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Calcium/Calmodulin-Dependent Protein Kinase II Beta (CaMKIIβ): A Regulator of Oligodendrocyte Maturation and Myelination

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Virginia Commonwealth University

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Calcium/Calmodulin-Dependent Protein Kinase II Beta (CaMKIIβ): A Regulator of Oligodendrocyte Maturation and Myelination

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

By

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<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>Actin related proteins 2/3</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>βIV-tubulin</td>
<td>Beta four tubulin</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Calcium/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CaMKIIβ</td>
<td>Calcium/calmodulin-dependent protein kinase II beta</td>
</tr>
<tr>
<td>CNP</td>
<td>2',3'-cyclic nucleotide 3'-phosphodiesterase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CtrlP</td>
<td>Myristoylated control peptide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>EAAT</td>
<td>Excitatory amino acid transporter</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>FN</td>
<td>Fibronectin</td>
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<tr>
<td>G-actin</td>
<td>Globular actin</td>
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<tr>
<td>Gal-C</td>
<td>Galactocerebroside</td>
</tr>
<tr>
<td>GLAST</td>
<td>Glutamate aspartate transporter</td>
</tr>
<tr>
<td>GLT1</td>
<td>Glutamate transporter 1</td>
</tr>
<tr>
<td>hpf</td>
<td>Hours post fertilization</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MAG</td>
<td>Myelin associated glycoprotein</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>MOG</td>
<td>Myelin-oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribose nucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>Myr-AIP</td>
<td>Myristoylated autacamtide-2 related inhibitory peptide</td>
</tr>
<tr>
<td>N</td>
<td>Number</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Neural-Wiskott Aldrich Syndrome protein</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium calcium exchanger</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NT3</td>
<td>Neurotrophin 3</td>
</tr>
<tr>
<td>OLG</td>
<td>Oligodendrocyte</td>
</tr>
<tr>
<td>OPCs</td>
<td>Oligodendrocyte progenitor cells</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PDGFR&lt;sub&gt;α&lt;/sub&gt;</td>
<td>Platelet derived growth factor receptor alpha</td>
</tr>
<tr>
<td>PLP</td>
<td>Proteolipid protein</td>
</tr>
<tr>
<td>pMN</td>
<td>Motor neuron progenitor domain</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative RT-PCR</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribose nucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase – polymerase chain reaction</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinases</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedge hog</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering ribose nucleic acid</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transcription growth factor beta</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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ABSTRACT

Calcium/Calmodulin-Dependent Protein Kinase IIβ (CaMKIIβ): A Regulator of Oligodendrocyte Maturation and Myelination

By Christopher Thomas Waggener

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University

Advisor: Babette Fuss, Ph.D., Professor, Department of Anatomy and Neurobiology

Oligodendrocytes are cells located in the central nervous system (CNS) that are responsible for the production of the lipid rich membrane, myelin. Myelin and the process of making and wrapping myelin around an axon (also known as myelination) are critical for normal development since they ensure proper signal conduction in the vertebrate CNS. The loss or damage of this myelin, which is typically associated with the demyelinating disease multiple sclerosis (MS), is associated with improper axonal protection along with disrupted nerve signaling which can lead to a variety of different debilitating phenotypic responses. It has been shown that there are MS lesions in which oligodendrocyte progenitors are present. However, while these cells are thought to possess the intrinsic ability to myelinate, they do not efficiently mature and/or repair the myelin sheath within the MS lesion. The reasons for this block in differentiation are currently not fully understood. A critical and thorough understanding of oligodendrocyte
development provides the foundation needed for future research to potentially provide therapeutic targets for stimulating proper maturation and efficient remyelination from the oligodendrocyte progenitors that are present within the MS brain.

In the search for regulators of oligodendrocyte development and potential therapeutic targets, the data generated as part of my thesis provided evidence that CaMKII (more specifically CaMKIIβ) is a regulator of oligodendrocyte myelination and maturation. Using pharmacological inhibitors or siRNA-mediated knockdown of this protein resulted in improper formation of the oligodendrocyte process network. Interestingly, siRNA-mediated knockdown of CaMKIIβ appeared to play no noticeable role in the genetic regulation of specific oligodendrocyte developmental markers. Furthermore, an overall reduction of the thickness of the compact myelin was observed in the ventral spinal cord of CaMKIIβ knockout mice. These findings emphasize the importance of CaMKIIβ in oligodendrocyte myelination and maturation.

To further investigate CaMKIIβ’s role in the regulation of CNS myelination, the effect of glutamate signaling on CaMKIIβ and in particular its actin binding site were assessed. These data showed that signaling via glutamate transporters promote an increase of process network in oligodendrocytes. This effect was associated with a transient increase in intracellular calcium concentration and a change in the phosphorylation of at least one serine residue present within CaMKIIβ’s actin binding site. Changes in phosphorylation of CaMKIIβ’s actin binding site suggested that CaMKIIβ detaches from filamentous F-actin and
allows for remodeling of the oligodendrocyte’s actin cytoskeleton. This was demonstrated by overexpressing CaMKIIβ actin binding mutant constructs to alter phosphorylation of serine residues to either always allow actin binding (CaMKIIβ\textsuperscript{allA}) or never allow actin binding (CaMKIIβ\textsuperscript{allD}). The overexpression of CaMKIIβ\textsuperscript{allD} alone demonstrated a decrease in the process network of oligodendrocytes and inhibited the effect of glutamate on the process network. In contrast, the overexpression of CaMKIIβ\textsuperscript{allA} and CaMKIIβ\textsuperscript{WT} alone showed normal process network formation along with a significant increase in the process network after stimulation of glutamate. The above data strongly suggest that there is a significant relationship between sodium dependent glutamate transporters/CaMKIIβ activation and the oligodendrocyte cytoskeleton in the role of regulation of oligodendrocyte differentiation and CNS myelination.

The data presented in this dissertation provides overwhelming evidence that CaMKIIβ plays a significant role in the proper formation of the oligodendrocyte complex process network and myelination. CaMKIIβ’s relationship with glutamate and the actin cytoskeleton could lay the foundation for future research not only for the signaling of oligodendrocyte process formation and remyelination but also for future targets for MS therapies.
CHAPTER 1
Introduction to Oligodendrocyte Biology and CaMKIIβ

Myelin and its functional significance

The myelin membrane is the most prominent membrane structure in the vertebrate nervous system. Myelin is a lipid rich multilamellar membrane that surrounds axons in a segmental fashion. Oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system are the cells that are responsible for producing the myelin membrane. Myelin was originally named by Rudolf Virchow in the mid 1850’s (Baumann and Pham-Dinh, 2001); however, oligodendrocytes were discovered by Rio Hortega almost 70 years later in 1921 (Helmut Kettenmann, 2005). The myelin membrane is highly insulative and has a low water content. Myelin has a unique segmental morphology that structurally allows for gaps in between the myelin segments. These gaps (also known as nodes of Ranvier) allow for fast and efficient propagation of nerve impulses called action potentials. This fast propagation of action potentials is due to a process named saltatory conduction, which allows for faster conduction over long distances and reliable signal transfer in smaller diameter axons (Baumann and Pham-Dinh, 2001).

Myelin was one of the later structures of the nervous system to evolve (Baumann and Pham-Dinh, 2001, Hartline and Colman, 2007). Evolutionary need for fast conduction of nerve fibers stems from the demand for animals to make quick decisions to avoid prey (Hartline and Colman, 2007). There are certain physiological properties that allow for increased speed of conduction. Increasing
the size of the axon decreases the overall resistance of the electrical signal and allows for faster conduction. This option is not practical in mammals, as nerve fibers would have to be extremely large in proportion to their body size and space requirements for the type of conduction speed that mammals require. Insulating the axon with a thick lipid myelin membrane increases the resistance of the electrical field in the longitudinal axis of the axon from the outside of the membrane and decreases the transverse capacitance between the inside and the outside nerve fiber (Hartline and Colman, 2007). By insulating the axon with myelin, sodium channels are clustered at the nodes of Ranvier while there are no action potentials generated along the segmental myelin sheath (internode). The clusters of sodium channels combined with the insulation of the myelin sheath greatly increase the conduction velocity of action potentials while keeping the axon diameter reasonably small (Baumann and Pham-Dinh, 2001, Helmut Kettenmann, 2005).

The importance of myelin has become evident not only in pathologies (or experimental paradigms) where it is not formed properly during development but also under conditions in which it is destructed such as in the demyelinating disease multiple sclerosis (MS) (Baumann and Pham-Dinh, 2001).

**Multiple sclerosis (MS), the major demyelinating disease in humans**

Understanding oligodendrocyte development is essential to gain more knowledge not only in the basic developmental process of myelination but also to treat diseases and disorders that affect oligodendrocytes and/or myelin. One of
the most studied demyelinating diseases is multiple sclerosis (MS). Currently, there is no cure for the disease and no treatment for stimulating remyelination by oligodendrocytes and/or their progenitors. MS affects 2.1 million people worldwide (Multiple Sclerosis Society 2013). There are over 400,000 diagnosed cases in the United States alone with more than 200 new cases diagnosed a day. The most common ages of diagnosis of MS is between the ages of 20 to 50 years of age. However, over the past few years, there has been an increase in the diagnosis of pediatric cases (Fox et al., 2006b, Yeh et al., 2009). MS presents as single or multiple lesions anywhere in the brain and results in symptoms that can range from a single neurological deficit to multiple neurological deficits (Fox et al., 2006b).

The cause of MS is still unknown; however, there are genetic and environmental factors that are thought to play a role in the development of the disease (Hauser and Oksenberg, 2006). One of the current environmental factors that could contribute to the disease is a vitamin D deficiency. An epidemiological study showed that MS cases tend to occur less frequently in populations of people who live closer to the equator. Researchers theorize that people living closer to the equator tend to have more sun exposure than those living in colder climates farther away from the equator (Freedman et al., 2000).

The genetic factors of MS are widely unknown and have not been strictly linked to heredity. However, an individual is much more at risk of developing MS than the general population if that individual has had a first-degree relative (parent or sibling) with MS. Furthermore, some researchers believe that
individuals are born with a genetic predisposition that makes them more sensitive to environmental agents that can cause the disease (Multiple Sclerosis Society 2013). All in all, there are many theories for the environmental and genetic causes for MS but researchers have not been able to pinpoint specific reasons why people develop MS.

MS is a complicated disease; currently there are 4 different disease courses with varying amounts of severity. The most common type of MS is relapsing-remitting with 85% of patients being diagnosed with this form of the disease. Relapsing-remitting MS is characterized by bouts of MS attacks with symptoms included but not limited to: sensory symptoms (numbness and tingling), motor symptoms (weakness and spasticity), coordination abnormalities, vision difficulties, bladder dysfunction, and fatigue; followed by periods of recovery (Fox et al., 2006b, Comabella and Martin, 2007). The second form of the disease is primary progressive. This form of the disease has been characterized by slow but gradually progressive neurologic symptoms from the onset with no time points of recovery. The primary progressive type is seen in about 10% of the population with MS (Fox et al., 2006b). Secondary progressive is the third form of MS that is seen in about 50% of the MS population that first exhibits the relapsing-remitting form of MS (Fox et al., 2006b). Secondary progressive MS is characterized by a continuous worsening of neurological deficits with differing amounts of severity over time. Lastly the most rare form of MS is progressive relapsing. With only 5% occurrence in the population, progressive relapsing MS is characterized by steadily worsening disease from
the beginning but with clear bouts of more severe neurological symptoms. Times of remissions are seen by some but not all of the patients experiencing this form of MS (Multiple Sclerosis Society 2013). Therefore, classifying MS can be difficult, especially given the varying symptoms and degrees of severity (Comabella and Martin, 2007).

Although the cause of MS is unknown, much progress has been made in understanding the pathology of MS. Since MS is classified as an autoimmune disease, inflammation is often associated with MS lesions and potentially plays a role in the damaging of oligodendrocytes. Whether or not the axon is affected by the inflammation or the physical loss of myelin is still under debate in the field. Current therapies are treating this inflammation and attenuating or lessening the relapsing portion of the disease. However, the loss of myelin and axonal damage still continues and is not treated by the current therapies on the market for MS patients.

Because there are no current therapies to correct or aid in the remyelination of axons by oligodendrocytes in MS lesions, the focus of this dissertation is on understanding oligodendrocyte development and myelination. This information is important because it is believed that the repair of the myelin sheath follows the same concepts as the generation of the myelin sheath during normal development. By researching oligodendrocyte development, researchers can identify oligodendrocytes found in MS lesions and use this information to target potential remyelination therapies. The oligodendrocytes in MS lesions are immature and lack the ability to remyelinate (Chang et al., 2002). The lack of
differentiation in oligodendrocytes present in the MS lesion leads to inhibition of proper remyelination. This could be due to an altered local environment and altered signals (Chang et al., 2002, Colognato, 2011, Lafrenaye and Fuss, 2010). Although some of these cells remyelinate axons, this remyelination is insufficient (Goldschmidt et al., 2009, Bradl and Lassmann, 2010, Emery, 2010).

Understanding oligodendrocyte development and myelination can provide a wealth of information that could lead to potential therapies to repair the damaged/absent myelin in MS and possibly also in other diseases displaying pathological features of demyelination (such as leukodystrophies, neuromyelitis optica, transverse myelitis, and optic neuritis) (Keyoung and Goldman, 2007, Goldman et al., 2012). One option for a potential therapy is stem cell replacement. Exogenous stem cell transplantation studies have shown promising results. However, current research resulted in a shift towards pharmacologically-based therapies. Pharmacologically-based therapies target the endogenous regenerative processes of oligodendrocyte maturation and myelination. This research has focused on the changes not only within the cell but also on changes within the environment around the cells to a younger more plastic environment to enhance remyelination. (Lindvall and Kokaia, 2006, Zawadzka et al., 2010, Ruckh et al., 2012, Stoffels et al., 2013). Studies have shown that by replacing macrophages and blood factors from younger animals into older animals that have demyelinating lesions, remyelination by the endogenous cells was achieved (Lindvall and Kokaia, 2006, Zawadzka et al., 2010, Ruckh et al., 2012, Stoffels et al., 2013). This result is significant because it shows that less plasticity in the
cells and local environment is observed in older animals. These cells, however, have the innate ability to remyelinate if prompted by the new altered local environment around them. This research and other research into oligodendrocyte development are leading to a better understanding of the molecular mechanisms that promote oligodendrocyte differentiation during development that have the potential to reveal novel targets for designing remyelinating therapies. This research is also important because there are no current therapies that address the issue of remyelination available for patients today.

Oligodendrocyte specification

Since the discovery of oligodendrocytes, their origins in the brain and spinal cord have been highly debated. Recent in vivo studies using transgenic mouse models to track the origins of oligodendrocytes have, for the most part, provided much insight into the origins of oligodendrocytes. The majority of this research has, however, focused on the spinal cord and only very recently has begun to explore the origins of oligodendrocytes in the brain (Richardson et al., 2006).

As the brain develops from its early embryonic form to its more mature form, it develops layers and zones of cell initiation and proliferation. These zones of development as well as all of the brain and spinal cord are of neuroectodermal origin. Each zone comprises and produces distinct cell types and cell morphologies (Pringle et al., 1996, Woodruff et al., 2001, Brazel et al., 2003, Marshall et al., 2003, Helmut Kettenmann, 2005, Richardson et al., 2006).
Neurons and some astrocytes originate from the ventricular zone of the brain, which is the first cell proliferative area to develop. The ventricular zone of the brain is a pseudostratified epithelial-like tissue that consists of neuroectodermal tissue (Helmut Kettenmann, 2005). During embryonic development, an area of tissue distal to the ventricular zone becomes highly proliferative and begins to thicken. This new proliferative area of the brain is known as the subventricular zone (Brazel et al., 2003). Since the cells within the ventricular zone lose their proliferative capability by birth (P0) in the mouse, the cells leave the ventricular zone and will eventually diminish into a thin wall of ependymal cells. In contrast, the subventricular zone remains actively proliferative early postnatally (Brazel et al., 2003). In the cortex, cortical progenitors follow a developmental sequence of neuronal development, then astrocyte development, and lastly oligodendrocyte development. The generation of each of these cell types occurs in a temporally distinct yet overlapping pattern (Spassky et al., 1998, Spassky et al., 2000, Sauvageot and Stiles, 2002).

From studies in the rodent system, oligodendrocyte progenitors are thought to be derived from discrete locations in the brain and spinal cord (Miller, 1996, Woodruff et al., 2001, Richardson et al., 2006). Research suggests that oligodendrocyte progenitor cells arise from the ventricular zone of the spinal cord and the subventricular zone of the developing brain (Privat and Leblond, 1972, Levison et al., 1993, Brazel et al., 2003, Marshall et al., 2003, Helmut Kettenmann, 2005, Baracskay et al., 2007). The majority of oligodendrocyte progenitor cells arise from this subventral region of the neuroaxis (Helmut
Kettenmann, 2005). Experiments conducted with rat spinal cords showed that initially most oligodendrocytes have a restricted subventral origination and only later are derived from more dorsal regions as well.

There are multiple areas along the ventral and dorsal axes of the spinal cord that produce oligodendrocyte progenitor cells at different time points. The so-called pMN (motor neuron progenitor) domain of the spinal cord is responsible for 85 percent of the oligodendrocyte production in the first wave of oligodendrocyte generations beginning around E12.5 (Richardson et al., 1988, Pringle and Richardson, 1993, Miller, 1996, Pringle et al., 1996, Woodruff et al., 2001, Richardson et al., 2006). The second wave of oligodendrocyte production in the spinal cord is found to originate from more dorsal regions in the area of the dorsal progenitor domains 3-5 around E15 (Cai et al., 2005, Fogarty et al., 2005, Vallstedt et al., 2005, Emery, 2010).

Oligodendrocyte progenitor cells in the brain can be found in various regions of the subventricular zone (Levison and Goldman, 1993, Helmut Kettenmann, 2005). More specifically, oligodendrocytes originate from the medial and lateral ganglionic eminences (Woodruff et al., 2001, Brazel et al., 2003, de Castro and Bribian, 2005, Kessaris et al., 2006, Richardson et al., 2006). These eminence structures are thought to be transient and are only present during embryonic development. Data from transgenic fate mapping suggests that oligodendrocyte progenitor cells migrate from these subventricular structures to the cortex in several waves (Figure 1.1). Oligodendrocyte progenitor cells of the first wave are first seen in the SVZ at E12.5 where they then migrate into the
cortex (Kessaris et al., 2006, Richardson et al., 2006). Oligodendrocytes in and throughout the entire cortex are observed around E18 (Woodruff et al., 2001, Kessaris et al., 2006, Richardson et al., 2006).

The second wave of movement from the subventricular zone comes from the more dorsal region of the lateral and caudal ganglionic eminences (Woodruff et al., 2001, Brazel et al., 2003, Kessaris et al., 2006, Richardson et al., 2006). Before E18, the source of oligodendrocyte progenitor cells originate from more ventral locations. After E18, the contribution of ventral cells decreases and a second wave of more dorsal locations sends cells to the cortex (Woodruff et al., 2001, Kessaris et al., 2006, Richardson et al., 2006).

The third wave of migration comes from the dorsolateral subventricular zone. This zone can be identified through adulthood (Woodruff et al., 2001, Brazel et al., 2003, Kessaris et al., 2006, Richardson et al., 2006). The later migration from the dorsolateral subventricular zone starts at P2 of development (Levison and Goldman, 1993, Brazel et al., 2003). Thus, spatiotemporal oligodendrocyte specification initially begins in the ventral regions of the brain and spinal cord and then migrates to dorsal regions as development progresses (Cai et al., 2005, Fogarty et al., 2005, Vallstedt et al., 2005, Richardson et al., 2006, Emery, 2010) (Figure 1.1).

From the areas of origin, oligodendrocyte progenitor cells migrate in a dorsal and lateral pattern in the brain and spinal cord (Levison and Goldman, 1993, Cayre et al., 2009). Dorsally migrating cells not only move in a dorsolateral pattern but also move radially as well, stopping in white matter areas
above their original location or moving through the white matter into the cortex (Cayre et al., 2009) (Figure 1.1).

Unlike developing neurons, oligodendrocyte progenitor cells still proliferate while undergoing the process of migration. Evidence from real time imaging of acute brain slices suggests that migration temporarily ceases when mitosis begins (Zerlin et al., 2004, Cayre et al., 2009). Temporarily stopping migration explains why oligodendrocyte progenitors can create clonal populations of cells that colonize multiple levels of white matter and cortex (Zerlin et al., 2004).

Interestingly, the above described ventral dorsal spatiotemporal oligodendrocyte progenitor cell specification is not seen in the chick embryo. Chick and quail grafting experiments show that all oligodendrocytes in the avian cortex are ventrally derived (Richardson et al., 2006). It seems to be that only the higher order animals are capable of dorsally derived oligodendrocytes progenitors.

As discussed above, oligodendrocyte progenitors arise from specific restricted locations and migrate in a controlled fashion throughout the central nervous system to populate future white matter areas of the brain.

**Oligodendrocyte differentiation – changes in gene expression**

As described above, oligodendrocyte progenitor cells are first specified in localized ventricular and subventricular zones in the spinal cord and brain. These cells then begin a radial dorsolateral migration pattern away from the highly proliferative zones of origination to populate the entire spinal cord and brain,
where they differentiate into mature myelinating oligodendrocytes. Specific cell markers (lipids, proteins, surface antigens, or transcription factors) have been found to characterize distinct stages of oligodendrocyte differentiation. These markers have specific functions when it comes to the gene regulatory mechanisms and protein interactions that allow for the oligodendrocyte to develop normally. There are several types of signals that regulate oligodendrocyte development. While there has been considerable debate about whether in vivo and in vitro models yield the same findings concerning the oligodendrocyte lineage, much of the signaling properties that have been characterized in vitro hold true to what naturally occurs in animal systems (Lazzarini, 2004, Nishiyama et al., 2009). These specific cell markers along with a change in the cellular morphology allow for proper identification and study of oligodendrocyte development (Pfeiffer et al., 1993, Baumann and Pham-Dinh, 2001, Bradl and Lassmann, 2010).

Markers of oligodendrocyte development consist of lipids and proteins expressed by one or more stages of the oligodendrocyte lineage. Olig2 is a transcription factor that has been implicated in regulating the specification of oligodendrocyte progenitor cells and remains expressed throughout the lineage in rodents (Liu et al., 2007). Sox10 is another transcription factor expressed and regulates development of oligodendrocyte progenitor cells once they become specified and remains expressed throughout the oligodendrocyte lineage (Liu et al., 2007). In the rodent model system, one of the early cell surface lipids for premature oligodendrocytes is identified by the antibody A2B5. This antibody
recognizes several gangliosides on the cell surface (Raff et al., 1983, Raff et al., 1984, Pfeiffer et al., 1993, Baumann and Pham-Dinh, 2001, Baracskay et al., 2007, Bradl and Lassmann, 2010). This antibody characterizes an early bipolar or unipolar morphology of an oligodendrocyte progenitor cell and the lipids it recognizes are downregulated as the cell differentiates into a more mature oligodendrocyte (Raff et al., 1983, Raff et al., 1984, Pfeiffer et al., 1993, Baumann and Pham-Dinh, 2001, Baracskay et al., 2007, Bradl and Lassmann, 2010) (Figure 1.2). These A2B5+ oligodendrocyte progenitor cells are highly migratory and proliferative in the presence of platelet derived growth factor (PDGF) (Richardson et al., 1988, Gard and Pfeiffer, 1993, Pringle and Richardson, 1993). In the in vivo system, PDGF is thought to be secreted by neuronal cell bodies and astrocytes (Richardson et al., 1988, Fruttiger et al., 2000, Rosenberg et al., 2007) (Figure 1.3). PDGF increases the proliferation and survival of oligodendrocyte progenitor cells (Hart et al., 1989a, Hart et al., 1989b). Since PDGF signals to these oligodendrocyte progenitor cells, the receptor that they express for PDGF is another marker often used to identify them (Richardson et al., 1988, Gard and Pfeiffer, 1993, Pringle and Richardson, 1993). This receptor is the platelet derived growth factor receptor alpha (PDGFRα). Basic fibroblast growth factor (bFGF) is a growth factor and mitogen to which early oligodendrocytes respond (Fok-Seang and Miller, 1994). The combination of both PDGF and bFGF promotes cell proliferation and inhibits differentiation (Gard and Pfeiffer, 1993, Pfeiffer et al., 1993).
As oligodendrocyte development progresses, mitogenic factors wane and oligodendrocytes are then introduced to the differentiating factor thyroid hormone (TH). TH and a lack of mitogenic factors are necessary for oligodendrocyte progenitor cell maturation (Barres et al., 1994, Franco et al., 2008) (Figure 1.3). As the oligodendrocyte progenitor cells change biochemically, changes in morphology are occurring simultaneously. Oligodendrocytes become multipolar and reactive with the mouse monoclonal antibody O4, which binds to an uncharacterized surface antigen (POA) and sulfatide (Bansal et al., 1989, Bansal et al., 1992)(Figure 1.2). This seems to be the last point where these O4 expressing cells have proliferative capabilities. From this point on, their postmigratory stage of life is slowing and will soon end (Warrington and Pfeiffer, 1992).

