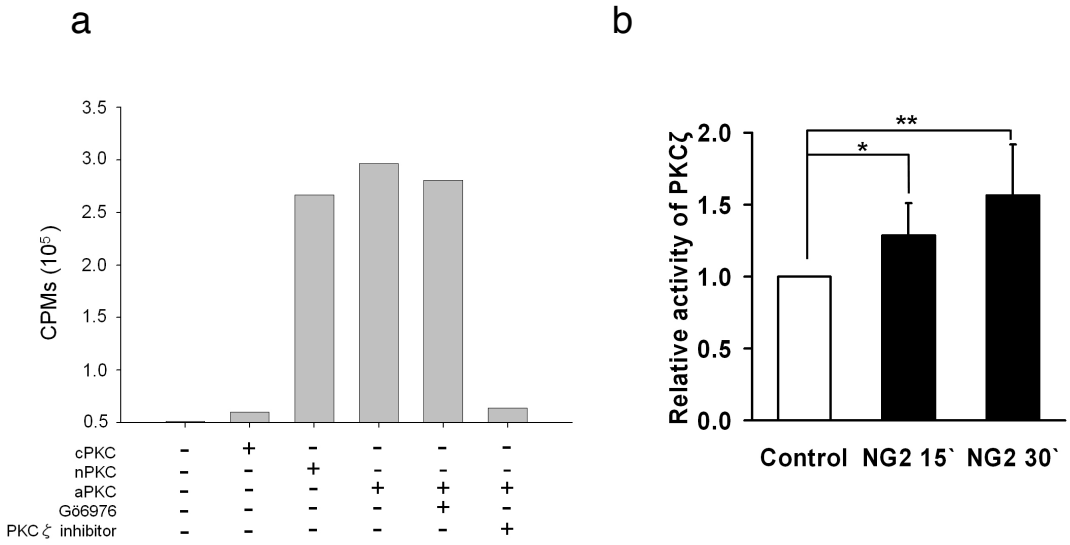


Figure 3.8. NG2 increases the activity of PKCζ in HT22 cells.

a) The *in vitro* kinase assay detects the activity of PKCζ with reasonable sensitivity and specificity. FLAG-PKCζ was immunoprecipitated from transfected HT22 cells and the immunoprecipitates were incubated with [Υ^{32}P] ATP, indicated peptide substrates and isoform-specific PKC inhibitors. The immunoprecipitates efficiently labeled the peptide substrate for atypical and novel PKC but not the peptide conventional PKC. PKCζ-specific inhibitor however reduced ^{32}P-incorporation to atypical and novel PKC peptide substrate, but not Gö6976, a conventional PKC inhibitor. b) PKCζ activity in HT22 cells measured using the in vitro kinase assay. Soluble NG2 (10 µg/ml) was bath-applied to FLAG-PKCζ-transfected HT22 cells for the indicated time. The recombinant kinase was immunoprecipitated and assayed as described in methods. The histogram in b shows the fold increase in the kinase activity of PKCζ ± SEM relative to control (n=3, normalized to control, * P= 0.16, ** P = 0.12, Students' t-test).

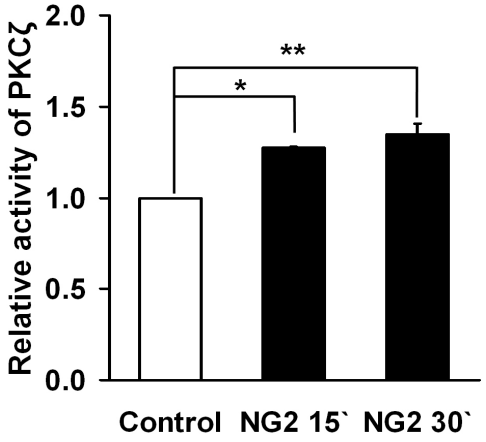


Figure 3.9. NG2 increases the activity of PKCζ in CGNs.

Rat CGNs were transfected with FLAG-PKCζ and soluble NG2 (10 μg/ml) was bath-applied for the indicated times. PKCζ activity was measured as described in Methods. The histogram shows the fold increase in the kinase activity of PKCζ ± SEM relative to control (n=3, normalized to control treatment; * $P < 0.01$; **$P < 0.05$, Students' t-test).

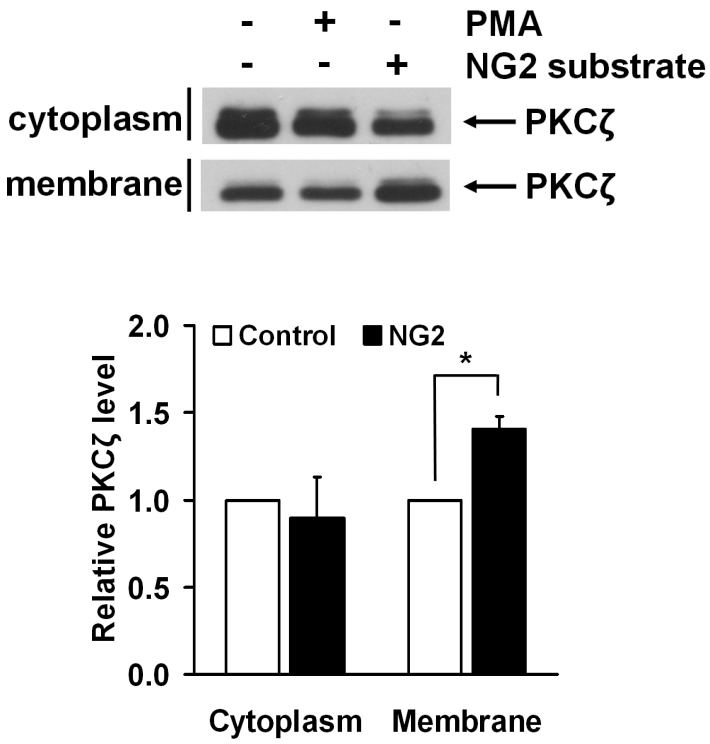


Figure 3.10. NG2 increases the translocation of PKCζ to the plasma membrane.

Enrichment of PKCζ in membrane fractions induced by NG2 treatment. CGNs were grown overnight on control or NG2-coated dishes. After cell lysis, cytoplasmic and membrane fractions were prepared as described in Methods and PKCζ in each fraction was detected by SDS-PAGE followed by immunoblotting with anti-PKCζ. Treatment of the cells with PMA had no effect on the compartmentalization of PKCζ. The histogram shows the fold increase in the level of PKCζ ± SEM relative to control (n=3, normalized to control treatment; * $P < 0.05$;, Students' t-test).

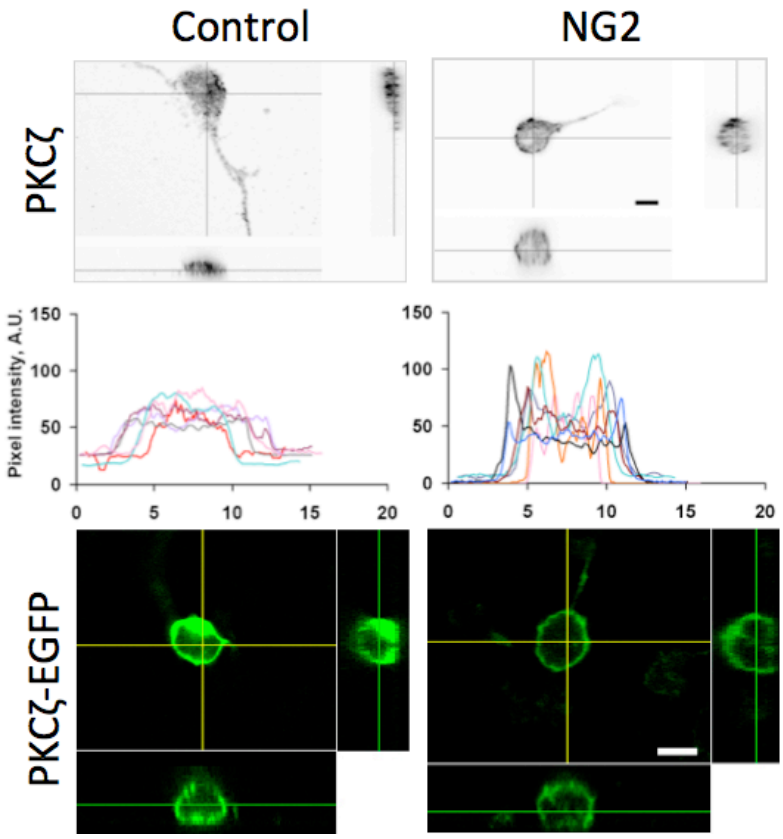


Figure 3.11. NG2 increases the translocation of PKCζ to the plasma membrane.

NG2 treatment translocates PKCζ to the membrane. Rat CGNs were plated on the surfaces coated either with laminin only or with laminin and NG2, grown for 24 hours, fixed and stained with anti-PKCζ antibody. Top panel shows that strong PKCζ immunoreactivity was found mainly at the membrane of cells grown on the NG2 coated substrate. Below is shown line scans of fluorescence intensity through the cell bodies (n= 6). Lower panel: Rat CGNs transfected with GFP-tagged PKCζ were grown on either control or NG2-coated substrates for 24 hours. The distribution of fluorescence was analyzed using confocal microscopy. Fluorescence was condensed at the membrane of cells grown on NG2. Scale bar 10 µm

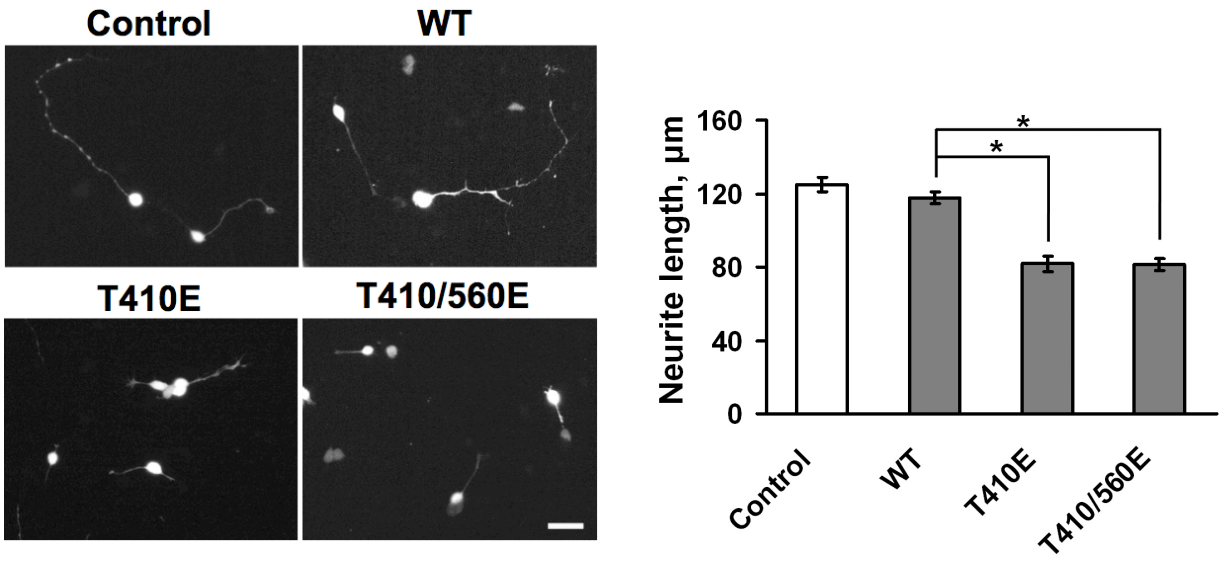
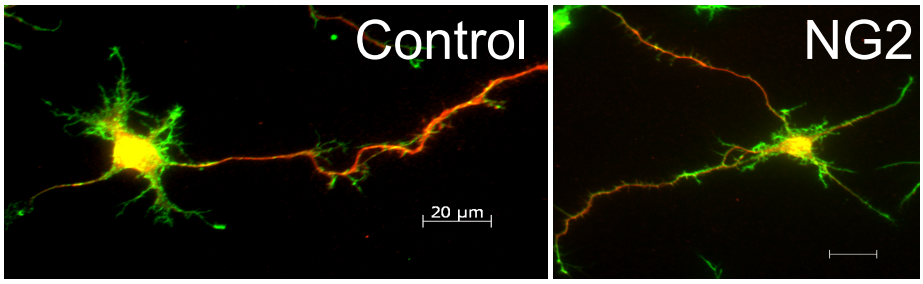


Figure 3.12. Phosphomimetic PKCζ induces axon growth inhibition.

The effect of phosphomimetic PKCζ on axon growth. 1 µg of indicated PKCζ expression plasmids along with 1 µg of dTomato plasmid were transfected into CGNs (Scale Bar 50 µm). The histogram shows average neurite lengths ± SEM (n=3, * $p < 0.001$; Dunn's post-hoc test).



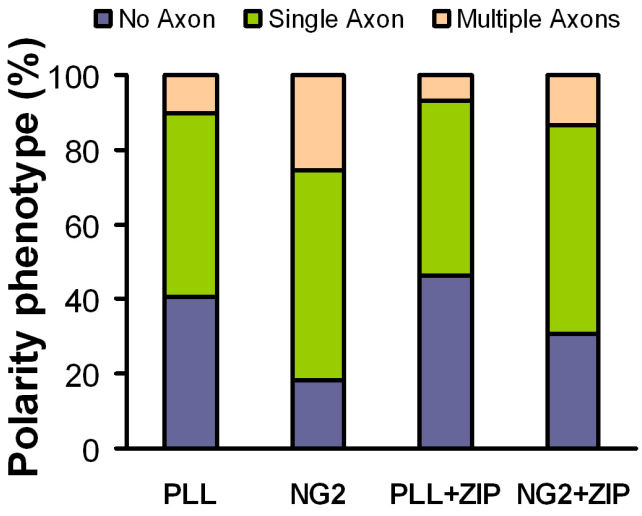


Figure 3.13. NG2 promotes axon formation in hippocampal neurons.

E18 hippocampal neurons were grown on PLL-coated or NG2-coated surfaces for 24~48 hours and cell polarization analyzed as described in Methods. The bar graph shows the percentage of neurons with the indicated number of axons (n=3, >136 cells were counted and scored in each condition). A typical stage 3/4 cell on control substrates and a multi-axonal cell grown on NG2 are shown immunofluorescently stained with anti-Tau antibodies (red) and AlexaFluor® 488 phalloidin. Scale bar 50 µm

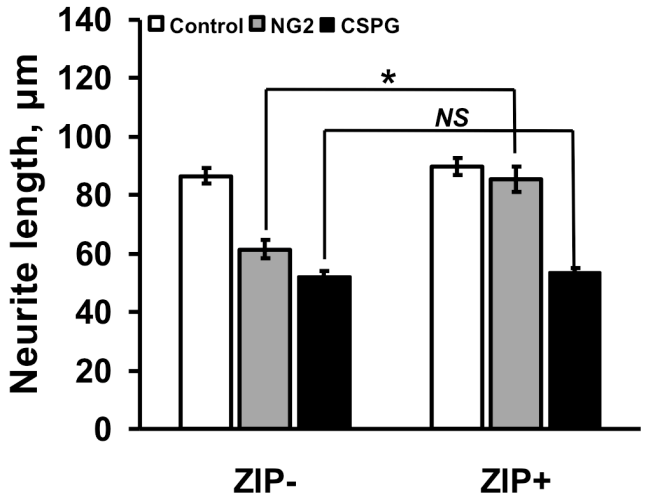


Figure 3.14. PKCζ inhibitor did not reverse the axonal growth inhibition by CSPG.

Dissociated cerebellar granule neurons (CGNs) from postnatal rats were grown either on control substrate (Laminin), on NG2 substrate (NG2) or CSPG substrate (CSPG) in the absence or presence of ZIP, a cell-permeable peptide inhibitor of PKCζ. ZIP potently reversed NG2-induced growth inhibition but not CSPG-induced inhibition. The histogram shows mean neurite lengths ± SEM (n=2, * $p < 0.001$; Dunn's post-hoc test).

