# ROLES OF CARBOXYPEPTIDASE E (CPE) IN REGULATION OF THE MICROTUBULE CYTOSKELETON, NEURONAL MIGRATION, AND DENDRITE MORPHOLOGY

By

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**ABSTRACT OF THE DISSERTATION**

ROLES OF CARBOXYPEPTIDASE E (CPE) IN REGULATION OF THE MICROTUBULE CYTOSKELETON, NEURONAL MIGRATION, AND DENDRITE MORPHOLOGY

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Carboxypeptidase E (CPE) is a member of the M14 metallocarboxypeptidases family and is responsible for the proteolytic processing of peptide intermediates in endocrine cells and neurons. The importance of CPE in the nervous system has been elucidated in recent years. $CPE^{-/-}$ mice display a variety of neuronal deficits, including abnormal dendritic structure, spine morphology, and degeneration and deficits in learning and memory. It remains unclear whether and how CPE may contribute to neurodevelopment in addition to its role in prohormone processing, sorting, and transport. In this dissertation, I show that the level of CPE expression in mouse brain increases during embryonic and early postnatal development, and I demonstrate that it is enriched in and expressed throughout neurons. By

overexpressing or knocking down CPE *in vivo* using the *in utero* electroporation technique, I find that CPE is required for proper cortical neuron migration. In hippocampal neuronal cultures, both overexpression and knockdown of CPE result in decreased dendrite branching, indicating that a balance of CPE protein level is required for proper dendrite morphogenesis. Importantly, I report that the interaction between the CPE carboxyl terminus and $p150^{Glued}$ is critical for regulation of the subcellular localization of $p150^{Glued}$, which may in turn affect the stability and dynamics of microtubule networks and may be responsible for the effects seen from CPE overexpression on dendrite morphology and neuronal migration. In addition, I identify a novel function for CPE in regulating tubulin polyglutamylation and show that the zinc-binding motif of CPE is required to mediate this activity. The role of CPE-mediated tubulin polyglutamylation in neuronal migration and dendrite branching is also examined, and my results show that this activity is not involved in regulation of these processes. Together, this study shows that CPE and its interactor, $p150^{Glued}$, are important players in neurodevelopment. Moreover, the zinc-binding motif of CPE and its function in regulating tubulin polyglutamylation play distinctive roles than does the CPE carboxyl terminus during early brain development.

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# INTRODUCTION

## 1. INTRODUCTION TO CPE PROTEIN

### Discovery of carboxypeptidase E (CPE) as a prohormone-processing enzyme

Carboxypeptidase E (CPE) was first identified as a prohormone-processing enzyme in endocrine cells and neurons, where it is responsible for the proteolytic processing of peptide intermediates, generating bioactive hormones and neurotransmitters (Fricker and Snyder 1982, Hook, Eiden et al. 1982). As a metallocarboxypeptidase, CPE hydrolyzes single carboxyl terminal amino acids from polypeptide chains with specificity for basic residues lysine or arginine and it does so by coordination with zinc ions.

### Structure and distribution

The CPE protein is a member of the N/E subfamily of the M14 family of metallocarboxypeptidases (Reznik and Fricker 2001) and is predicted to have the following functional domains (reviewed in (Cawley, Wetsel et al. 2012)): a zinc-binding site (conserved among M14 family members), a prohormone sorting signal binding site, an amphipathic α-helical transmembrane domain, and a cytoplasmic tail that interacts with microtubule (MT) binding proteins, such as the dynactin complex, dynein, kinesin-2 and kinesin-3 (Goldstein and Yang 2000, Berezuk and Schroer 2007). In the bovine pituitary gland, approximately 70% of CPE is soluble and 30% is membrane bound. Transmembrane CPE interacts with $p150^{Glued}$, a core component of the dynactin complex (Parkinson 1992). However, a

common carboxyl terminal sequence is shared between the soluble and transmembrane form of CPE (Parkinson 1992), suggesting that both of them can interact with the dynactin complex. Specific interaction of CPE and p150$^{Glued}$ has been verified by co-immunoprecipitation in AtT20 cells (Park, Cawley et al. 2008).

Distribution of CPE protein in various tissues has been studied extensively (reviewed in (Cawley, Wetsel et al. 2012)). CPE expression is detected in all major areas of the adult rat brain, with high levels of expression in pyramidal neurons of the hippocampus (Birch, Rodriguez et al. 1990). Expression patterns of CPE were examined by *in situ* hybridization of brain during rat embryonic development (Zheng, Streck et al. 1994). A low level of CPE mRNA is observed at embryonic day (E)10, mainly in the mantle layer of the neuroepithelium. Interestingly, expression of CPE is detected in neural tissues earlier than other known prohormone convertases, such as PC1 and PC2, indicating an unknown function of CPE at this developmental stage. As neurogenesis proceeds, CPE expression is observed in most brain regions and some peripheral tissues at a high level.

**Functions in the nervous system**

In addition to its carboxypeptidase activity, CPE function is far more complicated. Recent studies have reported its non-enzymatic functions in the endocrine and nervous systems, including prohormone sorting (Loh, Maldonado et al. 2002, Dhanvantari, Shen et al. 2003, Lou, Kim et al. 2005) and vesicle transport (Oiso,

Takeda et al. 2009, Zhang, Li et al. 2009, Lou, Park et al. 2010). Prohormones and neuropeptide precursors synthesized at the rough endoplasmic reticulum are transported to the trans-Golgi network (TGN) and subsequently sorted and packaged with their processing enzymes into granules to the regulated secretory pathway. In this process, CPE can recognize and bind to the sorting motifs of the peptide intermediates (Loh, Maldonado et al. 2002, Dhanvantari, Shen et al. 2003, Lou, Kim et al. 2005). In order to reach the secretion site, the granules are transported by MTs and motors, in which CPE is also involved by directly binding to motor protein complexes as mentioned earlier (Goldstein and Yang 2000, Berezuk and Schroer 2007). One neuropeptide sorted and transported by CPE is brain-derived neurotrophic factor (BDNF). Disruption of CPE binding to BDNF-containing vesicles results in reduced BDNF localization to neurites of hippocampal neurons (Lou, Kim et al. 2005, Park, Cawley et al. 2008).

Furthermore, recent studies suggest a neuroprotective role of CPE (Koshimizu, Senatorov et al. 2009). CPE acts extracellularly as a neurotrophic factor to protect neurons against oxidative stress-induced degeneration through Extracellular Signal-regulated Kinase (ERK) and AKT pathways (Cheng, Cawley et al. 2013). A single nucleotide polymorphism in the CPE gene was also discovered and *in vitro* studies suggest the loss of neuroprotective function with this mutation (Cong, Cheng et al. 2017).

**Studies in mouse models and human**

The importance of CPE function in the brain has been further supported by studies on $CPE^{-/-}$ mice and humans. The knockout mice exhibit aberrant dendritic architecture and spine morphology, deficits in learning and memory, and neurodegeneration under stress (Woronowicz, Koshimizu et al. 2008, Woronowicz, Cawley et al. 2010). Studies on a spontaneous mutant mouse model, $Cpe^{fat/fat}$, which lacks CPE enzymatic activity due to a point mutation in the *Cpe* gene (Coleman and Eicher 1990, Fricker, Berman et al. 1996), revealed anxiety and depressive-like behaviors in these mice (Rodriguiz, Wilkins et al. 2013). Similarly, a truncation mutation of CPE was identified in a morbidly obese human female with intellectual disability (Alsters, Goldstone et al. 2015), suggesting that this protein plays a role in brain function in both species.

More recently, a new mutation in the CPE gene from a patient with Alzheimer's Disease was identified and examined in cell culture and transgenic mice (Cheng, Cawley et al. 2016). Their findings indicate this human CPE mutation may be involved in Alzheimer's related neurodegeneration, memory deficits and depression.

## 2. INTRODUCTION TO P150$^{GLUED}$ AND MOTOR PROTEINS

### Structure and binding partners of p150$^{Glued}$

Dynactin is a multi-subunit protein complex, of which p150$^{Glued}$ is the largest subunit and binds directly to MTs and motors. It is a binding partner and regulator of dynein, a retrograde motor protein, and it is indispensible for the functional motor activity of dynein (Schroer 2004).

p150$^{Glued}$, otherwise called DCTN1 or Dynactin p150, contains a cytoskeleton-associated protein Glycine-rich (CAP-Gly) motif within its amino terminus, which can interact directly with MTs and the MT plus-end binding protein EB1 (Berrueta, Tirnauer et al. 1999, Askham, Vaughan et al. 2002), one coiled-coil domain in the middle region, which provides binding platform for dynein and other motor proteins, such as kinesin family motors (Blangy, Arnaud et al. 1997, Deacon, Serpinskaya et al. 2003), and a second coiled-coil domain within the carboxyl terminus, which is less well defined but may be required for association with Arp1 (McGrail, Gepner et al. 1995, Waterman-Storer, Karki et al. 1995), another subunit of the dynactin complex.

### Function in cargo transport

Dynein is the major molecular motor responsible for cargo transport along the MT track towards the minus-end, while kinesins mediate transport towards the plus-end of MTs. In peptidergic neurons, neuropeptides, such as BNDF, are packaged

into dense-core vesicles and transported to secretion sites in axons and dendrites, and excess vesicles can be retrogradely transported back to the cell body (Kwinter, Lo et al. 2009). Through direct interaction with motors, dynactin is involved in coordination of the bi-directional cargo transport by regulating dynein-mediated retrograde transport, and anterograde transport mediated by kinesin-2 (King and Schroer 2000, Berezuk and Schroer 2007). p150$^{Glued}$ is required for the initiation of dynein-mediated vesicular transport (Lloyd, Machamer et al. 2012, Moughamian and Holzbaur 2012) and may also regulate the processivity of dynein and kinesin-2 (Berezuk and Schroer 2007).

**Function in MT anchoring and cell cycle regulation**

In addition to regulation of motor activities, dynactin functions at the centrosome to anchor MTs and recruit cell cycle regulators independent of dynein (Quintyne, Gill et al. 1999, Quintyne and Schroer 2002). Loss of p150$^{Glued}$ at the centrosome results in delay in S phase entry without affecting dynein motility or its recruitment to the centrosome (Quintyne and Schroer 2002). The localization of p150$^{Glued}$ at the centrosome is also cell cycle-dependent (Chen, Syu et al. 2015). Specifically, p150$^{Glued}$ is concentrated at the mother centriole during G1/S phase, released towards the MT minus-end during G2/M stage, and extends to the mitotic spindle when the cell enters into mitosis.

**Function in maintaining MT stability**

In addition, p150$^{Glued}$ also accumulates at MT plus-ends, where it forms a complex with CLIP-170 and EB1 (Ligon, Shelly et al. 2006). Recent studies found that full-length p150$^{Glued}$, otherwise called p150$^{Glued}$ isoform 1A, is an anti-catastrophe factor that enhances MT stability by binding both to MTs and to tubulin dimers (Lazarus, Moughamian et al. 2013). In adult animal tissues, p150$^{Glued}$ 1A is expressed specifically in neurons, while alternatively spliced isoforms of p150$^{Glued}$ are found in both neurons and other cell types. Mutations in the *dynactin* gene (*DCTN1*), encoding p150$^{Glued}$, are associated with Perry syndrome, characterized by early-onset Parkinsonism, depression, weight loss, and hypoventilation (Farrer, Hulihan et al. 2009, Wider, Dachsel et al. 2010), and one of the causal mutations for Perry syndrome present in p150$^{Glued}$ abolishes this anti-catastrophe activity (Lazarus, Moughamian et al. 2013).

**Involvement in neuronal migration**

During neuronal migration, the dynein/dynactin complex is thought mediate nuclear movement, but where and how the complex exerts its effects has not been well-defined. Current models suggest that the dynein/dynactin complex may associate with the nuclear membrane and generate a pulling force to power the forward movement of nuclei, or alternatively, the complex may interact with the distal cell cortex to allow the nucleus and the associated centrosome to be pulled

along MTs (reviewed in (Tsai and Gleeson 2005, Marin, Valiente et al. 2010)). Some of the interactors and regulators of the dynein motor complex, such as Lis1 and Ndel1, have been studied extensively and shown to play important roles in the nucleus and centrosome coupling of migrating neurons (Smith, Niethammer et al. 2000, Dujardin, Barnhart et al. 2003, Li, Lee et al. 2005, Tsai, Bremner et al. 2007).

## 3. POST-TRANSLATIONAL MODIFICATIONS OF TUBULIN AND MTS

MTs are the largest component of cytoskeleton in eukaryotic cells. Assembled from α-tubulin and β-tubulin dimers, MTs are highly dynamic and undergo cycles of polymerization and depolymerization. They are involved in intracellular transport, cell cycle, morphology and motility, and all of these functions are largely dependent on MT "dynamic instability" (Mitchison and Kirschner 1984). To achieve diversity and complexity in function, the dynamics of MTs have to be controlled precisely. One of the most important controllers is post-translational modification (PTM) of tubulins and MTs.

PTM was first described in 1973 as the incorporation of tyrosine into the carboxyl terminus of α-tubulin by an RNA-independent enzyme (Barra, Rodriguez et al. 1973). Various types of tubulin modifications have been discovered since then, but correlating the temporal and spatial unique "codes", generated by different tubulin modifications, and the specific cellular function of MTs still remains a challenge. Here, in this dissertation, the work focuses on the major PTMs: detyrosination and tyrosination, acetylation and deacetylation, and polyglutamylation.

### Detyrosination and tyrosination

Detyrosination preferentially targets MTs rather than tubulin dimers (Gundersen, Khawaja et al. 1987). The enzyme that catalyzes the removal of terminal tyrosine

(detyrosination) is still unknown, but may be a member of the cytosolic carboxypeptidase (CCP) family (Kalinina, Biswas et al. 2007, Rodriguez de la Vega, Sevilla et al. 2007). Detyrosinated tubulins are subsequently tyrosinated by the tubulin tyrosine ligase (TTL) (Raybin and Flavin 1977), completing a detyrosination/tyrosination cycle. A complex network of detyrosinated tubulins and tyrosinated tubulins provide cues for MT interacting proteins, including the plus-end binding protein (+TIP) $p150^{Glued}$ and CLIP-170 (Bieling, Kandels-Lewis et al. 2008), which bind more efficiently to tyrosinated tubulins than detyrosinated tubulins. Detyrosination/tyrosination may also affect the functions of motor protein kinesins (Konishi and Setou 2009, Peris, Wagenbach et al. 2009, Ghosh-Roy, Goncharov et al. 2012), although a direct correlation remains unclear.

**Acetylation and deacetylation**

Although several acetylases (αTAT1, ELP3, ARD-1NAT1, GCN5) and deacetylases (HDACs, SIRTs) have been characterized (Witt, Deubzer et al. 2009, Akella, Wloga et al. 2010, Shida, Cueva et al. 2010, Perdiz, Mackeh et al. 2011, Yao and Yang 2011, Kalebic, Sorrentino et al. 2013), the regulation and function of acetylation on MTs are still not well understood. Acetylated-tubulin has long been considered as a marker of stable MTs; however, there is little direct experimental evidence indicating a correlation between acetylation and MT stabilization (reviewed in (Janke and Bulinski 2011)). Like other types of PTMs, MT acetylation can also affect protein

interaction with MTs and motor protein function (reviewed in (Song and Brady 2015)).

**Polyglutamylation**

Polyglutamylation of tubulins was first described in mouse brain tissues (Edde, Rossier et al. 1990), and represents a major PTM in the brain (Audebert, Koulakoff et al. 1994). Since 2005, several polyglutamylases have been identified as members of the tubulin tyrosine ligase-like (TTLL) protein family (Janke, Rogowski et al. 2005, Ikegami, Mukai et al. 2006, van Dijk, Rogowski et al. 2007). Depending on the preference of the specific enzyme, glutamates can be added onto a Glu residue in the carboxyl terminus of either α-tubulin or β-tubulin, initiating side chains or elongating existing chains to variable lengths (van Dijk, Rogowski et al. 2007). Other players controlling side chain length are the deglutamylases, which are characterized as members of the cytosolic carboxypeptidase (CCPs) family (Kalinina, Biswas et al. 2007, Rodriguez de la Vega, Sevilla et al. 2007). CCPs share a conserved zinc-binding motif, which is a consensus sequence within the M14 carboxypeptidase family that CPE belongs to (Gomis-Ruth, Companys et al. 1999). Similar to polyglutamylases, CCPs differ in their specificity for substrates and reaction, either shortening long side chains or removing the branch point glutamates from α-tubulin or β-tubulin (Rogowski, van Dijk et al. 2010).

Functionally, generating variable polyglutamyl chain length contributes to the

regulation of protein binding affinity to MTs. For example, the microtubule-associated proteins (MAP) Tau, MAP1B and MAP2, as well as kinesin motors, bind to MTs modified with three glutamyl units most efficiently, while the relative affinity decreases with a shorter or longer chain length (Boucher, Larcher et al. 1994, Larcher, Boucher et al. 1996, Bonnet, Boucher et al. 2001). More recently, quantitative evidence was provided to describe the graded control of spastin binding to MTs and its MT severing activity by polyglutamylation (Valenstein and Roll-Mecak 2016). In specific, modulation of spastin activity by polyglutamylation is biphasic and is sensitive to glutamyl chain length.

Together, various patterns of polyglutamylation can be generated on MTs and converted to sophisticated signals, conferring specificity to MAPs and motor proteins. Therefore, it is crucial to understand the spatial and temporal regulation of polyglutamylation.

**Roles of PTMs in undifferentiated cells and neurons**

In proliferating cells, tubulin detyrosination, acetylation, and polyglutamylation vary during the cell cycle. That is, they are enriched in the mitotic spindles and centrioles, and the net level of polyglutamylation increases during mitosis (Gundersen and Bulinski 1986, Piperno, LeDizet et al. 1987, Bobinnec, Moudjou et al. 1998, Regnard, Desbruyeres et al. 1999). Current experimental evidence indicates the involvement of PTMs, especially polyglutamylation, in regulating cell cycle and maintaining

centriole integrity (reviewed in (Janke and Bulinski 2011)).

PTMs are most abundant in neurons (Janke and Bulinski 2011). Increased MT acetylation occurs during neuronal differentiation, and reduction of acetylated-tubulin results in defective cortical migration and dendrite branching (Falconer, Vielkind et al. 1989, Creppe, Malinouskaya et al. 2009). Moreover, knockdown of a β-tubulin specific polyglutamylase, TTLL7, results in decreased polyglutamylation levels and reduced MAP2-positive neurite outgrowth (Ikegami, Mukai et al. 2006). Studies in mouse models with deficiency in polyglutamylation revealed an involvement of polyglutamylation in synaptic function and neuronal degeneration. ROSA22 mice that lack a functional PGs1 subunit of the tubulin polyglutamylase complex display impaired KIF1A-based synaptic transport (Ikegami, Heier et al. 2007). *Purkinje cell degeneration* (*pcd*) mice that lack a functional deglutamylase CCP1 display neuronal degeneration (Mullen, Eicher et al. 1976, Greer and Shepherd 1982), which can be rescued by downregulation of hyperglutamylation caused by the lack of CCP1 (Rogowski, van Dijk et al. 2010). In addition, during postnatal brain development, glutamylated α-tubulin is abundant at early stages, while glutamylated β-tubulin is found at lower levels in young neurons and increases during development (Audebert, Koulakoff et al. 1994), which may indicate a role for polyglutamylation in stabilizing MTs and the extension of stable neurites.

## 4. INTRODUCTION TO BRAIN DEVELOPMENT AND DISORDERS

Functional connectivity between neurons in the brain depends on where neurons are positioned and how their dendrites are shaped during development. Abnormal neuronal migration and dendrite patterning may result in brain disorders. Often, aberrant brain structure results from improper function or expression of a complex of proteins rather than of a single protein. Thus, studying the interaction between multiple proteins that regulate neuronal development will aid in our understanding of processes that result in abnormal brain function. Here, we mainly focus on the differentiation, migration and morphogenesis of excitatory pyramidal neurons.

### Cortical Development

#### Neurogenesis

As depicted in **Figure 1** (reproduced from (Woodworth, Custo Greig et al. 2012)), during development of the cerebral cortex, lower layer pyramidal neurons are generated in the ventricular zone (VZ), a proliferative neuroepithelial region that lies adjacent to lateral ventricles, and upper layer neurons are generated in the subventricular zone (SVZ), another proliferative compartment in between the VZ and the intermediate zone (IZ) (reviewed in (Dehay and Kennedy 2007)). Radial glial cells, the neural progenitors, divide within the VZ to generate both postmitotic neurons and intermediate progenitor cells. Intermediate progenitor cells then divide within the SVZ to give rise to pairs of postmitotic neurons (Noctor,

Martinez-Cerdeno et al. 2004).

**Neuronal migration**

The newly born neurons migrate towards the pial surface following an inside-out pattern (**Figure 1**): later-born neurons migrate and pass earlier-born neurons to the cortical plate (CP) and reside more superficially in the CP, while earliest-born neurons reside in the deep layers of the cortex (Angevine and Sidman 1961, Rakic 1974). The mammalian cerebral cortex consists of six layers, and neurons in different layers connect to distinct regions of the brain or spinal cord. Thus, disrupting their positioning may disrupt proper neuronal network circuitry and lead to defects in the functions of the cortex.

Three modes of radial migration have been described, including somal translocation, multipolar migration, and glial-guided radial migration or locomotion. Earliest-born neurons leave the progenitor cell pool by somal translocation with a leading process inherited from the radial progenitor cell (Miyata, Kawaguchi et al. 2001, Nadarajah, Brunstrom et al. 2001). Later-born neurons move through the SVZ towards the IZ in a distinct multipolar migration mode (Tabata and Nakajima 2003). These newly differentiated neurons possess multipolar morphology with neurites extending in random directions, and they undergo transition to bipolar morphology when they move through the SVZ to the IZ (Noctor, Martinez-Cerdeno et al. 2004) (**Figure 2B**, reproduced from (Sakakibara and Hatanaka 2015)). The multipolar to bipolar transition is critical for neuronal polarization and subsequent glial-guided

radial migration, and it relies heavily on MT dynamics (reviewed in (Ohtaka-Maruyama and Okado 2015)). Indeed, reduction or complete loss of Lis1 protein, which is associated with MTs and the centrosome and involved in maintaining MT stability, results in the accumulation of multipolar cells in the IZ and failure in neuronal migration to the CP (Tsai, Chen et al. 2005, Youn, Pramparo et al. 2009).

Bipolar cells possess a thick leading process pointing towards the pial surface and a thin trailing process pointing in the opposite direction and migrate along the radial glial fiber towards their final destinations in the CP (Noctor, Martinez-Cerdeno et al. 2004). This is called glial-guided radial migration or locomotion, which is the most standard movement of pyramidal neurons (Rakic 2006). It consists of three major steps: leading process extension, nucleokinesis (the forward movement of the nucleus), and rear end retraction (Sakakibara, Ando et al. 2013).

Neuronal migration is regulated by a variety of extracellular molecules, including BDNF, Ephrin-B1, and Reelin. (Ringstedt, Linnarsson et al. 1998, D'Arcangelo, Homayouni et al. 1999, Hiesberger, Trommsdorff et al. 1999, Assadi, Zhang et al. 2003, Beffert, Weeber et al. 2004, Gonzalez-Billault, Del Rio et al. 2005, Dimidschstein, Passante et al. 2013, Ortiz-Lopez, Vega-Rivera et al. 2017). Importantly, neuronal migration is also regulated intrinsically by cytoskeletal components. A network of MTs and associated proteins plays a critical role in leading process extension and nucleokinesis (reviewed in (Valiente and Marin 2010,

Jiang and Nardelli 2016)). Time-lapse imaging studies show that the centrosome is positioned in front of the nucleus during migration, and different models have been proposed to explain how the nucleus is pulled towards the leading edge (Tanaka, Serneo et al. 2004, Tsai and Gleeson 2005). Although the underlying mechanisms are not yet clear, the centrosome is generally thought to be critical in coupling the cell cortex and the nucleus (N-C coupling). Cytoplasmic MTs extend from the centrosome anteriorly to the leading edge and posteriorly to the nucleus. Upon dynamic changes to MTs, the centrosome advances into the leading process, and subsequently, the nucleus translocates forward. Several MAPs and motor proteins regulate nucleokinesis. For example, Lis1 and doublecortin (DCX) stabilize MTs and maintain N-C coupling in coordination with dynein (Smith, Niethammer et al. 2000, Bai, Ramos et al. 2003, Tanaka, Serneo et al. 2004, Tsai and Gleeson 2005, Tsai, Bremner et al. 2007). In addition, PTMs of MTs contribute to the regulation of neuronal migration by modulating protein interactions with MTs (Umeshima, Hirano et al. 2007, Baudoin, Alvarez et al. 2008). Therefore, perturbations in MT stability or MT anchoring to the centrosome, N-C coupling, or localization of the dynein motor complex may result in disrupted migration.

**Dendrite morphogenesis and function**

After bipolar neurons reach their postmigratory destination, they differentiate into a multipolar morphology (Takano, Xu et al. 2015). The trailing process of a migrating

neuron eventually develops into an axon, and the leading process becomes the dendritic arbor. Dendrites differ from axons in many aspects. For example, axons only have plus-end oriented MTs, whereas dendrites contain MTs in mixed orientations, facilitating their highly branched dendritic tree (Baas, Deitch et al. 1988, Burton 1988, Baas, Black et al. 1989, Baas and Lin 2011). In addition, dendrites are enriched in endoplasmic reticulum and the Golgi complex, which are transported by the minus-end MT motor dynein and contribute to dendrite growth (Horton, Racz et al. 2005, Cui-Wang, Hanus et al. 2012). As dendritogenesis proceeds, new branches form, are pruned, and mature to shape a complex dendritic tree. Even in adult life, dendritic arbors are dynamic structures and constantly change in response to environmental cues, neuronal activities, and stress and injury (reviewed in (Copf 2016)).

In cultured rodent hippocampal neurons, multiple neurites extend during the first 2 days after plating, and axons are specified at approximately day *in vitro* (DIV) 3 (**Figure 2A**, reproduced from (Sakakibara and Hatanaka 2015)). The remaining neurites then develop into primary dendrites and continue to grow until at least DIV 10. Secondary and higher order dendrites begin to extend from DIV 6 through DIV 12 and are pruned after DIV 12 until 3 weeks in culture (Dotti, Sullivan et al. 1988, Akum, Chen et al. 2004).