CNP (2',3'-cyclic nucleotide 3'-phosphodiesterase) is a marker for more mature, so-called premyelinating oligodendrocytes (Trapp et al., 1988) (Figure 1.2). CNP is a protein that catalyzes 2',3'-cyclic nucleotides to make 2'nucleotides. In addition to its presence in premyelinating oligodendrocytes, it is also seen in uncompacted myelin in the paranodal loops (Wells and Sprinkle, 1981, Nave and Trapp, 2008). The biological role of CNP in vivo is not well understood to date. However, when CNP is overexpressed in mice, premature and abnormal myelination is observed and when overexpressed in vitro, it promotes process outgrowth (Lee et al., 2005, Nave and Trapp, 2008). The roles in CNP regulating the cytoskeleton have been described in the “Oligodendrocyte
differentiation – changes in actin cytoskeletal organization” section of the introduction.

Cells of the oligodendrocyte lineage that are O4 positive and CNP positive are post mitotic and terminally differentiated cells that begin to express myelin markers such as myelin basic protein (MBP), proteolipid protein (PLP), myelin associated glycoprotein (MAG), myelin-oligodendrocyte glycoprotein (MOG), and the galactocerebrosides (GalC) that are recognized by the O1 antibody (Figure 1.2). At this stage, the beginnings of membranous sheets develop in culture and these cells are classified as mature oligodendrocytes (Bansal et al., 1989). The myelinating oligodendrocyte will remodel its process network and extensively produce myelin membranes to wrap axonal segments (Helmut Kettenmann, 2005, Bauer et al., 2009).

MBP is one of the major myelin protein components in the CNS (Baumann and Pham-Dinh, 2001, Helmut Kettenmann, 2005). One of MBP’s functions is the compaction and binding of opposing cytoplasmic myelin membranes (Readhead et al., 1990). MBP’s role in compaction is made evident in the shiverer mutant mouse line. In this mouse line, the Mbp gene is mostly deleted and the protein is absent resulting in hypomyelination and the formation of uncompacted myelin in the CNS (Readhead and Hood, 1990, Readhead et al., 1990, Shine et al., 1990).

MBP is expressed in multiple isoforms in the rodent and other mammalian systems. In the mouse, MBP contains 7 exons which through alternative splicing of exons 2, 5 and 6 account for the four major “classic “ MBP variants (Campagnoni, 1988, Campagnoni and Macklin, 1988, Baumann and Pham-Dinh,
2001, Helmut Kettenmann, 2005). The molecular weight of each one of the 4 classic MBP’s are: 21.5kDa, 18.5kDa, 17kDa, and 14kDa (Helmut Kettenmann, 2005). The splicing of these different transcripts is developmentally regulated. This can be seen in the larger 17 and 21.5kDa isoforms that contain exon2. Exon2 containing MBP is seen early on during oligodendrocyte differentiation, and interestingly, it is also seen in chronic MS samples where presumed limited and insufficient remyelination is occurring (Capello et al., 1997, Nagasato et al., 1997). The non-exon 2 containing isoforms are considered specific for the myelinating stages of the lineage (Campagnoni, 1988, Campagnoni and Skoff, 2001).

Proteolipid protein (PLP) is another major myelin protein component in the CNS. This highly conserved protein is a cell-surface protein that spans the membrane four times, forming two extracellular loops, which are thought to mediate adhesion between the outer (extra cellular side) membranes of the myelin sheath (Popot et al., 1991, Baumann and Pham-Dinh, 2001). PLP has a splice variant with the name DM20 (Campagnoni, 1988, Timsit et al., 1995, Nave and Trapp, 2008). Plp1/Dm20 is a 7 exon encoded gene, where use of an internal splice donor site within exon 3 results in a 35 amino acid deletion generating the splice variant DM20 (Helmut Kettenmann, 2005). The expression of each one of these splice variants is developmentally regulated, where DM20 is the earlier expressed isoform and PLP is expressed later as the oligodendrocyte matures (Timsit et al., 1995).
Functionally, the significance of PLP has been demonstrated by mouse mutant and knockout studies. The PLP mutant mouse *jimpy*, which has a mutant form of the protein, has a reduced number of oligodendrocytes and thin myelin resulting in premature death (Knapp et al., 1986, Duncan et al., 1989). However, in *Plp1* knockout mice, there is no oligodendrocyte loss or loss of myelin. Axonal swellings and axonal degeneration were noticed in these animals; therefore, the phenotypic response seen in the *jimpy* mice has been suggested to be a result of protein misfolding in the mutant PLP (Griffiths et al., 1998).

**The oligodendrocyte cytoskeleton**

From specification to myelination, oligodendrocytes not only progress through changes in gene expression but they also undergo extensive changes in morphology. Oligodendrocyte morphological development can be broken down into three steps. The first step is cell spreading and formation/extension of a complex process network. The second step is the establishment of axonal contact and the extension/spreading of the myelin sheath. Compaction and maintenance of the myelin sheath is the final stage (Bauer et al., 2009). All of the three steps mentioned above require large-scale morphological changes in the oligodendrocyte that will eventually make the myelin sheath. These large-scale changes are mediated to a large extent by changes in the cellular cytoskeleton (Bauer et al., 2009).

The oligodendrocyte cytoskeleton is composed of microtubules and microfilaments. These cytoskeletal components and many of their regulatory
elements are also found in other types of cells. This is in contrast to intermediate filaments, which are present in the non-oligodendroglia CNS cell types (astrocytes and neurons) but absent in cells of the oligodendrocyte lineage (Bauer et al., 2009). Microfilaments and microtubules are both part of the cytoskeleton but their roles differ somewhat when it comes to function. Process outgrowth is primarily mediated by microfilaments because of their location and their ability to directly connect via actin binding proteins to the plasma membrane (Bauer et al., 2009). Microtubules, on the other hand, are primarily thought to mediate process stability and localize to the cellular process once the microfilament network has moved the process forward (Bacon et al., 2007, Chan et al., 2009, Pollard and Cooper, 2009). Furthermore, both microtubules and microfilaments are polarized polymers and both can be associated with motor proteins such as kinesins, dyneins, and myosins for the transport of cellular organelles, vesicles, proteins, and mRNAs (Ross et al., 2008). The polarity of these cytoskeletal proteins is based on the direction and site of addition of subunits to the growing polymer. The fast growing end (or plus end) of these polymers is termed thusly because it is the site of addition of individual subunits while the opposite end (the minus end) is unstable and usually the site of depolymerization (Bauer et al., 2009, Pollard and Cooper, 2009).

Microtubules are comprised of alpha and beta-tubulin heterodimers. These heterodimers form a classic hollow cylindrical tube. In the cellular processes of oligodendrocytes, microtubules are arranged parallel to the orientation of the process (Lunn et al., 1997, Song et al., 2001, Bauer et al.,
In the cell body of the oligodendrocyte, however, the microtubule network is arranged in a mesh-like pattern (Lunn et al., 1997). It has been shown that oligodendrocytes express a unique form of beta tubulin (βIV-tubulin) that is apparently not expressed by any other CNS-derived cell (Terada et al., 2005). The function of this unique form of tubulin is still under investigation. Microtubules can go through post-translational modifications including acetylation. Acetylated α-tubulin seems to be associated with stable processes in more mature oligodendrocytes since it appears to be present in primary processes but not at the most distal tips of the process (Lunn et al., 1997, Song et al., 2001).

The other cytoskeletal component that oligodendrocytes possess is actin. Actin is found in oligodendrocytes in two distinct forms: globular monomer (G-actin) and filamentous (F-actin) (Song et al., 2001, Le Clainche and Carlier, 2008, Pollard and Cooper, 2009). F-actin is formed by the polymerization of G-actin subunits. Spontaneous nucleation of G-actin is not likely because of the stability of actin and the high threshold of nucleation that actin possesses (Bauer et al., 2009, Pollard and Cooper, 2009). Actin utilizes specific actin binding and regulatory proteins that act as catalysts for actin nucleation. Two examples for such proteins are the actin related protein 2/3 (Arp2/3) complex and the formin family of proteins (Bacon et al., 2007, Bauer et al., 2009, Chan et al., 2009, Pollard and Cooper, 2009).

**Oligodendrocyte differentiation – changes in actin cytoskeletal organization**
For the purposes of this dissertation, understanding the cytoskeletal changes that occur during oligodendrocyte differentiation is critical for a better understanding of the signaling pathways that regulate oligodendrocyte differentiation and that could be targeted to stimulate endogenous repair of the myelin sheath in MS. Process extension is the initial step in oligodendrocytes developing a complex process network and subsequently the myelin sheath. Once terminal differentiation has occurred, a complex process network is established by rearranging and reorganizing the cellular cytoskeleton (Bauer et al., 2009). This process requires well coordinated polymerization and depolymerization that regulates the changes that are occurring (Bradke and Dotti, 1999, Mallavarapu and Mitchison, 1999, Pollard and Cooper, 2009, Koskinen et al., 2012). The term named for this addition of subunits (polymerization) to the positive end of the microfilament and the removal of subunits (depolymerization) from the minus end of the subunit is called treadmilling. In general, process outgrowth and membrane sheet formation are mediated by filopodia and lamellipodia production; the generation of which is caused by stimulation of actin polymerization as well as actin filament branching and bundling (Song et al., 2001, Bacon et al., 2007, Pollard and Cooper, 2009). Filopodia are formed in the presence of parallel F-actin finger-like bundles that are the driving force of process extension. Lamellipodia are the lagging structures that fall into formation after filopodia have somewhat extended. It is generally understood that filopodia are formed first and then due to branching, the lamellipodia push out the leading
edge of the oligodendrocyte process (Song et al., 2001, Bacon et al., 2007, Pollard and Cooper, 2009).

The actin dynamics that control filopodia and lamellipodia formation and movement are regulated by actin binding proteins such as the Arp 2/3 complex and neural-Wiskott Aldrich Syndrome Protein (N-WASP) (Bacon et al., 2007, Delatour et al., 2008). Arp2/3 is a complex that binds to the sides of existing ("mother") filaments and initiates growth of a new ("daughter") filament at a distinctive angle from the mother filament and initiates branching of F-actin filaments (Pollard, 2007, Delatour et al., 2008). N-WASP has been shown to aid in actin filament branching by interacting with Arp2/3 (Bacon et al., 2007). Importantly, N-WASP has been shown to be required for lamellipodia formation in maturing oligodendrocytes (Bacon et al., 2007). Rho GTPases signal to many of the actin-associated proteins and promote actin polymerization or depolymerization. Rho GTPases are major regulators of the actin cytoskeleton and mediate process elongation and branching as well as inhibition of process formation (Bauer et al., 2009). The three most common and best studied members of the Rho GTPase family are CDC42, Rac1, and RhoA. Process retraction is often found mediated by the activation of RhoA. RhoA in turn activates ROCK, which signals to the actin cytoskeleton to initiate process retraction (Wolf et al., 2001). In contrast to RhoA, activation of CDC42 and Rac1 results in process outgrowth by stimulating actin polymerization and branching (Colognato et al., 2002, Bacon et al., 2007, Sloane and Vartanian, 2007). Interestingly, knockout of CDC42 or Rac1 in mice does not result in deficient
myelin formation. This could be due to the redundancy of the two actin polymerizing proteins that can compensate for each other (Thurnherr et al., 2006).

In addition to actin polymerization, the depolymerization or (breaking down) of the actin cytoskeleton by certain factors is equally important in the formation and extension of new and current oligodendrocyte processes. It is critical that the periods of actin destabilization be followed up with periods of actin stabilization to achieve a proper balance of actin dynamics (Ballestrem et al., 1998, Bradke and Dotti, 1999). Oligodendrocytes express the actin depolymerizing factor cofilin, which appears to be localized to the oligodendrocyte “growth cone” (Fox et al., 2006a). Other than localization, much of the function of cofilin has not been specifically described in oligodendrocytes as it has been in other cell types. However, based on the localization and conservation of the protein, it is assumed that cofilin functions as an actin depolymerizer similar to its role in Schwann cells and other cell types (Sparrow et al., 2012). This depolymerization of the actin cytoskeleton by cofilin allows the redistribution of actin filaments by breaking down longer filaments into shorter filaments and monomeric subunits (Lappalainen and Drubin, 1997, Sparrow et al., 2012).

In addition to actin binding proteins and Rho GTPases, there are several other proteins that are linked to process extension in more mature oligodendrocytes. Fyn, a member of the Src family kinases (SFK), promotes process extension and morphological differentiation by inactivating RhoA (Wolf et
al., 2001, Liang et al., 2004). This inactivation of RhoA is developmentally regulated and upon inactivation by Fyn, there is an increase in process extension (Wolf et al., 2001, Liang et al., 2004). Dominant negative constructs in vitro or knockout and mutants in vivo have shown that Fyn is necessary for proper myelination of the CNS. When Fyn is downregulated in vitro or knocked out in vivo, process outgrowth is inhibited and a hypomyelination phenotype is observed respectively (Wolf et al., 2001, Klein et al., 2002, Liang et al., 2004).

Fyn has many downstream effects on process extension. One of these downstream effects is the inhibition of Myosin II, which seems to increase process extension in oligodendrocytes. Actin-associated motor protein non-muscle myosin II (NMII) is regulated by phosphorylation of a specific regulatory chain and is a key regulator of actinomyosin assembly. More specifically, activity of NMII in oligodendrocytes is controlled by Fyn kinase via downregulation of RhoA-ROCK-NMII phosphorylation (NMII inhibition) (Conti and Adelstein, 2008, Wang et al., 2008, Wang et al., 2012). When myosin II is inhibited in culture, there is an increase in actin polymerization, branching, and the amount of myelinated segments formed in a co-culture system (Conti and Adelstein, 2008, Wang et al., 2012).

Kelch-related acting binding protein Mayven is also linked to process extension in differentiating oligodendrocytes (Jiang et al., 2005). Mayven is expressed and upregulated during oligodendrocyte differentiation where oligodendrocytes form a more complex process network. Mayven promotes process extension by binding actin to facilitate cytoskeletal rearrangement (Jiang
et al., 2005). Mayven protein binding domains suggest that Fyn and Mayven may interact and play a role in the dynamics of cytoskeletal rearrangement leading to process extension (Jiang et al., 2005).

Major myelin proteins have also been shown to affect the actin cytoskeleton and process extension. For example, MBP has been shown to act as a scaffolding protein that links the lipid bilayer to bind actin, induces F-actin polymerization, and bundles actin in a phosphorylation dependent manner (Boggs, 2006, Boggs et al., 2006, Boggs et al., 2012). MBP is not phosphorylated when located within the sheets of myelin; therefore it can not perform as a scaffolding protein (Boggs, 2006, Boggs et al., 2006, Boggs et al., 2012). When MBP is phosphorylated, it is localized in the cell body. This phosphorylated state of MBP prevents interaction with actin in the oligodendrocyte cell body (Boggs, 2006, Boggs et al., 2006, Boggs et al., 2012).

CNP is another myelin protein that affects the cytoskeleton of oligodendrocytes. Studies show that when CNP is upregulated before and during myelination, it can bind to both microtubules and microfilaments. More specifically, CNP acts in the processes of cells and aids the connections of the actin cytoskeleton to the membrane. Studies have suggested that CNP is important in filopodia formation in oligodendrocytes (De Angelis and Braun, 1996a, b).

All of the actions described above are internally derived in the cell. However, extracellular matrix (ECM) components and signaling molecules signal to internal proteins governing process formation and extension. The ECM
components laminin, fibronectin, and collagen all interact with membrane proteins such as integrins. These integrins are heterodimers made of an $\alpha$ and a $\beta$-chain. Oligodendrocytes express $\alpha\nu\beta1$, $\alpha\nu\beta3$, $\alpha\nu\beta5$, $\alpha6\beta1$, and $\alpha\nu\beta8$ (Colognato et al., 2002, Benninger et al., 2006, Colognato and Tzvetanova, 2011). Most of these integrins (especially the $\alpha\nu$ integrins) seem to be developmentally regulated and expressed at different stages of oligodendrocyte development (Colognato and Tzvetanova, 2011). It should be noted that $\alpha6\beta1$ integrin seems to play a key role in the axo-glial interaction that controls CNS myelin wrapping (Lee et al., 2006).

Simply put, oligodendrocyte process extension is assisted by a conglomeration of different actin binding proteins and signaling molecules that mainly function in two roles by either allowing actin to polymerize or to depolymerize and rearrange the actin cytoskeleton. Both of these functions can happen simultaneously and when combined, allow for constant fluidity in the actin cytoskeleton and proper process elongation. There has been a good amount of research based on the characterization of the molecular players that govern polymerization and depolymerization. However, little is known about the molecular players that regulate stabilization of the actin cytoskeleton. The data highlighted in this dissertation demonstrates the importance of the actin binding/stabilizing protein Calcium/calmodulin-dependent protein kinase II beta (CaMKII$\beta$) described below.

**Calcium/calmodulin-dependent protein kinase II (CaMKII)**
Calcium/calmodulin-dependent protein kinase II (CaMKII) has been mainly described as a fine-tuned calcium sensor and fundamental kinase for neuronal activity in the development of memory and synaptic plasticity in the CNS (Hudmon and Schulman, 2002b, a, Griffith, 2004). Much of the research on CaMKII’s role in neurons has focused on understanding the mechanism of synaptic plasticity and increases in long-term potentiation (LTP), which is critical in learning and memory (Hudmon and Schulman, 2002b, a, Griffith, 2004).

Research highlighting CaMKII’s relationship with oligodendrocytes is rare. Currently, there are only a couple of research articles in the literature. In one of the articles, CaMKII’s expression in oligodendrocytes was only mentioned as part of their supplemental microarray data (Dugas et al., 2006). Another mention of CaMKII being involved in oligodendrocytes comes from a publication in which the myelin proteome was characterized (Jahn et al., 2009). Since CaMKII has hardly been described in oligodendrocytes, little is known about CaMKII’s role and function in these cells. The lack of research into the function and expression of CaMKII in oligodendrocytes is likely not due to its lack of importance compared to neurons; rather, it is probably due to the overwhelming amount of researchers characterizing synaptic plasticity and learning and memory in neurons. Additionally, expression levels of CaMKII are much higher in neurons and may have been overlooked in oligodendrocytes. Given the similarities in certain aspects of morphological maturation between oligodendrocytes and neurons, it is plausible to apply some of the findings in CaMKII neuronal research to oligodendrocyte development (Fox et al., 2006a).
Calcium/calmodulin-dependent protein kinase II (CaMKII) is a serine/threonine kinase (Hudmon and Schulman, 2002b, a, Griffith, 2004) that phosphorylates substrates at a serine or threonine site (Hudmon and Schulman, 2002b, a, Griffith, 2004). CaMKII encodes four different genes in the mammalian system (Hudmon and Schulman, 2002b, a, Griffith, 2004) and seven different genes in teleosts (Rothschild et al., 2007, Rothschild et al., 2009). In the mammalian system, these genes are given the names Camk2a, Camk2b, Camk2g, and Camk2d. Multiple splice variants exist with each of these genes (Hudmon and Schulman, 2002b, a, Griffith, 2004).

**Basic structure and organization of CaMKII**

CaMKII contains several functional domains within its protein structure. The catalytic domain which is located at the N-terminal end of the protein is where ATP and substrate binding sites are located (Hudmon and Schulman, 2002b, a, Griffith, 2004) (Figure 1.4). The regulatory region is upstream of the catalytic domain and consists of two domains (Figure 1.4). The two domains in this regulatory region are an autoinhibitory domain and a Ca\(^{2+}\)/CaM binding domain (Hudmon and Schulman, 2002b, a, Griffith, 2004) (Figure 1.4). These two domains regulate the activation of CaMKII. (Hudmon and Schulman, 2002b, a). The association domain is located C-terminal to the Ca\(^{2+}\)/CaM binding domain (Hudmon and Schulman, 2002b, a) (Figure 1.4). In between the association domain and the regulatory domain is a variable domain or “linker domain” (Griffith, 2004, Kristensen et al., 2011). This domain contains isozyme-
specific regions and multiple splice variants (Hudmon and Schulman, 2002b, a, Griffith, 2004). The association domain allows CaMKII monomers to join together, forming a large functional 12-subunit holoenzyme (Schulman and Greengard, 1978a, b, Hudmon and Schulman, 2002b, a, Griffith, 2004) (Figure 1.5). They can form homo and hetero-oligomers containing more than one Camk2 gene. Hetero-oligomerization provides inter-subunit phosphorylation when CaMKII is being activated (Griffith, 2004, Lantsma and Tombes, 2005).

**Activation of CaMKII's catalytic activity**

After the binding of calcium (Ca\(^{2+}\)) to calmodulin (CaM), Ca\(^{2+}\)/CaM binds with high affinity to the Ca\(^{2+}\)/CaM binding site within the regulatory domain of CaMKII (Hudmon and Schulman, 2002b, a, Griffith, 2004) (Figure 1.5). After the binding of Ca\(^{2+}\)/CaM to its appropriate binding site, autophosphorylation occurs. The autoinhibitory domain releases the catalytic domain, allowing access to phosphorylation sites critical for inter-subunit phosphorylation of the Thr286 site (for CaMKII\(\alpha\)) (Hudmon and Schulman, 2002b, a, Griffith, 2004) (Figure 1.5). Once the subunits are phosphorylated, the kinase remains at 100 percent of its activity even after intracellular calcium levels fall (Hudmon and Schulman, 2002b, a, Griffith, 2004) (Figure 1.5). Ca\(^{2+}\)/CaM is tightly bound to the binding site, creating a state of activity known as “active-CaM trapped” (Hudmon and Schulman, 2002b, a, Griffith, 2004) (Figure 1.5). Over time, Ca\(^{2+}\)/CaM dissociates from the kinase and CaMKII will remain active at a lower state. This state is known as the active-Ca\(^{2+}\) independent state (Hudmon and Schulman,
2002b, a, Griffith, 2004) (Figure 1.5). After Ca^{2+}/CaM dissociates from CaMKII, two other threonine sites are exposed and can be autophosphorylated. Once these two other threonine (Thr306 and Thr307) sites (for CaMKII{a}) have been phosphorylated, the phosphates block the Ca^{2+}/CaM binding site and do not allow further association with Ca^{2+}/CaM (Hudmon and Schulman, 2002b, a, Griffith, 2004) (Figure 1.5). This state, called “active capped,” possesses only 20 to 80 percent of CaMKII’s full capacity of activity (Hudmon and Schulman, 2002b, a, Griffith, 2004) (Figure 1.5). Over time, phosphatases act and return the partially active CaMKII to its inactive state (Griffith, 2004) (Figure 1.5).