Chapter 4 Cdc42-PAR6 is required for axon growth inhibition

4.1 Introduction

In the previous chapter, I showed that NG2, an inhibitory proteoglycan induces the activation of PKCζ and this activation is required for axon growth inhibition induced by the proteoglycan. Atypical PKC, including PKCζ and PKCλ/ι interacts with diverse proteins, which link the kinase to a wide range of cell physiology and function, as reviewed previously. It is therefore important to know which molecules are linked to PKCζ in axon growth inhibition. Since the alteration of axonal growth implies changes in cytoskeleton structures, the kinase is likely associated with PAR complex proteins.

In the PAR complex, PAR6 functions as an adapter to link Cdc42 to PKCζ activation. An upstream signal triggers a Cdc42-specific GEF and thereby catalyzes the conversion of Cdc42 from GDP-bound state to GTP-bound state (Schmidt and Hall, 2002). This activation likely allows Cdc42 to bind to PAR6 and presumably results in a conformational change of PAR6 to cause the activation of PKCζ by an unknown mechanism (Etienne-Manneville and Hall, 2002; Garrard et al., 2003). These led me to a hypothesis that NG2 may activate Cdc42 and the resulting interaction of Cdc42 and PAR6 may induce PKCζ activation to mediate the inhibitory action of NG2.

4.2 Results

4.2.1 NG2 CSPG induces Cdc42 activation

PAR6 complex allows the kinase to participate in the regulation of the cytoskeleton. A major role of PAR6 in the PAR complex is to link Cdc42 activation to the PAR complex. Therefore, I examined first whether NG2 treatment causes the activation of Cdc42. When a small GTPase becomes active, it increases its binding to a downstream effector. Taking advantage of this feature, I measured the change in the association of Cdc42 and PAR6. To validate this approach, recombinant Cdc42 and PAR6 were overexpressed in HT22 cells. The cell lysates were subjected to *in vitro* loading of either GDP or GTPγS to induce Cdc42 inactivation and activation, respectively. Thereafter, the complex of recombinant Cdc42-PAR6 was pulled down using an anti-myc antibody and Cdc42 remaining in the complex was detected by

immunoblotting with anti-dsRed antibody. As shown in the left panel of Figure 4.1, GDP-loading prevented the formation of Cdc42-PAR6 complex but GTP-loading promoted the complex formation. A similar result was obtained using dominant negative or constitutive active form of Cdc42. As shown in Figure 4.2, DN Cdc42 ($Cdc42^{T17N}$) failed to interact with PAR6 whereas CA Cdc42 ($Cdc42^{Q61L}$) more strongly associated with PAR6 than wild type Cdc42. Together, these results confirm that an increase in the association of Cdc42 and PAR6 is a valid measure of Cdc42 activation.

Finally, I applied this approach to the cells treated with NG2. As shown in Figure 4.1, treatment of transfected cells with soluble NG2 increased the association of Cdc42 and PAR6. This result suggests that NG2 indeed induces the activation of Cdc42 and thereby increases its interaction with PAR6.

4.2.2 Cdc42 is required for NG2 CSPG-induced PKCζ phosphorylation

To determine whether the activation of Cdc42 is necessary for the NG2-induced increases in PKCζ activity, I examined the ability of NG2 to increase the phosphorylation of T410 in HT22 cells transfected with either wild type or dominant-negative Cdc42 ($Cdc42^{T17N}$).

Since dominant negative Cdc42 stays in the GDP-bound state, it will be unable to bind to downstream effectors such as PAR6 (Figure 4.2) while still maintaining the ability to interact with a GTP exchange factor (GEF). Therefore, it efficiently sequesters any upstream signal to prevent the activation of downstream molecules. Either CGNs or HT22 cells were transfected with FLAG-PKCζ along with either wild type Cdc42 or a dominant negative form of Cdc42 and treated with NG2. The transfection efficiency of HT22 cells was lower than 50% and anti-phospho-PKCζ does not distinguish endogenous and recombinant proteins. The signal of endogenous phospho-PKCζ from non-transfected cells could, therefore, override the signal of phospho-FLAG-PKCζ from transfected cell in western blot analysis of cell lysates. To resolve this problem, I immunoprecipitated recombinant PKCζ from cell lysates by using an anti-FLAG antibody and examined its phosphorylation by immunoblotting with anti-phospho-PKCζ antibody. As shown in Figure 4.3, expression of a dominant-negative Cdc42 abolished the ability of NG2 to induce the activation of PKCζ in both transfected HT22 cells and CGNs. This result suggests that NG2 signals through Cdc42 to induce the activation of PKCζ.

4.2.3 Cdc42 is required for the axon growth inhibition by NG2

The Cdc42-dependency of NG2 for PKCζ phosphorylation suggests that the activation of Cdc42 is required for axon growth inhibition. To test this hypothesis, I overexpressed a dominant negative form of Cdc42 ($Cdc42^{T17N}$) in CGNs and examined its effect on axon growth inhibition. The transfection of $Cdc42^{T17N}$ allowed the neurons to extend long axons on NG2 substrate whereas the transfection of wild type Cdc42 showed no effect (Figure 4.4), suggesting that Cdc42 activation is required for axon growth inhibition.

The activation of and the requirement for Cdc42 suggest that the activity of this small GTPase alone may phenotypically copy the inhibitory effect of NG2 on axon growth. To test this idea, I created constitutively active forms of Cdc42 ($Cdc42^{Q61L}$) by mutating Gln^{61} to Leu (Nalbant et al., 2004). To uncover the effect of $Cdc42^{Q61L}$ in axon growth, rat CGNs were transfected with 0.5 μg of pCAGJC-dTom-$Cdc42^{Q61L}$ plasmid, grown for 24 hours on control substrate and fixed for the measurement of the neurite length. As shown in Figure 4.5, the transfection of CA Cdc42 significantly reduced axon lengths on control substrate, suggesting that the activation of Cdc42 is sufficient for axon growth inhibition.

4.2.4 Cdc42-PAR6 complex is required for axon growth inhibition by NG2

The NG2-induced Cdc42 activation and its requirement for PKCζ phosphorylation suggests that the Cdc42-PAR6 complex plays an important role in axon growth inhibition. Therefore, I speculated that disrupting the complex formation may prevent axon growth inhibition by NG2. To test this hypothesis, I took advantage of the observation that PAR6 contains a semi-CRIB domain that is necessary for its binding to Cdc42. I created two mutant forms of PAR6; one containing a 26 amino acid deletion of the semi-CRIB domain and the other with deletion of a proline 135 (Nishimura et al., 2005). Both deletion mutant forms of PAR6 failed to interact with wild type of Cdc42 without affecting the interaction with PKCζ, confirming that these mutant forms efficiently block the formation of Cdc42-PAR6 complex only (Figure 4.6). To investigate the effect of these mutants in axon growth inhibition, I transfected each into CGNs and analyzed the axonal growth of the transfected neurons on either control or NG2 substrate. Transfection of PAR6 mutants did not affect axonal growth on control

substrate, which was comparable to transfection of wild type PAR6. When transfected into CGNs, these mutant forms of PAR6 significantly reversed NG2-induced growth inhibition (Figure 4.7). These data suggest a functional requirement of Cdc42-PAR6 complex in axon growth inhibition.

4.2.5 NG2 induces the formation of PKCζ-PAR6 complex

The stoichiometry of the PAR complex changes dynamically, depending on its function and context (Chen and Macara, 2005; Nagai-Tamai et al., 2002; Nakayama et al., 2008; Nishimura et al., 2005; Wang et al., 2003; Zhang and Macara, 2008). These observations suggest that NG2 may alter the association of each component within the PAR complex for axon growth inhibition. To determine this, I examined whether the treatment of NG2 alters the stoichiometry of PKCζ-PAR6 complex. In preliminary experiments, NG2 appeared to reproducibly increase the association of the endogenous PKCζ and PAR6. The PAR6 signal was, however, generally too low to normalize for accurate quantitation, presumably due to the low affinity and specificity of currently available PAR6 antibodies or the low expression levels of endogenous PAR6. To solve this problem, I overexpressed recombinant PKCζ and PAR6 in both CGNs and HT22 cells and treated the cells with NG2. PKCζ-PAR6 complex was immunoprecipitated by either anti-Myc antibody or anti-FLAG antibody and each protein was detected by immunoblotting with either anti-Myc antibody or anti-FLAG antibody. NG2 increased the association of PKCζ and PAR6 in both HT22 and CGNs (Figure 4.8), suggesting that NG2 alters the stoichiometry of PKCζ-PAR6 for axon growth inhibition.

4.2.6 PKCζ-PAR6 complex is required for the axon growth inhibition by NG2

The increased association of PKCζ with PAR6 suggests that the complex formation may be functionally important in axon growth inhibition. To test this hypothesis, I created PKCζ NT containing amino acid residues 1-129 of PKCζ, which includes the PB1 domain of PKCζ. Both PKCζ and PAR6 have PB1 domains in their N-terminal regions. These proteins interact with each other via homophilic interaction of their N-terminal PB1 domains (Nishimura et al., 2005). Therefore, overexpression of PKCζ NT competitively interrupts the interaction between these proteins, thereby preventing the formation of PKCζ and PAR6 complex (Figure 4.9). To determine the functional significance of the increased PAR6-PKCζ binding, I transfected CGNs

with PKCζ NT and grew the cells on control or NG2-coated surfaces. As shown in Figure 4.10, expression of PKCζ NT but not full-length PKCζ attenuated the inhibitory action of NG2. This result suggests that NG2 induces the formation of PKCζ-PAR6 complex and this interaction is functionally required for the outgrowth inhibition induced by NG2.

4.3 Discussion

Here, I showed that NG2 induces the activation of Cdc42 and thereby increases the interaction of Cdc42 and PAR6. I also showed that Cdc42 and its interaction with PAR6 are required for the inhibitory activity of NG2. Finally, I showed that the formation of PKCζ-PAR6 complex is necessary for the outgrowth inhibition by the inhibitory proteoglycan. These results suggest that NG2 signals through Cdc42 and PAR6 to activate PKCζ for axon growth inhibition.

The activation of Cdc42 in axon growth inhibition; How does NG2 induce the activation of Cdc42? In general, GEFs function to convert small GTPases from GDP-bound state to an active GTP bound state. Many GEFs contain a regulatory domain at the N-terminal region, a Dbl homology (DH) domain in the middle region and Pleckstrin homology (PH) domain at the C-terminal region (Schmidt and Hall, 2002). In the inactive state, through an intramolecular interaction, the regulatory domain prevents the Dbl homology (DH) domain from interacting with and activating small GTPases. GEF activation occurs through relief of the intramolecular inhibitory sequence. The phosphorylation of or binding of lipid messengers to the inhibitory domain reduces this intramolecular interaction. Therefore, the activation of some upstream kinases or lipid messengers is often required for GEF activation (Schmidt and Hall, 2002). This idea suggests that there may exist unidentified upstream signaling components that NG2 signals through to activate Cdc42.

How does Cdc42 activation induce axon growth inhibition? In addition to the requirement of Cdc42, the balanced activity of the small GTPase is also required for proper axonal out growth, as described in the previous chapter. I speculate that NG2 may dysfunctionally up-regulate the balanced activity of Cdc42 that may be established by laminin. In my *in vitro* culture system, I used laminin as a control substrate. To test inhibitory activity of NG2, I made uniform NG2 substrate by mixing NG2 ECD with LN and then coating this mixture onto surfaces. This manipulation is important since it provides a physiologically more relevant context to neurons by mimicking *in vivo* condition of glial scars. Inhibitory proteoglycans are

usually found on the surfaces of glial cells in normal condition. Upon the injury, they are massively released into the extracellular space. Once inhibitory proteoglycans are secreted, they are complexed with extracellular molecules such as laminin and neuro-glial adhesion molecule (NgCAM) and exist as a form of extracellular matrix, generating the glial scar. Interestingly, depending on these two conditions, proteoglycans seem to exhibit different biological functions. For example, either soluble NG2 or a substrate form of NG2 induces growth cone collapse and repulsion and inhibits axonal outgrowth (Chen et al., 2002; Dou and Levine, 1994; Tan et al., 2006; Ughrin et al., 2003). A recent study showed that NG2-expressing glial cells do not repel growing axons but promote their extension (Yang et al., 2006). Therefore, these results suggest that inhibitory proteoglycans may play two separate roles, depending on normal and pathological conditions. In this regard, it is important to test the inhibitory effect of NG2 complexed with ECM molecules, since ECM molecules may contribute to the inhibitory property of inhibitory proteoglycans.

Laminin is an extracellular matrix and cell adhesion molecule. It is known to trigger β-integrin and Ras. These components subsequently signal through PI3-kinase and Cdc42 to enhance axon elongation and neurite outgrowth in neuronal cells (Menager et al., 2004; Sarner et al., 2000). Therefore, it is possible that NG2 further elevates the activity of Cdc42 that is already upregulated by LN, thereby causing hyperactivity of Cdc42. Several reports describe that hyperactivity of Cdc42 attenuates axonal outgrowth (Kim et al., 2003; Kuhn et al., 1999; Nishimura et al., 2005). Kim et al showed that increasing the activity of Cdc42 prevents axon outgrowth *in vivo*. Consistent with this, Nishimura *et al.* showed that expression of constitutively active form of Cdc42 attenuates axonal growth in cultured hippocampal neurons. Similarly, I observed that expression of constitutively active form of Cdc42 phenotypically copied the inhibitory action of NG2 (Figure 4.5). These results suggest that hyperactivity of Cdc42 may be a mechanism by which extrinsic inhibitory molecules attenuate axon growth. Consistent with this idea, Kuhn et al showed that a dominant negative form of Cdc42 reverses axon growth inhibition induced by a repulsive guidance cue, collapsin-1. Since Cdc42 is linked to Rac1 through the PAR complex, hyperactivity of Cdc42 is anticipated to result in hyperactivity of Rac1. Consistent with this idea, a constitutive active form of Rac1 also phenotypically copies the axonal outgrowth inhibition induced by a constitutive active form of Cdc42 (Kim et al., 2003; Nishimura et al., 2005). Furthermore, similar with a dominant negative form of Cdc42, a

dominant negative form of Rac1 attenuates the inhibitory action of collapsin-1. Therefore, this expected Rac1 hyperactivity might cause dysfunctional alteration of actin cytoskeleton and thereby hamper axonal outgrowth, as previously discussed (Kuhn et al., 1999). Therefore, I speculate that NG2 causes the hyperactivity of Cdc42 and this dysfunctional activation may be transferred to Rac1, causing hyperactivity of Rac1.

In general, the activity of Cdc42 is necessary for axonal outgrowth. In contrast to this idea, however, expression of a dominant negative Cdc42 was ineffective on the basal growth of axon in CGNs. (Figure 4.4) One explanation is that laminin substrate activates Cdc42 for the basal growth of axon and this activation may override the inhibitory effect of dominant negative Cdc42. LN is an extracellular matrix and cell adhesion molecule. It is known to enhance axon elongation and neurite outgrowth in neuronal cells through PI3K activation (Menager et al., 2004; Sarner et al., 2000). LN also induces the activation of Cdc42 to mediate neurite outgrowth in PC12 cells (Weston et al., 2000). A LN receptor, integrin α induces the activation of PI3K and Cdc42 for the formation of filopodia (Chang et al., 2005). Together, these results suggest that LN activates an integrin receptor and thereby induces the activation of Cdc42 through PI3K signaling. Therefore, I speculate that the Cdc42 activity generated by LN might be sufficient to cancel out the inhibitory effect of dominant Cdc42 and support normal axonal outgrowth.

***In vivo* Cdc42 activation assay:** Currently, the activation of Cdc42 proteins is assayed with the binding of GTP-bound Cdc42 to the p21-binding domain (PBD) of p21-activated protein kinase (PAK). This method is based on the observation that the active, GTP-bound Cdc42 could bind to the PBD of PAK. The reproducibility of this method is, however, less than satisfactory. This is partially due to the hydrolysis of GTP to GDP during the assay procedure and the low binding affinity of PBD to Cdc42-GTP (Herrmann et al., 1995). As a configuration-specific antibody that specifically recognizes Cdc42-GTP, but not Cdc42-GDP became recently available, the activation assay could be performed in a much shorter time and with more consistent reproducibility. However, considering the low level of endogenous Cdc42 in neuronal cells, an alternative method should be considered to increase the basal level of the small GTPase.

Compared to the conventional GTPase pull-down assays described above, the method that I described here has a few advantages as follows. This technique is to target Cdc42-effector complex formed *in vivo* rather than *in vitro*. Furthermore, by examining the interaction with a

specific downstream effector, it provides a specific and quantitative readout to interpret which signaling pathways the activation of a small GTPase is directed to among pathways.