Proper development of dendrite morphology, including shape, size, and patterning of the dendritic arbor, is critical for many aspects of neuronal function,

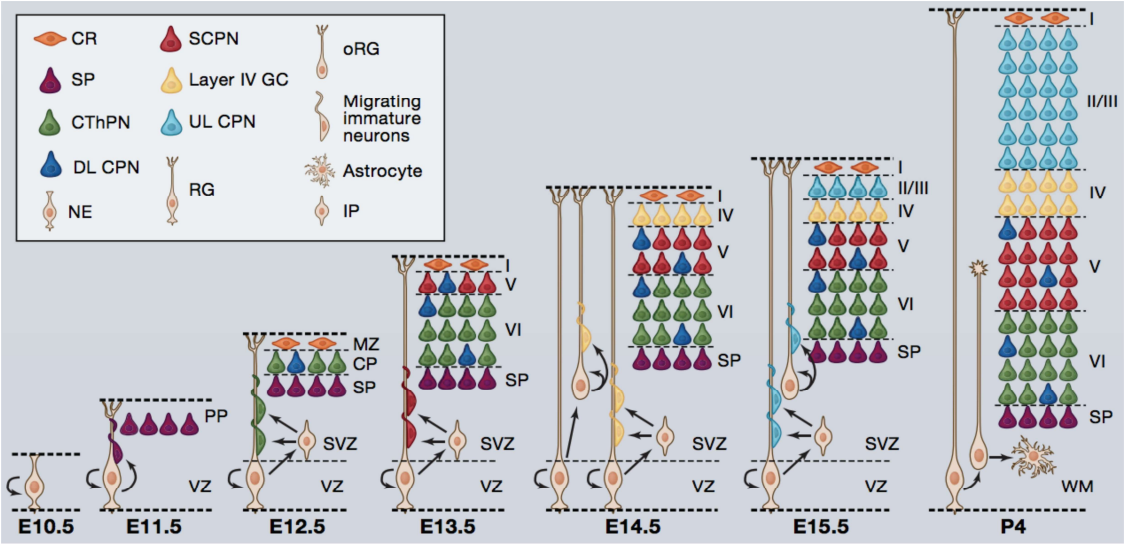
such as the number and type of synapses that will be formed along the dendrites, electrical properties of a neuron, and the positioning of a particular signal (London and Hausser 2005, Spruston 2008). The regulation of dendrite morphogenesis has been studied extensively both *in vivo* and *intro*. Molecules that shape the dendritic tree include transcription regulators, neurotrophic factors and receptors, cytoskeletal regulators and motors proteins, cell adhesion molecules, and other proteins involved in RNA localization and translation (reviewed in (Copf 2016)). For instance, brain-derived neurotrophic factor (BDNF), one of the major neurotrophic factors, increases proximal dendrites in pyramidal neurons (McAllister, Lo et al. 1995, Horch, Kruttgen et al. 1999). The effect is dependent on CREB-activated expression of cypin (cytosolic PSD-95 interactor), a protein that influences dendrite morphogenesis by regulating MT assembly (Akum, Chen et al. 2004, Kwon, Fernandez et al. 2011). The MT motors dynein and kinesins play a role in neurite outgrowth by functioning in cargo transport (Hoogenraad, Milstein et al. 2005, Sachdev, Menon et al. 2007, Satoh, Sato et al. 2008, Braunstein, Eschbach et al. 2010). Spastin, a MT-severing protein, also influences neurite outgrowth by regulating MT dynamics (Riano, Martignoni et al. 2009, Zhang, Li et al. 2012). Although a variety of factors control dendrite morphology, their endpoint of control is often cytoskeletal reorganization, emphasizing the importance of understanding the role of the MT network and its regulators in development.

**Developmental defects and brain disorders**

Various brain disorders have been linked to altered neurogenesis, neuronal migration, and post-migratory development (Barkovich, Guerrini et al. 2012). Impaired neuronal migration may result in brain malformations, such as lissencephaly, characterized by "smooth brain." Several genes involved in MT polymerization and nuclear and centrosome movement, including LIS1, DCX, RELN, DYNC1H1 and a subset of tubulin encoding genes, are responsible for these disorders (reviewed (Mirzaa and Paciorkowski 2014)). Since these genes are involved in multiple processes during cortical development, the disorders may result from a combination of abnormalities. Moreover, defects in interneuron migration may disrupt the excitatory/inhibitory balance of neural circuitries and result in psychiatric disorders, such as depression, anxiety, and schizophrenia (Di Cristo 2007).

Alterations in dendrite morphology have been observed in a number of neurodevelopmental disorders (reviewed in (Kulkarni and Firestein 2012)). Patients with autism spectrum disorders display decreased dendrite branching and increased spine density in the hippocampus (Raymond, Bauman et al. 1996, Hutsler and Zhang 2010), and neurons of patients with schizophrenia or Down syndrome show reduced dendritic arbor and spine density in different brain regions (Takashima, Ieshima et al. 1989, Becker, Mito et al. 1991, Garey, Ong et al. 1998, Glantz and Lewis 2000, Broadbelt, Byne et al. 2002, Kolluri, Sun et al. 2005, Hill,

Hashimoto et al. 2006). Given that many of the autism and schizophrenia high-risk genes actually regulate dendrite morphogenesis, it has been proposed that dendrite morphology may be essential in the etiology of neurodevelopmental disorders (Copf 2016).

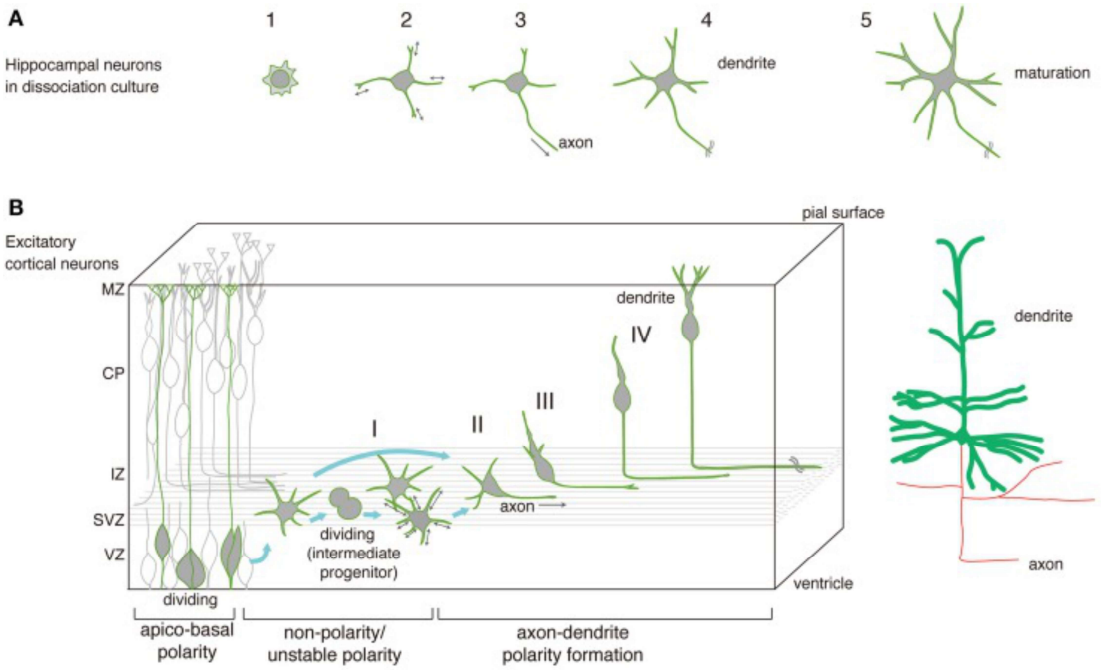



Woodworth et al., Cell, 2012

**Figure 1. Neurogenesis and neuronal migration in the developing mouse neocortex**

The schematic depicts the generation of cortical projection neurons and their migration to different cortical layers. Starting at E11.5, radial glial cells (RG), neural progenitors, divide within the ventricular zone (VZ) to generate both postmitotic neurons and intermediate progenitor cells (IP). IPs then migrate and divide within the subventricular zone (SVZ) to produce a pair of postmitotic neurons. These newly born neurons, from both VZ and SVZ, migrate along radial glial fibers to reach their final destinations in the cortical plate (CP). Distinct subtypes of projection neurons are born sequentially during neurogenesis and migrate to cortical layers following an “inside-out” fashion: later-born neurons migrate and surpass earlier-born neurons and reside more superficially in the CP, while earlier born neurons reside in

the deep layers of the cortex. CR, Cajal-Retzius cell; SP, subplate neuron; CThPN, corticothalamic projection neuron; CPN, callosal projection neuron; DL, deep layer (layers V and VI); oRG, outer radial glia; UL, upper layer (layers II/III and IV); NE, neuroepithelial cell; GC, granule cell. The schematic is reproduced from Woodworth et al., 2012.




Sakakibara and Hatanaka, Front Neurosci, 2015

**Figure 2. Stages of neuronal development *in vitro* and *in vivo***

Neuron developmental stages. **A**. In hippocampal neuron cultures, dendrites undergo 5 stages of development: Stage 1, formation of filopodia and lamellipodia in immature neurons; Stage 2, outgrowth of multiple minor neurites from the cell body; Stage 3, axon specification and extension; Stage 4, dendrite elongation and active branching; Stage 5, formation of spines and synapses. **B**. During embryonic brain development, (I) Immature neurons are generated in the ventricular zone (VZ) and subventricular zone (SVZ) and gain multipolar morphology; (II) A new process extends and becomes the axon; (III) Other neurites become dendrites and transform into a pia-directed leading process, and neurons gradually become bipolar; (IV)

Bipolar neurons migrate toward the pial surface and mature after reaching their final destination. The schematic is reproduced from Sakakibara and Hatanaka, 2015.

# CHAPTER I:

# THE ROLES OF CPE AND ITS INTERACTOR, P150$^{GLUED}$, IN REGULATION OF THE MICROTUBULE CYTOSKELETON, NEURONAL MIGRATION, AND DENDRITE MORPHOLOGY

**ABSTRACT**

Higher brain functions rely on proper development of the cerebral cortex, including correct positioning of neurons and dendrite morphology to ensure functional neuronal circuitry. Disruptions in the regulation of these processes may result in various neuropsychiatric disorders. Roles for carboxypeptidase E (CPE) in the nervous system have been reported, and mutations in the *CPE* gene have been linked to depression and intellectual disability. However it remains unclear whether CPE is involved in early brain development, and in turn, contributes to the pathophysiology of neuropsychiatric disorders. In this chapter, we investigate the expression profile of CPE in the developing mouse brain and explore the functional significance of the interaction between CPE and p150$^{Glued}$. Our results show that in COS-7 cells, overexpression of CPE redistributes p150$^{Glued}$ from the centrosome through interaction of p150$^{Glued}$ with the carboxyl terminus of CPE. Using *in utero* electroporation, we demonstrate that CPE is required for cortical neuron migration during embryonic development in a p150$^{Glued}$-dependent manner. In hippocampal neuronal cultures, both overexpression and knockdown of CPE result in decreased dendrite branching, suggesting that a balance of CPE levels is required for proper dendritogenesis. In summary, Chapter I identifies functions for CPE during early brain development and a potential involvement for CPE in the development of neuropsychiatric diseases. In addition, a model depicting the impact of CPE and p150$^{Glued}$ interaction in neurodevelopment is proposed.

## INTRODUCTION

Functional connectivity between neurons in the brain depends on where the neurons are positioned and how their dendrites are shaped during development. Abnormal neuronal migration and dendrite patterning may result in a various neurocognitive disorders due to developmental defects. Often, aberrant brain structure results from improper function or expression of a complex of proteins rather than of a single protein. Thus, studying the interaction between multiple proteins that regulate neuronal development will aid in our understanding of processes that result in abnormal brain function.

Carboxypeptidase E (CPE) was identified as a prohormone-processing enzyme (Fricker and Snyder 1982, Hook, Eiden et al. 1982). Recent studies have reported its non-enzymatic functions in the endocrine and nervous systems, including prohormone sorting and vesicle transport (reviewed in (Cawley, Wetsel et al. 2012)). One neuropeptide sorted and transported by CPE is brain-derived neurotrophic factor (BDNF). Disruption of CPE binding to BDNF-containing vesicles results in reduced BDNF localization to neurites of hippocampal neurons (Lou, Kim et al. 2005, Park, Cawley et al. 2008). The importance of CPE function in the brain has been further supported by studies on $CPE^{-/-}$ mice and humans. The knockout mice exhibit aberrant dendritic architecture and spine morphology, deficits in learning and memory, and neurodegeneration under stress (Woronowicz, Koshimizu et al. 2008, Woronowicz, Cawley et al. 2010). Studies on a spontaneous mutant mouse model,

*Cpe*$^{fat/fat}$, which lacks CPE enzymatic activity due to a point mutation in the *Cpe* gene (Coleman and Eicher 1990, Fricker, Berman et al. 1996), revealed anxiety and depressive-like behaviors in these mice (Rodriguiz, Wilkins et al. 2013). Similarly, a truncation mutation of carboxypeptidase E (CPE) was identified in a morbidly obese human female with intellectual disability (Alsters, Goldstone et al. 2015), suggesting that this protein plays a role in brain function in both species.

The mechanism by which CPE regulates neurodevelopment is still poorly understood, but one of its binding partners, p150$^{Glued}$, may play an important role in CPE function. The cytoplasmic tail of CPE interacts with p150$^{Glued}$ (Park, Cawley et al. 2008), the major subunit of the dynactin complex, which binds directly to microtubules and motor proteins (reviewed in (Schroer 2004)). Dynactin is involved in coordination of bi-directional cargo transport by regulating both retrograde transport mediated by dynein and anterograde transport mediated by kinesin-2 (King and Schroer 2000, Berezuk and Schroer 2007). In addition to regulation of motor activities, dynactin functions at the centrosome to anchor microtubules and recruit cell cycle regulators (Quintyne, Gill et al. 1999, Quintyne and Schroer 2002). Mutations in the *dynactin* gene (*DCTN1*), encoding for p150$^{Glued}$, are associated with Perry syndrome, characterized by early-onset Parkinsonism, depression, weight loss, and hypoventilation (Farrer, Hulihan et al. 2009, Wider, Dachsel et al. 2010). In addition, it was recently reported that full-length p150$^{Glued}$ is a neuron-specific anti-catastrophe factor of microtubules, and one of the causal mutations for Perry

syndrome present in p150$^{Glued}$ abolishes this anti-catastrophe activity (Lazarus, Moughamian et al. 2013). Thus, by regulating microtubule dynamics and molecular motors, p150$^{Glued}$, may play a role in proper brain structure and function.

As such, understanding the roles of CPE and p150$^{Glued}$ and their interaction in regulating brain development can provide insight into mechanisms underlying neurocognitive and neuropsychiatric disorders, such as major depressive disorder and Perry syndrome, as well as potential therapeutic targets these disorders. We previously reported the involvement of CPE in mediating Nitric Oxide Synthase 1 Adaptor Protein (NOS1AP)-mediated decreases in dendritic arborization, but an understanding of the mechanism by which CPE itself regulates dendrite branching has not been fully elucidated. In addition, since CPE interacts with p150$^{Glued}$, and members of the dynactin complex regulate cortical development (Smith, Niethammer et al. 2000, Tai, Dujardin et al. 2002, Dujardin, Barnhart et al. 2003), in the current study, we asked whether CPE plays a role in cortical neuron migration. We also investigated the effects of CPE overexpression or knockdown on dendrite branching and explored the role of its carboxyl terminal interaction with p150$^{Glued}$ in mediating these effects. We found that CPE is required for proper cortical neuron migration and that intermediate expression of CPE protein is needed to maintain dendrite morphology in hippocampal neurons. Furthermore, both of these CPE functions are dependent on the carboxyl terminus, and hence, interaction with p150$^{Glued}$. These results underscore the importance of CPE during brain

development and suggest that disruption of CPE and p150$^{Glued}$ function may result in impaired brain function, such as that observed in patients with major depressive disorder and Perry syndrome.

## METHODS AND MATERIALS

### DNA constructs and RNA interference

Plasmids encoding rat full-length CPE protein were subcloned into pEGFP-C1 and pCAG-GFP vectors. The CPEΔC10 construct was generated by deleting the carboxy terminal 10 amino acids of the CPE full-length construct, and CPE-C10 construct contains the carboxy terminal 10 amino acids of the CPE full-length construct. Mutant forms of CPE (CPEΔC10, CPE-C10) were subcloned into pEGFP-C1 and pCAG-GFP vectors. siRNA against CPE and negative control siRNA were purchased from Life Technologies (CPE siRNA, Mouse S64324; CPE siRNA Rat S220210; negative control #1, cat# 4390743). shRNAs targeting the CPE transcript (5'-GGTGGAATGCAAGACTTCA-3'), and an un-related sequence (5'-GAGCATTTGTATGAGCGCG-3') were designed and ligated into the pGE2hrGFPII vector (Agilent Technologies). pCAG-IRES-EGFP plasmid (pCIG) was a gift from Gabriella D'Arcangelo (Rutgers University) and pCAG-IRES-TagRFP plasmid (pCIR) was a gift from Marie-Catherine Tiveron (Institut de Biologie du Développement de Marseille, France).

### Antibodies and reagents

Mouse CPE (BD 610758) and mouse MAP2 (BD 556320) primary antibodies were purchased from BD Pharmingen. Mouse Dynactin $p150^{Glued}$ antibody (SC-135890) was from Santa Cruz. Rabbit anti-pericentrin (ab4448) was from Abcam. Chicken

anti-GFP (PA-9533) was from Thermo Fisher. Mouse GAPDH (MAB374) and chicken β-III tubulin (AB9354) primary antibodies were from EMD Millipore.

**COS-7 cell transfection and immunocytochemistry**

COS-7 cells were plated at 15,800 cells/cm$^2$ on 0.1mg/ml poly-d-lysine-coated coverslips and transfected 24hrs after plating with pEGFP-C1, pEGFP-C1-CPE, pEGFP-C1-CPEΔC10 or pEGFP-C1-CPE-C10 using Lipofectmine 2000 (Thermo Fisher) following the manufacturer's protocol. Cells were fixed 2 days after transfection with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 15min and immunostained for GFP, pericentrin, and Dynactin p150$^{Glued}$, followed by nuclear staining with Hoechst dye. Coverslips were mounted onto glass slides with Fluoromount G (Southern Biotechnology; Birmingham, AL).

**Immunofluorescent microscopy and p150$^{Glued}$ localization analysis**

Slides of COS-7 cells were prepared as described above. Images were taken at 600X using an Olympus Optical (Tokyo, Japan) IX50 microscope and fluorescent imaging system. For assessment of p150$^{Glued}$ localization, a straight line was drawn from one side of the cell through the centrosome (positive for pericentrin immunostaining) and nucleus (labeled by Hoechst dye) to the other end of the cell. To quantify the percentage of p150$^{Glued}$ concentrated on the centrosome, the intensity of p150$^{Glued}$ and pericentrin was plotted along the line. p150$^{Glued}$ intensity within the peak of

pericentrin was measured (value A), and its intensity within 1 micron away on each side was measured (value B). The ratio of A to B was calculated to get the percentage of $p150^{Glued}$ localized to the centrosome.

**Primary neuronal culture and dendrite branching analysis**

Neuronal cultures were prepared from hippocampi of rat embryos at 18 days of gestation as described previously (Firestein, Firestein et al. 1999). Cells dissociated from the hippocampi were plated at 10500 cells/$cm^2$ onto 0.1mg/ml poly-d-lysine-coated coverslips and maintained in Neurobasal medium (Thermo Fisher) supplemented with B27 (Thermo Fisher), GlutaMAX (Thermo Fisher), penicillin, and streptomycin. Cultures were co-transfected with pCAG-mOrange and indicated CPE constructs or siRNA at day *in vitro* (DIV) 7 using Lipofectamine LTX with Plus (Thermo Fisher), fixed at DIV 10 with 4% PFA in PBS, and immunostained for GFP and MAP2. Images of neurons were taken using Olympus Optical (Tokyo, Japan) IX50 microscope and fluorescent imaging system, and dendrite morphology was assessed as previously described (Kutzing, Langhammer et al. 2010, Langhammer, Previtera et al. 2010).

**Co-immunoprecipitation and Western blot analysis**

Adult rat cortices were dissected and homogenized in HEPES/Sucrose buffer (20 mM HEPES (pH 7.5), 320 mM sucrose, 1 mM EDTA, 5 mM DTT, 1 mM PMSF).

Neuro2a cells were transfected 24hrs after plating with pEGFP-C1, pEGFP-C1-CPE, or pEGFP-C1-CPEΔC10 using Lipofectmine LTX Plus, and then collected and lysed 2 days after transfection in HEPES/Sucrose buffer. Rat cortical brain extracts and Neuro2a cell lysates were centrifuged at 350 x *g* to pellet unbroken cells or tissue debris. The supernatant was collected and centrifuged at 100,000 x *g* at 4$^{o}$C for 1hr to fractionate cytosolic proteins. Equal amounts of cytosol proteins were pre-cleared with protein G agarose (50% slurry (GE Healthcare, Piscataway, NJ)) for 1 hour and then subjected to immunoprecipitation with monoclonal Dynactin p150$^{Glued}$ antibody at 4$^{o}$C overnight after addition of 0.05% bovine serum albumin (BSA) to the protein extract. Samples were then incubated with protein G agarose for 1 hour, and immunoprecipitates were washed with buffer (0.1% Triton X-100, 50mM Tris, PH 7.4, 300mM Nacl, 5mM EDTA, 0.02% $NaN_3$). Proteins were eluted with protein loading buffer and resolved by SDS-PAGE, and transferred to PVDF membranes. Membranes were probed for GFP or CPE, and Dynactin p150$^{Glued}$. Immunoreactive bands were visualized with HyGlo quick spray (Denville Scientific; South Plainfield NJ), and resulting films were quantified with ImageJ software (http://rsb.info.nih.gov/ij/; National Institutes of Health, Bethesda, Maryland).

### *In utero* electroporation, immunohistochemistry, and microscopy

*In utero* electroporation was performed as we previously described (Carrel, Hernandez et al. 2015). Pregnant Swiss mice at gestation day 14.5 (E14.5) were

anesthetized with isoflurane. The abdominal cavity was opened to expose the uterine horns. 1-3 µl of plasmids (2-2.5 µg/µl) with 1 mg/mL Fast Green (Sigma) were microinjected through the uterus into the lateral ventricles of embryos by pulled glass capillaries (Drummond Scientific, Broomall, PA). Electroporation was performed by placing the heads of the embryos between tweezer-type electrodes. Square electric pulses (35 V, 50 ms) were passed five times at 1-s intervals using a NEPA21 electroporator (SONIDEL Limited, Dublin, Ireland). Embryos were allowed to develop *in utero* for 3 days after electroporation (until E17.5). Mouse brains were dissected and fixed for 48 h in 4% PFA in PBS at 4°C. Brains were cryoprotected in 30% sucrose in PBS, frozen in PolyFreeze (Sigma), and sectioned coronally at 16 µm using a cryostat. Images of fluorescent mouse brain sections were taken on a Zeiss Axio Observer.Z1 microscope using a 20× numerical aperture (NA) 0.8 objective, with a Clara E CCD Camera (Andor Technology Limited, Belfast, UK).

**Neuronal migration analysis**

TagRFP positive cells were counted using the image analysis software ImageJ (http://rsb.info.nih.gov/ij/; National Institutes of Health, Bethesda, Maryland). For each section analyzed, cortical regions of interest containing positive cells were manually selected using Hoechst staining of the nuclei. Then, for each region, we used a combination of ImageJ built-in minimum and unsharp mask filters to enhance the signal due to fluorescent cell bodies while lowering the signal due to

fluorescent processes. Cells were automatically counted as local maxima, while keeping the same level of noise tolerance for a given set of experiments (and after validation of this level by manual counting of three to four sections). To normalize the analysis, we used median sections in a series of sections containing transfected cells (two sections per brain at E20, separated by at least 48 mm). Therefore, brains that did not meet these two criteria were discarded.

## RESULTS

### CPE expression in neurons of the developing rodent cortex

To address the role of CPE in brain development, we examined the expression profile of CPE protein at different developmental stages. We studied CPE expression in mouse brain from embryonic day (E)12 to postnatal day (P)10, which is a crucial time window for positioning of neurons in different layers of the neocortex and in single neuron development (Jiang and Nardelli 2016). Cortices from mice at E12, E14, E16, P0, P10, and P60-90 were isolated and examined by Western Blot analysis. CPE expression is detected as early as E12, and remains stable from E12 to E16. The expression level significantly increases from E16 to P0, and P0 to P10. When growing into adulthood, CPE protein level drops and maintains at a similar level as seen in P0. (**Figure I-1A, B**). These results suggest that CPE may play an important role during embryonic and postnatal brain development.

To further investigate CPE expression in specific cell types, we dissected brains from rats at E18 and immunostained for CPE and neuron-specific class III β -tubulin (Tuj1) in the lateral neocortex. We observed that cells expressing high levels of CPE protein are, for the most part, positioned in the cortical plate (CP), while cells in the intermediate zone (IZ) and ventricular zone (VZ) show much lower CPE expression levels (**Figure I-1C**). Moreover, cells expressing CPE are Tuj1-positive (**Figure I-1C**), indicating that CPE is expressed specifically in neurons of the neocortex.

Finally, we examined the subcellular distribution of CPE in cultured primary hippocampal neurons. CPE protein is evenly distributed throughout dendrites and co-localizes with β-III tubulin (**Figure I-1D**), suggesting a function for CPE other than its previously reported role in vesicle transport (Park, Cawley et al. 2008).

**CPE overexpression affects p150$^{Glued}$ localization and are mediated by the carboxyl terminus of CPE**

It has been reported that in pituitary corticotrope tumor (AtT20) cells (Park, Cawley et al. 2008), CPE interacts with p150$^{Glued}$, the largest subunit in the Dynactin protein complex. To confirm whether this interaction between CPE and p150$^{Glued}$ exists in neuronal cells, we performed a co-immunoprecipitation assay using rat brain lysates. As expected, p150$^{Glued}$ co-immunoprecipitates with CPE (**Figure I-2A,B**). In addition, CPE is thought to bind to p150$^{Glued}$ via its carboxyl terminus (Park, Cawley et al. 2008). To confirm that CPE lacking the carboxyl terminal 10 amino acids (CPEΔC10) cannot bind to p150$^{Glued}$, we transfected Neuro2a cells with constructs encoding GFP (control), GFP-tagged CPE, or GFP-tagged CPEΔC10. GFP antibody was used to detect the presence of GFP-tagged CPE (**Figure I-2C**). As predicted, CPE co-immunoprecipitates with p150$^{Glued}$, whereas CPEΔC10 does not (**Figure I-2D**), demonstrating that CPEΔC10 can be used to study the involvement of the interaction of CPE and p150$^{Glued}$ in CPE function.

p150$^{Glued}$ predominantly localizes to the centrosome and is involved in anchoring microtubules to the centrosome and recruiting cell cycle regulators (Quintyne, Gill et al. 1999, Quintyne and Schroer 2002). Given that CPE interacts with p150$^{Glued}$ (Park, Cawley et al. 2008), it is possible that CPE affects the subcellular localization of p150$^{Glued}$, which in turn alters the cytoskeleton and other downstream processes. To test this possibility, we overexpressed CPE in COS-7 cells, where CPE levels are low, and analyzed the subcellular distribution of p150$^{Glued}$. Centrosomes were labeled by immunostaining of pericentrin, an integral component of the pericentriolar material (Doxsey, Stein et al. 1994). In cells expressing GFP, we observed abundant co-localization of p150$^{Glued}$ with pericentrin at the centrosome, while CPE overexpression results in a significant decrease in the percentage of p150$^{Glued}$ localized at the centrosome (**Figure I-3A,B**).

We next asked whether the effect of CPE overexpression on p150$^{Glued}$ redistribution is dependent on the interaction of p150$^{Glued}$ with the carboxyl terminal 10 amino acids of CPE protein (Park, Cawley et al. 2008). Therefore, to answer the question, in addition to GFP-tagged CPEΔC10, we generated a construct encoding a GFP-tagged CPE protein of only the last 10 amino acids (CPE-C10). We hypothesized that expression of GFP-tagged CPEΔC10 would not affect p150$^{Glued}$ subcellular distribution, and expression of CPE-C10, serving as a dominant-negative control, would lead to a similar phenotype as observed with overexpression of CPE. As predicted, localization of p150$^{Glued}$ remains unaltered when CPEΔC10 is

expressed, and expression of CPE-C10 results in significantly less $p150^{Glued}$ localized to the centrosome, similar to what is observed with CPE overexpression. Taken together, these results support our hypothesis that CPE-promoted changes to $p150^{Glued}$ localization from the centrosome is dependent on the interaction between CPE and $p150^{Glued}$.

**CPE is required for proper cortical neuron migration**

It has been reported that dynactin and associated proteins regulate cortical cell radial migration (Tsai and Gleeson 2005). Since CPE associates with $p150^{Glued}$, we investigated whether CPE deficiency alters neuronal migration. Thus, we designed a short-hairpin (sh)RNA against CPE to examine the effect of CPE knock down *in vivo*. To test the specificity of the shRNA, we employed both Western blotting and immunohistochemistry. Rat cortical neurons expressing CPE shRNA demonstrate a 50% decrease in CPE levels compared to neurons expressing control shRNA (**Figure I-4A,B**). Immunostaining for CPE in coronal brain sections after *in utero* electroporation (IUE) further support knockdown of CPE in single transfected neurons (**Figure I-4C**).

