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Modulation of growth factor-induced ERK signaling by the microtubule associated protein tau

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MODULATION OF GROWTH FACTOR-INDUCED ERK SIGNALING BY THE MICROTUBULE ASSOCIATED PROTEIN TAU

by

Chad Jeremy Leugers

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Neuroscience
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Thesis Supervisor: Associate Professor Gloria Lee

ABSTRACT

The microtubule-associated protein tau is known for its ability to bind to and stabilize microtubules and for its ability to nucleate microtubule assembly. In neurodegenerative tauopathies such as Alzheimer's disease, tau becomes hyperphosphorylated and loses the capacity for microtubule binding, possibly contributing to microtubule destabilization and axonal degeneration. However, evidence now indicates that soluble forms of hyperphosphorylated tau might have a toxic gain of function linked to abnormal signal transduction and cell cycle events in normally post-mitotic neurons. In support of this hypothesis, tau has been found to associate with numerous signaling proteins such as tyrosine kinases, adaptor proteins, and scaffold proteins. During early brain development, fetal tau is also more phosphorylated than tau in the adult brain and weakly binds microtubules, suggesting tau has functions in addition to microtubule stabilization.

The aim of this dissertation research is to investigate the possible role of tau in neuronal signaling, using tau-expressing and tau-depleted cell lines. Here, we provide evidence that during growth factor stimulation of neuronal cells, tau functions in advance of the neurite elongation stage. Tau is required for neurite initiation in a manner that does not require its microtubule binding function, and in addition, tau potentiates AP-1 transcription factor activation in response to nerve growth factor (NGF). The effect of tau on AP-1 activation is mediated through the enhanced activation of extracellular signal-regulated kinase (ERK), in response to both NGF and epidermal growth factor (EGF). We show that phosphorylation of tau at Thr231 also occurs in response to NGF and is required for tau to impact on ERK signaling, whereas the ability of tau to bind to microtubules is not required. Together, these findings indicate a new functional role for tau in neuronal signal transduction and have implications for tau function during early brain development and in neurodegenerative disease.

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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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To Steph, Caleb, and Cayden

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The microtubule-associated protein tau is known for its ability to bind to and stabilize microtubules and for its ability to nucleate microtubule assembly. In neurodegenerative tauopathies such as Alzheimer's disease, tau becomes hyperphosphorylated and loses the capacity for microtubule binding, possibly contributing to microtubule destabilization and axonal degeneration. However, evidence now indicates that soluble forms of hyperphosphorylated tau might have a toxic gain of function linked to abnormal signal transduction and cell cycle events in normally post-mitotic neurons. In support of this hypothesis, tau has been found to associate with numerous signaling proteins such as tyrosine kinases, adaptor proteins, and scaffold proteins. During early brain development, fetal tau is also more phosphorylated than tau in the adult brain and weakly binds microtubules, suggesting tau has functions in addition to microtubule stabilization.

The aim of this dissertation research is to investigate the possible role of tau in neuronal signaling, using tau-expressing and tau-depleted cell lines. Here, we provide evidence that during growth factor stimulation of neuronal cells, tau functions in advance of the neurite elongation stage. Tau is required for neurite initiation in a manner that does not require its microtubule binding function, and in addition, tau potentiates AP-1 transcription factor activation in response to nerve growth factor (NGF). The effect of tau on AP-1 activation is mediated through the enhanced activation of extracellular signal-regulated kinase (ERK), in response to both NGF and epidermal growth factor (EGF). We show that phosphorylation of tau at Thr231 also occurs in response to NGF and is required for tau to impact on ERK signaling, whereas the ability of tau to bind to microtubules is not required. Together, these findings indicate a new functional role for tau in neuronal signal transduction and have implications for tau function during early brain development and in neurodegenerative disease.

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Aβ	amyloid beta
AD	Alzheimer's Disease
AP-1	activator protein 1
APP	amyloid precursor protein
ATP	adenosine triphosphate
CBD	corticobasal degeneration
CDK5	cyclin dependent kinase 5
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
DNA	deoxyribonucleic acid
DMPK1	dystrophia myotonica protein kinase 1
dsRNA	double stranded ribonucleic acid
Dyrk1A	dual specificity tyrosine phosphorylation regulated kinase 1A
FAT	fast axonal transport
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FTDP-17	frontotemporal dementia with Parkinsonism linked to chromosome 17
GEF	guanine nucleotide exchange factor
Grb2	growth factor receptor-bound protein-2
GSK3β	glycogen synthase kinase 3β
GTP	guanosine triphosphate
HRP	horse radish peroxidase

IEP	isoelectric point
IGF	insulin-like growth factor
IP	immunoprecipitation
MAP	microtubule associated protein
MAPK	mitogen activated protein kinase
MTBR	microtubule binding repeat
NE	norepinephrine
NFT	neurofibrillary tangle
NGF	nerve growth factor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC6-3	pheochromocytoma clone 6-3
PC12	pheochromocytoma clone 12
PDGF	platelet-derived growth factor
PHF	paired helical filaments
PI3K	phosphatidyl inositol 3 kinase
PLCγ	phospholipase Cγ
PP1	protein phosphatase 1
Pro	proline
PSP	progressive supranuclear palsy
RAGE	receptor for advanced glycation endproducts
RNA	ribonucleic acid
RNAi	RNA interference
s.e.m.	standard error of the mean
Ser	serine
SDS	sodium dodecyl sulfate
SFK	Src family kinase

SH2	Src homology 2
SH3	Src homology 3
SHP2	SH2 containing phosphatase 2
shRNA	short hairpin ribonucleic acid
SOS	Son of Sevenless
Syk	spleen tyrosine kinase
Tet-R	tetracycline repressor
Thr	threonine
TrkA	tropomyosin related kinase A
Tyr	tyrosine
VEGF-D	vascular endothelial growth factor D

CHAPTER I

INTRODUCTION

Neurodegenerative diseases are debilitating conditions that cause a progressive and irreversible loss of neurons in the brain, decreased cognitive function and quality of life, and contribute to ever increasing health costs in aging populations. Within the broad spectrum of neurodegenerative diseases is a subset of disorders collectively known as tauopathies, defined by the involvement of pathological forms of the microtubule-associated protein tau. Diseases with tau pathology include Alzheimer's disease (AD), frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), and Pick's disease, among others (reviewed in (Spillantini and Goedert, 1998, Binder et al., 2005, Goedert and Jakes, 2005, Ballatore et al., 2007)). Although these diseases may vary with respect to associated pathologies, the common pathological finding in all tauopathies is the abnormal hyperphosphorylation and aggregation of tau protein in the brain. Since the discovery of abnormal aggregates of tau in these diseases, the physiological and pathological functions of tau protein have become the subject of intense research efforts, in the hope of gaining a better understanding of brain development, and to gain insights that may lead to novel therapeutic options for disease sufferers.

Under normal conditions in the adult brain, tau protein can be found associated with microtubules (Binder et al., 1985). However, the interaction with microtubules is tightly regulated by phosphorylation (Biernat et al., 1993), and the hyperphosphorylated forms of tau found in neurodegenerative disease detach from microtubules and accumulate in the somato-dendritic compartment (Bancher et al., 1989). It is thought that this dysregulation of microtubule binding contributes to the degeneration of axonal processes and neuronal cell death, yet studies have also demonstrated a toxic gain of function for soluble hyperphosphorylated tau that is no longer associated with

microtubules. Interestingly, the fetal isoform of tau present in the developing brain is also highly phosphorylated and has less affinity for microtubules (Goedert and Jakes, 1990, Bramblett et al., 1993, Goedert et al., 1993, Watanabe et al., 1993). Several lines of evidence now indicate that these non-microtubule associated forms of tau may have additional functions during early brain development, and may contribute to the pathogenic mechanisms of neurodegenerative disease. Here, we focus on the evidence for additional roles for tau beyond microtubule stabilization in both neuronal development and in the adult diseased brain.

Tau in neuronal signal transduction

Tau is a member of a class of microtubule associated proteins (MAPs) including MAP2, and MAP4. MAPs such as tau are known primarily for their ability to nucleate microtubule assembly and stabilize existing microtubule networks. Tau expression is enriched in neurons of the central and peripheral nervous system, although it can be found in a number of other tissues throughout the body (Gu et al., 1996). Under normal physiological conditions, tau protein contains very little secondary structure and is predominantly unfolded (Mukrasch et al., 2009), although functionally, it can be divided into three major domains: the amino-terminal projection domain, the proline-rich domain, and the microtubule binding repeat domain (Figure 1.1). The projection domain of tau does not associate with microtubules and can project away from the microtubule surface or it can assume an additional conformation where it comes in closer contact with carboxy-terminal sequence (Hirokawa et al., 1988, Brandt et al., 1995, Jeganathan et al., 2006). The microtubule binding repeats (MTBR) are a series of three or four imperfect repeats required for the interaction between tau and microtubules (Lee et al., 1989). A proline-rich region upstream of the MTBR also contributes to microtubule binding and is involved in the nucleation of microtubule assembly (Brandt and Lee, 1993). Seven PXXP motifs are found in the proline-rich region, allowing for potential interactions with

SH3 domain-containing proteins (Figure 1.1). In addition, this region contains numerous phosphorylation sites in the form of serine/threonine-proline motifs which are the targets of many proline-directed kinases (reviewed in (Johnson and Stoothoff, 2004, Stoothoff and Johnson, 2005)).

In the adult human brain, six isoforms of tau are expressed through alternative splicing of the *MAPT* gene located on chromosome 17q21. These isoforms are distinguished by the presence of one or two amino-terminal inserts (0N, 1N, 2N), as well as three or four microtubule binding repeats (3R, 4R) (Figure 1.1). An additional isoform of tau, known as "high molecular weight tau", includes a longer splice variant of exon 4, exon 4a, and is preferentially expressed in the peripheral nervous system (Andreadis et al., 1992, Goedert et al., 1992). In contrast to adult brain, the fetal human brain expresses only the shortest three repeat isoform (0N3R). Fetal tau has a lower affinity for microtubules when compared to longer isoforms, and this tau species is found to be highly phosphorylated compared to adult tau from normal brains (Goedert and Jakes, 1990, Bramblett et al., 1993, Goedert et al., 1993, Watanabe et al., 1993, Yu et al., 2009). Many phospho-sites present in abnormally hyperphosphorylated tau are also found in fetal brain, leading to the hypothesis that at least some tau phosphorylation in disease is a recapitulation of developmental processes not normally occurring in adult brains (Kanemaru et al., 1992, Bramblett et al., 1993, Jicha et al., 1997).

These unique features of fetal tau seem to suggest that tau may have additional functions in early brain development that go beyond microtubule stabilization. In support of this idea, it has been demonstrated in the N2a neuroblastoma cell line that expression of mutant tau with alanine replacements at serine 262 and serine 356 prevented the development of neuritic processes when the cells were induced to differentiate (Biernat et al., 2002). Since phosphorylation at serine 262 and 356 within the MTBR dramatically reduces the affinity of tau for microtubules (Biernat and Mandelkow, 1999), these data would indicate a requirement for phosphorylated, non-microtubule associated tau during

neurite development. Furthermore, tau is enriched in the growth cones of developing neurites that contain dynamic and highly unstable microtubules (DiTella et al., 1994, Black et al., 1996, Liu et al., 1999). Growth cones are enriched in signal transduction proteins and also contain the machinery required for the dynamic responses of actin filopodia and lamellipodia to external guidance cues. This raises the questions of whether tau can interact with some of these components and in what capacity.

Signal transduction across the cell membrane is often concentrated in specialized membrane microdomains known as lipid rafts, regions heavily populated with anchored kinases and other adaptor proteins involved in signaling (reviewed in (Golub et al., 2004, Allen et al., 2007, Guirland and Zheng, 2007)). The amino terminus of tau has also been shown to interact with the plasma membrane and more specifically lipid rafts (Brandt et al., 1995, Klein et al., 2002, Kawarabayashi et al., 2004, Sui et al., 2006, Hernandez et al., 2009). Interestingly, this interaction can occur independent of intact microtubules, and expression of amino terminal rat tau truncated at serine 202 in differentiating PC12 cells interferes with neurite outgrowth, probably by competing with endogenous tau for binding at the plasma membrane (Brandt et al., 1995). These results suggest a critical role for tau in neurite outgrowth that requires an interaction with the plasma membrane. Taken together, the studies mentioned above implicate tau in the neuronal signal transduction that occurs in plasma membrane lipid rafts.

Non-receptor tyrosine kinases

The identification of an interaction between the amino-terminus of tau and the plasma membrane led to the search for possible protein interactors that might also associate with tau at the plasma membrane. The proline-rich domain of tau contains a number of PXXP motifs which are consensus binding sites for Src homology 3 (SH3) domains, a conserved and widely utilized protein-protein interaction module first characterized in the non-receptor tyrosine kinase Src. Co-sedimentation assays utilizing a

panel of GST-SH3 domain fusion proteins from a number of SH3-containing proteins identified an association between tau and the SH3 domains of the Src-family kinases (SFK) Src, Fyn, and Lck, and the interaction between Fyn and tau was further mapped to the sequence ^{233}pro-lys-ser-pro (Lee et al., 1998). The PXXP/SH3 binding was further shown to be regulated by phosphorylation; in particular, phosphorylation at threonine 231 appears to decrease the SH3 domain association between tau and Fyn (Zamora-Leon et al., 2001).

This interaction between tau and SFK fits well with the localization of tau at the plasma membrane, especially since SFK are tethered to the inner surface of the plasma membrane during various signaling events (van't Hof and Resh, 1997, Webb et al., 2000). The functional implications of these interactions have been studied in the context of myelin formation and differentiation in developing oligodendrocytes. Tau associates with both tubulin and Fyn in oligodendrocytic processes, and deletion of the PXXP binding site for Fyn SH3 disrupts process outgrowth, demonstrating a requirement for the tau/Fyn interaction in oligodendrocyte development (Klein et al., 2002). Subsequent studies in oligodendrocytes expressing a truncated tau incapable of binding microtubules showed that an association between tau and microtubules must also be maintained for proper process outgrowth and myelin formation (Belkadi and LoPresti, 2008). These studies envision a model in which tau acts as a bridge between Fyn tethered at the plasma membrane and the cytoskeleton. This interaction is then required for the stabilization of microtubule tracks within the process and eventual transport of vesicles to the site of myelin deposition. More recently, the Fyn/tau/tubulin interaction was shown to further play an important role in oligodendrocyte differentiation. By overexpression of mutant tau proteins in naïve oligodendrocytes stimulated to differentiate with apotransferrin, it was demonstrated that the formation of a complex between microtubule-associated tau and activated Fyn in lipid rafts was required for differentiation (Perez et al., 2009).

SH3/PXXP interactions can serve to bring substrates into close proximity with the

catalytic region of the SH3-containing kinase, and in fact, cellular and in vitro kinase assays have shown that tyrosine 18 of tau is a substrate for Fyn (Lee et al., 2004). We have developed antibodies specific for phosphorylated tyrosine 18, and experiments with these reagents have demonstrated that tyrosine phosphorylated tau is present during early brain development and in Alzheimer's disease brain tissue, but not in normal adult brain (Lee et al., 2004). Lck was similarly shown to phosphorylate human tau in vitro at tyrosine 29 (Williamson et al., 2002). The effects of tyrosine phosphorylation on tau function are not entirely clear. However, tyrosine phosphorylation does not seem to impact on the ability of tau to bind microtubules, since both soluble and microtubule associated tau is found to be tyrosine phosphorylated (Lee et al., 2004). Regardless of the role of tyrosine phosphorylation in tau function, PXXP/SH3 interactions between tau and SFKs can also serve to enhance tyrosine kinase activity. We have shown that tau enhances the tyrosine phosphorylation of tubulin by Fyn in *in vitro* kinase assays, and similarly demonstrated that tau enhances the activation of Src in platelet-derived growth factor (PDGF) -stimulated 3T3 fibroblasts (Sharma et al., 2007).

Tyrosine phosphorylated tau may have important functional roles in the brain, especially given the rarity and highly conserved nature of tyrosine phosphorylation in many intracellular signaling pathways. Interactions between other cellular components and tyrosine phosphorylated tau have not been well studied; however the sequence surrounding tyrosine 18 (amino acids 2-18) has been shown to be required for the ability of tau filaments to inhibit fast axonal transport (FAT) in squid axoplasm (LaPointe et al., 2009). The mechanism was further found to involve an inhibition of protein phosphatase 1 (PP1) activation and subsequent inhibition of the glycogen synthase kinase 3β (GSK3β) activity required for vesicle trafficking (LaPointe et al., 2009). Although these experiments involved aggregated tau filaments, it is possible that these findings might reflect a physiological role for phosphorylation at tyrosine 18 in fetal tau as a regulatory switch for interactions with PP1. Additional functions for tyrosine 18/29 phosphorylation

may yet be elucidated in the context of neuronal signal transduction through Src family kinases.

Tau has also been found to associate with the non SFK, non-receptor tyrosine kinases Abl, Arg, and Syk (Derkinderen et al., 2005, Lebouvier et al., 2008, Tremblay et al., 2009). Abl and Arg share close sequence homology, and like SFK, both contain SH3 and SH2 domains involved in regulating kinase activation. In contrast to SFK however, the Abl family kinases contain actin binding motifs, and in the case of Abl, nuclear localization sequence and a DNA binding domain (Koleske et al., 1998). Both kinases are expressed in the brain and are enriched in the presynaptic zone as well as the post-synaptic density where they may be involved in maintaining synapse morphology (Moresco and Koleske, 2003). In addition, activation of platelet-derived growth factor receptor (PDGFR), nerve growth factor receptor (TrkA), and cell surface integrins are all known to recruit Abl kinases (Plattner et al., 1999, Yano et al., 2000, Cui et al., 2009) implicating this family in a wide range of signal transduction events. As with SFKs, Abl and Arg are found to phosphorylate tau on tyrosines, primarily tyrosine 394 (Derkinderen et al., 2005, Tremblay et al., 2009) and to a lesser extent tyrosine 197 (Lebouvier et al., 2009). Phosphorylation at tyrosine 394 was identified in both fetal brain tissue and paired helical filaments (PHF) from Alzheimer's disease brain tissue using mass spectroscopy (Derkinderen et al., 2005); however, unlike SFKs, the association between tau and Abl did not seem to involve a PXXP/SH3 interaction. Additional experiments are needed to determine the nature of the interaction between tau and these non-receptor tyrosine kinases.

Spleen tyrosine kinase (Syk) has also been identified to interact with and phosphorylate tau on tyrosine 18 in vitro and after co-expression in CHO cells (Lebouvier et al., 2008). The Syk family of tyrosine kinases, comprised of Syk and ZAP-70, is characterized by non-receptor kinases that lack a membrane targeting motif and contain two Src homology 2 (SH2) domains which bind phospho-tyrosine motifs on

partner proteins (Yanagi et al., 2001). The interaction between tau and Syk appeared to be independent of the SH2 domains, and the efficacy of phosphorylation on tyrosine 18 seems to be similar to that of Fyn kinase (Lebouvier et al., 2008). Syk has been primarily studied in the context of the immune system where it is involved in signal transduction through immune response receptors and heterotrimeric G-protein coupled receptors (Kurosaki, 1999, Turner et al., 2000, Tohyama and Yamamura, 2009). However, Syk is also expressed in the brain and studies have shown Syk to be activated in the retinoic acid-induced differentiation of P19 embryonic carcinoma cells into a neuronal-phenotype (Tsujimura et al., 2001). Together, these findings raise the possibility that an interaction between tau and Syk might play a role in neurite development in the developing brain. To date, the functional implications of tyrosine-phosphorylated tau have not been well elucidated, but further studies may identify binding partners dependent on tyrosine phosphorylation in the context of neuronal signal transduction. Likely targets would be proteins containing SH2 domains or phospho-tyrosine binding (PTB) domains, and may include other kinases, adaptor proteins, or scaffolding proteins associated with the cytoskeleton.

Serine/threonine kinases, adaptor proteins, and scaffolding proteins

Although tyrosine kinase activity is highly involved in neuronal signal transduction, the activation of serine/threonine kinases and protein-protein interactions between adaptor proteins, scaffolding proteins, and downstream signaling effectors also has a significant impact on the transmission of intracellular signals. Evidence now suggests tau may have a wide range of interactions with some of these components in addition to the associations with non-receptor tyrosine kinases discussed above. Tau has long been known to be highly phosphorylated and many tau kinases are also involved in signal transduction. For example, tau has been reported to be phosphorylated in vitro by

members of the mitogen-activated protein kinase family: ERK1/2, JNK1/2, and p38 (Roder et al., 1993, Blanchard et al., 1994, Reynolds et al., 1997a, Reynolds et al., 1997b). Tau can also be phosphorylated by protein kinase A (PKA), protein kinase B (PKB/Akt), GSK3β, cyclin-dependent kinase 5 (CDK5), and microtubule affinity-regulating kinase (MARK), among others (reviewed in (Shahani and Brandt, 2002, Stoothoff and Johnson, 2005)). Many of the tau phosphorylation sites for these kinases are found to be phosphorylated during early brain development and abnormally phosphorylated in diseased brains (Bramblett et al., 1993, Goedert et al., 1993, Watanabe et al., 1993, Yu et al., 2009). Interestingly, while some phosphorylation events can affect the ability of tau to associate with microtubules, many have no appreciable effect on microtubule binding capacity, leaving unanswered the question of why these sites are being phosphorylated in the fetal brain. If these functions can be further elucidated in the context of neuronal signal transduction, it may also shed light on the actions of hyperphosphorylated tau in neurodegenerative disease.

Tau has also been found to interact with the signaling adaptor protein Grb2, the scaffolding protein 14-3-3ζ, and the p85α regulatory subunit of phosphatidyl-inositol-3 kinase (PI3K) (Agarwal-Mawal et al., 2003, Reynolds et al., 2008, Souter and Lee, 2009). Grb2 contains multiple SH3 domains and an SH2 domain for linking growth factor receptor activation to Ras activation by the guanine nucleotide exchange factor (GEF), SOS. Full length tau binds the N-terminal SH3 domain of Grb2 in *in vitro* pulldown assays although the precise PXXP motif responsible for the interaction has not been mapped (Reynolds et al., 2008). The N-terminal SH3 of Grb2 also associates with MAP2c (Lim and Halpain, 2000, Zamora-Leon et al., 2001), a microtubule associated protein sharing significant sequence homology with tau, and in MAP2c the interaction has been mapped to a region within the microtubule binding repeats that does not contain a PXXP consensus binding site for SH3 domains (Lim and Halpain, 2000). Thus it is possible that Grb2 may similarly associate with tau using sequences outside of a PXXP

motif. Interestingly, the N-terminal SH3 domain of Grb2 is also responsible for binding the Ras-GEF SOS during signal transduction (Simon and Schreiber, 1995), giving rise to the possibility that a physiological tau/Grb2 interaction might mutually exclude any association between Grb2 and SOS. Additional studies will need to be carried out to address these questions.

The 14-3-3 family of scaffolding proteins is also known to have diverse roles in signal transduction leading to cellular proliferation, differentiation, and apoptosis. 14-3-3 family members have been identified as components of neurofibrillary tangles (Layfield et al., 1996), and as proteins capable of forming protein complexes with tau and GSK3β (Hashiguchi et al., 2000, Agarwal-Mawal et al., 2003, Yuan et al., 2004). 14-3-3 proteins typically bind to phosphorylated serine and threonine residues and phosphorylation of tau at Ser214 by PKA or Akt has been shown to enhance the binding between 14-3-3 isoforms (β,η,ζ) and tau *in vitro* (Sadik et al., 2009a). However, 14-3-3 is also capable of interacting with tau in a phosphorylation-independent manner although binding to phospho-Ser214 is preferred. Surface plasmon resonance experiments found a three-fold increase in binding affinity between 14-3-3 and fetal 3R tau compared with 4R tau, and Ser214 phosphorylation even further increased the affinity of 14-3-3 for tau in both isoforms (Sadik et al., 2009b). Ser214 is known to be highly phosphorylated in the fetal brain (Andorfer and Davies, 2000, Sadik et al., 2009a), and, in fact, 14-3-3 co-immunoprecipitated with tau from fetal brain (Andorfer and Davies, 2000, Sadik et al., 2009a), suggesting a functional interaction between these proteins during brain development.

The SH3 domain of the p85α PI3K subunit was found to co-immunoprecipitate with tau in a prostate cancer cell line (Souter and Lee, 2009), and bind full length tau in co-sedimentation assays (Reynolds et al., 2008). Reynolds et al. further used tau peptides spanning individual PXXP motifs to show that amino acids 209-223 and 227-241 were both capable of binding the SH3 domain of the p85α regulatory subunit (Reynolds et al.,

2008). Furthermore, pseudo-phosphorylated tau was found to have a significantly decreased interaction with the SH3 domain of p85α (Reynolds et al., 2008). The PI3K/Akt pathway is highly involved in cell signaling processes, leading to cellular proliferation, protein synthesis, and inhibition of apoptosis, and thus, an interaction between tau and PI3K may have many effects. Typically, the p85 regulatory subunit of PI3K associates with the p110 catalytic subunit of PI3K, preventing the activation of the kinase. Dissociation of p85 from p110 is required for PI3K to become catalytically active, which is in turn necessary for the activation of downstream components of the pathway, such as Akt (reviewed in (Saji and Ringel, 2009)). Therefore, an interaction between tau and p85α might enhance the catalytic activity of PI3K, and the phosphorylation of tau by additional kinases could negatively regulate PI3K signaling by causing a dissociation of p85α from tau.

Actin

In addition to its established role in microtubule interactions, tau has been reported to interact with the actin cytoskeleton. Early experiments in vitro found an association between tau and actin filaments, and suggested the interaction was mediated through the carboxy-terminal domain of tau (Moraga et al., 1993, Yamauchi and Purich, 1993, Farias et al., 2002). Moreover, experiments with cultured tau-depleted cerebellar neurons found alterations in actin morphology in developing growth cones, indicating that tau has an impact on growth cone actin dynamics (DiTella et al., 1994). More recently, the carboxy-terminus of tau was found to interact with actin in PC12 cells differentiated with nerve growth factor, and the association was found to be regulated by amino-terminal tau sequence (Yu and Rasenick, 2006). In contrast, another study indicated that recombinant tau is not capable of directly binding actin filaments (Roger et al., 2004), suggesting that additional protein interactions or post-translational modifications of tau may be required. We have recently reported the ability of tau to

prolong actin stress fiber breakdown in PDGF-stimulated 3T3 cells (Sharma et al., 2007). Tau was able to mediate this effect by enhancing the activation of Src kinase, a signaling intermediate responsible for regulating actin stress fiber dynamics in response to PDGF. Similarly, tau has been shown to affect actin morphology through an indirect interaction with Gem GTPase (Oyama et al., 2004). As Gem GTPase is a negative regulator of the Rho GTPase signaling pathway responsible for controlling actin stress fiber formation and neurite retraction (Ward et al., 2002), tau may be able to influence actin dynamics indirectly through interactions with multiple upstream regulatory elements.