**CaMKII as an actin binding and actin cytoskeleton regulatory protein**

It has been recently shown that CaMKII can play a role in the regulation of the actin cytoskeleton (Shen et al., 1998, Fink et al., 2003, O'Leary et al., 2006, Okamoto et al., 2007, Lin and Redmond, 2008, 2009, Sanabria et al., 2009). The CaMKII isoform that has been best described for the binding to F-actin is the β isozyme (Shen et al., 1998). Further supporting the relationship between actin and CaMKII, studies using RNA interference targeted to CaMKII{β} resulted in a significant reduction of actin synaptic volume (Okamoto et al., 2007, Lin and Redmond, 2008, 2009, Okamoto et al., 2009). It has been shown that oligomerization of CaMKII{β} is needed for actin binding but catalytic activity is not essential for actin binding (Okamoto et al., 2007, Lin and Redmond, 2008). An actin binding domain that is N-terminal within the variable domain of CaMKII{β} is responsible for F-actin binding (O'Leary et al., 2006, Okamoto et al., 2007).
The structure and the composition of the variable domain play a key role in the protein’s regulation and binding abilities to actin. Expression of Camk2b in the adult brain is prevalent with the splice variants of exons 1, 2, 3, 4 and 5 in the variable region. These results further support the evidence that strict control over an important actin binding element is regulated with fine precision. Exon 1 in the variable domain seems to be the actin binding region that is unique to Camk2b (O’Leary et al., 2006). The first, third, and/or fourth exon in the variable region seem to control the targeting to the F-actin cytoskeleton. Deletion of exon 1 in the variable region inhibits actin bundling and colocalization with bundled actin, demonstrating that the first exon in the variable region is critical for binding and bundling of actin (O'Leary et al., 2006).

The function of CaMKIIβ is more complex than only its use as a multifunctional kinase. CaMKIIβ is responsible for the reorganization and stabilization of the actin cytoskeleton (Sanabria et al., 2009). While CaMKIIβ seems to possess a unique actin binding site, other CaMKII isoforms have been recently reported to also bind to actin via a location C-terminal to the variable domain (Hoffman et al., 2013).

CaMKIIβ has been shown to play an important role in the regulation of actin dynamics within the dendritic spine (Okamoto et al., 2007, Okamoto et al., 2009, Sanabria et al., 2009). The model that has been proposed by Okamoto et al. (2009) uses CaMKIIβ as a “gating” mechanism that will maintain spine structure at Ca$^{2+}$ resting levels. When Ca$^{2+}$ levels increase, modification of the actin cytoskeleton is observed. CAMKIIβ then preserves the new modification for
periods of time (Okamoto et al., 2009). This proposed model starts off with CaMKIIβ bundling actin in a resting basal state in the dendritic spine. Once Ca\(^{2+}\) enters the cell and activates CaMKIIβ, it disassociates from actin and allows for F-actin unbundling. Actin polymerization, F-actin rearrangement, and spine enlargement soon follow after dissociation of CaMKIIβ from f-actin bundles. Lastly, CaMKIIβ will once again accumulate in the dendritic spine and bundle the newly rearranged f-actin bundles, allowing for cytoskeletal stabilization (Okamoto et al., 2009).

The model of CaMKIIβ described by Okamoto et al. (2009) is supported by many experiments that show the relevance of CaMKIIβ location and its association with the actin cytoskeleton. By overexpressing Camk2b, researchers have observed an accumulation of the protein in spines. When CaMKII is inhibited (CaMKII inhibitor KN-93), researchers also observed alternations and spine remodeling. The overexpressed accumulation of CaMKIIβ in these dendritic spines demonstrates the fact that the protein is bundling actin and stabilizing the spine structure in an activity-dependent manner (Shi and Ethell, 2006, Okamoto et al., 2007, Okamoto et al., 2009).

The actin network is responsible for the outgrowth of dendrites. When CaMKIIβ is knocked down, it impairs outgrowth of these dendrites (Fink et al., 2003). In addition to binding and bundling F-actin, CaMKIIβ binds to monomeric actin (G-actin) and inhibits actin polymerization that is diminished by the binding of Ca\(^{2+}/\) CaM (Sanabria et al., 2009). This binding of G-actin enhances the structural rigidity of F-actin (Sanabria et al., 2009). Furthermore, CaMKIIβ has
been shown to control the rate and extension of actin polymerization through binding and sequestration of G-actin (Sanabria et al., 2009, Hoffman et al., 2013).

CaMKIIβ activation by autophosphorylation can facilitate actin unbinding (O’Leary et al., 2006, Okamoto et al., 2007, Okamoto et al., 2009, Sanabria et al., 2009). When using different mutant constructs, strong correlations with colocalization are seen in the A303R (calmodulin binding mutant) mutant and the K43R (autophosphorylation mutant) mutant. This demonstrates that CaMKIIβ binds to actin in a catalytic independent/dependent manner. Once the phosphorylation of the main autophosphorylation site is activated, CaMKIIβ appears unbound to actin. This result is shown when using the T287D (constitutively active mutant) mutant that did not show colocalization with actin in Cos-7 cells when transfected (O’Leary et al., 2006). CaMKIIβ binding to F-actin did not increase phosphorylation of a substrate peptide, indicating that the activation state of the kinase was not changed (Fink et al., 2003).

Although the CaMKIIβ information presented in this section is based on neuronal research, the findings can be applied to oligodendrocytes due to the commonalities in the actin cytoskeleton. In oligodendrocytes as well as neurons, the actin cytoskeleton plays a critical role in the formation, extension, and remodeling of processes that extend from the cell body. CaMKII’s involvement in oligodendrocyte development is potentially imperative, as oligodendrocytes are dependent on creating these complex processes and maintaining a regulation of the cytoskeleton similar to the model proposed in neurons.
**Figure 1.1**: Oligodendrocyte origination points and migratory patterns in brain and spinal cord. A: Red represents the first wave of development and migration in the rodent ventral spinal cord. Green represents the origination and migration of the second wave of development in more dorsally located positions of the spinal cord. B: Rodent brain displaying sagittal section (upper) showing the site of origination for oligodendrocytes and (orange) their rostral caudal radial migration to populate the cortex (green arrows). The rostral migratory stream is the specialized rout that allows cells to migrate to the olfactory bulb from the SVZ (red). Coronal sections of the rodent brain (lower) showing oligodendrocyte origination from the SVZ (orange) and their dorsolateral radial migration pattern from the SVZ into the cortex (green arrows). Adapted from (Cayre et al., 2009; Richardson et al., 2006).
A. Spinal Cord

B. Rodent Brain
Figure 1.2: Oligodendrocyte lineage and some specific markers that are expressed at different developmental stages. Adapted from (Baracskay et al., 2007; de Castro and Bribian, 2005; Fox et al., 2004; Pfeiffer, et al., 1993).
**Figure 1.3:** Signaling network of oligodendrocyte development. Green arrows indicate stimulation. Red bars indicate repression or inhibition. Blue arrows represent developmental time between stages of oligodendrocyte development. Adapted from (Nicolay et al., 2007).
OPC
Premyelinating Oligodendrocyte
Myelinating Oligodendrocyte

BMP
Irx3
Olig2
Nkx2.2
Sox10
Nkx6

Shh
Pax6
Nkx2.2

Sox17
Ngn1/2
SoxD
Hes5
TH
Nkx6.2

Olig1
Olig2

Zfp488

Specification

Maturation

Transcription Factors and Signaling Pathways in Oligodendrocyte Development
Figure 1.4: The structure and functional domains of CaMKII. Phosphorylation sites are representative for CaMKIIα. The phosphorylation sites depicted are conserved between CaMKII isozymes (Lisman et al., 2002).
Figure 1.5: Activation of CaMKII. A six subunit holoenzyme rather than a 12 subunit holoenzyme is shown for simplicity. Phosphorylation sites are representative for CaMKIIα (Griffith, 2004b).
CHAPTER 2

CaMKIIβ Regulates Oligodendrocyte Maturation and CNS Myelination

(This chapter was accepted as a paper in the Journal of Neuroscience in May of 2013. The work reported for this manuscript is based primarily on my own efforts. Assistance was provided by Dr. Jeffrey Dupree (electron microscope imaging Figure 2.4)

Introduction

During development, oligodendrocytes, the myelinating cells of the central nervous system (CNS), undergo a lineage progression during which bipolar progenitors give rise to cells with an extended process network that then transition into mature oligodendrocytes generating the myelin sheath (Pfeiffer et al., 1993, Baumann and Pham-Dinh, 2001). The morphological aspects of this progression are to a large extent regulated by changes in the cellular cytoskeleton (Bauer et al., 2009). However, the exact mechanisms by which the cellular cytoskeleton regulates oligodendrocyte maturation and CNS myelination are currently only poorly understood.

One of the molecular players that emerges as an important regulator of the actin cytoskeleton is calcium/calmodulin-dependent kinase type IIβ (CaMKIIβ). CaMKIIβ belongs to a family of highly conserved serine/threonine kinases, which in mammals is encoded by four different genes (Camk2a, Camk2b, Camk2g, Camk2d) giving rise to four isozymes (CaMKIIα, CaMKIIβ, CaMKIIγ, CaMKIIδ) (Tombes et al., 2003). Structure-functionally, CaMKII monomers are composed
of four domains, a kinase catalytic, an autoinhibitory (regulatory), an association (oligomerization) and a central variable domain that is subject to alternative splicing and located distal to the autoinhibitory domain (Hudmon and Schulman, 2002b). Interestingly, CaMKIIβ has been characterized to also possess a distinctive actin binding domain (Okamoto et al., 2009), which has been implicated in mediating actin filament stabilization/bundling (Shen et al., 1998, Fink et al., 2003, O'Leary et al., 2006, Okamoto et al., 2007, Lin and Redmond, 2008). While primarily characterized in neurons, CaMKII genes including CaMKIIβ appear to also be expressed by cells of the oligodendrocyte lineage (Cahoy et al., 2008). Thus, we examined here the role of CaMKII and in particular CaMKIIβ in regulating oligodendrocyte maturation and myelination.

**Materials and Methods**

*Animals.* Sprague-Dawley female rats with early postnatal litters were obtained from Harlan Laboratories, Inc. (Indianapolis, IN). *Camk2b*−/− and *Camk2b*A303R mice (both in the F2 129P2-C57BL/6 background; van Woerden et al., 2009; Borgesius et al., 2011) were generated and bred at Erasmus University Medical Center. Animal studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University or a Dutch Ethical Committee for animal experiments.

*Primary oligodendrocyte cultures.* Primary oligodendrocytes were isolated from P3 rat brains by A2B5 immunopanning (Barres et al., 1992) and then cultured in differentiation medium for at least 48 hours (Lafrenaye and Fuss,
Under these conditions, the majority of cells represented post-migratory, premyelinating oligodendrocytes as they expressed the O4 antigen (Sommer and Schachner, 1982, Warrington et al., 1993) (data not shown).

For CaMKII inhibition experiments, cells were cultured for 44 to 48 hours followed by incubation with 1) KN-93 or its inactive analog KN-92 (EMD Millipore, Billerica, MA), 2) myristoylated autocamtide-2 related inhibitory (Myr-AIP) or myristoylated control (scrambled AIP sequence) peptide (Enzo Life Sciences, Inc., Farmingdale, NY) or 3) KN93 or KN92 in combination with jasplakinolide (Enzo Life Sciences, Inc., Farmingdale, NY).

For siRNA-mediated gene silencing, cells were cultured for 20 to 24 hours and then transfected with siGLO Green transfection indicator along with either an siRNA SMARTpool directed against rat Camk2a, b, g or d or a control non-targeting siRNA SMARTpool (all from Thermo Fisher Scientific, Inc., Waltham, MA) (Lafrenaye and Fuss, 2010).

**Oligodendrocyte morphology analysis.** Oligodendrocyte morphology was assessed by determining the process index (total area found to be O4-positive minus the area occupied by the cell body) as previously described (Dennis et al., 2008). For the generation of representative images, confocal laser scanning microscopy was used (Zeiss LSM 510 META NLO; Carl Zeiss Microscopy, LLC, Thornwood, NY). Images represent 2D maximum projections of stacks of 0.4 µm optical sections.

**CaMKIIβ-F-actin co-localization analysis.** Cells of the oligodendroglia cell line CIMO (Bronstein et al., 1998) were nucelofected (Lonza Cologne GmbH,
Cologne, Germany) with a plasmid encoding GFP-CaMKIIβ (Okamoto et al., 2004), and F-actin was visualized using Acti-stain 555 phalloidin (Cytoskeleton, Inc., Denver, CO).

**PCR and Western blot analysis.** For the determination of alternative splicing profiles, endpoint PCR analysis was performed using the following gene-specific primer pairs:

Camk2a: Forward: 5'-TGGCCACCAGGAACCTTTCCGGAGG-3' and Reverse: 5'-TGCAGGCAAGCAGGAGGGGCGCCCAGA-3')

Camk2b: Forward: 5'-CACGGAATTTCCTCAGTGGGCAGACAG-3' and Reverse: 5'-CGCAGCTCTCAGCAGCGGGCCAC-3'

Camk2g: Forward: 5'-CGCTCCGGAAGGGTGCCATCCTACAACCATGC-3' and Reverse: 5'-TCCGGAGCGTCTCTCTGACTGACTGTGTCAGG-3'

Camk2d: Forward: 5'-CGCTCCGGAACGAGAAATTTTCAGCAGCAGCAAGA-3' and Reverse: 5'-TCCGGATCCTGGCTTGATGGGACTGTTGGAC-3'.

For the determination of relative mRNA expression levels, quantitative (q)RT-PCR was performed on a CFX96 Real-Time PCR Detection System (BioRad, Hercules, CA) using the following gene-specific primer pairs:

Camk2a: Forward: 5'-ACGGAAGAGTACCAGCTCTTCGAGG-3' and Reverse: 5'-CCTGGCCAGCCAGCACCTTCAC-3'

Camk2b: Forward: 5'-GTGTA CCTCCAGACAGACACTCAAAG-3' and Reverse: 5'-CCAGATACCTGGTTTGC-3'

Camk2g: Forward: 5'-ACGCAAGTCTCAGCAGCCGGAGAA-3' and Reverse: 5'-AGGCTCTCTGCGCTTGCCCC-3'
**Camk2d**: Forward: 5′-TGCCGTCTCTGAAGCACCCCA -3′ and Reverse: 5′-ACCAAGTAATGGAAGCCTCTCTTCGG-3′

**Mbp** (exon 2 containing isoforms): Forward: 5′-ACCTGGCAGCAATCACCATGGACC-3′ and Reverse: 5′-TTGTACATGTGGCAGCGCCGGAC-3′

**Mpb** (all isoforms): Forward: 5′-GTGACACCTCGTACACCCCCTCCAT-3′ and Reverse: 5′-GCTAAATCTGCTGAGGGACAGGCCT-3′

**Plp**: Forward: 5′-CCACACTAGTTTCCCTGCTCACCT-3′ and Reverse: 5′-GGTGCTCGGCCCATGAGTT-3′

**Cyclophilin** (as reference gene): Forward: 5′-GGAGACGAACCTGTAGGACG-3′ and Reverse: 5′-GATGCTCTTTCCTCCTGTGC-3′

For comparing the expression levels of the different Camk2 genes, $R_0$ values were determined as described by Peirson et al. (2003)(Peirson et al., 2003). To determine relative expression levels, the $\Delta \Delta C_T$ method was used (Livak and Schmittgen, 2001).

For Western blot analysis, anti-CaMKIIβ (Life Technologies, Grand Island, NY) and anti-GAPDH antibodies (Millipore, Billerica, MA) were used. Bound antibodies were detected using enhanced chemiluminescence (ECL) in combination with VersaDoc imaging (BioRad Laboratories, Hercules, CA).

**Electron Microscopic Analysis.** Spinal cord tissue was prepared and analyzed by electron microscopy as previously described (Dupree et al., 1998, Marcus et al., 2006, Forrest et al., 2009). Numbers of axons were determined manually per
field of view (14.6 µm²). G-ratios were determined as previously described (Dupree et al., 1998, Marcus et al., 2006, Forrest et al., 2009).

Results

*Camk2b* is the predominant *Camk2* gene expressed by differentiating oligodendrocytes

To determine the extent and alternative splicing pattern of *Camk2* gene expression in differentiating oligodendrocytes, RT-PCR analysis was performed using gene-specific primer pairs spanning the variable domain (Fig. 2.1A; (Hudmon and Schulman, 2002b, Tombes et al., 2003). Sequence analysis of the resulting amplification products revealed the expression of *Camk2a, b* and *d* but not *g*. All of the three oligodendrocyte-derived genes were found to give rise to multiple alternatively spliced isoforms in a gene-specific fashion. Interestingly, the majority of *Camk2b* isoforms contained the alternatively spliced exon I of the variable domain, which has been implicated in conferring actin binding/stabilizing properties to CaMKIIβ (O'Leary et al., 2006).

To determine the quantitative contribution of each of the three oligodendrocyte-derived *Camk2* genes to overall *Camk2* expression, qRT-PCR was performed using primer pairs located outside of the variable region and not affected by alternative splicing. This analysis revealed a quantitative expression of *Camk2b > Camk2d > Camk2a* (Fig. 2.1B).

Inhibition of CaMKII activity restrains the morphological maturation of
differentiating oligodendrocytes

The above expression analysis suggested that CaMKII and in particular CaMKIIβ may play an important functional role in differentiating oligodendrocytes. To assess such a potential role of CaMKII, differentiating oligodendrocytes were treated with KN-93, a membrane permeable pharmacological inhibitor of CaMKII activity, or its inactive derivative KN-92, and process morphology, as a measure for oligodendrocyte maturation, was determined (Dennis et al., 2008). Treatment with KN-93 caused a decreased process index (Fig. 2.2A,B) at 10 but not 1 µM. Such concentration-dependency is in agreement with a half-maximal inhibition of CaMKII at a KN-93 concentration of approximately 12 µM (Tombes et al., 1995). In addition, cells were treated with the membrane-permeable myristoylated-autocamtide-2-related inhibitory peptide (MyrAIP), which mimics the CaMKII autoinhibitory domain (Ishida and Fujisawa, 1995) and blocks activity at concentrations similar to KN-93 (Easley et al., 2006, Easley et al., 2008). Such treatment resulted similar to the KN-93 treatment in a decreased process index (Fig. 2.2C). CaMKII inhibition was not found to be associated with a change in cell viability (KN-92: 100±9%, KN-93: 96±12%).

Morphological maturation of oligodendrocytes occurs as a dynamic process that is characterized by process extension and retraction events (Kachar et al., 1986, Fox et al., 2006a). As shown in Fig. 2.2D, process indices were found to be significantly decreased 2 hours after initial KN93 treatment when compared to the process indices found at the beginning of the treatment. Thus, the decreased morphological maturation seen in response to CaMKII inhibition is likely due to
an increase in process retraction events rather than an inhibition of process outgrowth.

Retraction of cellular processes has been associated with de-stabilization of the actin cytoskeleton (Easley et al., 2006) Since KN-93 has been well described to inhibit not only CaMKII’s kinase catalytic but also CaMKIIβ’s actin binding/stabilizing activity (Fig. 2.2G; (Sumi et al., 1991, Lin and Redmond, 2008), its use alone precludes an analysis of specifically CaMKIIβ’s actin binding/stabilizing activity. However and in support of an actin de-stabilizing effect of KN-93 treatment in differentiating oligodendrocytes, co-treatment with jasplakinolide, which specifically and rapidly blocks actin filament disassembly (Boggs and Wang, 2004), abolished the effect of KN-93 on the oligodendrocyte’s process network (Fig. 2.2E,F). No evidence for a change in cellular viability was noted.

**Downregulation of Camk2b expression restrains the morphological maturation of differentiating oligodendrocytes**

To determine the extent to which specifically CaMKIIβ may be involved in regulating the morphology of the oligodendrocyte’s process network, an siRNA-mediated gene silencing approach was used. As shown in Fig. 2.3A,B, treatment with an siRNA pool to Camk2b led to a significantly decreased process index. Under the conditions used, siRNA treatment resulted in significantly reduced mRNA levels for Camk2b (Fig. 2.3C), Camk2a (60±4%) and Camk2d (74±9%). Use of an siRNA pool to Camk2g served as a control since expression of
Camk2g was undetectable in our original analysis (Fig. 2.1A). For cells treated with the siRNA pool to Camk2b, a reduction in CaMKIIβ protein levels could also be confirmed (Fig. 2.3C, inset). In neither case, was the gene-specific downregulation of Camk2 expression associated with an increase in mRNA levels for any of the other Camk2 genes (data not shown).

In vivo, morphological maturation of oligodendrocytes is associated with well described changes in gene expression (Pfeiffer et al., 1993; Baumann and Pham-Dinh, 2001; Emery, 2010). Under experimental conditions, however, molecular mechanisms regulating cellular morphology may be uncoupled from those that regulate gene expression (Buttery and ffrench-Constant, 1999, Osterhout et al., 1999, Kim et al., 2006, Lafrenaye and Fuss, 2010). To investigate a potential role of Camk2b in regulating gene expression in differentiating oligodendrocytes, expression levels for mRNAs encoding the major myelin genes myelin basic protein (Mbp) and proteolipid protein (Plp) (Fulton et al., 2010) were determined. No significant differences were noted (Fig. 2.3D). In addition, no difference was noted in the percentage of O4-positive cells that were also immuno-positive for MBP (siControl 52±4%, siCamk2b 49±2%).

**Systemic knock-out of Camk2b leads to significantly reduced myelination**

To determine the extent to which Camk2b may regulate developmental myelination in vivo, ventral spinal cords of systemic Camk2b knock-out (Camk2b−/−) mice (van Woerden et al., 2009) were analyzed. As shown in Fig. 2.4A-C, the myelin sheath g-ratio (axon diameter divided by the diameter of the entire
myelinated fiber) was significantly increased at postnatal day 21 (P21) in Camk2b<sup>−/−</sup> spinal cords. This effect on myelin thickness persisted up to at least 58 days of age (Fig. 2.4D,E) and was not associated with significant changes in the number of myelinated axons (P21: WT 39±2/14.6µm², Camk2b<sup>−/−</sup> 41±2/14.6µm²; P58: WT 35±2/14.6µm², Camk2b<sup>−/−</sup> 35±1/14.6µm²) or apparent signs of axonal damage (Fig. 2.4A). In addition, no significant changes in the number of oligodendrocytes were noted (P21: WT 100±6%, Camk2b<sup>−/−</sup> 112±5%).

To assess whether the mechanism by which CaMKIIβ regulates oligodendrocyte maturation and CNS myelination may be mediated by a non-enzymatic activity, developmental myelination was assessed in Camk2b<sup>A303R</sup> mutant mice. In these mice the wild-type Camk2b gene has been replaced by the mutated Camk2b<sup>A303R</sup> gene (Borgesius et al., 2011). This mutation has been characterized to lead to a loss of calcium/calmodulin binding and kinase catalytic activation but to preserve the ability of CaMKIIβ to bind to and bundle/stabilize actin filaments (Shen and Meyer, 1999, Fink et al., 2003, O'Leary et al., 2006, Lin and Redmond, 2008). As shown in Fig. 2.4F,G, Camk2b<sup>A303R</sup> mutant mice were devoid of the deficits in myelin thickness seen in Camk2b<sup>−/−</sup> mice.