To examine cortical neuron migration, we performed IUE in mice at E14.5, introducing either CPE shRNA or scramble control shRNA together with cDNA encoding red fluorescent protein (RFP) and analyzed the distribution of

RFP-positive cells in each cortical area at E17.5. In brain sections from mice expressing control shRNA, ~45% of transfected cells migrate to the cortical plate (CP), and ~40% of transfected cells are located in the intermediate zone (IZ). In contrast, only ~25% of transfected cells reach the CP when CPE is knocked down, and a significant amount of transfected cells stall in the IZ (**Figure I-5A, B**).

We next tested the role of the carboxyl terminus of CPE in regulating neuronal migration. GFP-tagged CPE, CPEΔC10, CPE-C10, or GFP (control) was expressed in neural progenitor cells using IUE at E14.5, and neuronal migration was examined at E17.5. We observed no change in migration when CPE or CPEΔC10 was expressed. However, expression of CPE-C10 results in a significant decrease in the percentage of cells reaching the CP (**Figure I-5C,D**), suggesting that binding of an interacting protein, such as $p150^{Glued}$, may be necessary for proper cortical migration. CPE-C10 acts in a dominant negative manner and disrupts the binding of $p150^{Glued}$ or another protein to CPE. Together, our data suggest that CPE is required for proper cortical neuron migration and its carboxyl terminus, and hence interaction with $p150^{Glued}$, may be involved in this process.

**CPE-promoted effects on dendrite branching are dependent on its carboxyl terminal**

We previously reported that CPE mediates the effect of NOS1AP on dendrite

morphology *in vitro* (Carrel, Du et al. 2009). To further explore possible mechanisms underlying CPE regulation of dendrite branching, and specifically whether the effect is dependent on interaction with $p150^{Glued}$, we overexpressed or knocked down CPE in primary hippocampal neuronal cultures. Dendritogenesis was examined between day in vitro (DIV) 7 and DIV 10, as both primary dendrite extention and higher order dendrite formation actively take place during this time window (Banker and Goslin 1988, Dotti, Sullivan et al. 1988). Cultured hippocampal neurons were co-transfected with cDNA encoding mOrange and GFP, GFP-tagged CPE, CPEΔC10, or CPE-C10 at DIV 7, and neurons positive for both mOrange and GFP were analyzed for dendrite branching at DIV 10. Overexpression of CPE and CPE-C10 result in significantly decreased dendrite branching close to soma (0-12μm and 0-18μm, respectively; **Figure I-6A-C**). Overexpression of CPEΔC10 has no effect on dendrite branching, suggesting that CPE affects dendrite branching via its carboxyl terminus.

To knock down CPE protein in cultures, we used a commercially purchased siRNA against rat CPE, and its specificity was demonstrated by immunocytochemistry (**Figure I-6F**). Transfected neurons show ~50% reduction in CPE protein levels (**Figure I-6G**). Interestingly, knockdown of CPE results in a similar effect on dendrite branching as does overexpression, decreasing dendrite branching at a distance of 0-42μm from the soma (**Figure I-6D,E**). These data suggest that a balance of CPE protein levels may be required to maintain proper dendritic arborization. Taken together, our results strongly indicate that CPE

regulates proximal dendrite branching and that this function is dependent on its interaction with $p150^{Glued}$ via it carboxyl terminus.

## DISCUSSION

### Expression of CPE during embryonic brain development

CPE expression is detected in all major areas of the adult rat brain (Birch, Rodriguez et al. 1990), and its roles in prohormone processing and sorting and vesicle transport have been well-studied (reviewed in (Cawley, Wetsel et al. 2012)). In addition to the adult nervous system, CPE expression has been reported in mammalian embryos (Zheng, Streck et al. 1994, Selvaraj, Xiao et al. 2017). Interestingly, studies of CPE expression during mouse brain development show that CPE is expressed in neural tissues earlier than other prohormone convertases, such as PC1 and PC2, suggesting a distinct role for CPE during embryonic neurodevelopment. In this study, we elucidate the expression profile of CPE in mouse brain at different developmental stages. In mouse cortices, CPE protein level increases during embryonic and early postnatal development with a peak of expression at P10, and higher CPE expression is detected in neurons in the CP than in the IZ or VZ. At the cellular level, endogenous CPE is evenly distributed throughout the soma and neurites, and it co-localizes with β-III tubulin throughout the neurites. It was previously reported that overexpressed CPE in hippocampal neurons localize in punctate vesicles and mediate vesicle transport (Park, Cawley et al. 2008). Our findings suggest CPE may have other functions besides sorting and transporting of vesicles in neurons. Taken together, we proposed that CPE might contribute to early brain development, and impairments in neuronal positioning and

morphology resulted by CPE gene mutations and protein level alterations may be etiologic for neuropsychiatric and neurocognitive deficits exhibited in $CPE^{-/-}$ mice and $Cpe^{fat/fat}$ mice models (Woronowicz, Koshimizu et al. 2008, Rodriguiz, Wilkins et al. 2013). In human subject, CPE gene mutation related mental disorders, such as dementia and depression in an Alzheimer's patient with CPE-QQ mutation (Cheng, Cawley et al. 2016), and intellectual disability in a morbidly obese female with a truncating mutation of CPE (Alsters, Goldstone et al. 2015), may also be explained by CPE-mediated neurodevelopment disruptions.

**Roles of CPE in cortical development and dendrite morphogenesis**

It has been well-established that abnormalities in cortical neuron migration and dendrite morphology may affect higher brain functions, such as learning, memory, emotion, and cognition, and may cause psychiatric disorders including depression, bipolar disorders, and schizophrenia (Valiente and Marin 2010, Barkovich, Guerrini et al. 2012, Brennand, Simone et al. 2012, Kulkarni and Firestein 2012). How do our data fit into the current understanding of neurodevelopment and relevant diseases? During development of the cerebral cortex, pyramidal neurons are generated by neural progenitor cells in the VZ or subventricular zone (SVZ), and migrate towards the pial surface. Here, we show that neurons electroporated with a CPE shRNA construct fail to migrate to the CP, suggesting that CPE is required for proper neuronal migration. Given the fact that CPE overexpression does redistribute

p150$^{Glued}$ from the centrosome in COS-7 cells, it is quite surprising that we do not see alterations in cortical neuron migration with CPE overexpression *in utero*. One possibility is that the sensitivity of neurons to elevated CPE protein level during embryonic development is less than that of neurons *in vitr*o, likely due to the existence of complicated regulatory cues for neuronal migration *in viv*o (Marin, Valiente et al. 2010).

As neurons reach their final destination, they differentiate into a multipolar morphology with defined axons and dendritic arbors (Takano, Xu et al. 2015). Proper development of dendrite morphology, including shape, size, and patterning of the dendritic arbor, is critical for many aspects of neuronal function, such as the number and type of synapses that will be formed along the dendrites, electrical properties of a neuron, and the positioning of a particular signal (London and Hausser 2005, Spruston 2008). In this study, we report that both CPE overexpression and knockdown result in decreased dendrite branching close to the soma in young hippocampal neuronal cultures, suggesting that a balance of CPE protein level is needed to maintain proper dendritogenesis. Previous study in 14-week-old CPE$^{-/-}$ mice exhibited increased dendritic branching proximal to the soma, and decreased branching in distal dendritic arbor (Woronowicz, Cawley et al. 2010). Our findings further demonstrate the importance of CPE in dendritogenesis, and indicate that loss of CPE at different developmental stages may have distinct impact on dendrite morphology. Taken together, the defects in neuronal migration

and dendritic architecture caused by alterations to CPE protein levels are in line with the phenotypes observed in CPE$^{-/-}$ and *Cpe*$^{fat/fat}$ mouse models, and individuals with CPE mutations, such as deficits in learning and memory, anxiety and depressive-like behaviors, and intellectual disability (Cawley, Zhou et al. 2004, Rodriguiz, Wilkins et al. 2013, Alsters, Goldstone et al. 2015, Cheng, Cawley et al. 2016).

**Mechanisms by which CPE regulates cortical development and dendrite morphology**

Neuronal migration and dendrite morphogenesis are dependent on continuous morphological changes mediated by the cytoskeleton, especially the centrosome and microtubule network (reviewed in (Dent 2017, Fukuda and Yanagi 2017)). A number of risk genes have been identified for psychiatric disorders, and many are involved in cytoskeletal regulation (Copf 2016, Fukuda and Yanagi 2017). p150$^{Glued}$ plays an important role in regulating the microtubule network (King and Schroer 2000, Askham, Vaughan et al. 2002, Deacon, Serpinskaya et al. 2003, Schroer 2004, Ligon, Shelly et al. 2006, Berezuk and Schroer 2007, Lloyd, Machamer et al. 2012, Lazarus, Moughamian et al. 2013). In the present study, we confirm the interaction between the CPE carboxyl terminus and p150$^{Glued}$ in rat brain lysate and identify, for the first time, a function for the CPE carboxyl terminus in regulating the localization of p150$^{Glued}$ at the centrosome. The observation that CPEΔC10, which does not bind

to p150$^{Glued}$, does not promote changes to dendrite branching caused by CPE overexpression strongly suggests an involvement of the CPE-p150$^{Glued}$ interaction in mediating the effects of CPE. Moreover, expression of CPE-C10, which may act in a dominant negative manner to disrupt the normal interaction between CPE and p150$^{Glued}$, alters p150$^{Glued}$ localization, neuronal migration, and dendrite branching to a greater degree than does overexpression of CPE. Multiple lines of evidence from previous studies support the idea that p150$^{Glued}$ regulates cortical development and dendrite branching, giving further evidence that CPE may act in a p150$^{Glued}$-dependent manner. First, accumulation of p150$^{Glued}$ at the centrosome and microtubule plus-end is critical for maintaining microtubule stability and dynamics (Quintyne, Gill et al. 1999, Lazarus, Moughamian et al. 2013). Therefore, disrupted p150$^{Glued}$ localization may affect microtubule organization, and in turn, alter neuronal migration and dendrite branching. The fact that mutations of the *DCTN1* gene, encoding p150$^{Glued}$, within the glycine-rich (CAP-Gly) domain render the affinity of dynactin for microtubules and cause Perry syndrome further supports a role for the CPE-p150$^{Glued}$ interaction in neurodevelopmental processes (Farrer, Hulihan et al. 2009). Second, several cytoskeletal regulators, such as Disrupted in Schizophrenia – (DISC1), regulate cortical migration and are associated with psychiatric disorders (Fukuda and Yanagi 2017). In fact, DISC1 is a risk gene for various neurodevelopmental disorders, regulates the localization of dynein-dynactin complex at the centrosome, and hence, contributes to cortical development (Kamiya,

Kubo et al. 2005). Lastly, CPE interacts with p150$^{Glued}$ to recruit motor proteins and transport BDNF-containing vesicles, which increases the number of proximal dendrites in pyramidal neurons (McAllister, Lo et al. 1995, Horch, Kruttgen et al. 1999, Kwinter, Lo et al. 2009).

Taken together, we propose a model (**Figure 7**) for understanding the mechanism underlying CPE-mediated effects on neuronal migration and dendrite branching. A balance of CPE-p150$^{Glued}$ protein complex and free CPE protein is required to maintain microtubule stability and dynamics, and in turn, to maintain proper neuronal migration and dendrite branching. Excess or decreased CPE protein levels may disrupt the balance of the CPE-p150$^{Glued}$ interaction and alter proper subcellular distribution of p150$^{Glued}$ to the centrosome or microtubules. This results in abnormal cytoskeletal function. In summary, our findings provide new insight for the functions of CPE during early brain development and potential involvement in neuropsychiatric diseases and demonstrate the importance of the interaction between the carboxyl terminus of CPE and p150$^{Glued}$ in mediating proper cortical neuron migration and dendrite morphogenesis.

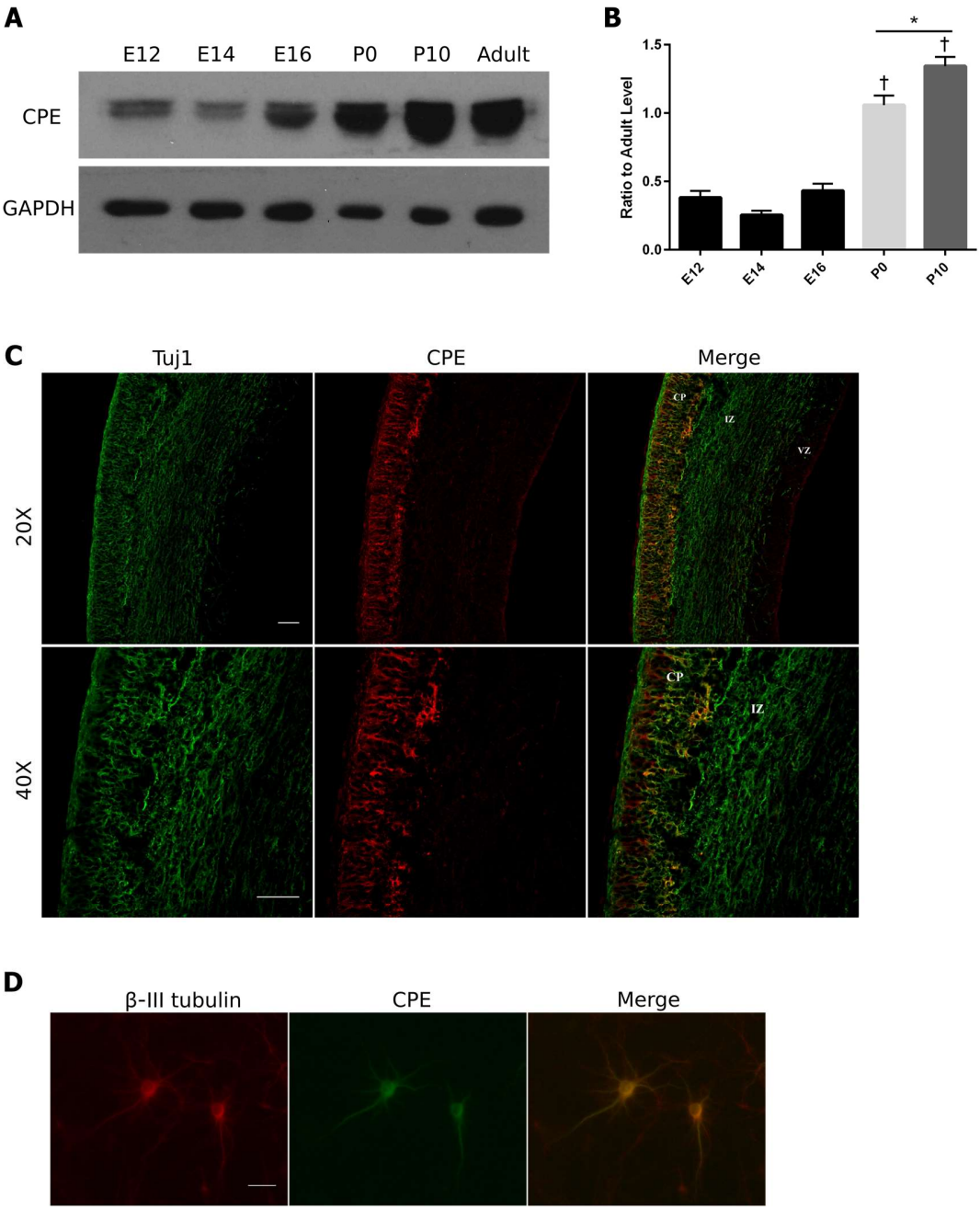



**Figure I-1. Expression profile of CPE in rodent brain**

(A) CPE protein expression increases developmentally in mouse cortex. Proteins were extracted from cortices of mice at the indicated developmental ages of

embryonic (E), postnatal (P) or adult and were resolved by SDS-PAGE. Proteins were transferred to membranes and immunoblotted for CPE and GAPDH. Representative blots are shown. (B) Quantitation of CPE expression level. Intensities of CPE bands were quantitated and normalized to GAPDH intensities. Ratio of CPE level at indicated developmental ages to adult level was taken. Error bars indicate ± S.E.M. n=3 for all conditions. $^{\dagger}p < 0.0001$ (E12, E14 and E16 vs. P0 or P10) as determined by one-way ANOVA followed by Tukey's multiple comparisons test. $^{*}p < 0.05$ as determined by one-way ANOVA followed by Tukey's multiple comparisons test. (C) CPE is expressed predominantly in neurons in the cortical plate (CP). Coronal sections of E18 rat lateral neocortex were immunostained with anti-CPE (red) and anti-Tuj 1 (neuron-specific Class III β-tubulin; green). Images were taken using confocal microscopy. Scale bar = 50μm. VZ, ventricular zone; IZ, intermediate zone; CP, cortical plate. (D) Representative images showing endogenous CPE protein distribution in rat hippocampal neurons at DIV 10. Neurons were immunostained for CPE (green) and β-III tubulin (red). Scale bar = 50μm.

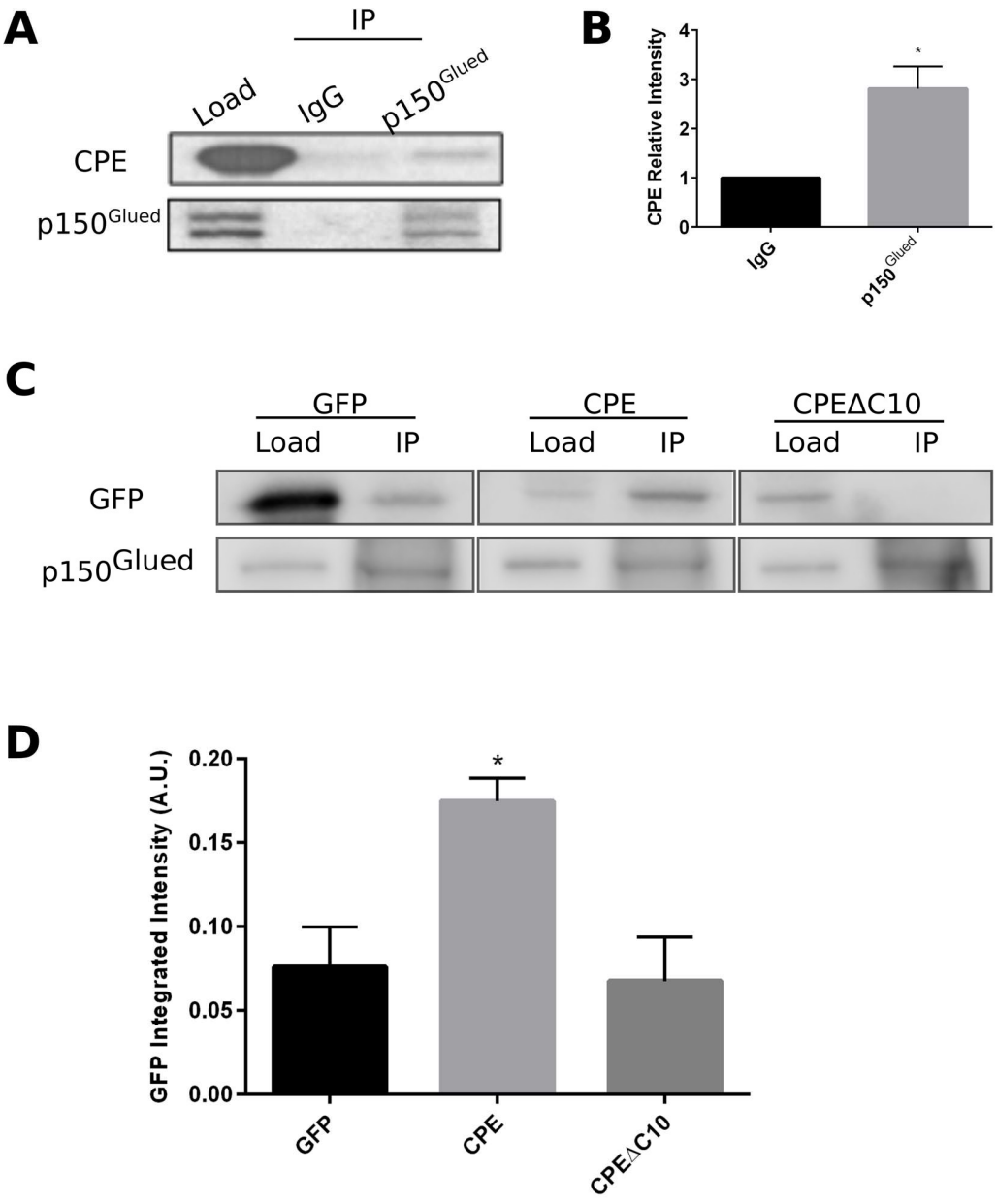
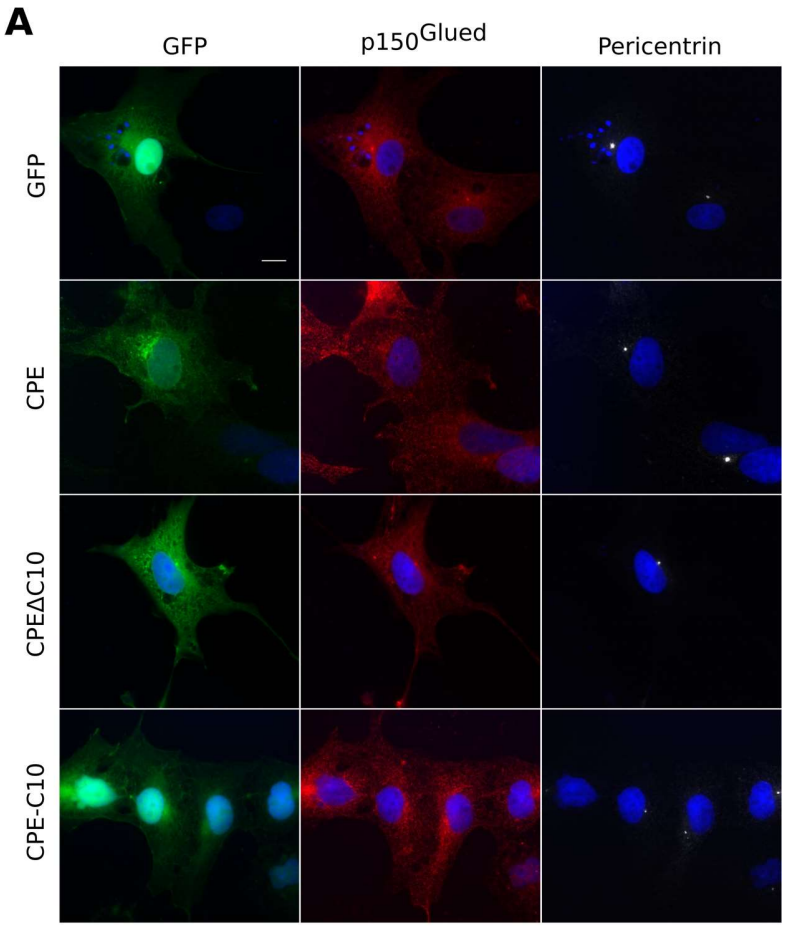



**Figure I-2. CPE interacts with p150$^{Glued}$ via its carboxyl terminus**

(A) CPE and p150$^{Glued}$ co-immunoprecipitate. Adult rat cortices were homogenized. Proteins were extracted and subjected to immunoprecipitation with anti-p150$^{Glued}$ or rabbit-IgG. Cortical protein extract (Load), p150$^{Glued}$ immunoprecipitates (IP;

p150$^{Glued}$) and rabbit-IgG immunoprecipitates (IP; IgG) were resolved by SDS-PAGE. Proteins were transferred to membrane and immunoblotted for CPE and p150$^{Glued}$. Load represents 5% of extracted proteins. Representative blots are shown. (B) Quantitation of CPE band intensities, represented in panel A. Error bars indicate ± S.E.M. n=3. $^{*}p < 0.05$ as determined by paired Student's t-test. (C) Neuro2a cells were transfected with pEGFP (GFP), pEGFP-CPE (CPE) or pEGFP-CPEΔC10 (CPEΔC10) as indicated. Cells were lysed after 48 h, cytosolic proteins were collected and subjected to immunoprecipitation with p150$^{Glued}$ antibody, and cytosol protein extract (Load) or p150$^{Glued}$ immunoprecipitates (IP) were resolved by SDS-PAGE. Proteins were transferred to membrane and immunoblotted for GFP and p150$^{Glued}$. Load represents 4% of extracted proteins. Representative blots are shown. (D) Quantitation of GFP band intensities for CPE constructs, represented in panel C. Error bars indicate ± S.E.M. n=5 for all conditions. $^{*}p < 0.05$ as determined by one-way ANOVA followed by Dunnett's multiple comparisons test. A.U. = arbitrary units.


A
GFP
p150Glued
Pericentrin
GFP
CPE
CPEΔC10
CPE-C10

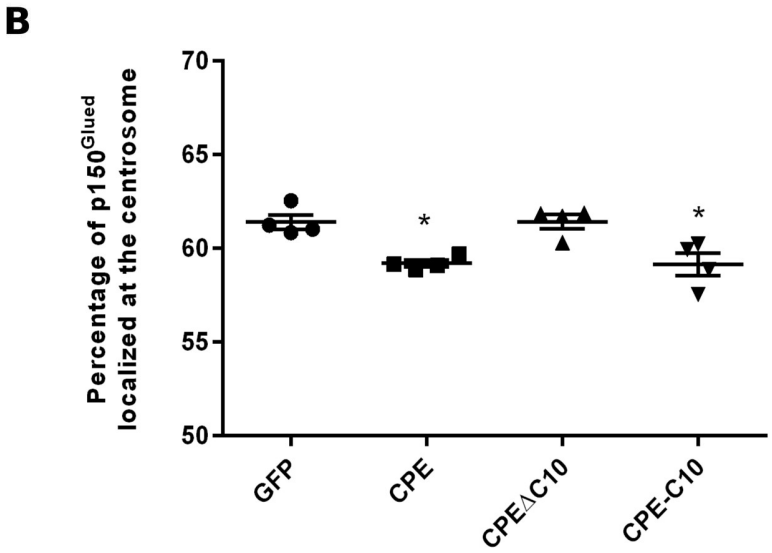

B
Percentage of p150Glued localized at the centrosome
70
65
60
55
50
*
*
GFP
CPE
CPEΔC10
CPE-C10

**Figure I-3. Overexpression of CPE alters p150$^{Glued}$ localization**

(A) Representative images showing p150$^{Glued}$ localization in COS-7 cells. COS-7 cells were transfected with pEGFP (GFP), pEGFP-CPE (CPE), pEGFP-CPEΔC10 (CPEΔC10), or pEGFP-CPE-C10 as indicated. Cells were fixed after 48 h and immunostained with anti-GFP (green), anti-p150$^{Glued}$ (red), anti-pericentrin (white), and nuclei were labeled with Hoechst dye (blue). Scale bar = 10μm. (B) Quantitation of the percentage of p150$^{Glued}$ protein (pixels) localized at the centrosome. For each independent experiment, 15-25 individual transfected cells of each condition were analyzed as described in *Methods and Materials*. Error bars indicate ± S.E.M. n=4 for all conditions. $^{*}p < 0.05$ as determined by repeated measures ANOVA followed by Turkey's multiple comparisons test.