The regulation of PAR6 and its function in axon growth inhibition; I demonstrated that PAR6-PKCζ complex is required for axon growth inhibition by showing that the expression of PKCζ PB1 domain reverses axon growth inhibition (Figure 4.10). The role of increased PKCζ-PAR6 complex in axon growth inhibition, however, remains undetermined (Figure 4.8).

One plausible explanation is that it may increase the ability of the PAR complex to activate PKCζ in response to upstream signals by promoting the recruitment of active Cdc42 (Etienne-Manneville and Hall, 2003; Lin et al., 2000; Yamanaka T Fau - Horikoshi et al.; Yamanaka et al., 2001). Depending on cell context, up-regulation of PKCζ-PAR6 complex seems to work unfavorably or favorably for the activation of PKCζ. In hippocampal neurons, overexpression of PAR6 results in a polarity defect with loss of axon structure presumably due to PAR6-mediated inhibition of PKCζ (Nishimura et al., 2005; Schwamborn and Puschel, 2004; Shi et al., 2003). Conversely, in a fibroblast cell line, overexpression of PAR6 promotes the activation of PKCζ and thereby stimulates cell transformation. Furthermore, co-expression of an active form of Cdc42 along with PAR6 further increases the kinase activity of PKCζ (Qiu et al., 2000). These results suggest that up-regulation of PKCζ-PAR6 complex may facilitate the activation of PKCζ in the context where active Cdc42 is enriched. Therefore, I speculate that the increased binding of PAR6 to PKCζ may synergistically works with NG2-induced Cdc42 activation to further up-regulate the activity of PKCζ, thereby causing hyperactivity of the kinase.

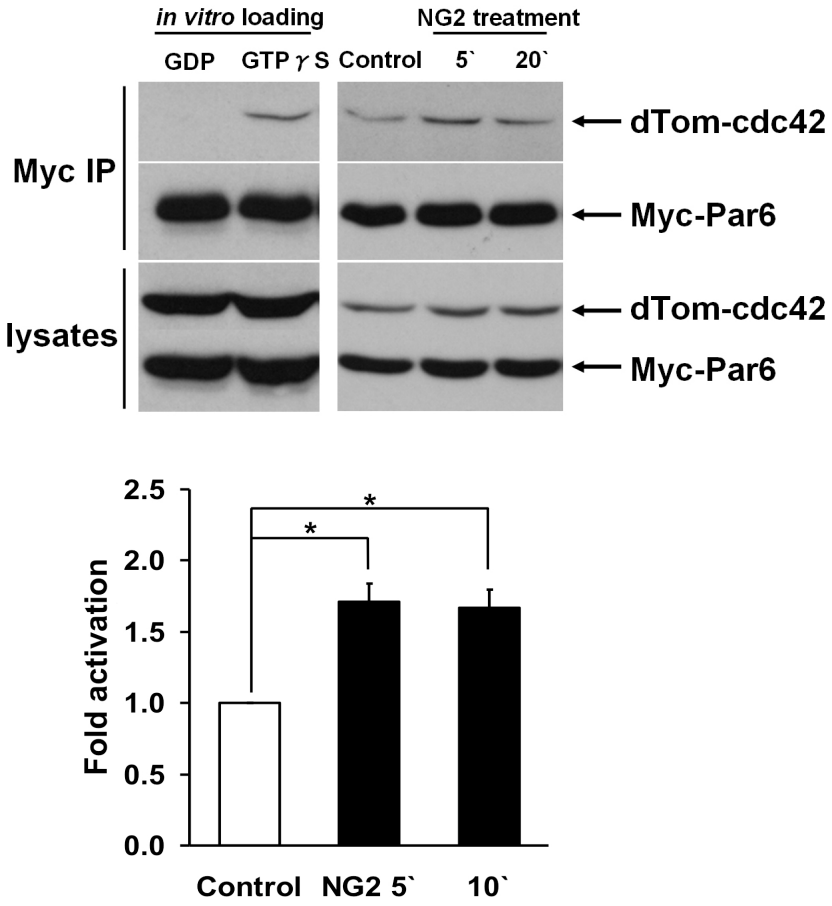


Figure 4.1. NG2 induces Cdc42 activation.

HT22 cells were transfected with dTomato-tagged Cdc42 (dTom-Cdc42) and Myc-tagged PAR6 (Myc-PAR6). For in vitro loading, cell lysates were incubated with either GDP or GTPγS as described in Methods. The cells were treated with soluble NG2 (35 μg/ml) for the indicated times and the interaction of Cdc42 and PAR6 was measured by immunoprecipitation with anti-Myc-tag antibody, followed by immunoblotting with either anti-dsRed or anti-Myc antibody. To quantitate Cdc42 activation, the ratio of Cdc42 to PAR6 was measured. The histogram below shows the mean of ratio of Cdc42 to Par6 ± SEM (n=3, normalized to control treatment; * $P < 0.05$, Student's t-test)

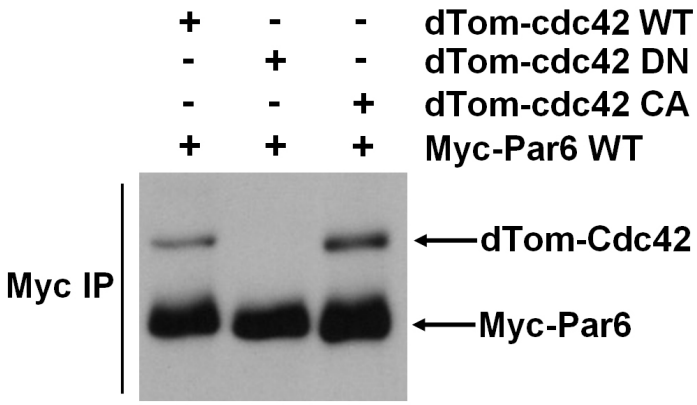


Figure 4.2. Cdc42 binds to PAR6.

HT22 cells were co-transfected with PAR6 and the indicated forms of Cdc42. After 16~24 hours, cell lysates were immunoprecipitated by anti-Myc antibody and blotted with anti-Myc or anti-DsRed antibody. PAR6 preferentially bound to constitutively active form of Cdc42 more than wild type of Cdc42 whereas it failed to interact with dominant negative form of Cdc42.

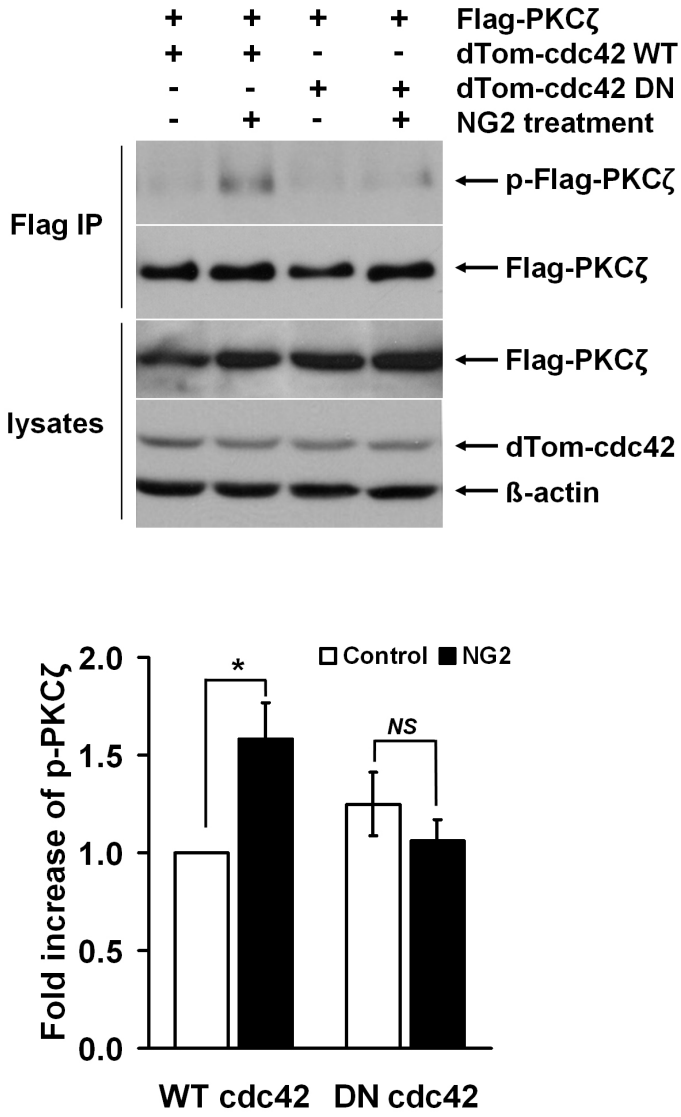


Figure 4.3. Cdc42 is required for NG2 CSPG-induced PKCζ phosphorylation.

DN-Cdc42 prevents the NG2-induced phosphorylation of PKCζ. NG2 (10 μg/ml) was bath applied for 30 minutes to HT22 cells transfected with either WT or DN forms of Cdc42 along with FLAG-PKCζ. The phosphorylation of the recombinant PKCζ was detected by immunoprecipitation with anti-FLAG antibody and by immunoblotting with specific anti-phospho-PKCζ antibody. The histogram shows the fold increase in PKCζ phosphorylation (average ± SEM, n=3, normalized to the control treatment of WT-Cdc42-transfected cells, * $P < 0.05$, Student's t-test).

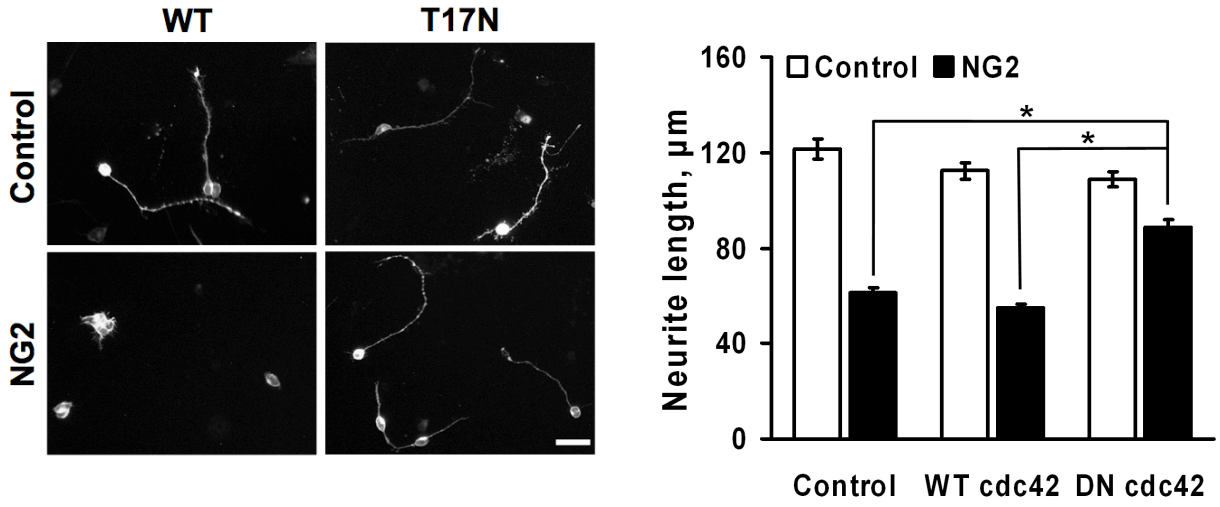


Figure 4.4. Cdc42 is required for axon growth inhibition.

DN-Cdc42 restores normal neurite growth. CGNs were transfected with either WT or DN-Cdc42 (T17N) expression plasmids and grown on either control or NG2-coated substrates (Scale bar 50 µm). The histogram shows mean neurite lengths ± SEM (n=3; *p < 0.001; Dunn's post-hoc test).

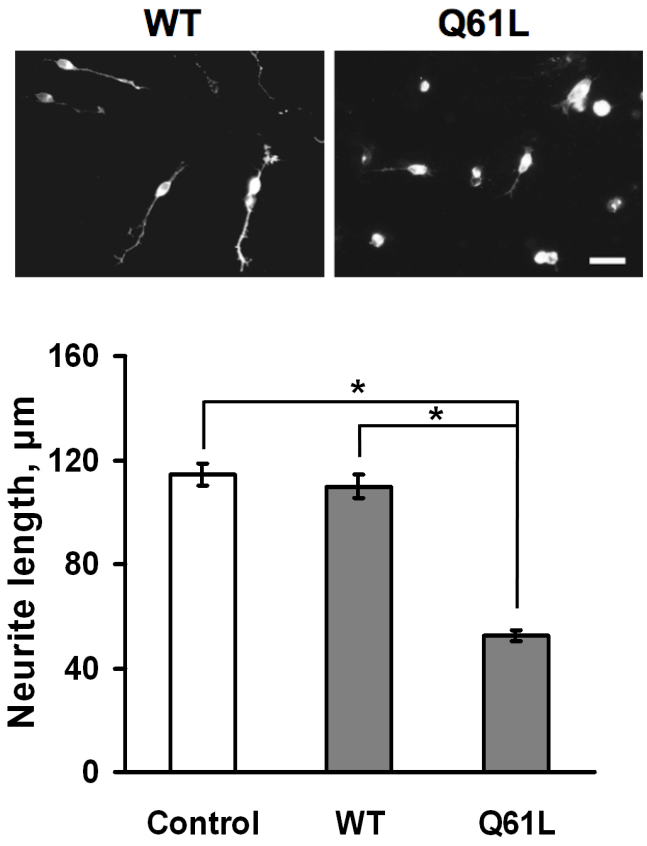


Figure 4.5. Constitutively active Cdc42 is sufficient for axon growth inhibition.

CA-Cdc42 inhibits neurite growth. CGNs were transfected with either WT- or CA-Cdc42 (Q61L) and grown on control substrate (Scale bar 50 µm). The histogram shows mean neurite lengths ± SEM (n=3; *$p < 0.001$; Dunn's post-hoc test).

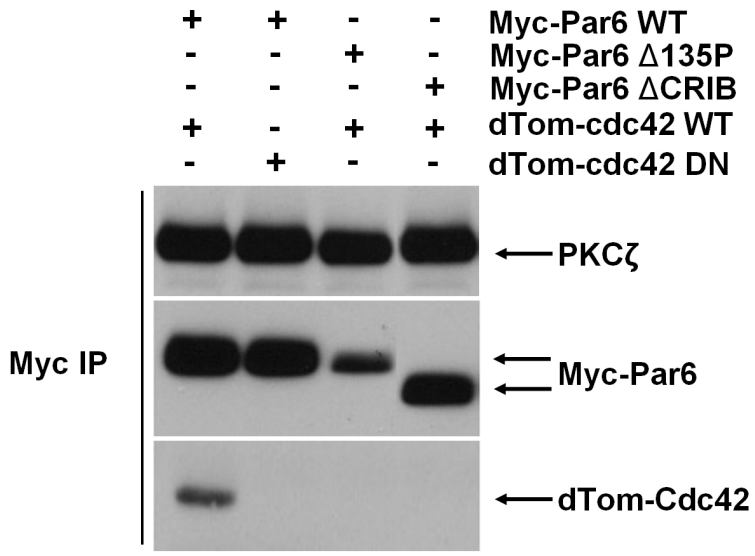


Figure 4.6. Semi-CRIB domain is required for the interaction of PAR6 and Cdc42.

HT22 cells were co-transfected with the indicated forms of PAR6 and Cdc42 were expressed along with Cdc42 in HT22 cells. After 16~24 hours, cell lysates were immunoprecipitated by anti-Myc antibody were blotted with antibodies targeted to Myc-epitope, FLAG- epitope and PKCζ. Semi-CRIB mutant forms of PAR6 failed to bind to Cdc42 whereas they are still able to associate with PKCζ.