The diversity of interactions between tau and signaling related proteins underscores the need for further research into the nature of tau's impact on neuronal development. Tau should be considered as a multifunctional protein, serving as both a stabilizer of microtubule networks, and as a potential scaffolding or adaptor protein capable of regulating interactions between signaling proteins. These additional functions for tau should also be considered for their putative impact on the pathogenesis of neurodegenerative diseases, which is to be considered in greater detail below.

The role of tau in neurodegeneration

The neurodegenerative tauopathies share a common pathology: the accumulation of hyperphosphorylated aggregates of tau protein within the brain. These aggregates, known as neurofibrillary tangles (NFTs) when occurring in the cell body, and neuropil threads when occurring in dendritic processes, are extremely insoluble and often persist as "ghost tangles" long after the original cell in which they were formed has died. While some tauopathies such as FTDP-17 are causally linked to mutations in the tau gene, others such as sporadic Alzheimer’s disease occur in the absence of tau mutations. The affected neuronal subtypes and brain regions can vary greatly amongst the tauopathies; nevertheless, hyperphosphorylation and the formation of insoluble tau aggregates are always found to occur. Most tauopathies occur rather infrequently in aging human

populations with the exception of Alzheimer's disease, which affects 15% of individuals over the age of 65 and nearly 50% of those over the age of 85 (Smith, 1998). For this reason, the biology of Alzheimer's disease has been the topic of significant research efforts and much progress has been made into understanding the molecular events that correlate with this disease. In spite of these advances, many questions still remain about the precise role of tau in the pathogenesis of AD.

Sporadic AD, also known as late-onset AD, is characterized by a progressive loss of memory and cognitive function, and a postmortem analysis of AD brain tissue reveals extensive neuronal loss accompanied by the presence of intracellular NFTs and extracellular deposits comprised of β-amyloid (Aβ) peptide. The aggregation of Aβ peptides in AD is now known to be caused by the abnormal processing and cleavage of the amyloid precursor protein (APP). Mutations in APP or in one of the two presenilin genes (PS1 and PS2) involved in APP processing can lead to a genetically inherited form of AD, also known as early onset AD (Hardy, 1997). Similarly, the genes encoding APP and the tau kinase Dryk1 are located on chromosome 21, leading to an early onset AD phenotype in cases of chromosome 21 trisomy (Down's syndrome) (Ferrer et al., 2005, Liu et al., 2008). Sporadic AD however, is largely unassociated with genetic components, although possession of the ε4 allele of apolipoprotein E confers an increased risk for developing AD (Corder et al., 1993, Saunders et al., 1993). The fact that mutations leading to altered APP processing are sufficient to cause AD has led to the formulation of the "amyloid cascade" hypothesis which places the abnormal processing of APP and accumulation of Aβ at the top of a cascade of events leading to tau hyper-phosphorylation, neuronal dysfunction, and cell death (Hardy and Allsop, 1991). Moreover, in contrast to the familial tau mutations leading to frontotemporal dementia with tau pathology, individuals with sporadic AD possess wild-type tau, and yet hyperphosphorylation and NFTs still develop. This observation further suggests that the increased activity of tau kinases or the decreased activity of tau phosphatases triggered by

upstream processes is sufficient to cause the hyperphosphorylation and aggregation found in the disease.

As the increased phosphorylation of tau in AD leads to a detachment of tau from microtubules, this loss of normal tau function plays an important role in degenerative pathology. Axons with destabilized microtubule tracks are no longer able to maintain structural integrity and are impaired in their ability to transport materials from the cell body to synaptic zones. While there can be little doubt that the effects of microtubule destabilization are significant, hyperphosphorylated tau can also exist for some time in a "pre-tangle" state in which it can impact on events within the cell prior to sequestration in NFTs (reviewed in (Ding and Johnson, 2008)). In fact, soluble forms of hyperphosphorylated tau may have toxic effects that serve to accelerate the disease process. Mice with inducible expression of human 4R P301L tau, an inherited FTDP tau mutation, were found to have improved cognitive function when the expression of soluble hyperphosphorylated tau was blocked, although NFTs continued to accumulate (Santacruz et al., 2005). These results suggested that tangle formation might be a neuroprotective mechanism whereby the cell attempts to neutralize the more toxic soluble species of tau. The toxic gain of function for soluble hyperphosphorylated tau might simply be due to a sequestration of normal microtubule associated proteins (Alonso et al., 1996, Alonso et al., 1997, Gong and Iqbal, 2008), which would further impair microtubule assembly and stability, or to soluble pre-tangle tau having additional functions. "Pre-tangle" tau exhibits many similarities to tau in the fetal brain including increased phosphorylation at specific fetal tau phosphorylation sites (Kanemaru et al., 1992, Bramblett et al., 1993, Jicha et al., 1997) and a decreased affinity for microtubules. Given that fetal tau might have a role in developmental neuronal signaling, it is also possible that these pathological forms of tau might have a similar role in abnormal cell signaling in the adult brain.

Interestingly, abnormal markers of cell cycle activation appear in the normally post-mitotic neurons of AD brain (reviewed in (Bonda et al., 2010)). The cell cycle consists of several distinct phases tightly regulated by cyclins, cyclin-dependent kinases, and other cell cycle-associated proteins. The cycle begins with a transitional or "gap" phase known as G1, leading up to DNA replication during S phase. S phase is followed by a second gap phase, G2, and finally mitosis (M-phase) in which chromosomal separation is completed, daughter nuclei are formed, and the cytokinetic fission into two daughter cells is completed. Differentiated cells, such as neurons in the brain, exit this division cycle and persist in a quiescent state known as G0. Maintenance of G0 is defined by the absence of cyclins A and D (Grana and Reddy, 1995, Zhu et al., 2004). However, increased expression of the cell cycle markers cyclin D and cdk4 can cause a differentiated neuron to exit G0 and re-enter the cell cycle at G1. Elevated levels of these proteins have been found in AD brains and mouse models of AD (McShea et al., 1997, Nagy et al., 1997a, Nagy et al., 1997b, Yang et al., 2003, Varvel et al., 2008). Progression of the cell cycle through S-phase and as far as G2, characterized by the replication of DNA and increased expression of the cyclin E/cdk2 complex, has also been observed in AD (Nagy et al., 1997b, Yang et al., 2001, Yang et al., 2003). Strangely, these cell cycle events are not observed to culminate in mitosis (Bowser and Smith, 2002, Ogawa et al., 2003), which has led to the hypothesis that at least some of the cell death in AD is caused by apoptosis after an aborted attempt to complete the cell cycle. Currently, the exact reasons for abnormal cell cycle activation in AD brain are not well understood, but several hypotheses have been put forward to explain these findings. The "Dr. Jekyll and Mr. Hyde" hypothesis posits that the cell cycle activation in AD neurons is caused by a regulatory failure of mechanisms normally involved in synaptic plasticity (Arendt, 2003, Arendt and Bruckner, 2007, Arendt, 2009). In this model, synaptic plasticity and cell proliferation are thought to be alternative effectors of the same pathways. Changes related to normal synaptic remodeling can therefore be abnormally linked to activation of

the cell cycle. An alternative hypothesis, known as the "two-hit" hypothesis, proposes that a combination of two “hits” is required for disease progression. These “hits”, abnormal cell cycle activity and oxidative stress, are two age-related risk factors that independently do not result in disease, but when occurring in the same cell, act synergistically to trigger the typical progression to AD (reviewed in (Zhu et al., 2001, Zhu et al., 2004, Zhu et al., 2007, Bonda et al., 2010)).

Irrespective of the upstream triggers of aberrant cell cycle activity, the question remains as to whether abnormally phosphorylated tau plays a role in these events leading to degeneration and cell death, independent of simply sequestering normal MAPs. Many markers of aberrant cell cycle activity have been linked with tau pathology in AD brains. An upregulation of the AP-1 transcription factors c-Fos and c-Jun, key regulators of cell cycle progression, have been reported to co-localize with NFT positive neurons in AD brain (Anderson et al., 1994, Marcus et al., 1998), as have increased levels of kinases upstream of AP-1 in the mitogen-activated protein kinase (MAPK) pathway such as ERK1/2, and MEK1/2 (Pei et al., 2002). Elevated expression levels of Ras GTPase, a key mediator of signal transduction, are also found to precede the formation of neurofibrillary tangles and Aβ pathology (Gartner et al., 1999). Over-expression of R406W mutant tau in a *Drosophila* model of tauopathy caused an activation of cell cycle events and neurodegeneration (Khurana et al., 2006). Similarly, mice expressing wild-type human tau on a null background show elevated levels of the cell cycle proteins, cyclin D1, ki67, and PCNA (Andorfer et al., 2005). Importantly, this latter study also found that most cell death was occurring in non-tangle positive cells, strengthening the idea that filament formation is a protective mechanism to reduce intracellular levels of soluble toxic tau species. Together, these findings point to an intriguing link between the onset of abnormal cell cycle events in post-mitotic neurons and the generation of hyper-phosphorylated tau.

The dysregulation of MAPK signaling and the related activation of cell cycle events is likely to be a key component linking the abnormal processing of APP to tau hyperphosphorylation (reviewed in (Zhu et al., 2002)). Aβ has been shown to induce activation of MAPK family members, especially JNK and ERK isoforms, both of which play important roles in cell proliferation and the regulation of programmed cell death. Aβ addition to primary neuronal cultures or to neuronal cell lines increases MAPK and/or AP-1 activation and leads to neurotoxicity (Ferreira et al., 1997, Rapoport and Ferreira, 2000, Frasca et al., 2004, Jang and Surh, 2005). Interestingly, tau depletion is neuroprotective for toxicity associated with Aβ-induced MAPK activation (Rapoport et al., 2002). This result can be explained by a role for tau in MAPK signaling downstream of Aβ. Supporting this idea are gene expression profiling studies carried out in differentiated SH-SY5Y neuroblastoma cells overexpressing P301L mutant tau (Hoerndli et al., 2007). Mutant tau expression in these cells induced an upregulation of genes associated with cell cycle reentry including cdk4, as well as genes regulated by AP-1 transcription factors such as vascular endothelial growth factor D (VEGF-D). Moreover, Aβ treatments induced the upregulation of cell cycle-related genes in cells expressing P301L tau (Hoerndli et al., 2007). The amino terminus of tau has also been shown to be required for Aβ-induced signaling (King et al., 2006), which is consistent with the idea that tau is involved in neuronal signal transduction through a mechanism that is independent of microtubule binding. Together, these findings point to a novel functional role for tau in cell signaling events, which may involve the MAPK signal transduction pathway.

Dissertation research focus

The evidence we have seen regarding a role for tau in neuronal signaling suggests a number of avenues for basic research into the multifunctional aspects of tau protein in the brain. Tau should be considered not only as a microtubule associated protein, but as a

signaling protein with important functions in neuronal differentiation and the regulation of the cell cycle. The growing body of data identifying the presence of abnormal cell cycle events in neurodegenerative tauopathies places particular importance on further understanding the role of tau in these processes. While it may be worthwhile to develop therapies aimed at preventing aberrant re-entry into the cell cycle and subsequent cell death, some of these processes may actually be required for normal neuronal function (Arendt, 2003). For this reason, the identification of interactions between phosphorylated tau species and components of neuronal signaling has the potential to yield more specific drug targets capable of improving the prognosis of those afflicted with Alzheimer's disease and other tauopathies.

The primary aim of the dissertation research is to investigate the potential roles of tau in neuronal signal transduction. We hypothesize that tau is able to impact on neuronal signal transduction events through a mechanism independent of its ability to bind and stabilize microtubules. To study the effects of tau on signal transduction, we have chosen to utilize the PC6-3 cell line, a subclone of the widely used PC12 cell line (Pittman et al., 1993) derived from a rat adrenal pheochromocytoma (Greene and Tischler, 1976). These cells make an excellent model to study neuronal signaling due to their ability to differentiate into a sympathetic neuronal phenotype upon exposure to NGF. Differentiation entails an exit from the cell cycle, an elaboration of neuritic processes, and an upregulation of proteins associated with neuronal function such as tubulin and the microtubule associated protein tau (Greene and Tischler, 1976, Drubin et al., 1985). The differentiation process has been well characterized and is known to require the activation of the mitogen-activated protein kinase (MAPK) pathway through the NGF receptor, TrkA (Figure 1.2) (Qui and Green, 1992, Huang and Reichardt, 2001, Kao et al., 2001, Eriksson et al., 2007). MAPK signaling is highly conserved and plays an important role in cellular responses to extracellular cues, leading to such diverse outcomes as activation of cell cycle events, differentiation, or apoptosis, all of which are

important in the processes of brain development and neurodegenerative disease. For these reasons, the activation of MAPK signaling in NGF-treated PC6-3 cells is an ideal system for investigating the physiological role of tau in neuronal signal transduction. Herein, we describe the generation of PC6-3 cell lines with altered tau expression and demonstrate a novel role for tau in growth factor-induced MAPK signaling that is independent of microtubule association. These findings provide important insights into tau function that may have broader implications for the role of tau in early brain development and in neurodegenerative disease.

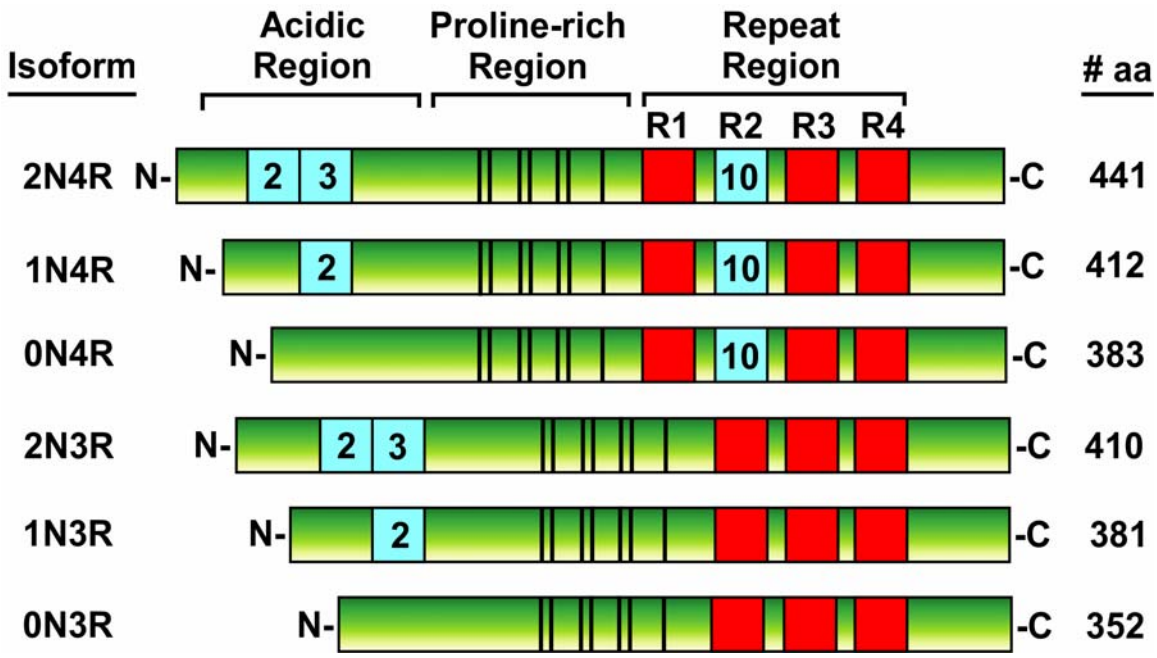

Figure 1.1 Functional domains of tau isoforms expressed in the central nervous system. The six alternatively spliced isoforms of human tau expressed in the central nervous system are shown with the standard nomenclature and number of amino acids (#aa). The 2N4R isoform corresponds to NCBI isoform 2, whereas the 0N3R isoform corresponds to NCBI isoform 4. Exons 2, 3, and 10 (shown in blue) are alternatively spliced, giving rise to six possible isoforms. The N-terminal region of tau is highly acidic and is able to interact with the plasma membrane. The proline-rich region contains numerous phosphorylation sites, as well as seven PXXP motifs (black bands) capable of binding SH3-domain containing proteins. The microtubule-binding repeats are shown in red (with the inclusion of exon 10 in 4R isoforms).

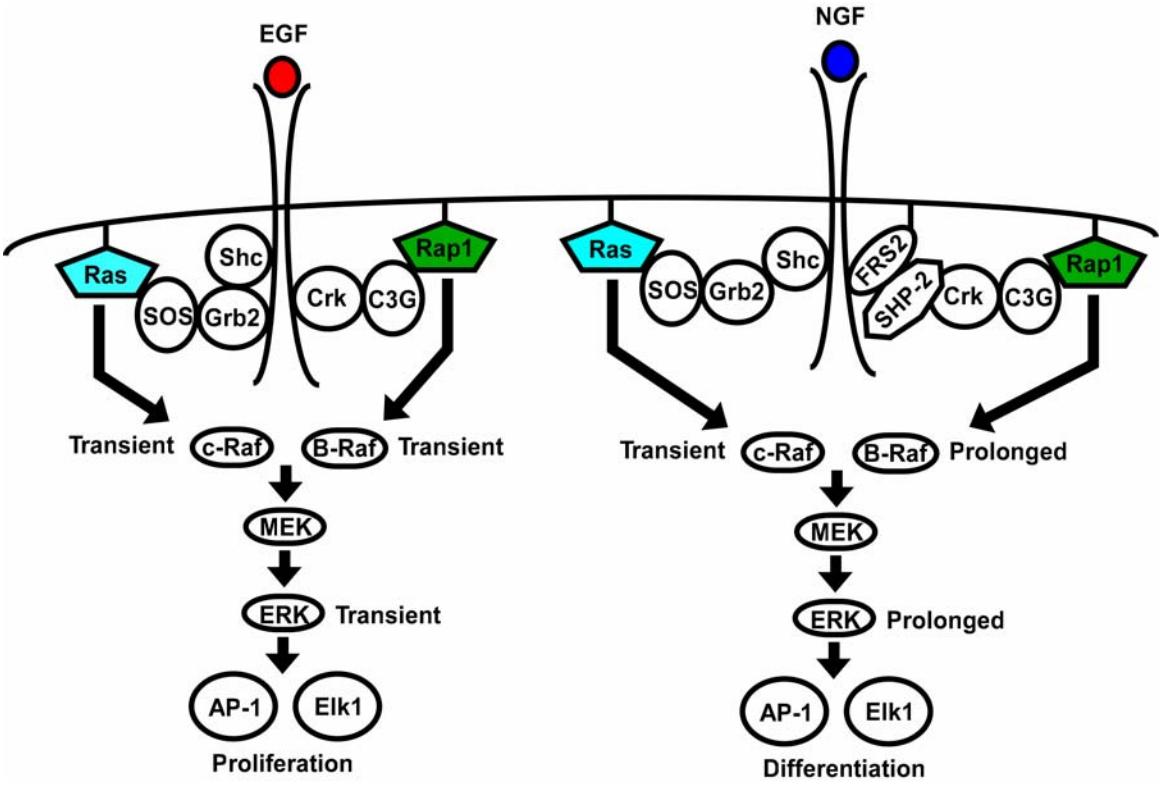

Figure 1.2 Model of EGF- and NGF-stimulated ERK activation. ERK activation in response to epidermal growth factor (EGF) or nerve growth factor (NGF) in PC12 cells requires the activation of the membrane bound small GTPases Ras and/or Rap1. NGF activation of the receptor tyrosine kinase TrkA induces the formation of a stable complex consisting of TrkA, FRS2, Crk, and C3G, leading to a prolonged activation of Rap1. Prolonged Rap1 activity results in a prolonged downstream activation of ERK (MAPK) and subsequent activation of transcription factors such as AP-1 and Elk1. In contrast, EGF-stimulated ERK activation is transient as a result of the transient, unstable coupling of the Crk-C3G complex to the EGF receptor (Adapted from (Kao et al., 2001)).

CHAPTER II

TAU IMPACTS ON EARLY SIGNAL TRANSDUCTION EVENTS ASSOCIATED WITH GROWTH FACTOR STIMULATION AND NEURITE INITIATION

Abstract

Microtubule-associated protein tau is known to bind to and stabilize microtubules, thereby regulating microtubule dynamics. However, recent evidence has indicated that tau can also interact with various components of intracellular signaling pathways, leading to the possibility that tau might have a role in signal transduction. Here we provide evidence that during growth factor stimulation of PC12 cells, tau has functions in advance of the neurite elongation stage. Using tau overexpressing and tau depleted PC6-3 cell lines, we demonstrate that tau is required for nerve growth factor (NGF)-stimulated neurite initiation in a manner that does not involve its microtubule binding function. We further show that tau potentiates AP-1 transcription factor activation in response to NGF. In addition, the effects of tau on AP-1 activation require upstream signaling through the ERK pathway, and tau is able to potentiate ERK activation in response to both NGF and epidermal growth factor (EGF). Together, these findings indicate a new functional role for tau in early neuronal development independent of its established role in microtubule stabilization.

Introduction

Microtubule-associated protein tau has been extensively studied due to its prominent role in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD) and the fronto-temporal dementias (reviewed in (Binder et al., 2005, Goedert and Jakes, 2005, Ballatore et al., 2007, Wolfe, 2009)). In general, research into the physiological functions of tau has focused on its interactions with microtubules.

However, additional roles for tau beyond those associated with microtubules have been suggested by numerous studies. For instance, we have previously demonstrated that the amino terminus of tau, a domain not involved in microtubule binding, is associated with the plasma membrane and can affect nerve growth factor (NGF)-induced neurite outgrowth (Brandt et al., 1995). Also, neurite outgrowth was shown to require phosphorylation of tau at Ser262, a modification that reduces the ability of tau to bind to microtubules (Biernat and Mandelkow, 1999). In addition, interactions between tau and various signaling proteins such as Src, Fyn, Abl, the p85 subunit of phosphatidyl-inositol 3-kinase, phospholipase Cγ, 14-3-3, and Grb2 have been described (Jenkins and Johnson, 1998, Lee et al., 1998, Yuan et al., 2004, Derkinderen et al., 2005, Reynolds et al., 2008, Souter and Lee, 2009). Such findings suggest that non-microtubule-associated tau species may be associated with signaling components at the plasma membrane during early neurogenesis and differentiation.

Recent reports have also indicated that tau can be linked to the increased expression of cell cycle proteins. Mice expressing human tau in the absence of mouse tau and mice expressing FTDP-17 mutant tau were found to have abnormal expression of cell cycle proteins such as cyclin D1, ki67, and PCNA (Andorfer et al., 2005, Delobel et al., 2006, Schindowski et al., 2008). Similarly, a cell culture model expressing P301L mutant tau was found to up-regulate numerous genes associated with cell cycle re-entry upon exposure to Aβ including cdk1, cdk4, and PCNA (Hoerndli et al., 2007). Cellular proliferation and differentiation are tightly regulated processes that involve the concerted efforts of a host of signaling proteins including cyclins, cyclin-dependent kinases, and nuclear transcription factors. These cell cycle regulators are in turn controlled by inputs from various signaling pathways, such as the mitogen-activated protein kinase (MAPK) cascade which transduces signals from the cell surface into the nucleus. In fact, many of the cell cycle proteins abnormally expressed in rodent models of tauopathy (e.g. cyclin D1, ki67, and PCNA) are known to be regulated by AP-1 transcription factors activated

during MAPK signaling. Similarly, abnormal activation of the MAPK signaling pathway occurs in AD brains and is associated with neurofibrillary tau pathology (reviewed in (Zhu et al., 2002)). Moreover, MAPK activity is upregulated during fetal brain development (reviewed in (Fukunaga and Miyamoto, 1998, Meloche and Pouyssegur, 2007)), in conjunction with an increase in tau phosphorylation and a weakened affinity for microtubules (Lindwall and Cole, 1984, Goedert and Jakes, 1990, Bramblett et al., 1993, Watanabe et al., 1993, Yu et al., 2009). These findings, which have been more extensively reviewed in Chapter I, have led to our hypothesis that tau is a multi-functional protein with a novel role in neuronal signal transduction during early brain development.

MAPK signaling pathway

To study the possible interactions between tau and the MAPK signaling pathway we have utilized the PC12 cell line as a model for neuronal signal transduction (Greene and Tischler, 1976). MAPK signaling in PC12 cells can be triggered by the addition of growth factors such as NGF and EGF, although there are distinct differences in the cellular response to each growth factor. NGF causes differentiation into a neuronal phenotype and requires a prolonged time course of MAPK activation, whereas EGF causes an increase in proliferation characterized by a transient activation of MAPK signaling (Qui and Green, 1992, Nguyen et al., 1993, Kao et al., 2001). These differences in MAPK signaling between growth factors can be attributed to differences in the formation of adaptor protein complexes with the growth factor receptors, although the core signaling module is similarly activated in each case. Ligand binding to receptor tyrosine kinases (i.e. NGF to TrkA, or EGF to EGFR) causes an activation of the intracellular tyrosine kinase domain and a subsequent recruitment of adaptor proteins linking receptor activation to GTPase activation (see Figure 1.2). Upon activation, the small membrane-bound GTPases Ras and Rap1 in turn activate a core MAPK module

composed of three kinases. In the context of NGF- or EGF-induced signaling, these kinases in sequential order of activation downstream of Ras/Rap1 are: c-Raf/B-Raf, MEK1/2, and ERK1/2 (MAPK) (Kao et al., 2001). Activation of the ERK1/2 serine/threonine kinases leads to the phosphorylation and activation of transcription factors required for differentiation, including c-Fos and c-Jun in the AP-1 transcription factor family (York et al., 1998, Leppa et al., 2001, Eriksson et al., 2007).

Ultimately, NGF exposure induces the expression of many proteins associated with a neuronal phenotype including tubulin, tau, sodium and calcium ion channels, and enzymes involved in neurotransmitter synthesis (Greene and Tischler, 1976, Drubin et al., 1985, Bouron et al., 1999). The upregulation of tau at later stages of differentiation (i.e. after three days NGF exposure) is known to coincide with increased tubulin synthesis and microtubule assembly during neurite elongation (Drubin et al., 1985, Black et al., 1986, Hanemaaijer and Ginzburg, 1991, Esmaeli-Azad et al., 1994). However, tau is also expressed at low levels in undifferentiated PC12 cells, and the function of tau in naive cells and during early stages of differentiation remains unclear.