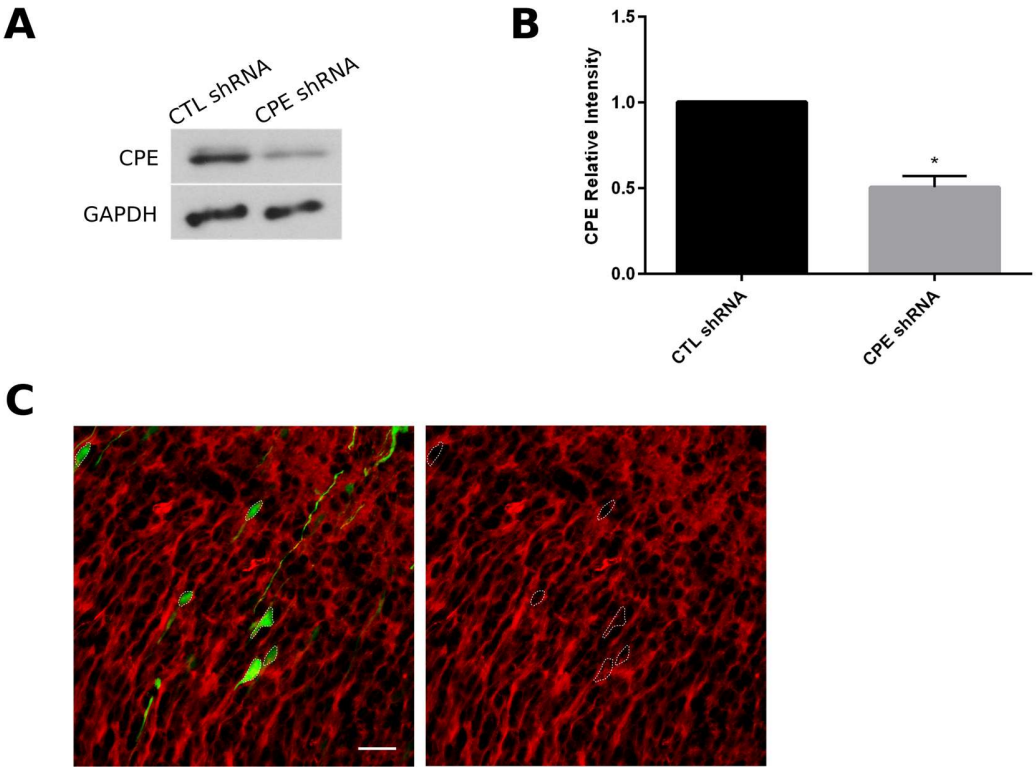



**Figure I-4. Knock down of CPE by shRNA in neurons**

(A) CPE shRNA knockdown efficiency in cortical neurons. E18 cortical neurons were electroporated with pGE-control shRNA (CTL shRNA) or pGE-CPE shRNA (CPE shRNA) before plating using Amaxa Rat Neuron transfection kit. After 96 h, cells were lysed, and extracted proteins were resolved using SDS-PAGE. Proteins were transferred to membrane and probed with antibodies to CPE and GAPDH. Representative blots are shown. (B) Quantitation of CPE protein levels relative to control (normalized to GAPDH). Error bars indicate ± S.E.M. n=3. $^{*}p < 0.05$ by Student's t-test. (C) CPE shRNA knockdown specificity in embryonic neocortex. CPE shRNA construct including GFP tag was electroporated into the lateral ventricle of

E14.5 mouse and analyzed 3 days later. Transfected cells were GFP-positive (green), CPE protein (red) was detected by immunostaining. Representative images of coronal sections of the lateral neocortex are shown. GFP-positive cells (marked by the dotted circles) in the left image showed low level of CPE protein as demonstrated on the right. Scale bar = 25μm.

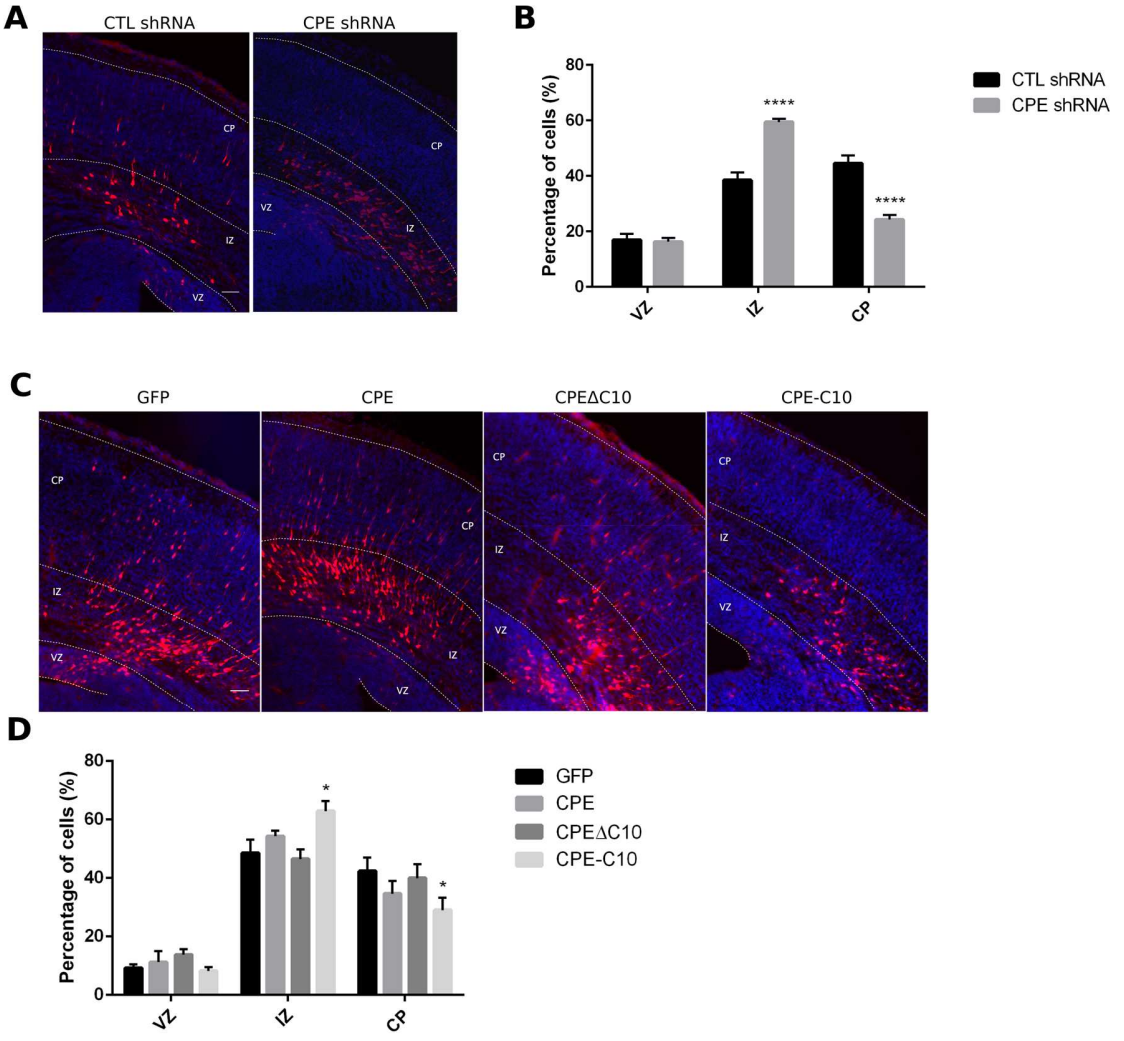



**Figure I-5. CPE is required for proper cortical neuron migration**

(A, C) Mice at E14.5 were electroporated *in utero* with constructs as indicated, and brains were analyzed at E17.5. Representative images of coronal brain sections are shown for each condition. Red, transfected cells; Blue, Hoechst dye. VZ, ventricular zone; IZ, intermediate zone; CP, cortical plate. Scale bar = 50μm. (B, D) Quantitation of the percentage of transfected cells in each cortical area. Error bars indicate ± S.E.M. n=7, CTL shRNA; n=7, CPE shRNA; n=8, GFP; n=4, CPE; n=5, CPEΔC10; n=6,

CPE-C10. $^{****}p < 0.0001$ as determined by two-way ANOVA followed by Sidak's multiple comparisons test. $^{*}p < 0.05$ as determined by two-way ANOVA followed by Tukey's multiple comparisons test.

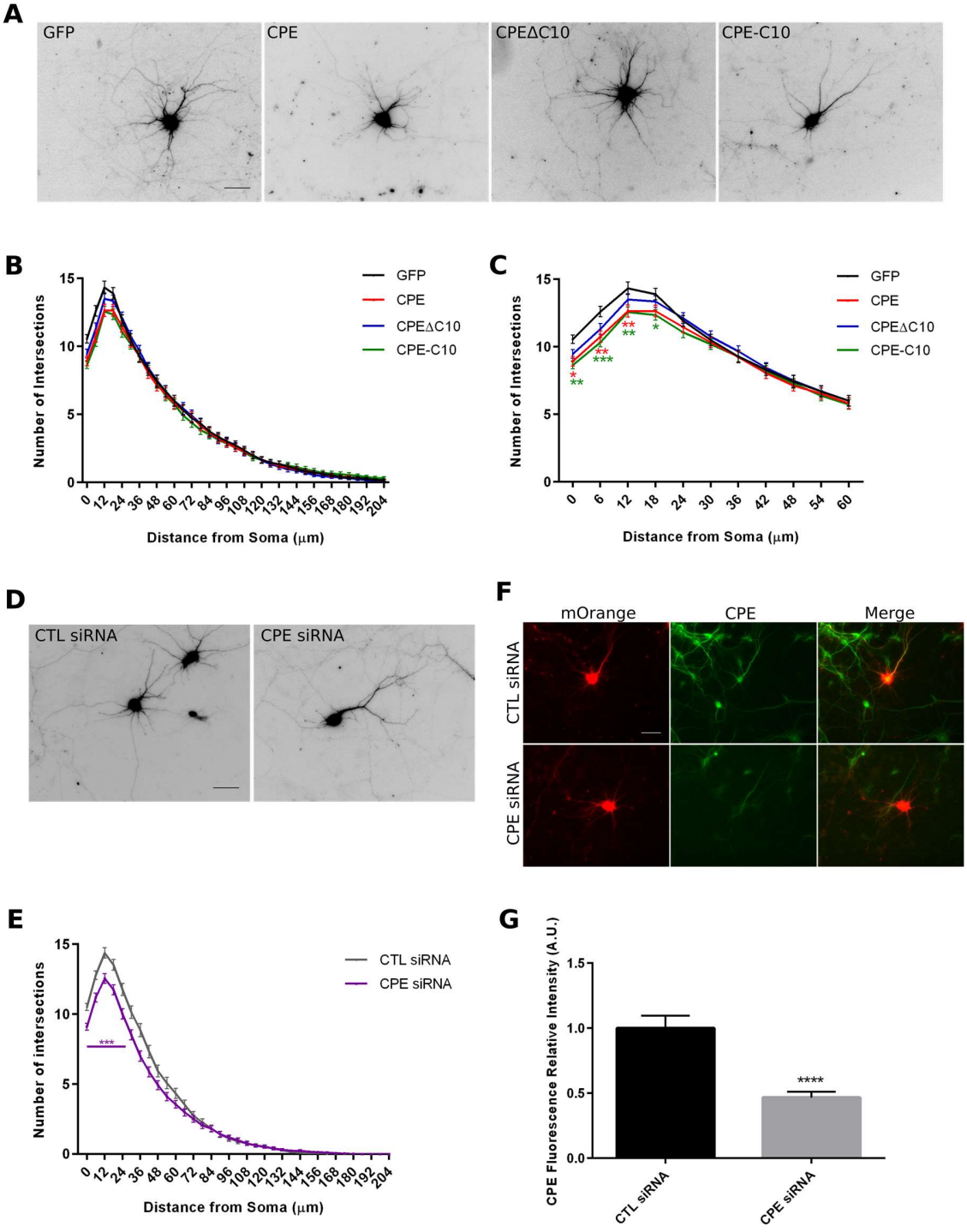

A
GFP
CPE
CPEΔC10
CPE-C10
B
Number of Intersections
Distance from Soma (μm)
GFP
CPE
CPEΔC10
CPE-C10
C
Number of Intersections
Distance from Soma (μm)
GFP
CPE
CPEΔC10
CPE-C10
D
CTL siRNA
CPE siRNA
F
mOrange
CPE
Merge
CTL siRNA
CPE siRNA
E
Number of intersections
Distance from Soma (μm)
CTL siRNA
CPE siRNA
G
CPE Fluorescence Relative Intensity (A.U.)
CTL siRNA
CPE siRNA

**Figure I-6. CPE regulates dendritic arborization in cultured hippocampal neurons**

(A) Hippocampal neurons were co-transfected with pCAG-mOrange and pEGFP (GFP), pEGFP-CPE (CPE), pEGFP-CPEΔC10 (CPEΔC10), or pEGFP-CPE-C10 (CPE-C10) as indicated at DIV 7. Neurons were fixed and immunostained for GFP and MAP2 at DIV 10. Neurons positive for both GFP and mOrange were assessed for Sholl analysis. Representative mOrange fluorescent images of neurons are shown as inverted black images. Scale bar = 50μm. (B) Sholl analysis of neurons overexpressing CPE and mutants on dendrite branching. (C) Sholl analysis from (B) within 60μm from soma. Error bars indicate ± S.E.M. n (neurons)=95, GFP; n=96, CPE; n=100, CPEΔC10; n=90, CPE-C10. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as determined by two-way ANOVA followed by Bonferroni multiple comparisons test. (D) Hippocampal neurons were co-transfected with pCAG-mOrange and negative control siRNA (CTL siRNA) or CPE siRNA as indicated at DIV 7. Neurons were fixed and immunostained for GFP and MAP2 at DIV 10. Neurons positive for both GFP and mOrange were assessed for Sholl analysis. Representative mOrange fluorescent images of neurons are shown as inverted black images. Scale bar = 50μm. (E) Sholl analysis of neurons transfected with the indicated siRNA. n=84, CTL siRNA; n=95, CPE siRNA. $^{***}p < 0.001$ as determined by two-way ANOVA followed by Bonferroni multiple comparisons test. (F) Representative images showing CPE siRNA knockdown efficiency in transfected neurons. Hippocampal neurons were co-transfected with pCAG-mOrange (red) and

either CTL siRNA or CPE siRNA at DIV 7, fixed at DIV 10, and immunostained for CPE (green). Scale bar = 50μm. (G) Quantitation of CPE fluorescence intensity in the cell body of transfected neurons. Error bars indicate ± S.E.M. n=12, CTL siRNA; n=19, CPE siRNA. $^{*}p < 0.05$ as determined by Student's t-test.

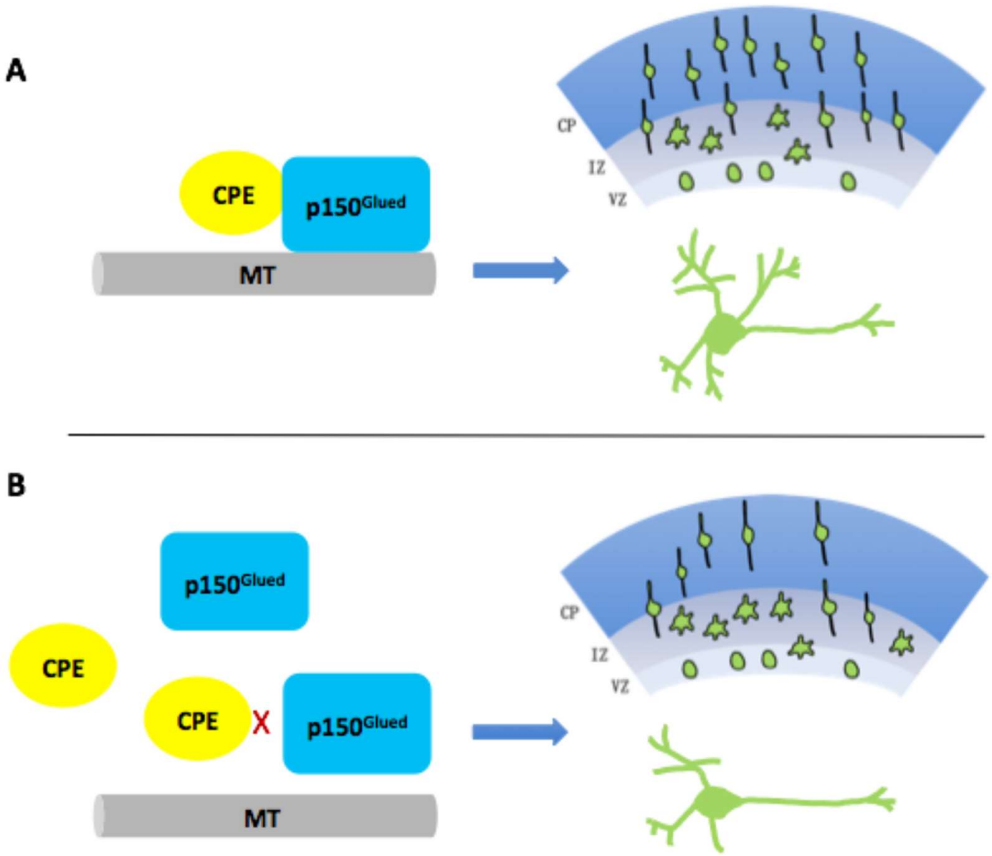



**Figure I-7. Model of CPE and p150$^{Glued}$ interaction in regulating cortical neuron migration and dendrite morphology**

(A) Under normal conditions, CPE regulates p150$^{Glued}$ localization via its carboxyl terminus to maintain necessary stability and dynamics of MTs, thus ensuring proper cortical migration and dendrite morphology. (B) When CPE protein levels are altered, the balance between CPE-p150$^{Glued}$ complexes and free CPE is disrupted, resulting in defects in neuronal migration and decreased dendrite branching.

# CHAPTER II:

# THE ROLES OF CPE IN REGULATION OF TUBULIN POLYGLUTAMYLATION AND THE INVOLVEMENT OF THE CPE ZINC-BINDING MOTIF IN NEURONAL MIGRATION AND DENDRITE MORPHOLOGY

**ABSTRACT**

Neuronal migration and dendritogenesis are dependent on dynamic changes to the microtubule (MT) network. Among various factors that regulate MT dynamics and stability, post-translational modifications (PTMs) play a critical role in conferring specificity of regulatory protein binding to MTs. Thus, it is important to understand the regulation of PTMs during brain development. In this chapter, we identified CPE as a regulator of tubulin polyglutamylation, a major PTM in the brain, and we examine the impact of CPE-mediated polyglutamylation on cortical neuron migration and dendrite morphology. We show, for the first time, that overexpression of CPE increases the level of polyglutamylated α-tubulin while knockdown decreases it. We also demonstrate that CPE-mediated polyglutamylation activity is dependent on the CPE zinc-binding motif and is necessary for CPE action on $p150^{Glued}$ localization. However, this activity may not be responsible for CPE-mediated effects on neuronal migration and dendrite morphology. Together with the findings in Chapter I, our data suggest that the zinc-binding and $p150^{Glued}$ interacting motifs of CPE play distinct roles in CPE function.

## INTRODUCTION

Carboxypeptidase E (CPE) is a member of the N/E subfamily of the M14 family of metallocarboxypeptidases (Reznik and Fricker 2001). It was discovered as a prohormone-processing enzyme in endocrine cells and neurons, where it is responsible for the proteolytic processing of peptide intermediates, generating bioactive hormones and neurotransmitters (Fricker and Snyder 1982, Hook, Eiden et al. 1982). CPE hydrolyzes single carboxyl terminal amino acids from polypeptide chains with specificity for basic residues, i.e. lysine or arginine, by coordinating with zinc ions. In addition to its enzymatic activity, CPE is involved in sorting and transporting hormone and neuropeptide intermediates, such as pro-insulin, pro brain-derived neurotrophic factor (BDNF), and pro-opiomelanocortin (Loh, Maldonado et al. 2002, Dhanvantari, Shen et al. 2003, Lou, Kim et al. 2005). In recent years, studies have elucidated the importance of CPE in the nervous system. $CPE^{-/-}$ mice display a variety of neuronal deficits, including abnormal dendritic structure and spine morphology, neuronal degeneration, and deficits in learning and memory (Woronowicz, Koshimizu et al. 2008, Woronowicz, Cawley et al. 2010). A more recent study identified a new mutation in the *CPE* gene from a patient with Alzheimer's disease, suggesting the involvement of this mutation in Alzheimer's-related neurodegeneration, memory deficits, and depression (Cheng, Cawley et al. 2016).

Microtubules (MTs) and microtubule-associated proteins (MAPs) play crucial roles in establishing proper neuronal morphology, intracellular transport, and migration in the cortex and hippocampus (Penazzi, Bakota et al. 2016). To achieve diversity and complexity in function, MT dynamics must be precisely controlled. One of the most important mediators of such control is post-translational modification (PTM) of tubulins. Polyglutamylation represents a major PTM in the brain (Audebert, Koulakoff et al. 1994), and it is unique from other PTMs in that polyglutamyl chains exhibit variable lengths and are involved in regulating protein binding affinity to microtubules. Specifically, the microtubule-associated proteins, Tau, MAP1B, and MAP2, and kinesin motors, most efficiently bind to MTs that are modified with three glutamyl units, while the relative affinity of the binding of these proteins decreases with shorter or longer glutamyl chain lengths on MTs (Boucher, Larcher et al. 1994, Larcher, Boucher et al. 1996, Bonnet, Boucher et al. 2001). More recently, it was reported that there is graded control of the binding of spastin to MTs, and spastin-mediated severing of MTs is regulated by polyglutamylation (Valenstein and Roll-Mecak 2016). In specific, modulation of spastin activity by polyglutamylation is biphasic and sensitive to glutamyl chain length.

Several polyglutamylases have been identified as members of the tubulin tyrosine ligase-like (TTLL) protein family (Janke, Rogowski et al. 2005, Ikegami, Mukai et al. 2006, van Dijk, Rogowski et al. 2007). Depending on the preference of the specific enzyme, glutamates can be added onto a Glu residue in the carboxyl

terminus of either α-tubulin or β-tubulin, initiating side chains or elongating existing chains to variable lengths (van Dijk, Rogowski et al. 2007). In contrast, deglutamylases regulate side chain length by removing glutamates from α-tubulin or β-tubulin. Recent data suggest that members of the cytosolic carboxypeptidase (CCPs) family act as deglutamylases (Kalinina, Biswas et al. 2007, Rodriguez de la Vega, Sevilla et al. 2007). CCPs share a conserved zinc-binding motif, a consensus sequence within the M14 carboxypeptidase family that includes CPE (Gomis-Ruth, Companys et al. 1999). Similar to polyglutamylases, CCPs differ in their specificity for substrates and activity, either shortening long side chains or removing the branch point glutamates from α-tubulin or β-tubulin (Rogowski, van Dijk et al. 2010).

In Chapter I, we report a role for CPE in the regulation of neuronal migration and dendrite morphology. We found that CPE carboxyl terminal interaction with $p150^{Glued}$, a MT-binding protein and anti-catastrophe factor, mediates effects on dendrite branching. Furthermore, CPE regulates the subcellular localization of $p150^{Glued}$. Since CPE shares a conserved zinc-binding motif with other reported deglutamylases, we asked whether CPE plays a role in regulating tubulin polyglutamylation. Interestingly, we found that CPE plays a role in tubulin polyglutamylation rather than acting as a deglutamylase. Additionally, expression of CPE protein with a mutated zinc-binding motif, which lacks glutamylation activity, demonstrates that the glutamylation activity of CPE is necessary to promote CPE

action on $p150^{Glued}$ localization, but it is not responsible for CPE-mediated effects on neuronal migration and dendrite morphology.

## METHODS AND MATERIALS

### DNA constructs and RNA interference

Full-length rat CPE protein was subcloned into pEGFP-C1 or pCAG. A mutant of CPE lacking amino acids required to bind zinc (CPE-H114A, E117A) was generated by site-directed mutagenesis of pEGFP-C1-CPE full-length construct, with His114 mutated to Ala and Glu117 mutated to Ala. cDNA encoding mutant forms of CPE-H114A, E117A was subcloned into pCAG-GFP. siRNA against CPE and the appropriate negative control were purchased from Life Technologies (CPE siRNA, Mouse S64324; CPE siRNA Rat S220210; negative control #1, cat# 4390743).

### Antibodies and reagents

Mouse anti-CPE (BD 610758) and mouse anti-MAP2 (BD 556320) were from BD Pharmingen. Mouse monoclonal antibody against Dynactin $p150^{Glued}$ (SC-135890) was from Santa Cruz. Rabbit anti-pericentrin (ab4448), rabbit anti-alpha-tubulin (ab18251), and rabbit anti-detyrosinated-tubulin (ab3201) were from Abcam. Mouse anti-polyglutamylated-tubulin (B3) (T9822) was from Sigma. Rabbit anti-acetyl-alpha-tubulin (5335s) was from Cell Signaling. Antibody against GFP (chicken; PA-9533) was purchased from Thermo Fisher.

### COS-7 cell transfection and immunocytochemistry

COS-7 cells (15,800 cells/$cm^2$) were seeded on coverslips previously coated with

0.1mg/ml poly-d-lysine. One day after plating, cells were transfected with pEGFP-C1, pEGFP-C1-CPE, or pEGFP-C1-CPE-H114A,E117A using Lipofectmine 2000 (Thermo Fisher) following the manufacturer's protocol. Forty eight hours after transfection, cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 15min and immunostained with indicated antibodies, followed by Hoechst dye nuclear staining. Using Fluoromount G (Southern Biotechnology; Birmingham, AL), coverslips were mounted onto glass slides for imaging.

**Immunofluorescent microscopy and p150$^{Glued}$ localization analysis**

Images of COS-7 cells were taken at 600X using an Olympus Optical (Tokyo, Japan) IX50 microscope and fluorescent imaging system. To determine p150$^{Glued}$ localization, a straight line was drawn from one side of the cell to the other. The line was drawn through the pericentrin-positive centrosome and nucleus. The percentage of p150$^{Glued}$ concentrated on the centrosome was determined by plotting the intensity of p150$^{Glued}$ and pericentrin along the line drawn. The intensity of p150$^{Glued}$ fluorescence was measured within the peak of pericentrin (value A), and p150$^{Glued}$ fluorescence within 1 micron of the pericentrin peak was measured (value B). Percentage of p150$^{Glued}$ localized to the centrosome was defined as the ratio of A to B.

**Fractionation and Western blot analysis of Neuro2a cell lysates**

Neuro2a cells were plated at 40%-50% confluence in 60mm dishes and transfected 24hrs after plating with pEGFP-C1, pEGFP-C1-CPE, or pEGFP-C1-CPE-H114A,E117A using Lipofectmine LTX Plus, or transfected with negative control siRNA or CPE siRNA with Lipofectmine RNAiMax, following the manufacturer's protocols. Cells were collected two days after transfection into pre-warmed (37$^{o}$C) microtubule stabilization and cell lysis buffer (100mM PIPES (pH 6.9), 5mM EGTA, 5mM $MgCl_2$, 20% glycerol, 0.5% Nonidet P40, 0.5% Triton X-100, and protease inhibitor cocktail (Cytoskeleton, Inc., cat# PIC02), modified from a previous study (Audebert, Desbruyeres et al. 1993). Cells were homogenized, and the extract was centrifuged at 350 x *g* at room temperature for 5min to pellet unbroken cells or tissue debris. The supernatant was collected and centrifuged at 100,000 x *g* at 37$^{o}$C for 1hr to fractionate soluble and cytoskeletal proteins. Pelleted cytoskeletal proteins were depolymerized and denatured by incubation with 8M Urea for 1hr. Equal volumes of protein samples were resolved by SDS-PAGE, and resolved proteins were transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were probed for polyglutamylated-tubulin, acetylated-tubulin, detyrosinated-tubulin, and alpha-tubulin. Immunoreactive bands were visualized with HyGlo quick spray (Denville Scientific; South Plainfield NJ), and resulting films were quantified with ImageJ software (NIH).

**Co-immunoprecipitation**

Twenty four hours after plating, Neuro2a cells were transfected with pEGFP-C1, pEGFP-C1-CPE or pEGFP-C1-CPE-H114A,E117A using Lipofectmine LTX Plus. Forty eight hours after transfection, cells were scraped into HEPES/Sucrose buffer (20 mM HEPES (pH 7.5), 320 mM sucrose, 1 mM EDTA, 5 mM DTT, 1 mM PMSF) and homogenized. Extracts were centrifuged at 350 x *g* to pellet unbroken cells or tissue debris, supernatant collected, and cytosolic proteins fractionated by centrifugation at 100,000 x *g* at 4$^{o}$C for 1hr. Cytosolic fractions were pre-cleared with protein G agarose (50% slurry (GE Healthcare, Piscataway, NJ)) for 1 hour, and after addition of 0.05% bovine serum albumin (BSA), proteins were immunoprecipitated with monoclonal Dynactin p150$^{Glued}$ antibody at 4$^{o}$C overnight. Protein G agarose was added, and the samples were incubated for 1 hour. Immunoprecipitates were washed with 0.1% Triton X-100, 50mM Tris, pH 7.4, 300mM NaCl, 5mM EDTA, 0.02% $NaN_3$, proteins were eluted, and eluates were resolved by SDS-PAGE. Proteins were transferred to PVDF membranes, which were probed for the indicated proteins. HyGlo quick spray (Denville Scientific; South Plainfield NJ) was used to visualize immunoreactive bands, and films were quantified using ImageJ software (NIH).