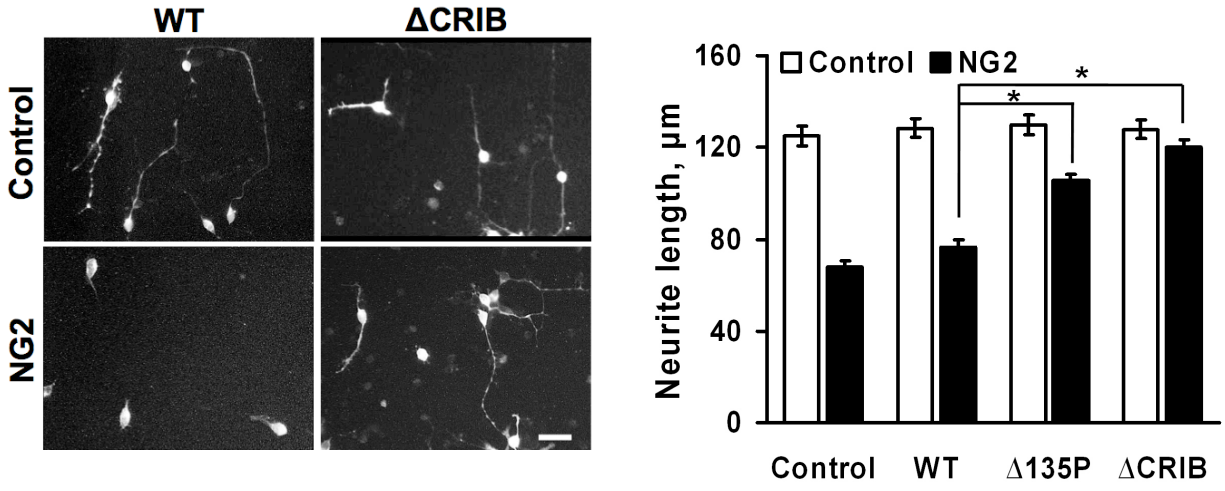


Figure 4.7. A Cdc42-PAR6 complex is required for axon growth inhibition.

CGNs were transfected with PAR6 mutants (**Δ**135P and **Δ** CRIB) and grown on either control or NG2-coated substrates (Scale bar 50 µm). The histogram shows mean neurite lengths ± SEM (n=3; *p < 0.001; Dunn's post-hoc test).

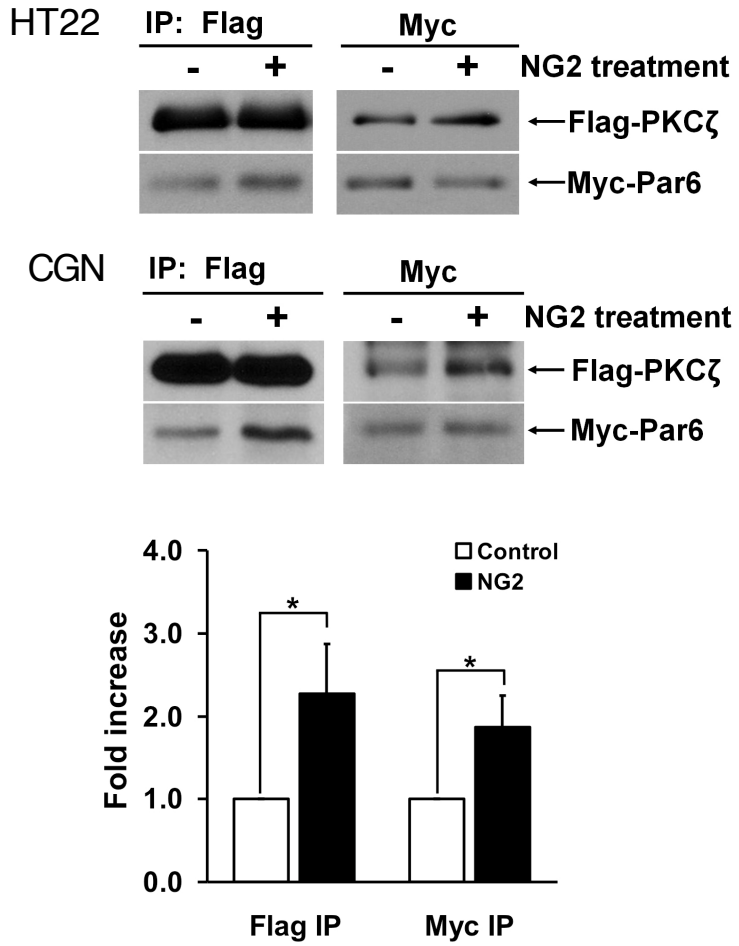


Figure 4.8. The regulation of a PKCζ-PAR6 complex by NG2

CGNs and HT22 cells were transfected with Flag-PKCζ and Myc-PAR6 and NG2 (10 μg/ml) was bath-applied to rat CGNs for 15 minutes or to HT22 cells for 30 minutes. The association of these epitope-tagged proteins was detected by immunoprecipitation followed by immunoblotting with epitope-specific antibodies. The histogram plots fold increase in PAR6-PKCζ binding in HT22 (mean ± SEM, n=3, * $p<0.05$, Student's t-test).

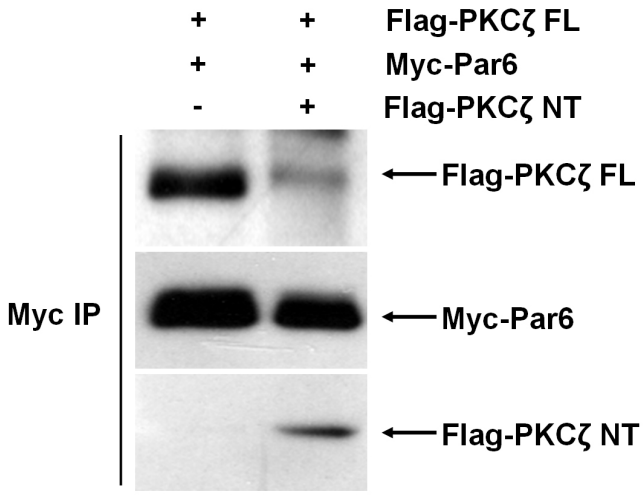


Figure 4.9. PKCζ and PAR6 interact with each other through their PB1 domains.

Expression of an N-terminal fragment of PKCζ (PKCζ NT) disrupts the interaction between PKCζ and PAR6. HT22 cells were co-transfected with the indicated sets of plasmids. After 16~24 hours, cell lysates were immunoprecipitated by anti-Myc antibody and blotted with antibodies targeted to FLAG- or Myc-epitope. Expression of PKCζ NT caused a reduction in the association of FLAG-PKCζ with Myc-PAR6.

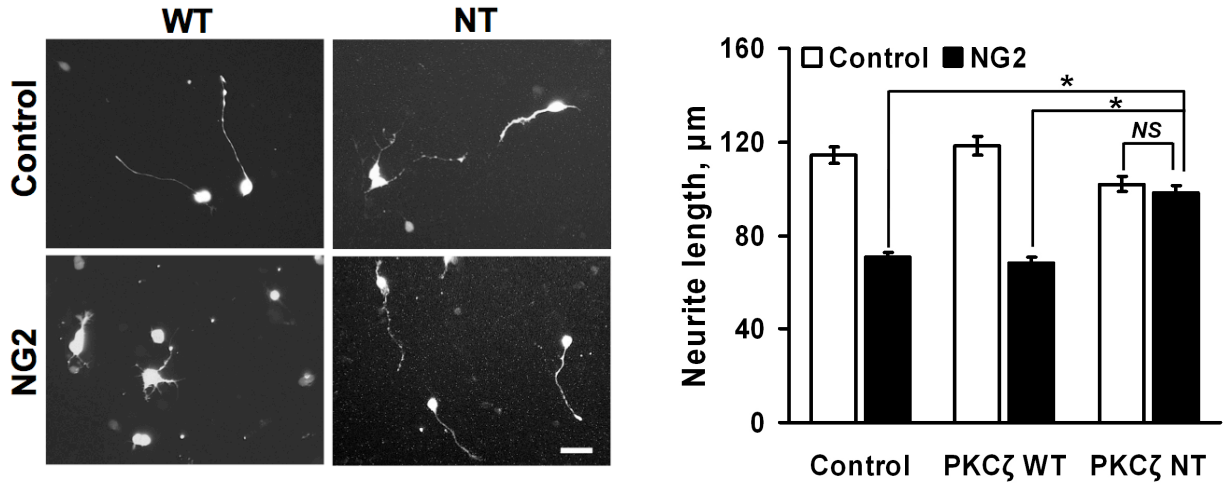


Figure 4.10. A PKCζ-PAR6 complex is required for NG2-induced growth inhibition.

CGNs were transfected with PKCζ PB1 domain-expressing plasmid (NT) and grown on control or NG2-coated substrates (Scale bar 50 µm). The histogram shows mean neurite lengths ± SEM (n=3; *$p < 0.001$; Dunn's post-hoc test).

Chapter 5 PAR3-Rac1 is required for axon growth inhibition

5.1 Introduction

In the polarity complex, PAR3 has multiple roles. First, PAR3 interacts with PKCζ and functions as a competitive inhibitor (Lin et al., 2000) of PKCζ. Activated PKCζ phosphorylates PAR3 and thereby reduces the interaction of these polarity proteins. This relief of intermolecular inhibition presumably allows the kinase to regulate GSK3β and MARK2 that are associated with microtubule regulation.

Another role of PAR3 is to activate Rac1 through TIAM/STEF, a Rac1-specific GTP exchanger. Rac1 is a key regulator of actin cytoskeleton since it regulates the signaling components that modulate actin polymerization and branching. Depending on the structure of actin filaments, the role of actin is permissive or non-permissive to microtubule invasion into growth cones (Bradke and Dotti, 2000; Lowery and Van Vactor, 2009). Actin filaments and bundles are required for axon elongation since these actin structures serve to guide microtubule into growth cones. Highly interlaced actin filaments hamper microtubule invasion, attenuating axon elongation. Rac1 is an important regulator to determine these actin structures. Therefore, balanced activity of Rac1 is required for normal axonal outgrowth and disturbing the balance often impairs axon growth. Since the PAR complex links Cdc42 to Rac1, NG2-induced hyperactivity of Cdc42 is anticipated to result in hyperactivity of Rac1. Therefore, in this chapter, I explore whether NG2 induces Rac1 activation for axon growth inhibition and what the role of the PAR complex is in the activation of Rac1.

The PAR complex is accumulated at the tips of growing axons. This asymmetric distribution is required for axon formation and elongation. PAR3 is responsible for this polarized subcellular localization of the PAR complex. The coiled-coil domain allows PAR3 to interact with KIF3, a plus-end-directed microtubule motor protein. The PAR complex tethered to KIF3 through PAR3 is transported to the tips of growing axons (Nishimura et al., 2004), where the complex coordinates the reorganization of actin and microtubules for axonal growth. Since interrupting the asymmetric localization of the polarity proteins impairs axon formation and development, it is possible that NG2 may induce the dislocation of the PAR complex, resulting

in axon growth inhibition. Therefore, in this chapter, I will explore whether or not NG2 induces any alteration in the distribution of PAR3.

5.2 Results

5.2.1 PKCζ-dependent phosphorylation of PAR3 is required for the axon growth inhibition by NG2

As shown previously in the example of PKCζ-PAR6 complex, the stoichiometric alteration of the PAR complex plays an important role in axon growth inhibition. This led me to a hypothesis that the interaction of PKCζ with PAR3 may be altered in response to NG2.

To test this, HT22 cells were transfected with recombinant PKCζ and PAR3 and treated with NG2. The complex of the recombinant proteins was pulled down by anti-FLAG or anti-Myc antibody; and each protein was detected by immunoblotting. As shown in Figure 5.1, treatment of transfected HT22 cells with NG2 reduced the interaction of PAR3 with PKCζ. To confirm that this is the case in the endogenous complex, I pulled down the complex from CGNs with anti-PKCζ antibody and subjected them to immunoblotting with anti-PKCζ and anti-PAR3 antibody. Similar to the results from the transfected HT22 cells, treatment with NG2 also reduced the interaction of PAR3 with PKCζ in CGNs (Figure 5.1).

The binding of PAR3 to PKCζ is regulated by protein kinases. For example, PKCζ and Rho kinase (ROCK) phosphorylate PAR3 at different sites and each of these phosphorylations reduces the affinity between PAR3 and PKCζ (Nagai-Tamai et al., 2002; Nakayama et al., 2008). Therefore, it is possible that NG2 may induce the activation of these kinases to decrease the interaction of these polarity proteins presumably through PAR3 phosphorylation. To test this hypothesis, the cells were transfected with recombinant PAR3 and PKCζ, NG2-treated along with the manipulation of the activity of PKCζ or ROCK. To knock down the activity of PKCζ, ZIP was used or a kinase-deficient form of PKCζ (PKCζ KD) was expressed. To inhibit the activity of ROCK, a ROCK-specific inhibitor, Y27632 was applied to the transfected cells. As shown in Figure 5.2, Y27632 had no effect on NG2-induced dissociation of PAR3 and PKCζ but both treatment with ZIP and expression of PKCζ KD prevented the dissociation. This result suggests that NG2 may signal through PKCζ to induce the phosphorylation of PAR3, resulting in the dissociation of PAR3 and PKCζ.

Since NG2 induces the dissociation of PKCζ and PAR3, I questioned whether or not this dissociation is sufficient to inhibit axon growth. To test this hypothesis, I overexpressed CR3, which is aPKC binding region of PAR3, to interrupt the interaction of these polarity proteins and investigate how its overexpression affects axonal outgrowth of CGNs. When I overexpressed CR3 in HT22 cells, I found the PAR3 signal was reduced in the PKCζ-PAR3 complex immunoprecipitated with using an anti-PKCζ antibody (Figure 5.3), suggesting that overexpression of CR3 disrupts the interaction of PKCζ and PAR3. Next, I overexpressed dTomato-tagged CR3 (dTom-CR3) in CGNs, grew them on laminin substrate, measured the average axonal length per cell and compared it with that of dTomato-transfected cells. I found that both dTomato and dTom-CR3 expression did not change the basal growth of axon in CGNs. Together, these results suggest that disruption of PKCζ-PAR3 alone is insufficient and another modification, presumably dependent on PKCζ activity, may be required for axon growth inhibition.

The insufficiency of the disruption of PKCζ-PAR3 led me to another question; is PAR3 phosphorylation by PKCζ required for axon growth inhibition? To evaluate this hypothesis, I converted Ser^{824} (the homologous residue to Ser^{827} of rat PAR3; ASIP) of human PAR3b to alanine; the phosphorylation of this residue by PKCζ causes a reduction in the interaction of the kinase with PAR3 (Nagai-Tamai et al., 2002). Thus, I overexpressed the non-phosphorylatable mutant form of PAR3 ($PAR3^{S824A}$) in CGNs and examined its effect on axon growth inhibition. Transfection of $PAR3^{S824A}$ allowed the neurons to extend long axons on NG2 substrate whereas transfection of wild type PAR3 showed no effect (Figure 5.4), suggesting that phosphorylation of PAR3 at the PKCζ site is required for the inhibitory action of NG2.

5.2.2 NG2 activates Rac1 in a PKCζ-dependent and PAR3-dependent manner

The possibility of PKCζ-dependent phosphorylation of PAR3 in axon growth inhibition suggests that certain signaling may occur through PAR3. One candidate is Rac1 since the PAR complex mediates Cdc42-induced Rac1 activation through STEF/TIAM. This led me to a hypothesis that NG2 may induce the activation of Rac1 through PKCζ and PAR3. To evaluate this idea, I measured Rac1 activation in response to NG2. Since Rac1 is a small GTPase, its activation strengthens the interaction with a downstream effector. Similar with the measurement of Cdc42 activity in previous chapter, I measured changes in the interaction of Rac1 with PAK1,

a downstream effector of this small GTPase (Brown et al., 1996). I transfected cells with myc-tagged PAK1 and dTomato-tagged Rac1, treated them with soluble NG2 and immunoprecipitated the recombinant protein complex using anti-Myc antibody. The proteins in the immunoprecipitated complex were detected by immunoblotting using anti-Myc and anti-dsRed antibodies. As shown in Figure 5.5, short-term treatment with NG2 increased the binding of Rac1 to PAK1. Either inhibition of PKCζ activity with ZIP or transfection of $PAR3^{S824A}$, however, blocked the effect of NG2. Together, these results suggest that NG2 induces the activation of Rac1 and this activation is dependent on the activity of PKCζ and PAR3 phosphorylation at the PKCζ site.