Here we have identified a role for tau in early events following NGF stimulation. We have used the well-studied PC12 system to develop cell lines with altered tau expression and demonstrate that tau is required for neurite initiation. Moreover, the effects of tau on neurite initiation do not require an association between tau and microtubules. In addition, we find that tau acts to potentiate the activation of AP-1 transcription factors through the extracellular signal-regulated kinase (ERK) pathway. The ability of tau to potentiate ERK activation involves a mechanism common to signaling downstream of both NGF and epidermal growth factor (EGF). These findings indicate a new role for tau in the context of neuronal differentiation, independent of its established role as a microtubule stabilizing protein.

Materials and methods

Cell culture

COS7 cells were grown on untreated tissue culture plastic and maintained in DMEM with 10% fetal bovine serum. The PC6-3 cell line (Pittman et al., 1993) was obtained from Dr. Henry Paulson (University of Michigan). PC6-3 cells were cultured on tissue culture plastic coated with 50 μg/ml Type I collagen (BD Biosciences) using RPMI 1640 media with 10% horse serum and 5% fetal bovine serum. Media for stable cell lines was supplemented with 200 μg/ml G418 and/or 2 μg/ml blasticidin.

RNA oligonucleotide synthesis and screening

To develop short hairpin RNA (shRNA) expressing plasmids for tau depleted stable cell lines, a panel of 21-mer oligonucleotides with homology to the rat tau coding sequence was developed according to the following criteria. First, each 21-mer was required to contain at least two mismatches with the corresponding human tau sequence. Second, each candidate was required to contain approximately 50% G/C content (30% minimum). Lastly, all candidate sequences were checked against the *Rattus norvegicus* genome for non-specific homology with other coding regions. The resulting panel of candidate rat dsRNA oligonucleotides was generated with the Ampliscribe T7 polymerase kit as previously described (Miller et al., 2003). Briefly, dsRNA was synthesized from template oligonucleotides using T7 RNA polymerase and RNase-free reagents, followed by precipitation with lithium chloride. To assess the level of tau repression, co-transfection assays were performed in COS7 cells by transfecting increasing amounts of dsRNA oligonucleotides (0-5 μg) along with rat tau cDNA (2N4R isoform, (Brandt et al., 1995)). Tau protein expression levels were quantified by densitometry (National Institutes of Health ImageJ analysis software) of Western blots probed with Tau5-HRP (1:20,000), which reacts to both human and rat tau (LoPresti et al., 1995). Tau5 conjugated to horse radish peroxidase (HRP) was prepared using the

EZ-Link Plus Activated Peroxidase Kit (Pierce). The expression of human tau in the presence of dsRNA was assessed in COS7 by co-transfecting increasing amounts of dsRNA oligonucleotides (0-5 μg) with human tau cDNA (1 μg, 0N3R isoform). The results from these screenings are summarized in Table 2.1.

Generation of stable cell lines

Clonal PC6-3 cell lines stably expressing human tau (352 residue, 0N3R isoform) were generated by transfecting PC6-3 cells with pRc/CMVn123c (Hall et al., 1997) followed by selection in 500μg/ml G418. Stable colonies were screened for human tau expression by Western blotting of cell lysates using Tau13 (1:1000), a human tau specific antibody (Garcia-Sierra et al., 2003). Clonality was confirmed by immunofluorescent analysis of glutaraldehyde fixed cells labeled with polyclonal anti-human tau, CR (1:1000, (Lee et al., 2004)). Relative tau expression level was quantified by densitometry (National Institutes of Health ImageJ analysis software) on Western blots probed with Tau5-HRP (1:20,000).

To develop stable cell lines with depleted endogenous tau, a panel of rat tau-specific dsRNA oligonucleotides was screened by transient co-transfection of dsRNA oligonucleotides and rat or human tau plasmids. Based on the results from COS7 screening assays, the sequence (5'- gatccccGTGTCCGCCTCTTTGGCCAttcaagagaTGG CCAAAGAGGCGGACACtttttggaaa-3') was subcloned into the pNTO vector ((Strack et al., 2004), generously provided by Dr. Stefan Strack, University of Iowa). The shRNA expressed from this plasmid (pNTO-rTau4) targets a sequence in rat tau exon 13, (5'-AAGTGTCCGCCTCTTTGGCCA-3'). A second sequence, (5'-gatccccGAGCACTC CAACTGCTGAAttcaagagaTTCAGCAGTTGGAGTGCTCtttttggaaa-3') was also subcloned into the pNTO vector and the shRNA expressed from this plasmid (pNTO-rTau5) targets a sequence on rat tau exon 2, (5'- AAGAGCACTCCAACTGCTGAA-3'). All pNTO plasmids were screened for shRNA efficiency by co-transfection assays in

Cos7 cells with rat or human tau plasmids as above, and by transfection into PC6-3 cells, followed by quantitative Western blotting with Tau5-HRP. pNTO-rTau4 and pNTO-rTau5 plasmids were transfected into PC6-3 cells using Lipofectamine 2000 (Invitrogen), and stable colonies were selected in media containing 500 μg/ml G418. Empty vector (EV) control cell lines were also generated in PC6-3 cells by stable transfection with the pNTO vector. The extent of tau depletion was determined by Western blotting with Tau5-HRP, using lysates from cells that had been induced to differentiate with 100 ng/ml NGF (2.5S, Research Diagnostics, Flanders, NJ) for three days.

To create cell lines with doxycycline-induced tau depletion, pNTO-rTau4 was transfected into TR156 cells, a cell line derived from PC6-3 stably expressing the tetracycline repressor protein (generously provided by Dr. Stefan Strack, (Strack, 2002)). Stable colonies were selected in media containing 500 μg/ml G418. For induction of rat tau shRNA in doxycycline-inducible cells, doxycycline (Sigma) was added to the culture media at a final concentration of 1 μg/ml for a minimum of three days.

Immunofluorescence

PC6-3 cells and stable cell lines were fixed in microtubule stabilizing buffer containing glutaraldehyde and 0.5% NP-40 as previously described (Leger et al., 1994). After fixation, cells were stained with anti-tubulin (DM1A, Sigma), polyclonal anti-tau (CR, (Lee et al., 2004)) or phalloidin-Alexa594. Secondary antibodies used for immunofluorescence were rabbit anti-mouse IgG-Alexa 488, goat anti-mouse IgG-Alexa 488, donkey anti-rabbit IgG-Alexa 488, or donkey anti-rabbit IgG-rhodamine. Confocal microscopy was performed on a Bio-Rad MRC-1024 system with a Nikon E600 microscope. Epifluorescence microscopy was performed on a Nikkon E800 microscope. Image analysis was carried out with the following software packages: MetaView, National Institutes of Health ImageJ, and Adobe Photoshop.

Neurite initiation assays

PC6-3 cells and stable cell lines were differentiated in 100 ng/ml NGF (2.5S, Research Diagnostics, Flanders, NJ) for 36 hours prior to glutaraldehyde fixation and permeabilization with NP-40 (Leger et al., 1994). In some experiments, 0.1 μM taxol (Calbiochem) was added simultaneously with NGF. After fixation, cells were stained with anti-tubulin (DM1A, Sigma), rhodamine-coupled anti-mouse secondary antibody, and viewed by epifluorescence or confocal microscopy. Neurite initiation for each cell was scored as the number of processes with a length greater than one cell diameter extending from the cell body. Random fields of cells were evaluated and a minimum of 200 cells per cell line was assessed in each experiment. All data is represented as the mean $\pm$ s.e.m. from three independent experiments. For neurite initiation assays performed on transfected cells, tau depleted cells were transfected with Lipofectamine 2000 (Invitrogen) and 0N3R human tau ("hTau") or S262/S356D mutant tau (amino acid numbering is based on the 2N4R isoform of tau, 441 residues) for 24 hours prior to NGF or NGF-taxol addition. Thirty-six hours after NGF addition, cells were fixed and double-labeled with anti-tubulin (DM1A) and polyclonal anti-tau (CR, (Lee et al., 2004)). A minimum of 200 CR-positive cells was scored for neurite initiation in each experiment. Statistical analysis of variance and post-hoc testing for neurite initiation assays was performed as with luciferase assays below (see "Statistical analysis").

AP-1 and ERK reporter assays

The level of endogenous AP-1 transcription factor activity was measured by an AP-1 reporter plasmid 3X-AP-1-Luc (generously provided by Paul Rothman, University of Iowa), which contains three AP-1 consensus binding sites (TGACTAA), in tandem, upstream of a minimal murine c-Fos promoter regulating the expression of firefly luciferase. The internal transfection control plasmid, pRL-SV40 (Promega), expresses

Renilla luciferase under the control of the SV40 early promoter. Used together, these two plasmids will be referred to as "AP-1 reporter system plasmids".

For ERK activation assays, the PathDetect *Trans*-Reporting System (Stratagene) comprising pFR-Luc and pFA2-ELK1, was used according to the manufacturer's protocol with the addition of the internal transfection control pRL-SV40 *Renilla* luciferase plasmid. Used together, these three plasmids will be referred to as "ERK reporter system plasmids".

Cells were grown on collagen coated 24-well plates to ~50% confluency and transfections were performed in triplicate with Lipofectamine 2000 (Invitrogen). For AP-1 assays, cells were transfected with 1.1 μg DNA (500 ng 3x-AP-1-Luc, 100 ng pRL-SV40, and 500 ng of either pRc/CMV control vector or 0N3R human tau plasmid ("hTau"). For ERK assays, cells were transfected with 1.1 μg DNA (500 ng pFR-Luc, 50 ng pFA2-ELK1, 50 ng pRL-SV40, and 500 ng of either pRc/CMV control vector or hTau. The pRc/CMV control vector was used as a control for human tau plasmids and to maintain equivalent amounts of total DNA in each transfection.

NGF (7S and 2.5S, Sigma) treatments were carried out 36-48 hours after transfection at 100 ng/ml or 50 ng/ml, respectively. EGF (Sigma) treatments were carried at 25 ng/ml after transfection. For both AP-1 and ERK reporter assays, a time course of growth factor treatment of up to 24 hours was carried out in preliminary experiments to determine the point of maximum reporter activation. In both assays, three hours growth factor induction proved to have the highest amount of reporter activity and therefore, this time point was used in all subsequent experiments. Cells were harvested, and AP-1 (or ERK) activation was assayed using the Dual Luciferase Assay Kit (Promega) according to the manufacturer's protocol, measuring Firefly and *Renilla* luciferase activities with a tube luminometer. For data analysis, firefly luciferase values were first normalized to *Renilla* luciferase values from the same sample to control for transfection efficiency. To calculate the fold increase in reporter activity after growth

factor treatment, the normalized firefly luciferase activity from the growth factor stimulated sample was divided by the normalized firefly luciferase reading from the non-stimulated control cells.

For experiments with the MEK1 inhibitor U0126, 50 μM U0126 (Promega) or DMSO vehicle control was added to the cells 15 minutes prior to NGF treatment.

Western blotting

Parental PC6-3 cells and stable cell lines were treated with NGF or EGF as above for various time points and lysed in 2X Laemmli buffer containing 10% β-mercaptoethanol. Concentration of total protein in cell lysates was determined by the bicinchoninic acid assay (BCA, Pierce). Samples were resolved in 8% SDS-PAGE gels, transferred to PVDF (Millipore), and probed with one of the following antibodies: Tau5, Tau13 (gifts from Lester Binder), phospho-ERK1/2 (Thr202/Tyr204, Cell Signaling 9101), ERK1/2 (Santa Cruz K-23), or GAPDH (Chemicon). Perkin-Elmer Western Lightning Plus-ECL was used for signal detection. Quantification of phospho-ERK1/2 and total ERK1/2 signals was performed by densitometry with ImageJ software. The phospho-ERK1/2 level at each time point was normalized to the total ERK1/2 level from the same sample and each normalized value was expressed as a percentage relative to the highest value, which was assigned 100%. Statistical significance was determined by Student's t-test.

Statistical analysis

For AP-1 and ERK luciferase assays, the results for each condition were reported as mean $\pm$ s.e.m. from three independent assays. In addition, each assay used transfections that were performed in triplicate. Statistical significance was determined by analysis of variance (linear mixed model) with the Statistical Analysis System (SAS) software package. Reporter activity from all assays (n=3) was analyzed as the random

effect, with each cell line/tau transfection/treatment as the fixed effect. In all figures, the data for each condition is shown as the mean from all assays ± s.e.m. However, for statistical analysis, the data was log transformed to account for proportional differences between groups. All p values <0.05 calculated from post-hoc Tukey comparisons between groups were considered to be statistically significant.

Results

Generation of tau over-expressing and tau depleted cell lines

To study the effects of tau on neuronal signal transduction, clonal cell lines with stable expression of human tau or short hairpin RNA (shRNA) against endogenous rat tau were generated in PC6-3 cells, a sub-clone of the well characterized PC12 rat pheochromocytoma cell line (Pittman et al., 1993). PC6-3 cells, like the parental cell line PC12, exit the cell cycle and differentiate into a sympathetic neuronal phenotype upon exposure to NGF (Greene and Tischler, 1976, Pittman et al., 1993). As the 0N3R isoform of tau is the only isoform expressed in the fetal brain, we chose to further investigate the role of this tau isoform in neuronal signaling. Four cell lines over-expressing the fetal isoform of human tau (352 residues, 0N3R isoform) were developed and one representative cell line, D5, was used for further studies (Figure 2.1, Table 2.2). Quantification of human tau expression in undifferentiated D5 cells showed that the human tau level was approximately four fold higher than the endogenous rat tau (Table 2.2). In contrast, a quantification of tau expression after differentiation with NGF for three days found that the human tau level was only two fold higher than the endogenous rat tau (Table 2.2). This difference is due to the upregulation of endogenous tau that is known to occur in PC12 cells after NGF stimulation for at least three days (Drubin et al., 1985).

To develop stable cell lines with depleted endogenous rat tau, we first identified rat tau dsRNA oligonucleotides that would effectively suppress rat tau expression without affecting human tau expression using a COS7 co-transfection assay (Figure 2.2A). Based on the results from this screening, we chose two dsRNA oligonucleotides as templates for the construction of plasmids expressing short hairpin RNA (shRNA) against rat tau. Subsequent co-transfections assays in COS7 cells with rat tau and shRNA plasmids confirmed the reduced expression of rat tau in the presence of shRNA (Figure 2.2B and Table 2.1). One of these constructs, pNTO-rTau4, was further used to generate stable cell lines with inducible or constitutive depletion of endogenous rat tau. PC6-3 Control cell lines were also generated using the empty pNTO vector (Figure 2.3A and Table 2.2). To determine the extent of tau depletion in shRNA-expressing cell lines, cells were differentiated with NGF for 3 days to up-regulate tau expression. Like the parental PC12 cells that are known to increase tau expression after NGF treatment (Drubin et al., 1985), empty vector control cells (EV) also up-regulated tau after NGF differentiation (Figure 2.3A, lanes 1-2). Other control cell lines acted similarly. In contrast, the tau depleted cell line rTau4 showed almost complete depletion of endogenous rat tau after NGF-induced differentiation (Table 2.2 and Figure 2.3A, lane 6), while the cell line rTau7 showed an intermediate level of tau depletion (Table 2.2 and Figure 2.3A, lane 4). In the undifferentiated state, both rTau4 and rTau7 had less tau than EV (Table 2.2 and Figure 2.3A, lanes 1,3,5).

A stable cell line with inducible expression of rat tau shRNA was also developed by stably integrating pNTO-rTau4 into TR156 cells, a PC6-3 cell line containing the tetracycline repressor (Tet-R) (Strack, 2002, Strack et al., 2004). The resulting inducible cell line, TR4-3, was found to up-regulate tau similar to control cells after NGF-induction (Table 2.2 and Figure 2.3B, lanes 1-2). However, simultaneous addition of doxycycline (to induce expression of rat tau-shRNA, Figure 2.3C) and NGF for three days reduced the levels of tau to approximately 35% of control levels (Table 2.2 and Figure 2.3B, lanes 3-

4). Tau levels in undifferentiated TR4-3 cells were also decreased to approximately 35% of control levels after a treatment with doxycycline for three days (Table 2.2 and Figure 2.3B, compare lanes 1 and 3).

Tau enhances NGF-induced neurite initiation without a requirement for microtubule binding

The differentiation of PC12 cells in response to NGF involves an early stage of neurite initiation in which a nascent process buds from the cell body, followed by a period of process elongation and stabilization. The rate of neurite elongation and neurite stability depend on the NGF-induced upregulation of tau that occurs approximately three days after NGF addition (Drubin et al., 1985, Hanemaaijer and Ginzburg, 1991, Esmaeli-Azad et al., 1994). However, tau is present at low levels before NGF addition as well as during the earliest phase of the NGF response. To determine if these low levels of tau have a role in the early NGF response, we first investigated neurite initiation. Neurite initiation takes place before the up-regulation of tau since process budding and nascent neurites are already visible in many cells within 36 hours after NGF addition.

To determine the effect of tau on neurite initiation, the morphologies of tau depleted and tau over-expressing cell lines were evaluated after 36 hours NGF induction, and processes greater than one cell diameter were scored as neurites. Neurite initiation, represented by the average number of neurites per cell, was decreased in tau depleted rTau4 cells compared to control PC6-3 cells (Figure. 2.4, 2.5A, 2.5C), while in tau over-expressing D5 cells, the average number of neurites per cell was increased (Figure. 2.4). Similar results were obtained with the tau depleted rTau7 cell line and with cells evaluated at an earlier time point (20 hrs, data not shown). Neurite initiation could also be restored in rTau4 after expressing human tau by transient transfection (Figure 2.4), although rTau4 rescued with human tau exhibited 42% less neurite initiation than PC6-3

(**$p<0.01$, Figure 2.4). These results suggest a role for tau in events leading to neurite initiation.

One possible explanation for the observed effect of tau depletion on neurite initiation is a requirement for tau in promoting microtubule assembly during process initiation. In order to address this possibility, we tested the ability of the microtubule stabilizing drug taxol to restore process initiation during NGF differentiation in rTau4. We have previously shown that a low concentration of taxol (0.1 μM), which allows microtubules to retain dynamicity (Derry et al., 1995), mimics the effects of tau expression on process outgrowth in PC12 cells treated with cytochalasin (Leger et al., 1994). Therefore, taxol treatment might be expected to restore neurite initiation in tau depleted cells if tau was required solely for microtubule stabilization during the early phase of process formation. Subsequently, we found that taxol treatment did not affect neurite initiation in control PC6-3 cells (Figure 2.5A and 2.5B, quantification in 2.5E), and did not increase the average number of neurites per cell in rTau4 after NGF differentiation (Figure 2.5C and 2.5D, quantification in Figure 2.5E). We did, however, observe a thickening of microtubule bundles in nascent neurites from control PC6-3 cells (Figure 2.5B), and increased microtubule bundling near the cell periphery in both control and rTau4 cells (Figure 2.5B and 2.5D). Neurite length in taxol-treated PC6-3 cells appeared to be greater than non-treated controls, as did neurite width, although these observations were not quantified. Together, these findings confirmed an increase in microtubule stabilization in the presence of taxol. Further, the ability of PC6-3 cells to differentiate normally in the presence of low concentrations of taxol confirmed the preservation of microtubule dynamicity under these conditions.

Since process initiation in tau depleted cell lines could not be restored by providing a microtubule stabilizing agent, these findings suggested that a tau function other than microtubule stabilization was involved in neurite initiation. A requirement for the microtubule stabilizing properties of tau in neurite initiation was further investigated

by testing a phosphomimetic tau mutant for its ability to restore neurite initiation in tau depleted cells. Expressing the tau mutant S262D/S356D, which has significantly reduced affinity for microtubules (Biernat et al., 1993, Drewes et al., 1995, Haase et al., 2004, Sharma et al., 2007), restored neurite initiation in taxol treated rTau4 cells 7.0 fold (Figure 2.5E), similar to the 8.8 fold increase obtained when neurite initiation was restored to rTau4 cells by expressing wild-type tau (Figure 2.4). Since S262D/S356D tau is still able to enhance neurite initiation similar to wild-type tau, the data indicates that the requirement for tau during neurite initiation is not likely to involve its ability to interact with microtubules.

Tau expression level affects NGF-induced AP-1 transcription factor activity

In PC12 cells, during the first 36 hours of NGF treatment, a sustained activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2), and a consequent up-regulation of the AP-1 transcription factor c-Fos accompanies neurite outgrowth (Pellegrino and Stork, 2006). Because gene expression profiling of tau-deficient mice had indicated that tau depletion affected the expression of AP-1 transcription factors (Oyama et al., 2004), we questioned whether tau would have a role in NGF-induced AP-1 activity during the early phase of NGF treatment. To investigate this possibility, we assayed AP-1 activity with a luciferase reporter system; activity was measured after three hours of NGF treatment as described in Materials and Methods.

As expected, control PC6-3 cells showed a 34 fold increase in AP-1 activity over non-stimulated cells following NGF treatment (Figure 2.6A). Interestingly, PC6-3 cells transiently over-expressing wild-type human tau (PC6-3 +hTau) were found to have a significant increase in NGF-induced AP-1 activity (1.8 fold) relative to control cells while the tau depleted line rTau4 had a significant decrease in AP-1 activity (3.5 fold) when compared to control EV cells (Figure 2.6B). Moreover, transient expression of

human tau in rTau4 was sufficient to significantly increase AP-1 activity (2.0 fold) relative to control rTau4 (Figure 2.6B; note that rTau4+hTau was 44% less than EV). Similar results were obtained in rTau7 (2.7 fold decrease relative to EV), as well as in assays performed at later time points of NGF treatment (12 and 24 hours, data not shown). Together, these data indicated that tau potentiates the NGF-mediated activation of AP-1 transcription factor DNA binding activity.

Tau potentiates NGF-induced ERK activation

AP-1 activity in response to NGF can be regulated through the Ras/ERK pathway (Pellegrino and Stork, 2006). To determine if tau affected the ERK pathway, cells were treated with 50 μM U0126, a highly specific inhibitor of the dual specificity kinase MEK1 that is responsible for activating ERK1/2. Pre-treatment of cells with U0126 almost completely abrogated AP-1 reporter activity in both control PC6-3 and rTau4 (Figure 2.7). Furthermore, the presence of human tau in either cell line, which had previously increased AP-1 activity (1.8-2.0 fold), failed to increase AP-1 activity when the cells were pre-treated with U0126. These data implicate the Ras/ERK pathway in mediating the effect of tau on AP-1 activity.

As these results suggested an interaction between tau and the ERK signaling pathway upstream of AP-1 activation, we tested the effects of tau on the activation of ERK using a luciferase reporter system to measure changes in ERK1/2 activation. We first compared control PC6-3 cells to PC6-3 cells over-expressing wild-type human tau and found that over-expression of human tau resulted in a significant increase in ERK reporter activity (2.1 fold, Figure 2.8A). In contrast, rTau4 and rTau7 showed a significant decrease in ERK activation compared to EV (Figure 2.8B) and PC6-3 (data not shown). In addition, when wild-type human tau was expressed in rTau4 or rTau7, although the restored level was 43% and 72%, respectively, of the EV level, both showed a significant recovery of reporter activity. These results indicated that, as with AP-1

activation, tau expression was capable of potentiating ERK signaling activity (Figure 2.8B)

An analysis of cell lysates harvested after 5, 60, and 180 minutes of NGF exposure showed that the level of active ERK1/2, as detected by anti-phospho-ERK1/2, was consistently reduced in rTau4 compared to PC6-3 (Figure 2.9A). Quantification showed significant decreases in ERK activation at all time points tested (average decrease 59.7%), whereas the overall time course of activation was similar in both cell lines (Figure 2.9B).

Tau potentiates EGF-induced ERK activation

ERK activation results from a cascade of phosphorylation events that can be triggered by a number of growth factors. Since tau affects ERK signaling activated by NGF, tau might also affect signaling induced by other growth factors. To investigate, we treated our cell lines with epidermal growth factor (EGF), which also activates the Ras/ERK pathway. A time course of EGF stimulation was first performed in PC6-3 cells expressing ERK reporter system plasmids to identify EGF time points with optimal luciferase reporter activity. Under our conditions, we found that stimulation with EGF for three hours yielded maximum levels of luciferase activity, similar to NGF stimulation (data not shown); however the magnitude of reporter activity was less than that obtained with NGF (compare Figure 2.8A with Figure 2.10A). This difference may be attributed to the transient EGF-induced activation of ERK upstream of transcription factor activation, as compared to prolonged ERK activation in response to NGF. Nevertheless, we found that in response to EGF, tau over-expression caused an increase in ERK reporter activity whereas tau depletion resulted in a significant decrease in reporter activity (Figure 2.10A). An analysis of cell lysates collected after EGF induction similarly showed a significant decrease in active ERK1/2 in rTau4 relative to PC6-3 after five minutes (Figure 2.10B, with quantification in Figure 2.10C). However, the pattern

of ERK1/2 activation did not change, showing that the NGF and EGF responses were distinct.

Stimulation of PC12 cells with EGF does not result in the prolonged ERK signaling required for neuronal differentiation as in the case of stimulation with NGF (Qui and Green, 1992, Traverse et al., 1992, Nguyen et al., 1993). However, the ability of over-expressed tau to potentiate ERK activation in D5 cells might conceivably lead to neurite outgrowth after stimulation with EGF. To test this possibility, we exposed control and D5 cells to EGF for 36 hours and observed neurite initiation. After 36 hours, we did not observe the development of neuritic processes in either cell line, indicating that tau over-expression is not sufficient to lengthen the time course of EGF-stimulated ERK activity required for neurite outgrowth (data not shown). An analysis of ERK1/2 phosphorylation in EGF-stimulated D5 cells showed a significant increase in ERK1/2 activation relative to control cells at both 5 minutes and 60 minutes after EGF treatment (Figure 2.11A, with quantification in Figure 2.11B). However, in D5 cells after 60 minutes EGF treatment, the phospho-ERK signal was decreased by over 90% relative to the level observed after 5 minutes EGF treatment (Figure 2.11B). Since ERK1/2 phosphorylation after a 60 minute NGF treatment is typically at least 60% of the 5 minute level (Figure 2.9B), the data suggests that tau has no effect on the rapid down-regulation of ERK signaling typical in EGF-stimulated cells. Together, these findings indicate that tau is affecting signaling at a point that is downstream of the receptor and common to both NGF and EGF signaling pathways.