**Discussion**

Using *in vitro* tissue culture as well as *in vivo* knock-out and knock-in strategies, we identified CaMKIIβ as a critical component of the molecular mechanism regulating oligodendrocyte maturation and CNS myelination. More specifically, our data point toward a role of CaMKIIβ in regulating the oligodendrocyte’s actin
cytoskeleton via a mechanism that may not require its kinase catalytic activity but may instead involve its actin binding/stabilizing activity.

Our *in vivo* analysis of developmental myelination demonstrates that CaMKIIβ is involved in the regulation of myelin thickness. Taken together with our *in vitro* tissue culture studies we propose that this regulatory role of CaMKIIβ is at least in part mediated by an oligodendrocyte-autonomous mechanism. In support of this idea, astrocytes are considered to not express considerable levels of *Camk2b* (Takeuchi et al., 2000, Vallano et al., 2000). In addition, CaMKIIβ protein levels in axons located within the ventral spinal cord have been described to be undetectable or very low (Terashima et al., 1994). Thus, systemic *Camk2b* knock-out is unlikely to cause a predominantly axon-mediated effect on myelination within the CNS region investigated here.

The lack of a myelination deficit in the spinal cord of *Camk2b*\(^{A303R}\) mutant mice strengthens the idea of a functional role of CaMKIIβ as an actin regulatory protein and via its actin binding activity. In neuronal dendritic spines, CaMKIIβ, via its actin binding activity, is thought to stabilize the actin cytoskeleton and thus overall spine shape. At the same time, however, calcium signaling has been implicated in promoting release of CaMKIIβ from the actin cytoskeleton and to thereby open a time window during which actin cytoskeleton remodeling events are favored (Okamoto et al., 2007; Okamoto et al., 2009). In analogy, oligodendrocyte maturation and CNS myelination may be regulated by CaMKIIβ-mediated alternating cycles of actin cytoskeleton stabilization and destabilization/remodeling. As CaMKIIβ-mediated regulation is dependent on
calcium signaling events, it is worth mentioning that an increase in calcium signaling has been reported to stimulate oligodendrocyte process outgrowth and thus morphological maturation (Yoo et al., 1999). Furthermore, it has been recently shown that balanced activation and de-activation of the actin filament severing and depolymerizing factor cofilin regulates Schwann cell function during peripheral nervous system myelination (Sparrow et al., 2012). This finding supports the idea that efficient myelination may be critically dependent on a well-balanced equilibrium between dynamic remodeling and kinetic stability of the actin cytoskeleton. Future studies will, however, be necessary to better define the role of CaMKIIβ in regulating the actin cytoskeleton during oligodendrocyte maturation and CNS myelination.
**Figure 2.1** In differentiating oligodendrocytes *Camk2b* is the predominantly expressed *Camk2* gene. **A**, Alternative splicing profile of oligodendrocyte-derived *Camk2* genes as determined by RT-PCR analysis. Conserved non-alternatively spliced “linker” exons within the variable region are depicted as black boxes labeled with the roman numerals II and VII. Alternatively spliced exons are depicted as white boxes labeled with roman numerals. Lines indicate alternative splicing events. P1 and P2 indicate the locations of the two primers used for RT-PCR amplification. **B**, *Camk2* mRNA expression levels as determined by qRT-PCR analysis. For the bar graph, total *Camk2* mRNA levels were set to 100% and the values for each of the three genes were adjusted accordingly. Data represent means ± SEM (n = 3 independent experiments, *p*<0.05, Student’s *t*-test).
A

Camk2a

Camk2b

Camk2d

B

mRNA level (in %)

Camk2a
Camk2b
Camk2d
**Figure 2.2** Inhibition of CaMKII activity in differentiating oligodendrocytes restrains the establishment of an expanded process network. A,F, Representative images of differentiating oligodendrocytes immunostained with the O4 antibody and treated for 6 hours as indicated. Scale Bars: 20 μm. B-E, Bar graphs representing quantitative analyses of process indices as described by Dennis et al. (2008). Cells in B,D,E were treated with the pharmacological CaMKII inhibitor KN-93 or its inactive derivative KN-92 as control, while cells in C were treated with the myristoylated autoinhibitory CaMKII peptide (Myr-AIP) or a myristoylated control peptide (CtrlP). Cells in E were co-treated with the actin stabilizing peptide jasplakinolide (10 μM) where noted and analyzed after 6 hours of treatment. Otherwise, final concentrations and duration of treatments are indicated within the bar graphs. In B,C,E experimental conditions were compared to control-treated cells cultured for an equivalent period of time. In D, experimental conditions were compared to control-treated cells at time-point 0. For all bar graphs, the mean values for control cells were set to 100% (horizontal grey line) and experimental values were calculated accordingly. At least 25 cells per condition and experiment were analyzed in three independent experiments (= total of at least 75 cells per condition). Data represent experimental means ± SEM (*p<0.05, Student’s t-test). G, Representative images of CIMO cells transfected with a plasmid encoding GFP-CaMKIIβ and stained for F-actin (phalloidin). Scale Bars: 5 μm.
Figure 2.3 Knock-down of Camk2b expression in differentiating oligodendrocytes restrains the establishment of an expanded process network. A, Bar graph representing quantitative analyses of process indices (Dennis et al., 2008) upon siRNA-mediated knock-down of individual Camk2 genes as indicated. The mean value for cells treated with the control siRNA pool was set to 100% (horizontal grey line) and experimental values were calculated accordingly. At least 25 cells per condition and experiment were analyzed in four independent experiments (= total of at least 100 cells per condition). Data represent experimental means ± SEM (*p<0.05, Student’s t-test). B, Representative images of differentiating oligodendrocytes immunostained with the O4 antibody and treated with a control (siControl) or Camk2b-specific (siCamk2b) siRNA pool. Scale Bars: 20 µm. C, Bar graph depicting the Camk2b mRNA level upon siRNA-mediated knock-down of Camk2b. The mean value for cells treated with the control siRNA pool was set to 100% (horizontal grey line) and the experimental value was calculated accordingly. The experimental mean ± SEM (*p<0.05, one sample t-test) is shown. The inset depicts a representative Western blot. CaMKIIβ and GAPDH protein levels are shown for cells treated with a control (siControl) or Camk2b-specific (siCamk2b) siRNA pool. D, Bar graph depicting Mbp (total and exon 2 containing) and Plp mRNA levels upon siRNA-mediated knock-down of Camk2b. The mean value for cells treated with the control siRNA pool was set to 100% (horizontal grey line) and experimental values were calculated accordingly. Data represent experimental means ± SEM (*p<0.05, one sample t-test).
Figure 2.4 Knock-out of Camk2b leads to an increase in the g-ratio (decrease in the thickness) of the myelin sheath, while myelination appears unaffected in Camk2\textsuperscript{A303R} mutant mice. A, inset in F, Representative electron micrographs of the ventral spinal cord of 21-day-old (P21) wild-type (WT) and Camk2b knock-out (Camk2b\textsuperscript{-/-}) mice (in A) or Camk2\textsuperscript{A303R} mutant mice (in F). Scale bars: 1mm. B,D,F, Scatter plots depicting g-ratios versus axon diameters for P21 Camk2b\textsuperscript{-/-} (B), adult Camk2b\textsuperscript{-/-} (D) or P21 Camk2\textsuperscript{A303R} (F) (black filled circles) and WT littermate (red open circles) ventral spinal cords. The lines represent linear fits to pooled data from all mice for each genotype. 100 axons per animal were measured and 3 animals per genotype were analyzed. C,E,G, Bar graphs depicting average slopes (g-ratio versus axon diameter) and average g-ratios from individual animals (n=3). Stars indicate statistically significant differences between wild-type and knock-out/mutant mice (*p < 0.05, Student’s t-test).
Introduction to Glutamate and Glutamate Transporters

Glutamate transporters and receptors in oligodendrocytes

Glutamate is a major excitatory neurotransmitter found in the CNS that governs a wide variety of activities from normal brain function to the development of cells in the CNS (Gallo and Ghiani, 2000, Kukley et al., 2007, Bakiri et al., 2009, Hamilton et al., 2010). During development, there are multiple sources of glutamate released in the CNS that could affect cells of the oligodendrocyte lineage. These sources range from cells such as unmyelinated axons and astrocytes to extracellular lipids and proteins such as prostaglandins and neuropeptides respectively (Parpura et al., 1994, Gallo et al., 1996, Gallo and Ghiani, 2000, Bezzi and Volterra, 2001, Kukley et al., 2007, Ziskin et al., 2007, Nave and Trapp, 2008, Bakiri et al., 2009, Hamilton et al., 2010). Glutamate release and signaling in oligodendrocytes are key in many aspects of oligodendrocyte progenitor migration, proliferation, and differentiation (Barres and Raff, 1993, Gallo et al., 1996, Ziskin et al., 2007, DeSilva et al., 2009).

In general, glutamate signals through the activation of specific glutamate receptors. These receptors are divided into metabotropic and ionotropic receptors. Metabotropic glutamate receptors are further subdivided into classes based on activation or inhibition of downstream proteins such as phospholipase C and adenylate cyclase (Bakiri et al., 2009, Martinez-Lozada et al., 2011). However, metabotropic receptors seem to be downregulated early on in
Examples of ionotropic receptors are \textit{N}-methyl-\textit{D}-aspartate (NMDA), \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), and Kainate receptors. All types of ionotropic receptors are expressed during oligodendrocyte differentiation. Although NMDA receptors and AMPA receptors are usually associated with neurons, they also play a critical role in mediating glutamate signaling and excitotoxicity in oligodendrocytes (Verkhratsky and Kirchhoff, 2007).

Oligodendrocytes express AMPA receptors in immature oligodendrocytes and mature oligodendrocytes throughout the brain (Gallo and Ghiani, 2000, Verkhratsky and Steinhauser, 2000, Verkhratsky and Kirchhoff, 2007). Activation of AMPA receptors in oligodendrocytes produces substantial \(\text{Ca}^{2+}\) influx and an increase in cytosolic \(\text{Ca}^{2+}\) signals in culture and acute brain slices (Burnashev et al., 1992; Verkhratsky and Kirchhoff, 2007).

NMDA receptors are expressed in oligodendrocytes in the corpus callosum, cerebellum, and optic nerve (Karadottir et al., 2005, Salter and Fern, 2005, Micu et al., 2006). They are glutamate-gated ion channels formed by oligomeric subunits (Wong, 2006), consisting of a NR1 subunit and various types of NR2 subunits. Both of these subunit types have been found in oligodendrocytes (Wong, 2006). In particular, the NR2 subunits NR2A, NR2B, NR2C, and NR2D are expressed in oligodendrocytes (Wong, 2006).
NR2C, and NR3A are the most abundant subunits in the optic nerve. NR2B subunits are present, although not as abundant (Wong, 2006).

With regard to their subcellular localization, NMDA receptors are found primarily in the cellular process of oligodendrocytes where they are thought to mediate responses that develop in the distal processes (Salter and Fern, 2005, Micu et al., 2006). They are much less prominent in the soma of oligodendrocytes (Micu et al., 2006; Salter and Fern, 2005).

By using electrophysiological recordings, research has shown the activation of NMDA receptors during all stages of the oligodendrocyte lineage (Karadottir et al., 2005). Although the function of NMDA receptors in oligodendrocytes is unclear, NMDA receptors are suggested to play a role in the targeting and interaction of the axon with the oligodendrocyte process (Salter and Fern, 2005; Verkhratsky and Kirchhoff, 2007; Wong, 2006).

Contradictory to the role NMDA receptors play during development, studies have shown that oligodendrocyte specific knockout of NMDA receptors NR1 subunit (which in turn functionally inactivates NMDA receptors) leads to normal physiology, morphology, numbers, and myelination of oligodendrocytes (De Biase et al., 2011, Guo et al., 2012). This brings into question the importance of NMDA receptors during development. De Biase et.al (2011) observed an increased expression of AMPA receptors after NMDA knockout. This observed increase in AMPA receptors could provide a compensatory response to the NMDA knockout (De Biase et al., 2011). In addition to a potential role during development, activation of NMDA receptors has also been found to lead to rapid
cell death during ischemic attacks by mediating excitotoxicity (Salter and Fern, 2005; Verkhratsky and Kirchhoff, 2007; Wong, 2006). Thus, the functional role of NMDA receptors may be more prominent under pathological conditions than during development.

Glutamate can activate other types of glutamate responsive transmembrane proteins. These proteins belong to a family of electrogenic sodium-dependent transporters. These transporters have been well characterized to remove glutamate from the extracellular environment (Domercq et al., 1999, DeSilva et al., 2009, Martinez-Lozada et al., 2011). The glutamate transporter family has five genes in mammals: excitatory amino acid transporters 1-5 (Eaat1-Eaat5); however, oligodendrocytes have been characterized to express three of these genes: Eaat1-Eaat3. These subtypes are also named glutamate aspartate transporter (GLAST), glutamate transporter 1 (GLT1), and EAAC1 respectively. (Domercq et al., 1999, DeSilva et al., 2009, Martinez-Lozada et al., 2011, Lee et al., 2012).

Several studies have examined the expression and the functional role of sodium-dependent glutamate transporters as they relate to oligodendrocyte development. A study using transgenic mice for each promoter of GLT1 and GLAST focused on the cellular identification of these glutamate transporters in different cell types of the CNS. The study used reporter mice and showed that GLT1 promoter activity is in the adult and for the most part restricted to astrocytes. GLAST promoter activity was, however, also seen in oligodendrocytes in the white matter of the spinal cord, striatum, and corpus
callosum (Regan et al., 2007). Furthermore, this study demonstrated by functional uptake experiments that oligodendrocytes likely express more than one glutamate transporter (Regan et al., 2007). This was furthermore demonstrated in a later study showing that all three transporters are found in rat oligodendrocytes \textit{in vitro}. Not only were they found present in oligodendrocytes, they were also found to be functionally active with EAAC1 apparently possessing the highest uptake activity (DeSilva et al., 2009). Based on these studies, the functional activity of these transporters appeared to be similar in the uptake capacity and the affinity for glutamate as seen for astrocytes (DeSilva et al., 2009). In several studies, protein expression of each transporter was seen at varying stages of the oligodendrocyte lineage (DeSilva et al., 2009). Interestingly, GLT1 expression seems to be upregulated as oligodendrocyte development progresses from pre-oligodendrocytes to mature oligodendrocytes (DeSilva et al., 2009). However, \textit{in vivo}, there seems to be a downregulation of GLT1 in more mature (P20) rat corpus callosum. This is in contrast to slight differences in expression profiles when comparing cells in culture to \textit{in vivo} tissues. All three subtypes of transporters are expressed by oligodendrocytes (Domercq et al., 1999, DeSilva et al., 2009, Martinez-Lozada et al., 2011).

In addition to glutamate clearance, research suggests that glutamate transporters could play a role in downstream signaling mechanisms. Activation of glutamate transporters allows for glutamate to enter the cell through the transporter’s pore. Along with the entrance of glutamate, 3 \(\text{Na}^+\) ions also pass through the transporter and enter the cell. This influx of sodium can activate the
reverse mode of the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) (Martinez-Lozada et al., 2011, Boscia et al., 2012b). This exchanger then takes the 3 Na\(^+\) ions out in exchange for Ca\(^{2+}\). Thus, activation of glutamate transporters can induce a Ca\(^{2+}\) influx into the cell (Martinez-Lozada et al., 2011, Boscia et al., 2012b). This Ca\(^{2+}\) influx has been shown in Bergmann glia cells to initiate signaling cascades (Martinez-Lozada et al., 2011). Oligodendrocytes express NCX1 and NCX3 and their expression seems to be developmentally regulated with NCX1 being downregulated and NCX3 being upregulated as oligodendrocytes mature (Boscia et al., 2012b) (Figure 3.1). Furthermore, the expression of NCX3 seems to be crucial for proper myelination and oligodendrocyte development (Boscia et al., 2012b). Thus the importance of glutamate transporters and its association with NXC may be critical for proper oligodendrocyte development.
Figure 3.1: The transport of glutamate through the glutamate transporter is coupled to the Na⁺/Ca²⁺ exchanger regulating the Na⁺ efflux in exchange for Ca²⁺ influx, which can provide localized Ca²⁺ signaling. Adapted from (Martínez-Lozada et al., 2011).
INTRODUCTION

Glutamate, the major excitatory amino acid, mediates a wide variety of cellular responses in the developing and adult central nervous system (CNS), not only by affecting signal transduction in neurons but also by regulating glia cells, including the myelinating cells of the CNS, oligodendrocytes (Bakiri et al., 2009, Kolodziejczyk et al., 2010). Interestingly, the primary mode of glutamate release affecting differentiating cells of the oligodendrocyte lineage during development and under physiological conditions appears to be vesicle exocytosis along unmyelinated axons (Kukley et al., 2007, Ziskin et al., 2007), even though glutamate release from active axons by reversal of glutamate uptake has also been proposed (Kriegler and Chiu, 1993). The prominent release of glutamate from unmyelinated axons raises the possibility that glutamate, in addition to mediating signaling events at synaptic junctions between NG2-positive glial progenitors and axons, may be able to trigger transient cellular responses in
differentiating oligodendrocytes that are involved in the regulation of oligodendrocyte maturation and myelination.

Cells of the oligodendrocyte lineage have been described to express members of all of the three major glutamate-responsive transmembrane protein families, namely ionotropic and metabotropic glutamate receptors as well as sodium-dependent glutamate transporters (Kolodziejczyk et al., 2010, Matute, 2011). Out of these, metabotropic glutamate receptors have been found downregulated as oligodendrocytes differentiate (Deng et al., 2004, Luyt et al., 2006), and ionotropic glutamate receptors have been primarily implicated in mediating excitotoxicity (Matute, 2011). Thus, sodium-dependent glutamate transporters emerge as good candidates for mediating glutamate-evoked responses related to the maturation of differentiating oligodendrocytes, i.e. of cells that are located in the vicinity of axonal segments to be myelinated and that are beyond the progenitor stage. Of the known five mammalian sodium-dependent glutamate transporters, also known as excitatory amino acid transporters (EAAT) or members of the solute carrier family 1 (SLC1), three have been found expressed by cells of the oligodendrocyte lineage, namely GLAST (EAAT1, SLC1A3), GLT-1 (EAAT2, SLC1A2) and EAAC1 (EAAT3, SLC1A1) (Domercq and Matute, 1999, Regan et al., 2007, Arranz et al., 2008, DeSilva et al., 2009, Kukley et al., 2010, Lee et al., 2012). These transporters have been well characterized to remove the excitatory amino acid glutamate from the extracellular environment (Danbolt, 2001, O'Shea, 2002, Beart and O'Shea, 2007). However, there is increasing evidence for a role of sodium-dependent
glutamate transporters beyond extracellular glutamate clearance (Chen et al., 2006, Martinez-Lozada et al., 2011, Lopez-Colome et al., 2012).

Interestingly, signaling initiated by the activation of sodium-dependent glutamate transporters has been proposed to activate calcium/calmodulin-dependent kinase type II (CaMKII) (Martinez-Lozada et al., 2011), and one of the four CaMKII genes, namely CaMKIIβ, has recently been implicated in the regulation of oligodendrocyte maturation and myelination (Waggener, 2013 #1214). More specifically, CaMKIIβ has been proposed to promote the morphological aspects of oligodendrocyte maturation, which are to a large extent regulated by changes in the cellular cytoskeleton (Bauer et al., 2009), primarily via its actin binding/stabilizing domain rather than its well-known kinase catalytic domain (Waggener et al., 2013). Thus and based on the above observations, we investigated here a possible role of a glutamate transporter-CaMKIIβ-actin cytoskeleton axis in the regulation of the morphological aspects of oligodendrocyte maturation.

**MATERIALS AND METHODS**

**Animals**

Sprague–Dawley female rats with early postnatal litters were obtained from Harlan Laboratories, Inc. (Indianapolis, IN). All animal studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.
**Antibodies**

Supernatants from cultured hybridoma cells (clone A2B5; ATCC, Manassas, VA) were used for immunopanning. Anti-GLAST, anti-GLT-1, anti-EAAC1 (Abcam, Cambridge, MA), anti-CaMKII, anti-pCaMKII T²⁸⁶⁷ (Cell Signaling Technology, Inc. Danvers, MA) and anti-GAPDH (EMD Millipore, Billerica, MA) antibodies in combination with horseradish peroxidase (HRP)-labeled secondary antibodies (Vector Laboratories, Burlingame, CA) were used for Western blot analysis. Antibodies specifically recognizing pCaMKIIβ S³⁷¹ were generated and characterized by us. Supernatants from cultured hybridoma cells (clone O4; gift from S.E. Pfeiffer) and anti-MBP antibodies (EMD Millipore, Billerica, MA) were used for immunocytochemistry to identify post-migratory, premyelinating oligodendrocytes (Sommer and Schachner, 1981, Bansal et al., 1989) and later stages of the oligodendrocyte lineage, respectively. Alexa 488- or Alexa 564-conjugated antibodies (Life Technologies, Grand Island, NY) were used as secondary antibodies.

**Cell Culture**

Primary rat oligodendrocyte progenitors were isolated from postnatal day 3 (P3) rat brains by A2B5 immunopanning (Lafrenaye and Fuss, 2011, Barres, 1992 #57). Oligodendrocyte progenitors were either used directly in plasmid nucleofection experiments or plated onto fibronectin (10 µM/mL)-coated tissue culture dishes or glass coverslips. Plated oligodendrocyte progenitors were then cultured in serum-free differentiation medium (DMEM containing 40 ng/ml tri-
iodo-thyronine (T3; Sigma, St Louis, MO) and 1x N2 supplement (Life Technologies Corp., Grand Island, NY; DMEM/T3/N2). In siRNA-mediated gene silencing experiments, differentiating oligodendrocytes were transfected with siRNAs 24h after plating. Otherwise, plated oligodendrocyte progenitors were allowed to differentiate for 48h. Under these conditions, the majority of cells represented post-migratory, premyelinating oligodendrocytes as they expressed the O4 antigen (Sommer and Schachner, 1982, Warrington et al., 1993) (data not shown). Such populations of differentiating oligodendrocytes were then either directly analyzed or treated with L-glutamate (Glu; R&D Systems, Inc. Minneapolis, MN), D-aspartate (Asp; R&D Systems, Inc. Minneapolis, MN), the competitive, non-transportable blocker of excitatory amino acid transporters TBOA (R&D Systems, Inc. Minneapolis, MN), the membrane permeable pharmacological inhibitor of CaMKII activity KN-93 or the inactive derivative of KN-93, KN-92 (EMD Millipore, Billerica, MA) as indicated. In the case of dual treatments, TBOA, KN-93 or KN-92 were added 30 min prior to the application of L-glutamate or D-aspartate. Cells were analyzed 6 hours after addition of L-glutamate or D-aspartate unless stated otherwise.

Cells of the immortalized mouse oligodendroglial cell line CIMO were cultured in DMEM/5% FCS/1 µg/mL interferon-γ (EMD Millipore, Billerica, MA) at 33 °C (Bronstein et al., 1998) and then used in plasmid nucleofection experiments.