5.2.3 Rac1 is necessary and sufficient for axon growth inhibition

The NG2-induced activation of Rac1 suggests that Rac1 activation may be required for axon growth inhibition. To test this hypothesis, I transfected a dominant negative form of Rac1 ($Rac1^{T17N}$) to suppress Rac1 activity and examined its effect on axon growth inhibition by NG2. As shown in Figure 5.6, transfection of $Rac1^{T17N}$ allowed axonal outgrowth of CGNs grown on NG2 substrate.

The activation of and requirement for Rac1 in axon growth inhibition led me to a hypothesis that Rac1 is sufficient for axon growth inhibition. To test this hypothesis, I transfected, a constitutively active form of Rac1 ($Rac1^{G12V}$) and examined its effect on axon growth on control substrates. As shown in Figure 5.7, the transfection of $Rac1^{G12V}$ attenuates axonal outgrowth whereas the transfection of wild type Rac1 did not affect the basal growth of axon on control substrate.

5.2.4 NG2 induces alteration of the subcellular localization of PAR3

The regulation of cytoskeleton stability in the growth cone is critical for axon elongation and path finding. It is widely accepted that the PAR complex regulates the dynamics of actin and microtubule at the axonal tip. This idea led me to a hypothesis that NG2 treatment alters the intracellular distribution of the PAR complex. To test this hypothesis, I grew CGNs on either control substrate or NG2 substrate and compared the distribution of endogenous PAR3. As shown in Figure 5.8, in CGNs grown on control surfaces, most PAR3 was found at the axonal compartment and tip. In the cells grown on NG2 substrate, PAR3 immunoreactivity in the cell

body increased whereas immunoreactivity in the axon shaft relatively reduced. This result suggests that NG2 substrate alters the subcellular localization of the PAR complex.

5.3 Discussion

Here, I demonstrated that NG2 induces the activation of Rac1 through the polarity complex. In addition, I showed that a dominant negative form of Rac1 prevents NG2-induced inhibition and a constitutive active form of Rac1 phenotypically mimics the inhibitory effect of NG2. In the previous chapter**,** I suggested that NG2 may further increase the activity of Cdc42 that is presumably upregulated by LN, resulting in hyperactivity of Cdc42. Since the PAR complex mediates Cdc42-induced Rac1 activation (Arimura and Kaibuchi, 2007; Nishimura et al., 2005), this hyperactivity of Cdc42 is likely transferred through the PAR complex to cause hyperactivity of Rac1 for axon growth inhibition. Consistent with this idea, expression of a constitutive active Rac1 attenuates axon growth of CGNs on LN substrate. Therefore, I speculate that hyperactivity of Rac1 is a plausible mechanism by which NG2 inhibits axon growth.

The role of Rac1 activation in axon growth; The role of Rac1 in the axon growth varies depending on cell types. The cells whose axonal outgrowth relies on growth factors or morphogenic factors require Rac1 activation for axon formation. For instance, in PC12 cells, expression of dominant negative Rac1 inhibits NGF-induced neurite outgrowth (Wang et al., 2007; Yasui et al., 2001). In a neuroblastoma cell line, SH-SY5Y, expression of the dominant-negative mutant Rac1 prevents retinoic acid-induced neurite outgrowth (Pan et al., 2005). Differentiated neurons, whose axonogenesis is independent of exogenous factors, however, require the balanced activity of Rac1 for proper axon growth. For instance, in cultured hippocampal neurons, either the activation of or inhibition of Rac1 reduces axon growth (Nishimura et al., 2005). In the chick embryo, disturbing Rac1 activity delays neurite outgrowth from motor neurons cultured on extracellular matrix (Kuhn et al., 1998). In *Drosophila*, motor neurons and giant fibers expressing a constitutively active form of Rac1 exhibit severe outgrowth impairments (Allen et al., 2000; Kaufmann et al., 1998; Kim et al., 2003). Similarly, both dominant active and negative mutants of Rac1 retard axon outgrowth of fly sensory neurons without polarity defects (Luo et al., 1994). In *Xenopus*, the expression of dominant negative or active Rac1 in neurons changes growth cone morphologies; and further significantly retards axon outgrowth (Ruchhoeft et al., 1999; Woo and Gomez, 2006). Consistent with these findings,

collapsin-1 (also known as semaphorin 3D) activates Rac1 and requires the activation for the inhibitory action (Jin and Strittmatter, 1997). In addition, as shown in Figure 5.6, expression of a constitutive active form of Rac1 phenotypically mimics the inhibitory effect of NG2. Together, these results emphasize the significance of the balanced activity of Rac1 for axonal development and suggest that hyperactivity of Rac1 is a main cause of axon growth inhibition induced by NG2.

Hyperactivity of Rac1 and actin cytoskeletons; How does the hyperactivity of Rac1 attenuate axon growth? Rac1 promotes actin polymerization through LIMK/Cofilin pathway and branching through WAVE/PAK1/ARF2/3 pathway (Arimura and Kaibuchi, 2007; Montenegro-Venegas et al., 2010; Ten Klooster et al., 2006), NG2 probably increases the stability of actin filament network and results in more packed and rigid actin filament structure. Straight and bundled F-actin structure in general serves to guide and promote microtubule progression into a growth cone, resulting in axonal elongation. Excessively meshed structure of F-actin network, however, prevents microtubule penetration, resulting in attenuation of axonal outgrowth (Bradke and Dotti, 1999, 2000). Therefore, NG2-induced hyperactivity of Rac1 probably increases both the rigidity and the meshwork of actin filaments to attenuate the microtubule progression into the growth cone.

The stoichiometric alteration of the PAR complex by NG2; I showed that NG2 reduces the interaction of PKCζ and PAR3 presumably through phosphorylation by PKCζ. Does it suggest that PAR3 is liberated out of the PAR complex upon the phosphorylation? Since PAR3 interacts with PAR6 independently of PKCζ, there is a possibility that PAR3 remains tethered to the complex through the interaction with PAR6. Through further experiments, however, it needs to be confirmed whether NG2-mediated disruption of PKCζ-PAR3 complex liberates the PAR3 out of the complex.

Depending on whether PAR3 interacts with PKCζ-PAR6, PAR3 can have a dual role as both a positive and negative regulator of Rac1. In the leading edge of a migrating cell, for instance, PAR3-TIAM2 complex associates with PKCζ-PAR6, resulting in Rac1 activation. In the trailing edge, PAR3-TIAM2 is dissociated from PKCζ-PAR6 due to PAR3 phosphorylation by RhoA kinase. In this status, PAR3 negatively regulates and thereby results in the inactivation of Rac1. Similar with this inhibitory role of PAR3, in kidney epithelial cells, PAR3 also

suppresses TIAM1, thereby leading to Rac1 inactivation and this function of PAR3 seems to be independent of PKCζ and PAR6. Together these results suggest that PAR3 may need to remain in the PAR complex to induce the activation of Rac1. Given that NG2 induces the activation of Rac1, it is possible that phosphorylated PAR3 is dissociated from PKCζ but may remain tethered to the PAR complex to activate TIAM in axon growth inhibition.

The spatial alteration of the PAR complex by NG2; I showed that NG2 induces PAR3 dislocation (Figure 5.7). Assuming that PAR3 remains in the PAR complex, this result suggests that NG2 may change the spatial localization of the polarity machinery. As I previously reviewed, a major work place of the PAR complex is the axonal tip, where it coordinates the dynamics of actin and microtubule cytoskeleton. The accumulation and transport of the complex to the growth cone is thus required for proper axonal development (Nishimura et al., 2004). Consistent with this data, in the control cells, PAR3 punctae, which likely represent axonal transport of the PAR complex, were found mainly at the axonal compartment. In the neurons grown on NG2, however, PAR3 signal were found mainly at the cell body. This result suggests that NG2 may hamper the axonal transport of the polarity machinery to the growth cone. Since the PAR complex is tethered to KIF3, a plus-end-directed microtubule motor protein, through PAR3, NG2 may target this interaction to interfere the axonal transport of the PAR complex. Such misregulation would direct Rac1 activity to inappropriate place, thereby attenuating normal axonal growth.

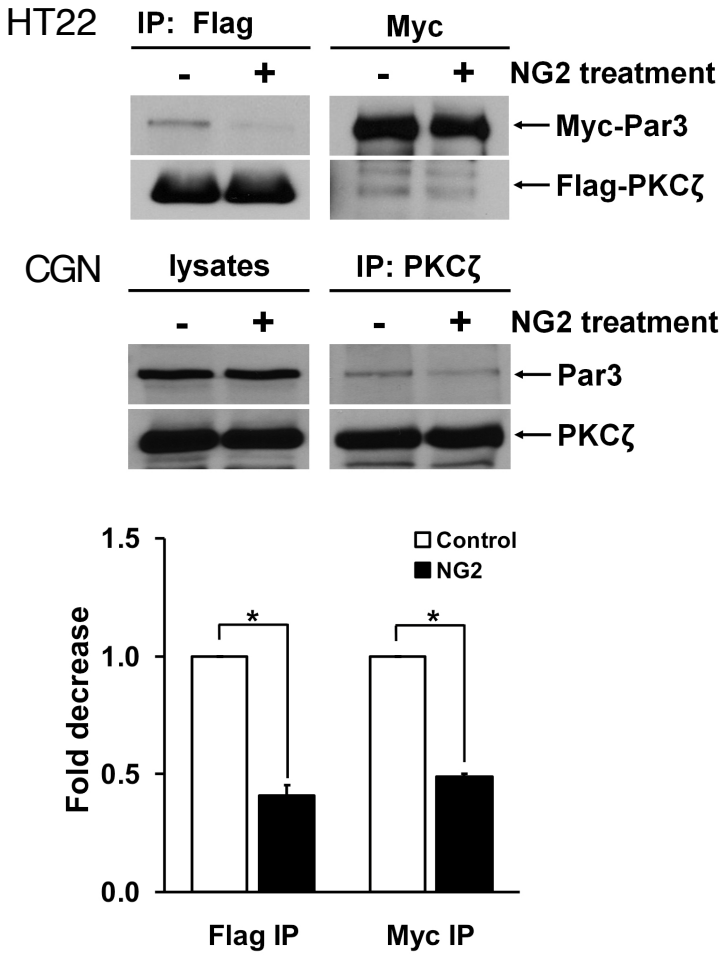


Figure 5.1. The regulation of a PKCζ-PAR3 complex by NG2

NG2 treatment dissociates a PKCζ-PAR3 complex. NG2 (10 μg/ml) was bath applied for 30 minutes to HT22 cells and CGNs transfected with epitope-tagged PKCζ and PAR3. The binding of these proteins was detected by immunoprecipitation followed by immunoblotting with epitope-specific antibodies. The histogram shows the mean ± SEM decreases in PAR3-PKCζ binding in HT22 cells (n=3, normalized to control, * $P < 0.05$, Student's t-test).

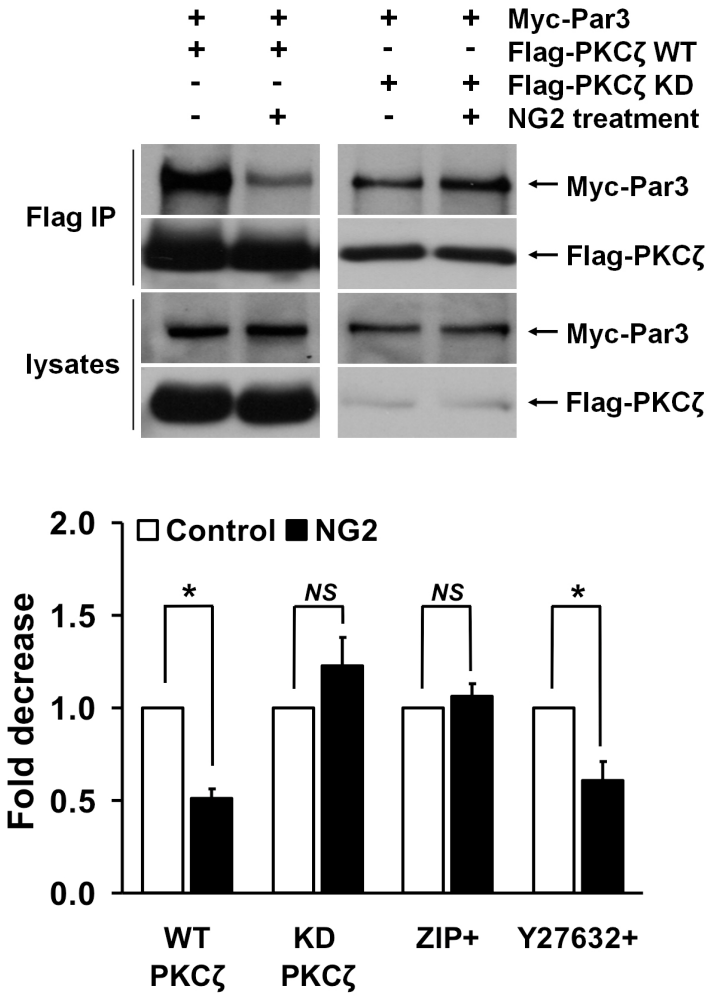


Figure 5.2. PKCζ activity is required for the dissociation of the PKCζ-PAR3 complex.

NG2 (10 μg /ml) was applied for 30 minutes to HT22 cells transfected with PAR3 and either wild type (WT) or kinase-deficient (KD) forms of FLAG-PKCζ in the absence or presence of ZIP (2 μM) or Y27632 (10 μM). The PKCζ-PAR3 complex was detected by immunoprecipitation with anti-FLAG antibody, followed by immunoblotting with specific anti-myc or FLAG antibody. Quantitation of the results is shown to the right (mean ± SEM, n=3, normalized to control treatment, $P<0.05$, Student's t-test).

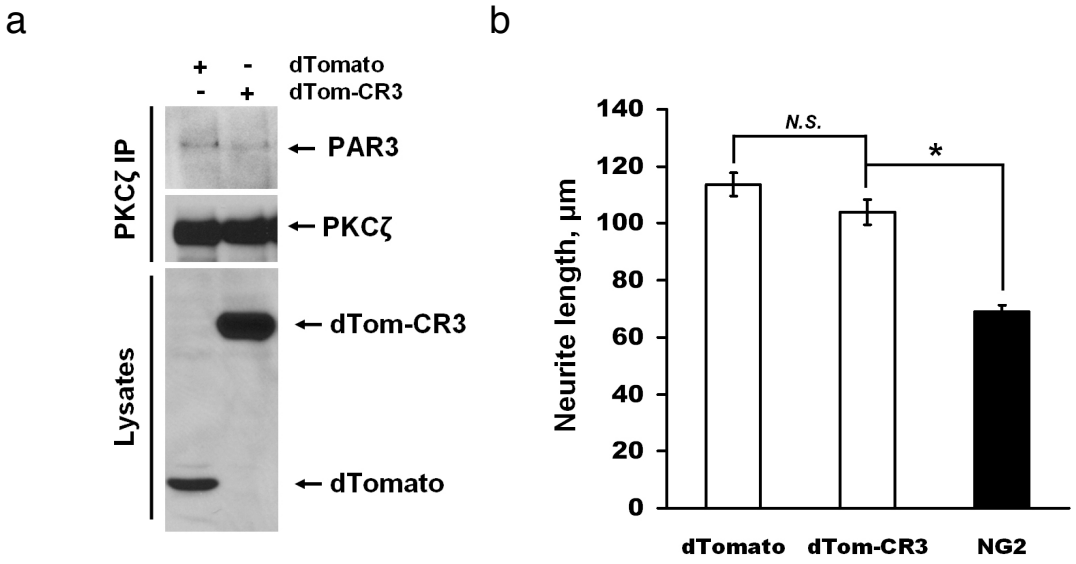


Figure 5.3. The disruption of PKCζ-PAR3 is insufficient for axon growth inhibition.

(a) Expression of CR3 fragment reduces the formation of PKCζ-PAR3 complex in HT22. (b) Expression of CR3 fragment of PAR3 does not affect the basal growth of axons on control substrate. CGNs were transfected with CR3 domain of PAR3 and grown on control (laminin) substrates. The histogram shows the quantitation of neurite lengths (mean ± SEM, n=3; *$p < 0.001$; Dunn's post-hoc test).