### *In utero* electroporation, histological procedures, microscopy and migration analysis

*In utero* electroporation was performed as we reported (Carrel, Hernandez et al. 2015). Briefly, at gestation day 14.5 (E14.5), pregnant Swiss mice were anesthetized

with isoflurane, and the abdominal cavity was opened to expose the uterine horns. Plasmids (1-3 µl of 2-2.5 µg/µl stock) were microinjected through the uterus into the lateral ventricles of embryos using pulled glass capillaries (Drummond Scientific, Broomall, PA). Embryos were co-injected with 1 mg/ml Fast Green (Sigma). The heads of the embryos were placed between tweezer-type electrodes, and square electric pulses (35 V, 50 ms) were passed five times at 1-s intervals using a NEPA21 electroporator (SONIDEL Limited, Dublin, Ireland). Embryos were allowed to develop *in utero* for the indicated number of days after electroporation, at which time the brains were dissected and fixed in 4% PFA in PBS for 48 h at 4°C and cryoprotected in 30% sucrose in PBS. Brains were then frozen in PolyFreeze (Sigma) and sectioned coronally at 16 µm. A Zeiss Axio Observer.Z1 microscope using a 20× numerical aperture (NA) 0.8 objective, with a Clara E CCD Camera (Andor Technology Limited, Belfast, UK) was used to image the sections. Migration analysis was performed as described in Chapter I Methods and Materials section.

**Primary neuronal culture and dendrite branching analysis**

Primary cultures of hippocampal neurons were prepared as we described previously (Firestein, Firestein et al. 1999, Akum, Chen et al. 2004). Tissue was mechanically dissociated, and cells were plated at a density of 10,500 cells/cm$^2$ on poly-d-lysine-coated coverslips and maintained in Neurobasal medium (Thermo Fisher) supplemented with B27 (Thermo Fisher), GlutaMAX (Thermo Fisher),

penicillin, and streptomycin. At day *in vitro* (DIV) 7, cultures were co-transfected with pCAG-mOrange and indicated CPE constructs using Lipofectamine LTX Plus (Thermo Fisher), fixed at DIV 10, and immunostained for GFP and MAP2. An Olympus Optical (Tokyo, Japan) IX50 microscope and fluorescent imaging system was used to image the neurons, and dendrite morphology was assessed using our Bonfire analysis program (Kutzing, Langhammer et al. 2010, Langhammer, Previtera et al. 2010).

## RESULTS

### CPE protein shares homology with reported tubulin deglutamylases

Since CPE belongs to the M14 carboxypeptidase family, and several members of the cytosolic carboxypeptidase family have been identified as tubulin deglutamylases (Rogowski, van Dijk et al. 2010), we performed multiple sequence alignment to identify conserved motifs. Interestingly, CPE shows homology to the previously reported deglutamylases, cytosolic carboxypeptidase (CCP) 1, CCP4, CCP5 and CCP6, with highest homology at a majority of the putative active sites for substrate binding and catalysis (**Figure II-1**; (Aloy, Companys et al. 2001)). Importantly, CPE shares a Zn-binding motif with these deglutamylases, and this motif is conserved among M14 family members and is crucial for their enzymatic activity (**Figure II-1**; (Gomis-Ruth, Companys et al. 1999)). Thus, we hypothesized that CPE may function as a tubulin deglutamylase.

### CPE protein regulates tubulin polyglutamylation

To investigate whether CPE is involved in regulating post-translational modification (PTM) of microtubules, and in specific, deglutamylation as predicted (**Figure II-1**), we overexpressed or knocked down CPE protein in Neuro2a cells and examined changes to PTMs, including polyglutamylation, acetylation, and detyrosination, in both polymerized microtubules and free tubulin pools. First, we confirmed CPE siRNA knockdown efficiency (**Figure 2A, B**). We then fractionated Neuro2a cell

lysates by ultracentrifugation. Surprisingly, in the cytoskeletal fractions of Neuro2a cells, we found that CPE overexpression increases the level of polyglutamylated α-tubulin, while knockdown of CPE decreases polyglutamylated α-tubulin (**Figure II-2C, D**). Polyglutamylated α-tubulin levels remain the same in the soluble fractions of Neuro2a cells (**Figure II-2E, F**). These results are novel and suggest that CPE plays a role, either directly or indirectly, in regulating the polyglutamylation of microtubules. Furthermore, the function of CPE is distinct from the functions previously reported for deglutamylases of the M14 carboxypeptidase family.

We next asked whether the effect of CPE on tubulin polyglutamylation is dependent on its Zn-binding motif, which is conserved among M14 carboxypeptidase family members (Gomis-Ruth, Companys et al. 1999). Although not much is known about the role of zinc in regulating the deglutamylation activity of these proteins, it was previously reported that activity of the tubulin glutamylase, Nna1, increases in the presence of zinc (Wu, Wang et al. 2012). Thus, we generated a mutant form of CPE predicted to lack the capacity to bind zinc by substituting histidine-114 and glutamate-117, both in the zinc-binding motif, with alanines. As a result, we observed that overexpression of CPE-H114A, E117A does not change the level of polyglutamylated α-tubulin. This result supports our hypothesis that CPE regulates the polyglutamylation of tubulin in microtubules and this activity of CPE is dependent on zinc binding.

To further investigate whether CPE regulates additional post-translational

modification of microtubules, we analyzed the cytoskeletal and soluble fractions of Neuro2a cell lysates by Western blotting for acetylated-α-tubulin and detyrosinated-α-tubulin. In contrast to that seen for polyglutamylation, we observed no significant changes in either acetylation or detyrosination of tubulin as a result of alterations in CPE protein levels (**Figure II-3**).

**Overexpression of CPE alters p150$^{Glued}$ localization at the centrosome and is partially dependent on zinc-binding**

We show in Chapter I that CPE regulates the localization of its interactor, p150$^{Glued}$, at the centrosome. Thus, we asked whether the CPE zinc-binding motif and resulting effect on tubulin polyglutamylation are involved in regulation of p150$^{Glued}$ localization. To confirm that the mutated CPE protein, CPE-H114A,E117A, does indeed bind to p150$^{Glued}$, we performed a co-immunoprecipitation assay. We expressed GFP, GFP-tagged CPE, or GFP-tagged CPE-H114A, E117A in Neuro2a cells and immunoprecipitated p150$^{Glued}$ from the cytosolic protein extract. We found that CPE-H114A,E117A co-immunoprecipitates with p150$^{Glued}$ as does wild type CPE (**Figure II-4A, B**). We then transfected COS-7 cells with constructs encoding GFP, CPE, or CPE-H114A,E117A and analyzed the percentage of p150$^{Glued}$ protein localized at the centrosome. As reported in Chapter I, overexpression of CPE decreases p150$^{Glued}$ localization at the centrosome (**Figure II-4C, D**). Interestingly, overexpression of the zinc-binding domain mutant (CPE-H114A,E117A) partially

attenuates the effect of CPE overexpression on p150$^{Glued}$ distribution (**Figure II-4C, D**), as the percentage of p150$^{Glued}$ localized at the centrosome when CPE-H114A, E117A is overexpressed does not significantly differ from that observed with either GFP or CPE overexpression. Our results suggest involvement of the zinc-binding motif and tubulin polyglutamylation in regulation of p150$^{Glued}$ localization by CPE. However, our data also indicate that although the motif is necessary, it is not sufficient to promote CPE action on p150$^{Glued}$ localization.

**Overexpression of CPE-H114A, E117A alters cortical neuron migration**

As demonstrated in Chapter I, CPE is required for proper cortical neuron migration. To investigate whether CPE-mediated tubulin polyglutamylation is involved in neuronal migration, we electroporated GFP (control), GFP-tagged CPE, or CPE-H114A, E117A constructs *in utero* into embryonic day (E)14.5 mouse brains and analyzed brain sections at E17.5. We observe a decrease in the percentage of neurons reaching the CP when CPE-H114A, E117A is overexpressed (**Figure II-5A, B**). In contrast, overexpression of CPE does not alter cortical neuron migration (**Figure II-5A, B**). These results suggest that the zinc-binding motif of CPE plays a role in cortical neuron migration. It may act in a dominant negative manner by either inhibiting normal glutamylation induced by CPE, or it may attenuate other processes that regulate neuronal migration.

**Overexpression of CPE-H114A, E117A alters dendritic arborization in cultured hippocampal neurons**

In Chapter I, we propose that a balance of CPE protein levels may be required to maintain proper dendritic arborization in hippocampal neurons. In specific, overexpression of CPE results in decreased dendrite branching proximal to soma, and the effect is dependent on its carboxyl terminus. To investigate whether CPE-mediated effects on dendritic arborization are dependent on CPE-mediated tubulin polyglutamylation, we overexpressed CPE or CPE-H114A,E117A in rat hippocampal neuronal cultures at day *in vitro* (DIV)7 and examined dendrite morphology at DIV 10, a time when active branching occurs in our cultures (Banker and Goslin 1988, Dotti, Sullivan et al. 1988, Charych, Akum et al. 2006). Sholl analysis demonstrates that overexpression of either CPE or CPE-H114A, E117A result in decreased dendrite branching proximal to the soma (0-12μm and 0-18μm, respectively; **Figure II-6A-C**), suggesting that the regulation of proximal dendrite number by CPE is independent of CPE-mediated tubulin polyglutamylation.

## DISCUSSION

### Novel function for CPE in regulating tubulin polyglutamylation

Various patterns of polyglutamylation can be generated on MTs and confer specificity of MAPs and motor protein binding to MTs (Boucher, Larcher et al. 1994, Larcher, Boucher et al. 1996, Bonnet, Boucher et al. 2001, Valenstein and Roll-Mecak 2016). Therefore, it is of importance to understand how polyglutamylation is regulated. Since CPE shares a conserved zinc-binding motif with currently known deglutamylases of the CCP family (Gomis-Ruth, Companys et al. 1999, Rodriguez de la Vega, Sevilla et al. 2007, Rogowski, van Dijk et al. 2010), we performed sequence alignment between CPE and several deglutamylases, and explored the role of CPE in regulating tubulin polyglutamylation. Surprisingly, we found that overexpression of CPE increases polyglutamylated α-tubulin, while knockdown of CPE decreases it. In addition, CPE-mediated effects on polyglutamylation levels are dependent on its zinc-binding motif. These results indicate a novel function for CPE in regulating tubulin polyglutamylation, either directly or indirectly. Previously identified polyglutamylases are members of the TTLL family, and sequence analysis shows that they share a conserved core TTL domain and an extended TTL domain (Janke, Rogowski et al. 2005, Ikegami, Mukai et al. 2006, van Dijk, Rogowski et al. 2007). Our findings suggest that polyglutamylases may not be limited to members of the TTLL family, as CPE is not a member of this family.

It should also be noted our results do not rule out the possibility that CPE may

act indirectly to regulate tubulin polyglutamylation. The molecular mechanism underlying CPE action remains unclear, but one possible explanation is that CPE may form a complex with polyglutamylases or deglutamylases and regulate substrate targeting or enzymatic activity. Indeed, immunoprecipitation of mouse brain polyglutamylase suggests that this enzyme may exist as multimetric complex and a subunit of the complex, PGs1, regulates localization of the enzyme (Regnard, Fesquet et al. 2003). In addition, we assessed the effects of CPE on polyglutamylated α-tubulin, and thus, it is possible that CPE may also affect polyglutamylation of β-tubulin.

**The zinc-binding motif of CPE is necessary but not sufficient to promote CPE action on p150$^{Glued}$ localization**

CPE expression begins early and increases dramatically during embryonic development (Birch, Rodriguez et al. 1990, Zheng, Streck et al. 1994). Roles for CPE in the endocrine and nervous systems as a prohormone-processing enzyme, sorting receptor, and mediator of vesicle transport have been reported by multiple groups (reviewed in (Cawley, Wetsel et al. 2012)). However, how CPE contributes to neuronal development is still largely unknown. We report in Chapter I that CPE and its interactor, p150$^{Glued}$ function to control cortical development and dendrite morphogenesis. The results suggest that CPE regulates neuronal function by impacting the MT network and MT-associated proteins. CPE overexpression alters

the localization of p150$^{Glued}$, a protein that anchors MTs at the centrosome and binds to MT plus-ends (Berrueta, Tirnauer et al. 1999, Quintyne, Gill et al. 1999, Askham, Vaughan et al. 2002), and this effect is dependent on the interaction between CPE carboxyl terminus and p150$^{Glued}$. Since polyglutamylation shapes protein binding to MTs, we hypothesized that CPE may affect p150$^{Glued}$ localization by regulating levels of MT polyglutamylation and thus changes to p150$^{Glued}$ binding affinity to centrioles or MTs. Interestingly, we found that the zinc-binding motif and CPE-mediated polyglutamylation activity are necessary, but not sufficient, for regulating p150$^{Glued}$ localization. Together with our previous findings in Chapter I, we provide new insights into CPE-mediated regulation of p150$^{Glued}$ localization, suggesting the involvement of the CPE zinc-binding motif.

**The zinc-binding motif of CPE plays a role in neuronal migration but not in the regulation of dendrite morphology**

We show that CPE is required for proper cortical cell migration and dendrite branching in Chapter I. Knockdown of CPE protein *in utero* of E14.5 mice results in an increased percentage of cells found in the intermediate zone (IZ) at E17.5 and fewer cells located in the cortical plate (CP) compared to control. In cultured neurons, both overexpression and knockdown of CPE protein lead to decreased dendrite branching, suggesting that a balance of CPE protein level is required for normal dendritogenesis. MT polyglutamylation is controlled in a graded manner

(Valenstein and Roll-Mecak 2016) and that disrupted polyglutamylation levels and resulting alterations in binding of MAPs and motor proteins to MTs may cause abnormalities in neuronal development. Indeed, knockdown of a polyglutamylase, TTLL7, in PC12 cells represses MAP2-positive neurite growth (Ikegami, Mukai et al. 2006). The observation that CCP1, a deglutamylase also known as Nna1, is specifically expressed in differentiating neurons during brain development and upregulated in regenerating motor neurons after injury (Harris, Morgan et al. 2000) also suggests a role for polyglutamylation in neurite outgrowth. Moreover, loss of α-tubulin polyglutamylation in ROSA22 mice results in deficits in synaptic vesicle transport (Ikegami, Heier et al. 2007), and the finding is further supported by another study showing that increased neuronal activity facilitates MT polyglutamylation and synaptic transport (Maas, Belgardt et al. 2009).

We observe that expression of CPE-H114A,E117A disrupts neuronal migration, indicating the necessity of CPE zinc-binding motif in normal cortical development. Two explanations may exist for this result. First, CPE-mediated tubulin polyglutamylation may not be involved in neuronal migration since overexpression of CPE does not show disrupted migration. The binding of zinc to CPE may regulate other functions of CPE. Alternatively, CPE-H114A,E117A may act in a dominant-negative manner and disrupt normal function of endogenous CPE protein. As discussed above, CPE may regulate that activity of other polyglutamylases or deglutamylases and regulate substrate targeting or enzymatic activity.

Surprisingly, in hippocampal neuronal cultures, overexpression of CPE-H114A, E117A results in the same phenotypes in dendrite architecture as does wild type CPE, suggesting that the zinc-binding motif is not involved in regulation of dendritogenesis. It is very likely that the decrease in dendrite branching caused by CPE overexpression is not related to the increase in MT polyglutamylation at the time point we examined, but is solely dependent on the interaction between the carboxyl terminus of CPE and $p150^{Glued}$. In fact, localization of brain-derived neurotrophic factor to neurites, which promotes proximal dendrite branching (McAllister, Lo et al. 1995, Horch, Kruttgen et al. 1999), is dependent on CPE- and dynein/dynactin complex-mediated vesicle transport (Lou, Kim et al. 2005, Park, Cawley et al. 2008). In addition, the influence of CPE on the subcellular localization of $p150^{Glued}$, an anti-catastrophe factor of MTs (Lazarus, Moughamian et al. 2013), may also affect MT stability and dendritic architecture.

During mouse brain development, polyglutamylation peaks during the early stages of development when neurons differentiate (Audebert, Koulakoff et al. 1994), indicating a role of polyglutamylation during early brain development. If polyglutamylation does not regulate neuronal migration or dendrite branching during this developmental stage, what could be its function? One possibility is that CPE-mediated polyglutamylation may contribute to neuronal protection. $CPE^{-/-}$ mice exhibit degeneration of pyramidal neurons in the hippocampus (Woronowicz, Koshimizu et al. 2008). CPE was later reported to be a neurotrophic factor, and it

protects neurons from oxidative stress-induced degeneration (Cheng, Cawley et al. 2013, Cheng, Rodriguiz et al. 2015). Recently, an additional splice variant of CPE that has neuroprotective effects against glutamate-induced toxicity during embryonic development was identified (Qin, Cheng et al. 2014). Furthermore, hyperglutamylation of MTs in *Purkinje cell degeneration (pcd)* mice that lack functional CCP1 is thought to be the cause of neurodegeneration observed in the mice (Mullen, Eicher et al. 1976, Rogowski, van Dijk et al. 2010, Wu, Wang et al. 2012). Together with our findings that CPE regulates tubulin polyglutamylation, we propose that the molecular mechanisms underlying the neuroprotective function of CPE may be dependent on this enzymatic activity of CPE.

In summary, our study reports the identification of a novel function for CPE in the regulation of tubulin polyglutamylation, and our data suggest that the zinc-binding motif is essential for mediating this effect. Moreover, we show that the CPE zinc-binding motif and polyglutamylation activity do not regulate dendrite morphogenesis but that an intact zinc-binding motif is necessary for proper cortical neuron migration. In addition, we propose that CPE-mediated tubulin polyglutamylation may contribute to CPE-mediated neuronal protection from glutamate-induced toxicity. Taken together with our previous results, the zinc-binding and $p150^{Glued}$ interacting motifs of CPE play distinct roles in CPE function.

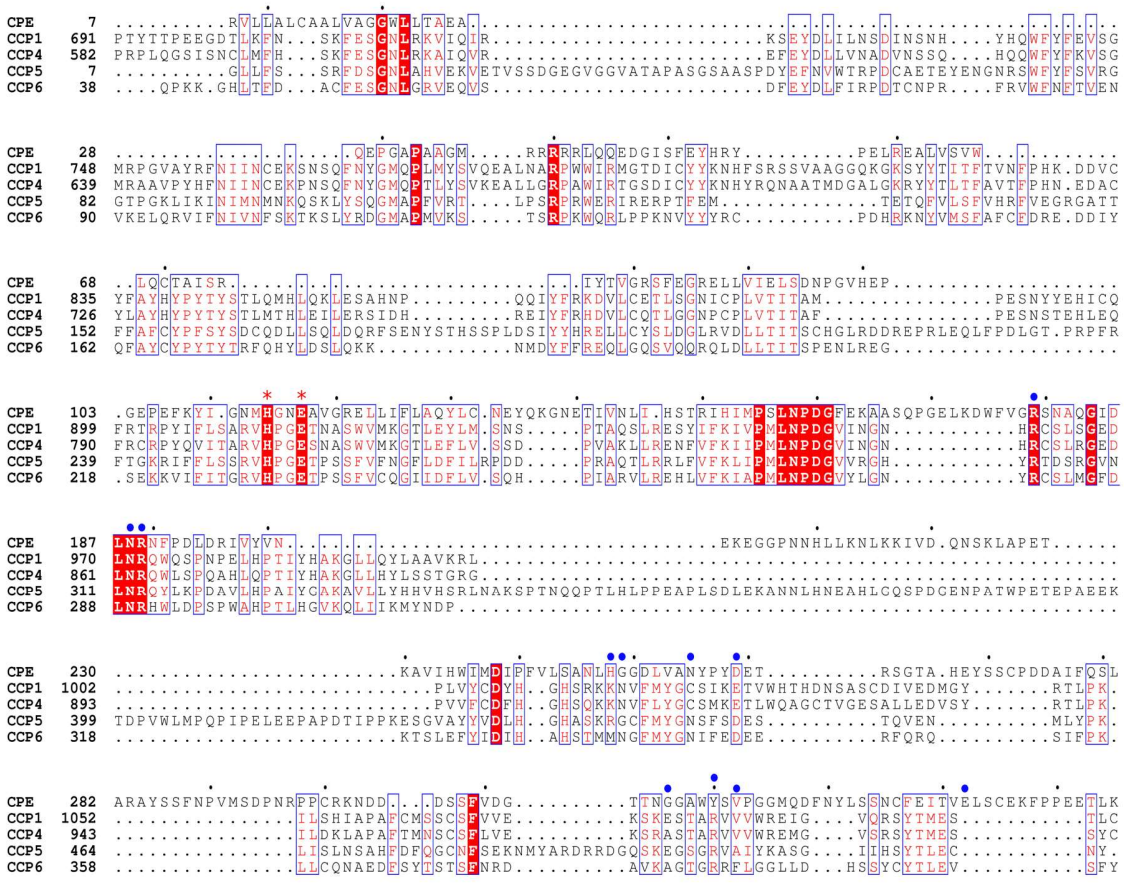


**Figure II-1. CPE protein shares homology with reported tubulin deglutamylases**

Sequence alignment of mouse CPE protein and four reported tubulin deglutamylases: cytosolic carboxypeptidase (CCP) 1, CCP4, CCP5, and CCP6. Sequences near the amino and carboxyl termini are not shown due to lack of homology with CPE. Conserved residues are shown in red boxes and white characters; conservatively changed residues are shown in red characters with blue frames. Red asterisks

indicate the conserved zinc-binding motif, which is mutated to generate the CPE-H114A,E117A mutant construct used in this work. Blue dots indicate the putative active sites of CPE protein.

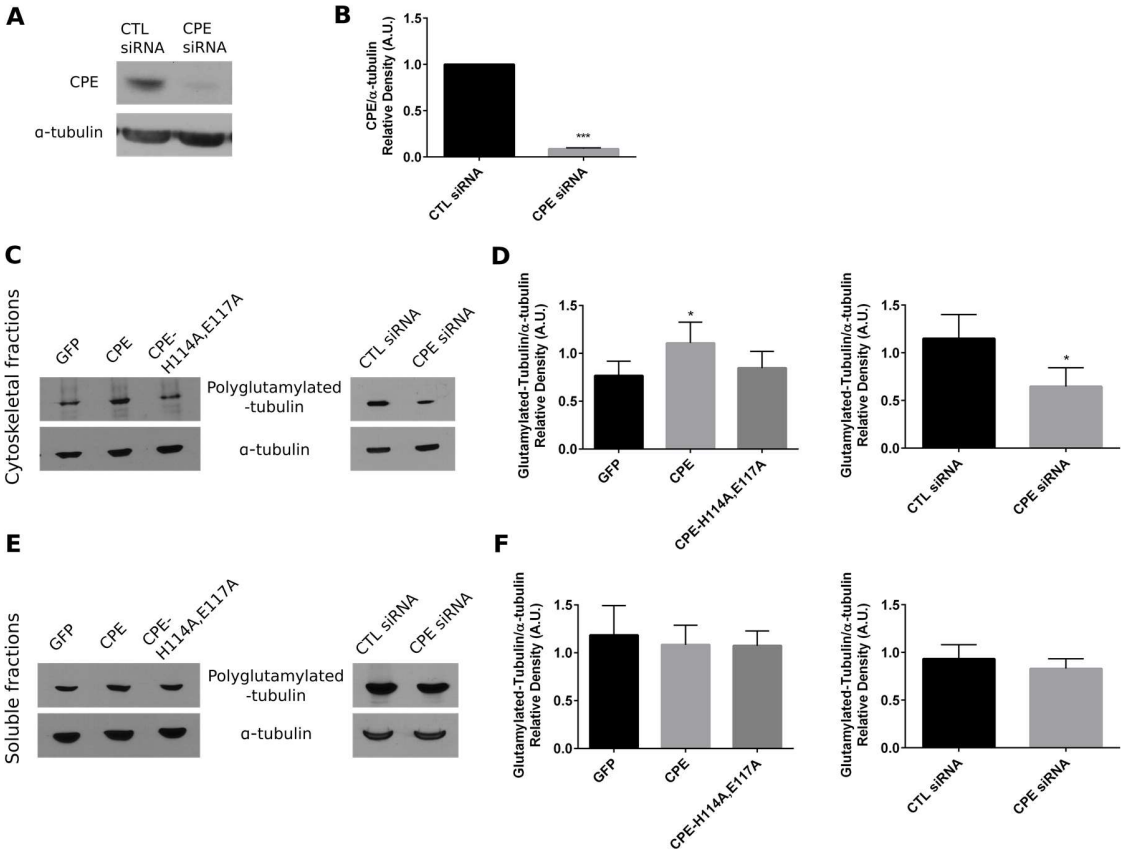



**Figure II-2. Alterations in CPE protein levels result in changes to polyglutamylation of α-tubulin**

(A) Knockdown efficiency of CPE siRNA in Neuro2a cells. Neuro2a cells were transfected with negative control siRNA (CTL siRNA) or CPE siRNA. Cells were collected 48h after transfection, and proteins were extracted and subjected to Western Blot analysis to detect CPE and α-tubulin. Representative blots are shown. (B) Quantitation of CPE protein levels/ total α-tubulin levels relative to control determined by Western blot analysis in A. Error bars indicate ± S.E.M. n=3 for all conditions. *** $p < 0.001$ as determined by paired Student's t-test. A.U. = arbitrary

units. (C) Western Blot analysis of polyglutamylation of tubulin in cytoskeletal fractions of Neuro2a cells. Cells were transfected with pEGFP, pEGFP-CPE, pEGFP-CPE-H114A,E117A, CTL siRNA, or CPE siRNA. Cells were collected and lysed 48h after transfection. Cytoskeletal and soluble proteins were fractionated and resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted for polyglutamylated α-tubulin and total α-tubulin. *Left*, representative blots of CPE overexpression and control conditions. *Right*, representative blots of CPE knockdown and control conditions. (D) Quantitation of polyglutamylated α-tubulin levels normalized to total α-tubulin levels relative to control in cytoskeletal fractions. (E) Western Blot analysis of polyglutamylation of tubulin in soluble fractions of Neuro2a cells. *Left*, representative blots of CPE overexpression and control conditions. *Right*, representative blots of CPE knockdown and control conditions. (F) Quantitation of polyglutamylated α-tubulin levels normalized to total α-tubulin levels relative to control in soluble fractions. Error bars indicate ± S.E.M. n=6 for all cytoskeletal fractions, n=3 for all soluble fractions. Outliers were excluded by Grubbs' test. $^{*}p < 0.05$ as determined by repeated measures one-way ANOVA followed by Tukey's multiple comparisons test (*left panels*) or paired Student's t-test (*right panels*). A.U. = arbitrary units.