Discussion

In the present study, we have used cell lines stably expressing tau shRNA to show that tau depletion reduced the number of neurites initiated per cell, suggesting that tau has a role in the NGF response prior to the stage of neurite elongation. Previous investigations of PC12 cells with tau depletion have reported an effect of tau on neurite

length but effects on neurite initiation have been less clear. In one study, PC12 cells were allowed to differentiate in media containing NGF and tau anti-sense oligonucleotides for several days (Hanemaaijer and Ginzburg, 1991). An initial reduction in neurite initiation was not reported in these anti-sense treated cells, although neurites were seen to retract from 48 to 96 hours after treatment with NGF and tau anti-sense, (Hanemaaijer and Ginzburg, 1991). A key difference between this study and our current findings should be noted, however. Here, we have used cell lines with stable expression of tau shRNA to constitutively deplete endogenous tau in naïve, undifferentiated cells; therefore tau levels are already minimal in our cells prior to NGF stimulation. In contrast, the PC12 cells treated with tau anti-sense received the anti-sense oligonucleotides simultaneously with NGF stimulation, which leaves insufficient time for the depletion of existing tau protein at the onset of NGF-induced differentiation. In a second study, stable PC12 cell lines were developed expressing either full length sense or anti-sense tau cDNA (Esmaeli-Azad et al., 1994). Among the cell lines expressing anti-sense tau cDNA, a reduction in average neurite length after NGF-stimulation was reported. However neurite initiation was not investigated (Esmaeli-Azad et al., 1994). These earlier studies, while clearly identifying a role for tau in neurite elongation, left open the question as to whether tau was involved in the earliest stages of neurite initiation. As our rTau4 and rTau7 cell lines have demonstrated a clear requirement for tau in neurite initiation, these models of tau depletion have proven to be useful tools for investigating tau function in neuronal differentiation. In addition, our development of a cell line with inducible tau depletion (Figure 2.3B) may provide additional information about the role of tau in neurite maintenance or in the signaling associated with trophic factor withdrawal, since tau expression can be selectively repressed at various times in fully differentiated cells.

Tau potentiates neurite initiation

Our experiments with taxol and the S262D/S356D mutant tau have revealed that a separate property of tau, other than microtubule stabilization, was required to aid neurite initiation following NGF stimulation. This finding is consistent with previous studies demonstrating that neurite outgrowth in N2a cells required tau phosphorylation at S262/S356 (Biernat et al., 2002). There are several possibilities concerning the role of non-microtubule associated tau in neurite initiation. First, the effects of tau on neurite initiation may be directly related to tau's ability to potentiate ERK1/2 and AP-1 activation as we have seen in our current study. It is well established that the AP-1 transcription factors c-Fos and c-Jun are activated during neuronal differentiation in PC12 cells and are required for neurite outgrowth (Greenberg et al., 1985, Leppa et al., 2001, Eriksson et al., 2007). Therefore, impaired neurite initiation in tau depleted cells may simply be due to an attenuation of AP-1 transcription factor activity after NGF induction. However, our neurite initiation data suggests the effects of tau depletion on neurite initiation are more severe than the effects of tau depletion on ERK signaling (compare Figure 2.4 with Figures 2.6 and 2.8). Thus, tau may impact on neurite initiation through multiple mechanisms that can include ERK signaling, or tau may impact on neurite initiation through a mechanism independent of the effect on upstream ERK signaling.

For example, tau may be involved in protein interactions near the cell periphery at individual sites of neurite budding. Neurite initiation requires rearrangements of the actin cortex, insertion of newly synthesized membrane as well as a precise regulation of microtubule dynamics. As discussed in Chapter I, tau is able to associate with the actin cytoskeleton, and has in fact been reported to co-localize with actin-rich extensions after NGF stimulation in PC12 cells (Yu and Rasenick, 2006). This interaction occurred independently of an association with microtubules, and in conjunction with our rescue experiments with S262D/S356D mutant tau (Figure 2.5E), strongly supports a functional role for microtubule-independent tau in neurite initiation. Actin dynamics in growth

cones and developing neurites are also regulated by the non-receptor tyrosine kinase Src (Thomas and Brugge, 1997, Robles et al., 2005), which as we have seen, also interacts with tau and phosphorylates tau on tyrosine 18 (Lee et al., 1998, Lee et al., 2004, Sharma et al., 2007). Moreover, we have previously demonstrated that the interaction between tau and Src leads to enhanced Src activation in PDGF-stimulated fibroblasts upstream of actin stress fiber breakdown (Sharma et al., 2007). Similarly, tau may be able to enhance the Src-dependent actin rearrangements associated with the initial stages of neurite outgrowth. A requirement for a tau/Src interaction may thus explain the effects of tau depletion on NGF-induced neurite initiation. Further experiments will be needed to address these possibilities.

Tau potentiates ERK activation

The first hint that tau expression might be linked to transcription factor activity came from microarray analysis showing that the AP-1 transcription factors *Fos* and *Fosb* were the two genes most highly up-regulated in mice genetically deficient in tau (Supplemental data in (Oyama et al., 2004)). However, our results indicated that tau depletion decreased, rather than increased, the NGF-induced activity of AP-1 transcription factors.

Given that the nature of the two experimental systems are vastly different (an undifferentiated neuronal cell line responding to growth factor addition versus adult mouse brain tissue), there could be several explanations for the apparent inconsistency in these results. Since upstream ERK activity has not been investigated in these mice, it is possible that the elevated levels of c-Fos and FosB mRNA in the tau deficient mouse do not necessarily correlate with increased levels of AP-1 activity. Alternatively, if AP-1 activity is indeed elevated, it may reflect increased signaling through pathways other than the ERK pathway (e.g. increased calcium influx through voltage gated calcium channels) which also converge on AP-1 transcription factors. We speculate that a compensatory

up-regulation of c-Fos and FosB was required for survival of the mouse during embryonic development, whereas tau is less critical for the viability of undifferentiated neuronal cells, rendering similar compensatory measures unnecessary. In spite of these differences between experimental systems, it is clear from our data that tau has a crucial role in the induction of AP-1 activity in response to growth factors in the PC6-3 cell line. Further experiments in neuronal stem cells or primary neuronal cultures will serve to expand on these findings and determine whether AP-1 activity in other cell types is similarly affected by tau expression level.

The potentiation of AP-1 activity by tau raises the issue of nuclear tau. Nuclear tau has been reported in a number of cell lines including human neuroblastoma cells, lymphocytes, and fibroblasts (Loomis et al., 1990, Thurston et al., 1996). In one study, tau was also found to localize to the nucleus in PC12 cells overexpressing myotonin protein kinase (DMPK1) after NGF treatment (Hernandez-Hernandez et al., 2006), although it was not found in PC12 controls. DMPK1 is known to disrupt the subcellular localization of tau and cause tau aggregation (Vermersch et al., 1996), therefore the altered localization of tau is likely due to DMPK1 overexpression. Accordingly, we did not observe nuclear tau in either control PC6-3 cells or tau overexpressing D5 cells under our growth conditions, nor did the localization significantly change after NGF addition (data not shown). Furthermore, in our previous PC12 work, we had found transfected tau mainly in the cytoplasm, rather than in the nucleus (Leger et al., 1994). These results suggest that the effects of tau on AP-1 activity are not due to a nuclear localization of tau after NGF stimulation. In Chapter III, we will further address the subcellular localization of tau as it pertains to ERK signaling.

Our pharmacological tests showed that tau affected AP-1 transcription factor activity by modulating signaling through the ERK pathway. In the tau depleted rTau4 cells, NGF-induced ERK reporter activity was reduced 5.5 fold relative to control EV cells whereas transiently expressing human tau in rTau4 was able to restore ERK reporter

activity 2.3 fold (Figure 2.8). The inability of the exogenously expressed tau to totally restore ERK (or AP-1) reporter activity to control levels resembled the inability of exogenous tau to completely restore neurite initiation to rTau4 (Figure 2.4 and 2.5). This might suggest that the effects of tau on ERK signaling and neurite initiation are mediated through a common mechanism that is similarly unable to completely restore function. For example, although high levels of exogenous tau are expressed during the rescue experiments, it may function less efficiently than endogenous tau. Tau expressed by transfection may differ from endogenous tau with respect to its subcellular localization (i.e. "mistargeting"). Moreover, tau expressed by transient transfection in neuronal cells usually migrates faster than the endogenous tau in SDS-PAGE gels (data not shown), suggesting that endogenous tau is more efficiently phosphorylated. If tau phosphorylation or subcellular localization is important for tau to impact on ERK signaling and/or neurite initiation, these differences could account for the partial restoration of function in rTau4 cells expressing exogenous human tau.

Tau was found to potentiate ERK activation in response to EGF (Figure 2.10) as well as NGF (Figure 2.8), suggesting tau impacts on ERK signaling through a mechanism common to both growth factor signaling pathways. Although EGF and NGF similarly lead to the activation of ERK, the cellular response in each case is quite different. Sustained activation of ERK in response to NGF is required for neurite initiation and differentiation in PC12 cells (Qui and Green, 1992, Nguyen et al., 1993, Kao et al., 2001), while the transient activation of ERK in response to EGF does not lead to differentiation (Huff et al., 1981). Since tau over-expression appears to increase ERK activity in response to EGF, there is a possibility that tau-overexpression might lead to differentiation in EGF treated cells. We tested this by observing neurite initiation in control and tau over-expressing D5 cells upon exposure to EGF. However, neurite initiation was not observed indicating that tau expression alone is not sufficient to induce differentiation in response to EGF. Moreover, these findings are in agreement with a

quantification of phosphorylated ERK1/2 levels in EGF-treated D5 cells. D5 cells did not show a prolonged activation of ERK in response to EGF as would be required for differentiation to occur (Figure 2.11). The EGF receptor is known to be ubiquitinated and targeted for proteasomal degradation by the E3 ubiquitin ligase, c-Cbl, in response to EGF (Levkowitz et al., 1999, Waterman et al., 1999), and the resulting loss of receptor tyrosine kinase activity leads to a rapid attenuation of EGF-induced ERK signaling (Kao et al., 2001). Therefore, we expect that the upstream inactivation of the EGFR would preclude any effects the over-expression of tau might have on the prolonged Rap1 signaling required for neurite initiation.

Our data implicating tau as a component of ERK signaling has broader implications for the roles of tau in brain development. ERK signaling is upregulated in the developing brain, coinciding with the presence of highly phosphorylated fetal tau lacking a strong affinity for microtubules (Goedert and Jakes, 1990, Bramblett et al., 1993). Given our current findings, we speculate that fetal tau is similarly able to potentiate ERK signaling during early brain development. Although our data is presently limited to PC12 cell lines, we feel that similarities between the two systems strongly suggest a role for fetal tau in the developing brain. Fetal brain specifically expresses the 0N3R isoform of tau (see Figure 1.1) and likewise we have demonstrated that this isoform is capable of potentiating ERK signaling in response to NGF and EGF. In addition, NGF- and EGF-induced activation of ERK signaling is known to occur in the developing brain (Fukunaga and Miyamoto, 1998, Benoit et al., 2001), coinciding with the active proliferation and differentiation of neuronal precursors. In spite of these similarities, further experiments will need to be performed in cultured primary neurons or neuronal stem cells to more precisely define the physiological role of fetal tau in neurons of the CNS. It will also be of interest to determine if tau is able to potentiate ERK signaling in response to other neurotrophic factors such as brain-derived neurotrophic

factor (BDNF), neurotrophin 3 (NT3), and neurotrophin 4/5 (NT4/5) which are also present during brain development.

Tau may also impact on ERK signaling in non-neuronal cell types, including tissues outside of the CNS. Although tau expression is enriched in the CNS, tau can be found in a number of tissues including heart, skeletal muscle, testes, and prostate epithelia (Gu et al., 1996). Preliminary experiments in our laboratory with ALVA-31 prostate epithelial cell lines indicate that tau can impact on EGF-stimulated ERK signaling in these cells as well. Specifically, ALVA-31 cells with depletion of endogenous tau show a reduction in EGF-stimulated ERK1/2 phosphorylation as compared to control cells (Skye Souter, unpublished data). These findings are in agreement with our present findings in tau depleted PC6-3 cells, and further underscore the significance of an interaction between tau and the ERK signaling pathway.

In conclusion, we have shown that tau is able to potentiate ERK activation in response to NGF and EGF, although the ability of tau to potentiate ERK activation upon exposure to EGF does not result in neuronal differentiation. Further, we have found that neurite initiation in response to NGF is modulated by the presence of tau. Our experiments with S262D/S356D mutant tau lacking an association with microtubules also demonstrate that microtubule binding is not required for tau to enhance neurite initiation. However, the effects of tau on neurite initiation may involve multiple mechanisms, and it remains to be seen if microtubule association or other modifications of tau are necessary for tau to enhance ERK signaling. In the next chapter we will investigate how phosphorylation of tau impacts on ERK signaling, and further investigate the interactions between tau and upstream components of the signaling cascade.

Table 2.1 Summary of rat tau oligonucleotides used for RNA interference.

siRNA/Plasmid Name	Oligonucleotide Sequence	Target Site	Efficiency*
si-rTau1	AAGACAGGACAGGAAATGAC	Exon 5	5%
si-rTau2	AGAAAGGCACATCCAATGCCA	Exon 7	50%
si-rTau3	AGTGTGGTTCCTTAGGGAACC	Exon 11	25%
si-rTau4	AAGTGTCCGCCTCTTTGGCCA	Exon 13	98%
si-rTau5	AAGAGCACTCCAACTGCTGAA	Exon 2	55%
si-rTauB4	AATGACGAGAAGAAAGCGAAG	Exon 4	46%
si-rTauB5	AAAGAACGTCAGGTCCAAGAT	Exon 9	47%
si-rTauB11	AAGATTGGCTCCTTGGATAAC	Exon 12	88%
pNTO-rTau4**	GATCCCC**GTGTCCGCCTCTTTGGCCA**TTCAAGAGA**TGGCCAAAGAGGCGGACAC**TTTTTGGAAA	Exon 13	94%
pNTO-rTau5***	GATCCCC**GAGCACTCCAACTGCTGAA**TTCAAGAGA**TTCAGCAGTTGGAGTGCTC**TTTTTGGAAA	Exon 2	95%

*Percent depletion of exogenously co-transfected 2N4R rat tau in Cos7 cells as determined by densitometric analysis of Western blots

**hairpin 64-mer sequence derived from si-rTau4

***hairpin 64-mer sequence derived from si-rTau5

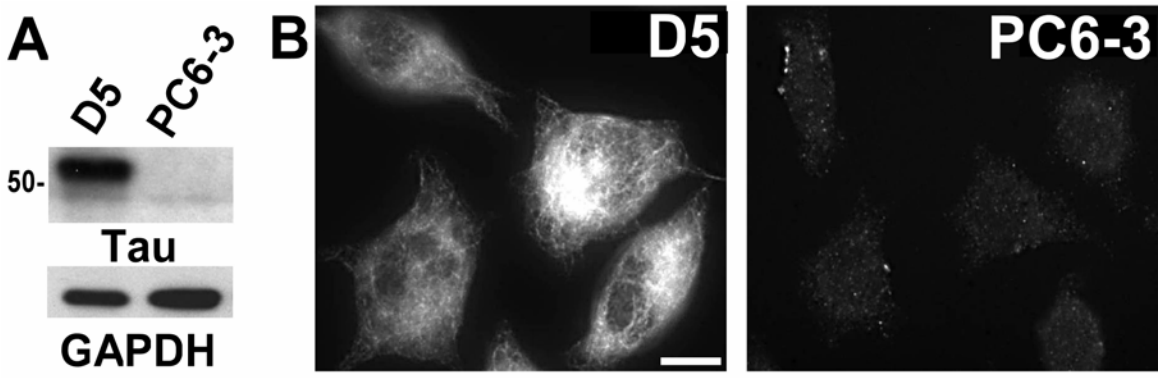

Figure 2.1 Generation of PC6-3 cell lines with over-expression of human tau. (A) Cell lysates from D5, a representative PC6-3 cell line over-expressing 0N3R human tau, and PC6-3 controls were probed with the human tau specific antibody, Tau13. **(B)** PC6-3 control and D5 tau over-expressing cells were fixed with glutaraldehyde labeled with polyclonal anti- human tau (CR), and viewed by epifluorescence microscopy. Control cells do not show labeling with the human tau specific antibody. Scale bar = 3 μm.

Table 2.2 Characteristics of PC6-3 cell lines with altered tau expression.

Cell Line	Parental Line	Stably Expressed Plasmid	Tau Expression Levels
PC6-3	PC12	None	Control
D5-16	PC6-3	pRc/CMVn123c	0N3R human tau is expressed 3 fold higher than endogenous tau in undifferentiated cells and 1.5 fold higher after differentiation.*
D5	PC6-3	pRc/CMVn123c	0N3R human tau is expressed 4 fold higher than endogenous tau in undifferentiated cells and 2 fold higher after differentiation.*
E1-19	PC6-3	pRc/CMVn123c	0N3R human tau levels not characterized.
E1-20	PC6-3	pRc/CMVn123c	0N3R human tau levels not characterized.
EV	PC6-3	pNTO Vector	Tau levels are equivalent to parental PC6-3 cells.
V4	PC6-3	pNTO Vector	Tau levels are equivalent to parental PC6-3 cells.
rTau4	PC6-3	pNTO-rTau4	No endogenous tau detected in undifferentiated or differentiated cells.*
rTau7	PC6-3	pNTO-rTau4	No tau detected in undifferentiated cells. Tau expression is 15% of control cells after differentiation.*
TR156	PC6-3	pCDNA6/TR**	Tau levels are equivalent to parental PC6-3 cells.
TR4-3	TR156	pNTO-rTau4	Tau expression after 3 days treatment with doxycycline is 35% of controls (-dox). After differentiation, tau expression in doxycycline treated cells is 35% of controls (-dox +NGF).

Note: Tau levels are determined by quantitative Western blotting.

*Tau expression in differentiated cells was measured after NGF treatment (100 μg/ml) for three days.

**The TR156 cell line is derived from PC6-3 cells and stably expresses the Tet repressor (Strack, 2002).

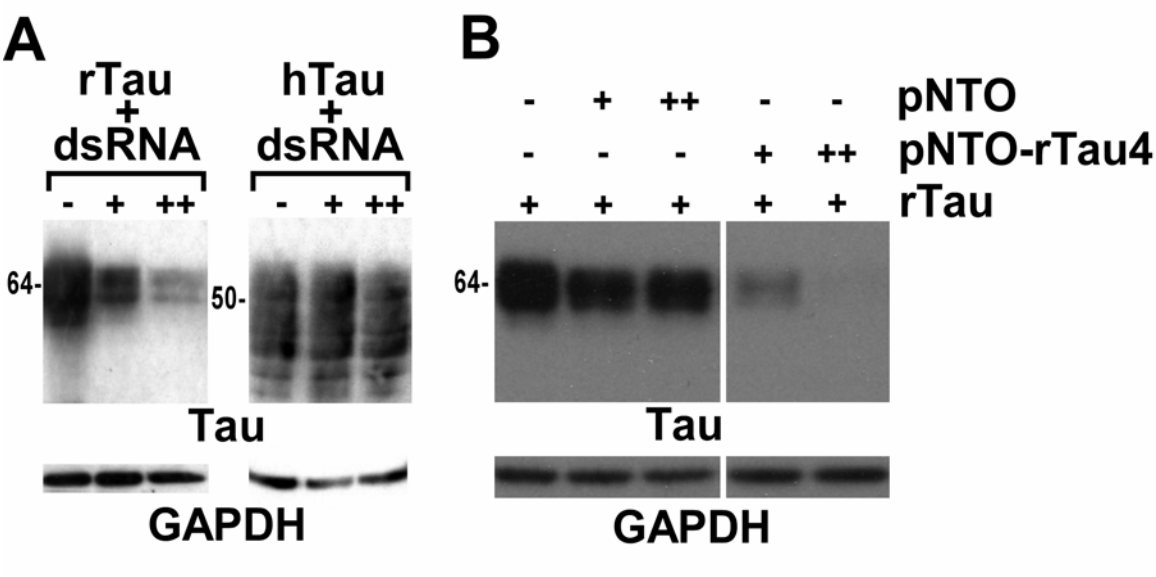

Figure 2.2 Rat tau-specific RNA interference does not affect human tau expression. **(A)** Cell lysates from COS7 cells co-transfected with increasing amounts of rat tau specific dsRNA oligonucleotides (si-rTau4) and either 2N4R rat tau (rTau, left panel) or 0N3R human tau (hTau, right panel) plasmids, were probed with Tau5-HRP. "-, +, and ++" indicate the addition of 0, 3, or 5 µg, respectively, dsRNA. Human tau expression (left panel) is not affected by co-transfection with rat tau-specific dsRNA. The presence of multiple tau bands in each lane can be attributed to differential phosphorylation of tau species and/or a partial degradation of the exogenously expressed tau. **(B)** Cell lysates from COS7 cells co-transfected with rat tau (rTau) and either empty pNTO vector or pNTO-rTau4 expressing shRNA against rat tau were probed with Tau5-HRP. "+ and ++" indicates the addition of 0.5 µg or 1 µg, respectively, plasmid DNA. 1 µg of lysate was loaded in each lane of (A) and (B).

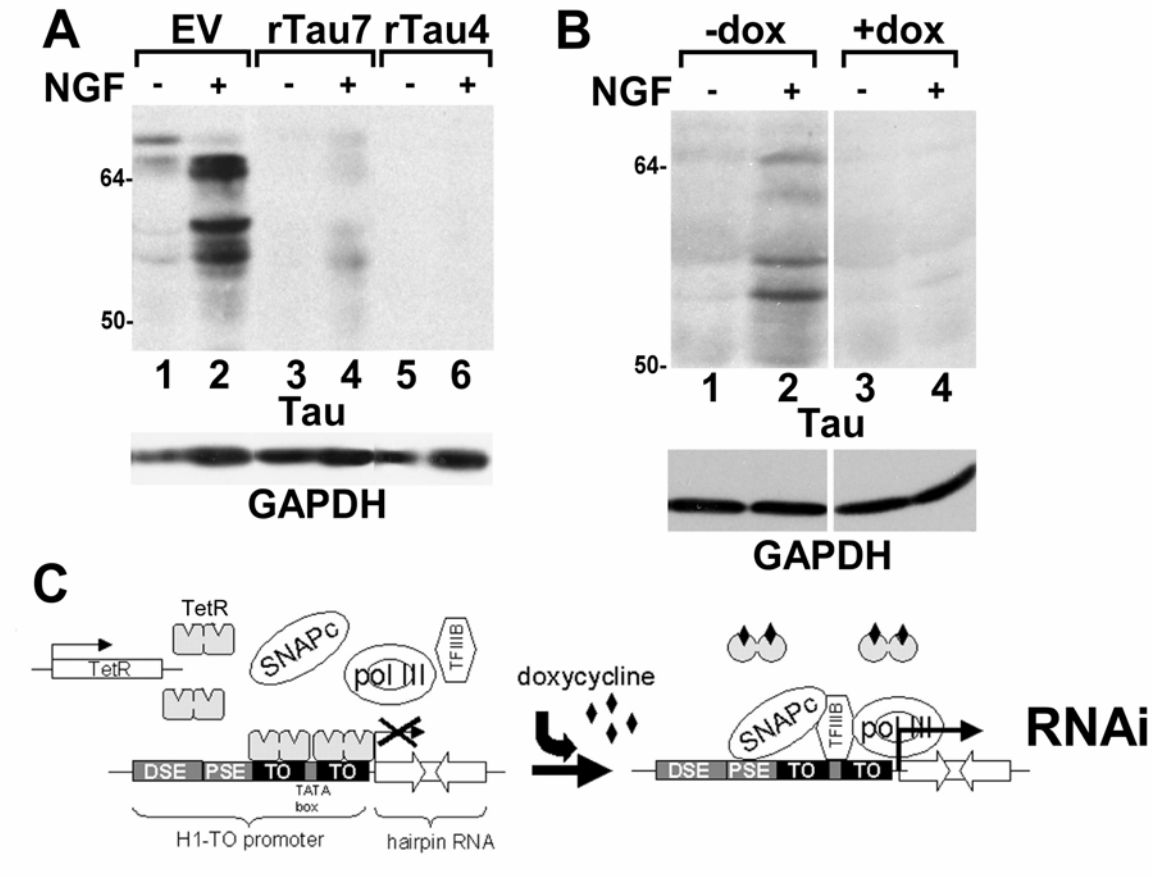

Figure 2.3 Generation of PC6-3 cell lines with depletion of endogenous rat tau. (A) Cell lysates from PC6-3 cell lines stably expressing shRNA against endogenous rat tau (rTau4, rTau7) or an empty pNTO vector control line (EV) were harvested before or after NGF treatment for three days as indicated. Lysates were probed with Tau5-HRP. GAPDH was used as a loading control. **(B)** Cell lysates from TR4-3 cells with inducible expression of shRNA against endogenous rat tau were treated with 1 μg/ml doxycycline for three days (+dox, lanes 3 and 4), harvested, and probed with Tau5-HRP. Where indicated, NGF treatment was carried out for three days prior to harvest (lanes 2 and 4). In lane 4, cells were treated simultaneously with NGF and doxycycline for 3 days. GAPDH was used as a loading control. 1 μg of lysate was loaded in each lane in (A) and (B). **(C)** Schematic of inducible RNA interference in TR4-3 cells. Cells contain the doxycycline-inducible pNTO plasmid expressing rat tau shRNA as well as a plasmid encoding the tetracycline repressor (TetR). In the absence of doxycycline, expression of shRNA is prevented by the action of TetR binding to the H1 promoter (adapted from (Strack et al., 2004)).

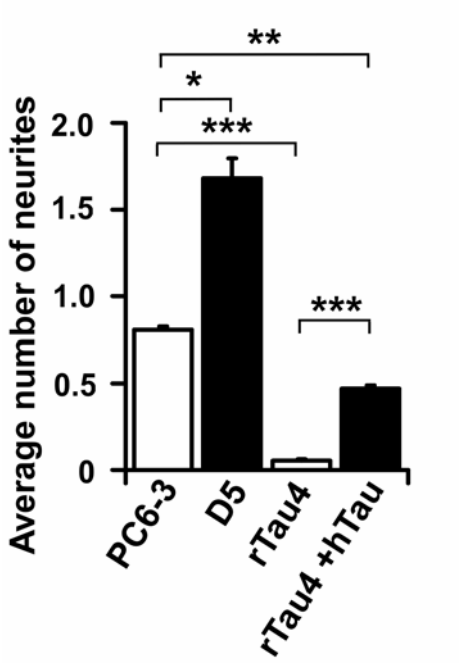

Figure 2.4 Tau expression level affects early neurite initiation induced by NGF
Average number of neurites per cell in PC6-3 or in stable cell lines D5 or rTau4 was determined after 36 hours NGF differentiation. "rTau4 + hTau" denotes rTau4 transfected with 0N3R human tau and scored for neurite initiation as described in Materials and Methods. Results are shown as the average $\pm$ s.e.m. from three independent experiments (*$p<0.05$, **$p<0.01$, ***$p<0.005$).