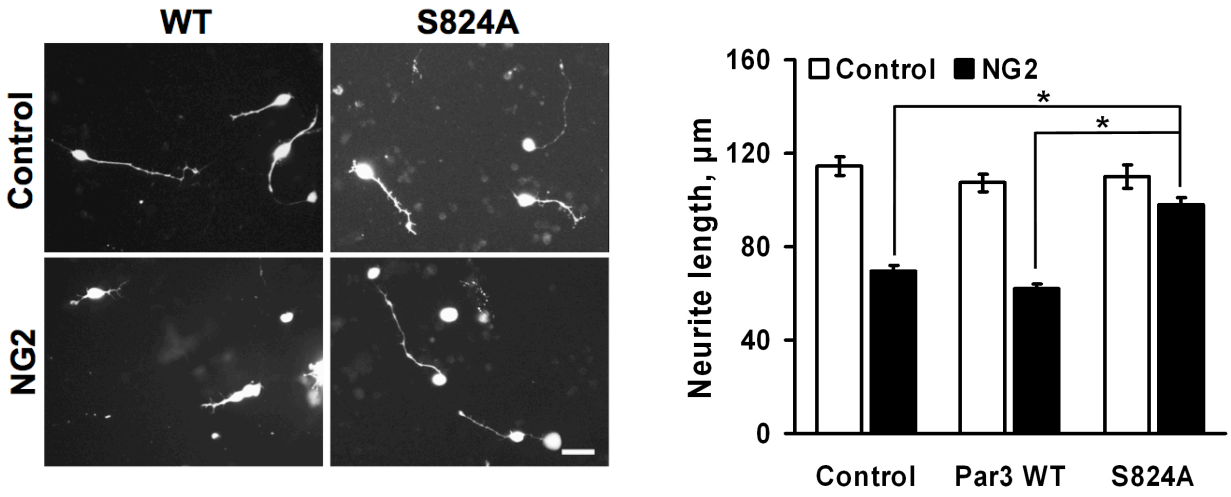


Figure 5.4. The phosphorylation of PAR3 site is required for axon growth inhibition.

Expression of a phosphorylation-deficient mutant PAR3^{S824A} prevents axon growth inhibition. CGNs were transfected with either WT or phosphorylation-deficient PAR3 and grown on control or NG2-coated substrates (Scale bar 50 µm). The histogram shows the quantitation of neurite lengths (mean ± SEM, n=3; *p < 0.001; Dunn's post-hoc test).

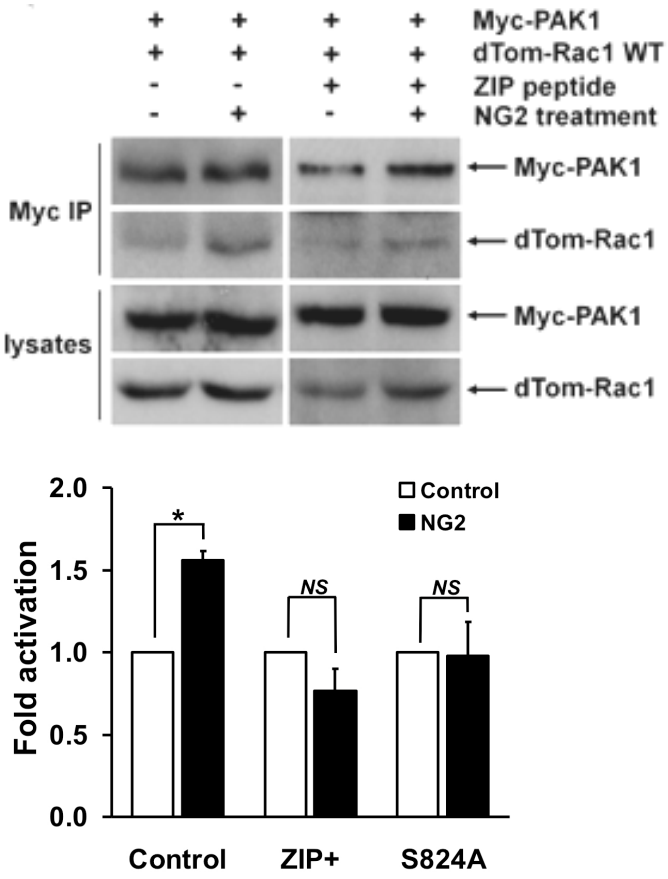


Figure 5.5. NG2 activates Rac1 to inhibit axon growth.

NG2 was bath-applied for 5 minutes to HT22 cells transfected with epitope-tagged PAK1 and Rac1 in the absence or presence of ZIP (2 μM) for 5 minutes. Rac1 binding to transfected PAK1 was measured by immunoprecipitation with anti-myc antibody followed by immunoblotting with anti-dsRed. The histogram to the right shows that NG2 increased Rac1 binding to PAK1 and this was prevented either by adding zip peptide or expressing the phosphorylation-deficient mutant form of PAR3 (mean ± SEM, n=3, normalized to control, * $P < 0.05$, Student's t-test).

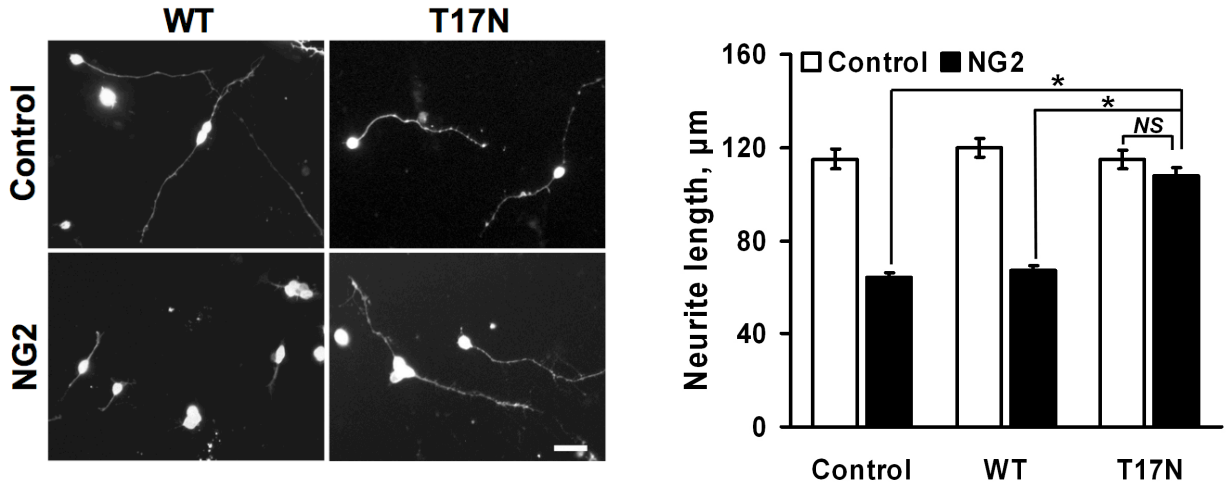


Figure 5.6. Rac1 activation is required for axon growth inhibition.

Rac1 activation is required for axon growth inhibition. CGNs were transfected with either wild type (WT) or dominant negative form (T17N) of Rac1 and grown on control or NG2-coated substrates. DN-Rac1-expressing CGNs were able to extend long axons on NG2 substrates, similar to the cells grown on control substrates (Scale bar 50 µm). The histogram shows mean neurite lengths ± SEM (n=3, *P < 0.001; Dunn's post-hoc test).

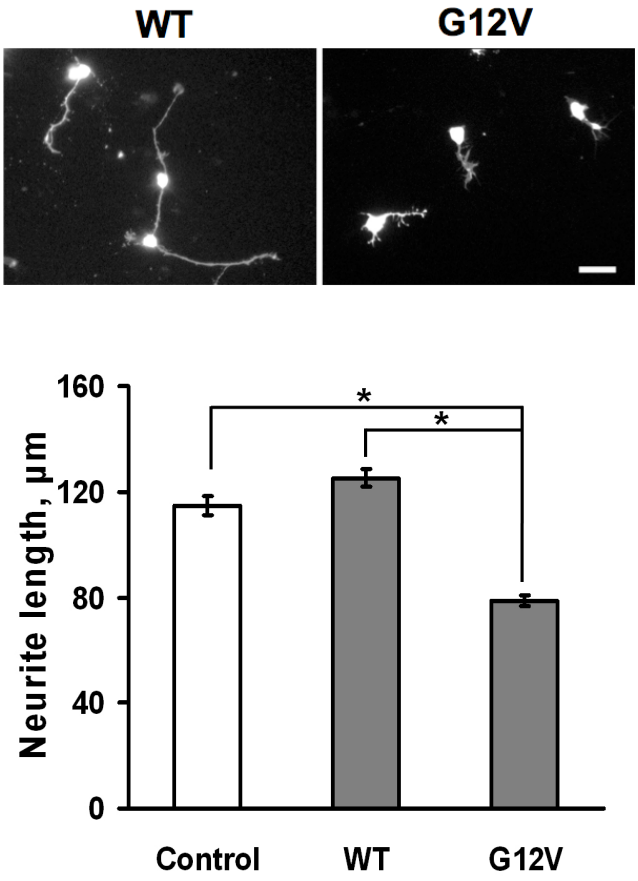


Figure 5.7. Constitutively active Rac1 is sufficient to inhibit axon growth from CGNs.

Wild type (WT) or constitutively active form (G12V) of Rac1 was transfected into CGNs. Constitutively active form of Rac1-expressing CGNs extended only short stubby axons on control substrates (Scale bar 50 µm). The histogram shows mean neurite lengths ± SEM (n=3; *$p < 0.001$; Dunn's post-hoc test).

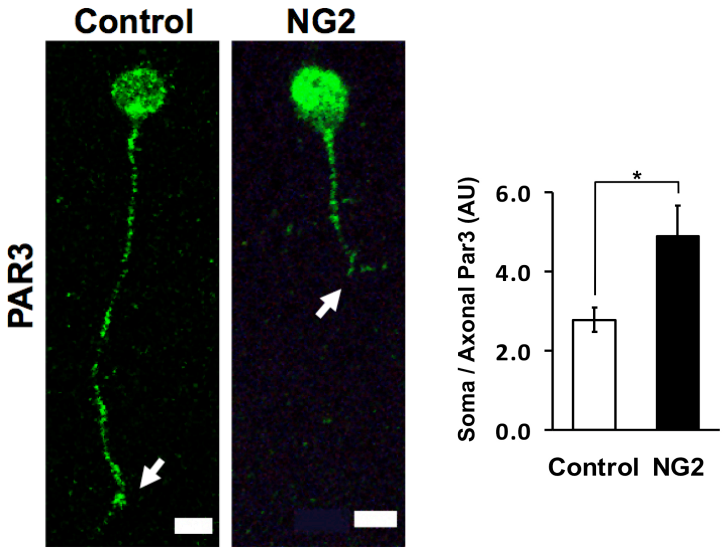


Figure 5.8. NG2 treatment alters the spatial distribution of PAR3 in CGNs.

CGNs were grown on either control or NG2 substrate for 24 hours and stained with anti-PAR3 antibody. Arrows point to the growth cones. The histogram shows the average ratio of PAR3 fluorescence in the cell body to that of the axonal compartment (mean ± SEM, n=15, for each condition $P<0.05$, Student's t-test). Scale bar 10 µm

Chapter 6 Ceramide signaling is involved in axon growth inhibition

6.1 Introduction

One of the fundamentals of cell biology is that cells respond to the environment. They should sense certain stimuli or external cues and thereby modulate their inner properties according to environmental change. To accomplish this purpose, cells are designed to have receptor systems including membrane receptors and receptor-associated enzymes to generate intracellular second messenger. In some cases, the enzymes exist as an integrated part of the receptors. In this way, an extracellular cue binds to and activates a receptor at the cell surface. The activated receptor then switches on the second messenger-generating enzymes. Through this process, cells couple extracellular information into intracellular biochemical event to alter the function of the cell. This initial signal transduction in general takes place at the cell surface. Therefore, the second messenger-generating enzymes often take advantage of lipid components in the plasma membrane to convert them into certain message molecules. In this section, I will discuss the lipid messengers related to cell polarity and how they are linked to extrinsic cues in axonal growth.

Phosphatidylinositol-3,4,5-trisphosphate; Phosphatidylinositol (3,4,5)-triphosphate (PIP3) is the product of phosphoinositide 3-kinases (PI3K) phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PIP2). PI3K and PIP3 exhibit a polarized activation and distribution during chemotaxis in neutrophils (Servant et al., 2000). This asymmetry is necessary for protrusion of the leading edge membrane in migrating cells. In cultured hippocampal neurons, PI3K activity accumulates at the tip of the newly specified axon. Either pharmacological inhibition of PI3K or PIP3 degradation by PTEN disrupts axon specification (Menager et al., 2004; Schwamborn and Puschel, 2004; Shi et al., 2003). Local activation of minor neurites using laminin-coated beads induces a concentration of PIP3 at the tips of the immature neurites and results in axonal growth from these neurites (Menager et al., 2004). Together, these results indicate that the activation of PI3K and the accumulation of PIP3 at the tip of a naïve neurite are required for axon specification.

PIP3 generation is triggered by some stimulatory extrinsic cues, such as BDNF and NT3 (Arimura and Kaibuchi, 2007; Yamada et al., 1997). These extrinsic cues likely activate PI3K through Ras since pharmacological inhibition of PI3K prevents Ras-induced formation of multiple axons (Yoshimura et al., 2006). The PIP3 triggers several signaling pathways to promote axonal development. One of them is RAP1B-Cdc42 pathway. Ras-related protein 1B (RAP1B), a Ras superfamily GTPase, concentrates at the tips of prospective axons before the localization of Cdc42 and the PAR complex. Overexpression of Rap1b results in formation of multiple axon-like neurites and the accumulation of the PAR complex in each neurite. RNAi-mediated knockdown of Rap1b prevents axon formation. Active form of Cdc42 rescues the effect of Rap1b suppression by RNAi whereas active form of Rap1B rescues the loss of axons induced by PI3K inhibition (Schwamborn and Puschel, 2004). Therefore, RAP1B appears to function in order to link PI3K signaling to Cdc42 and the PAR complex. Together, these results suggest that stimulatory extrinsic cues signal through Ras, PI3K and Rap1 to activate the PAR complex for axon specification and development.

Ceramide signaling in cell polarity; Ceramide is well known as a second messenger for the cell death signaling by TNF-α (Obeid et al., 1993; Schutze et al., 1992). It is generated through sphingomyelinase (SMase)-degradation of sphingomyelin. In general, ceramide signals through Ras/Raf cascade to trigger apoptotic cell death (Basu et al., 1998). In addition to an apoptotic effect, this lipid is involved in various cell polarity phenomena such as cilia formation, neuronal migration, and neurite outgrowth (Falluel-Morel et al., 2006; Wang et al., 2009; Wang et al., 2008; Wang et al., 1999). In these models, ceramide is associated with the polarity complex proteins for cytoskeleton remodeling. For example, ceramide synthesis from sphingomyelin is required for the formation of primary cilia. In this model, ceramide appears to have an instructive role in activating aPKC and spatially localizing the polarity complex to regulate microtubule dynamics (Wang et al., 2009). Ceramide is also required for the motility of neural progenitor cells. Wound-induced motility is accompanied by SMase activation. The resulting ceramide is enriched at the leading edge of the cell where it presumably recruits the polarity machinery for cytoskeleton reorganization. Consistent with these results, pharmacological depletion of ceramide results in several defects in embryonic brain, which is caused by motility defects of progenitor cells. These defects include loss of β-catenin apical-lateral membranes, unpolarized cellular morphology, disorganized distribution of cells and

mislocalization of Tuj1-positive cells. Together, these results suggest that ceramide is implicated to the distribution of neural progenitor cells and the cortical layer formation in embryonic mouse brain (Wang et al., 2008). Ceramide is also involved in the regulation of axonal outgrowth. Contrary to the stimulatory role of PIP3, exogenous application of ceramide attenuates axon outgrowth by reorganizing the cytoskeleton (de Chaves et al., 1997; de Chaves et al., 2001; Falluel-Morel et al., 2006; Schultz and Larsson, 2004). It, however, remains elusive whether there is any extrinsic cue to trigger ceramide signaling to inhibit axon growth.

The relevance of ceramide to cell polarity and its inhibitory effect on axon outgrowth suggest that ceramide signaling may be an upstream pathway to mediate axon growth inhibition. Left unanswered, however, is what are the extracellular cues that generate ceramide for axon growth inhibition. Therefore, in this chapter, I explore whether NG2 recruits ceramide signaling to mediate its inhibitory effect.