**Quantitative (q)RT-PCR Analysis**
Total RNA was isolated from oligodendrocyte cultures using the RNeasy Micro Kit (QIAGEN Inc., Valencia, CA). Purified RNA samples were quantified using the Nanodrop ND-1000 (Nanodrop Inc., Wilmington, DE) and oligo(dT)/random hexamer-primed cDNAs were synthesized using the Omniscript RT kit (QIAGEN Inc., Valencia, CA). qRT-PCR was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and the following gene-specific primer pairs:

**Glast:** forward (5′-AGCCTGGGGTGCTTCCACCA-3′), reverse (5′-ACCACAGCCTTGCACTTCAGTCT-3′);

**Glt-1:** forward (5′-TGCGGTCCCATCCACCCT-3′), reverse (5′-GGCGGCCGCTGGCTTTAGCA-3′);

**Eaac1:** forward (5′-GCCACAGCTCGGGATGCG-3′), reverse (5′-CACGATGCCCAGTACCACGGC-3′).

**Ppia** (reference gene): forward: (5′-GGAGACGAACCTGTAGGACG-3′) and reverse: (5′-GATGCTCTTTCTCCTGTGC-3′)

**Pgk1** (reference gene): forward: (5′-ATGCAAAAGACTGGCCAAGCTAC-3′) and reverse: (5′-AGCCACAGCCTCAGCATATTTTC-3′)

PCR conditions were as follows: 95°C for 3 min followed by 40 cycles of 95°C for 15s, 58°C for 30s, and 95°C for 10s. For comparing the expression levels of the different genes, \( R_0 \) values were determined as described by (Peirson et al., 2003). To determine relative expression levels the \( \Delta\Delta C_T \) method was used (Livak and Schmittgen, 2001).
Western blot analysis

Cells were homogenized in lysis buffer (150 mM NaCl, 10 mM KCl, 20 mM HEPES (pH 7.0), 1 mM MgCl₂, 20% Glycerol, 1% Triton X-100) including the complete protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL). Protein concentrations were determined using the micro BSA protein assay kit (Thermo Scientific, Rockford, IL). 12μg of protein were used for Western blot analysis (except when using anti-GLAST antibodies, in which case 30μg of protein were used). All samples were diluted 1:2 in Laemmli Sample Buffer (Bio-Rad, Hercules, CA) and denatured by a 5 min incubation at 95°C. Proteins were resolved on 10% SDS–polyacrylamide gels (Mini-Protean TGX Gels, Bio-Rad, Hercules CA) and then transferred to Immobilon-P PVDF membranes (EMD Millipore, Billerica, MA). Membranes were incubated in 5-10% fat-free milk powder in TBS-T (10 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 0.2% Tween 20) for 60 min to block the excess of non-specific protein binding sites. Membranes were then incubated overnight at 4°C with primary antibodies as indicated. Bound antibodies were detected using HRP-conjugated secondary antibodies in combination with the ECL Prime Western blotting detection reagent (GE Healthcare Life Sciences, Piscataway, NJ). Chemiluminescent signals were detected by exposure of photographic film (Kodak BioMax MR, Eastman Kodak Company, Rochester, NY) and quantified by densitometry using the ImageJ software package (Abramoff et al., 2004).

Immunocytochemistry
For immunocytochemistry using O4 hybridoma supernatants, cells were fixed in a solution containing 4% paraformaldehyde, nonspecific binding sites were blocked using a solution of 10% FCS in DMEM and cells were incubated with the supernatant (1:1 diluted in 10% FCS/DMEM) overnight. For immunocytochemical detection of MBP, cells were fixed in a solution containing 4% paraformaldehyde and then permeabilized using 0.5% Triton X-100/0.4 M sucrose in PBS. Subsequently, cells were incubated for 30 min in 10% FCS/DMEM and then overnight with anti-MBP antibodies (SMI99; Covance, Princeton, NJ) (1:250 diluted in 10% FCS/DMEM). Primary antibodies were detected using Alexa 488- or Alexa 564-conjugated secondary antibodies (Life Technologies Corp., Grand Island, NY) (1:250 diluted in PBS) and nuclei were counterstained using Hoechst (1 µg/mL; EMD Millipore, Billerica, MA).

To visualize the actin cytoskeleton, cells were fixed in a solution containing 4% paraformaldehyde, 0.5% glutaraldehyde and 0.4M sucrose and then incubated with Acti-stain 555 phalloidin (Cytoskeleton, Inc., Denver, CO).

**siRNA-mediated Gene Silencing**

Differentiating oligodendrocytes were transfected with ON-TARGETplus siRNA SMARTpools directed against rat *Glast*, *Glt-1* or *Eaac1* (Thermo Fisher Scientific Inc., Pittsburg, PA) using Lipofectamine 2000 (Life Technologies Corp., Grand Island, NY). As control, an ON-TARGETplus non-targeting siRNA pool (Thermo Fisher Scientific Inc., Pittsburg, PA) was used. Transfection medium containing siRNA-Lipofectamine complexes was replaced with serum-free differentiation
medium (DMEM/T3/N2) after 3h and cells were cultured for an additional 72h. Knock-down of gene expression was assessed by qRT-PCR and Western blot analysis.

**Process Morphology Analysis**

Oligodendrocyte morphology was analyzed as previously described (Dennis et al., 2008). Briefly, oligodendrocytes were immunostained using O4 hybridoma cell supernatants. Images of approximately 30 cells were taken randomly for each treatment group in each experiment (n≥3) using an Olympus BX51 inverted fluorescent microscope (Olympus America Inc., Center Valley, PA). IP Lab imaging software (BD Biosciences Bioimaging, Rockville, MD) was used to determine process index (total O4 immunopositive area minus the cell body) and network area (total area within the radius of the O4 immunopositive process network minus the cell body). For the bar graphs representing network area, the mean value for cells cultured under control conditions was calculated. This mean value was set to 100% and adjusted, i.e. normalized, values for all cells were averaged for each experimental condition. For the generation of representative images, confocal laser scanning microscopy was used (Zeiss LSM 510 META NLO; Carl Zeiss Microscopy, LLC, Thornwood, NY). Images represent 2D maximum projections of stacks of 0.4 µm optical sections.

**Cell Count Analysis**

Images of four fields were taken with a 20x objective from each coverslip (three
coverslips per condition) using an Olympus BX51 fluorescence microscope equipped with an Olympus DP72 CCD camera (Olympus America Inc., Center Valley, PA). The number of Hoechst-positive and MBP immuno-positive oligodendrocytes was then determined using the Cell Counter plugin to the ImageJ software package (Abramoff et al., 2004).

**Plasmid Nucleofection**

A2B5 immunopanned oligodendrocyte progenitors or CIMO cells were nucleofected (Lonza Cologne GmbH, Cologne, Germany) with the following constructs: a plasmid encoding eGFP-CaMKIIβ (Okamoto et al., 2004), a plasmid encoding eGFP-CaMKIIβ^allA in which all serine and threonine residues located within the variable domain (aa 317-396) were replaced with alanine, a plasmid encoding eGFP-CaMKIIβ^allD in which all serine and threonine residues located within the variable domain (aa 317-396) were replaced with aspartic acid, and a control plasmid encoding eGFP alone. All plasmids had the same plasmid backbone derived from the eukaryotic expression vector pEGFP-C1 (Clontech Laboratories, Inc., Mountain View, CA).

**Intracellular Calcium Measurement**

Intracellular calcium concentrations were determined in principle as previously described (Gryniewicz et al., 1985). Briefly, differentiating oligodendrocytes were loaded with the calcium indicator fura-2 AM ester (2.5µM) and pluronic (0.01%) (Life Technologies Corp., Grand Island, NY) in differentiation medium for
30 min at 37°C. Cells were washed and incubated in differentiation medium for an additional 30 min at 37°C. Ratiometric calcium measurements were made at 340 and 380 nm excitation and 510-520 nm emission wavelengths with cells cultured in differentiation medium (unless mentioned otherwise) using a Zeiss Observer.Z1 microscope in combination with the Axio VisionRel 4.8 software package (Carl Zeiss Microscopy, LLC, Thornwood, NY). Measurements were made before and after the application of the indicated compounds. To calculate intracellular free calcium concentrations (in nM), a calibration curve (Calcium Calibration Buffer Kit, Life Technologies Corp., Grand Island, NY) was used.

**Statistical Analysis**

For statistical analysis the GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA) was used. In the case of two or more groups of data composed of variable values, two-tailed Student’s t-tests or Kruskal–Wallis one-way analyses of variance (ANOVA) combined with post hoc Dunn’s or Student–Newman–Keuls tests were performed. When comparing a single control group with experimental groups of data, ANOVA with post hoc Dunnett tests were used. In the case data were compared to a set control value (1 or 100%) lacking variability, one-sample t-tests were used (Skokal and Rohlf, 1995, Dalgaard, 2008).

**RESULTS**

*Sodium-dependent glutamate transport, mediated primarily by GLAST and GLT-1, promotes the morphological maturation of differentiating*
oligodendrocytes.

As introduced above, the sodium-dependent glutamate transporter genes Glast, Glt-1 and Eaac1 have previously been described to be expressed by cells of the oligodendrocyte lineage. To confirm such expression in our culture system, qRT-PCR analysis was performed and revealed relative mRNA expression levels of 45±5%, 31±6%, and 12±5% for Glast, Glt-1 and Eaac1, respectively (Fig. 4.1A). Using Western blot analysis the expression of the three sodium-dependent glutamate transporters in differentiating oligodendrocytes could be further validated (Fig. 4.1B). To assess a potential role of sodium-dependent glutamate transport beyond extracellular glutamate clearance in differentiating oligodendrocytes, these cells were treated with 100 mM L-glutamate in combination with TBOA, a competitive and non-transportable inhibitor of sodium-dependent glutamate transport (Shimamoto et al., 1998, Shigeri et al., 2001), and morphological aspects of oligodendrocyte maturation were assessed by determining the cells’ network areas and process indices (Dennis et al., 2008). Increases in both parameters were observed upon treatment with L-glutamate (Fig. 4.2A,B and data not shown). This maturation-promoting effect of L-glutamate was seen as early as 2 hours and up to 6 hours of treatment (Fig. 4.2C). Importantly, it could be blocked by pre-treatment with TBOA and was thus found to be dependent on the activity of sodium-dependent glutamate transporters (Figs. 4.2A,B). It is of note that in agreement with previous studies, no obvious effects on cell survival were noted in the presence of the glutamate and TBOA concentrations used here and within the time frames analyzed (Deng
et al., 2006) and data not shown).

To further substantiate that sodium-dependent glutamate transporters play a predominant role in the observed maturation-promoting effect exerted by L-glutamate, the naturally occurring amino-acid D-aspartate was used as a glutamate-equivalent agonist. As L-glutamate, D-aspartate is efficiently taken up through the sodium-dependent glutamate transporter system (Davies and Johnston, 1976, Danbolt and Storm-Mathisen, 1986, Kanai and Hediger, 1992, Pines et al., 1992, Palacin et al., 1998), and it is then endogenously metabolized (Schell et al., 1997). In contrast to L-glutamate, however, D-aspartate is known to not activate non-NMDA receptor ionotropic glutamate receptors (Domercq et al., 2005, Errico et al., 2008), thus eliminating potential confounding effects due to an activation of AMPA/kainate receptors. As shown in Fig. 4.2D, the effect of D-aspartate on the oligodendrocyte’s process network was comparable to the effect seen upon treatment with L-glutamate (compare Figs. 4.2B and D).

It has been well demonstrated that morphological maturation of oligodendrocytes occurs during development concurrently with changes in gene expression (Pfeiffer et al., 1993, Baumann and Pham-Dinh, 2001, Emery, 2010). Under experimental conditions, however, molecular mechanisms regulating cellular morphology may be uncoupled from those that regulate gene expression (Buttery and ffrench-Constant, 1999, Osterhout et al., 1999, Kim et al., 2006, Lafrenaye and Fuss, 2011, Waggener et al., 2013). Thus and to assess a potential role of L-glutamate and the activity of sodium-dependent glutamate transporters on myelin gene expression, potential changes in myelin basic
protein (MBP) expression were assayed using immunocytochemistry. As shown in Fig. 4.2E, no significant differences in the number of MBP-positive cells were noted, thus suggesting that the sodium-dependent glutamate transporter-mediated effect on oligodendrocyte maturation is primarily associated with the morphological aspects of this process.

As shown in Fig. 4.1, differentiating oligodendrocytes express more than one of the known sodium-dependent glutamate transporter genes. To evaluate the role of individual transporter genes in the L-glutamate-mediated maturation-promoting effect observed here, differentiating oligodendrocytes were transfected with siRNA pools specifically silencing Glast, Glt-1 or Eaac1 expression. Knockdown of gene expression was confirmed by Western blot analysis, which revealed a specific reduction in transporter protein levels of at least 70% (Fig. 4.S1). Importantly, for none of the down-regulated genes a compensatory up-regulation of any of the other two transporter genes was noted (Fig. 4.S1). As shown in Fig. 4.2F, knock-down of either Glast or Glt-1 expression was found to eliminate the effect of L-glutamate on the oligodendrocyte’s process network. For the sodium-dependent glutamate transporter with much lower expression levels in differentiating oligodendrocytes when compared with Glast or Glt-1, namely Eaac1, knock-down of gene expression was seen to only attenuate the effect of L-glutamate on the oligodendrocyte’s process network (siCtrl+Glu: 157±7%, siEaac1: 136±6%, p=0.007, ANOVA). Taken together, these data demonstrate that L-glutamate can promote morphological maturation of differentiating oligodendrocytes via a sodium-dependent glutamate transporter-mediated
mechanism. In addition, they suggest that even a slight decline in oligodendrogial sodium-dependent glutamate transporter expression can significantly reduce the effect of L-glutamate on the morphological maturation of differentiating oligodendrocytes.

**Activation of sodium-dependent glutamate transporters in differentiating oligodendrocytes leads to a transient increase in intracellular calcium levels**

Having established that activation of sodium-dependent glutamate transporters promotes the morphological maturation of differentiating oligodendrocytes, we next wished to explore potential downstream signaling events involved in this process. These signaling-related studies were focused on calcium-mediated events, since it had previously been demonstrated that glutamate transport can activate the reverse mode of the sodium/calcium exchanger and thereby lead to a transient increase in intracellular calcium levels (Rojas et al., 2007, Martinez-Lozada et al., 2011, Lopez-Colome et al., 2012). To assess the effect of an activation of sodium-dependent glutamate transporters on intracellular calcium levels, D-aspartate was used as a glutamate-equivalent agonist. Using this strategy, a contribution of AMPA/kainate receptor activation to the observed transient increase in permeability to calcium (Fig. 4.3) could be excluded. The D-aspartate-induced transient increase in free intracellular calcium levels within differentiating oligodendrocytes was found to be dose-dependent (Fig. 4.3B) and to be absent in the presence of TBOA (Fig. 4.3C). In addition, no such increase
was observed in the absence of extracellular calcium (Fig. 4.3B). Taken together, these data support the idea that in differentiating oligodendrocytes L-glutamate (as D-aspartate) activates sodium-dependent glutamate transporters which in turn mediate a transient increase in intracellular calcium levels via entry from the extracellular environment. Furthermore, it is of note that the D-aspartate-mediated transient increase in intracellular calcium levels was observed first within cellular processes and then within the cell body, thus suggesting that the sodium-dependent glutamate transporter-evoked intracellular calcium response may initially occur locally within oligodendrocyte processes.

The maturation promoting effect of sodium-dependent glutamate transporters in differentiating oligodendrocytes is mediated by a transient phosphorylation event within CaMKIIβ’s actin binding domain

Our recent data provided good evidence for a critical role of the calcium sensor CaMKIIβ and in particular its actin binding/stabilizing domain in regulating oligodendrocyte maturation and myelination (Waggener et al., 2013). Thus, CaMKIIβ and in particular its actin binding/stabilizing domain may be directly involved in the sodium-dependent glutamate transporter-mediated effect described here. Indeed, pre-treatment of differentiating oligodendrocytes with KN93, a membrane-permeable pharmacological inhibitor of CaMKIIβ’s kinase catalytic as well as actin binding/stabilizing activity (Lin and Redmond, 2008, Sumi, 1991 #1025), was found to block the maturation-promoting effect of L-glutamate on the oligodendrocyte’s process network (Fig. 4.4A). It is of note that
KN93 treatment alone and thus a continuous inhibition of CaMKII activity attenuated the morphological maturation of differentiating oligodendrocytes (Fig. 4.4A). This effect has been previously described and was found to not be associated with a change in cellular viability (Waggener et al., 2013).

To further assess the effect of L-glutamate on CaMKII and in particular CaMKIIβ’s actin binding/stabilizing domain in differentiating oligodendrocytes, phosphorylation events at CaMKII’s T^{287/6} and CaMKIIβ’s S^{371} site were analyzed. CaMKII’s T^{287/6} site represents CaMKII’s autophosphorylation site, which due to sequence conservation could only be detected by pan pCaMKII T^{287/6} antibodies. CaMKIIβ’s S^{371} site is located within CaMKIIβ’s actin binding/stabilizing domain (Kim et al., 2011), and antibodies specifically recognizing the pCaMKIIβ S^{371} site have been generated by us. These antibodies were found to not recognize CaMKIIβ S^{371A}, a mutant form of CaMKIIβ, which cannot be phosphorylated at its S^{371} site (data not shown). As shown in Fig. 4.4B,C, treatment of differentiating oligodendrocytes with L-glutamate led to a significant increase in phosphorylation at CaMKIIβ’s S^{371} site. This effect was time-dependent and transient, since an increase in phosphorylation was not seen prior to 30 min of treatment and at 4 hours and beyond (Fig. 4.4C,F). Furthermore, no such increase in phosphorylation was observed at CaMKII’s autophosphorylation (T^{286/7}) site within 15-60 min of L-glutamate treatment (Fig. 4.4E), nor was a change in total levels of CaMKIIβ noted (Fig. 4.4D). Importantly, the effect of L-glutamate on the phosphorylation at CaMKIIβ’s S^{371} site could be blocked by pre-treatment with TBOA (Fig. 4.4G), thus demonstrating that activation of sodium-dependent
glutamate transporters mediates a phosphorylation event within CaMKIIβ’s actin binding/stabilizing domain. To further assess the contribution of individual sodium-dependent glutamate transporter genes in this L-glutamate-mediated increase in pCaMKIIβ S^{371} levels, differentiating oligodendrocytes were transfected with siRNA pools specifically targeting Glast, Glt-1 or Eaac1 expression. Similar to what was observed for the effect of gene-specific knock-down of sodium-dependent glutamate transporter expression on the oligodendrocyte’s network area (Fig. 4.2F), knock-down of Glast or Glt-1 expression eliminated the effect of L-glutamate on the phosphorylation of CaMKIIβ’s S^{371} site, while the effect of a knock-down of Eaac1 expression appeared less pronounced.

*Phosphorylation events within CaMKIIβ’s actin binding domain appear to regulate the association of CaMKIIβ with filamentous F-actin and the effect of glutamate on the oligodendrocyte’s process network*

Our data described so far demonstrated that in differentiating oligodendrocytes, L-glutamate activates sodium-dependent glutamate transporters, which in turn mediate a transient increase in intracellular calcium levels, a transient phosphorylation event at CaMKIIβ’s S^{371} site and a promotion of the morphological aspects of oligodendrocyte maturation. In addition, our recent data demonstrated that upon increases in intracellular calcium levels, serine, including S^{371}, and threonine residues are phosphorylated within CaMKIIβ’s actin binding domain, and that these phosphorylation events are sufficient to induce
detachment of CaMKIIβ from filamentous F-actin (Kim et al., 2011). Taken together, the above findings raised the possibility that sodium-dependent glutamate transporter-mediated phosphorylation within CaMKIIβ’s actin binding/stabilizing domain may affect the oligodendrocyte’s process network via a change in CaMKIIβ’s association with filamentous F-actin. To investigate this idea, mutant forms of eGFP fusion proteins of CaMKIIβ were generated that either represented CaMKIIβ in which the serine and threonine residues within in the actin binding/stabilizing domain are non-phosphorylatable (eGFP-CaMKIIβallA) or their phosphorylated state is mimicked (eGFP-CaMKIIβallD). In addition, eGFP-CaMKIIβWT and eGFP-CaMKIIβK43R, a mutant that is impaired in ATP binding and thus inactive with regard to its kinase catalytic but not actin binding activity (Okamoto et al., 2007), were used. Consistent with the previously characterized role of phosphorylation events within CaMKIIβ’s actin binding/stabilizing domain on CaMKIIβ’s association with filamentous F-actin, nucelofection studies using plasmids encoding the above described eGFP-CaMKIIβ forms revealed individual subcellular distribution patterns (Fig. 4.5A,B). More specifically, in cells of the oligodendroglial cell line CIMO, the non-phosphorylatable eGFP-CaMKIIβallA form was found to largely co-localize with filamentous F-actin (Fig. 4.5A). Similarly, it was detected in differentiating oligodendrocytes at subcellular locations at which filamentous F-actin has been previously described to be enriched (Fig. 4.5B), namely within oligodendrocyte growth cones and at process branching points (Song et al., 2001, Fox et al., 2006a). In contrast, the phospho-mimetic eGFP-CaMKIIβallD form showed little
co-localization with filamentous F-actin and appeared similar to eGFP distributed diffusely and throughout the cytoplasm (Fig. 4.5A,B). In agreement with a likely only partially phosphorylated state, eGFP-CaMKIIβWT displayed a somewhat intermediate subcellular distribution (Fig. 4.5A,B). It is of further note that the eGFP tag used here has been previously shown to not interfere with CaMKIIβ function (Okamato et al., 2004, Okamoto et al., 2007).

To correlate the phosphorylation state of CaMKIIβ’s actin binding/stabilizing domain und thus its level of association with filamentous F-actin with the morphological maturation of differentiating oligodendrocytes, the effect of the expression of the different eGFP-CaMKIIβ forms on the oligodendrocyte’s process network was evaluated. As shown in Fig. 4.5C, expression of the non-phosphorylatable eGFP-CaMKIIβallA form blocked the maturation-promoting effect of L-glutamate treatment. No such effect was observed when eGFP-CaMKIIβWT or eGFP-CaMKIIβK43R were expressed, suggesting that the L-glutamate-mediated maturation-promoting effect may not require CaMKIIβ’s kinase catalytic activity. Importantly and consistent with our previous findings (Waggener et al., 2013; Fig. 4.4A), constitutive expression of the phospho-mimetic eGFP-CaMKIIβallD form attenuated the morphological maturation of differentiating oligodendrocytes and eliminated the effect of L-glutamate treatment on the cell’s process network. Taken together, these data support the idea that phosphorylation within CaMKIIβ’s actin binding/stabilizing domain leading to detachment of CaMKIIβ from filamentous F-actin, is a required step in the mechanism promoting the morphological aspects of oligodendrocyte
maturation via activation of sodium-dependent glutamate transporters. Notably, constitutive (vs. transient) phosphorylation within CaMKIIβ’s actin binding/stabilizing domain appears to have an opposing effect.