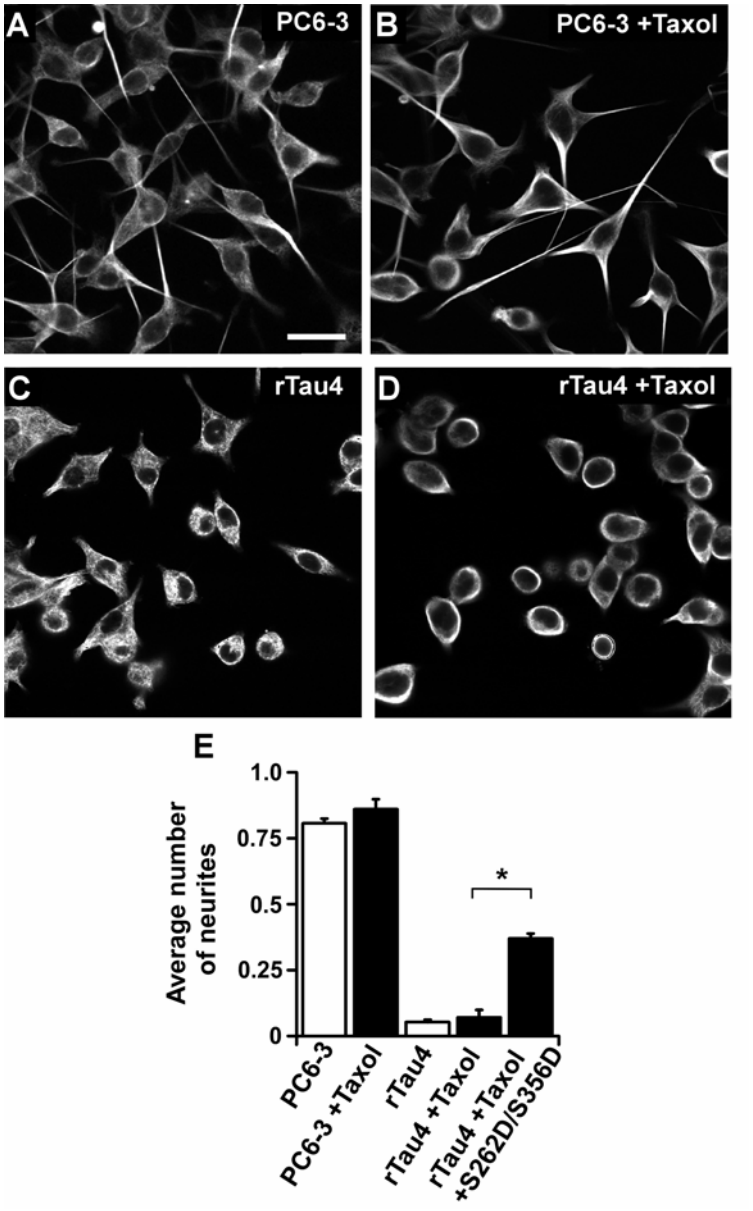

Figure 2.5 Microtubule stabilizing properties of tau are not required for tau to affect neurite initiation. (A, B, C, D) PC6-3 or rTau4 cells were differentiated with 100 ng/ml NGF for 36 hours in the presence or absence of 0.1 μM Taxol as indicated. Cells fixed with glutaraldehyde were labeled with anti-tubulin antibodies (DMA1), Alexa-488-conjugated anti-mouse secondary antibodies, and viewed by confocal microscopy as described in Materials and Methods. (Scale bar = 10 μm). Taxol does not restore neurite initiation in tau depleted cells. **(E)** Average neurites per cell was determined as in Figure 2.4B in PC6-3 or rTau4 cells differentiated in the presence of NGF, with or without the addition of 0.1 μM Taxol. "rTau4+Taxol+S262D/S356D" denotes rTau4 cells transfected with S262D/S356D mutant tau plasmid 24 hours prior to simultaneous Taxol-NGF treatment. Neurite initiation was scored as in Figure 2.4 and described in Materials and Methods (*$p<0.05$).

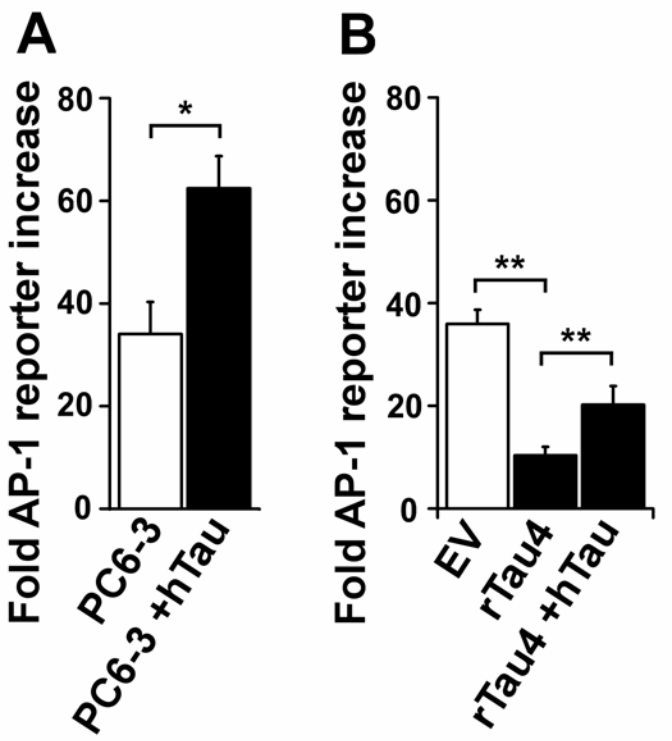

Figure 2.6 Tau expression level affects DNA binding activity of AP-1 transcription factors. (A) PC6-3 cells were transfected with AP-1 luciferase reporter plasmids and either control vector "PC6-3" or human tau plasmids "PC6-3+htau". 36 hours after transfection, NGF (50 ng/ml) was added for 3 hours prior to harvesting. Fold increase in reporter activity relative to minus NGF (naïve) values was calculated for each condition as described in Materials and Methods (*$p<0.01$). **(B)** Control (EV) and tau depleted rTau4 cells were transfected with AP-1 reporter plasmids and human tau (hTau) as indicated (EV and rTau4 received control vector DNA). NGF addition and fold increase in reporter activity was performed and determined as in (A), (**$p<0.005$). The data shown is the mean ± s.e.m. from three independent experiments where each cell line or condition was assayed in triplicate.

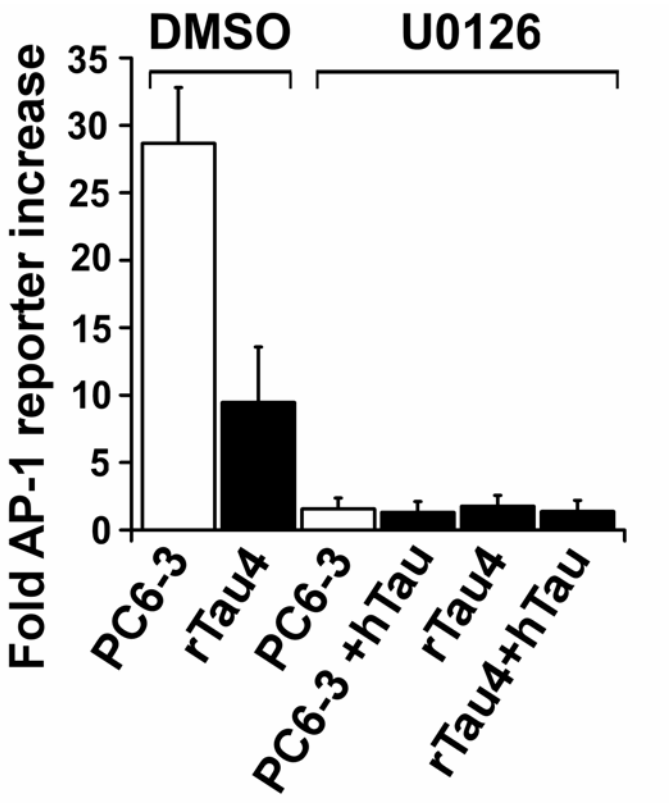

Figure 2.7 Tau potentiates AP-1 activation through the ERK pathway.
PC6-3 and rTau4 cells were transfected with AP-1 luciferase reporter plasmids as in Figure 2.6, adding human tau as indicated (+hTau). PC6-3 controls and rTau4 received control vector DNA. Where indicated, MEK1 inhibitor (U0126, 50 μM) or vehicle control (DMSO) was added to the cells for 15 minutes prior to induction with NGF for 3 hours. Fold increase in reporter activity was determined as in Figure 2.6 and the data is shown as the mean ± s.e.m. from three independent experiments where each cell line or condition was assayed in triplicate. MEK1 inhibition prevents enhanced AP-1 activity when tau is overexpressed.

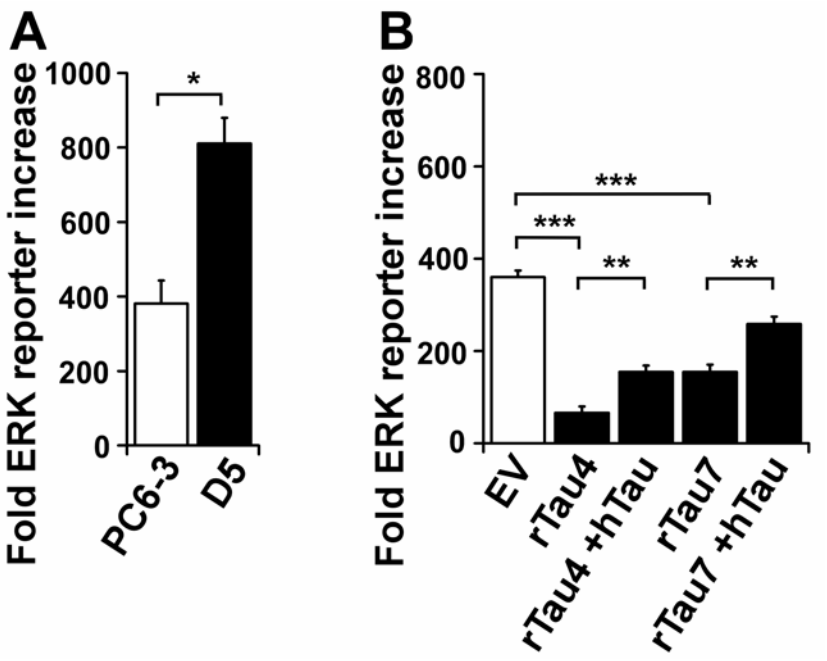

Figure 2.8 Tau potentiates NGF-induced ERK signaling. (A) PC6-3 cells or tau over-expressing D5 cells were transfected with ERK luciferase reporter plasmids for 36 hours followed by differentiation with 50 ng/ml NGF for 3 hours prior to harvest. Fold reporter increase was calculated relative to minus NGF (naïve) control as described in Materials and Methods (*$p<0.05$). **(B)** ERK activation in response to NGF was assayed in control EV cells, rTau4, and rTau7 as described in (A). ("+ hTau" indicates the addition of 0N3R human tau plasmid; other assays received control vector DNA, **$p<0.005$, ***$p<0.0001$). For panels (A) and (B), data shown is the mean ± s.e.m. from three independent experiments; for each experiment, transfections were performed in triplicate for each condition.

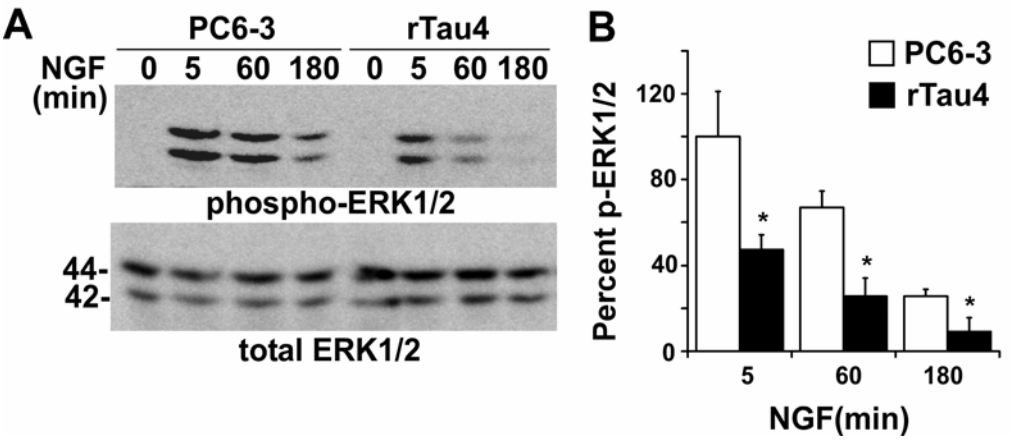

Figure 2.9 NGF-induced ERK activation is impaired in tau depleted cells. (A) Cell lysates from PC6-3 and rTau4 were harvested after treatment with NGF (50ng/ml) for the indicated time points and probed for activated ERK1/2, using anti-phospho-ERK1/2 (phospho-ERK1/2), and total ERK1/2. The blot shown is representative of three experiments. 1 μg of lysate was loaded in each lane. **(B)** The ratio of phospho-ERK/total ERK for each condition was calculated and shown relative to the highest value obtained (PC 6-3, 5min), which was assigned 100% (* indicates $p<0.05$ relative to PC6-3).

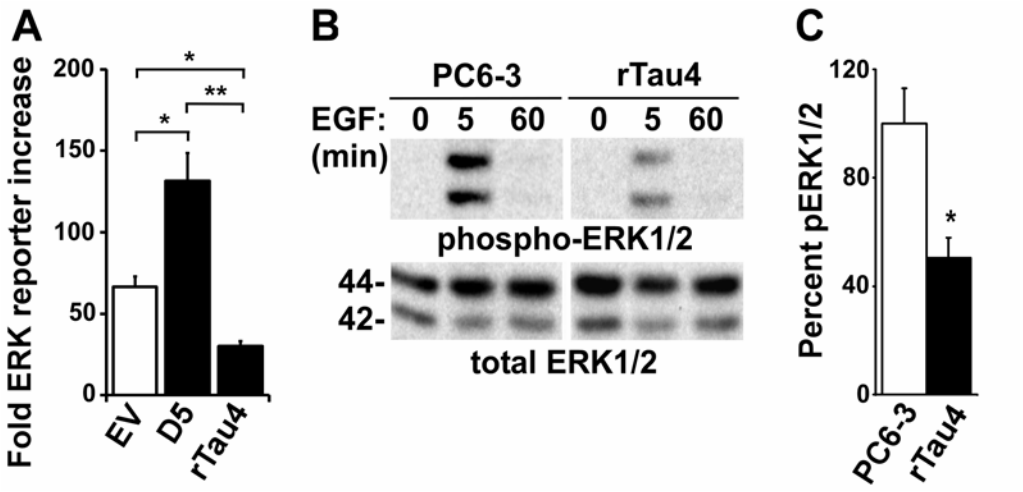

Figure 2.10 Tau potentiates EGF-induced ERK signaling. (A) EV cells, tau overexpressing D5 cells, or tau depleted rTau4 cells were transfected with ERK luciferase reporter plasmids for 36 hours followed by differentiation with 25 ng/ml EGF for 3 hours prior to harvest. Fold reporter increase was calculated relative to minus NGF (naïve) control as described in Materials and Methods (*$p<0.05$, **$p<0.005$). **(B)** Cell lysates from PC6-3 and rTau4 were harvested after treatment with EGF (25 ng/ml) for the indicated time points and probed for activated ERK1/2, using anti-phospho-ERK1/2 (phospho-ERK1/2), and total ERK1/2. The blot shown is representative of three experiments. 1 μg of lysate was loaded in each lane. **(B)** The ratio of phospho-ERK/total ERK for each condition was calculated and shown relative to the highest value obtained (PC 6-3, 5min), which was assigned 100% (* indicates $p<0.05$ relative to PC6-3).

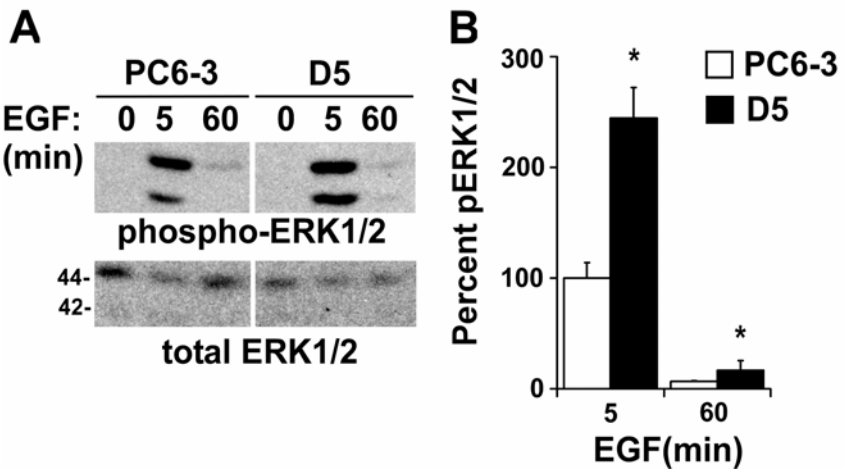

Figure 2.11 Tau overexpression enhances EGF-induced ERK1/2 phosphorylation but does not prolong ERK activation. (A) Cell lysates from PC6-3 and D5 were harvested after treatment with EGF (25 ng/ml) for the indicated time points and probed for activated ERK1/2, using anti-phospho-ERK1/2 (phospho-ERK1/2), and total ERK1/2. The blot shown is representative of three experiments. 1 μg of lysate was loaded in each lane. **(B)** The ratio of phospho-ERK/total ERK for each condition was calculated and shown relative to the highest control value obtained (PC 6-3, 5min), which was assigned 100% (* indicates $p<0.05$ relative to PC6-3).

CHAPTER III

TAU PHOSPHORYLATION MODULATES THE EFFECTS OF TAU ON ERK SIGNALING

Abstract

During early brain development, tau is highly phosphorylated and possesses a weaker affinity for microtubules compared to tau from adult brain. The phosphorylation state of tau is known to regulate the interaction between tau and microtubules. However, it is unclear if the novel interactions between tau and the ERK signaling pathway presented in Chapter II are also regulated by tau phosphorylation, or if an interaction between tau and microtubules is necessary for tau to potentiate ERK signaling. Moreover, the molecular mechanisms underlying the effects of tau on ERK signaling remain to be determined. Here, we show that an association between tau and microtubules is not required for tau to potentiate ERK signaling. In addition, we demonstrate that phosphorylation of tau at Thr231 occurs in response to nerve growth factor and is required for tau to potentiate ERK signaling. Our data further suggests that tau is impacting on ERK signaling at a point upstream of the non-receptor tyrosine kinase Src, and the small GTPase Ras. These results enhance our understanding of the multifunctional role of tau in neuronal signaling, and shed new light on the functions of tau in brain development and neurodegenerative disease.

Introduction

Tau phosphorylation and ERK signaling

During early brain development, the microtubule associated protein tau is known to play an important role in axonal elongation and in the development of axonal polarity (Ferreira and Caceres, 1989, Caceres and Kosik, 1990). In spite of these findings, a number of questions still remain regarding the physiological functions of fetal tau at these

early stages of neuronal differentiation. The later requirement for tau in axonal elongation and the development of axonal polarity is preceded by a phase of development in which fetal tau is highly phosphorylated and has a weakened affinity for microtubules (Goedert and Jakes, 1990, Bramblett et al., 1993, Goedert et al., 1993, Watanabe et al., 1993). Moreover, tau is found to be enriched in the growth cone (DiTella et al., 1994, Brandt et al., 1995, Black et al., 1996), a region of dynamic microtubule assembly and disassembly, suggesting a functional role for tau independent of microtubule stabilization. These early phases of brain development also correlate with increased amounts of signal transduction through the ERK signaling pathway. ERK signaling is highly regulated in the brain and is involved in numerous aspects of neuronal development including the proliferation of neuronal precursors, differentiation of precursors after the final round of mitosis, and the modulation of synaptic plasticity in maturing and adult neurons (Fukunaga and Miyamoto, 1998, Meloche and Pouyssegur, 2007). As we have seen in Chapter II, tau is able to potentiate signaling through the ERK pathway in response to growth factors such as NGF and EGF. This novel function of tau might provide a physiological role for the highly phosphorylated fetal tau during brain development, although the effects of tau phosphorylation on ERK signaling are unclear. Hyperphosphorylation of tau is also a hallmark of neurodegenerative tauopathies such as Alzheimer's disease, where again the increase in tau phosphorylation coincides with an abnormal increase in MAPK signaling and attempts by post-mitotic neurons to re-enter the cell cycle (Zhu et al., 2002, Zhu et al., 2004, Yang et al., 2006). Thus, an analysis of the effects of tau phosphorylation on ERK signaling is necessary to further our understanding of the interactions between tau and the MAPK pathway in brain development and disease.

Mechanism of tau interaction with the ERK signaling pathway

The underlying mechanism of the interaction between tau and the ERK signaling pathway also remains to be determined. Our experiments with AP-1 and ERK reporter assays in Chapter II suggested that tau was impacting on signaling upstream of ERK1/2 activation. In these experiments, we observed altered ERK1/2 phosphorylation in response to both EGF and NGF as early as five minutes after growth factor stimulation in our tau depleted cell lines. For tau to affect such rapid ERK1/2 phosphorylation, it is more likely to interact with signaling components upstream of ERK1/2 activation. Moreover, we did not observe tau in the nucleus in any PC6-3 cell lines, regardless of tau expression level, indicating that tau is not directly interacting with nuclear ERK1/2 or AP-1 transcription factors in the nuclear compartment. Previous studies have demonstrated that the amino terminus of tau is able to associate with the plasma membrane (Brandt et al., 1995), and with lipid rafts (Klein et al., 2002, Kawarabayashi et al., 2004, Sui et al., 2006, Hernandez et al., 2009), specialized regions of membrane enriched in signaling complexes (Golub et al., 2004, Guirland and Zheng, 2007). In addition, tau has been shown to associate with a number of proteins involved in growth factor-induced signal transduction including Src family non-receptor tyrosine kinases, the adaptor protein Grb2, the scaffolding protein 14-3-3, the p85α regulatory subunit of phosphitydlinositol-3 kinase (PI3K), phospholipase-Cγ, and the non-receptor tyrosine kinase c-Abl (Jenkins and Johnson, 1998, Lee et al., 1998, Lee et al., 2004, Yuan et al., 2004, Derkinderen et al., 2005, Reynolds et al., 2008, Souter and Lee, 2009). To date, physiological functions for these interactions have not been demonstrated and it is of interest to determine whether tau impacts on ERK activation through associations with one or more of these proteins. Although the precise node in the pathway at which tau is impacting on signaling remains unclear, our data thus far suggests a role for tau upstream of ERK activation. Accordingly, many of the aforementioned signaling proteins reported

to interact with tau are functional intermediates of ERK signaling downstream of receptor activation and upstream of the terminal ERK (Thomas and Brugge, 1997, Yano et al., 2000, Huang and Reichardt, 2003, Brummer et al., 2008).

Here we have further analyzed the effects of tau phosphorylation on ERK signaling in response to NGF and find that an association with microtubules is not required for tau to impact on signaling, nor is tyrosine phosphorylation at Tyr394. In contrast, we find that endogenous and over-expressed tau is phosphorylated at Thr231 in response to NGF and Thr231 phosphorylation is required for tau to potentiate ERK signaling. Our data further suggests that tau is impacting on ERK signaling at a position upstream of Ras activation and upstream of the non-receptor tyrosine kinase Src. These findings identify a novel function for Thr231 phosphorylated tau in ERK signaling that may have broader implications for the role of fetal tau in early brain development and hyperphosphorylated tau in neurodegenerative disease.

Materials and methods

Cell culture

PC6-3 cells (Pittman et al., 1993) were cultured on tissue culture plastic coated with 50 μg/ml Type I collagen (BD Biosciences) using RPMI 1640 media with 10% horse serum and 5% fetal bovine serum. Media for stable cell lines was supplemented with 200 μg/ml G418.

Plasmids

Plasmids expressing mutant tau (T231D, S235D, T231A/S235A, T231A and Y394F, numbered according to the 2N4R human tau isoform, also known as isoform 2 in the NCBI protein database) were synthesized by site-directed mutagenesis (QuikChange Mutagenesis Kit, Stratagene) using the 0N3R (NCBI isoform 4) human tau plasmid, pRc/CMVn123c, as template (Hall et al., 1997). Plasmids expressing the 0N3R human

tau mutants S262D/S356D and T231D/S235D have been previously described (Sharma et al., 2007).

ERK reporter assays

ERK luciferase reporter assays were performed on PC6-3 or rTau4 cells as described in Chapter II, with additional modifications to the protocol as described below.

For experiments with oncogenic Ras (G12V mutant, (Barbacid, 1987)), cells were co-transfected with ERK reporter system plasmids and FLAG-RasV12 (generously provided by Dr. Stefan Strack), and harvested after 36 hours in the absence of growth factors. The amount of FLAG-RasV12 DNA used was determined by preliminary experiments indicating the amount of plasmid required to yield reporter activation levels similar to those present after three hour NGF treatment. To calculate the fold increase in ERK reporter activity, the normalized firefly luciferase reading from the FLAG RasV12 containing condition was divided by the normalized firefly luciferase reading from the control vector containing condition.

For experiments with constitutively active Src, cells were co-transfected with ERK reporter system plasmids as above along with varying amounts of Src1.1 (CA-Src). CA-Src was generated by incorporating the activating mutation Y529F into the wild-type mouse c-Src expression plasmid (pUSEamp plasmid, Upstate). Transfections were performed in triplicate for 36 hours in the absence of growth factors. To calculate the fold increase in ERK reporter activity, the normalized firefly luciferase reading from the CA-Src containing condition was divided by the normalized firefly luciferase reading from the control vector containing condition.

For experiments with the Src family kinase inhibitor, PP2 (Calbiochem), cells were transfected with ERK reporter system plasmids for 36 hours as above, followed by a 45 minute treatment with 10 μM PP2 or DMSO vehicle control. After PP2 treatment,

cells were differentiated with either 50 ng/ml NGF (2.5S, Sigma) or 25 ng/ml EGF (Sigma) for three hours prior to harvest.