6.2 Results

6.2.1 PKCζ is required for ceramide-induced axon growth inhibition

The activation of PKCζ by ceramide and its connection to the polarity machinery led me to a hypothesis that ceramide signaling may be involved in axon growth inhibition. To test the hypothesis, I bath-applied C6 ceramide to CGNs grown on control substrate and examined its effect on axon outgrowth. As shown in Figure 6.1, the application of ceramide significantly prevented the axon elongation on control substrate, similar to the inhibitory effect of NG2. Furthermore, pharmacological suppression of PKCζ activity prevented the inhibitory action of ceramide. Altogether, these results suggest that ceramide inhibits axon outgrowth in a PKCζ-dependent manner.

6.2.2 Ceramide is required for axon growth inhibition by NG2

The requirement of PKCζ for inhibitory effect of axon growth inhibition by ceramide suggests that this lipid messenger may play a significant role in mediating the inhibitory action of extrinsic inhibitors. Since the ceramide that participates in signaling is derived from the hydrolysis of sphingomyelin by sphingomyelinase, suppression of sphingomyelin synthesis is a way to manipulate ceramide signaling in cells. Therefore, I applied fumonisin B1 (FB1), an

inhibitor of *de novo* sphingomyelin synthesis, to CGNs culture grown either NG2 or chick embryo CSPGs and examined the effect of FB1 on axon growth. As shown in Figure 6.2, the treatment of FB1 allowed axon outgrowth on NG2 substrate but not the CSPG substrate. This result suggests that ceramide signaling is required for the inhibitory effect of NG2 but not other CSPG species.

6.3 Discussion

Here, I showed that ceramide inhibits axonal outgrowth of CGNs in a PKCζ-dependent manner. I also showed pharmacological suppression of ceramide signaling attenuates the inhibitory effect of NG. These results suggest that NG2 recruits ceramide signaling to mediate its inhibitory action.

The inhibitory action of ceramide seems somewhat contradictory to the observation that Harel and Futerman made several years ago (Harel and Futerman, 1993). In this report, they showed that treatment of hippocampal neurons with FB1 prevents axonal elongation whereas application of ceramide abolishes the inhibitory effect of FB1. In fact, this promotive role of ceramide on axonal elongation, however, results from glycoceramide, a metabolic derivative of ceramide but not from ceramide itself. Therefore, the role of ceramide as a signaling component is likely to attenuate axonal elongation (Futerman et al., 1998).

It has long been known that exogenous application of ceramide attenuates axon outgrowth by reorganizing the cytoskeleton. (de Chaves et al., 1997; de Chaves et al., 2001; Falluel-Morel et al., 2006; Schultz and Larsson, 2004). A critique can be raised that ceramide-mediated inhibition could be a side effect of apoptosis since ceramide indeed induces apoptosis of neurons at high concentration. It, however, inhibits axon growth without apoptosis at low concentration (Futerman et al., 1998). Consistent with this, anti-apoptotic reagents, such as a DAG analogue are unable to reverse ceramide-induced growth inhibition (de Chaves et al., 1997; De Chaves et al., 1998). Another critique is that some metabolites of ceramide, not ceramide itself, may generate signaling for axon growth inhibition. To investigate this possibility, De Chasves et al injected C6-NBD-ceramide to sympathetic neurons and tracked down the fate of the lipid. They found that most C6 ceramide remained unchanged and only a little fraction of fluorescent-ceramide was converted to phosphorylated ceramide. These results suggest that

ceramide triggers a unique signaling pathway that is not fully characterized to induce axon growth inhibition.

It is anticipated that ceramide induces alterations of cytoskeleton for its inhibitory action on axon outgrowth. How does ceramide accomplish the cytoskeleton restructuring to attenuate axon outgrowth? As reviewed earlier, in several cell polarity models, ceramide is associated with the polarity complex proteins. The requirement of PKCζ for the inhibitory effect of ceramide (Figure 6.1), therefore, suggests that polarity complex proteins may play a role in linking ceramide signaling to cytoskeleton reorganization for axon growth inhibition.

Pharmacological depletion of sphingomyelin prevents the inhibitory effect of NG2 but not of chick embryo CSPG (Figure 6.2). The structural and biochemical difference of NG2 from other CSPGs indicates that these two inhibitory molecules may have distinctive receptors and intracellular mechanisms. Consistent with this idea, the connection of PKCζ and ceramide with RPTPσ, which is recently identified as a CSPG receptor, remains vague. I, therefore, speculate that NG2 triggers ceramide signaling to activate the PAR complex whereas CSPG recruits different signaling pathways, such as calcium, conventional PKC and RhoA signaling.

What is the role of ceramide in the regulation of the PAR complex? As reviewed earlier, ceramide stimulates PKCζ activation and the formation of the PAR complex. Suppression of ceramide synthesis results in atypical distribution of the PAR complex as well as polarity defects (Krishnamurthy et al., 2007; Wang et al., 2008). Ceramide, therefore, seems to have an instructive role to localize the PAR complex to the region where actin and microtubule interact with each other. Consistent with this speculation, NG2 induces PKCζ activation and translocation to the membrane as well as atypical localization of PAR3, suggesting that NG2-induced ceramide signaling may be related to the spatial alterations the PAR complex.

In this regard, it is noteworthy that ceramide has antagonistic effect on neural migration and neurite outgrowth; ceramide accelerates the motility of granule neuron progenitors whereas it inhibits neurite outgrowth. (Falluel-Morel et al., 2006). Ceramide is accumulated at the leading edges of neural progenitor cells and it recruits the PAR complex there for the motility of the cells. In general, the leading edges of neural progenitors differentiated into dendrites, whereas the trailing edges sprout axons. Together, these observations and ideas suggest that NG2 signals

through ceramide to induce the dislocation of the PAR complex from axonal compartment and this dislocation may be a mechanism by which NG2 inhibits axon growth.

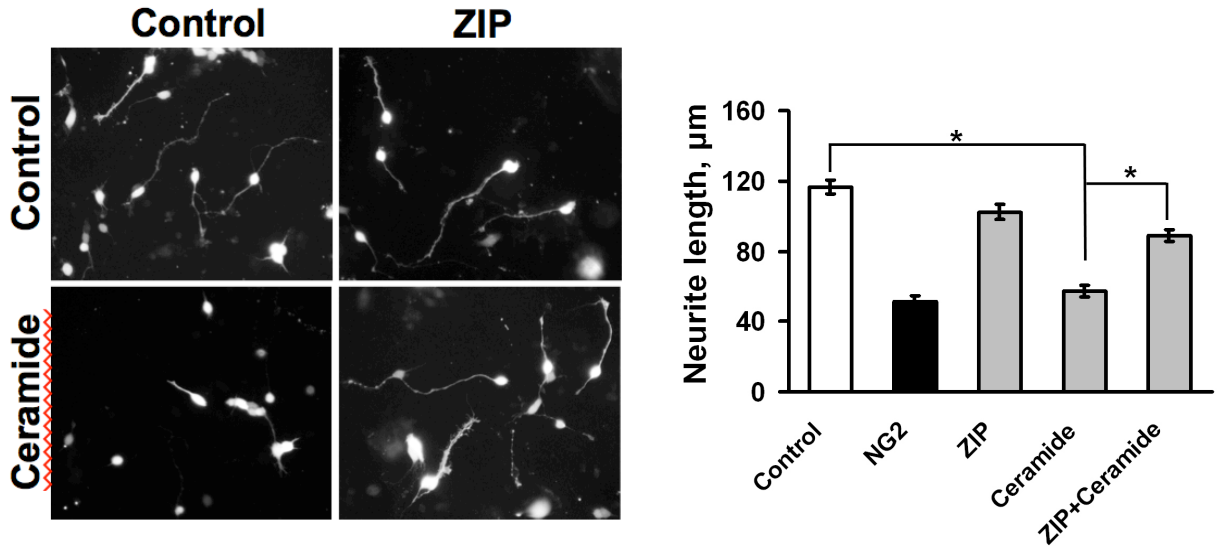


Figure 6.1. The effect of ceramide on axonal growth of CGNs

CGNs were grown on control or NG2 substrates in the presence of indicated reagents. 2.5 µM of bath-applied C6 ceramide were able to inhibit axonal growth on control substrates, similar to the inhibitory effect of NG2. Ceramide-inhibition was attenuated in the presence of PKCζ inhibitor. The histogram shows mean neurite lengths ± SEM, (n=2, > 118 cells were counted and scored in each condition and each experiment; *P < 0.001. Dunn's post-hoc test).

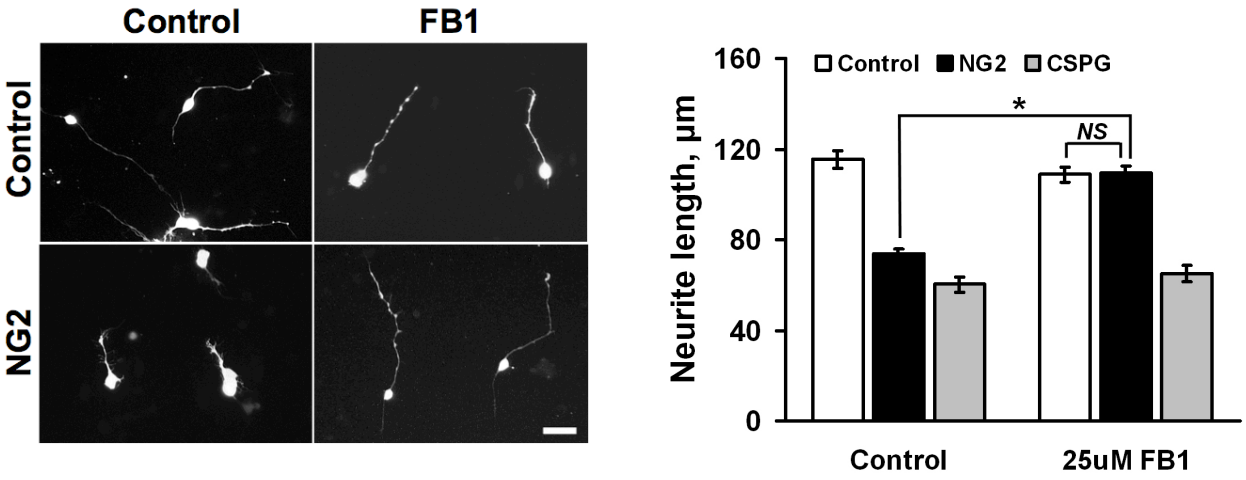


Figure 6.2. Ceramide signaling is required for axon growth inhibits by NG2.

CGNs were grown on control, NG2 or CSPG substrate in the presence of indicated reagents. 25 µM of bath-applied fumonisin B1 allowed axon growth on NG2 substrate but neither control nor CSPG substrates (Scale bar 50 µm). The histogram shows mean neurite lengths ± SEM (n=3, > 285 cells were counted and scored in each condition and each experiment; *P < 0.001; *P < 0.001. Dunn's post-hoc test).

Chapter 7 Conclusion and General Discussion

7.1 Conclusion

My research has shown that the polarity complex plays a central role as the intracellular machinery that mediates axon growth inhibition. Here, I used NG2 as a model for extrinsic inhibitory and repulsive cues. Using this model, I showed that NG2 substrate inhibits neurite outgrowth of both cerebellar granule neurons and dorsal root ganglia neurons. PKCζ is required for the inhibitory action of NG2 and sufficient for axon growth inhibition. I demonstrated that NG2 induces the activation of PKCζ with the increase of phosphorylation, kinase activity and membrane translocation of the enzyme. The activation of Cdc42 is required for the inhibitory effect of NG2 and sufficient for axon growth inhibition. NG2 activates Cdc42, resulting in the formation of Cdc42-PAR6 complex. This complex is considered to mediate NG2-induced PKCζ activation and required for the outgrowth inhibition induced by NG2. This activation of the kinase likely causes the phosphorylation of PAR3 at the PKCζ site, which is required for the inhibitory effect of NG2. I showed also that NG2 activates Rac1 and this activation is dependent on PKCζ and PAR3 phosphorylation. Rac1 is necessary and sufficient for axon growth inhibition. I also found that NG2 substrate induces an atypical accumulation of PAR3 in the cell bodies in CGNs. The requirement for Cdc42 activity suggests that NG2 may recruit further upstream second messengers. In this regard, I found that ceramide signaling is involved in axon growth inhibition by NG2.

Based on these results and relevant literature, I speculate that NG2 activates yet unidentified receptors and SMases to trigger ceramide signaling. Activated ceramide signaling may recruit a Cdc42-specific GEFs to trigger Cdc42, resulting in the formation of Cdc42-PAR6 complex. This complex likely activates PKCζ and this activation results in the activation of Rac1 and through PAR3 phosphorylation. Balance in the activity of Cdc42 and Rac1 is critical for normal axon growth, as reviewed previously. Suppressing these two GTPases attenuates the inhibitory effects of NG2 whereas upregulation of these GTPases induces axon growth inhibition. Therefore, I speculate that NG2 causes hyperactivity of Cdc42 and it is transferred to Rac1 through the PAR complex (Figure 7.1). This resulting hyperactivity of Rac1 may increase

meshwork and rigidity of actin filaments. This alteration likely hampers microtubule protrusion into growth cones, which is required for axon growth. In addition, due to the instructive role of ceramide, the elevated ceramide may contribute to dislocation of the polarity machinery from the axonal compartment. This dislocation may result in mislocalization of Rac1 activity. It may be another mechanism by which NG2 inhibits axon growth.

7.2 General Discussion

Extrinsic cues and the polarity machinery; The current understanding of the polarity machinery in general has been achieved from observations of spontaneously polarizing hippocampal neurons. This model provides us with a simple way to interpret molecular behavior regarding axon and dendrite specification. In this context, polarity appears to be an intrinsic property of the neurons. Numerous trophic factors and guidance cues, however, are required for the normal development of nervous system. This raises the question of whether there is any instructive role of extrinsic cues upon the polarity machinery. The effort to address these questions are well described in the work of Yi *et al.* where they showed the role of TGFβ in axon development and its connection to PAR6 (Yi et al., 2010). Chen *et al.* also showed that BDNF has an instructive role of axon specification through the polarity complex. Zhang et al. suggest that WNT is another extrinsic cue targeting polarity signaling to promote axonogenesis (Zhang et al., 2007). In addition to neuronal polarity, Wolf et al showed that WNT signals through PKCζ mediate its attractive guidance activity (Wolf et al., 2008). These researches revealed the significance of stimulatory cues and the polarity machinery in neuronal polarity and attractive guidance but they opened another question, whether the polarity machinery is also recruited by inhibitory cues. I demonstrated that an inhibitory cue such as NG2 also plays an instructive role in modulating the polarity machinery to attenuate axonal growth.

Ceramide as an intermediate messenger of inhibitory cues; Another gap in this field is what is the intermediate messenger that links cell surface signaling to the Par complex. A derivative of membrane lipid, phosphatidylinositol (3,4,5)-triphosphate (PIP3) has been proposed as such a messenger molecule and PIP3 is accumulated at the tip of a growing axon, where it regulates the polarity complex through Cdc42. The stimulatory role of PIP3 for axon development led me to explore other candidate lipids that may couple NG2 to intracellular signaling. Here, I showed a requirement for ceramide signaling for NG2-mediated inhibition.

The enrichment of some sphingolipids in the neuronal membrane suggests that they may have significant role for neuronal development. Among them, ceramide and its metabolite, glucosylceramide are related to neuronal polarity. Ceramide promotes the formation of minor neurites from lamellipodia at the earliest stage. Glucosylceramide is required for axon specification. Given that glucosylceramide is synthesized from ceramide, the following sequential roles of these lipids have been proposed in neuronal polarity. Ceramide is required for minor neurite formation and it likely keeps the neurites undifferentiated, with short processes filled with actin filament. In the neurite that will become an axon, ceramide likely is changed into glucosylceramide, initiating biochemical events for axonogenesis (Futerman et al., 1998). Therefore, it is possible that NG2-stimulated ceramide signaling may keep the immature neurites undifferentiated, delaying axonal outgrowth.