DISCUSSION

The study described here, investigated the role of sodium-dependent glutamate transporters in the regulation of oligodendrocyte differentiation. Our data demonstrated that activation of in particular GLAST and GLT-1 in differentiating oligodendrocytes promotes their morphological maturation. On a molecular level, this sodium-dependent glutamate transporter-mediated promotion of oligodendrocyte maturation was found to be associated with transient increases in intracellular calcium levels (from the extracellular environment) and phosphorylation at CaMKIIβ’s S371 site located within CaMKIIβ’s actin binding/stabilizing domain. In addition, our data revealed a requirement for phosphorylation events within CaMKIIβ’s actin binding/stabilizing domain. These phosphorylation events appeared furthermore to regulate CaMKIIβ’s association with filamentous F-actin. Based on these data and the ascribed function of CaMKIIβ’s actin binding/stabilizing domain in the regulation of dendritic spine morphology (Okamoto et al., 2009), we propose the following model for the role of sodium-dependent glutamate transporters in the regulation of oligodendrocyte maturation (Fig. 4.6). Under basal conditions, CaMKIIβ is in differentiating oligodendrocytes to a large extent associated with the actin cytoskeleton. Glutamate release from for example un-myelinated axonal segments activates
oligodendroglial sodium-dependent glutamate transporters, which in turn activate the reverse mode of oligodendroglial sodium/calcium exchangers resulting in a transient increase in intracellular calcium levels (Fig. 4.6A). This increase in intracellular calcium levels leads to phosphorylation events within CaMKIIβ’s actin binding/stabilizing domain (including CaMKIIβ’s S$_{371}$ site), inactivation of CaMKIIβ’s actin binding activity and detachment of CaMKIIβ from the actin cytoskeleton. Such transient inactivation of CaMKIIβ’s actin binding activity opens a time window during which actin cytoskeleton remodeling events and actin polymerization (Okamoto et al., 2007, Sanabria et al., 2009, Hoffman et al., 2013) are favored (Fig. 4.6B). This dynamic phase is then followed by a phase of actin cytoskeleton stabilization via re-activation of CaMKIIβ’s actin binding activity via de-phosphorylation (Fig. 4.6C). It is of note that the actin binding activity of CaMKIIβ appears to be independent of its kinase catalytic activity, a concept that is supported by recent in vivo studies (van Woerden et al., 2009, Borgesius et al., 2011). Also, actin binding properties have been described for other members of the CaMKII gene family, even though via a different structure-function domain (Caran et al., 2001, Hoffman et al., 2013). Further studies will be necessary to evaluate a potential contribution of CaMKII isoymes other than CaMKIIβ in the molecular mechanism described here. Importantly, constitutive inactivation of CaMKIIβ’s actin binding activity, in contrast to transient inactivation, appears to attenuate the morphological maturation of differentiating oligodendrocytes, an effect that is likely mediated by de-stabilization of the actin cytoskeleton over an extended period of time (Waggener et al., 2013). Thus, it is cycles of activation
and de-activation of CaMKIIβ’s actin binding activity that allow re-organization of the actin cytoskeleton and a promotion of the morphological aspects of oligodendrocyte maturation. This idea is further supported by recent findings demonstrating that balanced activation and de-activation of the actin filament severing and depolymerizing factor coflin regulates the function of the myelinating cells of the peripheral nervous system, namely Schwann cells (Sparrow et al., 2012).

Our studies revealed a prominent expression of the sodium-dependent glutamate transporter gene Glast in differentiating oligodendrocytes. This finding is in agreement with previous observations made in tissue culture as well as in vivo in both rodents and humans (Domercq and Matute, 1999, Pitt et al., 2003, Vallejo-Illarramendi et al., 2006, Regan et al., 2007, DeSilva et al., 2009). In addition, a low level expression of Eaac1 is consistent with the reported presence of few Eaac1-positive cells in the developing optic nerve (Domercq and Matute, 1999). In contrast, the expression of Glt-1 in cells of the oligodendrocyte lineage appears more complicated, since it has in particular in vivo been described to be developmentally regulated and to be predominant at stages at which oligodendrocytes are premyelinating (Domercq and Matute, 1999, Desilva et al., 2007, DeSilva et al., 2009). Our previous data revealed a prominent in vivo role of CaMKIIβ in the regulation of myelin thickness (Waggener et al., 2013) and thus likely not only in premyelinating but also myelinating oligodendrocytes. Thus, a developmentally regulated expression pattern of Glt-1 as described above would suggest that the signaling pathway proposed here (Fig. 4.6) may in
vivo be primarily mediated by GLAST and less so by GLT-1. However, in human tissue the expression of Glt-1 has been reported to occur in mature oligodendrocytes (Werner et al., 2001, Pitt et al., 2003). Thus, additional studies will be necessary to more precisely define individual sodium-dependent glutamate transporter contributions and potential species-specific differences. It is of further note that while development in mice with single knock-outs for Glast, Glt-1 or Eaac1 occurs without apparent CNS gross phenotypes (Peghini et al., 1997, Tanaka et al., 1997, Harada et al., 1998), double Glast/Glt-1 knockout mice die in utero and show multiple developmental brain defects (Matsugami et al., 2006). These data indicate that during CNS development the loss of one sodium-dependent glutamate transporter may functionally be compensated for by at least one of the remaining transporters, i.e. by a mechanism that could further complicate a delineation of individual roles for individual sodium-dependent glutamate transporters.

Our model proposed in Fig. 4.6 is suggestive of pivotal roles for oligodendroglial sodium/calcium exchangers and intracellular calcium levels in the regulation of oligodendrocyte maturation. It is thus noteworthy that the sodium/calcium exchanger NCX3 has recently been implicated in the regulation of oligodendrocyte differentiation (Boscia et al., 2012a). In addition, process outgrowth has been found to be regulated by intracellular calcium levels in differentiating oligodendrocytes (Yoo et al., 1999). Interestingly, in our studies (Fig. 4.3) intracellular calcium levels remained slightly above control levels after the initial rise. Such a calcium response is consistent with previously described
calcium responses mediated by signaling through sodium-dependent glutamate transporters in astrocytes (Rojas et al., 2007). In the case of astrocytes the initial rise in intracellular calcium concentrations was found to be amplified by calcium release from ryanodine sensitive calcium stores (Rojas et al., 2007), which have also been described to be expressed by cells of oligodendrocyte lineage (Simpson et al., 1998).

In the major demyelinating disease in human, Multiple Sclerosis (MS), a block in oligodendrocyte differentiation is considered one of the main causes of remyelination failure and repair under such pathological conditions (Chang et al., 2002, Fancy et al., 2010, Kremer et al., 2011). Interestingly, reduced sodium-dependent glutamate transporter protein levels have been reported for white matter areas surrounding MS lesions (Werner et al., 2001, Pitt et al., 2003). In addition, retrospective analysis of brain tissue from MS patients revealed oligodendrocyte differentiation block as the major determinant of remyelination failure within periplaque white matter regions (Kuhlmann et al., 2008). In light of our findings that knock-down of sodium-dependent glutamate transporter expression eliminates the effect of glutamate on the morphological maturation of differentiating oligodendrocytes (Fig. 4.2F), it is tempting to speculate that changes in sodium-dependent glutamate transporter expression may contribute to the differentiation block seen in MS. It is of further note that glutamate levels have been found elevated in MS brains (Werner et al., 2001, Srinivasan et al., 2005, Trapp and Stys, 2009). These changes in glutamate homeostasis have been implicated in mediating excitotoxicity (Pitt et al., 2003, Matute, 2011).
However, human oligodendrocytes appear to be largely resistant to such glutamate-mediated injury (Wosik et al., 2004) and thus changes in glutamate homeostasis and sodium-dependent glutamate transporter expression may, at least under certain circumstances, primarily limit repair under pathological conditions as they are seen in the CNS of MS patients.
FIGURE 4.1: Sodium-dependent glutamate transporters are expressed in differentiating oligodendrocytes. A: Bar graph representing sodium-dependent glutamate transporter mRNA levels as determined by qRT-PCR analysis. Total glutamate transporter mRNA levels ($Glast+Glt-1+Eaat1$) were set to 100% and the values for the individual gene-specific mRNA levels were adjusted accordingly. Data represent means ± SEM (n = 3 independent experiments, ***$p$≤0.001, ANOVA). B: Sodium-dependent glutamate transporter protein levels as determined by immunoblot analysis. Representative blots are shown. The numbers to the left indicate molecular weights in kDa.
FIGURE 4.2: The activation of sodium-dependent glutamate transporters promotes the morphological aspects of oligodendrocyte differentiation. A: Representative images of differentiating oligodendrocytes immunostained using O4 hybridoma supernatants and treated as indicated: control (Ctrl), L-glutamate (Glu; 100 µM), a non-transportable inhibitor of sodium-dependent glutamate transport (TBOA; 100 µM). Scale bars: 20 µm. B-D: Bar graphs representing quantitative analyses of network areas (Dennis et al., 2008). Cells were treated as indicated: control (Ctrl), L-glutamate (Glu, 100 µM), a non-transportable inhibitor of sodium-dependent glutamate transport (TBOA, 100 µM), D-aspartate (Asp, 100 µM). The mean values for control (non-treated) cells were set to 100% and experimental values were calculated accordingly. At least 30 cells per condition and experiment were analyzed in 3 independent experiments (i.e. a total of at least 90 cells per condition). Data represent means ± SEM (***p ≤ 0.001 compared to control, ANOVA). E: Bar graph depicting the number of MBP-immuno-positive cells normalized to the number of Hoechst-positive nuclei. The inset shows a representative image of cells cultured under control (non-treated) conditions and stained with an antibody specific for MBP (left panel) as well as with Hoechst to visualize nuclei (right panel). Cells were treated as indicated and control (non-treated) values were set to 100%. Experimental values were calculated accordingly. Data represent means ± SEM of 3 independent experiments in which a field of view of 508 µm² per coverslip and 3 coverslips per condition and experiment were analyzed. Three independent experiments were performed. ANOVA revealed no statistically significant difference (p ≤ 0.05). F: Bar graph depicting the network area upon siRNA-mediated knock-down of individual sodium-dependent glutamate transporters (as indicated) and subsequent treatment with L-glutamate (Glu, 100 µM). The mean value for control cells (siCtrl non-treated) was set to 100%. Experimental values were calculated accordingly. At least 30 cells per condition and experiment were analyzed in 3 independent experiments (i.e. a total of at least 90 cells per condition). Data represent means ± SEM (***p ≤ 0.001 compared to siCtrl non-treated, ANOVA).
FIGURE 4.3: The activation of sodium-dependent glutamate transporters increases intracellular calcium levels in differentiating oligodendrocytes. A: Representative pseudo-colored images of fura-2 AM ratio fluorescence measurements made in differentiating oligodendrocytes and upon treatment with D-aspartate (Asp). The bar to the left represents a relative color scale indicating low (L) and high (H) calcium levels. B-C: Time course of changes in free intracellular calcium concentrations \([\text{Ca}^{2+}]\) as detected by fura-2 AM ratiometric measurements made in differentiating oligodendrocytes and upon different treatments as indicated in the inset shown in the upper right in B and C. Start of treatment is indicated by the arrow. The graphs represent means ± SEM of measurements taken from 9 cells per treatment group and experiment and from 3 independent experiments (i.e. a total of 27 cells per treatment group).
FIGURE 4.4: Activation of sodium-dependent glutamate transporters leads to a transient phosphorylation event at CaMKIIβ’s S\textsuperscript{371} residue. A: Bar graph depicting a quantitative analysis of the oligodendrocyte’s network area (Dennis et al., 2008). Cells were first pre-treated with the pharmacological CaMKII inhibitor KN-93 or its inactive derivative KN-92 as control and then incubated in the absence or presence (+Glu) of 100 µM L-glutamate. The mean values for control cells (pre-treated with KN92 and incubated in the absence of L-glutamate) were set to 100% and experimental values were calculated accordingly. At least 30 cells per condition and experiment were analyzed in 3 independent experiments (i.e. a total of at least 90 cells per condition). Data represent means ± SEM (**p≤0.001 compared to control, ANOVA). The inset (upper right) depicts representative images of differentiating oligodendrocytes immunostained with O4 antibodies and treated with KN-92 plus L-glutamate (left) or KN-93 plus L-glutamate (right). Scale bars: 5µm. B, inset in H: Representative Western blots depicting CaMKII phosphorylation (pCaMKIIβ S\textsuperscript{371}, pCaMKII T\textsuperscript{286/7}) or total CaMKIIβ protein levels. GAPDH protein levels are shown representatively for the Western blot for which anti- pCaMKII T\textsuperscript{286/7} (B) or anti-pCaMKIIβ S\textsuperscript{371} (inset in H) antibodies were used. C-H: Bar graphs depicting the levels of pCaMKIIβ S\textsuperscript{371} (C,F-H), total CaMKIIβ (D) or pCaMKII T\textsuperscript{286/7} (E) at different time-points after addition of L-glutamate (Glu) (C-F), at time-point 60 min after addition of L-glutamate and prior pre-treatment with or without TBOA (G) or after transfection with siRNA pools as indicated and L-glutamate treatment for 60 min (H). Levels of phosphorylated or total CaMKII protein were normalized to GAPDH protein levels. The mean normalized values for control (non-treated) cells were set to 100% (horizontal line) and experimental values were calculated accordingly. Data represent means ± SEM of 3 independent experiments (*p≤0.01 compared to control, ANOVA).
FIGURE 4.5: Phosphorylation events within CaMKIIβ’s actin binding/stabilizing domain regulate the association of CaMKIIβ with filamentous F-actin and the glutamate-mediated promotion of the morphological aspects of oligodendrocyte maturation. A: Representative images of cells of the oligodendroglial cell line CIMO nucleofected with plasmids encoding eGFP fusion proteins of CaMKIIβ (WT and mutant forms as indicated) and stained for F-actin (phalloidin). Scale bars: 5 µm. B: Representative images of differentiating oligodendrocytes nucleofected with plasmids encoding eGFP fusion proteins of CaMKIIβ (WT and mutant forms as indicated). Arrowheads point toward eGFP-CaMKIIβallA localized at oligodendrocyte growth cones and process branching points. Scale bars: 5 mm. C: Bar graph depicting a quantitative analysis of the oligodendrocyte’s network area (Dennis et al., 2008). Cells were nucleofected as indicated and then incubated in the absence or presence (+Glu) of 100 µM L-glutamate. The mean values for control cells (nucleofected with an eGFP encoding plasmid and incubated in the absence of L-glutamate) were set to 100% and experimental values were calculated accordingly. At least 30 cells per condition and experiment were analyzed in 3 independent experiments (i.e. a total of at least 90 cells per condition). Data represent means ± SEM (***p≤0.001 compared to control, ANOVA).
FIGURE 4.6: Proposed model for a glutamate transporter-CaMKIIβ-actin cytoskeleton axis in the regulation of oligodendrocyte maturation. A: L-glutamate (Glu) stimulates the activity of sodium-dependent glutamate transporter activity, which in turn leads to an increase in intracellular sodium (Na\(^+\)) levels, activation of the reverse mode of the sodium/calcium exchanger and a transient increase in intracellular calcium (Ca\(^{2+}\)) levels. B: The transient increase in intracellular calcium levels leads to phosphorylation events within CaMKIIβ’s actin binding/stabilizing domain (pCaMKIIβ\(^{S371}\)), detachment of CaMKIIβ from filamentous F-actin and the opening of a time window during which cytoskeletal rearrangements and morphological remodeling can occur. C: Upon deactivation (dephosphorylation) of CaMKIIβ, CaMKIIβ binds to filamentous F-actin and thereby stabilizes the newly arranged cytoskeleton. Such cycles of activation and de-activation of CaMKIIβ’s actin binding activity allow re-organization of the actin cytoskeleton while at the same time preventing its uncontrolled disintegration. adapted from Okamoto et al, 2009 (Okamoto et al., 2009).
FIGURE 4.S1: siRNA-mediated gene silencing results in a significant knock-down of sodium-dependent glutamate transporter expression in differentiating oligodendrocytes. A: Bar graph depicting sodium-dependent glutamate transporter protein levels upon siRNA-mediated gene silencing and normalized to GAPDH protein levels. The mean value for cells treated with the control siRNA pool (siCtrl) was set to 100% (horizontal gray line) and experimental values were calculated accordingly. Data represent means ± SEM of 3 independent experiments (**p≤0.001,*p≤0.05, ANOVA). B: Representative Western blots depicting protein levels for GLAST, GLT-1, EAAC1 and GAPDH (as control). GAPDH protein levels are shown representatively for the Western blot, for which anti-EAAC1 antibodies were used.
CHAPTER 5
Final Conclusions

The myelin sheath that is produced by oligodendrocytes in the CNS serves a unique and critical function for proper conduction of nerve signals. Disruption of the myelin sheath leads to improper nerve conduction resulting in unpredictable debilitating afflictions for people as seen in the most well known demyelinating disease MS. As mentioned in Chapter 1, in the MS brain there are oligodendrocytes present within CNS-located lesions, however, they lack the ability to efficiently remyelinate (Chang et al., 2002). When remyelination does occur, the myelin sheath is thinner and is too inefficient to provide proper axonal protection and propagation of the electrical signal (Patrikios et al., 2006, Goldschmidt et al., 2009, Bradl and Lassmann, 2010, Emery, 2010). A better understanding of the molecular mechanisms that regulate oligodendrocyte maturation and development is thought to provide molecular and genetic clues to better understand not only developmental myelination but also how to promote remyelination under pathological conditions.

Unlocking the molecular secrets to myelination may enlighten researchers to a complex understanding of the morphological maturation of the oligodendrocyte. Morphological maturation along with gene regulation can go hand in hand or be separate, and to an extent one can drive the one or the other (Boggs, 2006, Boggs et al., 2006, Forrest et al., 2009, Lafrenaye and Fuss, 2010). Myelination requires the maturation of progenitor cells into cells that extend processes and
form a complex process network. These premyelinating oligodendrocytes then transition into mature myelinating oligodendrocytes (Song et al., 2001, Bacon et al., 2007, Bauer et al., 2009). These large scale morphological changes are mediated by precisely regulated changes in the actin cytoskeleton (Bauer et al., 2009). This fine actin regulation is highlighted by myelin defects seen in knockouts or knockdowns of cytoskeletal regulatory proteins such as WAVE1, cdc42, RAC1, Integrin-linked kinase (ILK), and Mayven (Jiang et al., 2005, Kim et al., 2006, Thurnherr et al., 2006, O'Meara et al., 2013). Interestingly, in MS lesions proteomic studies have revealed a large amount of misregulated cytoskeletal regulatory mechanisms (Jahn et al., 2009, Satoh et al., 2009).

As stated above, changes in oligodendrocyte development usually are characterized by large scale changes of the oligodendrocyte process network and the underlying actin cytoskeleton. In Chapter 2, by using in vitro tissue culture as well as in vivo knockout and knock-in models, CaMKIIβ has been shown to be a critical component of the molecular mechanisms regulating oligodendrocyte maturation and CNS myelination. CaMKIIβ’s properties in binding and stabilizing actin have been established in neuronal spine morphology but our finding of CaMKIIβ’s role in the regulation of oligodendrocyte development and the formation of a complex process network is novel. (Shen et al., 1998, O'Leary et al., 2006, Okamoto et al., 2007, Lin and Redmond, 2008, Okamoto et al., 2009, Sanabria et al., 2009).

In neurons and oligodendrocytes, the stabilization along with the destabilization of the actin cytoskeleton causes the fluidity of process elongation
along with membrane spreading (Bradke and Dotti, 1999, Okamoto et al., 2007, Okamoto et al., 2009). Furthermore, in neurons, the bundling and unbundling of actin acts to alter and stabilize the morphology of dendritic spines based on activity dependent plasticity (Okamoto et al., 2009). Evidence presented in Chapter 2 points towards the binding of actin to CaMKIIβ using colocalization studies. The evidence also suggests that once CaMKIIβ’s catalytic activity and actin binding is inhibited with a pharmacological inhibitor, there is no indication of colocalization (Chapter 2). Actin colocalization with CaMKIIβ suggests that the model of CaMKIIβ binding and dissociating from actin allows stabilization and alteration of the cytoskeleton respectively. Although this was developed for remodeling of the dendritic spine, it could also apply to the remodeling of oligodendrocyte protrusions. Based on previous data, one could assume that process outgrowth and maintenance of the process cytoskeleton is dependent on the balanced destabilization (unbinding) and stabilization (binding) of the actin cytoskeleton to a bundling molecule such as CaMKIIβ for controlled process outgrowth and myelination (Bradke and Dotti, 1999, Sparrow et al., 2012). Similarly, the activation and then deactivation of cofilin (the actin filament severing and depolymerizing factor) regulates Schwann cell myelination (Sparrow et al., 2012). This provides further support that well-balanced and controlled cytoskeletal reorganization and fluidity are critical for proper myelination.

In the CaMKIIβ knockout mice, differences in myelin thickness in the ventral spinal cord were noticed in young and adult mice indicating that CaMKIIβ is
critical for proper myelination of the CNS. A similar phenotype has been seen in conditional (oligodendrocyte specific) ERK1/ERK2 double knockout mice. Thinner myelin was seen with no changes in the numbers of oligodendrocytes; however, there were some changes seen in the expression of myelin genes (Ishii et al., 2012). The resulting similar phenotype seen between CaMKIIβ knockouts and ERK1/ERK2 double knockouts could potentially be attributed to overlapping signaling mechanisms. Once upstream signals (ERK1/ERK2 or CaMKIIβ) are knocked out, similar phenotypes arise. Conversely, these two signaling pathways could be different but provide a similar function since myelination is critical for normal development of the CNS. If we assume that CaMKIIβ is binding to actin and regulates actin binding and unbinding during the unbound state, it could potentially modulate ERK1/2 signaling as it has been described to do in other cell types (Lu et al., 2005). Additionally, ERK1/2 signaling in other cell types is linked to signaling that regulates actin polymerization and substrate detachment of the actin cytoskeleton when ERK1/2 signals to myosin light chain kinase or Calpain respectively (Katz et al., 2007). Therefore signaling mechanisms that regulate ERK1/2 dependent myelination could potently overlap with signaling related to CaMKIIβ dependent myelination. Changes in gene expression cannot be compared specifically between ERK1/2 knockouts and CaMKIIβ knockouts because gene expression has not been carefully assessed in the latter. However, by comparing studies done in vitro by knocking down CaMKIIβ with studies that knocked out ERK1/2, it could be assumed that these signaling mechanisms could be different but provide a similar outcome. In this proposed outcome,
ERK1/2 is regulating the genetic component of oligodendrocyte development and CaMKIIβ is regulating the morphological maturation component of oligodendrocyte development (Ishii et al., 2012, Waggener et al., 2013). Thus, two different developmental pathways are impacted but the same improper regulation of myelination could potentially be observed.

**Glutamate activates CaMKIIβ and increases the overall morphology of the oligodendrocyte’s process network**

As mentioned in Chapter 3, glutamate signaling in the brain is critical for normal brain development. Furthermore, as the nervous system develops and oligodendrocyte processes are looking to attach to axons, glutamate is being released in a vesicular fashion from unmyelinated axons (Kukley et al., 2007, Ziskin et al., 2007, Hamilton et al., 2010). The data presented in Chapter 4 suggest that maturing oligodendrocytes may respond to axonal released glutamate which would stimulate myelination.

In Chapter 4, the data demonstrated that sodium-dependent glutamate transporters are important for the regulation of oligodendrocyte differentiation. More specifically, the data showed that activation of sodium-dependent glutamate transporters promotes oligodendrocyte morphological maturation via transient increases in intracellular calcium and phosphorylation of CaMKIIβ’s S371 site found within CaMKIIβ’s actin binding/stabilization domain. These phosphorylation events seem to mediate CaMKIIβ’s association with F-actin and give further insight into the role that CaMKIIβ potentially plays during actin
binding and stabilization of the oligodendrocyte process network. These data show that there is a functional relationship between glutamate transporters and oligodendrocyte development. It has been shown that electrical activity via axons regulates myelination (Malone et al., 2013). More specifically, the glutamate released from electrically active unmyelinated axons has been implicated in increased development and then subsequent myelination of oligodendrocyte progenitor cells (Kukley et al., 2007, Ziskin et al., 2007, Bakiri et al., 2009, Kolodziejczyk et al., 2010).

Altering the binding properties of the serine/threonine residues within the actin binding domain changes the way CaMKIIβ binds to actin. This phosphorylation-dependent binding of actin is a novel finding and our data suggests the regulatory mechanisms mediated by CaMKIIβ’s actin binding site in oligodendrocytes are comparable to ones proposed in neurons and in particular in dendritic spines (Okamoto et al., 2009). These data are the first evidence that oligodendrocytes use CaMKIIβ to bind to actin and that the phosphorylation of serine/threonine residues within the actin binding site regulate the arrangement and organization of the oligodendrocyte actin cytoskeleton.