Western blotting

To ensure equal tau expression levels for rescue experiments in ERK reporter assays, wild-type human tau (0N3R) and various tau mutants were transfected into PC6-3 cells using Lipofectamine 2000 (Invitrogen) for 36 hours prior to harvest. Cells were grown under identical conditions as in ERK reporter assays and harvested in 2X Laemmli buffer containing 10% β-mercaptoethanol. Samples were resolved in 8% SDS-PAGE gels, transferred to a PVDF membrane (Millipore), and probed with the following antibodies: Tau13 (generously provided by Dr. Lester Binder) for human tau, and GAPDH (Chemicon). Perkin-Elmer Western Lightning Plus-ECL was used for signal detection. Quantification of Tau13 and GAPDH signals was performed by densitometry with National Institutes of Health ImageJ software. For quantification of mutant tau protein expression levels relative to wild-type, the average amount of protein expression was determined from multiple transfections to account for slight variations in protein expression between transfections at a given plasmid concentration.

Immunoprecipitation

PC6-3 cells stably expressing 0N3R human tau (D5) were plated in 150 mm dishes (1 x 150 mm dish per condition) and serum starved overnight prior to growth factor treatment (50 ng/ml NGF, 2.5S, Sigma). Cells were lysed in 1 ml of ice-cold RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS) containing protease and phosphatase inhibitors. After centrifugation at 6,200 g for 20 min. at 4°C, NP-40 was increased to 1.5% and supernatants were precleared with protein G-Sepharose beads for 20 min. The resulting supernatants were then incubated with either 3.5 μg non-specific mouse IgG or CP17 (anti-phospho-T231 tau, generously provided by

Dr. Peter Davies, (Weaver et al., 2000) for 1 hour. Protein G-Sepharose beads were added to the supernatants for an additional 1 hour. Immunoprecipitates were resolved by SDS-PAGE and transferred. Total tau (human and rat) was detected with Tau5-HRP (1:4000).

Flow cytometry

PC6-3 stable cell lines (EV, rTau4, or D5) were fixed and stained for flow cytometry following a previously reported protocol (Krutzik and Nolan, 2003). Briefly, cells were grown to near confluency in 75 cm^2 flasks and serum starved overnight (1% serum) prior to treatment with NGF (50 ng/ml, 2.5S, Sigma) for 5 minutes (naive controls did not receive growth factor). After NGF treatment, adherent cells were fixed by adding 16% paraformaldehyde directly to the culture medium (1.5% paraformaldehyde final concentration) for 10 minutes. Fixed cells were trypsinized, removed from the flask, and pelleted. Pelleted cells were then permeabilized by vigorous resuspension in 3 ml of ice-cold methanol for at least 10 minutes. Cells were washed with staining buffer (PBS with 1% BSA and 0.1% Tween-20) four times prior to labeling with primary antibody. Equivalent amounts of CP17 (anti- phospho-Thr231 tau, generously provided by Dr. Peter Davies, (Weaver et al., 2000)) or non-specific mouse IgG antibodies were diluted in staining buffer (70 µg/ml final concentration) and added to the cells for 1 hour. Cells were then washed twice more with staining buffer and incubated with secondary antibody (anti-mouse IgG Fab fragment coupled to R-phycoerythrin (R-PE), 1:200) for 20 min. After a final two washes in staining buffer, the cells were resuspended in staining buffer to a concentration of approximately 1 x 10^6 cells/ml and samples were analyzed with a Becton Dickinson LSRII cytometer.

For data analysis, a gated population of 10,000 fluorescing cells was collected from each condition. The Cell Quest Pro software package was used to generate a histogram plot representing the fluorescence intensity of each cell in the population. The

geometric mean fluorescence was calculated from each histogram. The fold change in fluorescence intensity was calculated by dividing the geometric mean fluorescence of CP17 labeled samples by that of IgG labeled control samples. Background fluorescence from secondary only (R-PE) controls was first subtracted from all samples within each cell line. Measurements from CP17 labeled samples were obtained in triplicate within each experiment. For statistical analysis, Student's t-tests were carried out on data from three independent experiments and significance values were defined as p values less than 0.05.

Statistical analysis

For ERK luciferase **r**eporter assays, the results for each condition were reported as mean $\pm$ s.e.m. from three independent assays. In addition, each assay used transfections that were performed in triplicate. Statistical significance was determined by analysis of variance (linear mixed model) with the Statistical Analysis System (SAS) software package. Reporter activity from all assays (n=3) was analyzed as the random effect, with each cell line/tau transfection/treatment as the fixed effect. In all figures, the data for each condition is shown as the mean from all assays $\pm$ s.e.m. However, for statistical analysis, the data was log transformed to account for proportional differences between groups. All p values <0.05 calculated from post-hoc Tukey comparisons between groups were considered to be statistically significant.

Results

Association with microtubules is not required for tau to enhance ERK activation

In Chapter II we described the generation of PC6-3 cell lines with stable expression of shRNA designed to deplete endogenous rat tau without affecting the expression of exogenous human tau. With these cell lines, and with cells overexpressing

human tau, we identified a novel function for tau in the potentiation of ERK signaling after growth factor stimulation. The attenuation of ERK signaling in tau depleted rTau4 cells could be rescued by expression of wild-type human tau, demonstrating that tau expression was sufficient to restore signaling. Following these initial rescue experiments, we next wished to identify structural features of tau necessary for the ability of tau to potentiate ERK signaling. Since the role of tau in neuronal development is often thought to involve its ability to bind to microtubules, we asked whether this activity would be necessary for tau to affect ERK signaling by testing the ability of the phosphomimetic tau mutant, S262D/S356D, to restore ERK reporter activity in tau depleted cells. It is well established that phosphorylation at Ser262 and Ser356, located within the microtubule binding repeats region of tau, abolishes the ability of tau to bind microtubules (Biernat et al., 1993, Drewes et al., 1995, Haase et al., 2004, Sharma et al., 2007). We observed that expression of S262D/S356D tau (0N3R isoform) in rTau4 was able to restore NGF-induced ERK reporter activity levels to those obtained using wild-type tau (Figure 3.1A), similar to our previous findings that S262D/S356D tau could restore neurite initiation in tau depleted cells as well as wild-type tau. These findings indicate that microtubule binding is not necessary for tau to affect signaling.

Phosphorylation of tau at Tyr394 is not required for tau to enhance ERK signaling

In addition to phosphorylation at Ser262/Ser356, fetal tau is known to be phosphorylated at Tyr394 during early brain development, as is the hyperphosphorylated adult tau found in Alzheimer's disease (Derkinderen et al., 2005). Tyr394 phosphorylation in fetal and AD brain was identified by mass spectroscopy, and the non-receptor tyrosine kinase c-Abl was further identified as the responsible kinase (Derkinderen et al., 2005, Lebouvier et al., 2009). Moreover, activated c-Abl is recruited to signaling complexes in response to growth factors such as NGF and EGF (Yano et al.,

2000, Tanos and Pendergast, 2006). To determine if the phosphorylation of 0N3R tau at Tyr394 was required for tau to potentiate ERK signaling, we attempted to rescue ERK reporter activity in rTau4 cells with the tau mutant Y394F. Y394F (0N3R isoform) was able to restore ERK signaling similar to wild-type tau (Figure 3.2A) indicating that phosphorylation at Tyr394 was not required for tau to enhance ERK signaling.

Phosphorylation of tau at Thr231 is required for tau to enhance ERK signaling

Phosphorylation of tau at the Thr/Pro and Ser/Pro motifs found at Thr231 and Ser235 (numbered according to 2N4R tau isoform) has also been shown to reduce its affinity for microtubules (Sengupta et al., 1998, Cho and Johnson, 2004), in addition to reducing its interactions with the SH3 domains of Src family tyrosine kinases (Zamora-Leon et al., 2001, Bhaskar et al., 2005, Reynolds et al., 2008). To test the effects of phosphorylation at these sites on ERK activity, we again performed rescue experiments with rTau4 cells expressing T231D/S235D mutant tau (0N3R isoform). We found that similar to S262D/S356D mutant tau, T231D/S235D mutant tau was not impaired in the ability to potentiate ERK activation, and surprisingly, the level of reporter activity in T231D/S235D expressing cells was significantly higher (1.7 fold) relative to the level obtained with wild-type tau (Figure 3.3A). This suggested that tau phosphorylation at T231/S235 might be involved in the interaction of tau with the ERK pathway and further confirmed that microtubule interactions were not required. We subsequently tested tau mutants with single amino acid replacements at either Thr231 or Ser235 (0N3R isoform) and found that the T231D mutation was sufficient to reproduce the effects seen with the double mutant T231D/S235D, while the S235D mutant resembled wild type tau in its ability to restore ERK reporter activity (Figure 3.4A). To corroborate these findings, we tested both T231A/S235A and T231A mutants (0N3R isoform) and found that neither restored NGF induced ERK reporter activity (Figure 3.5A). In fact, these mutants

affected ERK signaling in a dominant negative manner and impaired ERK signaling relative to rTau4, suggesting that phosphorylation of tau at Thr231 is required for the interaction with the ERK pathway.

To investigate the phosphorylation of Thr231 in response to NGF, we carried out immunoprecipitations in D5 cells using CP17, a monoclonal antibody specific for phospho-Thr231 tau (Weaver et al., 2000). Cells were treated with or without NGF for 5 minutes and CP17 immunoprecipitates were probed with the total tau antibody, Tau5. We detected human tau at short exposures (Figure 3.6, middle panel) as well as endogenous rat tau, at both high and low molecular weights, after longer exposures (Figure 3.6, right panel). The appearance of CP17-positive tau was consistent with a requirement for Thr231 phosphorylated tau in the context of ERK activation. Interestingly, solubilization of the cells for the immunoprecipitation required the use of a stringent buffer (RIPA buffer containing 1% NP40, 0.5% DOC, and 0.1% SDS) to recover all of the tau; solubilization with 1% Triton X-100 resulted in much of the NGF-induced CP17-reactive tau pelleting before the immunoprecipitation (data not shown). This may indicate that Thr231-phosphorylated tau is associated with a Triton-insoluble fraction such as the actin cytoskeleton after NGF exposure. Alternatively, Thr231-phosphorylated tau may be associated with Triton-insoluble lipid rafts, as has been previously reported for tau phosphorylated at Ser396/Ser404 (Klein et al., 2002, Kawarabayashi et al., 2004, Sui et al., 2006, Hernandez et al., 2009). These data suggested a novel function for Thr231-phosphorylated tau in response to NGF.

We further confirmed the phosphorylation of tau at Thr231 in response to NGF by flow cytometric analysis of fixed cells labeled with the CP17 antibody. We compared the mean CP17 fluorescence intensity in EV control cells, rTau4, and D5 with or without NGF stimulation for 5 minutes, and as expected, rTau4 cells displayed a minimal amount of non-specific fluorescence both before and after NGF treatment (Figure 3.7). In contrast, CP17 fluorescence in EV and D5 cells was increased in undifferentiated cells

and after stimulation with NGF (Figure 3.7). The increase in fluorescence intensity after NGF stimulation within EV and D5 cell lines was significant when compared to undifferentiated cells, indicating an increase in Thr231 phosphorylation in accordance with our CP17 immunoprecipitation experiments above. Moreover, the overall CP17 reactivity in D5 cells was increased relative to EV in both naïve cells and NGF-stimulated cells, confirming the phosphorylation of exogenously expressed human tau (Figure 3.7). Together with our immunoprecipitation data, the data suggests a novel function for tau phosphorylation at Thr231 in response to NGF.

Tau is likely to impact on ERK signaling upstream of Src and Ras

While we have established a role for tau phosphorylation in modulating the ability of tau to enhance ERK signaling, the nature of the interactions between tau and the ERK pathway remain unclear. Our immunoprecipitation data suggested that phospho-Thr231 tau might be localized to lipid rafts upon growth factor stimulation (Figure 3.6), possibly interacting with signaling components found in lipid rafts. As tau has been previously reported to interact with other lipid raft signaling components including Src family tyrosine kinases (Lee et al., 1998, Klein et al., 2002, Sui et al., 2006), we hypothesize that tau is impacting on ERK signaling through signaling complexes near the plasma membrane. Interestingly, phosphorylation at Thr231 has been shown to reduce the interaction between tau and Src (Zamora-Leon et al., 2001, Bhaskar et al., 2005), leading us to question whether tau was in fact impacting on ERK signaling through Src. Src is known to be involved in both EGF and NGF-induced signaling (Sato et al., 1995a, b, Thomas and Brugge, 1997, Obara et al., 2004); therefore, we chose to investigate the effects of tau on ERK signaling in the absence of Src activity. ERK reporter activity was measured in PC6-3 cells pretreated with the Src family tyrosine kinase inhibitor PP2 for 45 minutes prior to growth factor stimulation. PP2 treatment inhibited both NGF-

stimulated (Figure 3.8A) and EGF-stimulated (Figure 3.8B) reporter activity, regardless of tau overexpression, indicating that SFK activity is required for ERK activation in response to these growth factors. In light of this absolute requirement for Src activity leading to ERK activation, we were unable to determine whether tau impacts on ERK signaling independent of an association with Src.

Although the possibility of an association between tau and Src in the context of growth factor signaling in PC6-3 cells remains unclear, we have previously shown that tau is able to enhance the activation of Src in PDGF-stimulated 3T3 fibroblasts (Sharma et al., 2007). We therefore investigated whether tau impacted on ERK signaling upstream or downstream of Src activation in our cell lines. Control and rTau4 cells were transfected with a constitutively active Src mutant (CA-Src, Y529F activating mutation), along with ERK reporter plasmids in the absence of growth factor stimulation. Expression of CA-Src at varying levels led to a robust increase in ERK reporter activity in the absence of NGF (Figure 3.9A). Similarly, expression of CA-Src in rTau4 cells yielded equivalent levels of ERK reporter activity when compared to control cells (Figure 3.9A). Moreover, when we expressed a constitutively active Ras protein (CA-Ras, G12V mutant) at varying levels in control and rTau4 cell lines we found a similar level of ERK reporter activation in both cell lines, again indicating that tau is likely to impact on signaling upstream of Ras (Figure 3.9B). Since the impaired ERK activity in tau depleted rTau4 cells can be restored to control levels with these constitutively active signaling pathway intermediates, the data supports a role for tau upstream of Src and Ras activation.

In an effort to identify proteins interacting with tau during the activation of ERK signaling, we performed co-immunoprecipitation experiments with several candidate proteins known to associate with tau. The first of these, the adaptor protein Grb2, acts by bridging the gap between the intracellular tyrosine kinase tails of the TrkA or EGFR receptors and membrane-bound Ras. Grb2 contains one SH2 domain which binds

phosphorylated tyrosine residues on the activated receptor or other tyrosine phosphorylated adaptor proteins such as Shc (Huang and Reichardt, 2003). The protein also contains two SH3 domains for binding PXXP motifs on partner proteins, such as the guanine nucleotide exchange factor SOS (Simon and Schreiber, 1995, Huang and Reichardt, 2003). The N-terminal SH3 domain of Grb2 has also been reported to associate with tau in co-sedimentation assays between recombinant 2N4R tau and GST-N-SH3 Grb2 proteins (Reynolds et al., 2008), although a cellular interaction between tau and Grb2 has not been established. Accordingly, we attempted to co-immunoprecipitate tau and Grb2 in tau overexpressing D5 cells, both before and after stimulation with NGF using experimental conditions (1% Triton lysis buffer) previously reported to yield satisfactory Grb2 co-immunoprecipitations (Roccato et al., 2002). Under these conditions, we did not observe an interaction between tau and Grb2 in undifferentiated cells or after NGF stimulation (data not shown). While these results suggest that an association between tau and Grb2 is not required for NGF-induced ERK signaling, additional lysis conditions need to be tested to confirm this finding.

In an attempt to identify functional interactions between tyrosine phosphorylated tau and proteins with phospho-tyrosine binding domains, our laboratory has previously demonstrated an interaction between tau and an SH2 domain from the tyrosine phosphatase SHP2 in vitro (unpublished data). Moreover, overexpressed tau was found to co-immunoprecipitate with both a catalytically inactive "substrate trapping" SHP2 mutant and wild-type SHP2 from transfected COS7 cells (unpublished data); although a cellular interaction between endogenous proteins has not been established. Since SHP2 also positively regulates EGF- and NGF-stimulated ERK signaling upstream of Ras activation (Neel et al., 2003, D'Alessio et al., 2007, Dance et al., 2008), we asked whether a physiological interaction between tau and SHP2 might be occurring in NGF-stimulated D5 cells. Naïve and NGF-stimulated D5 cells were lysed under conditions identical to those previously used to demonstrate a co-immunoprecipitation between overexpressed

tau and wild-type SHP2 in COS7 cells (2% NP-40). However, we were unable to identify an interaction between tau and endogenous SHP2 in D5 cells under these conditions. While these results suggest that tau does not associate with SHP2 during NGF-induced ERK signaling, additional lysis conditions need to be tested to confirm this finding.

Discussion

Tau phosphorylation and ERK signaling

In this chapter, we investigated the effects of tau phosphorylation on growth factor-induced ERK signaling. We found that phosphorylation-mimicking mutations at any of four residues, Thr231, Ser235, Ser262, and Ser356, does not impair the ability of tau to restore ERK signaling in NGF-stimulated cell lines with tau depletion (Figure 3.1, 3.3, 3.4). In fact, phosphorylation at Thr231 is required for tau to potentiate ERK signaling (Figure 3.4, 3.5). All of these residues are known to be phosphorylated in fetal brain (Yu et al., 2009), and Ser262/Ser356 phosphorylation, in particular, significantly reduces the ability of tau to bind microtubules (Biernat et al., 1993, Drewes et al., 1995). Similarly, phosphorylation at Thr231/Ser235 impairs the association between tau and microtubules (Sengupta et al., 1998, Cho and Johnson, 2004). Our findings therefore support the idea that non-microtubule associated pools of tau in the fetal brain (i.e. cytosolic and/or membrane-associated) possess the novel ability to potentiate ERK activation. Moreover, we have seen that the effects of tau on ERK signaling are occurring in undifferentiated cells where tau is expressed at low levels and microtubule mass is low. Together, the data suggests that tau may impact on ERK signaling through a mechanism that does not require an association with microtubules. However, the possibility still remains that tau that is associated with microtubules is also capable of potentiating ERK signal transduction, and further experiments will be needed to investigate this.

Our rescue experiments performed with the tau mutant Y394F addressed the possible role of the non-receptor tyrosine kinase c-Abl in the tau-mediated potentiation of ERK signaling. c-Abl activity is increased in response to growth factors such as NGF and EGF (Yano et al., 2000, Tanos and Pendergast, 2006), and since Tyr394 of tau is a known substrate for c-Abl in the developing brain (Derkinderen et al., 2005), we questioned whether Tyr394 phosphorylation was necessary for tau to enhance ERK signaling. However, we did not observe decreased levels of ERK reporter activity in rTau4 cells rescued with Y394F tau as compared to wild-type tau, indicating that Tyr394 phosphorylation was not required for tau to potentiate ERK signaling. In spite of these findings, we cannot definitively say that tau is not being phosphorylated at Tyr394 in response to NGF, only that Tyr394 phosphorylation is not necessary.

Using additional tau mutants, we demonstrated that phosphorylation of tau at Thr231 is necessary for its effect on ERK reporter activity (Figure 3.3-3.5) and that the phosphorylation of exogenously expressed tau at Thr231 was increased in response to stimulation with NGF for 5 minutes (Figure 3.6 and 3.7). Phosphorylation of both high and low molecular weight endogenous tau was also increased. The phosphorylation of endogenous high molecular weight (HMW) tau is of particular interest since this isoform, containing the alternatively spliced exon 4a, is preferentially expressed in the peripheral nervous system (Georgieff et al., 1991, Oblinger et al., 1991) and very little is known about the function of this form of tau. Our finding that HMW tau is also phosphorylated at Thr231 in response to NGF indicates that this larger isoform is similarly involved in ERK signaling. However, it remains unclear how the additional sequence contributed by exon 4a affects the ability of tau to potentiate ERK signaling. Since HMW tau is not expressed in the brain, there may be as yet unidentified functions for this large isoform in other tissue types outside the central nervous system. HMW Tau is expressed in a number of other tissues including muscle, testes, and prostate epithelial cells (Gu et al., 1996, Souter and Lee, 2009), and thus our finding of a functional phosphorylation for

HMW tau opens up new avenues for further research into the nature of tau function in these cell types.

While our study does not identify the kinase responsible for phosphorylation at Thr231, Thr231 is a known substrate for several kinases including GSK3β (Ishiguro et al., 1992, Song and Yang, 1995, Cho and Johnson, 2004) and members of the MAPK family such as ERK, JNK, and p38 (Roder et al., 1993, Blanchard et al., 1994, Reynolds et al., 1997a, Reynolds et al., 1997b). Although GSK3β is a major tau kinase capable of phosphorylating tau at multiple sites, it also requires a "priming" phosphorylation event by another kinase such as cyclin-dependent kinase 5 (Cdk5). GSK3β-mediated phosphorylation of tau at Thr231 therefore requires a priming phosphorylation at Ser235 by Cdk5 (Goedert et al., 1994, Cho and Johnson, 2004). Since we have shown that Ser235 phosphorylation is not required for tau to impact on ERK signaling (Figure 3.4), our data suggests that GSK3β kinase activity is not required for phosphorylation at Thr231 in response to NGF. Moreover, GSK3β is typically inactivated in response to NGF or EGF signaling (Kleijn et al., 1998), and thus, the phosphorylation of tau at Thr231 in the context of growth factor signaling is likely to be mediated by ERK family members, leading to a positive feedback mechanism that would enhance signaling. This type of feedback phosphorylation is typical for ERK1/2, as it is known to regulate multiple upstream nodes in the ERK pathway through feedback phosphorylation (Yoon and Seger, 2006).

Phosphorylation of tau at Thr231 is one of the earliest phospho-epitopes to appear in Alzheimer's disease (Hasegawa et al., 1992, Vincent et al., 1998, Augustinack et al., 2002, Luna-Munoz et al., 2007) and one of several tau phosphorylation sites thought to "recapitulate" early brain development during neuropathogenesis (Kanemaru et al., 1992, Bramblett et al., 1993, Jicha et al., 1997). Interestingly, the levels of AP-1 transcription factors and multiple active components of the ERK signaling pathway (i.e. phospho-MEK1/2 and phospho-ERK1/2) are reported to be elevated in tangle positive neurons of

AD brain (Anderson et al., 1994, Marcus et al., 1998, Pei et al., 2002). This abnormal activation of MAPK signaling in AD has been linked to numerous factors including the accumulation of Aβ peptide, oxidative stress, or responses to inflammation (Zhu et al., 2002, Zhu et al., 2007). Our current findings might provide a correlation between dysfunctional MAPK signaling and the onset of tau hyperphosphorylation, in particular, phosphorylation of Thr231 in the earliest stages of neurodegeneration. Moreover, as we have seen that Thr231 phosphorylated tau potentiates ERK signaling, the abnormal tau phosphorylation in AD may exacerbate MAPK signaling, contributing to the aberrant cell cycle events also known to occur in AD brain (Yang et al., 2001, Yang et al., 2003, Yang et al., 2006, Varvel et al., 2008). Further experiments are needed to elaborate on these possibilities, in particular to determine whether ERK activation in response to Aβ and other upstream AD contributing factors also involves tau. Previous studies support the idea of an interaction between tau and ERK signaling in disease, such as a report that the Aβ-induced activation of ERK signaling and resulting neurotoxicity in cultured neurons could be prevented by tau depletion (Rapoport et al., 2002). In addition, in human neuroblastoma cells, the presence of P301L mutant tau altered the transcription patterns of AP-1 regulated genes (Hoerndli et al., 2007) and we speculate that this may be due to the effect of tau on AP-1 activation. Lastly, a requirement for the amino terminus of tau in Aβ-mediated signaling has been reported (King et al., 2006), consistent with our finding that the microtubule binding activity of tau is not required for its role in NGF activated signal transduction.

Although we have established a requirement for Thr231 phosphorylated tau in ERK signaling, the mechanism through which this phosphorylation impacts on signaling remains to be elucidated. Substituting Thr231 with alanine had a dominant negative effect on ERK reporter activity, which might indicate that the T231A tau sequesters a component of ERK signaling. We and others have previously shown that phosphorylation of tau at Thr231, as well as at other sites, decreases the binding of tau to

SH3 domains which commonly mediate protein-protein interactions during signaling (Lee et al., 1998, Zamora-Leon et al., 2001, Bhaskar et al., 2005, Reynolds et al., 2008). Therefore, preventing phosphorylation at Thr231 could alter the regulation of such interactions. Alternatively, phosphorylation at Thr231 might alter the conformation of tau, possibly via its interaction with the prolyl isomerase Pin1 (Lu et al., 1999), and if this conformational change were required to expose an area of tau critical for an interaction during signaling, T231A would exert a dominant negative effect. Tau sequence surrounding Thr231 contains multiple PXXP motifs capable of binding the SH3 domains of signaling proteins and phosphorylation at this site may regulate the association between tau and components of the ERK pathway. Although our results are limited to growth factor-induced ERK signaling in PC6-3 cell lines, we speculate that these findings may have functional implications for the role of tau in early brain development, and might explain the presence of increased levels of Thr231 phosphorylated tau in the fetal brain (Watanabe et al., 1993). Moreover, increased levels of phosphorylated ERK1/2 are found to occur in the fetal brain at the same time as tau phosphorylation at Thr231 (Watanabe et al., 1993, Yu et al., 2009), as would be predicted in light of our present findings. Further experimentation in primary neurons and neuronal stem cells will provide additional insights into the precise role of phosphorylated fetal tau during ERK signaling in the developing brain.

Tau association with ERK signaling upstream of Src and Ras

The ability of CA-Ras and CA-Src to restore ERK activity in tau depleted rTau4 cells suggests that tau might impact on signaling upstream of Ras activation. However, in interpreting the results from these experiments, it is important to note the possibility of signaling artifacts when over-expressing a constitutively active mutant such as CA-Ras or CA-Src. Since these mutants are such robust activators of signaling in the absence of an

upstream growth factor trigger, it is possible that the ERK signaling we observe does not correlate with the physiological activation of Ras or Src. For example, the restoration of ERK signaling in tau depleted cells expressing CA-Ras might mask a requirement for tau downstream of Rap1 signaling. To address this concern, we also tested lower amounts of CA-Ras in order to obtain levels of ERK reporter activation below those obtained with NGF (Figure 3.9B). In these experiments, the reporter activity in tau depleted cells was still restored to control levels, indicating that tau is impacting on signaling at a point upstream of Ras in the signaling cascade. The ability of CA-Src to restore signaling to tau depleted cells further suggests that tau potentiates signaling upstream of GTPase activation, especially since Src is reported to participate in both Ras and Rap1 activation (Ellis et al., 1990, Xing et al., 2000, Kao et al., 2001, Huang and Reichardt, 2003).