The significance of ceramide as an intermediate messenger of inhibitory cues is also highlighted by the following observations. The generation of the second messenger ceramide requires either acid sphingomyelinase (aSMase) or neutral sphingomyelinase (nSMase) activity to cleave sphingomyelin into ceramide (Jayadev et al., 1994; Spiegel et al., 1996). aSMase mainly is found in lysosome and presumably has a housekeeping role to recycle ceramide and its derivatives. nSMase appears to be regulated by ligand-receptor activation and involved in the generation of ceramide for signal transduction pathways. In addition, aSMase activity is restricted to cell body whereas nSMase is preferentially localized in axonal compartment in cultured sympathetic neurons (De Chaves et al., 1998). Consistent with these results, De Chaves et al cultured sympathetic neurons in compartmentalized chamber and observed that ceramide effectively inhibits neurite outgrowth only when applied to distal neurites but not when applied to cell bodies (de Chaves et al., 1997). Together, these results suggest that ceramide signaling pathway for axon growth inhibition is localized at the tips of axons. It is assumed that a growth cone interprets extrinsic cues and initiates biochemical events to induce proper response of growth cone. Thus, such local requirement of ceramide implies that a role of this lipid signaling is to enable growth cones to sense inhibitory guidance cues and to exhibit pausing or avoidance behaviors by triggering some downstream signaling events. Consistent with this speculation, the growth cones of chicken retinal ganglion cells avoid NG2 in an *in vitro* barrier assay that was performed by my collaborators, Weschenfelder and Bastmeyer (Figure 7.2). Pharmacological inhibition of PKCζ, a downstream component of ceramide signaling, reduced the avoidance of

NG2 stripes. Together, these suggest that growth cones may sense NG2 through unidentified receptor systems, trigger ceramide signaling and the PAR complex for the avoidance behavior. Therefore, ceramide signaling likely plays an important role in avoidance behavior of growth cones equivalently with growth inhibition in response to inhibitory cues.

Cytoskeleton dynamics and axon growth inhibition; Since the stability of actin filaments regulates axon elongation, balanced Rac1 activity is critical for proper axonal growth and path finding (Allen et al., 2000; Bradke and Dotti, 1999, 2000; Woo and Gomez, 2006). I have shown that NG2 induces hyperactivity of Cdc42 and results in dysfunctional activation of Rac1 through the PAR complex. This hyperactivity of Rac1 in response to NG2 led me to predict that it may promote actin polymerization and branched structure of actin filament. This idea was supported by the preliminary observation that NG2 induces clumps of actin filament at the growth cones of DRG neurons (Figure 7.3). These actin clumps are probably a highly polymerized meshwork of actin. These meshed actin filaments may hamper efficient microtubule protrusion into growth cones that is necessary for axon elongation. To confirm the involvement of actin stability in axon growth inhibition, one can ask whether actin-destabilizing reagents, such as cytochalasin and latrunculin, attenuate the inhibitory action of NG2.

Microtubule stability may be another target for axon growth inhibition. A damaged CNS axon forms a swollen structure at the tip. Such dystrophic growth cones or retraction bulbs are correlated with axonal degeneration at glial scar. Retraction bulbs contain disorganized microtubule structures but a normal growth cone is filled with microtubule bundles (Erturk et al., 2007). Furthermore, microtubule-stabilizing reagents restore disorganized microtubule structures and promote axon regeneration at the injury sites (Hellal et al., 2011).

NG2 possibly affects microtubule dynamics since PKCζ regulates the microtubule-associated proteins through GSK3β and MARK2 (Arimura and Kaibuchi, 2007; Chen et al., 2006b). Both GSK3β and MARK2 target microtubule-associated proteins, such as Tau and MAP1B. In addition, PAR3 regulates microtubule dynamics through Rac1 and Stathmin/OP18, a microtubule destabilizing protein. In this way, NG2 may induce changes in microtubule stability. To test this hypothesis, one can use either taxol to stabilize or nocodazole to destabilize microtubule and examine whether each of these microtubule drugs attenuates the inhibitory effect of NG2.

***In vivo* research;** All of my work relied on *in vitro* culture models. This approach has provided useful mechanistic information and identified a novel intracellular mechanism of axon growth inhibition. The success of reversing growth inhibition *in vitro* leads to the expectation that manipulating the polarity machinery may allow successful regeneration of damaged axons at the injury sites. Therefore, the physiological significance of my results needs to be re-examined using *in vivo* models. One approach would be to infuse ZIP peptide (a cell-permeable pseudosubstrate inhibitor of PKCζ) into the injured site of the animals as previously described (Tan et al., 2006). Alternatively, PKCζ-deficient animals can be used. A phenotypic analysis of PKCζ-/- mice showed that these mice are grossly normal except for some abnormality in immune system and hematopoietic system (Leitges et al., 2001). It has not been reported yet whether the genetic modification causes any physiological defect in nervous system. Altogether, these approaches are anticipated to provide us more practical information for therapeutic research to regenerate damaged neuronal tissue.

Clinical implication of the research; The failure of successful regeneration of severed axon in spinal cord injury is at least in part attributed to the inhibitory molecules. Therefore, a number of therapeutic strategies have been focused on neutralizing these molecules and these approaches improved axon regeneration *in vivo* (Bradbury et al., 2002; Tan et al., 2006). These successes, however, appear to be limited in terms of clinical application, since it is hardly possible to block such variety of inhibitory molecules simultaneously. It is, therefore, important to understand their intracellular signaling mechanism and even to find any pathway shared by the inhibitors. Such information will be valuable for clinical therapy of CNS injury. In that sense, the polarity complex and ceramide signaling would be important targets for further translational research for the future.

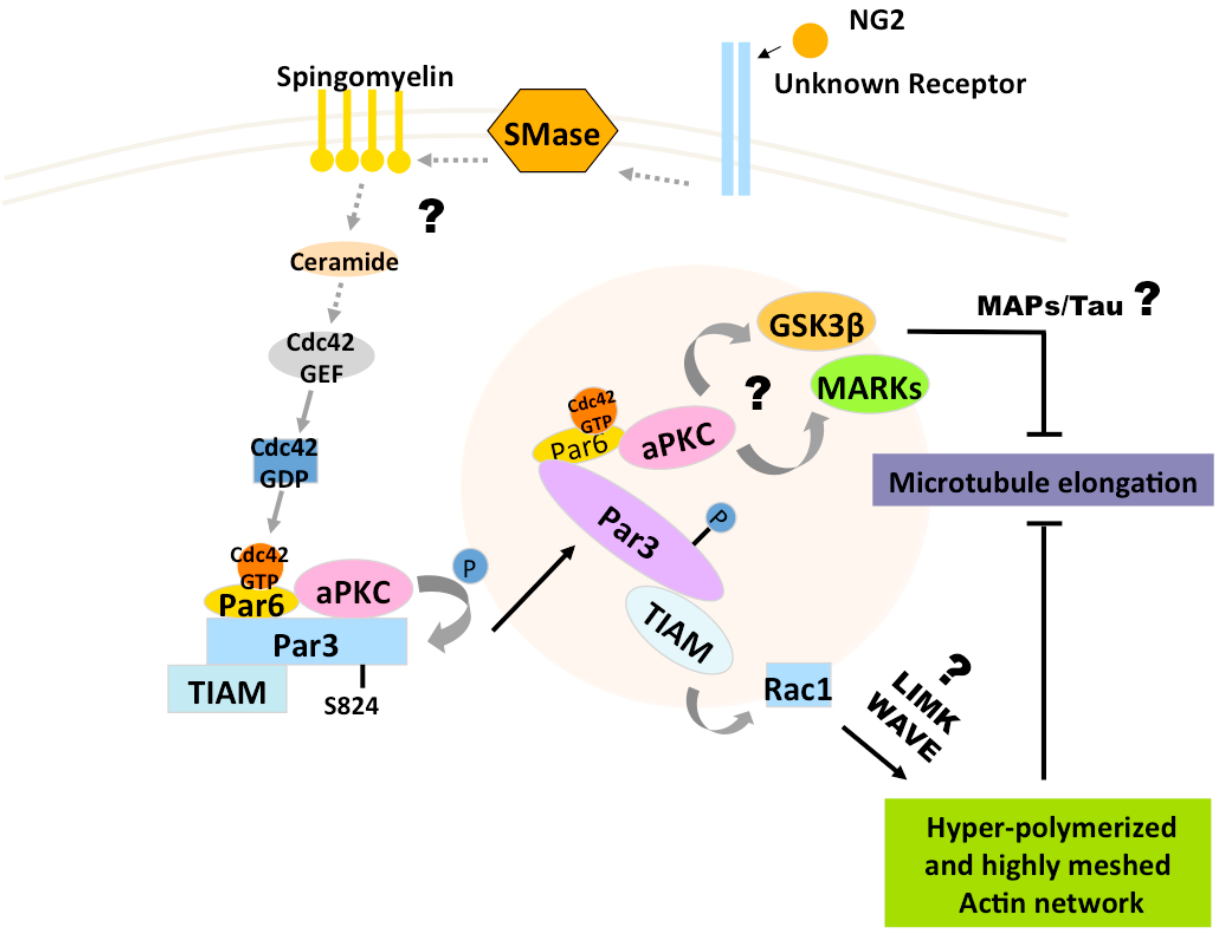


Figure 7.1. A proposed model of axon growth inhibition by NG2

NG2 triggers ceramide signaling through an unknown receptor system at cell surface level. The elevated ceramide may recruit the polarity proteins and Cdc42 to the membrane to activate aPKC. Activated aPKC phosphorylates PAR3. It alters the configuration of the PAR complex as illustrated here. This alteration may allow aPKC to regulate microtubule dynamics to attenuate axon growth. The PAR3 phosphorylation also triggers Rac1 signaling, promoting actin polymerization and branching. This alteration of actin cytoskeleton presumably prevents microtubule elongation in the growth cones, resulting in axon growth inhibition. The Rac1 activation may alter microtubule dynamics through Stathmin, a microtubule destabilizer.

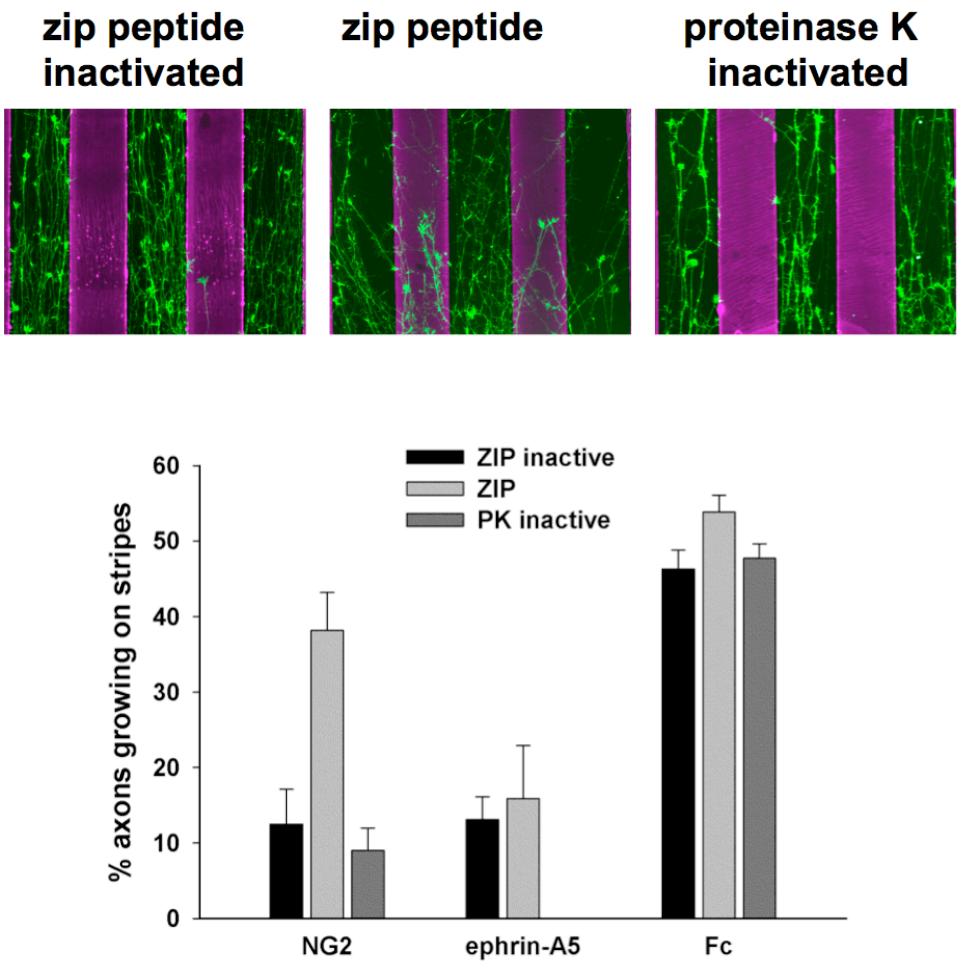


Figure 7.2. The avoidance of NG2 in the stripe assay requires PKCζ activity.

E7 chick retina was grown on striped surfaces coated with NG2 (purple) and living cultures were treated as indicated. The histogram below plots % of neurites growing on the stripes. PK-proteinase K

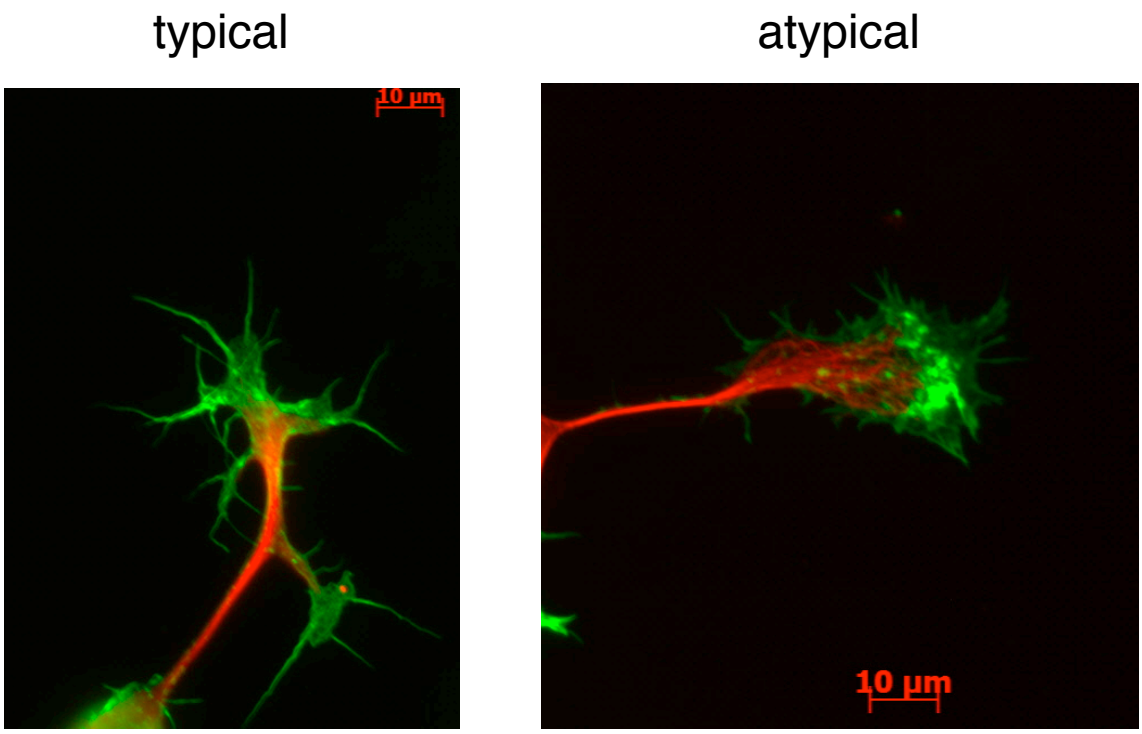


Figure 7.3. NG2 treatment alters the structure of actin cytoskeleton structures in the growth cones of DRG neurons.

DRG neurons were treated with either control or soluble form of NG2 substrate for 2 hours and stained with phalloidin (green) and anti-tubulin antibody (Red). NG2 treatment induced the formation of the clumps of phalloidin staining in the growth cone. Atypical clumps of phalloidin staining (green) was observed in 16.5% of counted growth cones (43/254) from control cultures and 25.4% (55/217) of growth cones from NG2-treated culture (Pooled data from 4 independent experiments).

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