If CaMKIIβ is critical for proper myelination, then alterations may be seen in diseases such as MS that have oligodendrocytes with simple process morphologies and limited/insufficient myelination. In some MS lesions, there seems to be an upregulation at the S371 site of CaMKIIβ (our own unpublished data).
Interestingly, the signaling mechanisms that potentially govern oligodendrocyte process guidance towards the axon may indeed be altered in MS. Higher levels of glutamate have been shown in normal appearing white matter and lesioned areas of MS brains (Werner et al., 2001, Trapp and Stys, 2009, Tisell et al., 2013). However, lower levels of glutamate transporter expression have also been reported for MS lesions (Werner et al., 2001, Pitt et al., 2003). Since our data show that glutamate stimulates the phosphorylation of the actin binding domain of CaMKIIβ and causes it to dissociate from F-actin, physiological higher concentrations of glutamate or more glutamate/Ca\(^{2+}\) being transported could cause destabilization of the actin cytoskeleton. This effect would be similar to the CaMKIIβ\(^{\text{AID}}\) (always actin unbound) mutant, expression of which results in a significant decrease in the process network of oligodendrocytes. If the environment around these immature oligodendrocytes is altered (higher glutamate concentrations), then constant destabilization of the actin cytoskeleton could lead to process retraction or process collapsing (Meng et al., 2010, Nagel et al., 2012). It is critical that the periods of actin destabilization be followed up with periods of actin stabilization to achieve a proper balance of actin dynamics (Sparrow et al., 2012).

Downstream activation of the reverse mode of NCX3 (sodium calcium exchanger) is one way to restore the Na\(^+\) equilibrium after glutamate transport. As previously discussed, the restoration of Na\(^+\) efflux allows for Ca\(^{2+}\) influx, at least in Bergmann glia cells (Martinez-Lozada et al., 2011). The subcellular location of NCX3 was described to be within the process network of MOG.
positive cells in culture and also within compacted myelin in MBP positive cross sections of axons in spinal cord white matter (Boscia et al., 2012b). It has been theorized and speculated but poorly understood for years that there are signals that are exchanged between the axon and the myelin sheath to communicate the regulation of thickness and maintenance of the myelin sheath (Nave and Trapp, 2008, Nave, 2010). This has been well demonstrated for the PNS but based on current research, the signals that control these events are more complex in the CNS (Nave and Salzer, 2006, Brinkmann et al., 2008, Taveggia et al., 2008). The CNS most likely requires additional uncharacterized signals to govern the regulation of myelin thickness. The mechanisms of glutamate signaling in oligodendrocytes regulating the cytoskeleton via CaMKIIβ could provide insight into this area of uncertainty.

This dissertation focuses on the role that CaMKIIβ plays on oligodendrocyte process network formation and myelination with regards to an association of CaMKIIβ with the actin cytoskeleton. What has not been mentioned or investigated is the multifunctional kinase role that CaMKIIβ or any of the expressed Camk2 genes can play in oligodendrocyte development. The neuronal model proposes that a catalytically active CaMKIIβ disassociates from actin and performs its traditional and well known role as a multifunctional kinase (Okamoto et al., 2007, Lin and Redmond, 2009, Lakhanpal et al., 2011). In relation to CaMKIIβ’s kinase role, it has been shown that CaMKIIβ is a regulator of LIMK1 by phosphorylation of LIMK1 Thr-508 site, which is then responsible for phosphorylation of the actin severing/depolymerizing factor cofilin (Saito et al.,
LIMK1 has been implicated in Schwann cells to activate cofilin and to thus be essential for PNS myelination (Sparrow et al., 2012). During the active unbound state of CaMKII\(\beta\), the phosphorylative effects of the kinase could be acting to aid in activating actin regulatory proteins such as cofilin via LIMK1.

The data presented in this dissertation give new insights into new molecular players responsible for morphological changes in the oligodendrocyte process network. A greater understanding of the molecular mechanisms that underlie process maturation could provide key clues and potential therapeutic targets for treating and repairing MS lesions. Oligodendrocyte progenitor cells that are found in the lesions of MS patients fight a host of mechanisms that keep them from the proper remyelination for which they are intrinsically designed. Researchers in the field still question whether immature oligodendrocytes in MS lesions have the ability to remyelinate. One of the current theories on this topic is that the addition of a new population of stem cells in the lesion could increase the ability of these new stem cells to remyelinate (Lindvall and Kokaia, 2006, Zawadzka et al., 2010). The second theory in the field is that the cells are intrinsically normal but the local environment is unable to support remyelination (Ruckh et al., 2012, Stoffels et al., 2013). In recent studies, it was shown that when adding a younger environment via new young macrophages and blood derived factors, the inhibitory environment was rejuvenated and allowed for proper remyelination of older oligodendrocyte progenitor cells (Ruckh et al., 2012). One of the reasons that remyelination could be occurring is that macrophages which are not normally expressed in the CNS can take up glutamate via the EAAT transporter system
(Rimaniol et al., 2000). This removal of higher levels of glutamate from the surrounding cells could restore the proper influx of glutamate, which would restore the balance of Ca2+ influx into the cells. This reduction or controlled elevation in Ca2+ levels would allow CaMKIIβ to normally move from an actin bound and unbound state with restored balance of destabilization to stabilization of actin.

This dissertation and the research presented here gives overwhelming support for the role that CaMKIIβ plays in the regulation of actin with regards to oligodendrocyte process morphology and myelination. Unveiling novel signaling mechanisms into the complex and poorly understood field of myelination pushes researchers closer to a better understanding of this principle. These findings are of great significance because there are no current therapies available for MS patients today that address the issue of remyelination. This research and other research into oligodendrocyte development are leading to a better understanding of the molecular mechanisms that promote oligodendrocyte differentiation during development that have the potential to reveal novel targets for designing remyelinating therapies.
CHAPTER 6
Supplementary Unpublished Data and Protocols

CaMKII Activity In Oligodendrocytes

CaMKII activity was measured in primary cells from P4 rat pups that were immunopanned with O4. Once immunopanned cells were either process right away or plated on glass coverslips coated with .1µg/ul of fibronectin and then harvested after a day. Cells were collected and rinsed centrifugally (2000×g for 5 min) with ice-cold PBS (phosphate-buffered saline) containing 2.5 mM EGTA. Cellular pellets were resuspended in 3 volumes of ice-cold homogenization buffer, which consisted of 20 mM Hepes (pH 7.4), 2.6 mM EGTA, 20 mM MgCl₂, 80 mM β-glycerol phosphate, 5 mM NaF, 0.1 µM okadaic acid (GIBCO-BRL), 0.1 µM calyculin A (GIBCO-BRL), 0.1 mM dithiothreitol, 0.01 mg/ml each chymostatin, leupeptin, aprotinin, pepstatin and soybean trypsin inhibitor (Sigma, St. Louis, MO). Samples were then sonicated (two 5-s bursts on ice), centrifuged at 12 000×g for 15 min at 4°C and either assayed immediately or frozen and stored at −80°C (Tombes et al., 1999).

For figure 6.1 P4 O4 immunopanned cells were harvested for CaMKII activity assay immediately after immunopanning. Cells from DRG cocultures were harvested after DRG explants were incubated for 1 week post explant. ATX and lacZ was added at 0.1µg/ml for 1 hour incubated at 37°C.
Figure 6.1: Premyelinating postmigratory oligodendrocytes express functional CaMKII. P4 O4 immunopanned rat oligodendrocytes express functional CaMKII. However, oligodendrocytes CaMKII activity is half of dorsal root ganglia neurons (DRG). However when DRGR cells cultures are incubated with ATX 0.1μg/ml or control LacZ 0.1μg/ml for 1 hour at 37°C before cells were harvested for activity assay (Tombes et al., 1999). oligodendrocytes N=2 DRG N=1.
Total CaMK-II Activity in Primary Oligodendrocytes and DRG Cocultures

pmoles/min/mg of protein

P4 O4 Day 0  ATX DRG  LacZ DRG
Figure 6.2: Premyelinating postmigratory oligodendrocytes express functional CaMKII that responds to stimulation with autotaxin and T3. P4 O4 panned oligodendrocytes were plated on 0.1µg/µl fibronectin coated coverslips. (A) Cells were incubated for 1 day and then stimulated with either 0.1µg/µl autotaxin or control LacZ for 1 hour at 37°C before harvesting for CaMKII activity assay. Cells stimulated with ATX had greater kinase activity when compared to LacZ stimulated cells. N=2 (B) Oligodendrocytes treated with differentiation media containing N2T3 had higher kinase activity compared to cells treated with mitogens PDGF/FGF N=2
Inhibition of CaMKII alters MBP expression in zebrafish

Treating fish with 10µM KN-93 for 24 hours alters the expression of MBP mRNA in the hind brain but not the posterior lateral line of 72 hour zebrafish. Fish treated in 35mm dish with a final concentration of 10µM KN-93, KN-92 or DMSO. Ran institu hybridization for MBP to detect altered MBP expression.

Protocol for insitu hybridization

**Zebrafish Whole Mount *In Situ* Hybridization**
(NBT/BCIP detection)

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**General embryo preparation:**
Stage embryos to desired age, anesthetize with MESAB (minimum 42µl/ml) and fix with 4% PFA in 1X PBS (new, never thawed) O/N at +4C
Wash embryos with 1x PBT 5min (x2)
Quick wash embryos with 100% MeOH, then change to fresh 100% MeOH.
Embryos can be stored in Methanol long term at -20C
(If need to use embryos sooner, put in 100% MeOH at RT for 15min, then transfer to -20C for 2 hrs)

**Day 1 - All steps during day 1 should be RNase-free (using DEPC water) and are carried out at RT except when noted**
Rehydrate embryos through methanol/PBT series - discard in MeOH waste!
66/33, 33/66, 0/100: 10’ each wash shaking -1mL
Wash with PBT 5min (x2) – 1mL
Treat with proteinase K if older than 24hpf (do not freeze/thaw stock more than 5x)

5µg/ml in PBT at 37C: 0.5min per hour age (ex. 30min for 60hpf) - 500µL
Treat with ice-cold acetone 8min at -20C (or on ice) – 500µL
Wash 1X PBT 5min x2 – 1mL
Post-fix with 4% PFA in 1xPBS 20min – 500µL (discard in PFA waste!)
Wash with PBT 5min x5 -1mL
Incubate embryos in pre-warmed 1xHyb- at 65C 5min – 500µL - discard all Hyb mixes in formamide waste!
Remove 1xHyb- and prehybridize in prewarmed 1xHyb+ for at least 1hr at 65C – 500uL
Dilute probe stocks: 1:100-1:300 in 1xHyb+ (if first time using)
**Note:** is some cases, it is best to prehybridize a newly diluted probe with embryos for one night before doing the *in situ*, this can lower background
Preheat probe to 65C
Aspirate prehybridization solution and replace with pre-warmed probe: 65C ~18 hr (minimum 50-100uL probe per tube – make sure all embryos are suspended)

Day 2
Prewarm solutions below at 65C before using
Remove diluted probe/hybridization solution and SAVE for re-use (use 200µL pipette tip) be sure to change tips when using different probes!
Washes (500µL per tube) - use drawn out Pasteur pipette or 1mL pipette to aspirate
Use 1mL volumes for all washes - Discard all solutions with Hyb mix in separate formamide waste!

- 1xHyb- (quick wash) (x1)
- 66% Hyb-/33% 2xSSC 10’ 65C (x1)
- 33% Hyb-/66% 2xSSC 10’ 65C (x1)
- 2xSSC 10’ 65C (x1)
- 0.2xSSC 20’ 65C (x2)
- 66% 0.2xSSC/33% 1xPBT 5’ RT (x1)
- 33% 0.2xSSC/66% 1xPBT 5’ RT (x1)
- 1xPBT 5’ RT (x1)

Block: Roche western blocking reagent 1:10 in 1XPBT for at least 1hr at RT on shaker – 500µL

Dilute antibodies in blocking buffer: (500µL per tube)
Note: do NOT use antibodies after they have expired!!!
- Anti-Dig-AP 1:10,000
- Anti-Fluorescein-AP 1:10,000

Incubate in primary antibody O/N at 4C (lay tubes on side)

Day 3
Washes (all 1mL volume and on shaker)
- 1xPBT (quick wash) (x1)
- 1xPBT 15’ RT (x6)
- 1x coloration buffer + 0.1% Tween-20 5’ RT (x3)

Coloration reaction: 1x coloration buffer + 0.1% Tween-20 + NBT/BCIP (4.5µL/3.5µL per 1mL of coloration buffer), let develop in dark and monitor signal, put at 4C or RT in eppendorf tubes for indicated times:
- ATX-dig 2 nights at 4C
- MBP-dig 1 night at 4C
- DM20-dig 1.5hrs at RT
- Olig1-dig 1 night at 4C
- Fli1-dig ~2hrs at RT
- Foxa2-dig 1 night at 4C
- Foxa2-fl 1 night at 4C
Olig2-dig  1 night at 4C
Sox10-dig  1 night at 4C+couple hrs @ RT

Note for NBT/BCIP: When solution gets older, it may precipitate out so heat to 37C for a few minutes to put back into solution
If first time using probe: Monitor color reaction under dissection scope frequently!

When coloration reaction completed:
- Aspirate coloration solution
- Quick was embryos with 1xPBT
- Dehydrate in methanol to clear out background for a few minutes then store in fresh methanol at -20C O/N up to a couple of months or minimum 2hrs at RT

Note: fish may look slightly pink but this will disappear after addition of Methanol

Rehydrate through methanol/PBT series then equilibrate in 90% glycerol/PBS for imaging
Store at +4C

**Imaging**
Glue coverslips to both sides of a slide to create a space in between; mount fish in the space using 90% glycerol. Move fish to desired position then place a long coverslip across the glued coverslips on each side. Nailpolish the long coverslip to keep in place; slides can be stored at +4C temporarily

Imaging fish from the side: mount fish using 2-3 coverslips on each side of slide; do not remove the yolk sacs. 3 coverslips works best for 36-48hpf fish; 2 coverslips works well for 60-72hpf fish
Imaging fish dorsally: remove yolk and mount fish with 2 coverslips on each side

Fish can be imaged on Zeiss dissecting microscope in microscopy core using extended focus software
Solutions:

PBT: 1X PBS + 0.1% Tween-20

<table>
<thead>
<tr>
<th>Component</th>
<th>Hyb mix (store at -20C)</th>
<th>Hyb+</th>
<th>Hyb-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formamide</td>
<td>25 ml</td>
<td>25 ml</td>
<td>(+4C)</td>
</tr>
<tr>
<td>20x SSC</td>
<td>12.5ml</td>
<td>12.5ml</td>
<td>(RT)</td>
</tr>
<tr>
<td>Heparin, 25 mg/ml</td>
<td>0.1ml</td>
<td>-</td>
<td>(-20C In situ hyb reagent box)</td>
</tr>
<tr>
<td>tRNA, 10mg/ml</td>
<td>2.5ml</td>
<td>-</td>
<td>(-20C In situ hyb reagent box)</td>
</tr>
<tr>
<td>tween-20, 20%</td>
<td>0.25ml</td>
<td>0.25ml</td>
<td>(RT)</td>
</tr>
<tr>
<td>citric acid, 1M</td>
<td>0.46ml</td>
<td>0.46ml</td>
<td>(RT)</td>
</tr>
<tr>
<td>nuclease-free dH2O</td>
<td>9.19ml</td>
<td>11.79ml</td>
<td>(RT)</td>
</tr>
<tr>
<td></td>
<td>50ml</td>
<td>50ml</td>
<td></td>
</tr>
</tbody>
</table>

Coloration buffer (store at RT)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCL, pH 9.5, 1M</td>
<td>5ml</td>
</tr>
<tr>
<td>MgCl2, 1M</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>NaCl, 5M</td>
<td>1ml</td>
</tr>
<tr>
<td>H2O</td>
<td>41.5ml</td>
</tr>
<tr>
<td></td>
<td>50ml</td>
</tr>
</tbody>
</table>

-Add Tween-20 immediately before use:
  Tween-20, 20% 5µl/ml

NBT stock: 50mg NBT + 0.7ml dimethyl formamide anhydride (71.4mg/mL) store at -20C protected from light

BCIP stock: 50mg BCIP + 1.0ml dimethyl formamide anhydride (50mg/mL) store at -20C protected from light

Reagents needed:

Methanol                        | Yeast RNA (Applied Biosystems/Ambion #AM7118)
PBT                              | 20X SSC (American Bioanalytical #AB1315601000)
Proteinase K (Invitrogen #25530015) | Formamide (Fisher #BP227-500)
4% PFA in PBS                  | Heparin (Fisher #AC41121-0010)
Acetone                         | RNase-free dH2O
Tween-20                        | 1M Citric acid
Roche western blocking reagent (Roche#11 921 673 001) | 1M Tris-HCl, pH 9.5
Anti-Dig-AP (Roche#11 093 274 910) | 1M MgCl2
Anti-Fluorescein-AP (Roche# 1 426 338) | 5M NaCl
NBT (Roche: 11 087 479 001)     | 50% Glycerol/ 1X PBS
BCIP (Roche: 10 760 994 001)    |
Figure 6.3: Inhibition of CaMKII by KN-93 alters the expression of mbp mRNA. (A) Fish treated with 10µM of the CaMKII inhibitor KN-93, the control KN-92, or vehicle control DMSO for 24hrs. Fish when treated with KN-93 showed a significant decreased or absent mbp mRNA expression compared to the two controls. However, in treatment groups and control groups the PNS structure of the posterior lateral line was not effected. (B) Statistical and graphical representation of the different binned categories of expression of mbp. In KN-93 treated fish absent or diminished mbp expression accounted for almost 90% (when taken together) of the fish seen in these groups. In the control groups, only 5% had an absent or diminished phenotype.
Inhibition of CaMKII alters \textit{in vivo} morphology of oligodendrocytes in the zebrafish spinal cord.

Nkx2.2a: meGFP fish were treated with 10\text{\textmu}M KN-93 or the control KN-92 for 24 hours. Fish were then fixed with 4\% PFA in 1X PBS (new, never thawed) O/N at +4C. Washed embryos with 1x PBT 5min (x2). Mounted on slides with coverslip spacers and imaged on a Zeiss 510 multiphoton microscope with a 40x objective and niquist sampling.
Figure 6.4: Inhibition of CaMKII alters the morphology to a much simpler morphology of oligodendrocytes in the zebrafish spinal chord. Fish treated for 24hrs with KN-93 or the control KN-92 were imaged at 72hpf. A distinct difference was noticed in the simplistic morphology in the KN-93 treated fish vs. the KN-92 fish. A li phenotype with thicker process (indicating myelin) was seen in the control vs. the treatment group with more immature looking cells that have a highly branched morphology but not myelinating.
Colocalization of DM20 positive cells with Camk2b1 in zebrafish ventral spinal cord.

In situ hybridization on 72hpf zebrafish with dm20 probe and Camk2b1 probe. In situ hybridization was performed then cryostat sectioning of fish embedded in OCT after cryoprotection was performed (20% sucrose) were cut at 20µM and mended on slides and imaged on Leica confocal microscope.

Methods for double in situ hybridization.

Zebrafish Whole Mount Double Fluorescent in situ Hybridization

Adapted from Julich et. al., 2005; Clay and Ramakrishnan, 2005; Brend and Holley, 2009; Jim Lister and Bruce Appel (3/14/06) lab protocols

General embryo preparation:
Stage embryos to desired age, anesthetize with MESAB (minimum 100ul/ml) and fix with 4% PFA in 1X PBS (new, never thawed) O/N at +4C
Wash embryos with 1x PBT 5min (x2)
Quick wash embryos with 100% MeOH, then change to fresh 100% MeOH.
Embryos can be stored in Methanol long term at -20C
(If need to use embryos sooner, put in 100% MeOH at RT for 15min, then transfer to -20C for 2 hrs)

Probes: Use both a Dig- and fluorescein-labeled probe; look at notes to decided which to use

Day 1 - All steps during day 1 should be RNase-free (using DEPC water) and are carried out at RT except when noted
Rehydrate embryos through methanol/PBT series
66/33, 33/66, 0/100: 10’ each wash shaking (1mL)
Pre-treat embryos with 1% H2O2 in PBT 30min to inactivate endogeneous peroxidase activity (500µL)
Fix in 4% PFA 20min – 500µL (discard in PFA waste!)
Wash with PBT 5min (x2) – 1mL
Treat with proteinase K if older than 24hpf (do not freeze/thaw stock more than 5x) (500µL)
10µg/mL in PBT at RT: 30min (for 36hpf to 5dpf); 10min (18-somite to 24hpf)
Treat with ice-cold acetone 8min at -20C (or on ice) - 500µL
Wash 1X PBT 5min x2
Post-fix with 4% PFA in 1xPBS 20min – 500µL (discard in PFA waste!)
Wash with PBT 5min x5 -1mL
Incubate embryos in pre-warmed 1xHyb- at 65C 5min – 500µL - discard all Hyb mixes in formamide waste!
Remove 1XHyb- and prehybridize in prewarmed 1xHyb+ for at least 1hr at 65C – 500uL
Dilute probe stocks: 1:100-1:300 in 1xHyb+ (if first time using)
**Note: is some cases, it is best to prehybridize a newly diluted probe with
embryos for one night before doing the in situ, this can lower background
Preheat probe to 65C
Aspirate prehybridization solution and replace with both pre-warmed probes
together: 65C ~18hr (minimum 50-100µL probe per tube – make sure all
embryos are suspended) also run single in situs on your experimental gene (see
“controls” section at end of protocol)
For negative controls for fluorescence do not add probes

Day 2
Prewarm solutions below at 65C before using
Remove diluted probe/hybridization solution and SAVE for re-use (use 200µL
pipette tip) be sure to change tips when using different probes!
Washes (500µL per tube) - use drawn out Pasteur pipette or 1ml pipette to
aspirate
**Note: Discard all solutions with Hyb mix in formamide waste

1xHyb- (quick wash) (x1)
66% Hyb-/33% 2xSSC 10’ 65C (x1)
33% Hyb-/66% 2xSSC 10’ 65C (x1)
2xSSC 10’ 65C (x1)
0.2xSSC 20’ 65C (x2)
66% 0.2xSSC/33% 1xPBT 5’ RT (x1)
33% 0.2xSSC/66% 1xPBT 5’ RT (x1)
1xPBT 5’ RT (x1)

Block: Roche western blocking reagent 1:10 in 1XPBT for at least 1hr at RT on
shaker – 500µL
(note: antibodies should be preincubated for an hour before use – read below*)
Antibodies: Optimize which order the antibodies should be added for your
probes – look at “notes” section below to optimize
Dilute antibodies in blocking buffer (500µL)
   Anti-Dig-POD (HRP) 1:1000
   Anti-Fluorescein-POD (HRP) 1:500
*Preincubate diluted antibodies in blocking buffer for at least 1hr before adding to
embryos
Incubate in antibody O/N at 4C (lay tubes on side and make sure all embryos are
submerged)

Day 3 (after TSA amplification all steps must be carried out in the dark)
Wash: 1xPBT (quick wash) (x1) - 1mL
Wash: 1X PBT 15min (x6) – 1mL
Prepare TSA/Cy3 or TSA/Fluorescein solution 1:50 in amplification reagent (spin down TSA substrate before using: 10,000rpm for 1min) and add to embryos for 30-60min at RT (may need to optimize time for each probe)
Try to use the least amount of volume possible! ~50µL per tube depending on how many embryos have; lay tubes on side, shake gently on orbital shaker and protect from light from this step on!!
Wash: 1X PBT 15min (x6) – 1mL
Block peroxidase activity of first antibody with 1% H2O2 in PBT 30min at RT - 500µL
Wash: 1X PBT 10min (x3) - 1mL
Block for second antibody: Roche western blocking reagent 1:10 in 1XPBT for at least 1hr at RT shaking - 500µL
Preincubate antibodies in blocking buffer for at least 1hr before adding to embryos:
- Anti-Dig-POD (HRP) 1:1000
- Anti-Fluorescein-POD (HRP) 1:500
Incubate in antibody O/N at 4C (lay tubes on side and make sure all embryos submerged)

Day 4
Wash: 1xPBT (quick wash) (x1)
Wash: 1X PBT 15min (x6)
Prepare TSA/Cy3 or TSA/Fluorescein solution 1:50 in amplification reagent (spin down TSA substrate before using: 10,000rpm for 1min) and add to embryos for 30-60min at RT (may need to optimize time for each probe)
Try to use the least amount of volume possible! ~50µL per tube depending on how many embryos have; Lay tubes on side, shake gently on orbital shaker
Wash: 1X PBT 15min (x6) – still protect from light!