There are multiple possibilities for how tau might be interacting with ERK signaling upstream of Ras and Src activation, including associations with signaling protein complexes at the plasma membrane that we have discussed above. Alternatively, tau might be affecting the trafficking of growth factor receptors to the cell surface in a microtubule-dependent manner. Studies performed in vitro and in cells have suggested a role for tau in axonal transport and vesicle trafficking where it has been found that the presence of tau inhibited motor-driven transport along microtubules (Seitz et al., 2002, Stamer et al., 2002, Vershinin et al., 2007, Dixit et al., 2008, Dubey et al., 2008, Vershinin et al., 2008, Stoothoff et al., 2009). In contrast, a study carried out in mice reported that the level of tau expression had no effect on axonal transport (Yuan et al., 2008). Based on these findings, whether tau acts to inhibit transport or has no effect on transport, one still would not predict that tau depletion would inhibit vesicle transport or lead to decreases in growth factor receptors (e.g. TrkA, EGFR) at the cell surface that would attenuate ERK signaling. Therefore, since our experiments showed that depleting tau decreased ERK signaling and adding tau increased signaling, the data did not support the involvement of altered receptor trafficking. In addition, our rescue experiments

(Figure 3.1, 3.3, 3.4) suggested that the mechanism did not involve the ability of tau to associate with microtubules. This also makes it unlikely that a microtubule-dependent mechanism such as vesicle transport is involved. Although receptor trafficking may not be affected by tau expression levels in the cell, the possibility still exists that tau might directly or indirectly interact with both TrkA and EGFR receptors. Such interactions might impact on receptor phosphorylation and/or recruitment of the adaptor proteins required for ERK signaling. Further experiments are necessary to investigate this possibility.

In conclusion, we have shown that tau potentiates signal transduction associated with growth factor stimulation in a manner that does not involve its microtubule binding activity but requires phosphorylation at Thr231. Phosphorylated tau is likely to associate with the lipid raft compartment upon growth factor stimulation, and our experiments with ERK reporter assays suggest that tau is impacting on ERK signaling upstream of Src and Ras activation. These findings shed new light on how tau may be affecting the differentiation and maturation of neurons in the developing brain, as well as the pathogenesis of neurodegenerative diseases such as Alzheimer's disease.

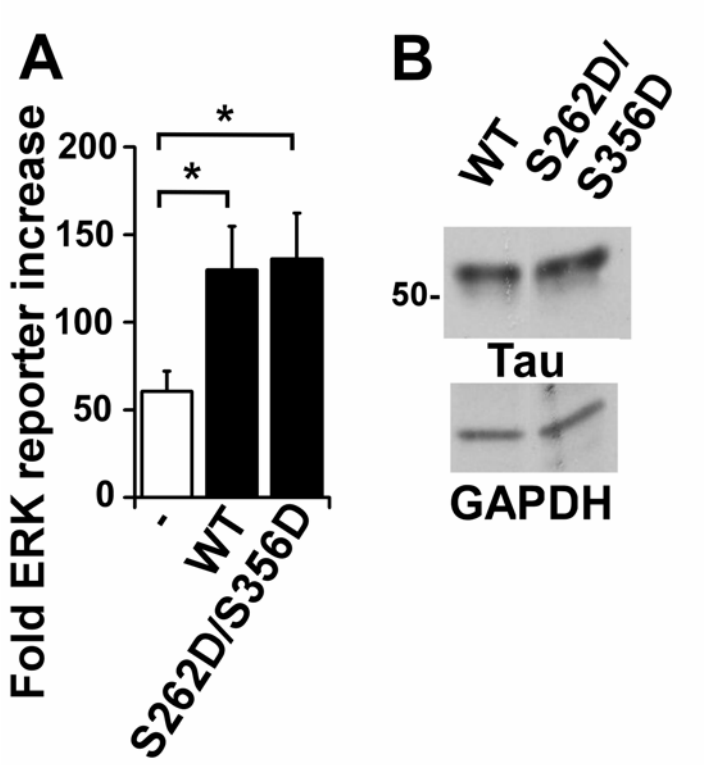

Figure 3.1 Microtubule association is not required for tau to potentiate ERK activation. (A) rTau4 cells were transfected with ERK reporter system plasmids and either 0N3R human tau (WT), or 0N3R S262D/S356D mutant tau (S262D/S356D). rTau4 control (white bar) received control vector DNA. 36 hours after transfection, NGF was added for 3 hours prior to cell harvest. The data shown is the fold increase in luciferase reporter activity as described in Materials and Methods and represents the mean ±s.e.m. from three independent experiments. Transfections were performed in triplicate for each condition in all assays. (*$p<0.05$) **(B)** To assure equal protein expression of wild-type and S262D/S356D mutant tau for luciferase assays shown in (A), lysates from rTau4 cells transfected under identical conditions were probed with the human tau antibody, Tau13. GAPDH levels are shown as a loading control. 1 µg of lysate was loaded in each lane.

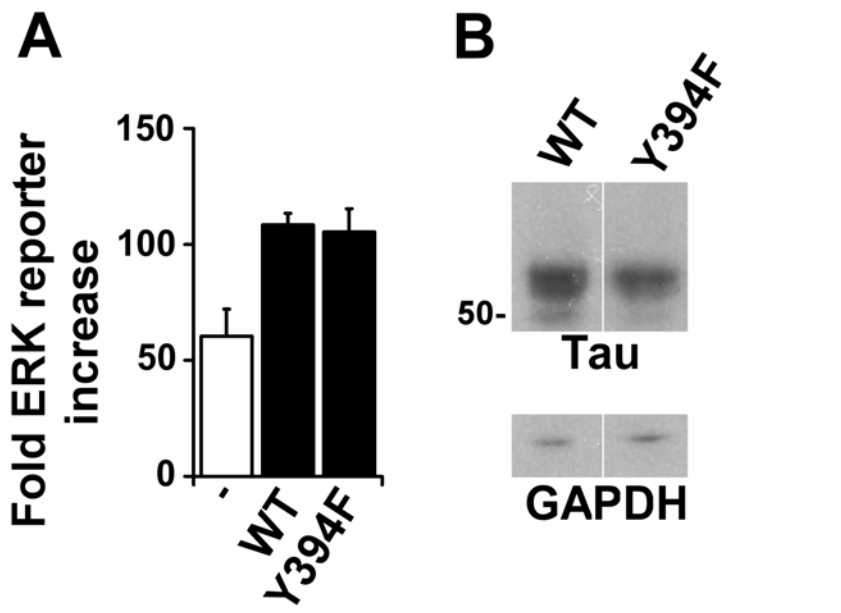

Figure 3.2 Phosphorylation at Tyr394 is not required for tau to enhance ERK activation. (A) rTau4 cells were transfected with ERK reporter system plasmids and either 0N3R human tau (WT) or 0N3R Y394F mutant tau (Y394F). rTau4 control (white bar) received control vector DNA. 36 hours after transfection, NGF was added for 3 hours prior to cell harvest. The data shown is the fold increase in luciferase reporter activity as described in Materials and Methods and represents the mean ± s.e.m. from three independent experiments. Transfections were performed in triplicate for each condition in all assays. **(B)** To assure equal protein expression of wild-type and Y394F mutant tau for luciferase assays shown in (A), lysates from rTau4 cells transfected under identical conditions were probed with the human tau antibody, Tau13. GAPDH levels are shown as a loading control. 1 μg of lysate was loaded in each lane.

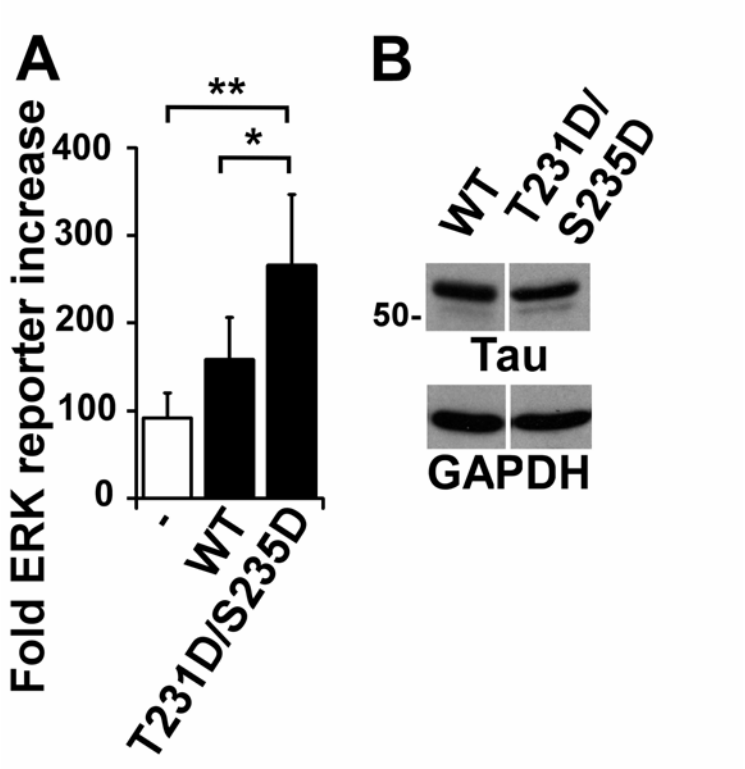

Figure 3.3 Pseudo-phosphorylation enhances the ability of tau to potentiate ERK activation. (A) rTau4 cells were transfected with ERK reporter system plasmids and either 0N3R human tau (WT), or 0N3R T231D/S235D mutant tau (T231D/S235D). rTau4 control (white bar) received control vector DNA. 36 hours after transfection, NGF was added for 3 hours prior to cell harvest. The data shown is the fold increase in luciferase reporter activity as described in Materials and Methods and represents the mean ±s.e.m. from three independent experiments. Transfections were performed in triplicate for each condition in all assays. (*$p<0.05$, **$p<0.005$) **(B)** To assure equal protein expression of wild-type and T231D/S235D mutant tau for luciferase assays shown in (A), lysates from rTau4 cells transfected under identical conditions were probed with the human tau antibody, Tau13. GAPDH levels are shown as a loading control. 1 μg of lysate was loaded in each lane.

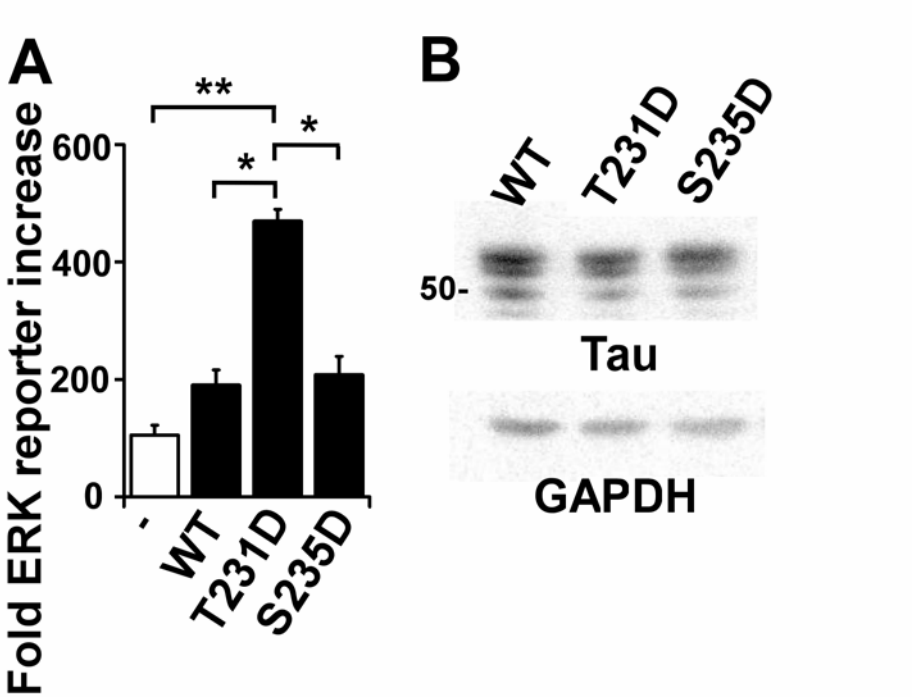

Figure 3.4 Pseudo-phosphorylation at Thr231 enhances the ability of tau to potentiate ERK activation. (A) rTau4 cells were transfected with ERK reporter system plasmids and either 0N3R human tau (WT), 0N3R T231D (T231D), or 0N3R S235D mutant tau (S235D). rTau4 control (white bar) received control vector DNA. 36 hours after transfection, NGF was added for 3 hours prior to cell harvest. The data shown is the fold increase in luciferase reporter activity as described in Materials and Methods and represents the mean ±s.e.m. from three independent experiments. Transfections were performed in triplicate for each condition in all assays. (*$p<0.05$, **$p<0.005$) **(B)** To assure equal protein expression of wild-type, T231D, and S235D mutant tau for luciferase assays shown in (A), lysates from rTau4 cells transfected under identical conditions were probed with the human tau antibody, Tau13. GAPDH levels are shown as a loading control. 1 µg of lysate was loaded in each lane.

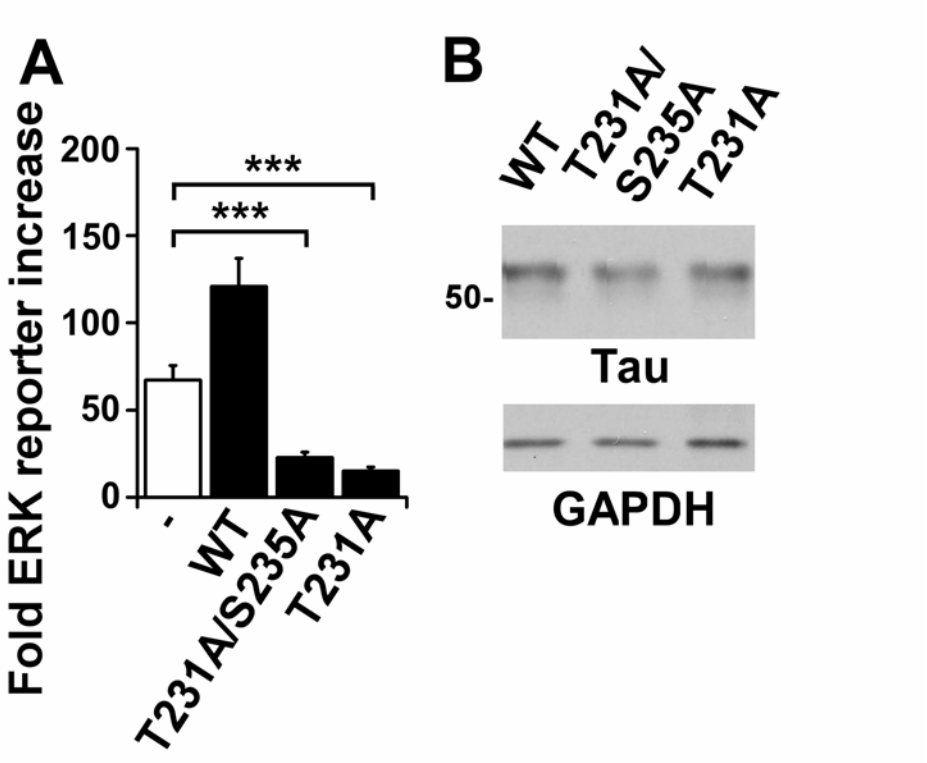

Figure 3.5 Phosphorylation at Thr231 is required for tau to potentiate ERK activation in response to NGF. (A) rTau4 cells were transfected with ERK reporter system plasmids and either 0N3R human tau (WT), 0N3R T231A/S235A (T231A/S235A), or 0N3R T231A mutant tau (T231A). rTau4 control (white bar) received control vector DNA. 36 hours after transfection, NGF was added for 3 hours prior to cell harvest. The data shown is the fold increase in luciferase reporter activity as described in Materials and Methods and represents the mean ±s.e.m. from three independent experiments. Transfections were performed in triplicate for each condition in all assays. (***$p<0.001$) **(B)** To assure equal protein expression of wild-type, T231A/S235A, and T231A mutant tau for luciferase assays shown in (A), lysates from rTau4 cells transfected under identical conditions were probed with the human tau antibody, Tau13. GAPDH levels are shown as a loading control. 1 µg of lysate was loaded in each lane.

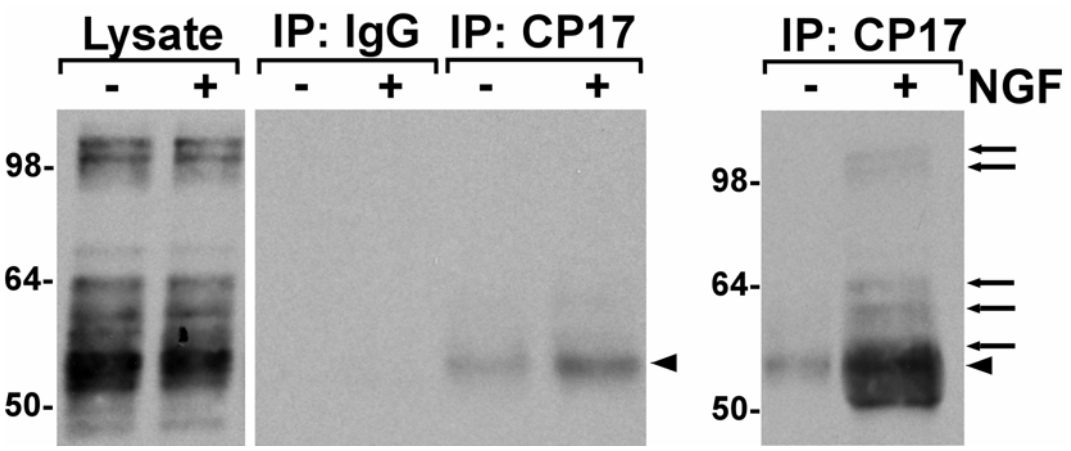

Figure 3.6 Tau is phosphorylated at Thr231 in response to NGF. Serum-starved D5 cells were stimulated with 50 ng/ml NGF for five minutes. After NGF exposure, cells were lysed in RIPA buffer containing 1% NP40, 0.5% DOC and 0.1% SDS. Tau was immuno-precipitated using the anti-phospho-Thr231 tau antibody, CP17, and analyzed by probing with HRP-coupled total tau antibody, Tau5. Input lysate lanes (left panel) were 1% of the total lysate and showed equal loading of protein. The middle and right panels reflect shorter and longer exposures, respectively. The data shown is representative of three independent experiments. Arrows indicate endogenous rat tau and arrowheads indicate human tau.

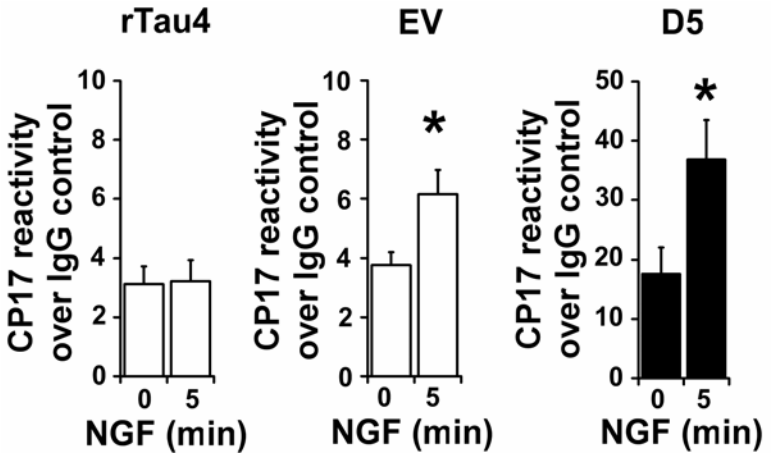

Figure 3.7 NGF induces tau phosphorylation at Thr231 in control and tau over-expressing cell lines. Tau phosphorylation at threonine 231 was analyzed by flow cytometry with the CP17 (anti-phospho-Thr231 tau) antibody. NGF treated (5 minutes, 50 ng/ml) or naïve cells from EV, rTau4, and D5 cell lines were fixed with paraformaldehyde and ice cold methanol as described in Materials and Methods, followed by labeling with CP17 primary and anti-mouse IgG secondary (conjugated to R-phycoerythrin) antibodies. The data shown represents the geometric mean fluorescence intensity of CP17 labeled cells divided by the geometric mean fluorescence intensity of cells labeled with non-specific IgG (background fluorescence from secondary-only controls was first subtracted from both CP17 and IgG samples). The data shown is the mean ± s.e.m. from three independent experiments. (*$p<0.05$)

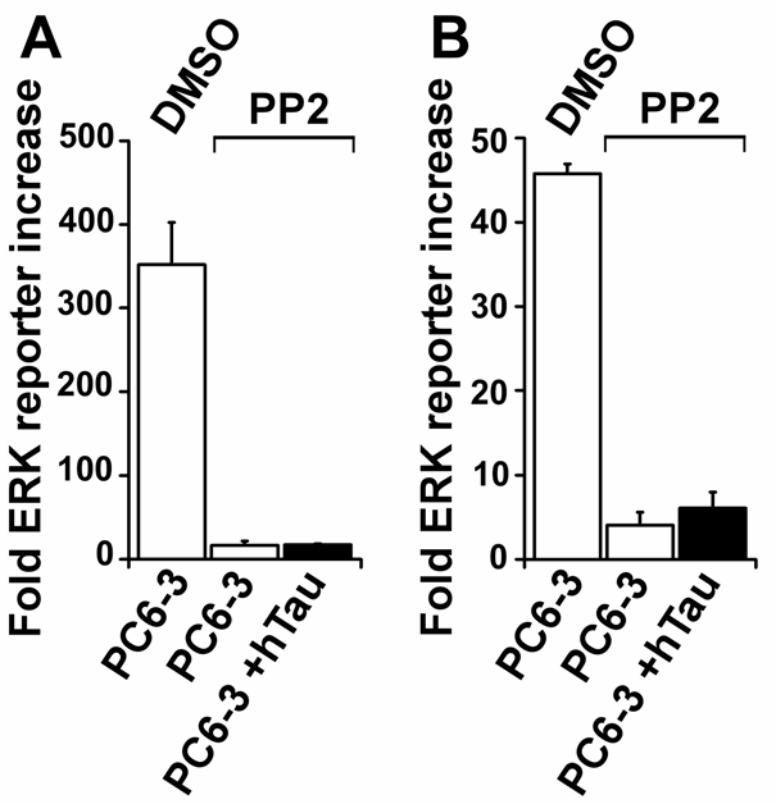

Figure 3.8 Src activity is required for tau to potentiate ERK activation in response to NGF or EGF. (A) PC6-3 cells were transfected with ERK reporter system plasmids and 0N3R human tau (+hTau, black bars) or control vector DNA (white bars) for 36 hours prior to treatment with the Src family kinase inhibitor, PP2. PP2 (10 μM) or DMSO vehicle control was added for 45 minutes, followed by NGF treatment (50 ng/ml) for three hours prior to cell harvest. The data shown is the fold increase in luciferase reporter activity as described in Materials and Methods and represents the mean ± s.e.m. from three independent experiments. Transfections were performed in triplicate for each condition in all assays. **(B)** PC6-3 were transfected with ERK reporter system plasmids as in (A) followed by PP2 treatment for 45 minutes prior to EGF treatment (25 ng/ml) for three hours.

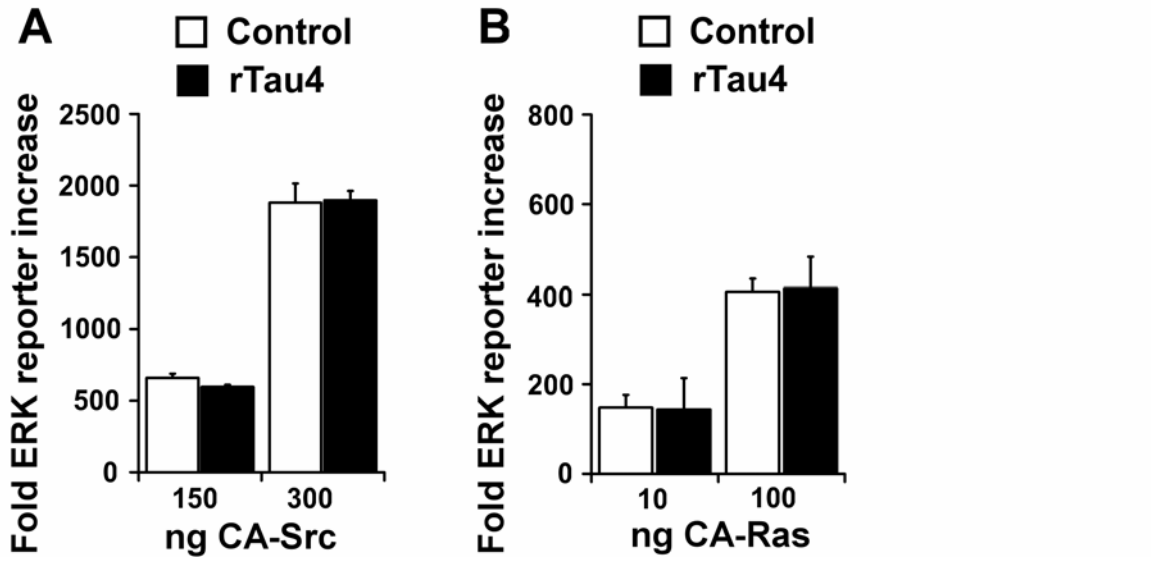

Figure 3.9 Tau is likely to impact on ERK signaling upstream of Ras and Src activation. (A) PC6-3 control and rTau4 cells were transfected with ERK reporter plasmids and constitutively active Src as described in Materials and Methods. 36 hours after transfection, cells were harvested in the absence of growth factor and luciferase activity was measured from cell lysates. The data shown represents the fold increase in reporter activity between CA-Src expressing cells and cells expressing CMV control plasmid. ERK signaling is restored to control levels in tau depleted rTau4 cells. **(B)** PC6-3 control and rTau4 cells were transfected with ERK reporter plasmids and constitutively active Ras as in (A). The data represents the fold increase in reporter activity between cells expressing CA-Ras and cells expressing CMV control plasmid. ERK signaling is again restored to control levels in tau depleted rTau4 cells. The data shown in (A) and (B) is the mean ± s.e.m. and is representative of n=3 assays.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation work has demonstrated a novel function for microtubule associated protein tau during growth factor-induced activation of the ERK signaling pathway. In Chapter II we showed that tau is able to potentiate ERK activation in response to NGF and EGF in PC12 cells, and in Chapter III we found that tau phosphorylation at Thr231 is required for tau to affect ERK signaling, and that tau most likely interacts with the signaling pathway upstream of GTPase activation. These findings raise a number of questions regarding the mechanism behind tau's association with ERK signaling and about the precise role of tau phosphorylation in ERK activation. Here we will describe additional experiments to probe these issues and further discuss the implications of tau function on MAPK signaling in neurodegenerative disease.

Mechanism of tau interaction with the ERK signaling pathway

Our current findings indicate that tau is interacting with the ERK signaling pathway at a position upstream of Ras and Src. Therefore, the immediate focus of our future experimentation will be to further identify the nature of tau's interaction with this part of the pathway. As we have described in Chapter III, there are a number of candidate proteins involved in ERK signaling that are also reported to associate with tau in vitro. Although we did not find an association between tau and the adaptor protein Grb2 or the protein tyrosine phosphatase SHP-2 in our initial experiments, the possibility still exists that tau does in fact interact with these proteins in the context of ERK signaling. Additional co-immunoprecipitation experiments will be performed after an optimization of antibody and lysis conditions to identify a possible functional interaction between tau and these proteins. However, rather than focusing all of our efforts entirely

on these several candidate proteins, we also plan to utilize additional techniques to identify tau interactors. The first technique will use mass spectroscopy to identify co-immunoprecipitating proteins from naïve and NGF-stimulated D5 cells after immunoprecipitation with a total tau antibody. Co-immunoprecipitation products will first be identified by silver staining, and then excised gel pieces will be analyzed by mass spectroscopy to identify putative binding partners of tau. Alternatively, total tau co-immunoprecipitations can be carried out from D5 cells metabolically labeled with ^{35}S-methionine. The resulting autoradiographs from these experiments will be able to identify the molecular weight of tau binding partners in NGF-stimulated cells. In addition, the isoelectric point (IEP) of ^{35}S-labeled binding partners can be determined by two-dimensional gel-electrophoresis as a further aid to identification.

Post-translational modifications of tau induced by NGF will also be investigated by mass spectroscopy. In addition to hyperphosphorylation, PHF tau in AD brain is known to be extensively modified, including such modifications as glycosylation, ubiquitination, glycation, polyamination, and nitration (reviewed in (Gong et al., 2005)). Although some of these changes have only been observed in diseased brain, others are found to occur under normal physiological conditions and may impact on normal tau function. For example, nitration of tau at Tyr197 has been demonstrated in NGF-stimulated PC12 cells with a peak occurrence at five days after NGF treatment and in normal mouse brain at six weeks of age (Nonnis et al., 2008). To further investigate the nature of tau post-translational modification in response to NGF, we will immunoprecipitate total tau (human and rat) from naïve and NGF-stimulated D5 cells at various time points. After silver staining to visualize immunoprecipitation products, excised gel pieces containing various tau species will be analyzed by mass spectroscopy to identify modifications that may be involved in NGF signaling. It will also be of interest to determine whether there are isoform specific differences in NGF-induced

modifications that may indicate unique functional roles in signaling (i.e. HMW tau compared to LMW tau).

In addition to potential interactions between tau and adaptor proteins/kinases involved in ERK signaling, tau might also directly or indirectly interact with TrkA and EGFR receptors. These interactions might impact on receptor autophosphorylation (activation), surface expression, or docking with associated signaling proteins. We have performed preliminary experiments to analyze cell surface expression of TrkA in control and tau depleted cells by flow cytometry with an antibody specific for the extracellular domain of TrkA. The results from these pilot studies suggest that there is no difference in surface expression of TrkA, indicating that tau does not affect surface expression of the receptor. These experiments can be further expanded by carrying out surface biotinylation experiments to further compare the levels of biotinylated receptors (including TrkA, EGFR, and the p75 low affinity neurotrophin receptor) present on the cell surface in control and tau depleted cell lines. In addition, an analysis of receptor phosphorylation in response to growth factor can be investigated by probing cell lysates from control and tau depleted cell lines with the appropriate commercially available phospho-specific antibodies.

In chapter III, we demonstrated the requirement for Thr231 phosphorylation of tau in the modulation of ERK signaling. The kinase responsible for this phosphorylation has not been identified, although we propose ERK1/2 as the candidate kinases, based on their known ability to phosphorylate Thr231 in vitro (Roder et al., 1993, Blanchard et al., 1994), and on the known NGF-induced downregulation of the major alternative Thr231 kinase, GSK3β (Kleijn et al., 1998). In order to prove this hypothesis, we will perform CP17 immunoprecipitations from D5 cells stimulated with NGF, with and without pretreatment with the MEK inhibitor U0126. A loss of CP17-positive tau signal in response to MEK inhibition will strengthen the case for ERK1/2 mediated tau phosphorylation in response to NGF. To definitively rule out a role for GSK3β in NGF-

induced Thr231 phosphorylation, the same experiments can be performed with an appropriate inhibitor such as lithium or XIII. Moreover, we plan to carry out the same series of CP17 immunoprecipitations after stimulation with EGF, as well as with both growth factors over longer time courses to more fully understand the scope of Thr231 phosphorylation after growth factor stimulation.

Our experiments using the ERK reporter in tau depleted cells is a convenient assay for screening the effects of various phospho-mimicking tau mutants on ERK signaling. We plan to extend these findings by testing additional phospho-mimicking mutants, as well as various structural mutants in the same assay. In addition, our findings thus far have focused on the contributions of fetal 0N3R tau to ERK signaling, and it is possible that the effects of the five additional isoforms of tau expressed in the brain (see Figure 1.1) may differ from the fetal isoform. We will test this possibility, especially since it may have relevance for the role of tau in MAPK signaling in the diseased AD brain (where all isoforms are present). Our first priority will be to continue to test the effects of tau pseudophosphorylation at sites known to be connected with components of the MAPK signaling pathway in some manner. For example, phosphorylation at Tyr18 and/or Tyr29 by the non-receptor tyrosine kinases Src, Fyn, Lck, and Syk has been demonstrated in vitro, in cells, and in the brain ((Williamson et al., 2002, Lee et al., 2004, Lebouvier et al., 2008) and unpublished data). However, the precise function of these phosphorylation events is unclear. Src in particular is necessary for growth factor induced ERK activity as we and others have shown and thus we will further focus on the possible effects of tyrosine phosphorylation on ERK signaling. Another site of interest is Ser214, one of several target sites for PKA and/or Akt (Ksiezak-Reding et al., 2003, Sadik et al., 2009a). Phospho-Ser214 is the preferred binding site for the scaffolding protein 14-3-3, although a second phosphorylation-independent interaction between tau and 14-3-3 has also been reported (Sadik et al., 2009a). Since 14-3-3 is involved in ERK signaling at multiple nodes, including upstream of GTPase activation through interactions

with the docking protein Gab2 (Brummer et al., 2008), we will test the effects of mimicking phosphorylation at Ser214 on ERK activation as well. Lastly, we will investigate the effects of point mutations known to cause inherited frontotemporal dementia, such as P301L, G272V, and V337M, among others (reviewed in (Wolfe, 2009)). The results from these assays will add to our understanding of the interaction between tau and the ERK signaling pathway, as well as shed further light on the function of abnormal tau in disease. The role of tau in abnormal MAPK signaling during the pathogenesis of neurodegenerative disease is discussed further below.

In this dissertation we have focused on the activation of ERK signaling in response to NGF and EGF. However, the ERK signaling pathway can be activated by a wide range of upstream triggers, including other growth factors, G-protein coupled receptors, L-type calcium channels, and glutamate receptors (reviewed in (Fukunaga and Miyamoto, 1998)). The question remains as to whether tau can participate in ERK signaling initiated by these diverse triggers, and our PC6-3 cell lines will serve as a useful model to further address some of these issues. In addition to stimulation with NGF and EGF, ERK signaling in PC12 cells can be induced by activating G-protein coupled α-adrenergic receptors with norepinephrine (NE), by activating G-protein coupled acetylcholine receptors with carbachol, and by activating purinergic receptors with adenosine triphosphate (ATP) (Xu et al., 1997, Williams et al., 1998, Zhong and Minneman, 1999). Similarly, PC12 cells can undergo ERK-dependent differentiation upon the activation of the fibroblast growth factor receptor (FGFR) with its ligand FGF (Togari et al., 1985, Kouhara et al., 1997). Due to the close similarities between adaptor protein complex formation in TrkA and FGFR signaling (e.g. recruitment of FRS-2 and SHP-2), we predict that tau would enhance ERK signaling initiated by FGF to the same extent as we have previously observed with NGF stimulation. However, ERK signaling through G-protein coupled receptors is less well understood and may involve mechanisms that do not include tau. We will test the effects of tau on ERK signaling

induced by FGF, NE, carbachol, and ATP using our established ERK reporter assays in tau depleted cells to answer these questions.

Based on the data we have gathered for this dissertation, and the data we hope to obtain from further experimentation, we propose the following putative model for an interaction between tau and the ERK signaling pathway in the context of growth factor-induced signal transduction (Figure 4.1). NGF-induced dimerization and auto-phosphorylation of the receptor tyrosine kinase TrkA recruits a complex of adaptor proteins including Grb2/SOS, Crk/C3G, SHP2, FRS2, SHC, and the associated non-receptor tyrosine kinase Src. These components in turn transduce a signal to the small GTPases Ras and Rap1, which are responsible for activation of the ERK cascade (Raf/MEK/ERK). Tau is phosphorylated at Thr231, most likely through the activity of ERK1/2, allowing tau to enhance signaling through interactions with adaptor protein complexes or through a direct association with the tyrosine kinase receptors upstream of Ras/Rap1. The mechanism of tau's association with the signaling machinery may involve PXXP/SH3 interactions, or conformational changes in tau induced by Thr231 phosphorylation. Finally, the ability of tau to potentiate signaling may proceed through the enhanced activation of downstream signaling proteins such as Src tyrosine kinase or SHP2 tyrosine phosphatase.

Implications for abnormal MAPK signaling in neurodegenerative disease

The effects of MAPK signaling on neurodegenerative disease, particularly Alzheimer's disease, are becoming increasingly more evident as many studies have shown that an abnormal reactivation of the pathway occurs in response to multiple upstream triggers (reviewed in (Zhu et al., 2002, Zhu et al., 2007)). Similarly, the many reports of aberrant cell cycle events in the post-mitotic neurons of AD brain (reviewed in (Arendt and Bruckner, 2007, Herrup and Yang, 2007, Bonda et al., 2010)) implicate

abnormal MAPK activation as a significant contributing factor in disease progression. The presence of activated ERK signaling components in neurofibrillary tangle-positive neurons (Anderson et al., 1994, Marcus et al., 1998, Pei et al., 2002) raises a number of interesting questions concerning the relationship between abnormal tau phosphorylation and the dysregulation of signal transduction in the disease state. Specifically, our findings in this dissertation concerning a role for phosphorylated tau in the potentiation of ERK signaling lead us to speculate that hyperphosphorylated tau in AD brain (and other tauopathies) might further contribute to abnormal MAPK signaling and exacerbate disease progression. To address these questions we propose a number of avenues for further research into the nature of the interaction between tau and the MAPK signaling pathway under disease conditions.

Although we have demonstrated a role for tau in growth factor-induced MAPK activation, MAPK activation in AD brain is likely to be initiated by additional triggers including Aβ peptide, oxidative stress, and inflammation (reviewed in (Zhu et al., 2002, Kim and Choi, 2010)). It is therefore important to conduct experiments to determine whether tau is able to potentiate MAPK activation in a similar fashion downstream of these various triggers. Aβ-induced MAPK activation has been documented in primary neuronal cultures, SH-SY5Y neuroblastoma cells, as well as PC12 cells (Rapoport and Ferreira, 2000, Rapoport et al., 2002, Frasca et al., 2004, Jang and Surh, 2005). Beginning with our tau depleted and tau overexpressing PC12 cell lines, we will test the ability of tau to potentiate ERK signaling in response to Aβ peptide using our previously described ERK reporter assays. Since the effects of Aβ on MAPK activation may proceed through multiple MAPK family members (i.e. ERK, JNK, p38), it may be necessary to investigate the activity of each of these MAPK members in response to Aβ in our PC6-3 cell lines. We will make use of commercially available antibodies and luciferase reporter reagents for these experiments. In addition to Aβ treatments, our established PC6-3 cell lines will be subjected to heat shock, irradiation, or hydrogen

peroxide treatment to determine whether tau can impact on MAPK activation induced by these cell stressors as well.

The effects of tau on growth factor-induced MAPK activation might also involve signaling through the p75 neurotrophin receptor. In addition to TrkA, PC12 cells express the p75 receptor which possesses low affinity for all neurotrophins, including NGF (Radeke et al., 1987, Roux and Barker, 2002). While neurotrophins such as NGF have the highest affinity for their respective Trk receptor tyrosine kinase (i.e. NGF for TrkA), they are also capable of binding with lower affinity to the p75 receptor, resulting in the activation of intracellular signaling, including MAPK signaling (Roux and Barker, 2002). In fact, activation of p75 in the absence of Trk activation can lead to JNK activation and apoptosis, which serves as a contributing factor to the cell death occurring in AD brain (Coulson, 2006, Diarra et al., 2009). Aβ can also activate the p75 receptor at low concentrations (Arevalo et al., 2009), as can immature prodomain-containing forms of the various neurotrophins. Immature proneurotrophins, namely proNGF and proBDNF, are known to be upregulated in highly susceptible regions of AD brain, and since these neurotrophins lack the ability to activate their corresponding high affinity receptors (i.e. TrkA and TrkB), they contribute to apoptotic cell death through activation of the p75 receptor (Sotthibundhu et al., 2008). Combined with the ability of Aβ to act as a ligand for p75, these findings serve to make abnormal JNK signaling through p75 of particular interest to our current findings. As we have hypothesized above, tau might also potentiate JNK signaling, which would further link hyperphosphorylated tau to abnormal MAPK signaling in neurodegenerative disease. To address these possibilities, we propose experiments in our PC6-3 cell lines (which also express the p75 receptor) in which the effects of tau on p75 signal transduction and JNK activation are investigated. We will expose cells to proBDNF to activate p75 independent of TrkA, and measure JNK activation in tau overexpressing and tau depleted cells by Western blotting and/or

luciferase reporter assays. The results from these experiments will lend further insights into the interaction between tau and p75-induced JNK signaling.

To expand on our experiments in PC12 cell lines, we plan to further investigate the effect of tau on Aβ-induced MAPK activation in primary neuronal cultures using a previously generated mouse model with tau depletion (Dawson et al., 2001). Cultured hippocampal neurons from these mice have been reported to exhibit an increased resistance to Aβ-mediated neurotoxicity, and this finding is also correlated with decreased MAPK activation (Rapoport et al., 2002). These results can be explained by our current findings which link tau depletion with attenuated ERK signaling. We propose experiments in which cultured neurons from tau deficient mice are exposed to Aβ and transfected with tau mutants such as T231A or T231D to investigate the putative role of tau in MAPK signaling in this system. Alternatively, we can make use of our existing RNAi developed against rat tau to suppress the expression of rat tau in cultured rat hippocampal neurons. After exposure to Aβ, tau depletion by RNAi in these cells can be accompanied by human tau "rescue" mutants to observe the effects of pseudo-phosphorylated tau mutants on MAPK-mediated neurotoxicity. It should be noted that the precise mechanism by which extracellular Aβ application leads to MAPK activation in any of the aforementioned cell systems remains unclear. Some evidence indicates that Aβ can act as a ligand for the surface receptor RAGE (Receptor for Advanced Glycation Endproducts), leading to the activation of the cell stress response mediated by the MAPK family member p38 (Yan et al., 2009). On the other hand, it has also been reported that Aβ treatment of cultured hippocampal neurons leads to the upregulation of NGF and TrkA receptors, followed by subsequent NGF-induced activation of TrkA signaling (Bulbarelli et al., 2009). Interestingly, combining Aβ application with anti-NGF antibodies to block NGF-mediated TrkA activation did not completely abrogate downstream signaling, indicating that TrkA can also be activated by Aβ through an NGF-independent mechanism (Bulbarelli et al., 2009). In fact, co-immunoprecipitation

experiments have identified the formation of a complex containing TrkA, p75 neurotrophin receptor, Aβ peptides, and presenilin 1 (PS1) in rat hippocampal neurons, leading to the abnormal activation of TrkA signaling in the absence of NGF (Matrone et al., 2009).

Results from these experiments will yield important data that could add to our understanding of the link between upstream Aβ accumulation and tau hyper-phosphorylation. Based on the data we have obtained from this dissertation work, and the results we hope to obtain from the experiments outlined above, we propose a putative model for the role of tau in Alzheimer's disease as depicted in Figure 4.2. Briefly, various upstream triggers such as oxidative stress, inflammation, and dysregulation of the cell cycle lead to an upregulation of the MAPK signaling pathway and other kinases associated with aberrant cell cycle re-entry. Concurrently, normal regulatory phosphatase activity is downregulated and the combination of these factors leads to tau hyperphosphorylation. Hyperphosphorylated tau detaches from microtubules, causing axonal destabilization and degeneration of synaptic networks. Soluble hyper-phosphorylated tau then exists in an intermediate stage, or "pre-tangle" stage, in which it is able to exert positive feedback on to the abnormally active MAPK signaling pathway. Neurofibrillary tangles are formed in a last ditch effort to sequester the abnormally active soluble tau species, at the expense of space-occupying lesions within the cell. Ultimately, neuronal death occurs in these degenerating neurons through various mechanisms, although tangle-positive neurons may persist for long periods of time (Morsch et al., 1999). Evidence suggests that cell cycle dysregulation is one of the earliest events in disease progression and apoptotic cell death is likely to be caused by a combination of cell cycle dysregulation and subsequent "hits" such as hypoxia or oxidative stress within the same cell (Zhu et al., 2001). Other neurons may die from non-apoptotic mechanisms including glutamate-mediated excito-toxicity (reviewed in (Hynd et al., 2004)) and inflammatory necrosis (Holmes et al., 2009).

One caveat to our proposed model is the possibility that ERK activation in AD brains may be neuroprotective rather than a mediator of Aβ-induced neurotoxicity. While a large body of evidence exists to support a neurotoxic outcome from abnormal ERK activation, some data also suggest that abnormal ERK activation might be neuroprotective. One study, performed in SH-SY5Y neuroblastoma cells, found that Aβ-induced toxicity was mediated through JNK rather than ERK activation (Wei et al., 2002). The authors further found that treating the cells with insulin-like growth factor (IGF) led to a strong activation of ERK which exerted a protective effect on the Aβ-induced toxicity mediated through apoptotic JNK signaling. In this respect, ERK activation in AD brain may represent an attempt to counteract abnormal activation of other MAPK pathways such as JNK, and thus the increased phosphorylation of tau would initially serve to potentiate the beneficial ERK signaling. On the other hand if tau is able to similarly potentiate JNK signaling, hyperphosphorylated pre-tangle tau would also be contributing to an exacerbation of apoptotic signaling through the JNK pathway. Further experiments, such as those outlined in this chapter, are required to investigate these possibilities.

Concluding remarks

As populations of elderly individuals around the world continue to increase in number, both due to increasingly effective healthcare and life-prolonging medicines and as a result of general population increases, we are poised to see a rise in neuro-degenerative diseases of epidemic proportions. By the year 2050, over 14 million people are projected to be suffering with Alzheimer's disease in the United States alone (Kelley and Petersen, 2007), underscoring the urgent need for therapeutic options to treat these patients. Increasing evidence supports a role for aberrant cell cycle re-entry as a significant contributor to the neuronal loss associated with AD, triggered by the abnormal activation of signaling pathways such as the MAPK pathway. Herein, we have described

a novel function for the microtubule associated protein tau as a modulator of growth factor-induced ERK signaling that specifically requires tau phosphorylation and does not involve microtubule association. Given the nature of abnormal tau phosphorylation in AD brain, and the correlated changes in MAPK signaling triggered by upstream factors such as Aβ and oxidative stress, we hypothesize a role for tau in exacerbating MAPK signaling in the diseased brain. Additional experiments to elucidate the precise role of phosphorylated tau in AD-associated MAPK signaling may lead to the design of novel therapeutics targeting tau-potentiated MAPK signaling. Such therapeutics might not have the side effects of attenuating the normal MAPK activity required for healthy neuronal function.

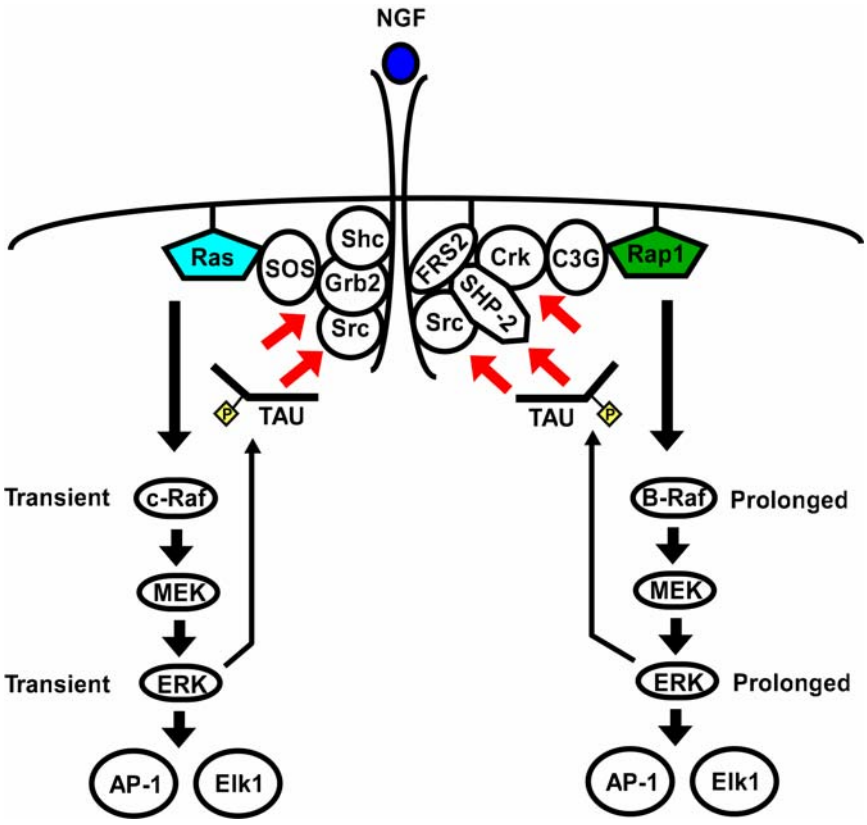

Figure 4.1 Putative model of ERK signaling modulated by tau. NGF activation of TrkA in PC12 cells leads to a recruitment of adaptor protein complexes (only partially represented here) to the activated receptor. The adaptor proteins Grb2 and Crk in turn recruit the Ras and Rap1 GEFs, SOS and C3G, required for Ras/Rap1 GTPase activation. GTPase activation induces signaling through a kinase cascade composed of Raf, MEK, and ERK kinases; culminating in the activation of ERK1/2 (MAPK) and downstream transcription factors. Tau associates with the ERK signaling pathway upstream of GTPase activation (red arrows) through a mechanism that may involve PXXP/SH3 domain interactions between tau and one or more signaling proteins such as the tyrosine kinase Src, the adaptor protein Grb2, or the tyrosine phosphatase SHP-2. Tau phosphorylation at Thr231 is required for tau to potentiate ERK signaling and is likely to be mediated by ERK1/2 positive feedback (thin black arrows).

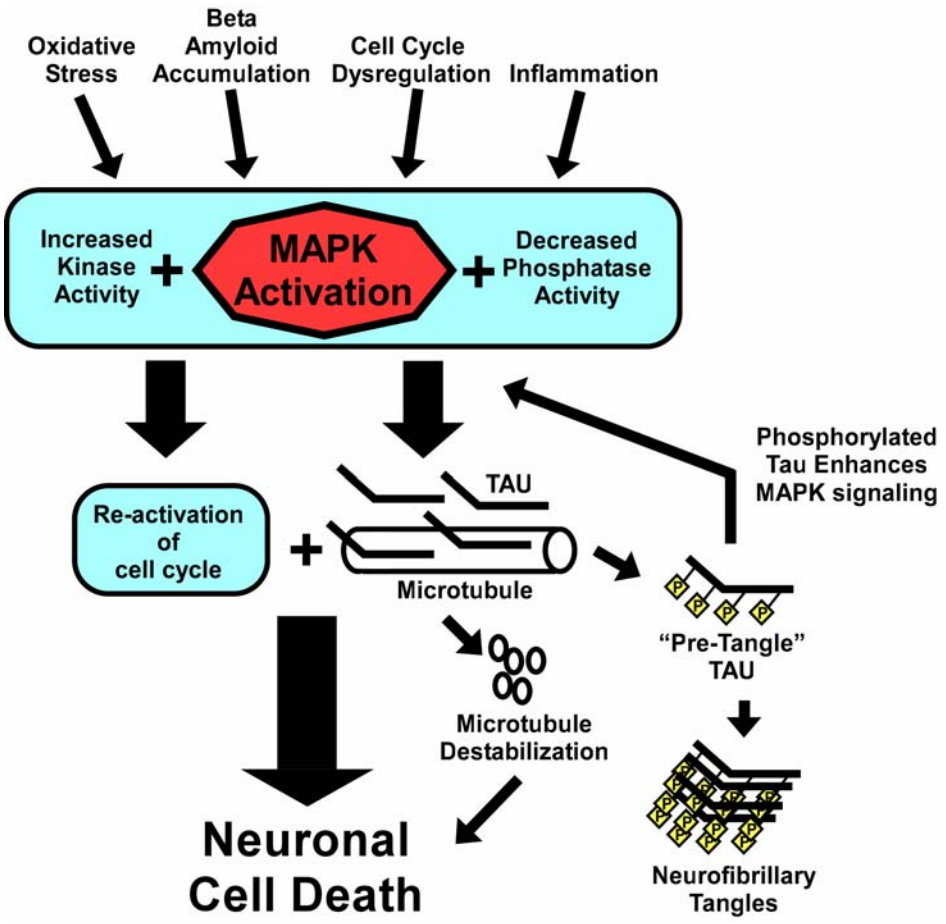

Figure 4.2 Putative model for the role of tau in abnormal MAPK signaling in Alzheimer's disease. A combination of upstream triggers lead to the abnormal activation of MAPK signaling in the early stages of AD. MAPK activation along with increased kinase activity and decreased phosphatase activity can lead to the hyper-phosphorylation of tau as well as aberrant re-entry into the cell cycle. Hyper-phosphorylated tau detaches from microtubules in early AD, and exists in a "pre-tangle" soluble state in which it is free to exacerbate MAPK signaling through a positive feedback mechanism. Neurofibrillary tangles are formed as a neuroprotective measure in an attempt to sequester the abnormally functioning protein. Neuronal cell death ultimately occurs downstream of failed attempts to complete the cell cycle, as well as through chronic inflammation, oxidative stress, loss of trophic support, and other factors. Microtubule destabilization further contributes to neuronal cell death through impairment of axonal function and axonal degeneration.

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