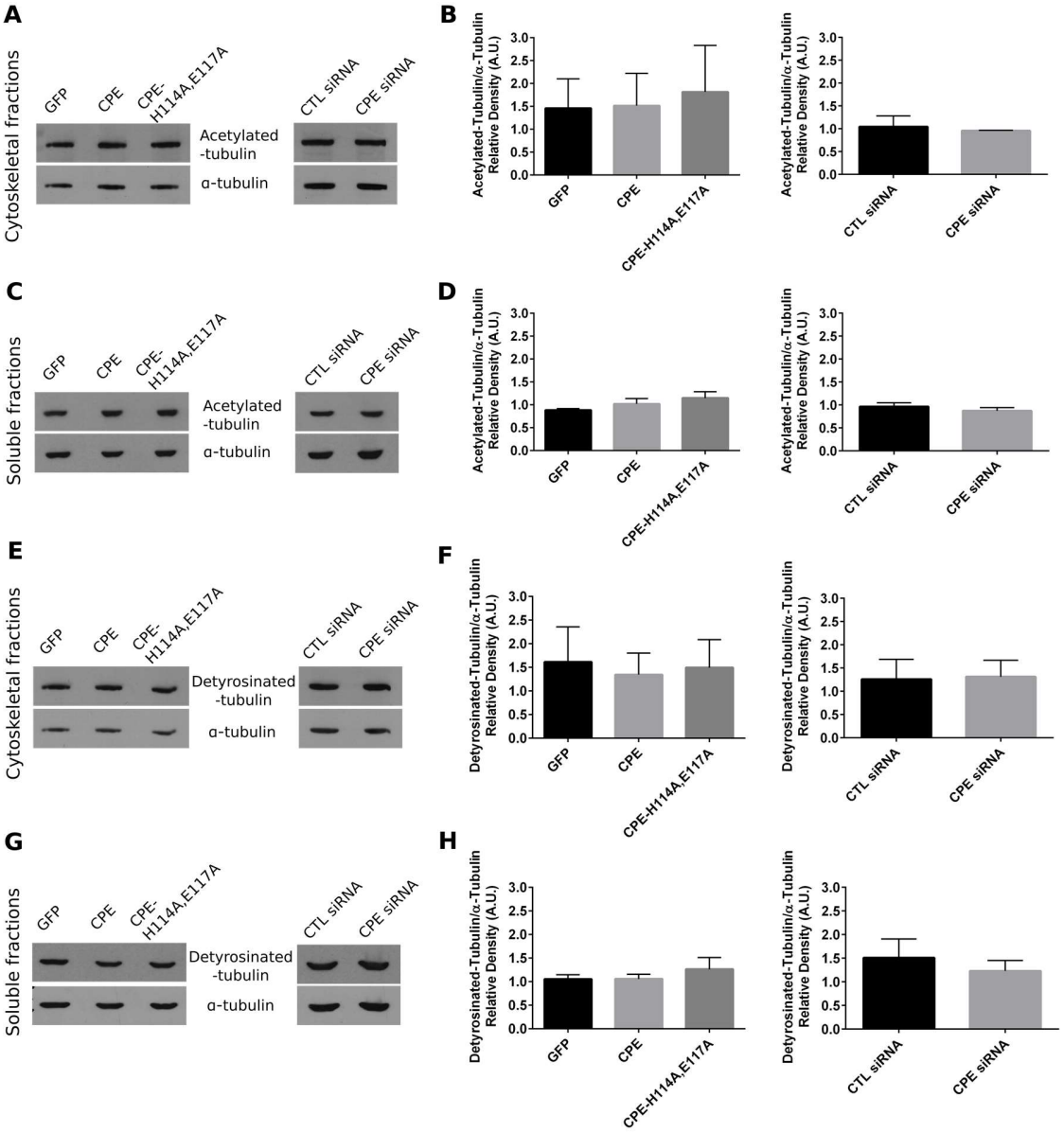



**Figure II-3. Alterations in CPE protein levels do not affect levels of acetylated or detyrosinated α-tubulin**

(A) Western blot analysis of acteylated α-tubulin in cytoskeletal fractions of Neuro2a cells. Cells were transfected with pEGFP, pEGFP-CPE, pEGFP-CPE-H114A,E117A, control (CTL) siRNA, or CPE siRNA. Cytoskeletal proteins were fractionated and

resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted for acetylated α-tubulin and total α-tubulin. Representative blots are shown. (B) Quantitation of acetylated α-tubulin levels/ total α-tubulin levels relative to control in cytoskeletal fractions. Error bars indicate ± S.E.M. n=3 for all conditions. A.U. = arbitrary units. (C) Western blot analysis of α-tubulin acetylation in soluble fractions of Neuro2a cells. Representative blots are shown. (D) Quantitation of acetylated α-tubulin levels / total α-tubulin levels relative to control in soluble fractions. Error bars indicate ± S.E.M. n=3 for all conditions. A.U. = arbitrary units. (E) Western blot analysis of detyrosinated α-tubulin in cytoskeletal fractions of Neuro2a cells. Cells were transfected with pEGFP, pEGFP-CPE, pEGFP-CPE-H114A,E117A, CTL siRNA, or CPE siRNA. Representative blots are shown. (F) Quantitation of detyrosinated α-tubulin levels / total α-tubulin levels relative to control in cytoskeletal fractions. Error bars indicate ± S.E.M. n=3 for all conditions. A.U. = arbitrary units. (G) Western blot analysis of detyrosinated α-tubulin in soluble fractions of Neuro2a cells. Representative blots are shown. (H) Quantitation of detyrosinated α-tubulin levels / total α-tubulin levels relative to control in soluble fractions. Error bars indicate ± S.E.M. n=3 for all conditions. A.U. = arbitrary units.

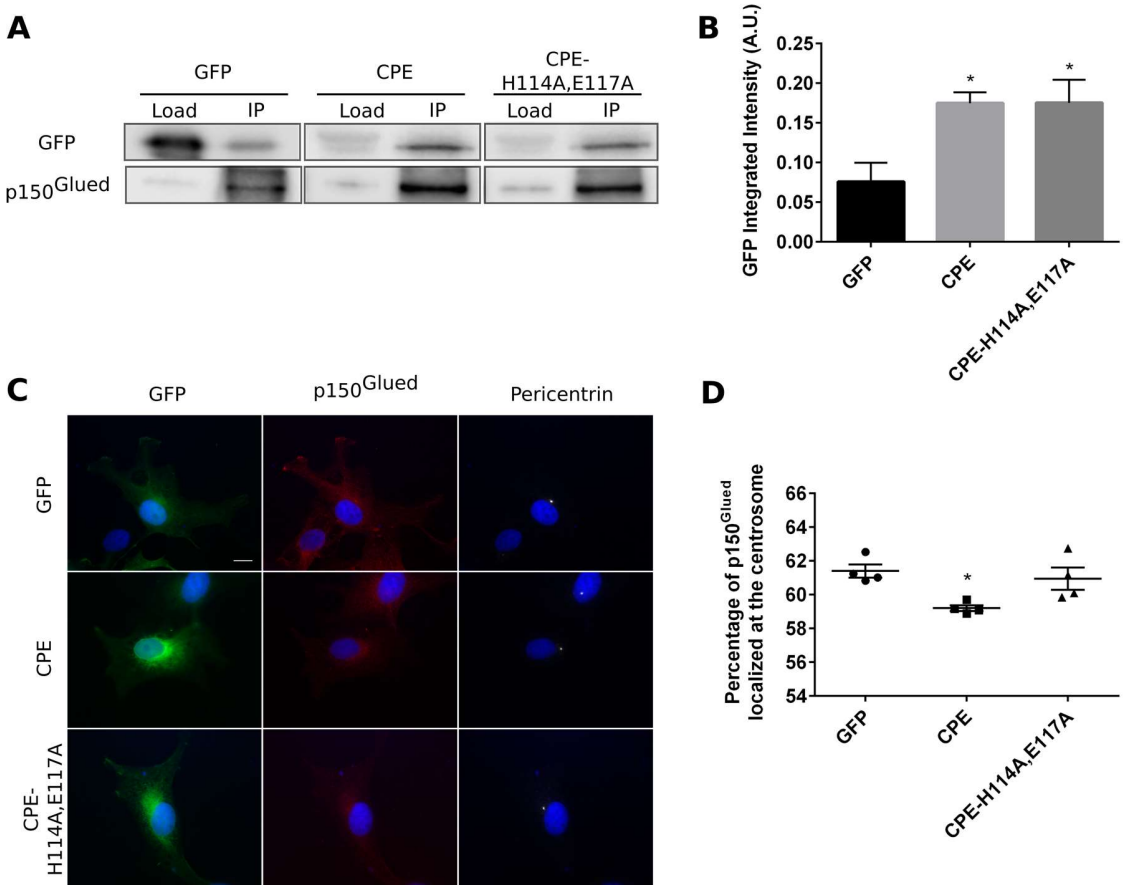



**Figure II-4. Overexpression of CPE alters p150$^{Glued}$ localization at the centrosome and is partially dependent on zinc-binding**

(A) CPE and CPE-H114A,E117A co-immunoprecipitate with p150$^{Glued}$. Neuro2a cells were transfected with GFP or CPE constructs. Cells were collected after 48h, and cytosolic proteins were extracted and subjected to immunoprecipitation with p150$^{Glued}$ antibody. Loading control (Load) and immunoprecipitates (IP) were resolved by SDS-PAGE. Proteins were transferred to PVDF membranes and immunoblotted for GFP and p150$^{Glued}$. Load represents 4% of extracted proteins.

Representative blots are shown. (B) Quantitation of GFP band intensities. Error bars indicate ± S.E.M. n=5 for all conditions. $^{*}p < 0.05$ as determined by one-way ANOVA followed by Dunnett's Multiple Comparisons Test. A.U. = arbitrary units. (C) Representative images showing $p150^{Glued}$ localization at the centrosome in COS-7 cells. COS-7 cells were transfected with GFP or CPE constructs. Cells were fixed 48h after transfection and immunostained with anti-GFP (green), anti-$p150^{Glued}$ (red), and anti-pericentrin (white), and nuclei were labeled with Hoechst dye (blue). Scale bar = 10μm. (D) Quantitation of the percentage of $p150^{Glued}$ proteins localized at the centrosome. For each independent experiment, 15-25 transfected cells of each condition were analyzed as described in *Methods and Materials*. Error bars indicate ± S.E.M. n=4 for all conditions. $^{*}p < 0.05$ as determined by repeated measures one-way ANOVA followed by Tukey's multiple comparisons test.

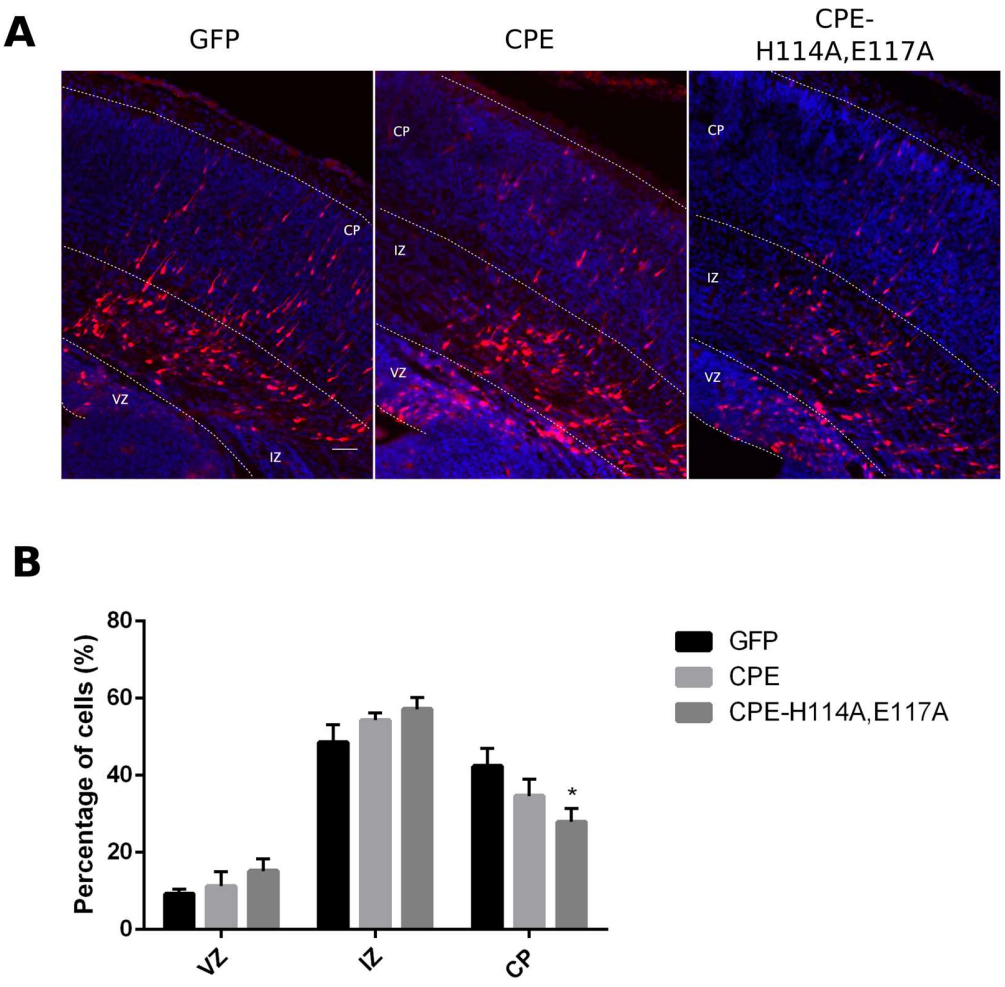



**Figure II-5. Overexpression of CPE-H114A,E117A alters cortical neuron migration**

(A) Mice at E14.5 were electroporated *in utero* with GFP or CPE constructs, and brains were analyzed at E17.5. Representative images of coronal brain sections are shown for each condition. Red, transfected cells; Blue, Hoechst dye. VZ, ventricular zone; IZ, intermediate zone; CP, cortical plate. Scale bar = 50μm. (B) Quantitation of the percentage of transfected cells in each cortical area. Error bars indicate ± S.E.M.

n=8, GFP; n=4, CPE; n=5, CPE-H114A,E117A. $^{*}p < 0.05$ as determined by two-way ANOVA followed by Tukey's multiple comparisons test.

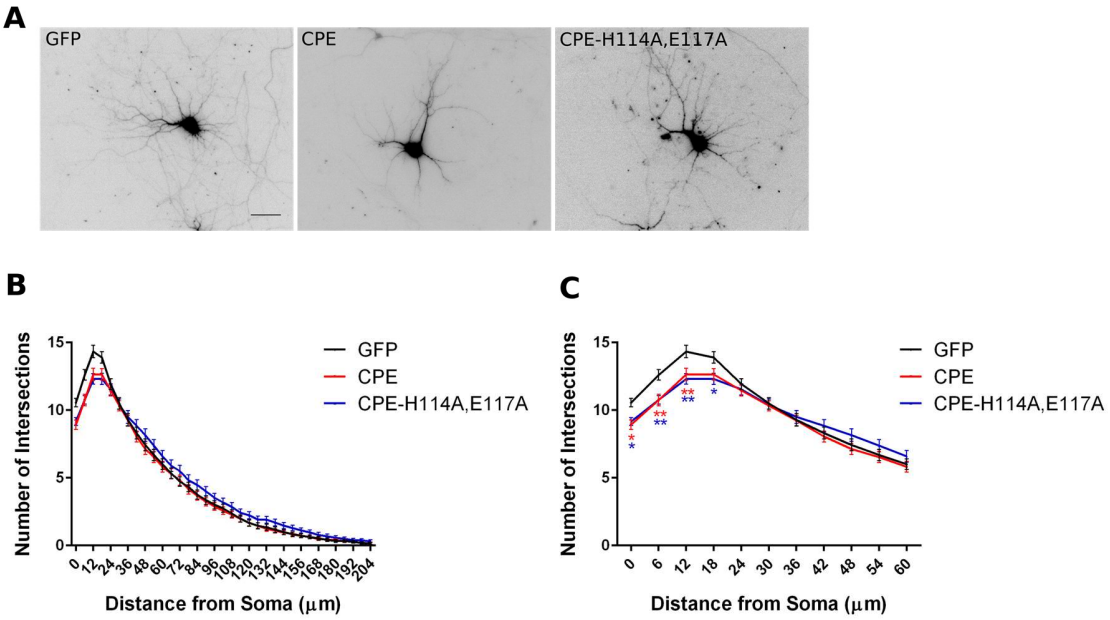



**Figure II-6. Overexpression of CPE decreases proximal dendritic arborization in cultured hippocampal neurons and is not dependent on zinc-binding**

(A) Rat hippocampal neurons were co-transfected with pCAG-mOrange (to visualize dendrites) and pEGFP (GFP), pEGFP-CPE (CPE), or pEGFP-CPE-H114A,E117A (CPE-H114A,E117A) at DIV 7. Neurons were fixed and immunostained for GFP and MAP2 at DIV 10. Neurons positive for both GFP and mOrange were used for Sholl analysis. Representative mOrange fluorescent images of neurons are shown as inverted black images. Scale bar = 50μm. (B) Sholl analysis of neurons overexpressing CPE or CPE-H114A, E117A. (C) Sholl analysis from (B) within 60μm from the soma. Error bars indicate ± S.E.M. n(neurons)=95, GFP; n=96, CPE; n=93, CPE-H114A,E117A. $^{*}p < 0.05$, $^{**}p < 0.01$ as determined by two-way ANOVA followed by Bonferroni multiple comparisons test.

# SUMMARY AND FUTURE DIRECTION

**SUMMARY**

This study aims at understanding the roles of CPE and its interactor, p150$^{Glued}$, in regulating the microtubule cytoskeleton and the resulting impact on neuronal development. In Chapter I, we report that overexpression of CPE or its carboxyl terminus, CPE-C10, redistributes p150$^{Glued}$ from the centrosome, while expression of CPE lacking the last 10 amino acids, CPEΔC10, does not affect the subcellular localization of p150$^{Glued}$. These data suggest that the carboxyl terminus of CPE is responsible for regulating p150$^{Glued}$ localization. We further investigated the roles of CPE in cortical neuron migration and dendrite morphology and the potential involvement of the interaction between CPE and p150$^{Glued}$ in mediating these effects. We show that CPE regulates neuronal migration and dendrite branching via a p150$^{Glued}$-dependent mechanism, and propose a model depicting the importance of a balance between CPE-p150$^{Glued}$ complex and free CPE protein level in regulating neurodevelopment. In Chapter II, We show that CPE regulates tubulin polyglutamylation and the effect is dependent on its zinc-binding motif. We observe that the zinc-binding motif is necessary for proper localization of p150$^{Glued}$ and neuronal migration, but may not be responsible for the phenotypes seen in dendrite branching.

The experiments in this proposal and those of others do not conclusively show that CPE and p150$^{Glued}$ interact directly. The interaction between CPE and Dynactin has been reported by other groups (Park, Cawley et al. 2008, Park, Cawley et al.

2008), and p150$^{Glued}$ was shown to co-immunoprecipitate with endogenous CPE and dynein from cytosol of AtT20 cells. The observation that excess CPE-C10 competes endogenous CPE but not motor proteins that bind to p150$^{Glued}$ strongly suggests that CPE directly interacts with p150$^{Glued}$ via its carboxyl terminus. However, current evidence cannot rule out the possibility of an indirect interaction with other proteins that exist in a complex with p150$^{Glued}$. Thus, CPE may regulate the localization of p150$^{Glued}$ indirectly by influencing other components of the dynactin and motor protein complex.

Furthermore, in Chapter I, we performed co-immunoprecipitation analysis of cytosol from rat brain lysate to examine the interaction between CPE and p150$^{Glued}$. Our results confirm the interaction; however, we found that less than 1% of CPE protein co-immunoprecipitates with p150$^{Glued}$. This could be explained by several scenarios. First, CPE is a multifunctional protein that serves different intracellular and extracellular roles (Cawley, Wetsel et al. 2012). Within the cytoplasm, CPE acts as a prohormone processing enzyme and sorting receptor and contributes to vesicle transport. On the other hand, approximately 29% of cellular CPE protein is secreted from neurons per hour as reported in hypothalamic neuronal cultures (Vilijn, Das et al. 1989). A recent study suggested that CPE acts as an extracellular trophic factor to protect neurons (Cheng, Cawley et al. 2013). Therefore, it is likely that only a subset of CPE can interact with p150$^{Glued}$ and function in vesicle transport or in maintaining MT stability. Second, we immunoprecipitated p150$^{Glued}$ from cytosolic protein

extract, and the vesicles containing CPE-p150$^{Glued}$ complex may have been spun down when we performed ultracentrifugation. In bovine pituitary gland, approximately 70% of total CPE protein is soluble and 30% is membrane bound, and both forms of CPE share a common carboxyl terminal sequence, which is responsible for interaction with p150$^{Glued}$. This suggests that both forms may interact with p150$^{Glued}$ (Parkinson 1992). To further investigate the interaction between CPE and p150$^{Glued}$ in different subcellular fractions, we could fractionate cytoskeletal, vesicular and cytosolic proteins and conduct co-immunoprecipitation in each fraction. Lastly, the affinity of CPE binding to p150$^{Glued}$ may be affected by post-translational modifications of CPE via a similar mechanism as reported for snapin (Chheda, Ashery et al. 2001, Chen, Lucas et al. 2005). For example, phosphorylation of snapin decreases its binding affinity to cypin, but it enhances its association with SNAP-25. It is possible that phosphorylation of CPE may regulate its binding to p150$^{Glued}$. Since we did not use phosphatase inhibitors in our co-immunoprecipitation experiments, CPE may not have maintained its phosphorylation. In addition, the fact that p150$^{Glued}$ exists in a large protein complex and that CPE may not directly bind to p150$^{Glued}$, the efficiency of immunoprecipitation may be low.

Full length CPE protein contains a signal peptide at the amino terminus that normally directs it into the secretory pathway (Cawley, Wetsel et al. 2012). A splice variant form of CPE, CPE-ΔN, that lacks a partial region of the amino terminus,

including the signal peptide, has been identified in both cancer cells and neurons. Expression of CPE-ΔN is elevated in various human metastatic tumor cell lines and is thought to be translocated to the nucleus, whereby it regulates the expression of neural precursor cell expressed, developmentally downregulated gene 9 (Nedd9) (Lee, Murthy et al. 2011). Recently, it was reported that CPE-ΔN is transiently expressed in mouse embryonic brain through early postnatal ages, and it protects neurons against glutamate-induced toxicity by enhancing the transcription of FGF2 mRNA (Qin, Cheng et al. 2014). In our CPE overexpression experiments, we tagged full length CPE with GFP at its amino terminus, which may affect recognition of the signal peptide and targeting of the protein (Benham 2012), resulting in mislocalization of the GFP-tagged CPE protein. Thus, overexpressed GFP-CPE protein may not be targeted to the secretory pathway but may be localized to the cytoplasm. Therefore, the phenotypes observed when overexpressing CPE *in vitro* and *in utero* may not represent the cellular function of full length CPE. There are two main explanations for the phenotypes resulting from CPE overexpression. One possibility is that the overexpressed GFP-CPE protein may act in a dominant-negative manner to compete with endogenous CPE for $p150^{Glued}$ interaction, resulting in redistribution of $p150^{Glued}$ from the centrosome and decreased dendritic branching. Another possibility is that GFP-CPE may act similarly to CPE-ΔN in the cytoplasm and may contribute to transcriptional regulation, cellular proliferation, and neuroprotection as reported by other groups (Lee, Murthy

et al. 2011, Qin, Cheng et al. 2014). Regardless, the hypothesis that CPE regulates $p150^{Glued}$ localization and neurodevelopment via its carboxyl terminus is still supported by our results since expression of CPE-C10 results in a similar phenotype to that of overexpressed full length CPE, while expression of CPEΔC10 has no effect. In addition, based on demonstration of CPE shRNA and CPE siRNA knockdown efficiency by Western blot analysis and immunocytochemistry (**Figure I-4**, **Figure I-6F-G**) and the CPE shRNA target sequence we designed, knockdown of CPE by shRNA and siRNA targets both the full length and N-terminal truncated CPE isoforms. Additionally, the observation that knockdown of CPE decreases the level of polyglutamylated α-tubulin indicates the necessity of CPE in mediating tubulin polyglutamylation, although we cannot differentiate which isoform of CPE is responsible for the effect with our present results.

To investigate the effect of CPE knockdown on neuronal migration, we utilized the technique of *in utero* electroporation and RNA interference. We observed that neurons electroporated with CPE shRNA fail to migrate to the CP compared to neurons electroporated CTL shRNA. To examine potential off-targeting effects caused by the CPE shRNA construct, we attempted to rescue the migration defects by co-electroporation with an shRNA-resistant CPE construct. However, the two constructs that we used failed to rescue the migration defects caused by CPE shRNA. The reason for failure to rescue may be due to a few reasons. One possibility is that a different isoform of CPE may be involved in the regulation of early brain

development. For instance, CPEΔN is transiently expressed during mouse embryonic development from E5.5 to P1 (Lee, Murthy et al. 2011, Qin, Cheng et al. 2014), suggesting a function primarily at the developmental stage that we examine. In addition, the rescue CPE construct is from rat, while the *in utero* experiments were conducted in mice. Although the mRNA sequences of rat and mouse are 99.2% identical, it is possible that a rescue construct from a difference species may affect its ability to rescue migration defects.

In addition to regulation of the microtubule cytoskeleton, several alternative mechanisms may explain how CPE is involved in neurodevelopment. First, CPE may regulate neurodevelopment by influencing BDNF transport. BDNF is a major neurotrophic factor and increases proximal dendrites in pyramidal neurons (McAllister, Lo et al. 1995, Horch, Kruttgen et al. 1999, Kwon, Fernandez et al. 2011). As discussed in Chapter I, CPE mediates transport of BDNF-containing vesicles by interacting with dynactin and motor proteins (Park, Cawley et al. 2008). Disruption of CPE binding to BDNF-containing vesicles results in reduced BDNF localization to the neurites of hippocampal neurons. The involvement of BDNF and its receptor TrkB in neuronal migration has also been reported in different brain regions (Ringstedt, Linnarsson et al. 1998, Borghesani, Peyrin et al. 2002, Medina, Sciarretta et al. 2004, Chiaramello, Dalmasso et al. 2007). BDNF promotes migration of cerebellar granule cells and neural precursors of the olfactory bulb. In the cerebral cortex, overexpression of BNDF causes aberrant cortical lamination. Furthermore,

CPEΔN regulates the expression levels of Nedd9 and FGF2 proteins (Lee, Murthy et al. 2011, Qin, Cheng et al. 2014), both of which are involved in various neurodevelopmental processes, including neurogenesis, neurite outgrowth, neuronal migration, and neuroprotection (Szebenyi, Dent et al. 2001, Bargon, Gunning et al. 2005, Jin, LaFevre-Bernt et al. 2005, Knutson and Clagett-Dame 2008, Vergano-Vera, Mendez-Gomez et al. 2009, Vogel, Ahrens et al. 2010, Cheng, Rodriguiz et al. 2015, Knutson, Mitzey et al. 2016). Therefore, Nedd9 and FGF2 may also be downstream targets of CPE and mediate the effects of it on neurodevelopment.

It is also important to note that we observe that the expression of CPE increases dramatically at later developmental stages from E16 to P10 in mice, and it localizes predominantly in the CP in differentiating neurons. These observations suggest that CPE may play important roles in development of later-born neurons or in later processes of neuronal development, including neuronal differentiation, dendrite morphogenesis, spine formation, and synaptogenesis. Thus, examination of the effects of CPE on neurodevelopment at later developmental time points will shed light on the understanding of CPE functions.

## FUTURE DIRECTIONS

### Role of CPE in dendritic spine development

Dendritic spines are small membrane protrusions from dendritic shafts that receive input from axons. They are highly dynamic and are diverse in number, size, and shape, which correlate directly with synaptic function (reviewed in (Kasai, Fukuda et al. 2010)). Formation and maturation of dendritic spines are dependent on cytoskeletal components: actin, MTs, and associated proteins. Although the role of actin in regulating spine dynamics has been well-studied, the presence of MTs and MT-binding proteins in dendritic spines were only revealed recently (Gu, Firestein et al. 2008, Hu, Viesselmann et al. 2008, Jaworski, Kapitein et al. 2009), and many of their functions in spine development remain to be explored. In the current study, we identified a role for CPE in the regulation of tubulin polyglutamylation and localization of the MT-binding protein $p150^{Glued}$, suggesting a potential role for CPE in regulating dendritic spines. In fact, CPE knockout mice exhibit abnormal spine morphology with increased number of D-type spines that are not fully functional (Woronowicz, Cawley et al. 2010). Thus, it would be of interest to investigate the influence of alterations in CPE protein levels on spine density and morphology and the molecular mechanisms underlying this influence both *in vitro* and *in vivo*. *In vitro* studies could be accomplished by overexpression or knockdown of CPE and expression of CPE mutants in neuronal cultures following the established methods in the present study. *In utero* electroporation could be performed to examine spine

development *in vivo*. Neurons on coverslips and in brain sections could be analyzed through immunofluorescence and confocal microscopy to compare number, length, width, and types of spines.

**Potential involvement of CPE in proliferation and differentiation of neural progenitor cells**

The functions of CPE during early brain development may not be limited to regulating neuronal migration and maturation as demonstrated in the first two chapters. In fact, the involvement of CPE in cellular proliferation has been studied extensively in cancer cells. Elevated expression of CPE protein has been reported in various types of cancer cells, and downregulation of CPE inhibits cancer cell proliferation and migration (Liang, Li et al. 2013, Liu, Shao et al. 2014, Fan, Li et al. 2016). More recently, it was reported that CPE, also known as Neurotrophic Factor-α1 (NF-α1), is an anti-proliferation factor and induces differentiation of neural stem cells into astrocytes (Selvaraj, Xiao et al. 2017). As described in Chapter I, we show that CPE regulates $p150^{Glued}$ localization at the centrosome. Importantly, loss of centrosomal $p150^{Glued}$ inhibits cell cycle progression from $G_1$ into S phase and may affect recruitment of cell cycle regulators to the centrosome (Quintyne and Schroer 2002). In addition, the fact that polyglutamylated MTs are enriched in the mitotic spindle and that polyglutamylation increases during mitosis (Bobinnec, Moudjou et al. 1998, Regnard, Desbruyeres et al. 1999) underlines the importance of

polyglutamylation in regulating cell division. Thus, it is reasonable to hypothesize that CPE regulates neural progenitor cell proliferation and differentiation during early embryonic development. Neural progenitor cell proliferation in embryonic brains could be assessed by labeling proliferating cells with 5-bromo-2-deoxyuridine (BrdU) following *in utero* electroporation of plasmids to overexpress or knock down CPE. CPEΔC10, CPE-C10, and CPE-H114A, E117A constructs could be utilized to examine the effect of the interaction between CPE and $p150^{Glued}$ and the potential involvement of the zinc-binding motif in the regulation of this process. To examine cell differentiation, several markers, such as Tbr2, Tbr1, NeuN, Tuj1, and GFAP, can be used to label progenitor cells, differentiated neurons, and astrocytes.

**Neuroprotective role of CPE and the potential involvement of zinc-binding motif and polyglutamylation activity**

Research on the neuroprotective function of CPE has been accumulating in recent years. CPE, or NF-α1, was reported as a neurotrophic factor, activating the Extracellular Signal-regulated Kinase (ERK) and AKT pathways to protect neurons from oxidative stress-induced degeneration (Cheng, Cawley et al. 2013). CPEΔN, a splice variant of CPE, has neuroprotective effects against glutamate-induced toxicity during embryonic development (Qin, Cheng et al. 2014). A single nucleotide polymorphism in the CPE gene was also discovered, and *in vitro* studies suggest a

loss of neuroprotective function with this mutation (Cheng, Cawley et al. 2016). In addition, studies of CPE knockout mice show degeneration of pyramidal neurons in the hippocampus (Woronowicz, Koshimizu et al. 2008). In addition, polyglutamylation peaks during the early stages of development when neurons differentiate (Audebert, Koulakoff et al. 1994), indicating a role for polyglutamylation during early brain development. Interestingly, hyperglutamylation of MTs in *Purkinje cell degeneration (pcd)* mice that lack functional CCP1 is proposed to be the cause of neurodegeneration in the *pcd* mice (Mullen, Eicher et al. 1976, Rogowski, van Dijk et al. 2010, Wu, Wang et al. 2012). Together with our novel finding that CPE regulates tubulin polyglutamylation, we propose that the mechanisms underlying the neuroprotective function of CPE may be dependent on its regulation of MT polyglutamylation. An *in vitro* glutamate-induced excitotoxicity model that was previously described (Tseng and Firestein 2011) can be used to examine the neuroprotective effects of CPE and potential involvement of the zinc-binding motif. Rat cortical neuronal cultures could be transfected with control, CPE, or CPE-H114A,E117A constructs and exposed to sublethal amounts of NMDA to induce excitotoxicity. Percentage of cell death could be analyzed after fixation and immunostaining of the neurons with MAP2 antibody.

**Identification of the mechanisms underlying CPE regulation of tubulin polyglutamylation**

Although the findings in this dissertation strongly suggest that CPE acts as a polyglutamylase, it is possible that CPE may regulate MT polyglutamylation indirectly. For example, as it was suggested for PGs1 (Regnard, Fesquet et al. 2003), CPE may exist in a complex with polyglutamylases and regulate subcellular localization of the complex, or it may be involved in the regulation of substrate recognition and enzymatic activity. To provide direct evidence for CPE function as a polyglutamylase, a cell-free assay for tubulin polyglutamylase activity could be performed. Enzyme activity can be measured by incorporation of [$^3$H]glutamate into tubulin with or without addition of recombinant CPE protein as described by other groups (Janke, Rogowski et al. 2005). Moreover, polyglutamylases have preference for α-tubulin and/or β-tubulin and exhibit cooperative effects (van Dijk, Rogowski et al. 2007). In our study, we find that CPE regulates polyglutamylation of α-tubulin, and it would be of interest to further examine the substrate specificity of CPE by the cell-free assay.

### *In utero* electroporation of CPE knockout mice to confirm changes in neuronal migration and morphology

In this study, knocking down CPE by *in utero* electroporation in mouse embryonic brains reveals the effect of transient loss of CPE. Previous studies have shown that CPE knockout mice exhibit deficits in learning and memory (Woronowicz, Koshimizu et al. 2008), but it remains unclear whether neuronal migration is

disrupted in CPE knockout mice and whether their behavioral deficits are linked to potential abnormal cortical development. Thus, it is important to investigate neuronal migration and morphogenesis in CPE knockout mice at different time points during brain development using *in utero* electroporation. It should be noted that long-term loss of CPE in the knockout mice may result in less severe phenotypes compared to transient loss due to compensatory mechanisms. In addition, since CPE can function as an extracellular neurotrophic factor (Cheng, Cawley et al. 2013), complete loss of CPE may lead to different phenotypes than knocking down the protein in individual cells. Abnormal dendritic architecture was previously observed in 14-week-old CPE knockout mice (Woronowicz, Cawley et al. 2010), but neuronal morphology at an earlier development point in CPE knockout mice has not been examined. By performing *in utero* electroporation of E14.5 CPE knockout mice, we can examine neuronal positioning and the multipolar to bipolar transition in the IZ at E17.5, and migration and dendrite morphology of neurons in layer V at P7. These experiments could provide further insight into the role of CPE *in vivo* during early brain development.

# REFERENCES

Akella, J. S., D. Wloga, J. Kim, N. G. Starostina, S. Lyons-Abbott, N. S. Morrissette, S. T. Dougan, E. T. Kipreos and J. Gaertig (2010). "MEC-17 is an alpha-tubulin acetyltransferase." Nature **467**(7312): 218-222.

Akum, B. F., M. Chen, S. I. Gunderson, G. M. Riefler, M. M. Scerri-Hansen and B. L. Firestein (2004). "Cypin regulates dendrite patterning in hippocampal neurons by promoting microtubule assembly." Nat Neurosci **7**(2): 145-152.

Aloy, P., V. Companys, J. Vendrell, F. X. Aviles, L. D. Fricker, M. Coll and F. X. Gomis-Ruth (2001). "The crystal structure of the inhibitor-complexed carboxypeptidase D domain II and the modeling of regulatory carboxypeptidases." J Biol Chem **276**(19): 16177-16184.

Alsters, S. I., A. P. Goldstone, J. L. Buxton, A. Zekavati, A. Sosinsky, A. M. Yiorkas, S. Holder, R. E. Klaber, N. Bridges, M. M. van Haelst, C. W. le Roux, A. J. Walley, R. G. Walters, M. Mueller and A. I. Blakemore (2015). "Truncating Homozygous Mutation of Carboxypeptidase E (CPE) in a Morbidly Obese Female with Type 2 Diabetes Mellitus, Intellectual Disability and Hypogonadotrophic Hypogonadism." PLoS One **10**(6): e0131417.

Angevine, J. B., Jr. and R. L. Sidman (1961). "Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse." Nature **192**: 766-768.

Askham, J. M., K. T. Vaughan, H. V. Goodson and E. E. Morrison (2002). "Evidence that an interaction between EB1 and p150(Glued) is required for the formation and maintenance of a radial microtubule array anchored at the centrosome." Mol Biol Cell **13**(10): 3627-3645.

Assadi, A. H., G. Zhang, U. Beffert, R. S. McNeil, A. L. Renfro, S. Niu, C. C. Quattrocchi, B. A. Antalffy, M. Sheldon, D. D. Armstrong, A. Wynshaw-Boris, J. Herz, G. D'Arcangelo and G. D. Clark (2003). "Interaction of reelin signaling and Lis1 in brain development." Nat Genet **35**(3): 270-276.

Audebert, S., E. Desbruyeres, C. Gruszczynski, A. Koulakoff, F. Gros, P. Denoulet and B. Edde (1993). "Reversible polyglutamylation of alpha- and beta-tubulin and microtubule dynamics in mouse brain neurons." Mol Biol Cell **4**(6): 615-626.

Audebert, S., A. Koulakoff, Y. Berwald-Netter, F. Gros, P. Denoulet and B. Edde (1994). "Developmental regulation of polyglutamylated alpha- and beta-tubulin in mouse brain neurons." J Cell Sci **107 ( Pt 8)**: 2313-2322.

Baas, P. W., M. M. Black and G. A. Banker (1989). "Changes in microtubule polarity

orientation during the development of hippocampal neurons in culture." J Cell Biol **109**(6 Pt 1): 3085-3094.

Baas, P. W., J. S. Deitch, M. M. Black and G. A. Banker (1988). "Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the dendrite." Proc Natl Acad Sci U S A **85**(21): 8335-8339.

Baas, P. W. and S. Lin (2011). "Hooks and comets: The story of microtubule polarity orientation in the neuron." Dev Neurobiol **71**(6): 403-418.

Bai, J., R. L. Ramos, J. B. Ackman, A. M. Thomas, R. V. Lee and J. J. LoTurco (2003). "RNAi reveals doublecortin is required for radial migration in rat neocortex." Nat Neurosci **6**(12): 1277-1283.

Banker, G. and K. Goslin (1988). "Developments in neuronal cell culture." Nature **336**(6195): 185-186.

Bargon, S. D., P. W. Gunning and G. M. O'Neill (2005). "The Cas family docking protein, HEF1, promotes the formation of neurite-like membrane extensions." Biochim Biophys Acta **1746**(2): 143-154.

Barkovich, A. J., R. Guerrini, R. I. Kuzniecky, G. D. Jackson and W. B. Dobyns (2012). "A developmental and genetic classification for malformations of cortical development: update 2012." Brain **135**(Pt 5): 1348-1369.

Barra, H. S., J. A. Rodriguez, C. A. Arce and R. Caputto (1973). "A soluble preparation from rat brain that incorporates into its own proteins ( 14 C)arginine by a ribonuclease-sensitive system and ( 14 C)tyrosine by a ribonuclease-insensitive system." J Neurochem **20**(1): 97-108.

Baudoin, J. P., C. Alvarez, P. Gaspar and C. Metin (2008). "Nocodazole-induced changes in microtubule dynamics impair the morphology and directionality of migrating medial ganglionic eminence cells." Dev Neurosci **30**(1-3): 132-143.

Becker, L., T. Mito, S. Takashima and K. Onodera (1991). "Growth and development of the brain in Down syndrome." Prog Clin Biol Res **373**: 133-152.

Beffert, U., E. J. Weeber, G. Morfini, J. Ko, S. T. Brady, L. H. Tsai, J. D. Sweatt and J. Herz (2004). "Reelin and cyclin-dependent kinase 5-dependent signals cooperate in regulating neuronal migration and synaptic transmission." J Neurosci **24**(8): 1897-1906.

Benham, A. M. (2012). "Protein secretion and the endoplasmic reticulum." Cold Spring Harb Perspect Biol **4**(8): a012872.

Berezuk, M. A. and T. A. Schroer (2007). "Dynactin enhances the processivity of kinesin-2." Traffic **8**(2): 124-129.

Berrueta, L., J. S. Tirnauer, S. C. Schuyler, D. Pellman and B. E. Bierer (1999). "The APC-associated protein EB1 associates with components of the dynactin complex and cytoplasmic dynein intermediate chain." Curr Biol **9**(8): 425-428.

Bieling, P., S. Kandels-Lewis, I. A. Telley, J. van Dijk, C. Janke and T. Surrey (2008). "CLIP-170 tracks growing microtubule ends by dynamically recognizing composite EB1/tubulin-binding sites." J Cell Biol **183**(7): 1223-1233.

Birch, N. P., C. Rodriguez, J. E. Dixon and E. Mezey (1990). "Distribution of carboxypeptidase H messenger RNA in rat brain using in situ hybridization histochemistry: implications for neuropeptide biosynthesis." Brain Res Mol Brain Res **7**(1): 53-59.

Blangy, A., L. Arnaud and E. A. Nigg (1997). "Phosphorylation by p34cdc2 protein kinase regulates binding of the kinesin-related motor HsEg5 to the dynactin subunit p150." J Biol Chem **272**(31): 19418-19424.

Bobinnec, Y., M. Moudjou, J. P. Fouquet, E. Desbruyeres, B. Edde and M. Bornens (1998). "Glutamylation of centriole and cytoplasmic tubulin in proliferating non-neuronal cells." Cell Motil Cytoskeleton **39**(3): 223-232.

Bonnet, C., D. Boucher, S. Lazereg, B. Pedrotti, K. Islam, P. Denoulet and J. C. Larcher (2001). "Differential binding regulation of microtubule-associated proteins MAP1A, MAP1B, and MAP2 by tubulin polyglutamylation." J Biol Chem **276**(16): 12839-12848.

Borghesani, P. R., J. M. Peyrin, R. Klein, J. Rubin, A. R. Carter, P. M. Schwartz, A. Luster, G. Corfas and R. A. Segal (2002). "BDNF stimulates migration of cerebellar granule cells." Development **129**(6): 1435-1442.

Boucher, D., J. C. Larcher, F. Gros and P. Denoulet (1994). "Polyglutamylation of tubulin as a progressive regulator of in vitro interactions between the microtubule-associated protein Tau and tubulin." Biochemistry **33**(41): 12471-12477.

Braunstein, K. E., J. Eschbach, K. Rona-Voros, R. Soylu, E. Mikrouli, Y. Larmet, F. Rene, J. L. Gonzalez De Aguilar, J. P. Loeffler, H. P. Muller, S. Bucher, T. Kaulisch, H. G. Niessen, J. Tillmanns, K. Fischer, B. Schwalenstocker, J. Kassubek, B. Pichler, D. Stiller, A. Petersen, A. C. Ludolph and L. Dupuis (2010). "A point mutation in the dynein heavy chain gene leads to striatal atrophy and compromises neurite outgrowth of striatal

neurons." Hum Mol Genet **19**(22): 4385-4398.

Brennand, K. J., A. Simone, N. Tran and F. H. Gage (2012). "Modeling psychiatric disorders at the cellular and network levels." Mol Psychiatry **17**(12): 1239-1253.

Broadbelt, K., W. Byne and L. B. Jones (2002). "Evidence for a decrease in basilar dendrites of pyramidal cells in schizophrenic medial prefrontal cortex." Schizophr Res **58**(1): 75-81.

Burton, P. R. (1988). "Dendrites of mitral cell neurons contain microtubules of opposite polarity." Brain Res **473**(1): 107-115.

Carrel, D., Y. Du, D. Komlos, N. M. Hadzimichalis, M. Kwon, B. Wang, L. M. Brzustowicz and B. L. Firestein (2009). "NOS1AP regulates dendrite patterning of hippocampal neurons through a carboxypeptidase E-mediated pathway." J Neurosci **29**(25): 8248-8258.

Carrel, D., K. Hernandez, M. Kwon, C. Mau, M. P. Trivedi, L. M. Brzustowicz and B. L. Firestein (2015). "Nitric oxide synthase 1 adaptor protein, a protein implicated in schizophrenia, controls radial migration of cortical neurons." Biol Psychiatry **77**(11): 969-978.

Cawley, N. X., W. C. Wetsel, S. R. Murthy, J. J. Park, K. Pacak and Y. P. Loh (2012). "New roles of carboxypeptidase E in endocrine and neural function and cancer." Endocr Rev **33**(2): 216-253.

Cawley, N. X., J. Zhou, J. M. Hill, D. Abebe, S. Romboz, T. Yanik, R. M. Rodriguiz, W. C. Wetsel and Y. P. Loh (2004). "The carboxypeptidase E knockout mouse exhibits endocrinological and behavioral deficits." Endocrinology **145**(12): 5807-5819.

Charych, E. I., B. F. Akum, J. S. Goldberg, R. J. Jornsten, C. Rongo, J. Q. Zheng and B. L. Firestein (2006). "Activity-independent regulation of dendrite patterning by postsynaptic density protein PSD-95." J Neurosci **26**(40): 10164-10176.

Chen, M., K. G. Lucas, B. F. Akum, G. Balasingam, T. M. Stawicki, J. M. Provost, G. M. Riefler, R. J. Jornsten and B. L. Firestein (2005). "A novel role for snapin in dendrite patterning: interaction with cypin." Mol Biol Cell **16**(11): 5103-5114.

Chen, T. Y., J. S. Syu, T. Y. Han, H. L. Cheng, F. I. Lu and C. Y. Wang (2015). "Cell Cycle-Dependent Localization of Dynactin Subunit p150 glued at Centrosome." J Cell Biochem **116**(9): 2049-2060.

Cheng, Y., N. X. Cawley and Y. P. Loh (2013). "Carboxypeptidase E/NFalpha1: a new neurotrophic factor against oxidative stress-induced apoptotic cell death mediated

by ERK and PI3-K/AKT pathways." PLoS One **8**(8): e71578.

Cheng, Y., N. X. Cawley, T. Yanik, S. R. Murthy, C. Liu, F. Kasikci, D. Abebe and Y. P. Loh (2016). "A human carboxypeptidase E/NF-alpha1 gene mutation in an Alzheimer's disease patient leads to dementia and depression in mice." Transl Psychiatry **6**(12): e973.

Cheng, Y., R. M. Rodriguiz, S. R. Murthy, V. Senatorov, E. Thouennon, N. X. Cawley, D. K. Aryal, S. Ahn, B. Lecka-Czernik, W. C. Wetsel and Y. P. Loh (2015). "Neurotrophic factor-alpha1 prevents stress-induced depression through enhancement of neurogenesis and is activated by rosiglitazone." Mol Psychiatry **20**(6): 744-754.

Chheda, M. G., U. Ashery, P. Thakur, J. Rettig and Z. H. Sheng (2001). "Phosphorylation of Snapin by PKA modulates its interaction with the SNARE complex." Nat Cell Biol **3**(4): 331-338.

Chiaramello, S., G. Dalmasso, L. Bezin, D. Marcel, F. Jourdan, P. Peretto, A. Fasolo and S. De Marchis (2007). "BDNF/ TrkB interaction regulates migration of SVZ precursor cells via PI3-K and MAP-K signalling pathways." Eur J Neurosci **26**(7): 1780-1790.

Coleman, D. L. and E. M. Eicher (1990). "Fat (fat) and tubby (tub): two autosomal recessive mutations causing obesity syndromes in the mouse." J Hered **81**(6): 424-427.

Cong, L., Y. Cheng, N. X. Cawley, S. R. Murthy and Y. P. Loh (2017). "A Novel Single Nucleotide T980C Polymorphism in the Human Carboxypeptidase E Gene Results in Loss of Neuroprotective Function." PLoS One **12**(1): e0170169.

Copf, T. (2016). "Impairments in dendrite morphogenesis as etiology for neurodevelopmental disorders and implications for therapeutic treatments." Neurosci Biobehav Rev **68**: 946-978.

Creppe, C., L. Malinouskaya, M. L. Volvert, M. Gillard, P. Close, O. Malaise, S. Laguesse, I. Cornez, S. Rahmouni, S. Ormenese, S. Belachew, B. Malgrange, J. P. Chapelle, U. Siebenlist, G. Moonen, A. Chariot and L. Nguyen (2009). "Elongator controls the migration and differentiation of cortical neurons through acetylation of alpha-tubulin." Cell **136**(3): 551-564.

Cui-Wang, T., C. Hanus, T. Cui, T. Helton, J. Bourne, D. Watson, K. M. Harris and M. D. Ehlers (2012). "Local zones of endoplasmic reticulum complexity confine cargo in neuronal dendrites." Cell **148**(1-2): 309-321.

D'Arcangelo, G., R. Homayouni, L. Keshvara, D. S. Rice, M. Sheldon and T. Curran

(1999). "Reelin is a ligand for lipoprotein receptors." Neuron **24**(2): 471-479.

Deacon, S. W., A. S. Serpinskaya, P. S. Vaughan, M. Lopez Fanarraga, I. Vernos, K. T. Vaughan and V. I. Gelfand (2003). "Dynactin is required for bidirectional organelle transport." J Cell Biol **160**(3): 297-301.

Dehay, C. and H. Kennedy (2007). "Cell-cycle control and cortical development." Nat Rev Neurosci **8**(6): 438-450.

Dent, E. W. (2017). "Of microtubules and memory: implications for microtubule dynamics in dendrites and spines." Mol Biol Cell **28**(1): 1-8.

Dhanvantari, S., F. S. Shen, T. Adams, C. R. Snell, C. Zhang, R. B. Mackin, S. J. Morris and Y. P. Loh (2003). "Disruption of a receptor-mediated mechanism for intracellular sorting of proinsulin in familial hyperproinsulinemia." Mol Endocrinol **17**(9): 1856-1867.

Di Cristo, G. (2007). "Development of cortical GABAergic circuits and its implications for neurodevelopmental disorders." Clin Genet **72**(1): 1-8.

Dimidschstein, J., L. Passante, A. Dufour, J. van den Ameele, L. Tiberi, T. Hrechdakian, R. Adams, R. Klein, D. C. Lie, Y. Jossin and P. Vanderhaeghen (2013). "Ephrin-B1 controls the columnar distribution of cortical pyramidal neurons by restricting their tangential migration." Neuron **79**(6): 1123-1135.

Dotti, C. G., C. A. Sullivan and G. A. Banker (1988). "The establishment of polarity by hippocampal neurons in culture." J Neurosci **8**(4): 1454-1468.

Doxsey, S. J., P. Stein, L. Evans, P. D. Calarco and M. Kirschner (1994). "Pericentrin, a highly conserved centrosome protein involved in microtubule organization." Cell **76**(4): 639-650.

Dujardin, D. L., L. E. Barnhart, S. A. Stehman, E. R. Gomes, G. G. Gundersen and R. B. Vallee (2003). "A role for cytoplasmic dynein and LIS1 in directed cell movement." J Cell Biol **163**(6): 1205-1211.

Edde, B., J. Rossier, J. P. Le Caer, E. Desbruyeres, F. Gros and P. Denoulet (1990). "Posttranslational glutamylation of alpha-tubulin." Science **247**(4938): 83-85.

Falconer, M. M., U. Vielkind and D. L. Brown (1989). "Establishment of a stable, acetylated microtubule bundle during neuronal commitment." Cell Motil Cytoskeleton **12**(3): 169-180.

Fan, S., X. Li, L. Li, L. Wang, Z. Du, Y. Yang, J. Zhao and Y. Li (2016). "Silencing of

carboxypeptidase E inhibits cell proliferation, tumorigenicity, and metastasis of osteosarcoma cells." Onco Targets Ther **9**: 2795-2803.

Farrer, M. J., M. M. Hulihan, J. M. Kachergus, J. C. Dachsel, A. J. Stoessl, L. L. Grantier, S. Calne, D. B. Calne, B. Lechevalier, F. Chapon, Y. Tsuboi, T. Yamada, L. Gutmann, B. Elibol, K. P. Bhatia, C. Wider, C. Vilarino-Guell, O. A. Ross, L. A. Brown, M. Castanedes-Casey, D. W. Dickson and Z. K. Wszolek (2009). "DCTN1 mutations in Perry syndrome." Nat Genet **41**(2): 163-165.

Firestein, B. L., B. L. Firestein, J. E. Brenman, C. Aoki, A. M. Sanchez-Perez, A. E. El-Husseini and D. S. Bredt (1999). "Cypin: a cytosolic regulator of PSD-95 postsynaptic targeting." Neuron **24**(3): 659-672.

Fricker, L. D., Y. L. Berman, E. H. Leiter and L. A. Devi (1996). "Carboxypeptidase E activity is deficient in mice with the fat mutation. Effect on peptide processing." J Biol Chem **271**(48): 30619-30624.

Fricker, L. D. and S. H. Snyder (1982). "Enkephalin convertase: purification and characterization of a specific enkephalin-synthesizing carboxypeptidase localized to adrenal chromaffin granules." Proc Natl Acad Sci U S A **79**(12): 3886-3890.

Fukuda, T. and S. Yanagi (2017). "Psychiatric behaviors associated with cytoskeletal defects in radial neuronal migration." Cell Mol Life Sci.

Garey, L. J., W. Y. Ong, T. S. Patel, M. Kanani, A. Davis, A. M. Mortimer, T. R. Barnes and S. R. Hirsch (1998). "Reduced dendritic spine density on cerebral cortical pyramidal neurons in schizophrenia." J Neurol Neurosurg Psychiatry **65**(4): 446-453.

Ghosh-Roy, A., A. Goncharov, Y. Jin and A. D. Chisholm (2012). "Kinesin-13 and tubulin posttranslational modifications regulate microtubule growth in axon regeneration." Dev Cell **23**(4): 716-728.

Glantz, L. A. and D. A. Lewis (2000). "Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia." Arch Gen Psychiatry **57**(1): 65-73.

Goldstein, L. S. and Z. Yang (2000). "Microtubule-based transport systems in neurons: the roles of kinesins and dyneins." Annu Rev Neurosci **23**: 39-71.

Gomis-Ruth, F. X., V. Companys, Y. Qian, L. D. Fricker, J. Vendrell, F. X. Aviles and M. Coll (1999). "Crystal structure of avian carboxypeptidase D domain II: a prototype for the regulatory metallocarboxypeptidase subfamily." EMBO J **18**(21): 5817-5826.

Gonzalez-Billault, C., J. A. Del Rio, J. M. Urena, E. M. Jimenez-Mateos, M. J. Barallobre, M. Pascual, L. Pujadas, S. Simo, A. L. Torre, R. Gavin, F. Wandosell, E. Soriano and J.

Avila (2005). "A role of MAP1B in Reelin-dependent neuronal migration." Cereb Cortex **15**(8): 1134-1145.

Greer, C. A. and G. M. Shepherd (1982). "Mitral cell degeneration and sensory function in the neurological mutant mouse Purkinje cell degeneration (PCD)." Brain Res **235**(1): 156-161.

Gu, J., B. L. Firestein and J. Q. Zheng (2008). "Microtubules in dendritic spine development." J Neurosci **28**(46): 12120-12124.

Gundersen, G. G. and J. C. Bulinski (1986). "Distribution of tyrosinated and nontyrosinated alpha-tubulin during mitosis." J Cell Biol **102**(3): 1118-1126.

Gundersen, G. G., S. Khawaja and J. C. Bulinski (1987). "Postpolymerization detyrosination of alpha-tubulin: a mechanism for subcellular differentiation of microtubules." J Cell Biol **105**(1): 251-264.

Harris, A., J. I. Morgan, M. Pecot, A. Soumare, A. Osborne and H. D. Soares (2000). "Regenerating motor neurons express Nna1, a novel ATP/GTP-binding protein related to zinc carboxypeptidases." Mol Cell Neurosci **16**(5): 578-596.

Hiesberger, T., M. Trommsdorff, B. W. Howell, A. Goffinet, M. C. Mumby, J. A. Cooper and J. Herz (1999). "Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation." Neuron **24**(2): 481-489.

Hill, J. J., T. Hashimoto and D. A. Lewis (2006). "Molecular mechanisms contributing to dendritic spine alterations in the prefrontal cortex of subjects with schizophrenia." Mol Psychiatry **11**(6): 557-566.

Hoogenraad, C. C., A. D. Milstein, I. M. Ethell, M. Henkemeyer and M. Sheng (2005). "GRIP1 controls dendrite morphogenesis by regulating EphB receptor trafficking." Nat Neurosci **8**(7): 906-915.

Hook, V. Y., L. E. Eiden and M. J. Brownstein (1982). "A carboxypeptidase processing enzyme for enkephalin precursors." Nature **295**(5847): 341-342.

Horch, H. W., A. Kruttgen, S. D. Portbury and L. C. Katz (1999). "Destabilization of cortical dendrites and spines by BDNF." Neuron **23**(2): 353-364.

Horton, A. C., B. Racz, E. E. Monson, A. L. Lin, R. J. Weinberg and M. D. Ehlers (2005). "Polarized secretory trafficking directs cargo for asymmetric dendrite growth and morphogenesis." Neuron **48**(5): 757-771.

Hu, X., C. Viesselmann, S. Nam, E. Merriam and E. W. Dent (2008). "Activity-dependent dynamic microtubule invasion of dendritic spines." J Neurosci **28**(49): 13094-13105.

Hutsler, J. J. and H. Zhang (2010). "Increased dendritic spine densities on cortical projection neurons in autism spectrum disorders." Brain Res **1309**: 83-94.

Ikegami, K., R. L. Heier, M. Taruishi, H. Takagi, M. Mukai, S. Shimma, S. Taira, K. Hatanaka, N. Morone, I. Yao, P. K. Campbell, S. Yuasa, C. Janke, G. R. Macgregor and M. Setou (2007). "Loss of alpha-tubulin polyglutamylation in ROSA22 mice is associated with abnormal targeting of KIF1A and modulated synaptic function." Proc Natl Acad Sci U S A **104**(9): 3213-3218.

Ikegami, K., M. Mukai, J. Tsuchida, R. L. Heier, G. R. Macgregor and M. Setou (2006). "TTLL7 is a mammalian beta-tubulin polyglutamylase required for growth of MAP2-positive neurites." J Biol Chem **281**(41): 30707-30716.

Janke, C. and J. C. Bulinski (2011). "Post-translational regulation of the microtubule cytoskeleton: mechanisms and functions." Nat Rev Mol Cell Biol **12**(12): 773-786.

Janke, C., K. Rogowski, D. Wloga, C. Regnard, A. V. Kajava, J. M. Strub, N. Temurak, J. van Dijk, D. Boucher, A. van Dorsselaer, S. Suryavanshi, J. Gaertig and B. Edde (2005). "Tubulin polyglutamylase enzymes are members of the TTL domain protein family." Science **308**(5729): 1758-1762.

Jaworski, J., L. C. Kapitein, S. M. Gouveia, B. R. Dortland, P. S. Wulf, I. Grigoriev, P. Camera, S. A. Spangler, P. Di Stefano, J. Demmers, H. Krugers, P. Defilippi, A. Akhmanova and C. C. Hoogenraad (2009). "Dynamic microtubules regulate dendritic spine morphology and synaptic plasticity." Neuron **61**(1): 85-100.

Jiang, X. and J. Nardelli (2016). "Cellular and molecular introduction to brain development." Neurobiol Dis **92**(Pt A): 3-17.

Jin, K., M. LaFevre-Bernt, Y. Sun, S. Chen, J. Gafni, D. Crippen, A. Logvinova, C. A. Ross, D. A. Greenberg and L. M. Ellerby (2005). "FGF-2 promotes neurogenesis and neuroprotection and prolongs survival in a transgenic mouse model of Huntington's disease." Proc Natl Acad Sci U S A **102**(50): 18189-18194.

Kalebic, N., S. Sorrentino, E. Perlas, G. Bolasco, C. Martinez and P. A. Heppenstall (2013). "alphaTAT1 is the major alpha-tubulin acetyltransferase in mice." Nat Commun **4**: 1962.

Kalinina, E., R. Biswas, I. Berezniuk, A. Hermoso, F. X. Aviles and L. D. Fricker (2007).

"A novel subfamily of mouse cytosolic carboxypeptidases." FASEB J **21**(3): 836-850.

Kamiya, A., K. Kubo, T. Tomoda, M. Takaki, R. Youn, Y. Ozeki, N. Sawamura, U. Park, C. Kudo, M. Okawa, C. A. Ross, M. E. Hatten, K. Nakajima and A. Sawa (2005). "A schizophrenia-associated mutation of DISC1 perturbs cerebral cortex development." Nat Cell Biol **7**(12): 1167-1178.

Kasai, H., M. Fukuda, S. Watanabe, A. Hayashi-Takagi and J. Noguchi (2010). "Structural dynamics of dendritic spines in memory and cognition." Trends Neurosci **33**(3): 121-129.

King, S. J. and T. A. Schroer (2000). "Dynactin increases the processivity of the cytoplasmic dynein motor." Nat Cell Biol **2**(1): 20-24.

Knutson, D. C. and M. Clagett-Dame (2008). "atRA Regulation of NEDD9, a gene involved in neurite outgrowth and cell adhesion." Arch Biochem Biophys **477**(1): 163-174.

Knutson, D. C., A. M. Mitzey, L. E. Talton and M. Clagett-Dame (2016). "Mice null for NEDD9 (HEF1alpha) display extensive hippocampal dendritic spine loss and cognitive impairment." Brain Res **1632**: 141-155.

Kolluri, N., Z. Sun, A. R. Sampson and D. A. Lewis (2005). "Lamina-specific reductions in dendritic spine density in the prefrontal cortex of subjects with schizophrenia." Am J Psychiatry **162**(6): 1200-1202.

Konishi, Y. and M. Setou (2009). "Tubulin tyrosination navigates the kinesin-1 motor domain to axons." Nat Neurosci **12**(5): 559-567.

Koshimizu, H., V. Senatorov, Y. P. Loh and I. Gozes (2009). "Neuroprotective protein and carboxypeptidase E." J Mol Neurosci **39**(1-2): 1-8.

Kulkarni, V. A. and B. L. Firestein (2012). "The dendritic tree and brain disorders." Mol Cell Neurosci **50**(1): 10-20.

Kutzing, M. K., C. G. Langhammer, V. Luo, H. Lakdawala and B. L. Firestein (2010). "Automated Sholl analysis of digitized neuronal morphology at multiple scales." J Vis Exp(45).

Kwinter, D. M., K. Lo, P. Mafi and M. A. Silverman (2009). "Dynactin regulates bidirectional transport of dense-core vesicles in the axon and dendrites of cultured hippocampal neurons." Neuroscience **162**(4): 1001-1010.

Kwon, M., J. R. Fernandez, G. F. Zegarek, S. B. Lo and B. L. Firestein (2011).

"BDNF-promoted increases in proximal dendrites occur via CREB-dependent transcriptional regulation of cypin." J Neurosci **31**(26): 9735-9745.

Langhammer, C. G., M. L. Previtera, E. S. Sweet, S. S. Sran, M. Chen and B. L. Firestein (2010). "Automated Sholl analysis of digitized neuronal morphology at multiple scales: Whole cell Sholl analysis versus Sholl analysis of arbor subregions." Cytometry A **77**(12): 1160-1168.

Larcher, J. C., D. Boucher, S. Lazereg, F. Gros and P. Denoulet (1996). "Interaction of kinesin motor domains with alpha- and beta-tubulin subunits at a tau-independent binding site. Regulation by polyglutamylation." J Biol Chem **271**(36): 22117-22124.

Lazarus, J. E., A. J. Moughamian, M. K. Tokito and E. L. Holzbaur (2013). "Dynactin subunit p150(Glued) is a neuron-specific anti-catastrophe factor." PLoS Biol **11**(7): e1001611.

Lee, T. K., S. R. Murthy, N. X. Cawley, S. Dhanvantari, S. M. Hewitt, H. Lou, T. Lau, S. Ma, T. Huynh, R. A. Wesley, I. O. Ng, K. Pacak, R. T. Poon and Y. P. Loh (2011). "An N-terminal truncated carboxypeptidase E splice isoform induces tumor growth and is a biomarker for predicting future metastasis in human cancers." J Clin Invest **121**(3): 880-892.

Li, J., W. L. Lee and J. A. Cooper (2005). "NudEL targets dynein to microtubule ends through LIS1." Nat Cell Biol **7**(7): 686-690.

Liang, X. H., L. L. Li, G. G. Wu, Y. C. Xie, G. X. Zhang, W. Chen, H. F. Yang, Q. L. Liu, W. H. Li, W. G. He, Y. N. Huang and X. C. Zeng (2013). "Upregulation of CPE promotes cell proliferation and tumorigenicity in colorectal cancer." BMC Cancer **13**: 412.

Ligon, L. A., S. S. Shelly, M. K. Tokito and E. L. Holzbaur (2006). "Microtubule binding proteins CLIP-170, EB1, and p150Glued form distinct plus-end complexes." FEBS Lett **580**(5): 1327-1332.

Liu, A., C. Shao, G. Jin, R. Liu, J. Hao, Z. Shao, Q. Liu and X. Hu (2014). "Downregulation of CPE regulates cell proliferation and chemosensitivity in pancreatic cancer." Tumour Biol **35**(12): 12459-12465.

Lloyd, T. E., J. Machamer, K. O'Hara, J. H. Kim, S. E. Collins, M. Y. Wong, B. Sahin, W. Imlach, Y. Yang, E. S. Levitan, B. D. McCabe and A. L. Kolodkin (2012). "The p150(Glued) CAP-Gly domain regulates initiation of retrograde transport at synaptic termini." Neuron **74**(2): 344-360.

Loh, Y. P., A. Maldonado, C. Zhang, W. H. Tam and N. Cawley (2002). "Mechanism of

sorting proopiomelanocortin and proenkephalin to the regulated secretory pathway of neuroendocrine cells." Ann N Y Acad Sci **971**: 416-425.

London, M. and M. Hausser (2005). "Dendritic computation." Annu Rev Neurosci **28**: 503-532.

Lou, H., S. K. Kim, E. Zaitsev, C. R. Snell, B. Lu and Y. P. Loh (2005). "Sorting and activity-dependent secretion of BDNF require interaction of a specific motif with the sorting receptor carboxypeptidase e." Neuron **45**(2): 245-255.

Lou, H., J. J. Park, N. X. Cawley, A. Sarcon, L. Sun, T. Adams and Y. P. Loh (2010). "Carboxypeptidase E cytoplasmic tail mediates localization of synaptic vesicles to the pre-active zone in hypothalamic pre-synaptic terminals." J Neurochem **114**(3): 886-896.

Maas, C., D. Belgardt, H. K. Lee, F. F. Heisler, C. Lappe-Siefke, M. M. Magiera, J. van Dijk, T. J. Hausrat, C. Janke and M. Kneussel (2009). "Synaptic activation modifies microtubules underlying transport of postsynaptic cargo." Proc Natl Acad Sci U S A **106**(21): 8731-8736.

Marin, O., M. Valiente, X. Ge and L. H. Tsai (2010). "Guiding neuronal cell migrations." Cold Spring Harb Perspect Biol **2**(2): a001834.

McAllister, A. K., D. C. Lo and L. C. Katz (1995). "Neurotrophins regulate dendritic growth in developing visual cortex." Neuron **15**(4): 791-803.

McGrail, M., J. Gepner, A. Silvanovich, S. Ludmann, M. Serr and T. S. Hays (1995). "Regulation of cytoplasmic dynein function in vivo by the Drosophila Glued complex." J Cell Biol **131**(2): 411-425.

Medina, D. L., C. Sciarretta, A. M. Calella, O. Von Bohlen Und Halbach, K. Unsicker and L. Minichiello (2004). "TrkB regulates neocortex formation through the Shc/PLCgamma-mediated control of neuronal migration." EMBO J **23**(19): 3803-3814.

Mirzaa, G. M. and A. R. Paciorkowski (2014). "Introduction: Brain malformations." Am J Med Genet C Semin Med Genet **166C**(2): 117-123.

Mitchison, T. and M. Kirschner (1984). "Dynamic instability of microtubule growth." Nature **312**(5991): 237-242.

Miyata, T., A. Kawaguchi, H. Okano and M. Ogawa (2001). "Asymmetric inheritance of radial glial fibers by cortical neurons." Neuron **31**(5): 727-741.

Moughamian, A. J. and E. L. Holzbaur (2012). "Dynactin is required for transport initiation from the distal axon." Neuron **74**(2): 331-343.

Mullen, R. J., E. M. Eicher and R. L. Sidman (1976). "Purkinje cell degeneration, a new neurological mutation in the mouse." Proc Natl Acad Sci U S A **73**(1): 208-212.

Nadarajah, B., J. E. Brunstrom, J. Grutzendler, R. O. Wong and A. L. Pearlman (2001). "Two modes of radial migration in early development of the cerebral cortex." Nat Neurosci **4**(2): 143-150.

Noctor, S. C., V. Martinez-Cerdeno, L. Ivic and A. R. Kriegstein (2004). "Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases." Nat Neurosci **7**(2): 136-144.

Ohtaka-Maruyama, C. and H. Okado (2015). "Molecular Pathways Underlying Projection Neuron Production and Migration during Cerebral Cortical Development." Front Neurosci **9**: 447.

Oiso, S., Y. Takeda, T. Futagawa, T. Miura, S. Kuchiiwa, K. Nishida, R. Ikeda, H. Kariyazono, K. Watanabe and K. Yamada (2009). "Contactin-associated protein (Caspr) 2 interacts with carboxypeptidase E in the CNS." J Neurochem **109**(1): 158-167.

Ortiz-Lopez, L., N. M. Vega-Rivera, H. Babu and G. B. Ramirez-Rodriguez (2017). "Brain-Derived Neurotrophic Factor Induces Cell Survival and the Migration of Murine Adult Hippocampal Precursor Cells During Differentiation In Vitro." Neurotox Res **31**(1): 122-135.

Park, J. J., N. X. Cawley and Y. P. Loh (2008). "A bi-directional carboxypeptidase E-driven transport mechanism controls BDNF vesicle homeostasis in hippocampal neurons." Mol Cell Neurosci **39**(1): 63-73.

Park, J. J., N. X. Cawley and Y. P. Loh (2008). "Carboxypeptidase E cytoplasmic tail-driven vesicle transport is key for activity-dependent secretion of peptide hormones." Mol Endocrinol **22**(4): 989-1005.

Parkinson, D. (1992). "Carboxypeptidase H in bovine pituitary gland: soluble forms are not processed at the C-terminus." Mol Cell Endocrinol **86**(3): 221-233.

Penazzi, L., L. Bakota and R. Brandt (2016). "Microtubule Dynamics in Neuronal Development, Plasticity, and Neurodegeneration." Int Rev Cell Mol Biol **321**: 89-169.

Perdiz, D., R. Mackeh, C. Pous and A. Baillet (2011). "The ins and outs of tubulin acetylation: more than just a post-translational modification?" Cell Signal **23**(5):

763-771.

Peris, L., M. Wagenbach, L. Lafanechere, J. Brocard, A. T. Moore, F. Kozielski, D. Job, L. Wordeman and A. Andrieux (2009). "Motor-dependent microtubule disassembly driven by tubulin tyrosination." J Cell Biol **185**(7): 1159-1166.

Piperno, G., M. LeDizet and X. J. Chang (1987). "Microtubules containing acetylated alpha-tubulin in mammalian cells in culture." J Cell Biol **104**(2): 289-302.

Qin, X. Y., Y. Cheng, S. R. Murthy, P. Selvaraj and Y. P. Loh (2014). "carboxypeptidase E-DeltaN, a neuroprotein transiently expressed during development protects embryonic neurons against glutamate neurotoxicity." PLoS One **9**(11): e112996.

Quintyne, N. J., S. R. Gill, D. M. Eckley, C. L. Crego, D. A. Compton and T. A. Schroer (1999). "Dynactin is required for microtubule anchoring at centrosomes." J Cell Biol **147**(2): 321-334.

Quintyne, N. J. and T. A. Schroer (2002). "Distinct cell cycle-dependent roles for dynactin and dynein at centrosomes." J Cell Biol **159**(2): 245-254.

Rakic, P. (1974). "Neurons in rhesus monkey visual cortex: systematic relation between time of origin and eventual disposition." Science **183**(4123): 425-427.

Rakic, P. (2006). "A century of progress in corticoneurogenesis: from silver impregnation to genetic engineering." Cereb Cortex **16 Suppl 1**: i3-17.

Raybin, D. and M. Flavin (1977). "Enzyme which specifically adds tyrosine to the alpha chain of tubulin." Biochemistry **16**(10): 2189-2194.

Raymond, G. V., M. L. Bauman and T. L. Kemper (1996). "Hippocampus in autism: a Golgi analysis." Acta Neuropathol **91**(1): 117-119.

Regnard, C., E. Desbruyeres, P. Denoulet and B. Edde (1999). "Tubulin polyglutamylase: isozymic variants and regulation during the cell cycle in HeLa cells." J Cell Sci **112 ( Pt 23)**: 4281-4289.

Regnard, C., D. Fesquet, C. Janke, D. Boucher, E. Desbruyeres, A. Koulakoff, C. Insina, P. Travo and B. Edde (2003). "Characterisation of PGs1, a subunit of a protein complex co-purifying with tubulin polyglutamylase." J Cell Sci **116**(Pt 20): 4181-4190.

Reznik, S. E. and L. D. Fricker (2001). "Carboxypeptidases from A to z: implications in embryonic development and Wnt binding." Cell Mol Life Sci **58**(12-13): 1790-1804.

Riano, E., M. Martignoni, G. Mancuso, D. Cartelli, F. Crippa, I. Toldo, G. Siciliano, D. Di

Bella, F. Taroni, M. T. Bassi, G. Cappelletti and E. I. Rugarli (2009). "Pleiotropic effects of spastin on neurite growth depending on expression levels." J Neurochem **108**(5): 1277-1288.

Ringstedt, T., S. Linnarsson, J. Wagner, U. Lendahl, Z. Kokaia, E. Arenas, P. Ernfors and C. F. Ibanez (1998). "BDNF regulates reelin expression and Cajal-Retzius cell development in the cerebral cortex." Neuron **21**(2): 305-315.

Rodriguez de la Vega, M., R. G. Sevilla, A. Hermoso, J. Lorenzo, S. Tanco, A. Diez, L. D. Fricker, J. M. Bautista and F. X. Aviles (2007). "Nna1-like proteins are active metallocarboxypeptidases of a new and diverse M14 subfamily." FASEB J **21**(3): 851-865.

Rodriguiz, R. M., J. J. Wilkins, T. K. Creson, R. Biswas, I. Berezniuk, A. D. Fricker, L. D. Fricker and W. C. Wetsel (2013). "Emergence of anxiety-like behaviours in depressive-like Cpe(fat/fat) mice." Int J Neuropsychopharmacol **16**(7): 1623-1634.

Rogowski, K., J. van Dijk, M. M. Magiera, C. Bosc, J. C. Deloulme, A. Bosson, L. Peris, N. D. Gold, B. Lacroix, M. Bosch Grau, N. Bec, C. Larroque, S. Desagher, M. Holzer, A. Andrieux, M. J. Moutin and C. Janke (2010). "A family of protein-deglutamylating enzymes associated with neurodegeneration." Cell **143**(4): 564-578.

Sachdev, P., S. Menon, D. B. Kastner, J. Z. Chuang, T. Y. Yeh, C. Conde, A. Caceres, C. H. Sung and T. P. Sakmar (2007). "G protein beta gamma subunit interaction with the dynein light-chain component Tctex-1 regulates neurite outgrowth." EMBO J **26**(11): 2621-2632.

Sakakibara, A., R. Ando, T. Sapir and T. Tanaka (2013). "Microtubule dynamics in neuronal morphogenesis." Open Biol **3**(7): 130061.

Sakakibara, A. and Y. Hatanaka (2015). "Neuronal polarization in the developing cerebral cortex." Front Neurosci **9**: 116.

Satoh, D., D. Sato, T. Tsuyama, M. Saito, H. Ohkura, M. M. Rolls, F. Ishikawa and T. Uemura (2008). "Spatial control of branching within dendritic arbors by dynein-dependent transport of Rab5-endosomes." Nat Cell Biol **10**(10): 1164-1171.

Schroer, T. A. (2004). "Dynactin." Annu Rev Cell Dev Biol **20**: 759-779.

Selvaraj, P., L. Xiao, C. Lee, S. R. Murthy, N. X. Cawley, M. Lane, I. Merchenthaler, S. Ahn and Y. P. Loh (2017). "Neurotrophic Factor-alpha1: A Key Wnt-beta-Catenin Dependent Anti-Proliferation Factor and ERK-Sox9 Activated Inducer of Embryonic Neural Stem Cell Differentiation to Astrocytes in Neurodevelopment." Stem Cells

**35**(3): 557-571.

Shida, T., J. G. Cueva, Z. Xu, M. B. Goodman and M. V. Nachury (2010). "The major alpha-tubulin K40 acetyltransferase alphaTAT1 promotes rapid ciliogenesis and efficient mechanosensation." Proc Natl Acad Sci U S A **107**(50): 21517-21522.

Smith, D. S., M. Niethammer, R. Ayala, Y. Zhou, M. J. Gambello, A. Wynshaw-Boris and L. H. Tsai (2000). "Regulation of cytoplasmic dynein behaviour and microtubule organization by mammalian Lis1." Nat Cell Biol **2**(11): 767-775.

Song, Y. and S. T. Brady (2015). "Post-translational modifications of tubulin: pathways to functional diversity of microtubules." Trends Cell Biol **25**(3): 125-136.

Spruston, N. (2008). "Pyramidal neurons: dendritic structure and synaptic integration." Nat Rev Neurosci **9**(3): 206-221.

Szebenyi, G., E. W. Dent, J. L. Callaway, C. Seys, H. Lueth and K. Kalil (2001). "Fibroblast growth factor-2 promotes axon branching of cortical neurons by influencing morphology and behavior of the primary growth cone." J Neurosci **21**(11): 3932-3941.

Tabata, H. and K. Nakajima (2003). "Multipolar migration: the third mode of radial neuronal migration in the developing cerebral cortex." J Neurosci **23**(31): 9996-10001.

Tai, C. Y., D. L. Dujardin, N. E. Faulkner and R. B. Vallee (2002). "Role of dynein, dynactin, and CLIP-170 interactions in LIS1 kinetochore function." J Cell Biol **156**(6): 959-968.

Takano, T., C. Xu, Y. Funahashi, T. Namba and K. Kaibuchi (2015). "Neuronal polarization." Development **142**(12): 2088-2093.

Takashima, S., A. Ieshima, H. Nakamura and L. E. Becker (1989). "Dendrites, dementia and the Down syndrome." Brain Dev **11**(2): 131-133.

Tanaka, T., F. F. Serneo, C. Higgins, M. J. Gambello, A. Wynshaw-Boris and J. G. Gleeson (2004). "Lis1 and doublecortin function with dynein to mediate coupling of the nucleus to the centrosome in neuronal migration." J Cell Biol **165**(5): 709-721.

Tsai, J. W., K. H. Bremner and R. B. Vallee (2007). "Dual subcellular roles for LIS1 and dynein in radial neuronal migration in live brain tissue." Nat Neurosci **10**(8): 970-979.

Tsai, J. W., Y. Chen, A. R. Kriegstein and R. B. Vallee (2005). "LIS1 RNA interference

blocks neural stem cell division, morphogenesis, and motility at multiple stages." J Cell Biol **170**(6): 935-945.

Tsai, L. H. and J. G. Gleeson (2005). "Nucleokinesis in neuronal migration." Neuron **46**(3): 383-388.

Tseng, C. Y. and B. L. Firestein (2011). "The role of PSD-95 and cypin in morphological changes in dendrites following sublethal NMDA exposure." J Neurosci **31**(43): 15468-15480.

Umeshima, H., T. Hirano and M. Kengaku (2007). "Microtubule-based nuclear movement occurs independently of centrosome positioning in migrating neurons." Proc Natl Acad Sci U S A **104**(41): 16182-16187.

Valenstein, M. L. and A. Roll-Mecak (2016). "Graded Control of Microtubule Severing by Tubulin Glutamylation." Cell **164**(5): 911-921.

Valiente, M. and O. Marin (2010). "Neuronal migration mechanisms in development and disease." Curr Opin Neurobiol **20**(1): 68-78.

van Dijk, J., K. Rogowski, J. Miro, B. Lacroix, B. Edde and C. Janke (2007). "A targeted multienzyme mechanism for selective microtubule polyglutamylation." Mol Cell **26**(3): 437-448.

Vergano-Vera, E., H. R. Mendez-Gomez, A. Hurtado-Chong, J. C. Cigudosa and C. Vicario-Abejon (2009). "Fibroblast growth factor-2 increases the expression of neurogenic genes and promotes the migration and differentiation of neurons derived from transplanted neural stem/progenitor cells." Neuroscience **162**(1): 39-54.

Vilijn, M. H., B. Das, J. A. Kessler and L. D. Fricker (1989). "Cultured astrocytes and neurons synthesize and secrete carboxypeptidase E, a neuropeptide-processing enzyme." J Neurochem **53**(5): 1487-1493.

Vogel, T., S. Ahrens, N. Buttner and K. Krieglstein (2010). "Transforming growth factor beta promotes neuronal cell fate of mouse cortical and hippocampal progenitors in vitro and in vivo: identification of Nedd9 as an essential signaling component." Cereb Cortex **20**(3): 661-671.

Waterman-Storer, C. M., S. Karki and E. L. Holzbaur (1995). "The p150Glued component of the dynactin complex binds to both microtubules and the actin-related protein centractin (Arp-1)." Proc Natl Acad Sci U S A **92**(5): 1634-1638.

Wider, C., J. C. Dachsel, M. J. Farrer, D. W. Dickson, Y. Tsuboi and Z. K. Wszolek (2010).

"Elucidating the genetics and pathology of Perry syndrome." J Neurol Sci **289**(1-2): 149-154.

Witt, O., H. E. Deubzer, T. Milde and I. Oehme (2009). "HDAC family: What are the cancer relevant targets?" Cancer Lett **277**(1): 8-21.

Woodworth, M. B., L. Custo Greig, A. R. Kriegstein and J. D. Macklis (2012). "SnapShot: cortical development." Cell **151**(4): 918-918 e911.

Woronowicz, A., N. X. Cawley, S. Y. Chang, H. Koshimizu, A. W. Phillips, Z. G. Xiong and Y. P. Loh (2010). "Carboxypeptidase E knockout mice exhibit abnormal dendritic arborization and spine morphology in central nervous system neurons." J Neurosci Res **88**(1): 64-72.

Woronowicz, A., H. Koshimizu, S. Y. Chang, N. X. Cawley, J. M. Hill, R. M. Rodriguiz, D. Abebe, C. Dorfman, V. Senatorov, A. Zhou, Z. G. Xiong, W. C. Wetsel and Y. P. Loh (2008). "Absence of carboxypeptidase E leads to adult hippocampal neuronal degeneration and memory deficits." Hippocampus **18**(10): 1051-1063.

Wu, H. Y., T. Wang, L. Li, K. Correia and J. I. Morgan (2012). "A structural and functional analysis of Nna1 in Purkinje cell degeneration (pcd) mice." FASEB J **26**(11): 4468-4480.

Yao, Y. L. and W. M. Yang (2011). "Beyond histone and deacetylase: an overview of cytoplasmic histone deacetylases and their nonhistone substrates." J Biomed Biotechnol **2011**: 146493.

Youn, Y. H., T. Pramparo, S. Hirotsune and A. Wynshaw-Boris (2009). "Distinct dose-dependent cortical neuronal migration and neurite extension defects in Lis1 and Ndel1 mutant mice." J Neurosci **29**(49): 15520-15530.

Zhang, C., D. Li, Y. Ma, J. Yan, B. Yang, P. Li, A. Yu, C. Lu and X. Ma (2012). "Role of spastin and protrudin in neurite outgrowth." J Cell Biochem **113**(7): 2296-2307.

Zhang, H., S. Li, M. Wang, B. Vukusic, Z. B. Pristupa and F. Liu (2009). "Regulation of dopamine transporter activity by carboxypeptidase E." Mol Brain **2**: 10.

Zheng, M., R. D. Streck, R. E. Scott, N. G. Seidah and J. E. Pintar (1994). "The developmental expression in rat of proteases furin, PC1, PC2, and carboxypeptidase E: implications for early maturation of proteolytic processing capacity." J Neurosci **14**(8): 4656-4673.