Nuclear stain (2 options)
1. (TO-PRO-3 iodide 642/661) (protect embryos from light at all steps)
   Notes: Fish get very sticky during this stain so be very careful to not get them stuck into the pipette tip!!
Wash embryos with 2xSSC 5min (x2) at RT 500µL
Incubate in RNase 100µg/mL in 2x SSC (+0.1% Tween-20) for 30min at 37C (50µL per tube)
   (Stock solution of RNaseA is 10mg/mL)
Wash with 2X SSC 3min (x6) at RT - 500µL
Add TO-PRO-3 at 33µg/mL in 2x SSC for 20-30min at RT (50µL per tube)
   (Stock solution is 671.42µg/mL)
Wash 2X SSC 3min (x6) - 500µL
Fix with 4% PFA 20min at RT - 500µL
Wash with PBT 5min (x2) – 1mL
2. Hoechst (BisBenzimide H 33258) UV 346/460nm (intercalates A-T regions of DNA)
Incubate with Hoechst 33258 (1µg/mL) in ddH2O 10min at RT
Wash with 1X PBS 5min (x2)
Wash with ddH2O 5min (x1)
Clear O/N in 90% glycerol/PBS at -20C (it is easier to deyolk fish after the embryos have cleared O/N)

Imaging
Glue coverslips to both sides of a slide to create a space in between; mount fish in the space using Vectashield. Move fish to desired position then place a long coverslip across the glued coverslips on each side. Nailpolish the long coverslip to keep in place; slides can be stored at -20C temporarily

Imaging fish from the side: mount fish using 2 coverslips on each side of slide; the yolk sacs do not have to be removed. Can be imaged using lower objectives (10x and 20x)

Imaging fish dorsally: remove yolk and mount fish with 1 coverslip on each side (for higher objectives (40x and 63x) or can use 2 coverslips for lower objectives (10x and 20x)

Note: fish mounted with 1 coverslip on each side can be imaged using all objectives. Fish mounted using 2 coverslips cannot be imaged on higher objectives because the objective pushes on the top coverslip and moves the fish

Note: yolk can cause background fluorescence

If cutting fish with cryostat:
   Fix with 4% PFA 20min, wash with PBT 5min (x5)
   Soak embryos in 30% sucrose (in 1X PBS) at +4C until they sink and then leave an extra day or 2 (~1 week total) – protect from light!
   Transfer fish to cryomold and fill with O.C.T. (be sure to remove majority of sucrose)
   Transfer block to -80C to freeze and store in sealed bag at -80C

CONTROLS – (must run these controls to make sure there is no unspecific overlap and that the antibodies are not binding unspecifically)
1. Single fluorescent in situ of both probes
2. Negative control: do not add probes and continue with the protocol
3. Double fluorescent in situ without adding second antibody on day 3 (leave in block solution O/N) and continue with TSA amplification: testing whether H202 is blocking HRP activity of first antibody. If it is not then you will see both green and red fluorescence (I have tested this and the H2O2 has always quenched the first HRP!)

NOTES:
(From Brend and Holley, 2009)
- Fluorescein-labeled probes are weaker than dig-labeled probes, best to detect the fluorescein-labeled probe first
- If one of the genes is expressed at a low level, make a dig-labeled probe for the weak gene and detect it first
- TSA/Fluorescein should only be used with fluorescein-labeled probe, NOT dig-labeled probe! (use TSA/Cy3 for dig-labeled probe)
- Perkin Elmer TSA vs. Invitrogen Alexa TSA kits: Most protocols use the Perkin Elmer kits, but if decide to use the Alexa-Tyramides, the buffer supplied does not work; they work only with the Perkin Elmer amplification diluent

(From Clay and Ramakrishnan, 2005)
- Roche western blocking reagent consists of 10% casein protein in maleic acid buffer
Sometimes can detect fluorescent background signal in the neuromasts and along the fin (Roche western blocking reagent minimizes the majority of the background but can still see sometimes). I have seen this with the ATX in situs
**Solutions:**

PBT: 1X PBS + 0.1% Tween-20

<table>
<thead>
<tr>
<th>Hyb mix (store at -20C)</th>
<th>Hyb+</th>
<th>Hyb-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formamide</td>
<td>25 ml</td>
<td>25 ml</td>
</tr>
<tr>
<td>20x SSC</td>
<td>12.5ml</td>
<td>12.5ml</td>
</tr>
<tr>
<td>Heparin, 25 mg/ml</td>
<td>0.1ml</td>
<td>-</td>
</tr>
<tr>
<td>tRNA, 10mg/ml</td>
<td>2.5ml</td>
<td>-</td>
</tr>
<tr>
<td>tween-20, 20%</td>
<td>0.25ml</td>
<td>0.25ml</td>
</tr>
<tr>
<td>citric acid, 1M</td>
<td>0.46ml</td>
<td>0.46ml</td>
</tr>
<tr>
<td>nuclease-free dH2O</td>
<td>9.19ml</td>
<td>11.79ml</td>
</tr>
<tr>
<td>50ml</td>
<td>50ml</td>
<td></td>
</tr>
</tbody>
</table>

**Reagents needed:**

- Methanol
- Yeast RNA (Applied Biosystems/Ambion #AM7118)
- PBT (20X SSC (American Bioanalytical #AB1315601000)
- Proteinase K (Invitrogen #25530015)
- Formamide (Fisher #BP227-500)
- 4% PFA in 1X PBS
- Heparin (Fisher #AC41121-0010)
- Acetone
- RNase-free dH2O (DEPC)
- H2O2
- 1M Citric acid (Fisher #A940-500)
- Tween-20
- 90% Glycerol/ 1X PBS
- Roche western blocking reagent (Roche#11 921 673 001)
- Hoechst SAFC #B2883
- Anti-Dig-POD (HRP) (Roche#11 207 733 901)
- O.C.T.
- Anti-Fluorescein-POD (HRP) (Roche#1 426 346)
- TSA Plus Fluorescein system (Perkin Elmer #NEL741001KT)
- TSA Plus Cyanine 3 system (Perkin Elmer #NEL744001KT)
- TO-PRO-3 iodide 642/661 (Invitorgen #T3605)
Figure 6.5: Double fluorescent In situ hybridization of dm20 and camk2b1 mRNA. dm20 positive (green) cells are labeled in the ventral spinal cord of zebrafish along with the colocalized camk2b1 (red). First conformation that oligodendrocyte cells in the fish express camk2b. Bar. 10µm
Colocalization of active pCaMKII and O4 at the oligodendrocyte cell surface.

Cells immunopanned from P3 rat pups and plated on fibronectin coated coverslips (0.1µg/µl) were fixed with 4% paraformaldehyde and blocked with DMEM 065 containing 10%FCS for 30 min. O4 was placed 1:1 with DMEM06510%FCS overnight at 4 degrees. Cells were then washed with PBS and Anti mouse igµ Alexa 594 was used to stain at 1:250 for 1hr at room temperature. Cells were washed with PBS and then probed with anit pCaMKII (upstate) 1:100 overnight at 4 degrees. Next day cells were washed with PBS and stained with alexa 488 anti rabbit at 1:250 for 1 hour at room temperature. Cells were washed with PBS and then mounted with vectashield on glass slides. Cells were imaged with the Zeiss 510 mutiphoton microscope
Figure 6.6: Positive active pCaMKII (green) immunostaining of O4 (red) positive OLG. Colocalization of active pCaMKII at the membrane surface along with the surface marker for premyelinating postmigratory oligodendrocytes. Scale bar: 20 μM.
Inhibition of CaMKII alters the expression of oligodendrocyte myelin markers but not early transcription factors.

P3 A2B5 immunopanned oligodendrocytes were plated on 6 well plates 1 million cells per well and treated with KN-93 or MyrAIP for 6 hours. KN-92 and a Control Myr Peptied were used as controls. Cell were allowed to sit for 24 hours and then treated for 6 hours at 10µm for each treatment. Once treatment of KN-93 or MyrAIP was over, cells were harvested for RNA using the qiagen MINI kit. RNA was measured and then DNAsed using the ambion turbo DNAsed kit. cDNA was made from the treated RNA using omniscript. With the use of random hexomers. qRTPCR was performed on cDNA refer to protocol for quantitative approach and materials.

Generate cDNA using Omniscript Reverse Transcription Kit (Qiagen)

(For use with 50ng to 2µg RNA)

- In separate PCR size tubes add RNA and RNase-free water to a total volume of 13µL.
- Heat 65°C for 5 min in thermocycler then place on ice immediately!
- Make master mix: (recipe is for one reaction, multiply by number of samples running + 10% extra)
  All components come with kit except RNase inhibitor and OligodT:
  - RNase inhibitor – be sure to dilute to 10 units/µL in RNase-free dH2O (if comes at different concentration)
  - OligodT (Roche) comes as 40µg (8nmol) solid. The stock is diluted in 80µL RNase-free dH2O to 100µM (500ng/µL). The working stock is diluted 1:5 in RNase-free dH2O to 20µM (100ng/µL).

<table>
<thead>
<tr>
<th>+RT master mix</th>
<th>no RT master mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>2µL Buffer RT</td>
<td>2µL</td>
</tr>
<tr>
<td>2µL 5mM dNTPs</td>
<td>2µL</td>
</tr>
<tr>
<td>1µL 20µM random Hexomers</td>
<td>1µL</td>
</tr>
<tr>
<td>1µL 10u/µL RNase inhibitor</td>
<td>1µL</td>
</tr>
<tr>
<td>1µL Omniscript RT</td>
<td>-----</td>
</tr>
<tr>
<td>----- dH2O (RNase-free)</td>
<td>1µL</td>
</tr>
</tbody>
</table>

- Vortex master mixes and spin down. Place on ice until ready.
- Add 7µL master mix to appropriate tubes (+ and – RT) – change tips in between samples! Final volume of reaction = 20µL
- Incubate at 37°C for 1 hour in thermocycler (make sure to put a +4°C forever step at end)
(Can stop here and leave cDNA at +4°C for a day or two, do not store in freezer!!)

qRT-PCR (using SYBR GREEN) – Fuss lab

Before setting up your experiment, need to optimize primers:
Note: Primer design: generate a ~100bp product
Fluorescence with SYBR green has a linear relationship with DNA mass (make sure primers comparing generate same size product!! – or very similar)
1. To determine annealing temp. of primers: use end-point PCR (+ and - RT) and run on agarose gel
2. To determine primer efficiency: Run standard curve – Use dilution series using RNA with known expression: 2ng, 20ng, 200ng, 2ug
   - The difference in the primer efficiency between the gene of interest and endogenous control primer set should not exceed 5%
     (More info under analysis section)

Setup:
1. **Make cDNA from RNA**
   We use Sensiscript RT kit (Qiagen) for small amounts of RNA (up to 50ng) and SuperscriptII (Invitrogen) for more than that
   - Determine amount of RNA to be used for your experiment from dilution series of primers
   - Make enough to run duplicates of both +RT and no RT control (a single no RT control can also be run)
   - Add same amount of RNA to each cDNA reaction
   - Ex. If using 2ng RNA per reaction and running 3 primers you will need 12ng RNA (2ng x2duplicates x3primers) so start with about 20ng RNA for your + and –RT reactions

2. **Making the SYBR green master mix:**
   - The number of master mixes to make is the number of primers running
   - Make one master mix for one primer set for all samples to be run with that primer; cDNA will be added directly to wells separately after
   - Make 10% extra of master mix
   Ex. If have 4 samples to be run in duplicates, then will need to make a master mix for 8 wells: multiply each master mix component by 8.8 (make 10% extra so do not come short). This is done for each primer
   SYBR green master mix comes as a 2X mix. (BioRad)
   Each reaction is 50µl total volume
   Master mix recipe for single reaction: multiply all reagents by x8.8 (from example above) (a separate master mix needs to be made for each set of primers)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYBR green mix</td>
<td>25µl</td>
</tr>
<tr>
<td>Forward primer</td>
<td>x ul (0.15uM final)</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>x ul (0.15uM final)</td>
</tr>
<tr>
<td>ddH2O (do not use DEPC!)</td>
<td>x ul</td>
</tr>
<tr>
<td>cDNA*</td>
<td>x ul</td>
</tr>
<tr>
<td>Total</td>
<td>50 ul</td>
</tr>
</tbody>
</table>

   *cDNA is not added to supermix, it is added to wells separately after master mix but need to calculate its volume into the mix to determine how much ddH2O to add to get to a total volume of 50 ul

**ex. If concentration of RNA used for cDNA reaction is 1ug/ul and using 2ng per reaction, then 2ul of cDNA will need to be added per well and the total volume of the master mix will be 48ul - that is the volume of master mix to be pipetted into wells.
   NOTE: It is suggested to add cDNA to master mixes for better reproducibility, but this requires making a master mix for each sample – this is a lot of work depending on how many samples you have. We have had great reproducibility with duplicate samples by pipetting cDNA directly into the wells and chose to continue to do it this way.

   - Add master mix and samples to appropriate wells in strip
   1. Number the tube strips first so that you do not mix them up!!
2. Add master mix to appropriate wells in tube strips (48ul using example from above**)
3. Add cDNA to appropriate wells separately – (change tips for each well!) (add 2ul using example from above**)
4. Put strip caps on tubes and spin to collect liquid at bottom

-Protocol setup menu: Click “edit”
  Adjust conditions as necessary
  Cycles: 95C 15min
           94C 15sec
           XC 20sec – annealing temp variable - depends on primers using
           72C 20sec
           Read Plate
           Go to line #2 x 34
           Melting curve 70C to 90C
           END
           No need to put a 4C step at end!!

Make sure that reaction volume = 50ul (top right of screen)

-When done setting up, put sample strips into thermocycler in Karen’s room and use metal “thing” to push down all caps and make sure they are tightly on

4. Analysis
   - To compare single gene expression between different samples, analyze using the comparative Ct method - remember that to run this analysis method, the difference in the primer efficiency between the gene of interest and the endogeneous control primer set should not exceed 5%)

Set fluorescence threshold to where all sample amplification curves are in their linear (exponential phase) and record their CT values at this threshold. Do not move the threshold while recording numbers. Note: the threshold value can be recorded and reused if desired; the value is located in the quantitation section on the right side under the threshold.

Equation: (note CT value is inversely proportional to expression)
ΔCT (Control sample) = (CT experimental gene) – (CT endogeneous control gene)
ΔCT (Experimental sample) = (CT experimental gene) – (CT endogeneous control gene)

ΔΔCT = ΔCT (Control sample) - ΔCT (experimental sample)
2^-ΔΔCT = fold increase or decrease of control (control will be 1; 2^0 = 1)


-Comparing expression of different genes within a single sample
   Need primer efficiencies for this calculation since we are using the same sample for the comparison, endogeneous normalizing controls do not need to be run (read below under section “determining primer efficiencies”
   Equation rationale:
   \[ R_0 = R_{CT} \times (1+E)^{CT} \]
\[ R_o = \text{starting fluorescence (proportional to starting template quantity)} \]
\[ R_{CT} = \text{Fluorescence at cycle } C_T \text{ is same for all samples so becomes a constant} \]
\[ E = \text{Primer efficiency (100\% = 1)} \]
\[ C_T = \text{Cycle at threshold } T \]

Final equation to use: \[ R_o = (1+E)^{CT} \]
Compare \( R_o \) values from all different primers

- Determining Primer efficiencies: (100\% efficiency is doubling of product)
If did not already specify standards and their quantities when setting up protocol, go to “master” and do it there.
Under quantification menu, select wells that are standards. To the right of the plate click “manage” and then select all wells using for curve and name it and exit. Select that newly made group then above quantitation curve, select log scale and move smooth all the way to the left.
There is a graph to the right that will show Ct vs. log RNA. Toggle axis so the x-axis is the log RNA and y-axis is the Ct. An equation will be displayed in form of \( y = mx + b \) (\( m = \text{slope} \)); \( R^2 \) value is also displayed which is a statistical term that says how good one value is a predicting another value; if \( R^2 = 1 \) then it is perfect; \( R^2 \) value > 99\% is good

Plug in slope into equation: \[ \text{Efficiency} = 10^{\left(1/\text{slope}\right)} - 1 \]
(refer to website: \text{http://www.uic.edu/depts/rrc/cgf/realtime/data.html})
Note: a 10-fold difference in sample is shown as 3.3 cycles in the log scale \( (\log_2 10 = 3.3 \text{ or } 2^{3.3} = 10) \)
A slope of -3.3 in the graph (x-axis: log quantity; y-axis: Ct value) = 100\% efficiency because every 3.3 cycles is a 10-fold (or log quantity of 1) change; slope = -3.3/1
Note: recommended efficiency is between 90-100\% and the slope between -3.1 and -3.6

- Comparing how much material between samples using Ct values:
Every 3.3 cycles is a 10-fold difference; \( \log_2 10 = 3.3 \text{ or } 2^{3.3} = 10 \); \( \log_2 100 = 6.6 \text{ or } 2^{6.6} = \sim 100 \)

- Comparing different runs:
\( C_T \) values generated from two different amplification runs can be directly compared only if an identical \( F_T \) (fluorescent threshold) is used for each run
\( F_T \) must be fixed if data from multiple runs are to be directly compared
Reagents/Supplies needed:

- Reverse transcriptase of choice - We use: Sensiscript (Qiagen #205211) – up to 50ng SuperscriptII (Invitrogen #18064-014) (Not included in the RT kits: you will also need an RNase inhibitor and either an OligodT primer or random primers)
- SYBR green mix of choice - We use: BioRad iQ Supermix #170-8882
- Forward and Reverse Primers for both experimental and control genes (~100bp product)
- ddH2O autoclaved (not DEPC!)
- Flat cap strips – BioRad #TCS0803
- Low tube strips, white – BioRad #TLS0851
- qPCR machine

Other considerations:

- Use filter-barrier pipette tips
- It is best to dilute template to between 2-10ul to avoid inaccuracies in pipetting low volumes
- Mix all reagents well prior to making reaction mixes (inversion then quick spin)

Helpful reference papers:


Helpful websites:

- [http://www.uic.edu/depts/rrc/cgf/realtime/data.html](http://www.uic.edu/depts/rrc/cgf/realtime/data.html)
- [http://pathmicro.med.sc.edu/pcr/realtime-home.htm](http://pathmicro.med.sc.edu/pcr/realtime-home.htm)
Figure 6.7: Inhibition of CaMKII by alters the expression of oligodendrocyte myelin markers but not developmental transcription factors. Inhibition with either KN-93 or MyrAIP decreases the expression of mbp, early expression exon 2 containing mbp, and PLP. However, there is no statistical reduction of the transcription factor olig2. Black bar =100% of control values. N=3. Oneway ANOVA P< 0.05
Knocking out CaMKIIβ alters myelin thickness but not over all numbers of myelinated axons or numbers of myelinated axons based on diameter in the ventral spinal cord.

Camk2b<sup>−/−</sup> and Camk2b<sup>A303R</sup> mice (both in the F2 129P2-C57BL/6 background; van Woerden et al., 2009; Borgesius et al., 2011) were generated and bred at Erasmus University Medical Center. Spinal cord tissue was prepared and analyzed by light microscopy as previously described (Dupree et al., 1998, Marcus et al., 2006, Forrest et al., 2009). Used Nikon scope with 100x objective and imaged 4 different fields of view in the ventral spinal cord per animal per condition. Used Image J to calculate caliber and circularity.

Protocol for determining axon caliber and circularity using Image J
1. Open image in Imag J
2. Make it an 8-bit (image, type, 8-bit)
3. Threshold (control, shift, T) this is automatic threshold (works for most images; you can alter it from here if you want)
4. Set image to give you area and circularity (analyse, set measurements, click area and circularity).
5. Set measurements for analysis (Analyse, Analyze Particles)
   a. Min size: 46 pix (circle with 1µm diameter)
   b. Show: chose Outlines
   c. click Display results
   d. click exclude edge particles
6. In your result box click edit, copy all
7. Paste this into excel
8. Delete all axons with a circularity of less than 0.4: Circularities should have come out as the C column (A column is axon #, B column is area) when you pasted the results into excel. Highlight that column click ‘sort ascending’ (AZ box next to the Sigma sign), then delete all axons until you hit 0.4 circularity
9. Discount any ‘red areas’ that are not really axons that Image J picked up finding the corresponding axon# on the ‘outlines’ window. Once you have already remove all axons the circularity less than 0.4, highlight column A and click ‘sort ascending’. Now you can delete the corresponding axons you did not want to be counted.

Converting area (pixels to Diameter (µm))

<table>
<thead>
<tr>
<th>Area (pixels)</th>
<th>Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>46-183</td>
<td>1-1.9</td>
</tr>
<tr>
<td>184-413</td>
<td>2-2.9</td>
</tr>
<tr>
<td>414-782</td>
<td>3-3.9</td>
</tr>
<tr>
<td>783-1150</td>
<td>4-4.9</td>
</tr>
<tr>
<td>1151-1796</td>
<td>5-5.9</td>
</tr>
<tr>
<td>1797-2442</td>
<td>6-6.9</td>
</tr>
<tr>
<td>2443-3088</td>
<td>7-7.9</td>
</tr>
<tr>
<td>3089-3734</td>
<td>8-8.9</td>
</tr>
<tr>
<td>3734+</td>
<td>9+</td>
</tr>
</tbody>
</table>
Figure 6.8: There is no statistical difference in the number of myelinated axons based on axon diameter after knock out of CaMKIIβ at a young or adult age. A. P21 camk2b-/- mice show no statistical difference in the number of myelinated axons when grouped by axon size. There is a trend towards a loss of large diameter axons in the KO group; however, this is not statistically significant. B. Same results seen in juvenile as in adult 56 day old mice that here is no total loss or loss based on axon diameter. C. Camk2bA303R mice show no difference in myelinated axon number when Ca^{2+}/ calmodulin is unable to bind allowing this to be a catalytically altered mutant.
References


functional GABA(B) receptors that stimulate cell proliferation and migration. J Neurochem 100:822-840.


kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells. Biochemical and biophysical research communications 181:968-975.


Christopher Thomas Waggener was born in Waco TX, December 26th 1979. He graduated from Northside High School in Roanoke VA in 1999. Christopher continued his education at Hampden-Sydney College in Hampden-Sydney VA, where he received a B.S. in Biology and the Jazz Hewit outstanding biology student award in 2003. Christopher then earned a M.S. in Biology at Virginia Commonwealth University in 2005, where he worked in the lab of Dr. Jennifer Stewart. From 2005 to 2007 Christopher served as a visiting instructor of biology at Randolph-Macon College. Christopher entered the Integrative Life Sciences Ph.D. program in the fall of 2007 and joined the laboratory of Dr. Babette fuss in the Department of Anatomy and Neurobiology in the spring semester of 2008. Throughout his research career there he was given many opportunities to present his research via posters at national meetings such as the American society for Neurochemistry. At VCU, Christopher participated in two Central Virginia Chapter Society for Neuroscience annual fall poster sessions. Christopher also earned various travel awards such as the Young Investigator Educational Enhancement Award from the American Society of Neurochemistry and graduate travel awards through VCU.

Manuscripts resulting from Christopher's work at Virginia Commonwealth University:

