ROLES OF CLASS II HISTONE DEACETYLASES IN

MUSCLE AND BRAIN

APPROVED BY SUPERVISORY COMMITTEE

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To my parents

To my husband Chanhee Kang

To my son Daniel Kang

ACKNOWLEDGEMENT

During my graduate school training, there have been numerous people that have helped me to develop myself as a scientist. I deeply appreciate my mentor, Dr. Eric Olson, for the opportunity to work in such a great environment and laboratory. Eric's guidance throughout my graduate years have driven me to perform better research and his enthusiasm and passion for science have opened my eyes and heart to the beauty of science.

My gratitude goes to Dr. Rhonda Bassel-Duby. She was always available when I need her for questions and discussion at any time of day. Without her support, encouragement, and advice in science and life, it would be much harder to accomplish my research.

I would like to thank my committee members, Drs. Michelle Tallquist, Zhijian Chen, and Joseph Garcia for their advice and support through my training.

I would like to thank Dr. James Richardson, John Shelton and the entire histology core for their work on histological sections. I also would like to thank Drs. Lisa Monteggia and Ege Kavalali, Megumi Adachi, Mohd Akhtar for collaboration with the brain study, Dr. Chris Sinton, Della Koovakkattu, Kathleen Godwin for collaboration with sleep study, and Dr. Kimberly Huber for discussion and reagents. I would like to thank Sasha Qi for ES cell targeting, John McAnally for transgenic injection, Cheryl Nolen for help with mouse study, and

Joel Mendez for technical help. Special thanks go to Jose Cabrera for help with figures and graphics and Jennifer Brown for assistance with manuscripts.

I would like to thank all past and present members of the Olson lab for all of their help and encouragement. In particular, I would like to thank Igor Rybkin, Johannes Backs, Ana Barbosa, Jens Fielitz, Mei Xin, Andrew Williams, Eric Small, David Patrick, Svetlana Bezprozvanny, Robert Frost, Lillian Sutherland, Daniel Quiat, Ning Liu, Kunhua Song, Guo Huang, Mark Hatley, Drazen Sosic, Mayssa Mokalled, Aaron Johnson, Gaile Vitug, Michele Carrer, Arin Aurora, Bret Johnson, Shusheng Wang, Viviana Moresi, Chad Grueter, Jiyeon Oh, Matthew Potthoff, Rusty Montgomery, Mike Haberland, Nik Munshi, and Yuri Kim. Their support and friendships have made the Olson lab a wonderful place to learn.

I thank the Korean Community at UT Southwestern for their friendship and support, especially Euiseok Kim for his friendship and help with brain study.

I also would like to thank my family, my grandmother, my parents, my sister and brother for their unconditional love, support, and encouragement. My deepest gratitude goes to my beloved husband, Chanhee Kang and my son, Daniel. I am forever indebted to my husband and son for endless support and love. Without their support, I could never stand my graduate training, much less finish. Having them with my life is the greatest blessing I could ever have wanted.

ROLES OF CLASS II HISTONE DEACETYLASES IN MUSCLE AND BRAIN

by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

September, 2009

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The University of Texas Southwestern Medical Center at Dallas, 2009

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A defining feature of brain and muscle is their ability to remodel their phenotypes in response to extracellular stimuli to maintain the balance between physiological demand and functional capacity. Successful adaptation to the environment is essential for the survival and this plasticity is achieved by activation of intracellular signaling pathways and subsequent activation of gene expression, the so-called "extrinsic genetic programs". Although it has been well known that calcium-dependent signaling is critical to regulate this extrinsic genetic program, little is known about how calcium-dependent signaling is propagated to the nucleus to induce the transcription of specific genes responsible for tissue plasticity. Furthermore, physiological and behavioral consequences of failure of plasticity are still poorly known. Here, I demonstrate that class II HDACs and MEF2 transcription factors are essential for tissue plasticity, and that defects of

this signaling pathway in muscle and brain cause muscle-fatigue susceptibility and a pronounced neurological deficit.

Protein kinase D1, a potent class II HDAC kinase, in skeletal muscle promotes transformation to type I myofibers through activation of MEF2. Conversely, genetic deletion of PKD1 in type I myofibers increases susceptibility to fatigue, suggesting that PKD1 is a key regulator of skeletal muscle plasticity.

Deletion of the class II HDAC4 in neurons impairs memory formation and synaptic plasticity in mice, while mice lacking class II HDAC5 exhibit normal memory formation. Furthermore, deletion of both HDAC4 and HDAC5 in neurons produced a more pronounced neurological deficit, including severe seizure activity, suggesting distinct and redundant roles for HDAC4 and HDAC5 in memory formation and in brain homeostasis.

While deletion of MEF2C in brain causes impairments in memory formation, mice lacking MEF2A and MEF2D exhibit no such deficits. Furthermore, deletion of MEF2A, MEF2C, and MEF2D results in decreased REM sleep, brief spontaneous seizures, and postnatal lethality accompanied by increased apoptosis, suggesting distinct and redundant roles for MEF2A, MEF2C, and MEF2D in brain homeostasis.

Taken together, these series of studies provide important clues to understanding the mechanism by which extrinsic genetic programs are regulated *in vivo*, especially focusing on the regulation of muscle and brain functions.

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LIST OF ABBREVIATIONS

ADCY1	adenylyl cyclase 1
BDNF	brain-derived neurotrophic factor
CaMK	calcium/calmodulin-dependent kinase
CNS	central nervous system
EEG	electroencephalogram
EMG	electromyogram
EGR3	early growth response gene 3
Fbxl3	F-box and leucine-rich protein 3
fEPSP	field excitatory postsynaptic potential
GABRD	gamma-aminobutyric acid (GABA) A receptor, delta
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
H&E	hematoxylin and eosin
HAT	histone acetyltransferase
HDAC	histone deacetylase
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MEF2	myocyte enhancer factor 2
MyHC	myosin heavy chain

NFAT	nuclear factor of activated T cell
NREM	non-rapid eye movement
NRSF	neuron-restrictive silencing factor
PGC-1	peroxisome-proliferator-activated receptor-gamma coactivator-1
PKD1	protein Kinase D 1
PKIG	protein kinase inhibitor gamma
PPP1R1A	protein phosphatase 1, regulatory subunit 1a
REM	rapid eye movement
RT-PCR	reverse transcriptase-polymerase chain reaction
SAHA	suberoylanilide hydroxamic acid
Tg	transgenic
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
WT	wild-type

Chapter I

General Introduction

Introduction

Development of various tissues in most animals can occur by both intrinsic and extrinsic genetic programs. Intrinsic genetic programs mainly consist of a complex transcriptional circuit, which regulates tissue-specific and stage-specific gene expression, so as to shape both early and postnatal tissues development, and maintain their morphologies, physiological properties, and patterns of gene expression throughout life. Extrinsic genetic programs include signaling pathways, which sense environmental changes and relay the information to the nucleus, where several activity-dependent transcriptional regulators induce gene transcription so as to modify tissues to adapt to changes in their environment. During the last two decades, intrinsic genetic programs have been extensively studied, yet relatively little is known about the detailed mechanisms of extrinsic genetic programs.

Since it has been shown that skeletal muscle and brain display a high degree of plasticity in response to environmental cues, these tissues are particularly valuable to study extrinsic genetic programs. In skeletal muscle,

exercise evokes elevation of intracellular calcium and subsequent activation of calcium-regulated signaling pathways (Bassel-Duby and Olson, 2006). These calcium-regulated signaling pathways regulate specific transcriptional regulators by several phosphorylation and dephosphorylation events, which cause changes in gene transcription and eventually lead to skeletal muscle fiber-type switching. In brain, experience-driven synaptic activity causes calcium influx into specific neurons, which leads to the remodeling of their synapses through activation of new gene transcription (Flavell and Greenberg, 2008). Common and distinct components of calcium-dependent signaling pathways and transcriptional regulators have been implicated in the plasticity of muscle and brain.

Among these transcriptional regulators, MEF2 transcription factors and class II HDACs are good candidates for playing a role in both intrinsic and extrinsic genetic programs in muscle and brain, based on the following observations: 1) MEF2 proteins and their repressor, class II HDACs are highly expressed in both muscle and brain. 2) MEF2 proteins are key regulators of skeletal muscle development. 3) *In vitro*, MEF2 proteins regulate neuronal

survival and differentiation. 4) Diverse calcium-regulated signaling pathways converge on MEF2 proteins/class II HDACs in the nucleus, leading to changes in expression of their target genes. 5) MEF2 target genes harboring mutations are associated with neurological disorders such as epilepsy and autism spectrum disorders. In fact, a growing body of evidence suggests that MEF2 proteins/class II HDACs play a critical role in muscle remodeling and brain function. Here I review the current view of signaling pathways in skeletal muscle remodeling, and present the roles of MEF2/class II HDACs proteins in skeletal muscle remodeling and brain function.

Skeletal Muscle Remodeling

Skeletal muscle is comprised of heterogeneous myofibers that display different metabolic and contractile properties. In response to environmental demands, skeletal muscle remodels by activation of intracellular signaling pathways to reprogram gene expression, adjusting muscle performance with physiological demands. An increasing body of evidence indicates that various components of calcium-dependent signaling pathways and several transcriptional regulators

including transcription factors, coactivators, and corepressors play important roles in skeletal muscle remodeling, and more importantly in the pathogenesis of human diseases such as muscular dystrophy and muscle atrophy. The detailed mechanistic understanding of skeletal muscle remodeling is therefore critical to find potential therapeutic targets. In the following sections, I discuss the diversity and plasticity of myofibers, and the signaling pathways that regulate skeletal muscle remodeling.

Myofiber Diversity and Plasticity

Although skeletal muscle appears histologically uniform, it is comprised of heterogenous myofibers which differ in metabolism, contractility and endurance. Myofibers are classified into type I and type IIa, type IId/x and type IIb fibers based on the expression of specific myosin heavy chain (MyHC) isoforms (Bassel-Duby and Olson, 2006; Potthoff et al., 2007a). Type I fibers, also termed slow-twitch fibers, are rich in mitochondria, exhibit oxidative metabolism, exert a slow contraction, and are resistant to fatigue. Type II fibers, termed fast-twitch

fibers, have less mitochondria content, exhibit oxidative (IIa) or glycolytic (IId/x and IIb) metabolism, exert quick contraction, and are susceptible to fatigue.

In response to physiological and environmental cues, adult myofibers exhibit a high degree of plasticity, switching fiber types from “fast” to “slow” or vice versa. For example, different neural input can change the properties and functions of skeletal muscle fibers: Reinnervation of slow-twitch muscle with nerve fibers that normally supply fast-twitch muscle results in an increase in contractile speed. Conversely, fast-twitch muscle reinnervated with nerve fibers that supply slow-twitch muscle displays slower contraction (Bassel-Duby and Olson, 2006). Fiber-type switching can be also achieved by electrical stimulation, which mimics specific nerve activity: A fast-to-slow switch transformation can be induced by tonic low-frequency (10-20 Hz) electrical stimulation, resembling the firing pattern of slow motoneurons, whereas a slow-to-fast switch transformation can be induced by phasic high-frequency (100-150 Hz) electrical stimulation, resembling the firing pattern of fast motoneurons (Schiaffino et al., 2007). Environmental cues, such as exercise training, are also known to promote an

adaptive muscle fiber transformation, increasing oxidative metabolism and slow-twitch fibers. These physiological- and environmental-induced muscle type switching are known to be regulated by various signaling pathways, mainly calcium-dependent signaling pathways, which culminate in changes of fiber-type specific gene transcription.

Signaling Pathways in Fiber Type Remodeling

Factors Regulating Slow-Twitch Fiber Formation

Calcineurin-NFAT pathway

Calcineurin is a Ca^{2+}/calmodulin-regulated protein phosphatase that acts on the NFAT (nuclear factor of activated T-cell) transcription factor family. Calcineurin is a heterodimeric protein comprised of a calmodulin-binding catalytic A subunit (CnA), and a calcium binding regulatory B subunit (CnB). In response to sustained and low frequency calcium waves, calcineurin is activated, and dephosphorylates its substrates including NFAT, resulting in translocation of

NFAT from the cytoplasm to the nucleus where it activates muscle remodeling genes (Olson and Williams, 2000).

Several lines of *in vivo* evidence indicate that the calcineurin-NFAT pathway plays an important role in the maintenance of slow-twitch fibers in skeletal muscle. Overexpression of activated-calcineurin in skeletal muscle drives fast to slow myofiber transformation, whereas overexpression of RCAN1, an inhibitory protein of calcineurin, leads to a decrease of type I fibers (Naya et al., 2000; Oh et al., 2005). In addition, rats treated with a calcineurin inhibitor, cyclosporine A, display a slow-to-fast fiber transformation, such as induction of glycolytic enzymes and a decrease in slow type I contractile proteins (Chin et al., 1998). Conditional knockout mice lacking calcineurin A or calcineurin β1 display a decrease in the number of slow fibers, further supporting the conclusion that calcineurin activity regulates the slow-twitch fiber program (Parsons et al., 2004; Parsons et al., 2003).

PGC-1alpha, -1beta and PPAR beta/delta

PGC-1α is a transcriptional co-activator that stimulates mitochondrial biogenesis and oxidative metabolism (Puigserver et al., 1999; Wu et al., 1999). PGC-1α is preferentially expressed in type I myofibers and induced by endurance exercise in both rodents and humans (Baar et al., 2002; Pilegaard et al., 2003). The importance of PGC-1α in the regulation of fiber type remodeling is seen in transgenic mice overexpressing PGC-1α in fast muscle, showing an increase in oxidative metabolism and type I fibers (Lin et al., 2002). However, PGC-1α is not required for embryonic development of slow fibers, based on results showing that soleus muscle from PGC-1α knock-out mice displayed a normal distribution of slow fibers (Arany et al., 2005).

While PGC-1α drives slow fiber transformation, PGC-1β promotes the formation of type IIx fibers. Overexpression of PGC-1β in skeletal muscle results in increased mitochondrial biogenesis with a simultaneous increase of type IIx fibers, whereas PGC-1β null mice show a reduced mitochondrial content (Arany et al., 2007; Lelliott et al., 2006).

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily encoded by three genes in human: alpha, gamma and beta/delta. PPAR isoforms are activated by lipids and modulate a lipid metabolism in different tissues. PPARβ/δ is the predominant PPAR isoform present in skeletal muscle and preferentially expressed in type I fibers. Overexpression of PPARβ/δ in skeletal muscle leads to an increases in oxidative myofibers and enhancement of running endurance (Wang et al., 2004). Conversely, mice lacking PPARβ/δ in skeletal muscle display slow-to-fast fiber type switching, resulting in reduced running endurance (Schuler et al., 2006). Furthermore, PPARβ/δ agonists and exercise training synergistically increase oxidative myofibers and running endurance in adult mice, suggesting that PPARβ/δ plays an important role in myofiber remodeling (Narkar et al., 2008).

Myocyte Enhancer Factor 2

Myocyte enhancer factor 2 (MEF2) transcription factors are key regulators of skeletal muscle development (Potthoff and Olson, 2007). MEF2 proteins belong to the evolutionarily conserved MADS (MCM1, agamous, deficens, SRF) family

of transcription factors. MEF2 proteins are encoded by four genes in vertebrates, MEF2-A, -B, -C, and –D, showing distinct, but overlapping, expression patterns during embryogenesis and in adult tissues. MEF2 transcription factors contain a conserved N-terminal MADS-box domain and adjacent MEF2 domain, which together mediate DNA binding, dimerization, and cofactor interaction. The C-terminal region of MEF2 proteins, which is subject to complex patterns of alternative splicing, contains a transcriptional activation domain and several regulatory domains, including multiple phosphorylation sites. MEF2 proteins bind to the consensus DNA sequence YTA$(W)_4$TAR as homo- or heterdimers. It is well known that activity of MEF2 proteins is regulated by interactions with class II histone deacetylases (HDACs). In response to various signals, HDAC kinases are activated and phosphorylate HDACs, which creates a docking site for 14-3-3 chaperone proteins and subsequently leads to nuclear export of HDACs, resulting in activation of MEF2-dependent gene expression (McKinsey et al., 2002).

Increasing evidence suggests that MEF2 transcription factors play a critical role in myofiber remodeling. Using a MEF2 reporter mouse(Naya et al.,

1999), which harbors a lacZ transgene under the control of three tandem MEF2 binding sites, it has been shown that MEF2 is chronically activated in slow postural muscles and that MEF2 activity is increased by exercise (Wu et al., 2001a). Furthermore, a previous study showing that administration of a calcineurin inhibitor blocked exercised-induced MEF2 activity, together with the fact that constitutively active calcineurin dramatically increases MEF2 activity in skeletal muscle, suggests a possible cross talk between the MEF2 and calcineurin signaling pathways (Wu et al., 2001a). The importance of MEF2 signaling in skeletal muscle remodeling is highlighted *in vivo* using conditional mice lacking MEF2 in skeletal muscle (Potthoff et al., 2007b). Deletion of MEF2C in skeletal muscles results in a reduction of slow-twitch fibers within the soleus muscle. Conversely, overexpression of superactive MEF2 (MEF2-VP16) in skeletal muscle is sufficient to drive the formation of slow-twitch fibers. Furthermore, MEF2-VP16 transgenic mice display an increase in oxidative gene expression and exercise endurance. Taken together, these results demonstrate that MEF2 is

necessary and sufficient to drive the formation of slow myofibers in skeletal muscle.

Class II HDACs

Class II HDACs (HDAC4, -5, -7, and -9) are related to the yeast protein HDA1 and are predominantly expressed in heart, brain, and skeletal muscles, acting as a signal-dependent repressors of MEF2. Class II HDACs have an extended N-terminal domain, which mediates interactions with other transcriptional cofactors and repressors, and confers responsiveness to a variety of extracellular signals. Class II HDACs shuttle between the nucleus and cytoplasm, regulated by HDAC kinases (McKinsey et al., 2002). Known HDAC kinases such as calcium /calmodulin-dependent kinase (CaMK) (McKinsey et al., 2000) or protein kinase D (PKD) (Vega et al., 2004a) phosphorylate class IIa HDACs, which recruits 14-3-3 chaperone protein and results in nuclear export of the HDAC/14-3-3 complex in a Crm1-dependent manner. A recent study showing posttranscriptional regulation of class II HDAC proteins among different muscle groups suggests a potential role of class II HDACs in regulating fiber-type identity (Potthoff et al.,

2007b). Indeed, deletion of any four alleles of class II HDACs results in a dramatic increase of slow-twitch fibers, whereas overexpression of class II HDACs in skeletal muscle blocks an exercised-induced slow fiber type switch, demonstrating that class II HDAC proteins regulate the formation of slow myofibers through the modulation of MEF2 activity (Potthoff et al., 2007b).

Class II HDAC Kinases

Since the phosphorylation of class II HDACs is a critical step in the regulation of MEF2 activity and transcription of MEF2 target genes, there has been intense interest in identifying the kinases responsible for the phosphorylation and the subsequent nuclear export of class II HDACs in skeletal muscle. One of the candidates of HDAC kinase is CaMK. *In vitro* studies showed that CaMK activates MEF2 by promoting the nuclear export of class II HDACs (McKinsey et al., 2000). It was also shown that CaMK inhibitors block the nuclear export of class II HDACs in response to tonic low-frequency electric stimulation in isolated myofibers (Liu et al., 2005). More importantly, *in vivo* evidence showing overexpression of CaMKIV in skeletal muscle activates MEF2, resulting in a fast-

to-slow fiber switching with an enhanced muscle oxidative capacity, indicates a possible role of CaMK as a HDAC kinase in skeletal muscle remodeling (Wu et al., 2002). However, the fact that endogenous CaMKIV is not expressed in skeletal muscle, together with the finding that mice lacking CaMKIV display a normal fiber type composition, suggests the possibility that other class II HDAC kinases play a major role in skeletal muscle remodeling under physiological conditions (Akimoto et al., 2004).

AMP activated protein kinase (AMPK), a master regulator of cellular metabolism, has been shown to contribute to physiological changes induced by exercise (Carling, 2004). Exercise activated AMPK stimulates energy-generating processes, such as glucose uptake and fatty acid oxidation through regulation of gene expression. A recent study showing that AMPK regulates GLUT4 expression by phosphorylating HDAC5 in human primary myotubes, suggests AMPK functions as a potential class II HDAC kinase in skeletal muscle (McGee et al., 2008). Salt-inducible kinase 1 (SIK1), a member of the AMPK family, also acts as a class II HDAC kinase (Berdeaux et al., 2007). Viral expression of SIK1

in dystrophic skeletal muscle was shown to enhance MEF2 activity by phosphorylating and inhibiting class II HDACs, promoting survival of skeletal myocytes, and partially rescuing a dystrophic phenotype in mice. These studies suggest the possibility that AMPK and SIK1 might play a role in myofiber remodeling through regulating the HDAC-MEF2 pathway. However, to date it

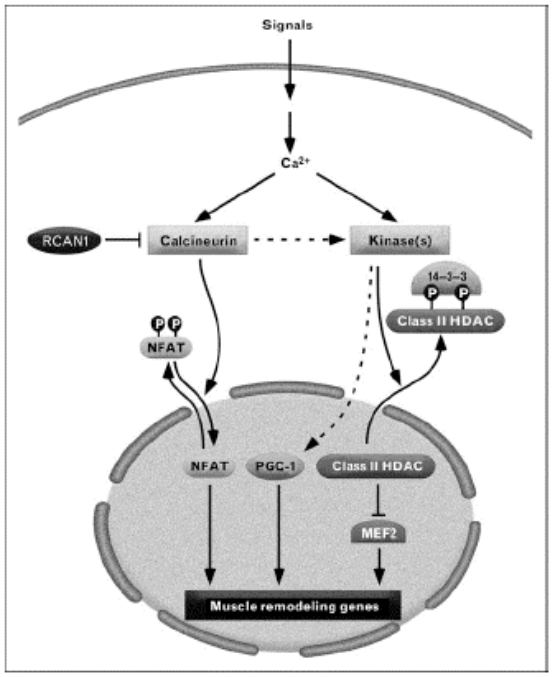

Figure 1.1 Signaling pathways regulating skeletal muscle remodeling
In skeletal muscle, extracellular stimuli evoke elevation of intracellular calcium and the subsequent activation of calcium-regulated signaling pathways, including calcineurin-nuclear factor of activated T cell (NFAT) and class II histone deacetylases (HDACs)-myocyte enhancer factor 2 (MEF2) signaling. (Adapted from Potthoff, 2007)

has not been tested.

Factors Regulating Fast-Twitch Fiber Formation

MAPK

Mitogen-activated protein kinase (MAPK) is a Ser/Thr protein kinase that responds to extracellular stimuli and regulates various cellular processes, such as gene expression, proliferation/differentiation, and cell survival/apoptosis. The role of MAPK in the modulation of fiber-type switching remains subject to debate. Initially, it was suggested that the Ras/MEK/ERK signaling cascade promotes a slow-type fiber formation in regenerating muscles (Murgia et al., 2000). Activation of ERK in regenerating muscles leads to the expression of slow-fiber specific genes, whereas inhibition of ERK results in the expression of fast-fiber specific genes. However, a recent study showed that pharmacological inhibition of ERK1/2 increases slow fiber type-specific reporter activity, whereas overexpression of a constitutively active ERK2 increases fast fiber type-specific reporter activity. Furthermore, overexpression of MAPK phosphatase 1 (MKP1)

that dephosphorylates and inhibits MAPKs in mouse and rat muscle fibers induces slow fiber formation, whereas activation of ERK2 induces the fast-twitch fiber program in soleus muscle. These *in vitro* and *in vivo* data suggest that MAPK signaling (ERK) is necessary to maintain fast-twitch fiber phenotype (Shi et al., 2008). The reason for the discrepancy between these two studies has yet to be elucidated.

Six/Eya

Six/Eya genes are the mouse homologues of *Drosophila sine oculis/eyes absent* genes, which are essential for compound-eye formation. Six1/Eya1 are enriched in fast-twitch muscle fibers. When overexpressed in slow-twitch muscle fibers, Six/Eya induces a fiber-type transition toward fast-twitch muscle fibers, suggesting that the Six/Eya pathway plays a role in the establishment and maintenance of the fast-twitch skeletal muscle phenotype (Grifone et al., 2004). In addition, a recent study showed that Six1a is required for fast muscle differentiation in zebrafish, supporting the view that the Six/Eya pathway promotes fast-twitch muscle fiber formation (Bessarab et al., 2008).

Sox6

Sox6, a member of the Sox family of transcription factors, has been implicated in fast fiber formation, especially in fetal skeletal muscle (Hagiwara et al., 2007). Mice lacking functional Sox6 display a decrease in fast fibers formation in late fetal skeletal muscle. Knockdown of Sox6 in WT myotubes results in an increase expression of the slow isoform of MyHC, suggesting that Sox6 promotes a fetal fast-twitch muscle phenotype with a concomitant repression of fetal slow-twitch fiber differentiation.

The Role of MEF2 in the Central Nervous System

Although MEF2 was first identified as a regulator of muscle gene expression, it is now apparent that the MEF2 transcription factor plays central roles in the development and function of many cell types, including heart, neural crest, immune cells, bone and neurons (Potthoff and Olson, 2007). MEF2 proteins are highly enriched in the central nervous system (CNS) and exhibit a unique temporal expression pattern in different regions of the brain. In addition, the timing of MEF2 expression is well correlated with the cell cycle exit of neurons and the subsequent acquisition of a differentiated phenotype (Lyons et al., 1995). More importantly, the calcium-regulated signaling pathway, one of the most prominent signaling events induced by neurotransmitter reception in the postsynaptic neuron, is also one of the most important regulatory components in the regulation of MEF2 proteins. Taken together, this suggests that MEF2 proteins play a critical role in the central nervous system. Indeed, a growing body of evidence, mostly *in vitro*, indicates that MEF2 proteins regulate neuronal survival and differentiation, as well as activity-dependent synapse development.

In vitro function of MEF2

Initial evidence suggesting that MEF2 transcription factors regulate neuronal survival came from studies which elucidated the molecular mechanisms underlying the calcium-dependent survival of neurons. In cultured cerebral neurons and cerebellar granule neurons with a depolarizing concentration of KCl,

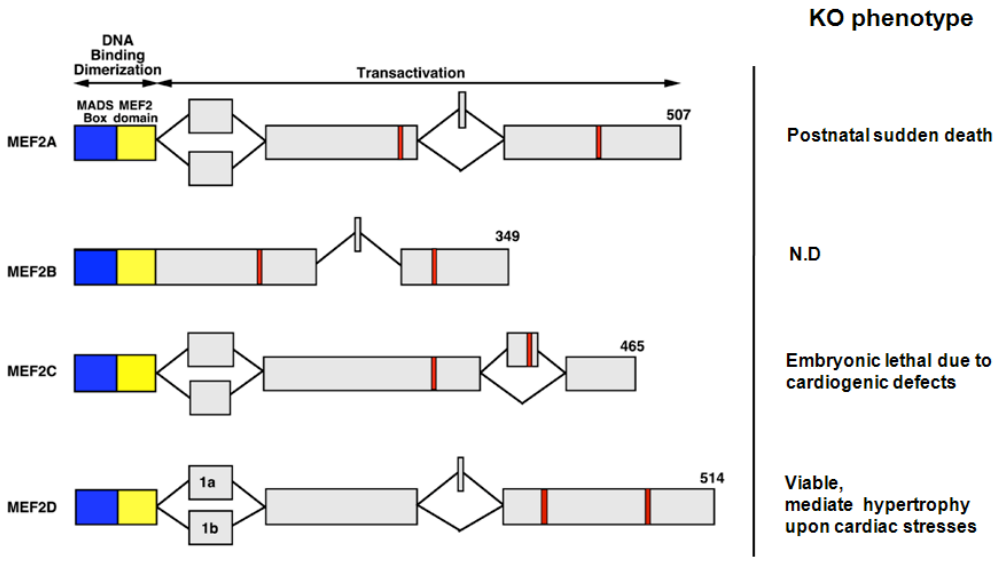

Figure 1.2 The family of Myocyte enhancer factor 2 (MEF2) transcription factor, showing protein domains and loss-of-function phenotypes in mice
Vertebrate MEF2 proteins are encoded by four genes, *Mef2a, b, c* and *d*. MADS (MCM1, agamous, deficens, SRF) box and MEF2 domain mediate DNA binding, dimerization, and cofactor interactions. The C-terminus region of MEF2 functions as a transcriptional activation domain.

inhibition of MEF2 function by introducing a dominant-interfering form of MEF2 or RNA interference results in apoptotic cell death. Conversely, overexpression of a constitutively active form of MEF2 (MEF2-VP16) promotes survival of cerebellar granule neurons grown in the presence of a low concentration of KCl that normally induces apoptosis. These data suggest that MEF2 proteins are necessary and sufficient for activity-dependent neuronal survival (Gaudilliere et al., 2002; Mao et al., 1999).

MEF2 proteins have also been implicated in the regulation of neuronal differentiation *in vitro*. Overexpression of MEF2C in P19 neuronal precursor cells induces neurogenesis by upregulation of MASH1, a neuronal-specific bHLH transcription factor which plays an important role in neuronal differentiation (Skerjanc and Wilton, 2000). In addition, MEF2C interacts with Sp1, and synergistically activates the NMDA receptor subunit 1 (*NR1*) gene, which plays important roles in neuronal development, plasticity, and cell death in cerebral cortical cultures (Krainc et al., 1998).

During synapse formation and remodeling, the expression of MEF2A and 2D is increased, suggesting a potential role of MEF2A and MEF2D in synapse development. MEF2A and MEF2D were shown to regulate excitatory synapse number and postsynaptic differentiation of dendrites in a neuronal activity-dependent manner during synaptic development *in vitro* (Flavell et al., 2006; Shalizi et al., 2006). It was shown that activity-dependent calcium signaling induced calcineurin-mediated dephosphorylation and activation MEF2, which in turn promotes the remodeling of synapses by inducing a subset of genes, including Arc (activity-regulated cytoskeletal-associated protein), synGAP (synaptic RAS GTPase-activating protein) Nur77 (orphan nuclear receptor), and miR-134. Genome-wide analysis of the MEF2 transcriptional program uncovered several target genes that were previously known to have diverse functions at synapses and to be mutated in human neurological disorders including epilepsy and autism spectrum disorder (Flavell et al., 2008), further supporting the notion that MEF2 proteins regulate activity-dependent synapse development.

In vivo function of MEF2

Despite significant progress in understanding the role of MEF2 proteins at the cellular level, the function of MEF2 proteins in brain is not well defined. Genetic deletion of MEF2C or MEF2A in mice causes embryonic or postnatal lethality due to cardiac defects, precluding the analysis of these proteins in adult brain (Lin et al., 1997; Naya et al., 2002). Using conditional knockout mice, it was demonstrated that MEF2C is involved in learning and memory (Barbosa et al., 2008). Mice lacking MEF2C in the CNS displayed deficits in behavioral plasticity with a concomitant increase in excitatory synapse number. Conversely, neuronal expression of a constitutively active form of MEF2 (MEF2-VP16) leads to a reduction of excitatory synapse number although it does not affect learning and memory. MEF2 proteins are also suggested to play a role in neuronal differentiation in the brain. Deletion of MEF2C in nestin-expressing neural stem/progenitor cells impairs neuronal differentiation, displaying abnormal compaction of neurons and smaller soma size (Li et al., 2008). Furthermore, recent studies showed that MEF2 regulates structural and behavioral plasticity in

the nucleus accumbens (NAc) in response to cocaine exposure. Overexpression of MEF2-VP16 in NAc blocks the cocaine-induced spine density increase and enhances behavioral responses to cocaine, whereas reduction of endogenous MEF2A and MEF2D by RNAi shows the opposite phenotype (Pulipparacharuvil et al., 2008). Taken together, these results demonstrate that MEF2 plays a critical role in the brain, as shown by neuronal survival, differentiation, and development *in vitro*.

The Role of Histone Deacetylases in the Central Nervous System

Histone deacetylases (HDACs) repress gene expression by modifying chromatin structure and interacting with specific transcription factors, such as MEF2. There are four classes of HDACs in mammals. Class I HDACs (HDAC1, -2, -3, -8) share high homology to the yeast protein RPD3, are expressed ubiquitously, and are localized in the nucleus. Class II HDACs (HDAC4, -5, -7, 9) are related to the yeast protein HDA1 and are predominantly expressed in heart, muscle and brain. Class II HDACs shuttle between the nucleus and cytoplasm in response to extracellular signals. Class III HDACs (Sirt1-7) are homologs of the yeast repressor Sir2 and require NAD^+ for catalytic activities. Class IV HDAC (HDAC11) shows homology to class I and II HDAC domains and is predominantly localized to the nucleus. Since MEF2 proteins play important roles in the brain, it is plausible that its repressor, class II HDACs, also function in the brain. Furthermore, it has become apparent that chromatin remodeling, which is also regulated by HDACs, acts as a key regulatory mechanism in several brain functions. In fact, genetic evidence, together with pharmacological inhibitor

studies suggests that HDACs play crucial roles in the maintenance of the central nervous system. Evidence suggesting a role of HDACs in neuronal development, neurodegeneration, learning and memory, and neuropsychiatric disorders, is summarized below.

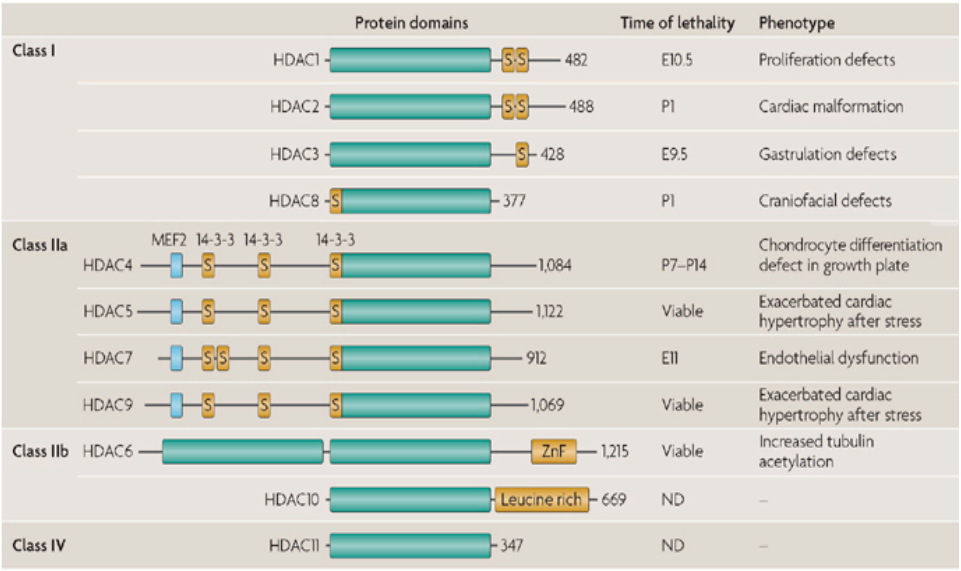

Figure 1.3 Domain organization of histone deacetylase (HDAC) superfamily and loss-of-function phenotypes in mice

The HDACs are categorized into different classes based on the sequence similarity to yeast prototypes. Deacetylase domain is marked by a green box, MEF2 interaction domain is marked by a blue box. S, serine phosphorylation site; ZnF, Zn finger (Adapted from Haberland, 2009)

Development

HDACs regulate fundamental neurodevelopmental processes, such as cell fate specification and neurogenesis. HDACs can associate with the neuron-restrictive silencing factor (NRSF) complex, so as to regulate neuronal development (Hsieh and Gage, 2004). NRSF binds to conserved NRS elements (NRSEs) in promoter regions of neural-specific genes. In non-neuronal cells, NRSF associates with repressor complexes, including the transcriptional co-repressor mSIN3, nuclear receptor co-repressor 1 (N-CoR1), and HDAC2. In this way, NRSF-HDAC2 represses expression of neural-specific genes in non-neuronal cells. Strong evidence for the role of HDACs in neuronal development came from pharmacological HDAC inhibitor studies. It was shown that HDAC inhibitors induce neural differentiation in both embryonic and adult cortical cells (Balasubramaniyan et al., 2006; Hsieh et al., 2004; Shaked et al., 2008). In addition, administration of HDAC inhibitors results in hypomyelination with delayed expression of differentiation markers and prolonged expression of progenitor markers in myelin-forming oligodendrocyte progenitor cells,

suggesting that HDACs regulate the timing of oligodendrocyte differentiation and myelination (Shen et al., 2005). HDAC inhibitors also induce neuronal differentiation of adult hippocampal neural progenitors and inhibit astrocyte and oligodendrocyte differentiation, at least in part, through the induction of neurogenic transcription factors, including NeuroD (Hsieh et al., 2004), suggesting that HDACs are involved in adult neural stem cell fate specification.

Recent genetic evidence further supports the essential role of HDACs in neural development. It was shown that HDAC1 and HDAC2 redundantly regulate neuronal development and are required for neuronal specification (Montgomery et al., 2009). Mice lacking HDAC1 and HDAC2 in developing neurons show severe neural abnormalities, including hippocampal abnormalities, absence of cerebellar foliation, and disorganization of cortical neurons. These abnormalities can be attributed to a failure of neuronal precursors to differentiate into mature neurons and to excessive cell death. HDAC1 and HDAC2 also regulate oligodendrocyte specification and differentiation (Ye et al., 2009). Genetic deletion of both *Hdac1* and *Hdac2* in the oligodendrocyte lineage cells leads to differentiation defects, in

part by stabilizing the β-catenin-TCF7L2 complex, which negatively regulates oligodendrocyte differentiation by repressing olig2 expression.

Neurodegenerative Disease

Polyglutamine (polyQ)-repeat diseases are inherited neurodegenerative disorders that are caused by an expansion of CAG repeats in different genes causing diseases such as Huntington's disease and spinocerebellar ataxias (SCAs). One of the possible pathological mechanisms of action of polyQ-expanded proteins is impairment of transcriptional regulation. It has been proposed that polyQ-expanded proteins interact with transcription factors, such as CBP, TAF4 (TATA-binding protein-associated factor 4) and Sp1 (specificity protein 1), and sequester these transcription factors within nuclear inclusions (NIs), resulting in inhibition of their normal function, thus contributing to pathogenesis (Kazantsev and Thompson, 2008). It has been shown that pharmacological inhibition of HDAC activity provides a therapeutic benefit in several neurodegenerative diseases including polyglutamine-repeat diseases (Abel and Zukin, 2008). HDAC

inhibitors might counteract a decrease in transcription induced by polyQ-expanded proteins, so as to provide a therapeutic benefit. *In vivo* studies showing that HDAC inhibitors modulate the toxicity of polyQ proteins in *Drosophila* and mouse models of Huntington's disease and other polyQ expanded diseases support a neuroprotective action of HDAC inhibitors (Ferrante et al., 2003; Hockly et al., 2003; Steffan et al., 2001; Thomas et al., 2008).

Learning and Memory

There is strong evidence that HATs and HDACs play important roles in learning, memory and synaptic plasticity. Mutations in CREB binding protein (CBP) or its homolog p300 cause Rubinstein-Taybi syndrome (RTS), a disorder associated with mental retardation (Blough et al., 2000; Petrij et al., 1995). Mouse models of RTS, such as mutant heterozygous CBP mice, display defects in chromatin acetylation and impairment in long-term memory and synaptic plasticity (Alarcon et al., 2004; Korzus et al., 2004; Vecsey et al., 2007). Treatment with HDAC inhibitors ameliorates the deficits in synaptic plasticity and cognition in RTS

mouse models through its effect on CBP and CREB (Alarcon et al., 2004; Korzus et al., 2004; Vecsey et al., 2007). Furthermore, inhibition of HDAC in the hippocampus with the HDAC inhibitor, TSA, results in enhancement in long term memory for contextual fear in wild type mice. Using mouse genetic models, a recent study showed that HDAC2 negatively regulates memory formation and synaptic plasticity (Guan et al., 2009). Neuron-specific over-expression of HDAC2 decreased dendritic spine density, synapse number, synaptic plasticity and memory formation, whereas mice lacking Hdac2 displayed increased synapse number and memory facilitation, which is reminiscent of the reported effects of treatment with HDAC inhibitors (Guan et al., 2009). Indeed, the effects of SAHA, an HDAC inhibitor, are abolished in HDAC2 knockout mice, suggesting that HDAC2 is the major target of SAHA in eliciting memory enhancement.

Neuropsychiatric Disorders

It is increasingly recognized that targeting histone acetylation may provide benefit for the treatment for neuropsychiatric disorders including depression and drug

addiction. In animal studies, two types of emotional stimuli, such as chronic social defeat stress (depression) and chronic cocaine (drug addiction) induce long-lasting changes in histone acetylation (Renthal et al., 2007; Tsankova et al., 2006). The activity-dependent class II HDAC, HDAC5, is a central regulator of the actions of chronic social defeat stress and chronic cocaine in the nucleus accumbens (NAc) (Renthal et al., 2007; Tsankova et al., 2006). Chronic, but not acute, exposure to cocaine or stress decreases HDAC5 function in the NAc, which allows for increased histone acetylation and expression of target genes suppressed by HDAC5. This regulation is important for behavioral adaptation, as mice lacking HDAC5 are hypersensitive to chronic, not acute, cocaine or stress exposure, suggesting that HDAC5 contributes to normal behavioral adaptation to these stimuli through the regulation of histone acetylation.

In light of the importance of the MEF2/class II HDACs signaling pathway in regulating muscle remodeling and brain function, the specific aims of my study are:

1) To investigate the role of the class II HDAC kinase, PKD1 in skeletal muscle remodeling
2) To examine the *in vivo* function of HDAC4 in the brain
3) To explore the *in vivo* function of MEF2 in the brain

Chapter II

Protein kinase D1 stimulates MEF2 activity in skeletal muscle and enhances muscle performance

Abstract

Skeletal muscle consists of type I and type II myofibers, which exhibit different metabolic and contractile properties. Type I fibers display an oxidative metabolism and are resistant to fatigue, whereas type II fibers are primarily glycolytic and suited for rapid bursts of activity. These properties can be modified by changes in workload, activity and hormonal stimuli, facilitating muscle adaptation to physiological demand. The MEF2 transcription factor promotes the formation of slow-twitch (type I) muscle fibers in response to activity. MEF2 activity is repressed by class II histone deacetylases (HDACs) and is enhanced by calcium-regulated protein kinases that promote the export of class II HDACs from the nucleus to the cytoplasm. However, the identities of skeletal muscle class II HDAC kinases are not well defined. Here we demonstrate that protein kinase D1 (PKD1), a highly effective class II HDAC kinase, is predominantly expressed in type I myofibers and, when mis-expressed in type II myofibers, promotes transformation to a type I, slow-twitch, fatigue-resistant phenotype. Conversely, genetic deletion of PKD1 in type I myofibers increases susceptibility to fatigue. PKD1 cooperates with calcineurin to facilitate slow-twitch fiber transformation. These findings identify PKD1 as a key regulator of skeletal muscle function and phenotype.

Introduction

Skeletal muscles consist of a heterogeneous mixture of myofibers that display different metabolic, contractile, and endurance properties. Based on the expression of myosin heavy chain (MyHC) isoforms, myofibers can be classified as either type I or type II fibers; type II fibers are further categorized into type IIa, type IId/x, and type IIb (Bassel-Duby and Olson, 2006). Type I fibers are oxidative, slow-twitch fibers with high endurance, whereas type IIb fibers are glycolytic, fast-twitch fibers with low endurance. Type IIa fibers can use both aerobic and anaerobic metabolism but display fast-twitch contracting properties. Type I myofibers play a major role in regulating whole body energy metabolism and insulin sensitivity. Slow-twitch oxidative skeletal muscle has greater insulin binding capacity, insulin receptor kinase activity, and a higher content of glucose transporter 4 (GLUT4) compared with fast-twitch glycolytic skeletal muscle (Bassel-Duby and Olson, 2006). In fact, type 2 diabetes mellitus is associated with defects in insulin signaling in skeletal muscle(Krook et al., 2000), and insulin resistance correlates with a reduced percentage of slow oxidative type I fibers and reduced oxidative enzyme capacity (Oberbach et al., 2006).

In response to various stimuli, including work load, hormonal influences, and innervation, myofibers modify their phenotype to maintain a balance between physiological demand and functional capacity. This muscle plasticity is achieved

through activation of intracellular signaling pathways that modulate myofiber gene expression (Schiaffino et al., 2007). Several calcium-dependent signaling pathways have been implicated in the modulation of myofiber phenotypes. In particular, the calcium, calmodulin-dependent protein phosphatase calcineurin, which responds selectively to sustained calcium waves (Dolmetsch et al., 1997), promotes the formation of slow, type I myofibers (Chin et al., 1998; Naya et al., 2000; Oh et al., 2005; Parsons et al., 2003), and blockade of calcineurin signaling promotes the formation of fast myofibers (Oh et al., 2005). Forced expression of calcium, calmodulin-dependent protein kinase (CaMK) IV in skeletal muscle also stimulates the formation of slow myofibers (Wu et al., 2002). However, CaMKIV is not expressed in skeletal muscle (Akimoto et al., 2004), suggesting that other endogenous kinases may normally play this role.

The myocyte enhancer factor-2 (MEF2) transcription factor acts as a transcriptional regulator of skeletal muscle remodeling and fiber type specification (Potthoff et al., 2007a; Potthoff et al., 2007b; Wu et al., 2001a). In adult skeletal muscle, the activity of MEF2 is tightly regulated through association with class II histone deacetylases (HDACs), which act as signal dependent repressors of gene expression (McKinsey et al., 2002). In response to differentiation signals or motor innervation, class II HDACs are phosphorylated, which provides docking sites for the 14-3-3 chaperone protein and leads to nuclear export of HDACs accompanied by derepression of MEF2 and activation

of MEF2 target genes (McKinsey et al., 2000; McKinsey et al., 2002; Verdin et al., 2003). Because phosphorylation of class II HDACs governs the activity of MEF2 and the downstream gene programs it regulates, identifying the kinases responsible for phosphorylation and nuclear export of class II HDACs in skeletal muscle may provide opportunities for manipulating skeletal muscle function.

Recently, we showed that protein kinase D1 (PKD1) acts as an HDAC export kinase in cardiomyocytes that controls hypertrophic growth in response to G-protein receptor-coupled agonists (Vega et al., 2004a). When excessively activated in the heart, PKD1 drives adverse cardiac remodeling, heart failure, and death (Harrison et al., 2006). Conversely, genetic deletion of PKD1 in the heart diminishes hypertrophy and pathological remodeling in response to pressure overload and chronic adrenergic signaling (Fielitz et al., 2008).

Three PKD isoforms, PKD1, 2 and 3, encoded by different genes (Rykx et al., 2003), share homology with the CaMK and protein kinase C (PKC) families. However, unlike CaMK and PKC, which are directly regulated by calcium, PKD activity is calcium-independent and, instead, is activated through phosphorylation by PKC (Rykx et al., 2003; Van Lint et al., 1995; Zugaza et al., 1996). PKD1 has been implicated in the regulation of a variety of biological processes, including membrane trafficking, cell survival, proliferation, differentiation, and migration (Rykx et al., 2003).

The potential involvement of PKD in the control of skeletal muscle development, growth and remodeling has not been investigated. Here we show that PKD1 is preferentially expressed in type I myofibers. Forced expression of constitutively active PKD1 (caPKD1) in type II fibers of transgenic mice potently stimulates the transcriptional activity of MEF2, as revealed by expression of a MEF2-dependent transgene, and in turn promotes their conversion toward a slow myofiber phenotype. Consistent with this fiber type switch, skeletal muscles derived from caPKD1 transgenic mice are resistant to fatigue during repetitive contractions. In contrast, skeletal muscle-specific deletion of PKD1, using a conditional PKD1 null allele, increases the fatigueability of type I soleus muscle, but not type II extensor digitorum longus muscle (EDL), where PKD1 expression is low. Calcineurin enhances the ability of PKD1 to activate slow-twitch and oxidative myofiber specific gene expression. We conclude that PKD1 signaling plays an important role in the control of skeletal muscle fiber type via its stimulatory activity toward MEF2, and that PKD1 and calcineurin act in a cooperative manner to modulate skeletal muscle function and phenotype.

Results

PKD1 is enriched in slow-twitch type I muscle fibers

Soleus muscle is comprised exclusively of slow-twitch type I and fast-twitch, oxidative type IIa myofibers, whereas EDL is predominantly comprised of fast-twitch, glycolytic type IIb myofibers. Tibialis anterior (TA), gastrocnemius and plantaris (G/P) muscles contain heterogenous myofiber types, but are comprised primarily of type II myofibers. Prior to exploring the potential involvement of PKD1 in the modulation of muscle fiber type, we examined the expression of PKD1 in each of the above muscles by Western blot analysis (Fig. 2.1A) and real time PCR (Fig. 2.1B). PKD1 protein and mRNA were highly enriched in soleus muscle. Despite detectable PKD1 mRNA expression, only a trace of PKD1 protein expression was detectable in EDL, and no PKD1 protein was detected in TA and G/P, suggesting a post-transcriptional mechanism for limiting PKD1 protein expression. Overall, the level of PKD1 expression correlated closely with the oxidative, slow-twitch fiber phenotype.

Forced expression of constitutively active PKD1 in skeletal muscle

To determine whether PKD1 signaling was sufficient to modulate skeletal muscle fiber types, we generated transgenic mice overexpressing constitutively active PKD1 (caPKD1) under the control of the muscle creatine kinase (MCK) promoter, which is preferentially active in fast-twitch, type IIb and type IIa

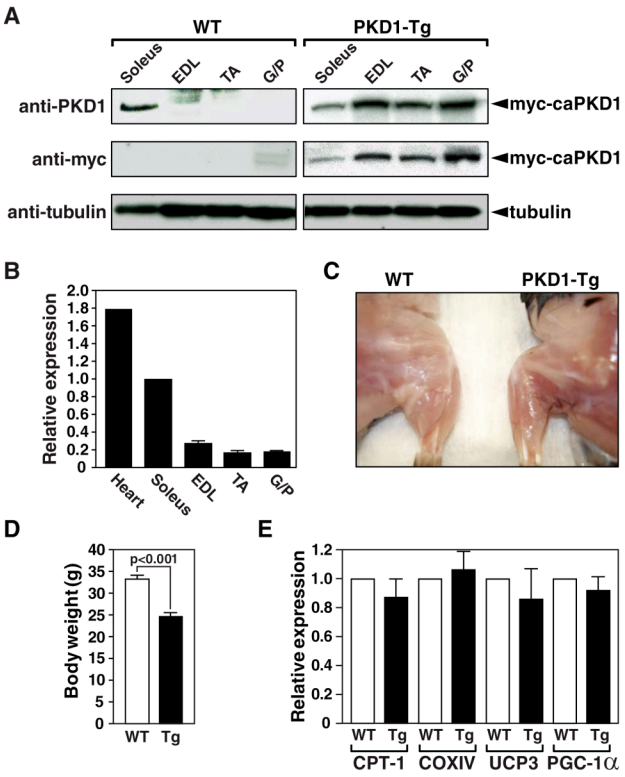

Figure 2.1 Expression of PKD1 in skeletal muscle

A, Immunoblots of skeletal muscle lysates isolated from MCK-myc-caPKD1 transgenic mice (PKD1-Tg) and wild-type (WT) mice using anti-PKD1, anti-Myc and anti-tubulin antibodies. Tubulin is used as a loading control. *B*, Real-time PCR showing expression of endogenous PKD1 in striated mouse muscles of 8-week old male mice. Muscles from 3 mice were pooled for each sample. *C*, Gross morphology of hindlimb from a 2 month-old WT and PKD1-Tg showing a lean phenotype of the transgenic mouse. *D,* Measurement of body weight of WT and PKD1-Tg mice shows 25% reduction in Tg mice (n=4 for each group). *E*, Real-time PCR showing no change in the expression of carnitine palmitoyl transferase (CPT-1), subunit IV of cytochrome c oxidase (COX IV) and uncoupling protein-3 (UCP3), peroxisome-proliferators-activated receptor-gamma coactivator-1 alpha (PGC-1α) enzymes involved in fatty acid synthesis and oxidative metabolism (n=3 for each group). Error bars indicate ± SEM.

myofibers (Chin et al., 1998), which express endogenous PKD1 at diminished levels relative to soleus. Examination of transgene expression by immunoblot with anti-Myc antibody, which recognizes the epitope-tagged caPKD1 protein, showed, as expected, the highest expression of caPKD1 in EDL, TA, and G/P, muscles primarily composed of type II fibers and less expression of the transgene in the soleus muscle (Fig. 2.1A).

MCK-caPKD1 mice were readily distinguishable from wild type littermates by their unusually lean appearance and weighed approximately 25% less than normal (Fig. 2.1C and D). Given the importance of skeletal muscle in the regulation of whole-body glucose and fatty acid metabolism, we examined the expression of enzymes involved in fatty acid metabolism so as to determine if the lean phenotype of the MCK-caPKD1 transgenic mice might reflect enhanced metabolic activity. RNA analyses showed no increase in mRNAs encoding CPT-1, COXIV, UCP3 or PGC-1α (Fig. 2.1E). Furthermore, studies using metabolic cages to measure food and liquid intake, ambulation, oxygen and carbon dioxide consumption showed no alteration in the respiratory exchange ratios, indicating that therewas no overall change in the metabolic properties of MCK-caPKD1 mice (data not shown). These findings suggest that the lean phenotype of MCK-caPKD1 transgenic mice does not result from altered metabolism.

MCK-caPKD1 transgenic mice display fiber type conversion

Type I fibers can be distinguished from type II fibers by their red color, which primarily reflects the high content of myoglobin. Muscles of MCK-caPKD1 transgenic mice appeared redder than those of wild type mice, suggesting an increase in type I fibers (Fig. 2.1C). We analyzed fiber type composition of MCK-caPKD1 transgenic mice using a metachromatic ATPase staining method, which allows identification of individual fiber types. Using the metachromatic ATPase

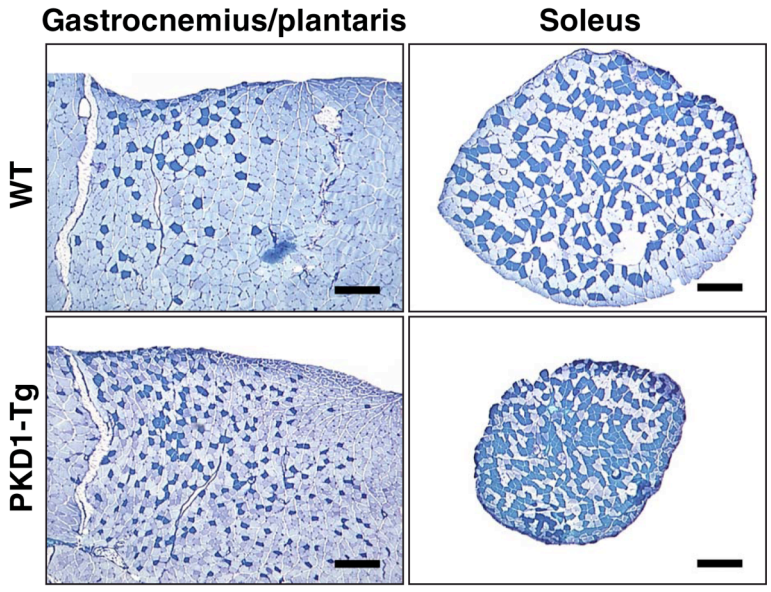

Figure 2.2 Skeletal muscle of MCK-caPKD1 transgenic mice have an increase in type I fibers
Gastrocneminus and plantaris (G/P) and soleus muscles were isolated from wild-type (WT) and MCK-myc-caPKD1 transgenic (PKD1-Tg) mice. Metachromatic ATPase fiber type analysis of skeletal muscle cross-sections shows an increase in type I fibers (dark blue) compared to type II fibers (light blue) in PKD1-Tg mice. Scale bar=300 μm.

stain, type I fibers stain dark blue and type II fibers stain different shades of lighter blue. Type I fibers were concentrated in specific regions of the G/P mice displayed an increase in type I fibers throughout the muscle (Fig. 2.2). In addition, both the EDL and soleus muscles displayed an increased number of type I and type IIa fibers in MCK-caPKD1 transgenic mice (Fig. 2.2). In wild type mice, the soleus muscle is comprised of ~45% type I fibers and the remaining fibers are type IIa (Fig. 2.2). In contrast, up to 60% of the fibers from the soleus of MCK-caPKD1 transgenic mice were type I fibers. Notably, the individual sizes of fibers, as well as the overall size of the muscle, were reduced in MCK-caPKD1 transgenic mice regardless of fiber type. Cross-sectional measurements of the soleus showed that the total number of fibers was decreased by only ~10% in MCK-caPKD1 transgenic mice. Therefore, a decrease in the overall muscle size reflects primarily a reduction in the size of individual myofibers. These findings show that over-expression of caPKD1 in myofibers promotes slow-twitch fiber formation in adult skeletal muscle and suggest that the lean phenotype might be explained, at least in part, by a reduction in myofiber size.

MCK-caPKD1 mice show an increase in specific contractile proteins

To further examine the fiber type switch in the skeletal muscle of MCK-caPKD1 transgenic mice, we examined the composition of MyHC isoforms in EDL, TA, and G/P muscles by silver staining-high resolution glycerol gels. Two major MyHC protein bands were present in muscle extracts of EDL, TA and G/P

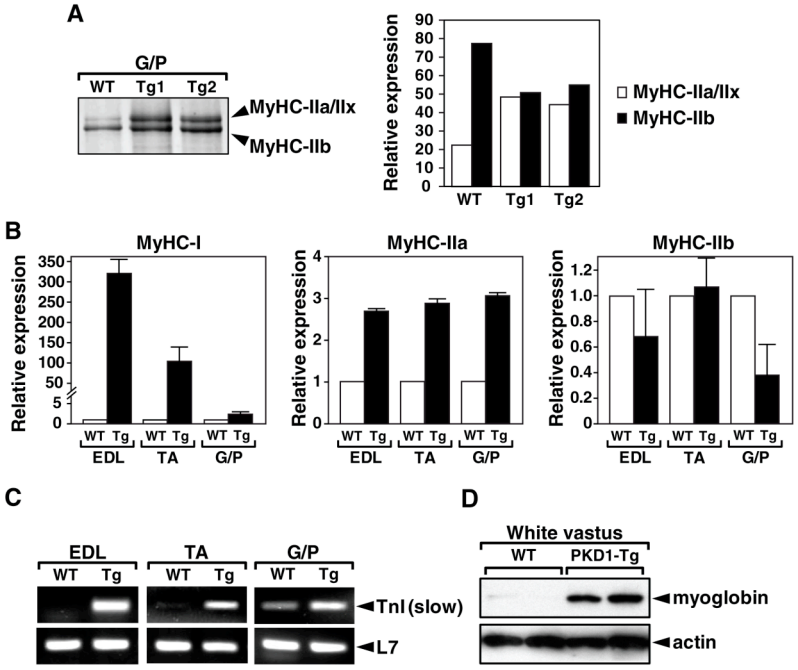

Figure 2.3 Type I and type IIa fibers are increased in MCK-myc-caPKD1 transgenic mice

A, Silver-stained, high-resolution glycerol gel resolving myosin heavy chain (MyHC) isoforms, MyHC-IIa, MyHC-IIx and MyHC-IIb, in protein lysates from extensor digitorum longus (EDL), tibialis anterior (TA) and gastrocnemius/plantaris (G/P) muscles from MCK-myc-caPKD1 transgenic mice (Tg) and wild-type (WT) mice. Graph shows relative expression of MyHC isoforms in Tg and WT muscles. *B*, Real-time PCR showing expression profile of MyHC isoforms in EDL, TA and G/P muscles isolated from PKD1-Tg and WT mice. (n=3). *C*, Semi-quantitative RT-PCR shows an increase in expression of troponin I (TnI) slow, a type I fiber-specific isoform of TnI, in EDL, TA and G/P skeletal muscle of PKD1-Tg compared to WT mice. *D*, Immunoblot of EDL, G/P, and white vastus (WV) lysates isolated from WT and PKD1-Tg using antibodies to myoglobin and actin revealed an increased expression of myoglobin in PKD1-Tg mice compared to WT mice. Actin was used as a loading control. Error bars indicate ± SEM

(data not shown).

muscles isolated from wild type mice, the faster migrating MyHC IIb protein and the slower migrating MyHC IIa/IIx proteins. In wild type mice, MyHC IIb comprises ~90% of the total MyHC in G/P muscles, whereas the MyHC IIb and IIa/IIx proteins in the G/P muscles isolated from MCK-caPKD1 transgenic mice comprise ~65% and ~35 % respectively (Fig. 2.3A). The MyHC IIa and IIx proteins were not separable on glycerol gels, so we examined their relative abundance by real time PCR, which revealed increased expression of MyHC I and IIa RNA and unaltered or decreased expression of MyHC IIb in the EDL, TA and G/P muscle of MCK-caPKD1 transgenic mice relative to wild type littermates (Fig. 2.3B). The expression of MyHC IIx was not significantly different from normal in EDL, TA and G/P muscle isolated from MCK-caPKD1 transgenic mice The expression of troponin I (slow), another type I fiber-specific contractile protein, was up-regulated in MCK-caPKD1 transgenic muscles (Fig. 2.3C). Myoglobin, a protein highly expressed in the soleus muscle and absent in the white vastus (WV) of wild type mice, was expressed in the WV of MCK-caPKD1 transgenic mice, as well as the EDL and G/P muscles as revealed by immunoblot and real time PCR analysis (Fig. 2.3D and data not shown). These findings show that over-expression of caPKD1 in skeletal muscle induces a switch from fast- to slow-twitch myofibers.

Skeletal muscle of MCK-caPKD1 transgenic mice display fatigue resistance

Fatigue resistance during repetitive contraction is a hallmark of type I fibers. To assess if fiber type conversion was accompanied by changes in the physiological properties of skeletal muscle in MCK-caPKD1 transgenic mice, we measured the susceptibility to fatigue during repetitive contractions. EDL and soleus muscles were isolated from wild type and MCK-caPKD1 transgenic mice and subjected to continuous electrical stimulation, which mimics muscle contraction during exercise. We measured the time required to lose 70% of the initial contractile force as an index of muscle fatigue. EDL and soleus muscles derived from MCK-

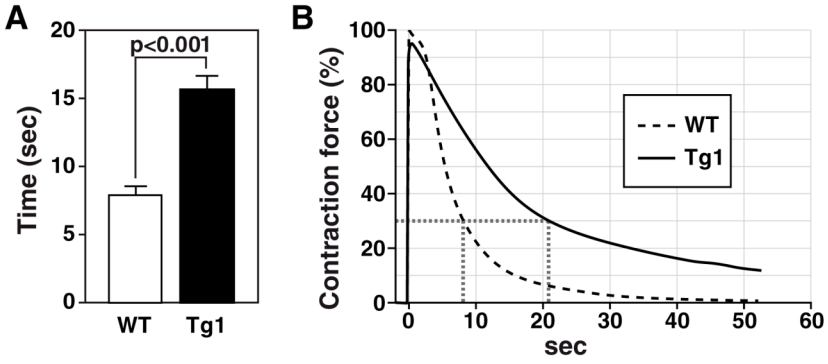

Figure 2.4 MCK-myc-caPKD1 skeletal muscle is more resistant to fatigue during repetitive contraction

Extensor digitorum longus (EDL) muscle isolated from WT mice and PKD1-Tg was subjected to continuous electrical stimulation. *A*, Time in seconds measured to reach 30% of initial contraction is defined as the fatigue index (n=5 WT and n=6 Tg) ($P<0.001$ by unpaired two-tailed Student's t test). *B*, Overlay of representative traces showing the percent contraction force over time in PKD1-Tg and WT EDL muscles. Error bars indicate ± SEM

caPKD1 transgenic mice fatigued slower than those from wild type mice (Fig. 2.4 and data not shown).

Deletion of PKD1 in skeletal muscle enhances fatigueability

To further investigate the potential involvement of PKD1 in muscle fatigue, we measured the susceptibility to fatigue during repetitive contractions in the soleus and EDL muscles of mice in which PKD1 was deleted in skeletal muscle. Skeletal muscle-specific deletion of PKD1 was achieved by breeding mice harboring a conditional PKD1 null allele (Fielitz et al., 2008) with transgenic mice expressing Cre recombinase specifically in skeletal muscle (Li et al., 2005). Mice with skeletal muscle-specific deletion of PKD1 (referred to as PKD1 skKO) were grossly indistinguishable from their wild type littermates. Quantification of PKD1 mRNA by real time RT-PCR revealed a 5-fold reduction of PKD1 mRNA in PKD1 skKO skeletal muscle (Fig. 2.5A). The observed residual expression of PKD1 mRNA most likely reflects PKD1 expression in fibroblasts, endothelial, smooth muscle and immune cells within the skeletal muscle. *Prkcm2* (encoding PKD2) and *Prkcn* (encoding PKD3) are expressed in skeletal muscle (Fig. 2.5B), but were not up-regulated in PKD1 skKO mice (data not shown). Consistent with the PKD1 over-expression experiments, indicating a role for PKD1 in muscle endurance, the soleus muscles from PKD1 skKO mice fatigued faster than those from wild type littermate controls (Fig. 2.5C), whereas no differences in fatigue

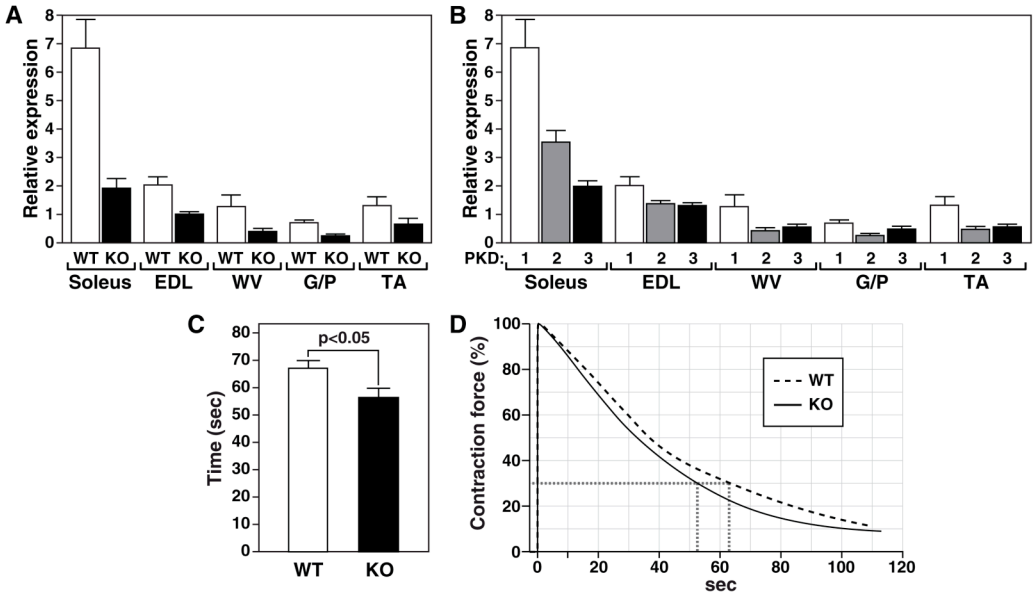

Figure 2.5 Deletion of PKD1 in soleus muscle leads to an increase in fatigue during repetitive contraction

A, Expression of *PKD1* transcripts detected by quantitative PCR. Total RNA isolated from soleus, extensor digitorum longus (EDL), white vastus (WV), gastrocnemius/plantaris (G/P) and tibialis anterior (TA) skeletal muscles of 8-week-old male mice was used for cDNA synthesis and subsequent quantitative RT-PCR of wild type (WT) and PKD1 skKO (KO) mice (n = 5). *B*, PKD1, 2 and 3 were quantified in the muscle groups of WT mice (n = 5) using quantitative RT-PCR. *C*, Soleus muscles were isolated and subjected to continuous electrical stimulation. Time to reach 30 % of initial contraction was measured as an index of fatigue. PKD1 skKO mice (n = 9) showed a significant increase in susceptibility to fatigue compared to WT littermate controls (n = 5) ($P<0.05$ by two-way ANOVA). *D*, Overlay of representative traces showing the percent contraction force over time in PKD1 skKO and WT soleus muscles. Error bars indicate ± SEM.

response were found in EDL muscle between PKD1 skKO and wild-type mice (data not shown). To assess if this phenotype was due to an effect of skeletal muscle-specific deletion of PKD1 on fiber type composition, we performed metachromatic ATPase staining, silver staining-high resolution glycerol gels and

RT-PCR for MyHC I, IIa, IIb and IIx. Surprisingly, no differences in fiber type composition of slow or fast skeletal muscle were found between PKD1 skKO and wild type littermates (data not shown), suggesting that fiber type switching does not account for the difference in fatigability seen in response to PKD1 deletion.

Activation of MEF2 in skeletal muscle of MCK-caPKD1 transgenic mice

Previous studies have shown that PKD1 signaling stimulates MEF2 activity by promoting the phosphorylation and export of class II HDACs from the nucleus (Dequiedt et al., 2005; Vega et al., 2004a). To determine whether overexpression of caPKD1 in skeletal muscle is sufficient to phosphorylate class II HDACs in vivo, we compared the phosphorylation status of HDAC4 and HDAC5 in skeletal muscle of wild type and MCK-caPKD1 transgenic mice by immunoprecipitation and Western blot analysis with antibodies against phospho-serine-467 in HDAC4 and phospho-serine-498 in HDAC5, which mediate nuclear export (McKinsey et al., 2000). We observed increased phosphorylation of HDAC4 (S467) and HDAC5 (S498) in soleus, TA and G/P muscles of the transgenic mice (Fig. 2.6A). To determine whether increased phosphorylation of class II HDACs in MCK-caPKD1 transgenic mice leads to MEF2 activation in vivo, we bred MCK-caPKD1 transgenic mice with MEF2 indicator transgenic mice harboring a lacZ transgene linked to three copies of the MEF2 consensus sequence from the desmin promoter (3XMEF2-lacZ) (Naya et al., 1999). In 3XMEF2-lacZ mice,

expression of lacZ depends on the activity of MEF2 (Wu et al., 2001a). We examined the expression of the lacZ transgene in wild type and MCK-caPKD1 transgenic muscles by β-galactosidase staining (Fig. 2.6B). In wild-type mice, MEF2 activity is normally detected preferentially in the soleus (Wu et al., 2000). In order to compare expression of the MEF2-lacZ transgene in soleus with type II fast fibers in transgenic mice, we allowed the β-galactosidase staining reaction to proceed for a relatively limited period of time, such that staining of the soleus was not detectable in wild-type mice. We previously reported that lacZ activity was only detectable within soleus muscle of a subset of animals (10-15%), and uniformly undetectable in EDL, plantaris, and white vastus muscles (Wu et al., 2000). Consistent with previous studies (Wu et al., 2000), only a basal level of MEF2 activity was detected in most adult skeletal muscle from wild type mice. However, skeletal muscle derived from MCK-caPKD1 transgenic mice displayed intense lacZ expression, indicative of the activation of MEF2 by caPKD1. The expression level of the lacZ transgene was higher in muscles comprised of type II fibers compared to soleus where the caPKD1 transgene is expressed at a lower level (Fig. 2.1B).

PKD1 synergizes with calcineurin in skeletal muscle

MEF2 is stimulated by calcineurin, which promotes the formation of slow-twitch

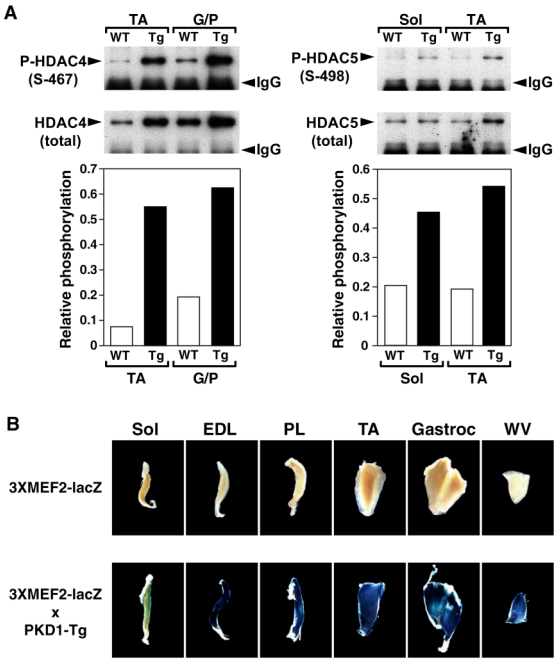

Figure 2.6 caPKD1 activates MEF2 in skeletal muscle through phosphorylation of class II HDACs

A, Western blot analysis of immunoprecipitated endogenous HDAC4 or HDAC5 from tibialis anterior (TA), gastrocnemius/plantaris (G/P), or soleus (Sol) muscles isolated from PKD1-Tg and WT mice. Blots were probed using an antibody that recognizes the phosphorylated form of HDAC4 (P-HDAC4 S-467) and HDAC5 (P-HDAC5 S-498). Blots were re-probed using commercial HDAC4 or HDAC5 antibodies. Graphs show the relative phosphorylation of HDAC4 and HDAC5 in wild type (WT) and MCK-caPKD1 (Tg) muscles. Relative phosphorylation was calculated as the ratio of phospho-HDAC to total-HDAC densitometric signals. *B,* β-galactosidase stain of soleus (Sol), extensor digitorum longus (EDL), plantaris (PL), tibialis anterior (TA), gastrocnemius (gastroc) and white vastus (WV) muscles isolated from two month old MEF2 indicator mice (3XMEF2-lacZ) or PKD1-Tg crossed with MEF2 indicator mice (3XMEF2-lacZ x PKD1-Tg). The expression of the lacZ transgene depends on MEF2 activity.

myofibers (Chin et al., 1998; Oh et al., 2005; Parsons et al., 2003; Wu et al., 2001a). To examine whether PKD1 cooperates with the calcineurin signaling

pathway to drive the slow fiber phenotype, we transfected C2C12 myogenic cells with a luciferase reporter gene controlled by the *myoglobin* promoter, which is activated by MEF2 (Chin et al., 1998), and a combination of expression plasmids encoding caPKD1 and/or constitutively active calcineurin. Cells were transferred to differentiation media 24 hrs following transfection and luciferase activity was measured 48 hrs following transfection. We observed that caPKD1 and calcineurin activated the myoglobin-luciferase reporter gene by 3- and 15-fold respectively. When co-expressed in C2C12 cells, the combination of caPKD1 and calcineurin activated the *myoglobin* promoter synergistically to approximately 50-fold, whereas no synergy was seen between calcineurin and a kinase-dead mutant of PKD1 (kdPKD1) (Fig. 2.7A). PKCθ has also been shown to cooperate with calcineurin in the activation of slow skeletal muscle genes in cultured muscle cells (D'Andrea et al., 2006). Since PKD1 is a downstream target of PKCθ (Yuan et al., 2002), we examined if the effect of PKCθ on slow skeletal muscle gene

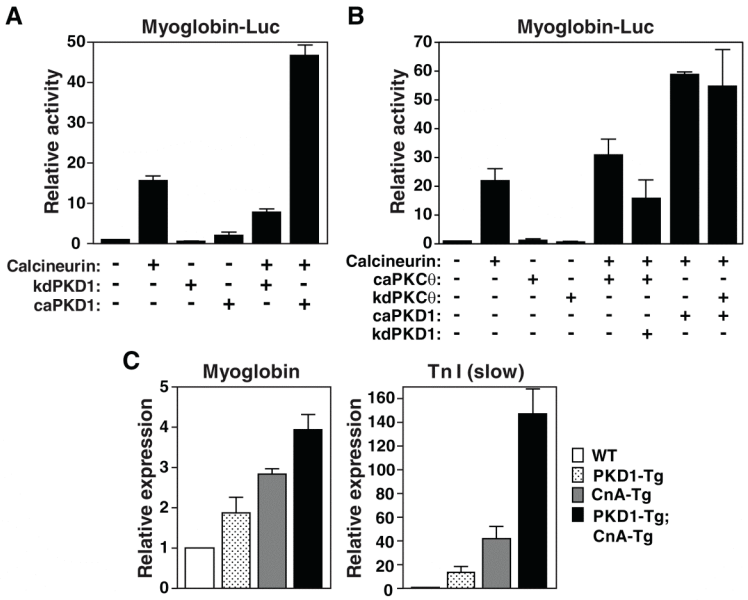

Figure 2.7 PKD1 acts synergistically with calcineurin
A, C2C12 cells were co-transfected with a reporter plasmid consisting of the *myoglobin* promoter driving luciferase (myoglobin-luc), and expression plasmids encoding the constitutively active form of calcineurin, the dominant negative form of PKD1 (kdPKD1), and/or the constitutively active form of PKD1 (caPKD1). CMV-lacZ was included in all transfections and the relative expression of luciferase activity was normalized to β-galactosidase expression. The values represent the mean ± SEM (n=3). *B,* C2C12 cells were co-transfected with myoglobin-luc and a combination of the expression plasmids encoding the constitutively active form of calcineruin, the constitutively active form of PKCθ (caPKCθ), the dominant negative form of PKCθ (kdPKCθ), caPKD1, and/or kdPKD1. T (n=3). *C*, Real-time PCR showed expression of myoglobin or troponin I slow (TnI slow) in EDL muscle isolated from wild-type (WT), MCK-myc-caPKD1 transgenic mice (PKD1-Tg), MCK-calcineurin transgenic mice (CnA-Tg), and MCK-myc-caPKD1 transgenic mice crossed with MCK-calcineurin transgenic mice (PKD1-Tg;CnA-Tg). The expression of myoglobin and TnI slow is upregulated synergistically in the PKD1-Tg;CnA-Tg double transgenic mice (n=3).

expression is mediated by PKD1. C2C12 myogenic cells were co-transfected with the myoglobin-luciferase reporter and a combination of expression plasmids

encoding calcineurin, constitutively active PKCθ (caPKCθ), kinase-dead PKCθ (kdPKCθ), caPKD1 and/or kdPKD1. Activation of the *myoglobin* promoter by calcineurin and caPKCθ was abolished by co-expression of the kinase inactive PKD1 (Fig. 2.7B). In contrast, the synergistic effect of PKD1 and calcineurin on the *myoglobin* promoter was not inhibited by co- expression of kdPKCθ. These findings suggest that PKCθ-PKD1 signaling activates slow muscle genes through cooperation with calcineurin. To confirm this cooperation in vivo, we bred MCK-calcineurin transgenic mice with MCK-caPKD1 transgenic mice. Analysis of myoglobin and troponin I (slow) expression showed similar enhancement in the EDL muscle of the double transgenic mice (Fig. 2.7C)**.**

Discussion

The results of this study demonstrate that PKD1 signaling promotes the slow-twitch skeletal muscle fiber phenotype and diminishes muscle fatigueability in vivo. Activation of slow-twitch fiber gene expression by PKD1 is accompanied by the stimulation of MEF2, a transcription factor shown to be sufficient to promote slow fiber gene expression and muscle endurance (Potthoff et al., 2007b).

Signaling pathways involved in slow-twitch fiber gene expression

Prior studies showed that calcineurin and CaMKIV are sufficient to promote the formation of slow-twitch myofibers (Chin et al., 1998; Delling et al., 2000; Naya et al., 2000; Wu et al., 2002). Similarly, peroxisome proliferator-activated receptor-gamma coactivator 1 (PGC-1), peroxisome proliferator-activated receptor-delta (PPARδ) (Lin et al., 2002; Wang et al., 2004) and MEF2 activate the expression of slow-twitch myofiber genes and serve as transcriptional targets of the upstream signaling pathways involved in specification of the slow myofiber phenotype (Czubryt et al., 2003). The ability of PKD1 to activate MEF2 and increase the abundance of type I fibers resembles the action of CaMKIV (Wu et al., 2001b). The PKD1 and CaMK signaling pathways also cooperate with calcineurin. However, PKD1 appears to differ from these other regulators of the

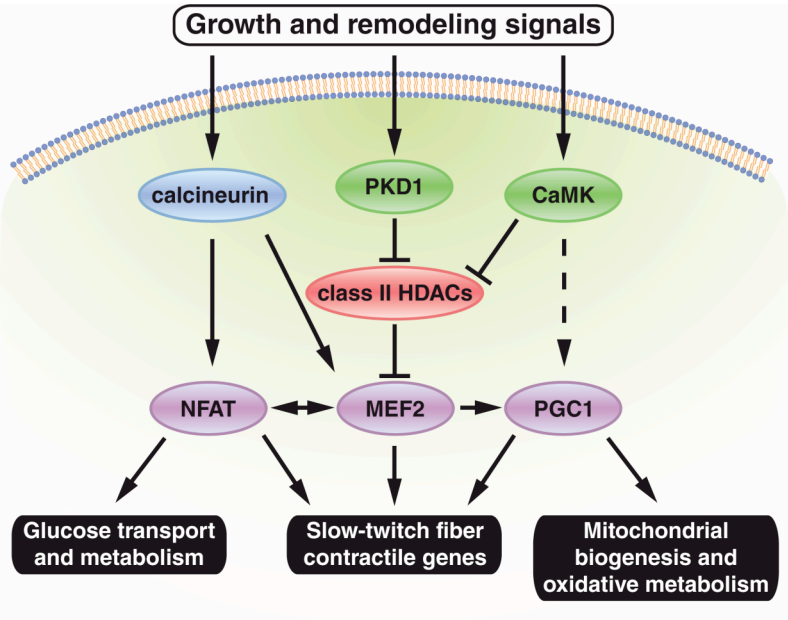

Figure 2.8 A model for the PKD1/Class II HDAC/MEF2 signaling pathway in skeletal muscle
Activation of PKD1 in skeletal muscle induces expression of the slow-twitch fiber specific contractile gene program and a subset of oxidative genes via phosphorylation of class II HDACs, which blocks their inhibitory influence on MEF2 as a consequence of their nuclear export. The calcineurin/NFAT pathway promotes the slow-twitch fiber gene program and up-regulates a subset of oxidative metabolism genes (Chin et al., 1998; Delling et al., 2000; Long et al., 2007; Oh et al., 2005; Ryder et al., 2003). The CaMK signaling pathway activates slow-twitch fiber contractile genes and the oxidative metabolism program via activation of MEF2 and co-activator PGC-1 (Lin et al., 2002; Wu et al., 2002).

slow-twitch myofiber phenotype in that it selectively promotes the expression of slow-twitch contractile protein genes and myoglobin, but not genes encoding

Mice over-expressing a constitutively active form of the lipid-activated nuclear receptor PPARδ in skeletal muscle show increased type I fibers formation, mitochondrial biogenesis, exercise endurance, resistance to high-fat-induced obesity and improved metabolic profile (Wang et al., 2004). We therefore

investigated whether transformed fibers in caPKD1 mice also confer improved metabolic properties. Although the expression of myoglobin was up-regulated, the MCK-caPKD1 mice displayed no overall metabolic changes. Furthermore, the expression of PGC-1α, a transcriptional coactivator responsible for the regulation of mitochondrial biogenesis and oxidative metabolism (Lin et al., 2002), was not changed in MCK-caPKD1 mice. These data, together with the fact that caPKD1 and PGC-1α showed no cooperation on the *myoglobin* promoter in C2C12 muscle cells (data not shown), suggest that the PKD1-MEF2 signaling pathway is dedicated to the control of slow contractile protein gene expression, rather than metabolic gene expression (Fig. 7). It is interesting to note that PKD1 itself is preferentially expressed in slow-twitch myofibers, suggesting that the *PKD1* gene may be regulated by PKD1 signaling through a positive amplification loop.

Another class II HDAC kinase, salt-inducible kinase-1 (SIK1), was recently reported (Berdeaux et al., 2007). SIK1, a direct target of CREB, was shown to disrupt class II HDAC repression of MEF2, promote survival of skeletal myocytes and partially rescue a dystrophic phenotype in mice. Whether SIK1 plays a role in myofiber remodeling remains to be determined.

Recently, several kinases were shown to mediate activity-dependent and -independent translocation of HDAC4 from the nucleus to the cytoplasm of adult skeletal muscle (Liu et al., 2005). Repetitive electrical stimulation of slow-twitch myofibers caused nuclear export of HDAC4 in cultured myofibers and HDAC4

translocation was blocked by the CaMK inhibitor, KN-62. In unstimulated fibers, nucleo-cytoplasmic shuttling of HDAC4 was unaffected by KN-62, but was blocked by the general serine-, threonine- kinase inhibitor staurosporine. These findings suggest that two different pathways mediate nuclear export of HDAC4; an activity-dependent CaMK signaling pathway and an activity- and CaMK-independent pathway. PKD1 may play a role in mediating nuclear efflux of HDAC4 in unstimulated resting fibers.

Control of muscle fatiguability by PKD1

Muscle fatigue is manifested by a decline of muscle performance with activity. Several factors contribute to muscle fatigue, such as failure of excitation-contraction coupling, alteration in metabolism, and reactive oxygen species (Allen et al., 2008). A major determinant of fatigue resistance is the density of mitochondria and the capacity of the muscle to use oxidative metabolism. Since type I myofibers are metabolically oxidative with high mitochondria content, they are more fatigue-resistant than type II myofibers. Although MCK-caPKD1 transgenic mice showed no overall metabolic changes at the whole-body level, the expression of a subset of oxidative genes, such as *myoglobin* was upregulated in skeletal muscle of these mice, which likely accounts for their fatigue resistance.

Skeletal muscle-specific deletion of PKD1 led to an increase in susceptibility to fatigue in soleus but not EDL muscles, consistent with the higher

protein expression of PKD1 in soleus compared to EDL muscle. However, despite highly efficient deletion of PKD1 in skeletal muscles, no changes in fiber type composition were observed, suggesting that the increased muscle fatigue in PKD1 skKO soleus muscle is not due to an altered expression of contractile proteins. This phenotype might reflect the absence of phosphorylation of myofilament proteins by PKD, as shown for cardiomyocytes (Haworth et al., 2004). Functional redundancy between different PKD isoforms might also have prevented changes in fiber type composition in PKD1 skKO mice. In this regard, PKD1 and 3 function redundantly to phosphorylate and export class II HDACs in B-lymphocytes (Matthews et al., 2006).

A striking phenotype of PKD1 transgenic mice is their reduction in muscle mass and myofiber size. The cross-sectional area of the individual fibers and the weight of the muscles of MCK-caPKD1 transgenic mice were decreased by 60% relative to wild type mice. The reduced fiber size is unlikely to be due to atrophy for several reasons. First, the functional properties, such as fatigue resistance, are improved in MCK-caPKD1 transgenic muscle. Second, the expression profile of muscle specific E3 ubiqutin ligases, such as Muscle RING-finger 1 (Murf1) which mediates muscular atrophy (Bodine et al., 2001), is not changed (data not shown). Finally, histological cross-sections of the muscles of MCK-caPKD1 transgenic mice revealed no increase in the number of central nuclei, an indicator of muscle injury (data not shown).

Therapeutic implications

The realization that PKD1 modulates the susceptibility of skeletal muscle to fatigue suggests potential opportunities for therapeutically manipulating PKD1 activity as a means of enhancing skeletal muscle function in the settings of human disease (Bassel-Duby and Olson, 2006). For example, a reduced slow myofiber population in skeletal muscle could contribute to insulin resistance, resulting in type 2 diabetes, based on the fact that insulin-stimulated glucose transport in skeletal muscle directly correlates with the percentage of slow-twitch myofibers. However, a challenge to the manipulation of PKD1 activity in vivo is that, while stimulation of PKD1 activity in skeletal muscle may improve muscle function, PKD1 activation in the heart causes dilated cardiomyopathy and heart failure (Harrison et al., 2006). Thus, an important challenge for the future will be to develop tissue-specific approaches to manipulate PKD activity in a tissue-specific manner.

Material and Methods

Generation of MCK-caPKD1 transgenic mice

An MCK-caPKD1 transgene was generated by placing a myc-tagged human constitutively active PKD1 (S738E/S742E) cDNA (Storz and Toker, 2003) downstream from the 4.8 kb muscle creatine kinase (MCK) promoter (Johnson et al., 1989). The construct contained a downstream human growth hormone poly A signal. DNA isolation and oocyte injections were performed as described (Molkentin et al., 1998). Genomic DNA was isolated from mouse tail snips and analyzed by PCR using a PKD1 specific primer (5'-GTGGTGGGTACCCCCGCTTAC-3') and a primer specific for human growth hormone poly A (5'-CACTCCGCTTGGTTCCCGAATAGAC-3'). Non-transgenic littermates were used for comparison with MCK-caPKD1 mice in all experiments. Mice 8-11 weeks of age were used for all experiments. All experimental procedures involving animals in this study were reviewed and approved by the Institutional Animal Care and Research Advisory Committee at University of Texas Southwestern Medical Center.

Skeletal muscle specific deletion of PKD1

The generation of a conditional PKD1 (*Prkcm*) allele has been described previously (Fielitz et al., 2008). We generated a skeletal myocyte-specific deletion of PKD1 using myogenin-Cre transgenic mice (Li et al., 2005) that

express Cre recombinase specifically in myocytes of the skeletal muscle and heterozygous PKD1$^{(loxP/KO)}$ mice, which were obtained using CAG-Cre transgenic mice which expresses Cre recombinase in the embryo at the zygote stage (Sakai and Miyazaki, 1997). The breeding scheme involved crossing PKD1$^{(loxP/loxP)}$; myogenin-Cre and PKD1$^{(loxP/KO)}$ mice to obtain PKD1$^{(loxP/KO)}$; myogenin-Cre mice (referred to as PKD1 skKO) and PKD1$^{(loxP/loxP)}$ (referred to as WT) as controls. For analysis, 8-10 week old male mice were used. Genotyping was performed by PCR using specific primers as described (Fielitz et al., 2008).

RNA isolation and RT-PCR

Total RNA was isolated from skeletal muscles using TRIZOL reagent (Invitrogen) following the manufacturer's instructions. Two micrograms of RNA was converted to cDNA using random primers and Superscript III reverse transcriptase (Invitrogen). Gene expression was analyzed using either semi-quantitative PCR or Real-time PCR. Real-time PCR was performed using the ABI PRISM 7000 sequence detection system with TaqMan primers (Applied Biosystems) or with SYBR Green Master Mix reagent (Applied Biosystems) and the primer set listed below. Real-time PCR values were normalized with L7 and GAPDH expression. Primer sequences are:

Myh2 (type IIa) forward: 5'- CCAGCTGCACCTTCTCGTTTGCCAG-3',

Myh2 reverse: 5'-CATGGGGAAGATCTGGTCTT CTTTCACGGTCAC-3',

Myh1 (type IIx) forward: 5'-GCGCAACGTGGAAGCTATCAAGGGTCTG-3',

Myh1 reverse: 5'- GATCTTCACATTTTGCTCATCTTTTGGTCACT-3',

Myh4 (type IIb) forward: 5'-CCTGGAACAGACAGAGAGGAGCAGGAGA-3',

Myh4 reverse: 5'-GTGAGTTCCTTCACTCTGCGCTCGTGC-3',

Troponin I (slow) forward: 5'-GTGCCTGGAACATCCCTAAT-3',

Troponin I (slow) reverse: 5'-TGAGAGGCTGTTCTCTCTGC-3',

Myoglobin forward: 5'-CATGGTTGCACCGTGCTCACAG-3',

Myoglobin reverse: 5'-GAGCCCATGGCTCAGCCCTG-3',

Cpt1 forward: 5'- ATCATGTATCGCCGCAAACT-3'

Cpt1 reverse: 5'-ATCTGGTAGGAGCACATGGGC-3'

COXIV forward: 5'-GTTCAGTTGTACCGCATCCA-3'

COXIV reverse: 5'-TTGTCATAGTCCCACTTGGC-3'

UCP3 forward: 5'-TTTCTGCGTCTGGGAGCTT-3'

UCP3 reverse: 5'-GGCCCTCTTCAGTTGCTCAT-3'

L7 forward: 5'-GGAGGAAGCTCATCTATGAGAAGGCA-3',

L7 reverse: 5'-AAGATCTGTCGAAGACGAAGGAGCT-3'.

β-galactosidase staining of skeletal muscle

Dissected muscles from MCK-caPKD1 transgenic mice and wild type littermates were fixed in 2% formaldehyde/0.2% glutaraldehyde in PBS for 45-60 minutes on ice. After fixation, muscles were washed twice with PBS for 10 minutes on ice,

and stained in X-gal solution containing 5 mM ferrocyanide, 5 mM ferricyanide, 2 mM $MgCl_2$, 1 mg/ml X-gal, 0.01% sodium deoxycholate, and 0.02% NP-40 for 1 hour at room temperature.

Fiber type analysis by metachromatic ATPase staining

Individual muscles were dissected from wild type and PKD1 transgenic mice and embedded in medium containing gum tagacanth (Sigma, St. Louis, MO) and tissue freezing medium (Triangle Biomedical Sciences, Durham, NC). Muscles were quickly frozen in liquid nitrogen-cooled isopentane and cut at 200μm interval using a cryotome and put on glass slides. For fiber type analysis, metachromatic ATPase staining was performed as previously described (Ogilvie and Feeback, 1990).

Electrophoretic analysis of myosin heavy chain isoforms

Dissected muscles were homogenized in extraction buffer (300 mM KCl, 100 mM KH_2PO_4, 50 mM K_2HPO_4, 10 mM EDTA, 1 tablet of protease inhibitor cocktail (Roche), pH 6.5). After 30 minutes of incubation on ice, homogenized tissues were subjected to centrifugation at 18,000*g* for 5 minutes at 4 °C. Supernatant fractions were diluted in 2X Laemmli buffer and 0.5 μg of protein was separated by 8% SDS-PAGE containing 30% glycerol for 40 hours at 60 volts and 4 °C. Gels were stained using a silver staining kit (Bio-Rad) following the

manufacturer's instructions. The relative expression of MyHC isoforms was calculated by densitometry using Image J (version 1.38X, National Institutes of Health).

Immunoblotting of muscle extracts

Tissues were homogenized in extraction buffer and subjected to centrifugation at 12,000*g* for 10 minutes at 4°C. The soluble fractions were collected and 20 μg of proteins were subjected on SDS-PAGE and analyzed by immunoblotting with antibodies against c-Myc (1:1000; Santa Cruz Biotechnology Inc.), PKD1 (1:1000; Santa Cruz Biotechnology Inc.), myoglobin (1:3000; DACO), actin (1:2000; Sigma-Aldrich) and β-tubulin (1:2000; Sigma-Aldrich). To detect HDAC phosphorylation, immunoprecipitation was performed using the ExactaCruz reagent (Santa Cruz Biotechnology Inc.) with HDAC4 and HDAC5 antibodies (McKinsey et al., 2000). The relative level of P-HDAC and total HDAC was calculated by densitometry using Image J (version 1.38X, National Institutes of Health).

Muscle performance assay

Mice were euthanized and extensor digitorum longus (EDL) and soleus muscles were excised and mounted on a Grass FT03 force transducer and bathed in an oxygenized (95% oxygen and 5% CO_2) physiological salt solution (120.5 mM

NaCl, 4.8 mM KCl, 1.2 mM Na_2HPO_4, 20.4 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$) at 30°C. Muscles were adjusted to optimal length showing maximal isometric twitch tension (Lo) and rested for 30 minutes. To measure skeletal muscle performance, muscles were continuously stimulated at 100 Hz until the force output reached 10% of the initial force. The time taken to reach 30% of the initial force output is the fatigue index (Grange et al., 2001).

Transient transfection assays

C2C12 myoblast cells were co-transfected with a luciferase reporter plasmid controlled by the 2kb *myoglobin* promoter (Chin et al., 1998), an expression vector encoding constitutively active calcineurin (Chin et al., 1998), an expression vector encoding the inactive form of PKD1 (K614W) (Storz and Toker, 2003), an expression vector encoding the constitutively active form of PKD1 (S738E/S742E), an expression vector encoding the constitutively active form of PKCθ (R145I/R146W) (Liu et al., 2001), and an expression vector encoding the inactive form of PKCθ (K409W) (Liu et al., 2001) as indicated. Twenty four hours following transfection, cells were changed to differentiation medium containing 2% horse serum. Cells were harvested 48 hours following transfection and lysed in passive lysis buffer (Promega). Luciferase activity was measured using the luciferase assay system (Promega) following the manufacturer's

instruction. The values were normalized with β-galactosidase expression using FluoReporter lacZ Quantitiation Kit (Molecular Probes).

Chapter III

Loss of histone deacetylase 4 in the brain compromises memory formation and synaptic plasticity

ABSTRACT

Administration of histone deacetylase (HDAC) inhibitors enhances learning and memory formation following brain injury. However, a detailed understanding of the molecular mechanism regulating HDAC inhibitor-mediated memory formation remains elusive. Recent studies showed that the class I HDAC2 negatively regulates memory formation, but it is unknown whether class II HDACs are also involved in memory. Here we show that mice lacking class II HDAC4 display a profound impairment in learning and memory processes. In contrast, mice lacking class II HDAC5 exhibit no overt behavioral abnormalities. Accompanying the learning and memory deficit of *Hdac4* knockout mice is impairment in synaptic plasticity and a decrease in expression of Arc/Arg3.1, an important regulator in memory formation. Deletion of both HDAC4 and HDAC5 in neurons produced a more pronounced neurological deficit, including severe seizure activity. Taken together, our results suggest that HDAC4 positively regulates learning and memory formation and that HDAC4 and HDAC5 function in the maintenance of CNS homeostasis, in a partially redundant manner. These findings suggest that class I and II HDACs play opposing role in the modulation of learning and memory.

INTRODUCTION

Memory formation depends on neuronal adaptative responses, including activity-dependent changes in synaptic connectivity, which require transcription and translation of new proteins (Greer and Greenberg, 2008). Experience-driven synaptic activity causes calcium influx into specific postsynaptic neurons, leading to the remodeling of their synapses through the activation of new gene transcription and subsequent regulation of translation and post-translational modification of proteins. Although physiological effects of experience-driven synaptic activity are well known, the molecular mechanisms underlying this process still remain elusive. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) regulate histone acetylation, leading to changes in chromatin structure and gene expression (Goldberg et al., 2007). Recent studies also indicate that HATs and HDACs can modify a large number of non-histone proteins to regulate their stability and function (Martin et al., 2007). These observations, together with recent findings showing that treatment with non-selective HDAC inhibitors facilitate learning and re-establish the access to long-term memory in mice, suggest that HDACs may play a critical role in memory formation by regulating transcription or post-translational modification of essential proteins involved in learning and memory (Fischer et al., 2007; Guan et al., 2009; Korzus et al., 2004). Until recently, however, the functions of HDACs in learning and memory have been largely inferred from studies using

pharmacological inhibitors, which inhibit a broad spectrum of classical HDACs, preventing the functional analyses of specific HDAC isoforms *in vivo*. Thus, it is important to examine specific roles of individual HDACs in learning and memory by deleting specific HDACs using genetically modified mice to understand the underlying molecular mechanism for memory enhancement.

We previously showed that the class I HDACs, HDAC1 and HDAC2, redundantly regulate neuronal development and are required for neuronal specification (Montgomery et al., 2009). Mice lacking HDAC1 and HDAC2 in developing neurons show severe neural abnormalities, including hippocampal abnormalities, absence of cerebellar foliation, and disorganization of cortical neurons. These abnormalities result from a failure of neuronal precursors to differentiate into mature neurons and excessive cell death. Another recent study elegantly demonstrated that HDAC2 negatively regulates memory formation and synaptic plasticity (Guan et al., 2009). They showed that neuronal specific over-expression of HDAC2 decreased dendritic spine density, synapse number, synaptic plasticity and memory formation, whereas mice lacking HDAC2 displayed increased synapse number and memory facilitation, which is a reminiscent of the reported effects of treatment with HDAC inhibitors. Indeed, they found that the effects of SAHA, a pan-HDAC inhibitor, on memory formation are abolished in HDAC2 knockout mice, suggesting that HDAC2 is the major target of SAHA in eliciting memory enhancement. Together, these data

provide genetic evidence supporting a role for class I HDACs in learning and memory.

Unlike class I HDACs, which are localized almost exclusively in the nucleus, class IIa HDACs (HDAC4, 5, 7, and 9) shuttle between the nucleus and cytoplasm in response to certain cellular signals (Haberland et al., 2009). Neuronal activity stimulates calcium flux, which in turn induces the translocation of HDAC4 and HDAC5 from the nucleus to the cytoplasm, probably by regulating calcium/calmodulin kinase-dependent phosphorylation of HDAC4 and HDAC5 (Chawla et al., 2003). These properties of activity-dependent regulation of class II HDACs lead to the intriguing possibility that class II HDACs play a critical role in learning and memory. However, the involvement of class II HDACs in these processes has not been explored.

To examine the functions of class II HDACs in learning and memory, we generated mice lacking HDAC4 and HDAC5 in the forebrain. We show that mice lacking HDAC4 display global behavioral abnormalities, including motor coordination deficit, less anxiety, and learning and memory defects, whereas mice lacking HDAC5 show no behavioral abnormalities. We also demonstrate that learning and memory deficits in brain-specific *Hdac4* knockout mice are accompanied by impairments in long-term potentiation (LTP) induction and a decrease in expression of the activity-regulated cytoskeleton-associated protein, Arc/Arg3.1, an immediate-early gene induced by numerous forms of neuronal

activity. Furthermore, deletion of both HDAC4 and HDAC5 in the forebrain resulted in gross histological abnormalities, postnatal lethality, seizure, and reduced dendritic arborization. These findings suggest that HDAC4 plays important roles in memory formation and synaptic plasticity, and HDAC4 and HDAC5 are functionally redundant in the maintenance of CNS homeostasis.

RESULTS

Deletion of HDAC4 in the forebrain

Among the class II HDACs, HDAC4 and HDAC5 are highly enriched in the brain (Fig. 3.1A and B). Global deletion of Hdac4 results in perinatal lethality due to skeletal abnormalities (Vega et al., 2004b). Therefore, to investigate the potential role of HDAC4 in learning and memory processes, we generated mice lacking HDAC4 specifically in forebrain neurons by mating *Hdac4*$^{loxP/loxP}$ females (Potthoff et al., 2007b) to *Hdac4*$^{loxP/+}$ heterozygote male mice harboring a transgene that expresses Cre recombinase under the control of the calcium/calmodulin dependent protein kinase II promoter (*CaMKII-Cre*). The CaMKII-Cre transgenic mouse expresses Cre recombinase from postnatal day 10 in the neocortex and hippocampus but not in the cerebellum (Luikart et al., 2005). Mice with homozygous deletion of *Hdac4* in the forebrain (referred to as *Hdac4*^{BKO}) were born at expected Mendelian ratios and showed normal body weight, gross brain morphology and lifespan. Histological analysis by H&E staining of brain sections did not reveal any detectable aberrations in the size, morphology, or density of neurons in various regions of the *Hdac4*^{BKO} brain (Fig. 3.1F). Deletion of the *Hdac4*^{loxP} allele in the brain of mutant mice was validated by in situ hybridization, Western blot analysis, and RT-PCR (Fig. 3.1C, D and E). In wild type mice, *Hdac4* is expressed throughout the brain such as the cortex, hippocampus, thalamus, amygdala, and cerebellum. In *Hdac4*^{BKO} mice, *Hdac4*

expression was abolished in the forebrain, including the cortex, amygdala and hippocampus but not the cerebellum (Fig. 3.1D). mRNA levels of HDAC5, HDAC7 and HDAC9 were not significantly altered in the hippocampus of *Hdac4*^{BKO} mice compared to control animals, suggesting that there are no significant compensatory changes in expression of these functionally related genes (Fig. 3.1E).

Hdac4^{BKO} mice are hyperactive and show deficits in learning and memory

To determine whether *Hdac4*^{BKO} or previously generated *Hdac5*$^{-/-}$ (*Hdac5* KO) (Chang et al., 2004) mice display any alterations in global behavior, we subjected these mice to several behavioral paradigms. *Hdac5* KO mice show no abnormalities in overall locomotor activity or anxiety-related behaviors (Fig. 3.2A and data not shown). In contrast, we found that *Hdac4*^{BKO} mice display hyperactivity, motor coordination deficit, and decreased anxiety-related behavior (Fig. 3.2A and data not shown). To assess the potential involvement of HDAC4 and HDAC5 in learning and memory, we examined *Hdac4*^{BKO} and *Hdac5* KO mice for context and cue-dependent fear conditioning. Context-dependent fear conditioning requires an intact hippocampus and amygdala, whereas cue-dependent fear conditioning only depends on the amygdala (Monteggia et al., 2004). *HDAC5 KO* mice and control mice showed comparable levels of freezing

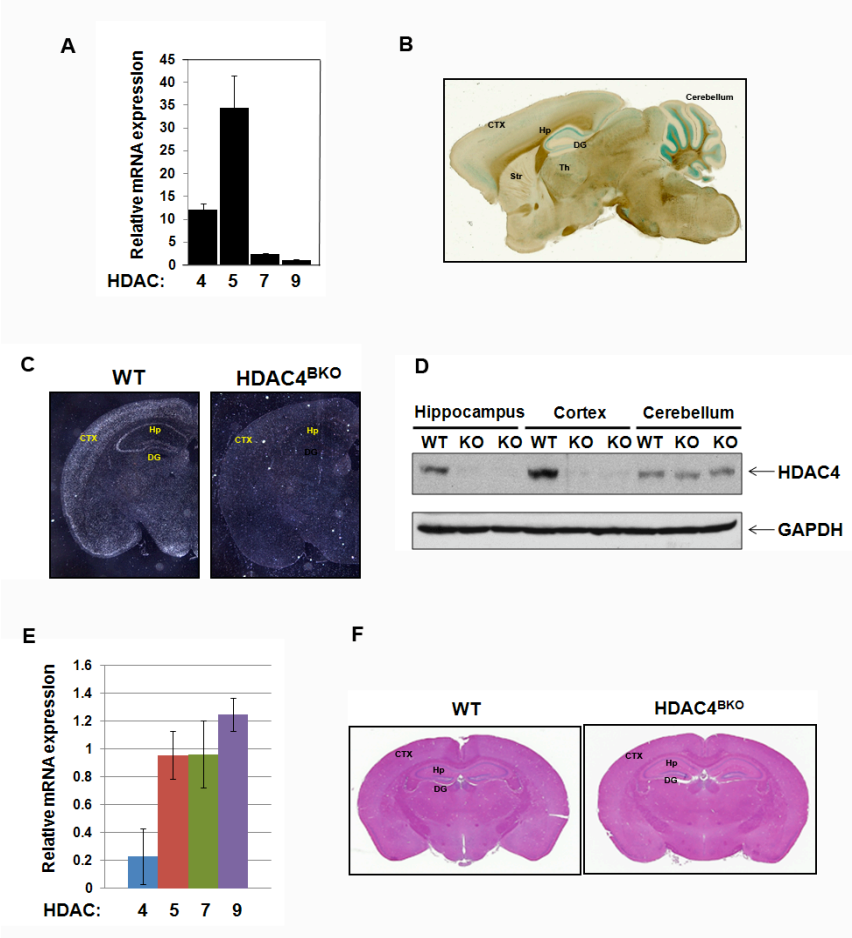

Figure 3.1 Generation of brain-specific HDAC4 knockout mice
(A) Expression level of class II HDACs in the hippocampus of wild type mice detected by quantitative RT-PCR. Error bars indicate ± SEM. (B) β-galactosidase staining was performed on adult brain section from HDAC4-LacZ knock-in mouse. LacZ expression, which represents the expression of endogenous HDAC4 in the adult brain, is observed in several brain regions including cortex (CTX), hippocampus (Hp) and cerebellum. Str, striatum; Th, thalamus (C) Detection of *Hdac4* transcripts by in situ hybridization to coronal sections from wild type and HDAC4^{BKO} mice at 4-month-old age. (D) Western blots from brain lysate showed deletion of HDAC4 in the forebrain ((cortex and hippocampus), but not cerebellum. GAPDH protein was used as a loading control. (E) Expression level of class II HDACs detected by quantitative PCR. Error bars indicate ± SEM. (F) H&E staining showed no obvious changes in brain architecture of 4-month-old HDAC4^{BKO} mice.

behavior in context- and cue-dependent fear conditioning (Fig. 3.2B). In contrast, the *Hdac4*^{BKO} mice displayed decreased freezing behavior compared to control mice when measured at 24h after training (Fig. 3.2B). No difference in baseline freezing behavior or pain sensation were observed between *Hdac4*^{BKO} and control mice (data not shown), suggesting that the impairments of fear conditioning are due to a deficit in associative learning rather than nonspecific effects on freezing behavior.

Although *Hdac4*^{BKO} mice exhibit a deficit in associative learning in a fear-conditioning paradigm, it is possible that the hyperactivity in locomotion observed in the *Hdac4*^{BKO} mice could skew the results. To rule out this possibility, we used the Morris water maze paradigm, which is independent of locomotor activity (Vorhees and Williams, 2006). We pre-exposed *Hdac4*^{BKO} mice and wild type mice to the water maze with a visible platform (6 trials/day for 5 days) prior to testing them with visible platform as rapidly as wild type mice on all 6 trials, indicating that the basic neurological functions needed for swimming and sight are normal. In hidden platform trials, *Hdac4*^{BKO} and wild type mice were trained for 11 days using 4 trials/day. On day 12, the submerged platform was removed and mice were allowed to swim freely for 60 s. Wild type mice showed a marked preference for the target quadrant where the submerged platform had been located, while *Hdac4*^{BKO} mice spent an equal amount of time in all quadrants,

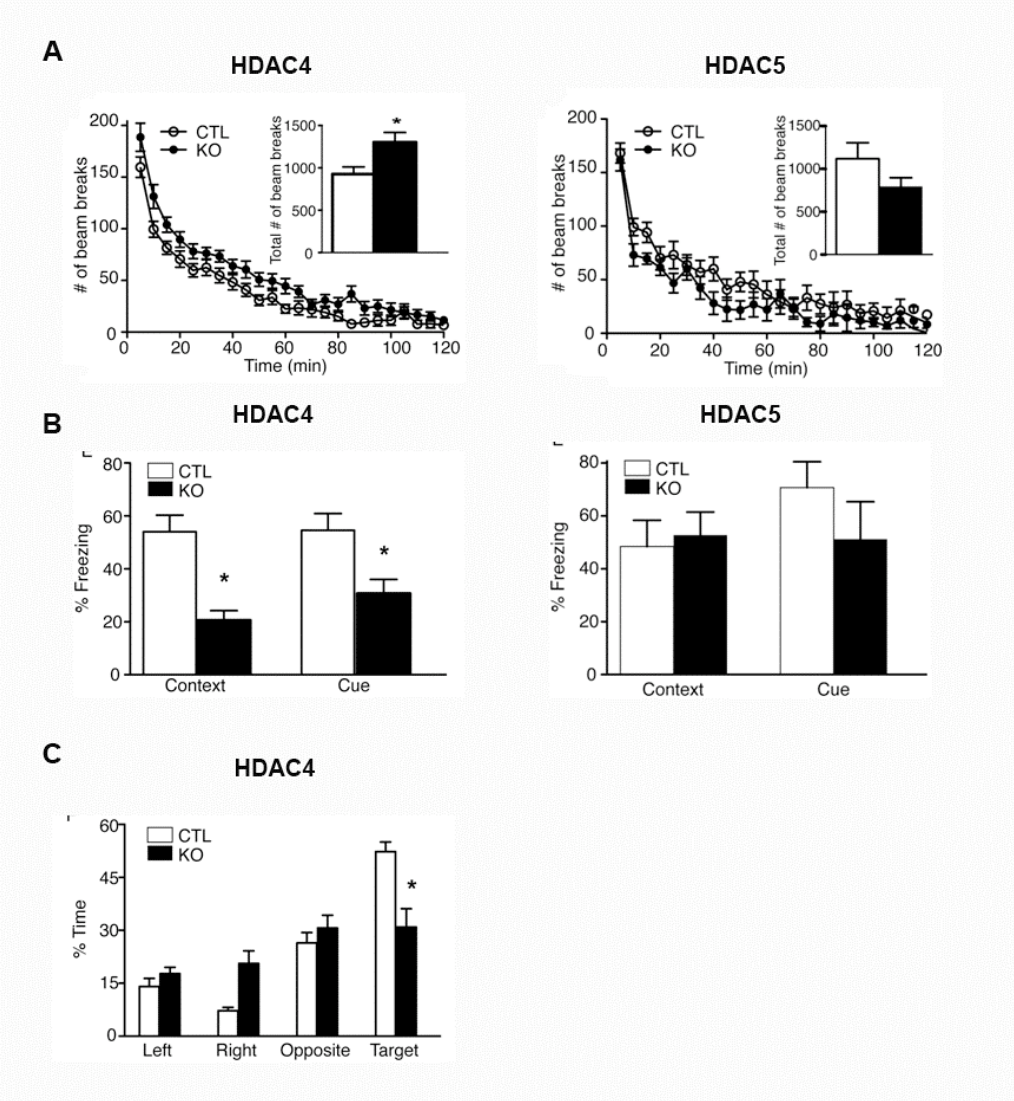

Figure 3.2 Mice lacking HDAC4, but not HDAC5, show behavior abnormalities including impaired memory formation
(A) HDAC4^{BKO} mice, but not HDAC5 KO mice, exhibit an increase in locomotor activity (ambulation), as assessed by a locomotor habituation paradigm. (B) Reduced freezing behavior of HDAC4^{BKO} mice during the context- and cue-dependent memory tests. *Hdac5* KO mice exhibit normal context- and cue-dependent fear conditioning. (C) HDAC4^{BKO} mice are impaired in spatial learning in the Moris water maze.

indicating that *Hdac4*^{BKO} mice have an impairment in spatial learning (Fig. 3.2C). Together, these findings imply that HDAC4, but not HDAC5, regulates memory formation in mice.

Recent studies have shown that HDAC4 promotes survival of cerebellar granule neurons and retinal neurons (Chen and Cepko, 2009; Majdzadeh et al., 2008). To address whether the deficit in learning and memory observed in HDAC4^{BKO} mice results from an increase in apoptosis, we performed TUNEL staining on brain sections from 4-month-old control and *Hdac4*^{BKO} mice. No difference in apoptosis was observed between control and *Hdac4*^{BKO} mice, suggesting that dysregulated apoptosis is not responsible for the impairment in learning and memory observed in *Hdac4*^{BKO} mice (data not shown).

Impaired LTP formation in Hdac4^{BKO} mice

Since synaptic plasticity is widely assumed as a cellular basis for learning and memory, we examined whether the impaired learning and memory seen in *Hdac4*^{BKO} mice is associated with changes in synaptic plasticity. We performed electrophysiological recordings on hippocampal slices prepared from *Hdac4*^{BKO} mice and their control littermates. Long-term potentiation (LTP) was induced in the CA1 of the hippocampus by two tetanic stimulations on the Sahaffer collateral pathway (Monteggia et al., 2004). Both control and *Hdac4*^{BKO} mice developed an immediate increase in the excitatory postsynaptic potential (EPSP) slope (fEPSP),

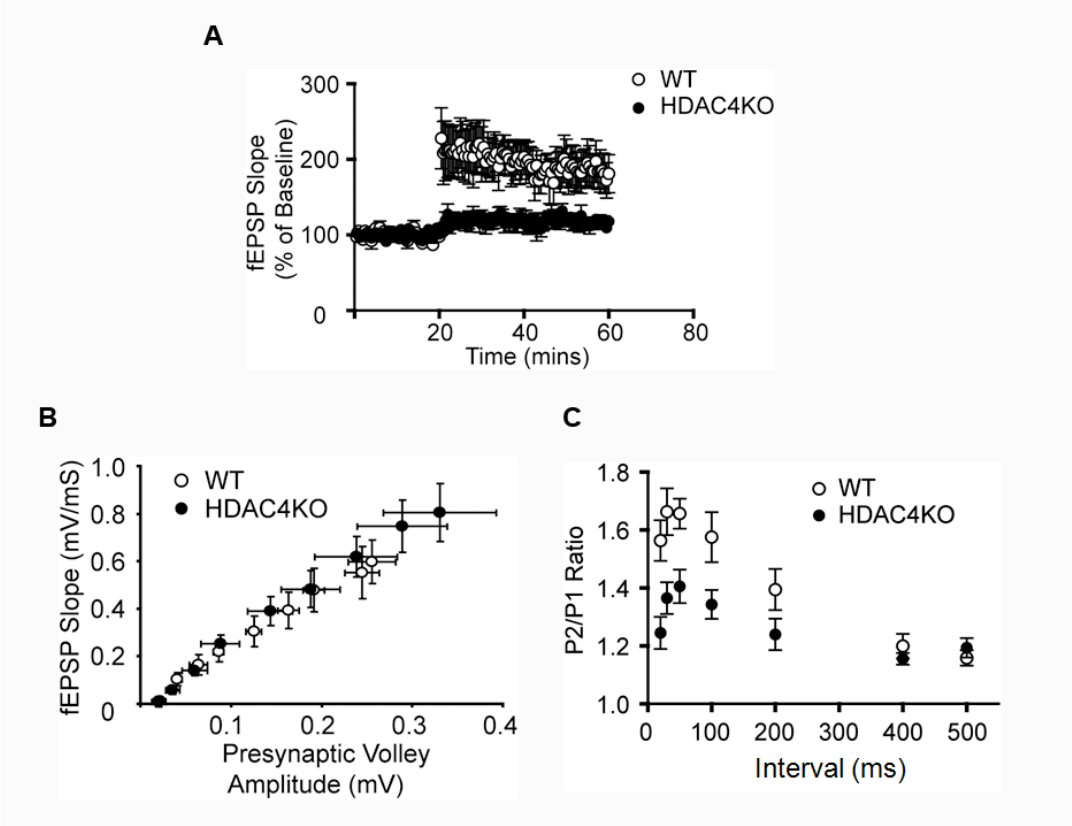

Figure 3.3 Impaired hippocampal plasticity in HDAC4^{BKO} mice
(A) Altered LTP at Schaffer collateral(SC)/CA1 pyramidal cell synapses. (B) Normal input/output function of the CA1-fEPSP in HDAC4^{BKO} mice. (C) Paired-pulse facilitation at SC-CA1 synapses is decreased in HDAC4^{BKO} mice.

yet the magnitude of enhancement of LTP was reduced in *Hdac4^{BKO}* mice, suggesting a dramatic modulatory action of HDAC4 expression on LTP induction (Fig. 3.3A). Next, we tested whether the decreased LTP observed in *Hdac4^{BKO}* mice might be due to a difference in basal synaptic properties. Input-output curves generated by plotting fEPSP slopes against the slopes of fibers volley were not changed in HDAC4^{BKO} mice, indicating that basal synaptic transmission is

unaltered in HDAC4^{BKO} mice (Fig. 3.3B). However, paired-pulse facilitation (PPF), a short-term form of synaptic plasticity associated with the probability of neurotransmitter release, was decreased in HDAC4^{BKO} mice, suggesting that loss of HDAC4 leads to alterations in presynaptic function (Fig. 3.3C).

Deletion of Hdac4 and Hdac5 in brain

Because HDAC4 and HDAC5 function redundantly in other tissues (Potthoff et al., 2007b), we assessed the possibility of functional redundancy between HDAC4 and HDAC5 in the brain by generating mice lacking both genes in the brain (herein referred to as DKO). DKO mice were viable but died prematurely starting at one month of age (Fig. 3.4A). DKO mice weighed approximately 25% less than control mice by 12 weeks of age (Fig. 3.4B) and showed gross histological abnormalities in the brain. The hippocampi of DKO mice were compacted and reduced in size even when normalized to the total brain size (Fig. 3.4C). Although cortical migration appeared unaffected in DKO mice, the cortex was thinner compared with control mice (Fig. 3.4C). TUNEL staining showed no difference between control and DKO brain, suggesting that the thinner cortex is not due to a decrease in cell number (data not shown). Immunostaining with NeuN and GFAP antibodies revealed an astrocytic reaction in the DKO brain, suggesting possible brain damage (Fig. 3.4D). Interestingly, we noticed that after tail lifting the DKO

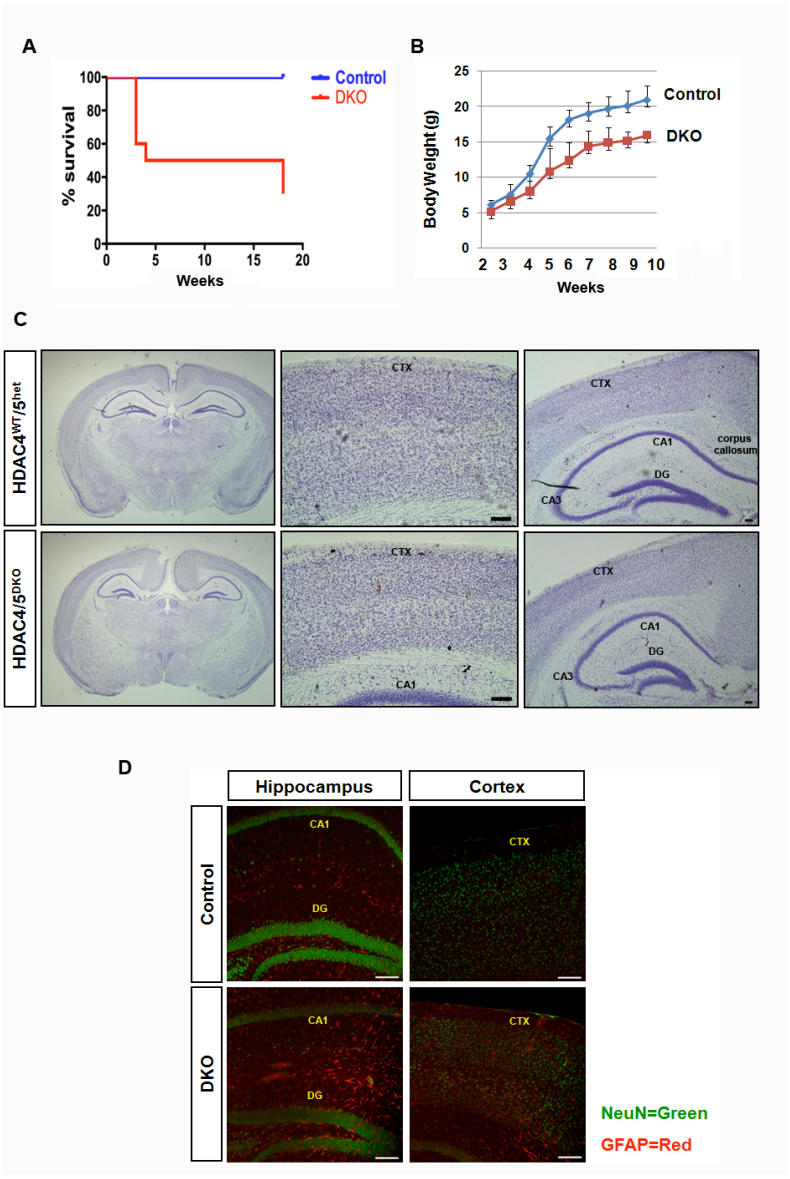

Figure 3.4 Brain deletion of HDAC4 and HDAC5 causes postnatal lethality and neuroanatomical defects

(A) Kaplan-Meier survival curves for mice with brain-specific deletion of HDAC4 and HDAC5 by CaMKII-Cre. (B) Measurement of body weight show decreased body weight in DKO mice. (C) Nissl stained sections of DKO and littermate control mice at 8 months of age. Nissl staining reveals thin cortex and deformed hippocampus in DKO. DG, dentate gyrus; CTX, cortex (D) NeuN and GFAP staining showed astrocytic reaction in the DKO brain.

mice developed seizures, which were captured using electroencephalogram-/electromyogram (EEG/EMG) recordings (Fig. 3.5). Repetitive spike-wave patterns were noted on the EEG, sometimes accompanied by rhythmic slow activity and continuous spike-wave bursting. The mean duration of seizures in DKO mice was 5.34 minutes. Due to the severe seizure activity, behavior testing could not be performed on these mice.

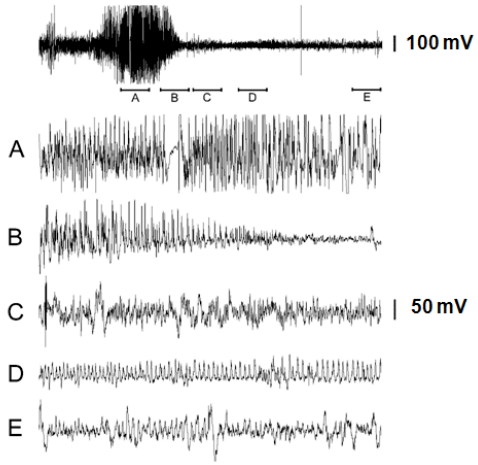

Fig. 3.5 Electroencephalogram/electromyogram (EEG/EMG) recording revealed seizure activity in DKO mice
Top panel: Representative EEG recording showing the onset of seizure activity in DKO mice. Panel A and B represent seizure activity.

Regulation of dendritic arborization in vivo by HDAC4 and HDAC5

spines are the major site of excitatory synaptic input in the mammalian CNS and their morphological changes have been linked to many neuronal processes

including learning and memory. To investigate whether neurological deficits observed in DKO mice were accompanied by physical changes in dendritic morphology, we performed Golgi staining on brain samples from DKO and control littermates and found that the sprouting of dendrites was dramatically reduced in DKO brain (Fig. 3.6A). To measure the number of excitatory synapse in hippocampal neurons, image stacks from z-series of Golgi-stained tissues were acquired, and the density of dendritic spines was calculated. Control neurons had 0.816 ± 0.048 versus 0.782 ± 0.071 spines per mm in DKO hippocampal pyramidal neurons, showing no significant difference in spine density (Fig. 3.6B). Together, these results suggest that HDAC4 and HDAC5 positively regulate dendritic arborization required for the establishment of proper synaptic connectivity in the brain.

Decreased expression of Arc/Arg3.1 in HDAC4^{BKO} mice

To gain insight into the mechanism by which HDAC4 regulates memory formation, we performed a microarray analysis of RNA isolated from hippocampus of DKO and control mice. Surprisingly, we observed very few changes in the overall pattern of gene expression in the DKO hippocampus (Fig. 3.7A). As HDAC4 is expressed both cytoplasmic and nucleus, we surmised that it might modulate protein expression and modification. We used Western blot analysis to profile the expression of candidate proteins reported to regulate

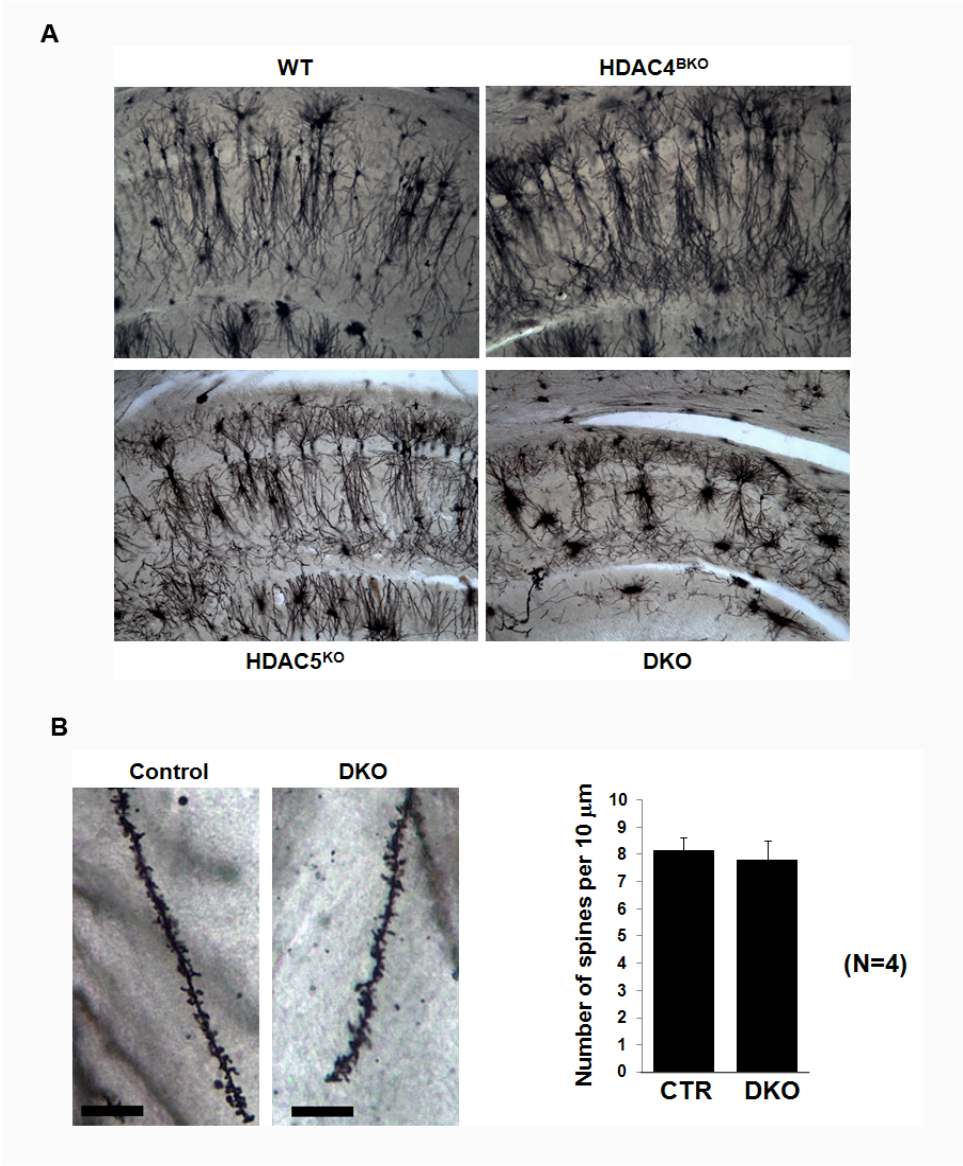

Figure 3.6 Decreased length of dendritic sprouting in DKO brain
(A) Golgi-stained sections on brains from WT, HDAC4^{BKO}, HDAC5 KO, and DKO mice revealed that the dendritic sprouting is decreased in DKO mice. (B) Spine density of CA1 pyramidal neurons' dendrites was determined from Golgi-stained section of Control and DKO mice.

activity or implicated in synaptic remodeling and plasticity, such as postsynaptic density protein 95 (PSD95), synapsin 1, NR2B and activity-regulated cytoskeleton-associated protein (Arc) (Fig. 3.7B and C). Among the candidates examined, only Arc protein expression was down-regulated in the hippocampus and cortex of HDAC4^{BKO} and DKO mice (Fig. 3.7D). Arc is an activity-regulated immediately early gene that undergoes rapid transport to dendrites where it is translated upon synaptic activity (Steward et al., 1998) and is essential for learning and memory (Guzowski et al., 2000; Plath et al., 2006). Although ARC protein expression was decreased, expression of Arc mRNA was not changed in HDAC4^{BKO} and DKO mice, suggesting that HDAC4 regulates Arc expression at the post-transcriptional level (Fig. 3.7E). Together, these data suggest that HDAC4 regulates memory formation, at least in part, through down-regulation of Arc protein.

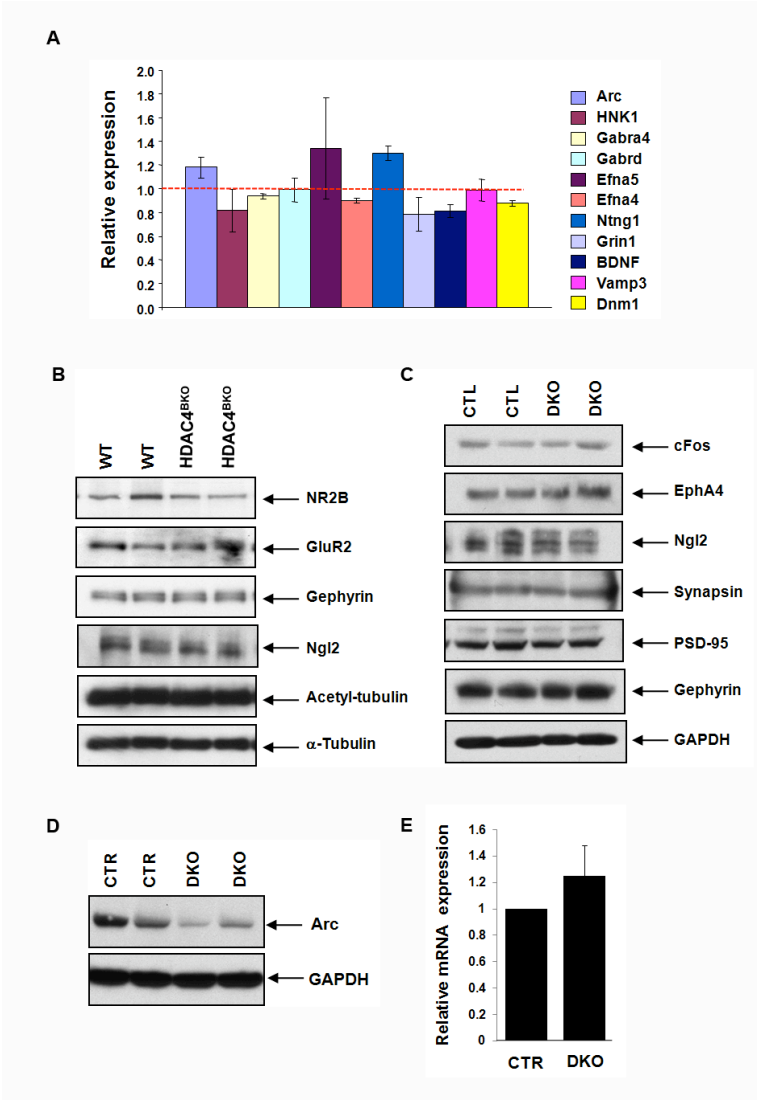

Figure 3.7 Decreased expression of Arc in HDAC4^{BKO} and DKO brain
(A) Quantative RT-PCR showed no changes in the expression level of neuronal-activity regulated genes and genes involved in synaptic plasticity, synaptic formation. Error bars indicate ± standard deviation. (B and C) Western blot analysis from brain lysate showed no change in the expression level of proteins involved in synapse formation and synaptic plasticity in HDAC4^{BKO} and DKO. (D) Western blot analysis from brain lysate revealed the decreased expression of Arc protein in DKO mice. GAPDH was used for loading control. (E) Expression level of Arc mRNA detected by quantitative RT-PCR. Error bars indicate ± SEM.

DISCUSSION

Our results demonstrate that HDAC4 positively regulates memory formation in mice. Although HDAC4 and HDAC5 have redundant functions in the regulation of dendritic arborization, the finding that *Hdac5* KO mice do not show deficits in memory formation indicates that HDAC4 plays a critical role in the regulation of learning and memory among class II HDACs.

Given the fact that HDAC inhibitors enhance learning and memory, together with the recent finding that HDAC2 negatively regulates memory formation, our observations reveal an unexpected positive role of HDAC4 in memory formation (Guan et al., 2009). Whereas HDAC2 is predominantly localized to the nucleus, HDAC4 shuttles between the nucleus and cytoplasm in an activity-dependent manner, suggesting the possibility that cytoplasmic function of HDAC4 is important for the positive regulation of memory formation. This possibility is supported by our finding that deletion of HDAC4 in the forebrain does not induce significant changes in gene expression, at least in the case of neuronal activity-regulated genes (Fig. 3.7A). Consistent with our findings of a cytoplasmic function of HDAC4 in the hippocampus, another group has shown that HDAC4 regulates the survival of retinal neurons due to cytoplasmic activity (Chen and Cepko, 2009). Indeed, we identified a *k*inase that *i*nteracts with *s*tathmin (KIS) as an interaction partner of HDAC4 using a yeast-two hybrid screen. KIS is a serine/threonine kinase functionally related to microtubule

dynamics and axon development. It is of particular interest because recent studies showed that KIS interacts with ribonucleo-protein particles (RNPs)-transported mRNA, including Arc mRNA and enhances their local translation in a kinase activity-dependent manner. We confirmed the interaction between HDAC4 and KIS by coimmunoprecipitation and colocalization experiments (data not shown). To determine if HDAC4 regulates kinase activity of KIS, we performed a kinase assay. As previously reported (Boehm et al., 2002), KIS is autophospohrylated and the level of autophosphorylation is increased when coexpressed with HDAC4, suggesting that HDAC4 potentiates the kinase activity of KIS. These observations, together with our data showing that HDAC4 regulates the expression of Arc at the protein level, leads to the intriguing possibility that HDAC4 regulates the translation of Arc by interacting with and modulating activity of KIS. A detailed molecular mechanism by which HDAC4 regulates the function of KIS needs to be further investigated.

It has been shown that non-selective HDAC inhibitors facilitate learning and memory in wild-type mice as well as in several mouse models of neurodegenerative disease (Fischer et al., 2007; Guan et al., 2009; Korzus et al., 2004). Tsai and colleagues recently identified HDAC2 as a major target of HDAC inhibitors for facilitating learning and memory. Since non-selective HDAC inhibitors can also block the function of HDAC4, it is likely that some beneficial effects of non-selective HDAC inhibitors on memory formation are masked by

the negative effects of HDAC inhibitors through HDAC4. Thus, it will be interesting to develop and examine whether class I HDACs (or HDAC2)-selective inhibitors show improved effects on memory enhancement compared to non-selective HDAC inhibitors.

MATERIALS AND METHODS

Generation of HDAC4 Conditional Knockout Mice

Hdac4 conditional mice (*Hdac4*$^{loxP/loxP}$) were generated by flanking exon 5 with loxP sites, which results in an out-of-frame mutation in the *Hdac4* allele(Potthoff et al., 2007b). To generate mice lacking *Hdac4* in brain, mice heterozygous for the *Hdac4*^{loxP} allele were mated to *CaMKII-Cre* transgenic mice (Luikart et al., 2005) that express cre recombinase specifically in the forebrain after birth. The resulting mice were mated to homozygous *Hdac4* floxed mice (*Hdac4*$^{loxP/loxP}$) to generate forebrain specific conditional null mice (*Hdac4*$^{loxP/loxP}$;*CaMKII-Cre*). *Hdac4* conditional null mice were genotyped by polymerase chain reaction (PCR) using the following primers:

HDAC4-fwd 5’ ATC TGC CCA CCA GAG TAT GTG 3’

HDAC4-rev 5’ CTT GTT GAG AAC AAA CTC CTG CAG CT 3’

nLacZ-rev 5’ GAT TGA CCG TAA TGG GAT AGG TTA CG 3’

Cre A 5’ AGG TTC GTT CAC TCA TGG A 3’

Cre B 5’ TCG ACC AGT TTA GTT ACC C 3’

All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Research Advisory Committee at University of Texas Southwestern Medical Center.

Histology

Mice were perfused and the brains were post-fixed overnight in DEPC-treated 4% paraformaldehyde. The brains were paraffin-embedded, sectioned at 5μm, and mounted on coated slides. Sections were stained with Nissl or Hematoxylin and eosin (H&E) using standard procedures. For in situ hybridization, riboprobes were labeled with [α-35S]-UTP using the MAXIscript in vitro transcription kit (Ambion, Austin, Texas) following the manufacturer's instructions. In situ hybridization of sectioned tissues was performed as previously described (Shelton et al., 2000). TUNEL assay was performed according to manufacturer's instructions (Roche).

RNA isolation and quantitative RT-PCR

RNA was isolated from cortex, hippocampus and cerebellum using RNasy kit (Qiagen) following the manufacturer's instructions. Two μg of RNA was converted to cDNA using random primers and Superscript III reverse transcriptase (Invitrogen). Quantitative analysis was performed by real-time PCR PCR using the ABI PRISM 7000 sequence detection system with TaqMan primers (Applied Biosystems) or with SYBR Green Master Mix reagent (Applied Biosystems).

Golgi Staining

Golgi staining was performed using FD Rapid GolgiStain Kit (FD NEuro Technologies) following the manufacturer's instructions. Briefly, animals were deeply anesthetized and brains were removed from the skull and rinsed in double distilled water. Brains were immersed in the impregnation solution, and stored at room temperature for 2 weeks in the dark. The impregnation solutions were replaced on the next day. Brains were transferred into Solution C and stored at 4 ºC for 1 week in the dark. Solution C was replaced the next day. Brain sections (100 µm thickness) were cut on a cryostat (Leica 430) with the chamber temperature set at -22 ºC. Each section was mounted with Solution C on saline-coated microscope slides. After absorption of excess solution, sections were naturally dried at room temperature. Dried sections were processed following the manufacturer's instructions.

Immunohistochemistry

The fixed brain sections (50 µm thickness) were cut on a cryostat (Leica 430) with the chamber temperature set at -20 °C. Tissues were permeabilized with PBS containing 0.5 % Triton X-100 for 10 min, incubated with blocking solution (PBS containing 0.5% Triton X-100 and 10% goat serum) for 1h at room temperature. Tissues were incubated with primary antibodies, anti-NeuN (1:1000; Affinity Bioreagents) and anti-GFAP (1:200; Sigma) for overnight at 4 °C. Tissues were

washed with PBS containing 0.5% Triton X-100 and incubated with secondary antibodies, anti-mouse-FITC (1:500) and anti-rabbit-Texas RED (1:500) for 1hr at room temperature. Tissues were washed with PBS containing 0.5% Triton X-100, and then mounted on glass slide with Vectashield (Vecta Laboratories). Images were taken using Zeiss confocal microscope.

Western blotting

Tissues were homogenized in extraction buffer and subjected to centrifugation at 12,000*g* for 10 minutes at 4°C. The soluble fractions were collected and 40 μg of proteins were subjected on SDS-PAGE and analyzed by immunoblotting with antibodies against Arc (1:20000; Synaptic systems), Synapsin-1 (1:20000, Synaptic systems), Gephyrin (1: 5000, Synaptic systems), c-Fos (1:1000, Santacruz), EphA4 (1:1000, Santacruz), NR2B (1:1000, Neuromab), GluR2 (1:1000, Neuromab), Ngl2 (1:1000, Neuromab), PSD-95 (1:500, Applied biosystems), acetyl-tubulin (1;2000, Sigma), alpha-tubulin (1:2000, Sigma), and GAPDH (1;3000, Sigma).

Electrophysiology

Electrophysiology was performed as previously described (Monteggia et al., 2004). Briefly, acute hippocampal slices (400 μm) were prepared as coronal sections using vibratome. Slices were transferred to a chamber at room

temperature in an oxygenated artificial cerebrospinal fluid (ACSF), and allowed to recover for 90 min before the recordings.

Behavioral Overview

All behavior testing was done on male mice at least 2 months old, with *Hdac4* floxed mice (n=23) or *Hdac4* floxed mice with *CaMKII-Cre* (n=23). Prior to all testing, mice were allowed to habituate in the behavior room for one hour. All data were analyzed and scored by an observer blind to the genotype. $P<0.05$ was considered as significance.

Locomotor Activity

Locomotor activity was measured as described previously (Monteggia et al., 2004). Briefly, locomotor activity was measured as animals were placed in a fresh home cage and locomotor activity was measured for two hours by four photocell beams linked to computer data acquisition software (San Diego Instruments, San Diego, CA). Total activity was scored as all movement of the animal within the two hours of testing.

Rotarod

Rotarod tests were carried out as previously described (Barbosa et al., 2008). Briefly, each mouse was placed on the rotarod (IITC Life Science, Woodland Hills, CA). The rotarod was activated and its speed ramped up from 0 to 45 revolutions per minute in 60 seconds. The time to fall off the rotarod or turn one

full revolution was measured for three consecutive trials. After the test, the mouse was returned to its original cage for two hours. The test was repeated for a total of 3runs/day for 4 consecutive days.

Elevated Plus Maze

Elevated plus maze was performed as described previously (Monteggia et al., 2004). Briefly, mice were placed in the center of an elevated plus-maze under dim lighting and their behavior was monitored for 5 min using a video tracking system (Ethovision 3.0, Noldus, Leesburg, VA). The time spent in the closed and open arms as well as the latency to enter the open-arms was used as the measure of anxiety-like behavior.

Open Field

Open field was carried as previously described (Monteggia et al., 2004). Briefly, mice were videotaped for their activity during 6 min in a 72 cm diameter open-field under dim lighting. Locomotor activity of the animal, as well as the time spent in the center and borders of the open field, was determined. Anxiety-like behavior was measured as the total time in the center of the open field as well as the latency to enter the center of the field.

Fear Conditioning

Fear conditioning paradigm was performed as described previously (Powell et al., 2004). Briefly, mice were placed in individual chambers (Med Associates, St. Albans, VT) with four mice tested at once. Mice were placed in the chambers for

2 minutes followed by a loud tone (90 dB) for 30 seconds then immediately followed by a 0.8 mA footshock for 2 seconds. Mice remained in the box for one minute at which time they received the same tone-paired footshock. The mice were immediately removed and placed back into their home cages. To test for context-dependent fear conditioning, 24 hours later, mice were placed back in the same boxes without a tone or shock and their behavior was videotaped for 5 min. The amount of time the animal spent freezing was assessed by the FreezeFrame program (Actimetrics, Wilmette, IL). Freezing behavior was defined as no movement except for respiration. Four hours later, the cue test was performed. To test for cue-dependent fear conditioning, mice were placed in a novel environment scented with vanilla odor with no tone or shock for three minutes followed by three minutes of the tone. The amount of time the mice spent freezing was assessed as described above. Cue-dependent fear conditioning was determined by subtracting the three minute baseline freezing from the freezing during the tone.

Pain Sensitivity

Pain sensitivity was measured as previously described (Monteggia et al., 2004). Briefly, mice were placed in individual chambers (MedAssociates) for two minutes to habituate. The animals were then shocked (0.05 mA for 1 sec) and their behavior was scored as no movement, flinching, or jumping. Every 30 seconds, the shock was increased by 0.05 mA, with a maximum shock of 0.6 mA, until the animal flinched and jumped in response to the shock.

Moris water maze

Moris water maze tests were performed as previously described (Powell et al., 2004). Time spent in each quadrant was obtained using automated video tracking software from Noldus (Ethovision 2.3.19).

Electroencephalogram/electromyogram (EEG/EMG) recording

DKO(n=4) and control mice (n=4) were anesthetized and surgically implanted for long-term EEG/EMG monitoring as previously described (Chemelli et al., 1999). Mice were housed individually under a 12 hr light-dark cycle at 24±1°C, with food and water being replenished as necessary each day, but mice were not otherwise disturbed. They were habituated to the recording condition for 2 weeks before EEG/EMG signals were recorded over a period of 3 days, beginning at lights-off (CT 12:00). During the light period on the third day of the recording, sound and tactile stimuli were used to examine sensitivity to induced seizures. Mice were exposed to 10 min of an intense sound stimulus (keys were shaken vigorously 50cm above the floor of an open-topped observation cage) followed by 3 min of tactile stimulus. Subsequently, the EEG/EMG record was visually screened for seizures. Seizure was characterized as a spike-wave pattern on the EEG, accompanied by atonic periods or sustained rhythmic contractions on the EMG. Each seizure lasting for 2 second or more was noted.

Chapter IV

Roles of myocyte enhancer factor 2 (MEF2) in brain

Abstract

Neuronal activity modulates neuronal circuit development by regulating gene expression and ultimately mediating behavioral responses of the animal. Myocyte enhancer factor 2 (MEF2) transcription factors have been shown to be key regulators of neuronal gene expression in an activity-dependent manner *in vitro*. An increase in neuronal activity activates MEF2 transcriptional activity through various calcium-regulated signaling pathways. Activated MEF2 promotes the transcription of a set of genes that suppress excitatory synapse number. However, the function of MEF2 *in vivo* is largely unknown. Here I show that MEF2 transcription factors function distinctively and redundantly in the regulation of memory formation and sleep. I found that deletion of MEF2C in the central nervous system causes impairments in memory formation, whereas deletion of MEF2A and MEF2D does not, suggesting that MEF2C is the major isoform of MEF2 responsible for memory formation. Furthermore, we found that deletion of MEF2A, MEF2C, and MEF2D causes a decrease in REM sleep, brief spontaneous seizures, and postnatal lethality accompanied by increased neuronal apoptosis. Consistent with these findings, genome-wide transcriptional analysis showed that MEF2 isoforms redundantly regulate target genes involved in synapse development. Taken together, these results demonstrate that MEF2 transcription factors function distinctively and redundantly in the maintenance of the central nervous system.

Introduction

In response to sensory stimuli, neurons display a high degree of plasticity to regulate proper development of neural circuits that underlie complex behaviors, including learning and memory (Greer and Greenberg, 2008). Transcriptional regulation is one important mechanism by which sensory experience induces changes in neural circuits. Experience-driven synaptic activity leads to calcium influx into neurons and subsequent activation of calcium-dependent signaling pathways, resulting in the activation of a gene expression program that coordinates synaptic development and function. Several transcription factors are involved in neuronal activity-dependent transcription in neurons, including members of the myocyte enhancer factor 2 (MEF2) family of transcription factors (Flavell et al., 2006; Greer and Greenberg, 2008).

MEF2 transcription factors are known to regulate the development and function of a wide range of tissue types, including skeletal muscle, heart, neural crest, bone, and vasculature (Potthoff and Olson, 2007). Several lines of evidence, mostly from *in vitro* studies, suggest that MEF2 also regulates the development and function of the brain. First, MEF2 is highly expressed in the brain with distinct, but overlapping, temporal and spatial expression patterns of each isoform (*Mef2*a, b, c, and d) (Lyons et al., 1995). Second, MEF2 regulates neuronal activity-dependent cell survival (Mao et al., 1999). Third, in response to neuronal activity, MEF2 promotes the transcription of a set of genes, including Arc

(activity-regulated cytoskeletal-associated protein) and synGAP (synaptic RAS GTPase-activating protein), that suppress excitatory synapse number (Flavell et al., 2006). Fourth, *in vitro* genome-wide analysis of MEF2 transcriptional activity identified several activity dependent MEF2 target genes that regulate a variety of aspects of synaptic function, including excitatory synapse weakening, excitatory synapse maturation, inhibitory synapse development, and presynaptic vesicle release (Flavell et al., 2008). Finally, brain-specific deletion of MEF2C in mice impairs hippocampus-dependent learning and memory, accompanied by a marked increase in the number of excitatory synapse (Barbosa et al., 2008). In spite of these findings, the precise roles of MEF2 isoforms in the development and function of the brain *in vivo*, remain largely unknown.

Synaptic activity can regulate a number of functions in the brain other than learning and memory, such as sleep. Recent genetic studies suggest that decreased neuronal excitability of large sets of neurons increases sleep, whereas increased neuronal excitability decreases it (Cirelli, 2009). For example, flies carrying a mutation in *Shaker,* encoding *Drosophila* α-subunit of a tetrameric potassium channel, sleep only 2-4h every day rather than the normal 8-10 h (Koh et al., 2008). Mice lacking mammalian homologues of *Shaker*, Kv1.2 or both Kv3.1 and Kv3.2, also sleep less than wild-type mice (Douglas et al., 2007; Espinosa et al., 2004). In addition, it has been shown that several neurotransmitters, which eventually modulate synaptic activity within neural circuits, affect sleep quantity

and quality (Cirelli, 2009). The finding that MEF2 regulates synaptic activity in a neuronal activity-dependent manner and that MEF2 is expressed in the crucial brain regions responsible for the regulation of sleep, such as cortex and thalamus, leads to the intriguing possibility that MEF2 may regulate sleep quality or quantity.

To examine the roles of each MEF2 isoform in the development and function of the brain, we generated mice with conditional deletions of *Mef2a* and *Mef2d* in the brain. We found that deletion of both *Mef2a* and *Mef2d* in the brain of mice results in no apparent behavioral abnormalities, including learning and memory. Consistent with this, *in vivo* genome-wide analysis of the MEF2 transcriptional program indicated that there is little, if any, change in the expression of genes involved in synaptic function. We also found that mice with deletion of *Mef2a*, *Mef2c* and *Mef2d* in the brain showed a decrease in REM sleep, brief spontaneous seizures, and postnatal lethality accompanied by increased apoptosis, suggesting redundant roles of MEF2 transcription factors in the regulation of brain function. Together, our findings suggest that MEF2C, but not MEF2A and MEF2D, is important for the regulation of learning and memory, whereas other MEF2 isoforms function redundantly in other aspects of brain function.

Result

Generation of a conditional Mef2a allele

Mice homozygous for a *Mef2a* null mutation die suddenly within the first week of life due to defects in cardiac function (Naya et al., 2002), precluding an analysis of potential functions of *Mef2a* in the adult brain. To investigate the potential roles of *Mef2a* in the adult brain, we generated a conditional *Mef2a* null allele (referred to as $Mef2a^{loxP}$) that contains loxP sites in the introns flanking the second coding exon encoding the MADS- and MEF2-specific domains, which mediate DNA binding, dimerization and cofactor interactions (Fig. 4.1A). Deletion of the genomic region between the loxP sites inactivates the *Mef2a* gene. Mice homozygous for the $Mef2a^{loxP}$ allele showed no apparent abnormalities.

Brain-specific deletion of MEF2A

We deleted *Mef2a* specifically in the central nervous system (CNS) by breeding $Mef2a^{loxP/loxP}$ mice with transgenic mice that express Cre recombinase under the control of the human GFAP promoter (hGFAP::Cre), which is expressed in neuronal progenitor cells during late embryogenesis (Zhuo et al., 2001). Mice with homozygous deletion of *Mef2a* in the brain (herein referred to as $Mef2a^{BKO}$) were viable, fertile, and reached the same age and body weights as their littermates. Deletion of the $Mef2a^{loxP}$ allele in the brains of mutant mice was

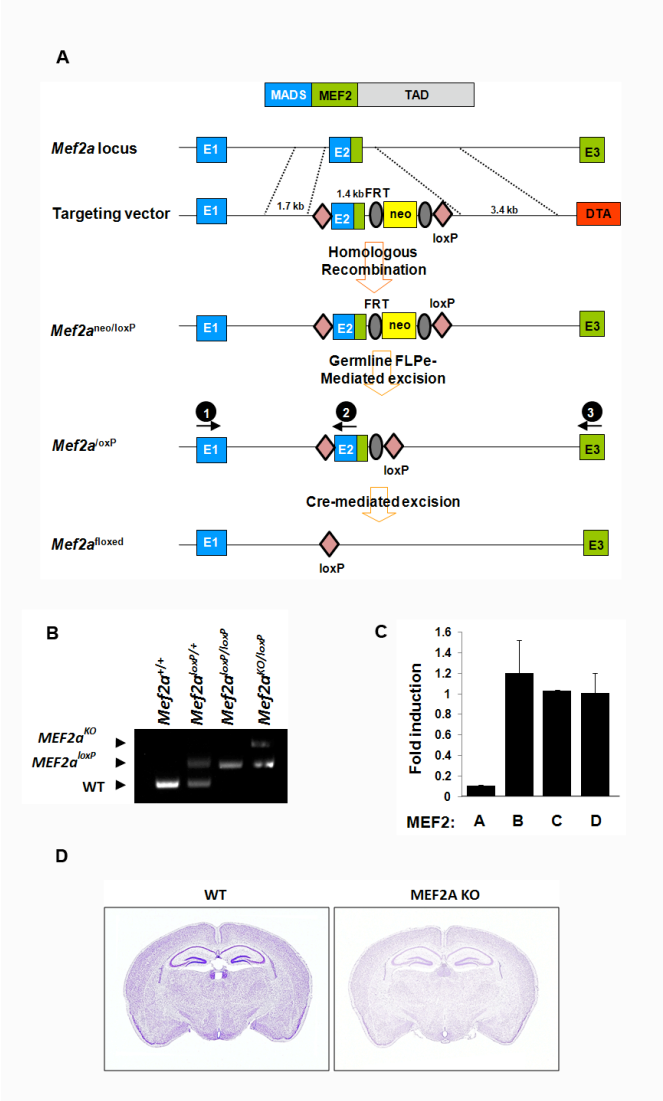

Figure 4.1 Generation of mice with a conditional *Mef2a* mutation
(A) Strategy to generate a conditional MEF2A allele. *loxP* sites were inserted into introns 1 and 2 through homologous recombination. Cre-mediated excision results in one *loxP* site in the place of exons 1-3. (B) PCR genotyping to distinguish different Mef2a alleles. The positions of primers are labeled (1, 2 and 3) and are circled in A. (C) Expression level of Mef2 detected by quantitative PCR. Error bars indicate standard deviation. (D) Histological section and Nissl staining of the brain of WT and $MEF2A^{loxP/loxP}$; *hGFAP-Cre* mice at 2 months of age. Brain-specific deletion of MEF2A was achieved by crossing $MEF2A^{loxP/loxP}$ mice with mice harboring transgene for *hGFAP-Cre.*

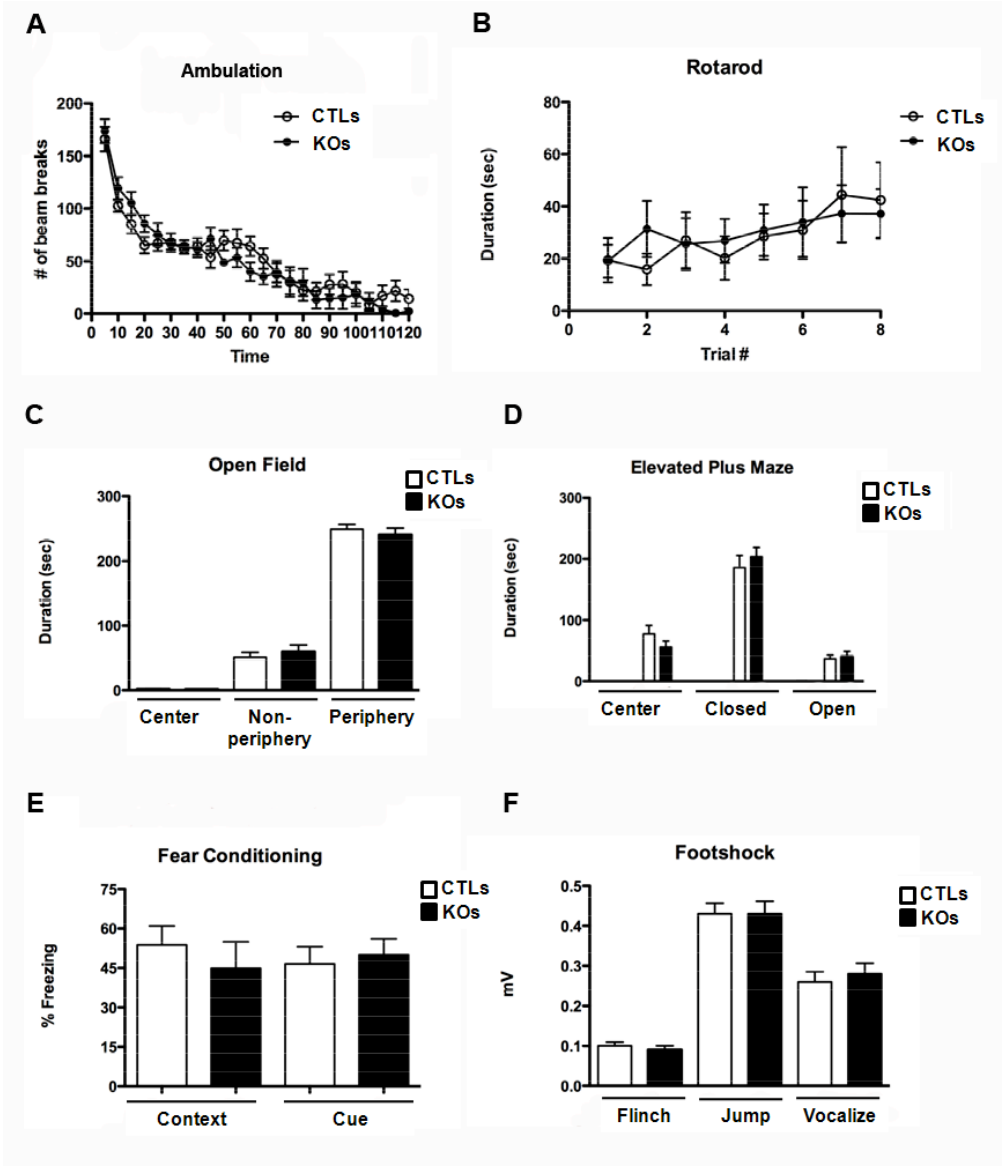

Figure 4.2 *Mef2a*^{BKO} mice show normal behavior
(A) *Mef2a*^{BKO} mice show no significant difference in locomotor activity, as assessed by consecutive beam breaks over two hours (WT, n=8; *Mef2a*^{BKO} mice, n=9). (B) *Mef2a*^{BKO} mice display normal motor coordination. (C and D) *Mef2a*^{BKO} mice exhibited normal anxiety-like behavior, as assessed in the open field and elevated plus maze. (E) Context- and cue-dependent fear conditionings are unaltered in *Mef2a*^{BKO} mice. (F) Baseline freezing behavior or pain sensation is unaltered in *Mef2a*^{BKO} mice.

confirmed by real-time PCR (Fig. 4.1C). Histological analysis by Nissl staining of brain sections revealed no obvious abnormalities in size, morphology, or density of neurons in various brain regions (Fig. 4.1D). Since MEF2A and MEF2D have been shown to regulate many neuronal activity-regulated genes *in vitro* (Flavell et al., 2006; Flavell et al., 2008), we investigated the potential involvement of *Mef2a* in global behavior using several behavioral paradigms. $Mef2a^{BKO}$ mice exhibited no apparent abnormalities in several behavior paradigms, including locomotor activity, anxiety-related behavior, and context- and cue-dependent fear conditioning (Fig. 4.2). These results suggest that MEF2A is dispensable for the regulation of global behavior, including learning and memory.

Brain-specific deletion of MEF2A and MEF2D

The fact that knockdown of MEF2A or MEF2D alone did not cause a significant increase in excitatory synapse numbers, but the knockdown of both MEF2A and MEF2D did increase excitatory synapse number *in vitro* (Flavell et al., 2006), suggests that MEF2A and MEF2D function redundantly. Therefore, the lack of behavioral abnormalities in $Mef2a^{BKO}$ mice may be due to functional redundancy between MEF2A and MEF2D. To examine this possibility, we generated *Mef2a* and *Mef2d* double brain knockout mice by breeding $Mef2a^{BKO}$ mice with *Mef2d* conditional mice ($Mef2d^{loxP/loxP}$) (Kim et al., 2008). Mice homozygous for a *Mef2d* null allele are viable and showed no neurological deficits (unpublished

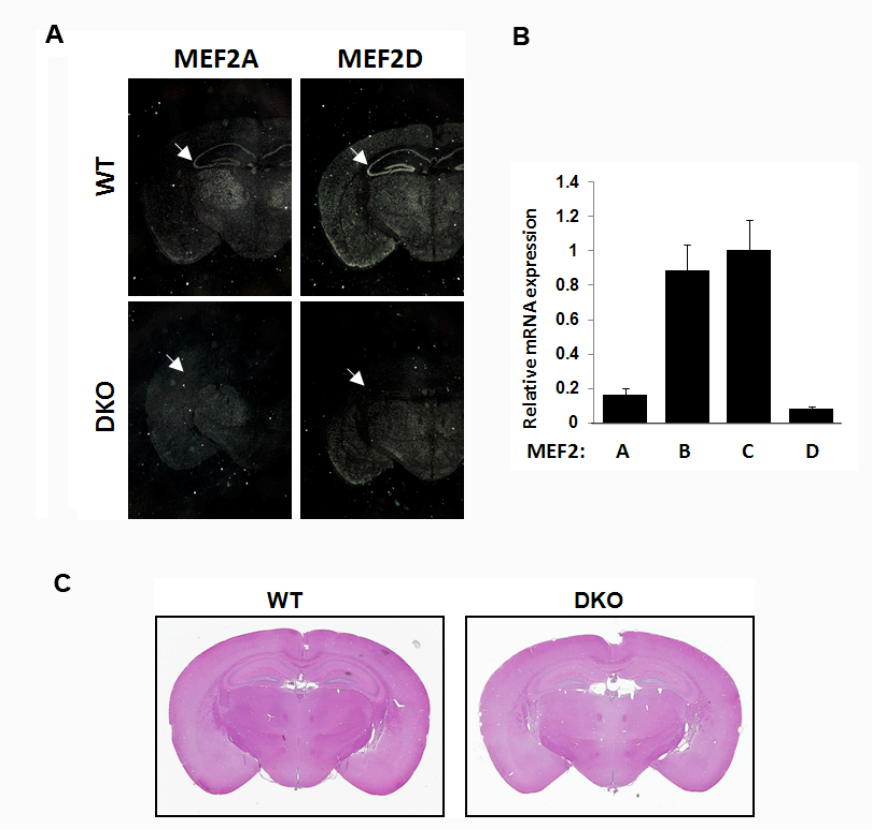

Figure 4.3 Analysis of brain-specific MEF2A and MEF2D knockout mice
(A) Detection of *Mef2a* and *Mef2d* transcripts by in situ hybridization in wild type (WT) and *Mef2a;Mef2d* double brain-specific knockout mice (DKO). Arrow indicates hippocampus. (B) Expression level of MEF2 transcription factors as detected by quantitative PCR. Error bars indicate standard deviation. (C) H&E staining on brain sections from WT and DKO mice at 2 months old age shows normal gross brain morphology.

data). Deletions of *Mef2a* and *Mef2d* alleles in the brain were confirmed by *in situ* hybridization and RT-PCR (Fig. 3A and B). *Mef2a;Mef2d* double brain-specific knockout mice (referred to as DKO) were viable to adulthood and weighed similar to their littermates. Histological analysis by H&E staining revealed no apparent change in size, morphology, or density of neurons (Fig. 4.3C). To

investigate the role of MEF2A and MEF2D in global behavior, DKO mice were subjected to a variety of behavioral paradigms. Unexpectedly, DKO mice revealed no apparent behavioral abnormalities including locomoter activity, anxiety related activity, and context- and cue-dependent fear conditioning (Fig. 4.4). These results, in contrast to the critical role of MEF2A and MEF2D in synaptic development *in vitro*, suggest that MEF2A and MEF2D are not essential for memory formation *in vivo*.

Deletion of MEF2A and MEF2D does not alter synaptic number and function

To determine whether the development of synapses was affected in DKO mice, we investigated the dendritic spine density for CA1 pyramidal neurons by Golgi staining. The number of spines per dendrite in pyramidal neurons was not significantly changed in DKO mice (Fig. 4.5A). To determine whether MEF2A and MEF2D regulate the synaptic plasticity *in vivo*, we performed electrophysiological recordings on hippocampal slices prepared from DKO mice and their littermates. No significant difference between wild type and DKO mice was observed in long-term potentiation (LTP) of CA1 neurons induced by stimulating the Schaffer collateral pathway (Monteggia et al., 2004) (Fig. 4.5B). We next examined whether MEF2A and D regulate basal synaptic properties *in*

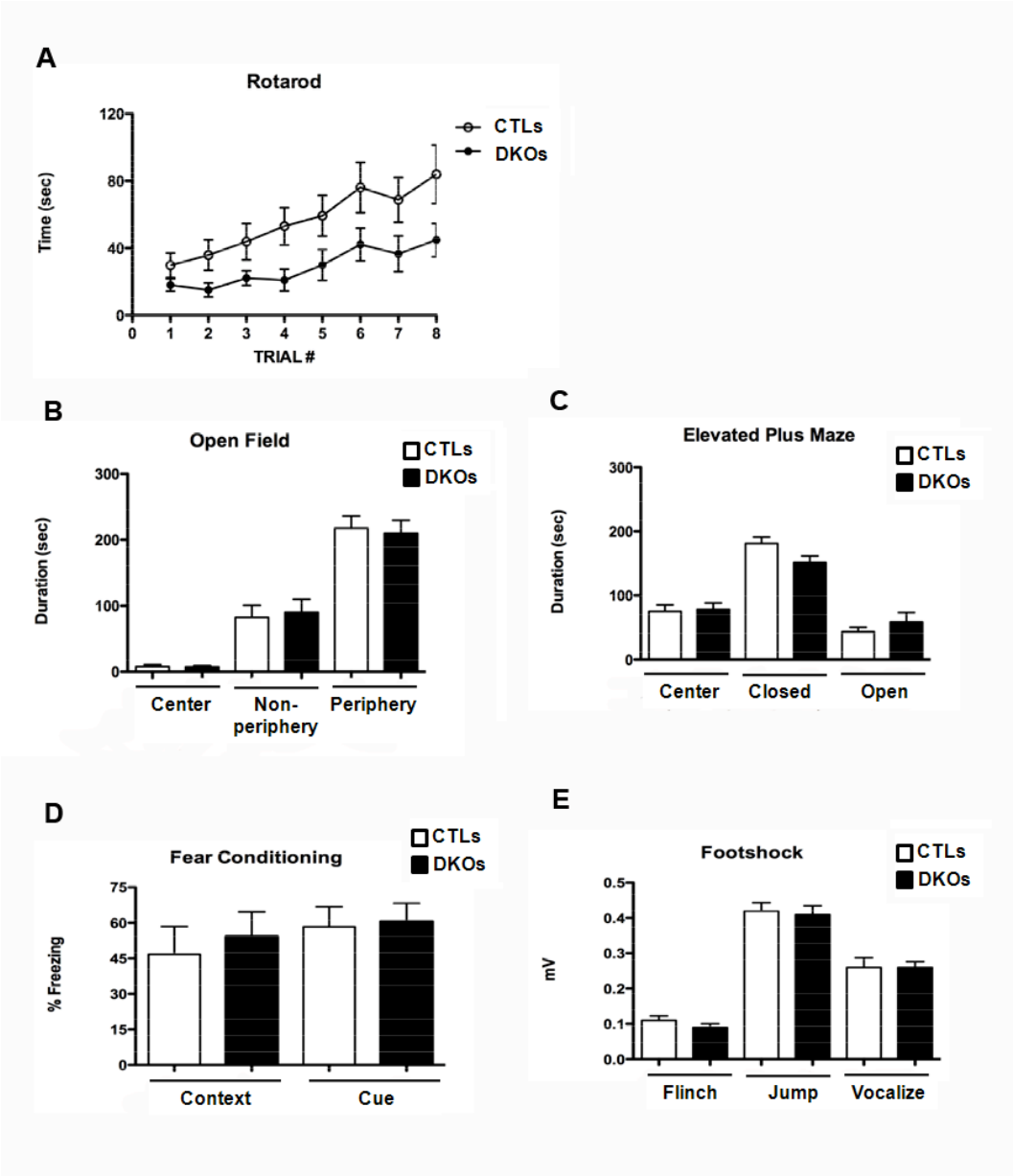

Figure 4.4 Normal global behavior in *Mef2a;Mef2d* double brain-specific knockout (DKO) mice

(A) DKO mice exhibit motor coordination deficit seen as falling off faster from an accelerating rotarod (WT, n=8; DKO, n=10). (B and C) DKO mice show normal anxiety-like behavior. (D) DKO mice show normal associative learning, assessed by context- and cue-dependent fear conditioning.

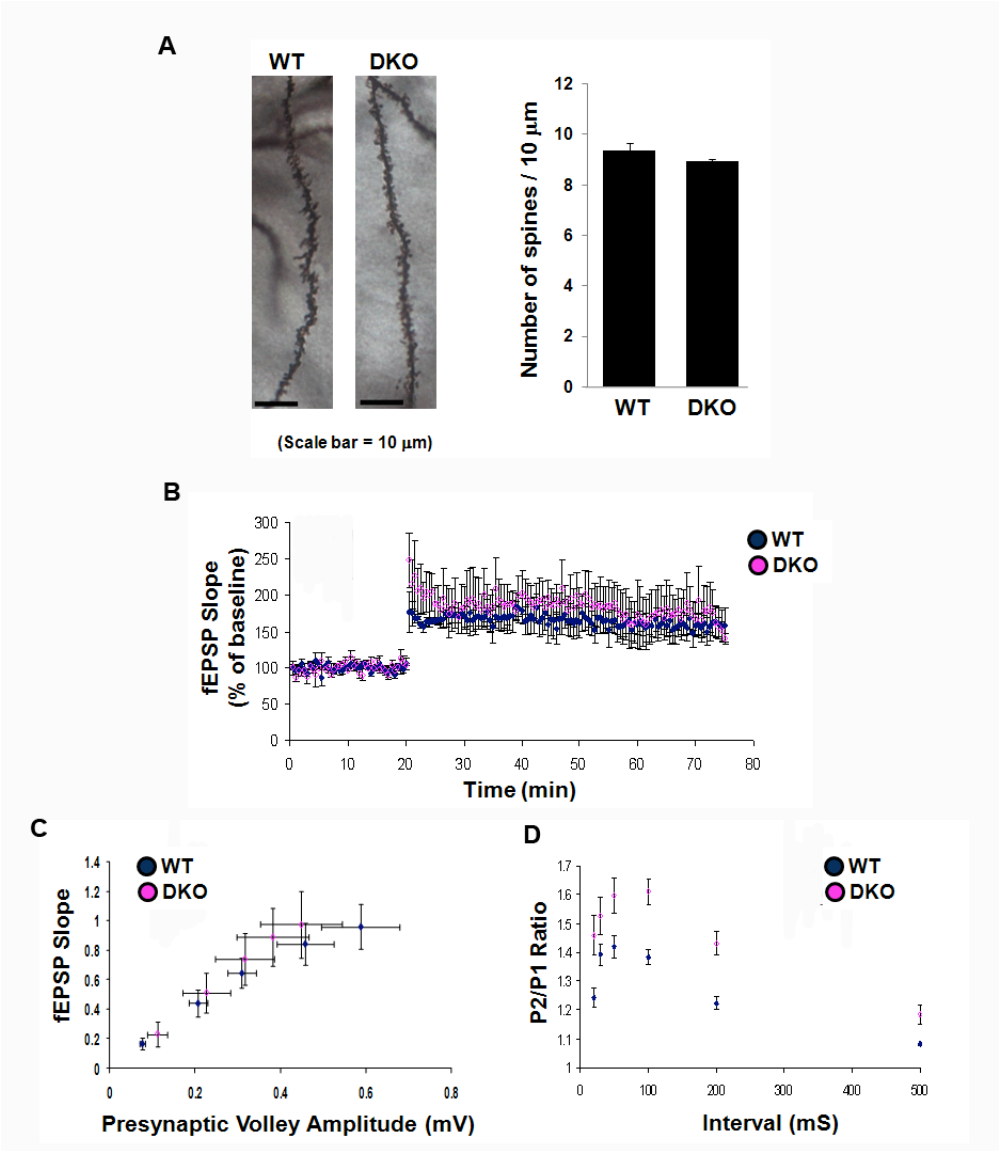

Figure 4.5 MEF2A and MEF2D are dispensable for synapse formation and plasticity

(A) Representative images of Golgi-stained CA1 pyramidal neurons from wild type (WT) and *Mef2a;Mef2d* double brain-specific knockout (DKO) mice (WT, n=2; DKO, n=4). (B) Schaffer collateral LTP is normal in DKO mice (WT, n=8; DKO, n=7). (C) Input-output curve is normal in DKO mice. (D) paired-pulse facilitation is increased in DKO mice.

vivo. The field excitatory post-synaptic potential (fEPSP) input-output function was unchanged in DKO mice. However, paired-pulse facilitation, an index of release probability, was increased in DKO mice, suggesting that MEF2A and MEF2D regulate neurotransmitter release at presynaptic sites. Taken together, these results demonstrate that MEF2A and MEF2D do not regulate synapse development and plasticity *in vivo*.

Brain specific deletion of MEF2A, MEF2C and MEF2D

Mice lacking MEF2A and MEF2D in the developing CNS show no deficit in learning and memory, suggesting that other MEF2 isoforms may play an important role in memory formation. In fact, MEF2C is highly expressed in the brain regions responsible for memory formation, such as cortex and dentate gyrus, and mice lacking MEF2C in the CNS display a deficit in context-dependent associative learning. However, brain-specific MEF2C knockout mice showed normal cue-dependent associative learning, raising the question whether MEF2 transcription factors function redundantly in the brain. To address this possibility, we generated brain-specific deletions of MEF2A, MEF2C, and MEF2D using *GFAP-Cre*. Triple deletion (designated TKO for triple conditional brain knockout) resulted in decreased body weight and partial postnatal lethality by 5 weeks old age (Fig. 4.6A and B). Histological analysis by H&E staining of

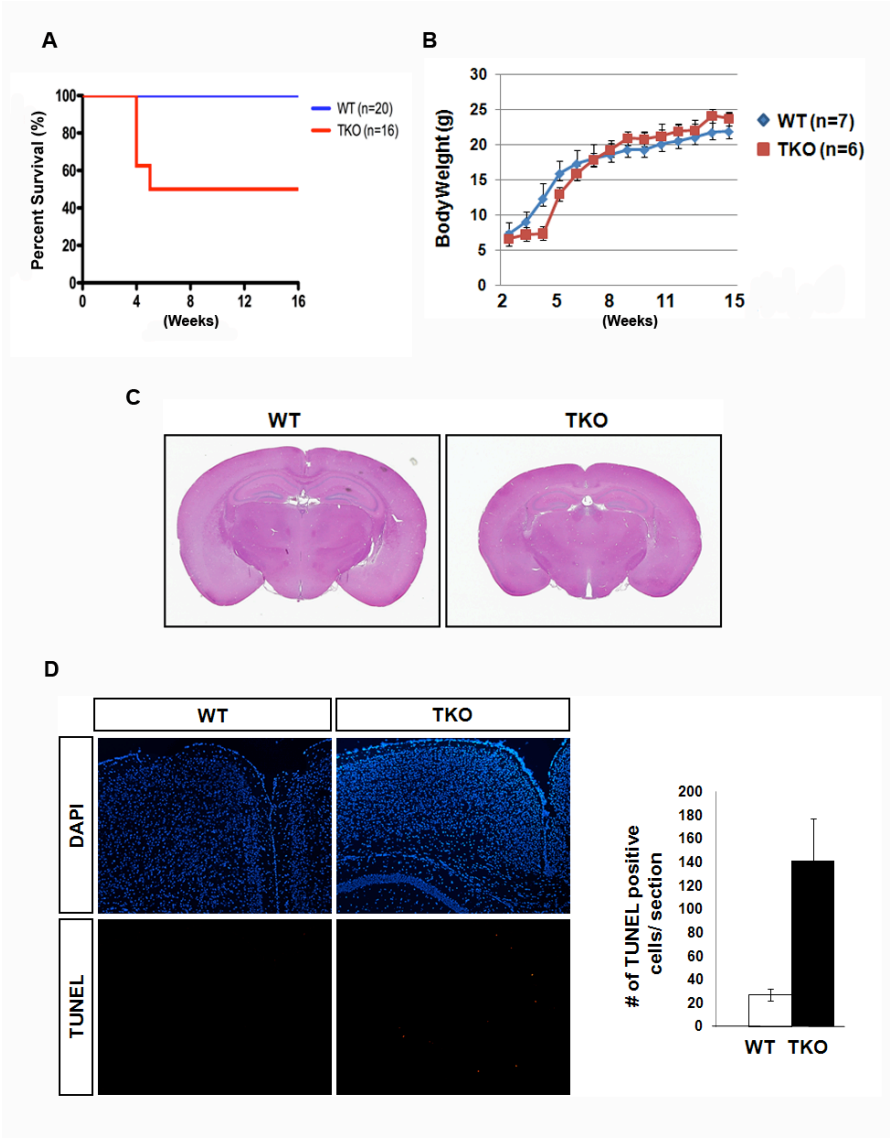

Figure 4.6 Generation of brain-specific MEF2A, C and D triple knockout (TKO) mice

(A) Kaplan-Meier survival curves for TKO mice. (B) Measurement of body weight of WT and TKO mice show a decreased weight before 5 weeks old age in TKO mice (WT, n=7; TKO, n=6). (C) H&E staining of coronal sections from WT and TKO mice at 1 month old age. (D) TUNEL staining of sections from WT and TKO brains at 1 month old age shows an increased apoptosis in TKO mice (WT, n=2; TKO, n=2). Error bars indicate standard deviation.

brain sections showed decreased brain size, but no obvious abnormalities in morphology of neurons in various brain regions (Fig. 4.6C). However, TUNEL staining revealed an approximately 5-fold increase in the levels of apoptosis in TKO mice compared to wild type or DKO mice ($P<0.014$), suggesting that MEF2A, C, and D redundantly regulate neuronal survival (Fig. 4.6D).

MEF2 regulates sleep behavior

Since MEF2 transcription factors mediate stress- or activity-dependent remodeling in other tissues (Kim et al., 2008; Potthoff et al., 2007b), we speculated that MEF2 might mediate stress responses in the brain. To investigate the potential role of MEF2 in epilepsy, we performed the electroencephalography /electromyography (EEG/EMG). During 72 h of continuous EEG monitoring, two out of four TKO mice displayed brief spontaneous seizures (30-40 sec in duration with a 3 min post-ictal period, n=4). Unexpectedly, we also observed a decrease of sleep duration in TKO mice. TKO mice have significantly less rapid-eye-movement (REM) sleep (58.8 ± 2.3 min/24h versus 72.8 ± 4.6 min/24h in TKO versus WT mice, respectively; $P<0.01$) (Fig. 4.7A). The reduced REM sleep occurs during both the light and dark phases. The mean REM sleep episode duration is not different from that of WT mice, indicating that the reduced time spent in REM sleep reflects fewer REM sleep episodes and the mean interval between REM sleep episodes is greater in the TKO mice (Fig. 4.7C). Consistent

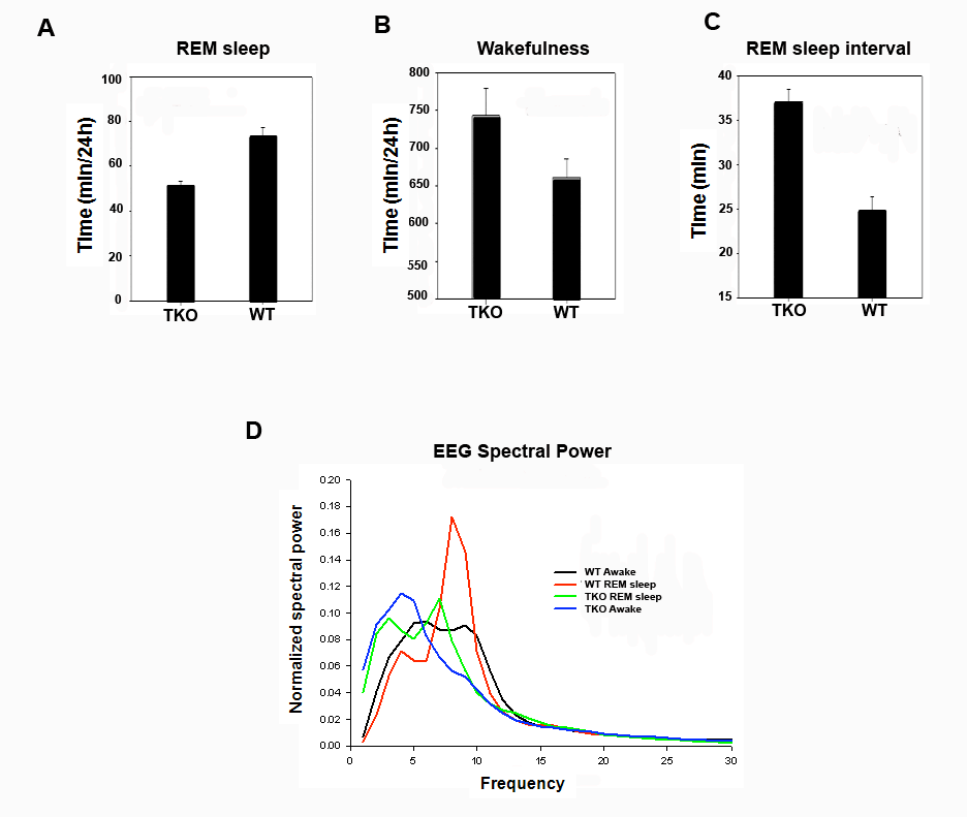

Figure 4.7 Characterizations of sleep-wake behavior in WT and TKO mice
(A) Duration of REM sleep is decreased in TKO mice (n=4 for each genotype). (B) Duration of wakefulness is significantly increased in TKO mice. (C) Interval of inter-REM sleep is increased in TKO mice. (D) EEG spectral power showed decreased REM theta power in TKO mice.

with this observation, the wakefulness is greater in TKO mice (747 ± 53 min/24h versus 661 ± 25 min/24h in TKO versus WT mice, respectively; $P<0.05$) (Fig. 4.7B). There was no significant difference in non-REM sleep time (Data not shown). Furthermore, EEG spectral analysis revealed a significant reduction in the power of the theta frequency band (8-10 Hz) during REM sleep in the TKO

mice (Fig. 4.7D). Since EEG theta power in mice is generated in the hippocampus, this finding suggests that the sleep disorder observed in TKO mice may result from a functional deficit in the hippocampus.

Down-regulation of genes involved in neuronal activity

To further understand the molecular mechanisms of postnatal lethality and sleep abnormalities observed in TKO mice, we performed expression profiling of 4 week-old TKO brains. Surprisingly, only a small portion of the total transcripts was dysregulated with 1.0% (198 genes) of unique and annotated transcripts down-regulated and 1.1 % (219 genes) up-regulated when a cutoff of 1.5-fold change was applied. Gene ontology analysis of down-regulated transcripts in TKO brain revealed that the most significantly enriched down-regulated genes are classified as signal transduction (Fig. 4.8A). We found that most of the genes involved in activity-dependent synaptic development are downregulated in TKO mice, but not in either DKO or MEF2C KO mice (data not shown), suggesting that MEF2 redundantly regulates gene expression *in vivo* (Fig. 4.8B). Furthermore, although the previous *in vitro* genome-wide analysis of the MEF2 transcriptional program revealed MEF2 target genes mutated in human neurological disorders including *c3orf58, Ube3a, Lgi1,* and *PCDH10* (Flavell et al., 2008), these genes were not downregulated in either TKO or DKO mice (data not shown). Taken together, our results demonstrate that MEF2 transcription

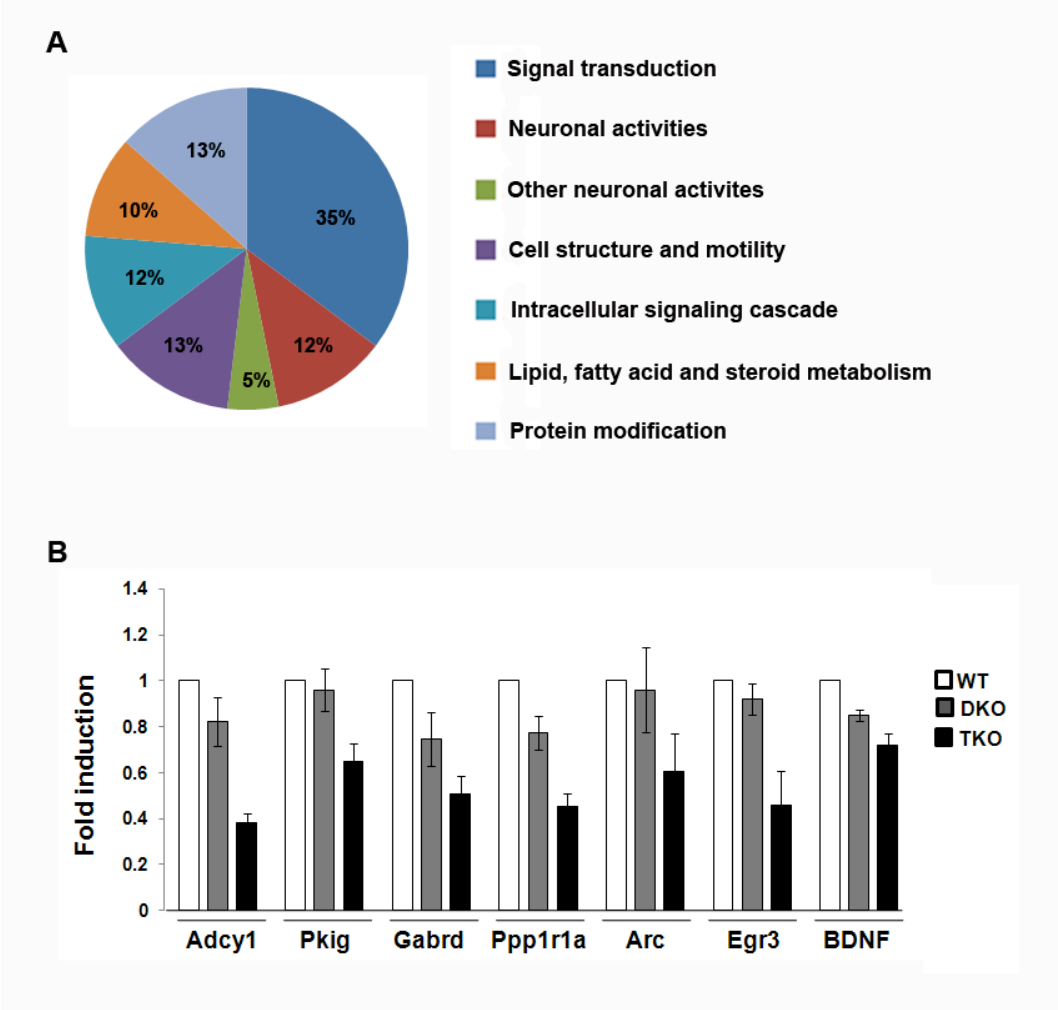

Figure 4.8 Analysis and validation of MEF2 target genes
(A) Gene ontology analysis was performed with PANTHER. Significantly ($P<0.05$) enriched biological processes are shown. (B) Quantitative RT-PCR analysis of transcript levels from WT, DKO, and TKO hippocampi at 1 month old age. Neuronal activity-regulated genes are dysregulated in the TKO brain.

factors function redundantly to regulate gene expression and synaptic function in the brain.

Discussion

Distinctive and redundant roles of MEF2 transcription factors in synapse development and brain function

Given the previous *in vitro* studies showing that *Mef2a* and *Mef2d* are the major isoforms that negatively regulate excitatory synapse (Flavell et al., 2006), it is striking to find that mice lacking *Mef2a* and *Mef2d* in the CNS have intact memory formation and synaptic plasticity. Consistent with this, we found that there are few, if any, changes in the expression of genes involved in synapse development in DKO mice. These contradictory results could be due to the possibility that other MEF2 transcription factors, for example MEF2C, may contribute redundantly to the regulation of synapse development *in vivo*. In fact, several lines of evidence indicate that this is the case. Whereas MEF2A and MEF2D are predominantly expressed in cultured hippocampal neurons (Flavell et al., 2006), MEF2C is highly expressed in the adult brain regions responsible for memory formation including cortex and dentate gyrus. Furthermore, our previous studies showed that deletion of MEF2C in the developing CNS results in deficits in context-dependent associative learning, accompanied by an increase in the number of excitatory synapses (Barbosa et al., 2008), suggesting that MEF2C is the major isoform that contributes to synapse development and brain function *in vivo*.

Although MEF2A or MEF2D alone does not regulate synapse development and brain function in *vivo*, it seems that they function redundantly with MEF2C to regulate gene expression involved in synapse development and brain function. Our genome-wide analysis of the MEF2-dependent transcriptional program revealed that most of the genes involved in activity-dependent synaptic development are down-regulated in TKO mice, but not in either DKO or MEF2C KO mice (data not shown). MEF2C KO mice showed deficits in memory formation, limited only to a specific type of memory, context-dependent associative learning. In contrast, TKO mice showed more severe behavioral abnormalities including brief spontaneous seizures and shortened sleep duration. These data strongly suggest that MEF2 transcription factors function redundantly in synapse development and brain function. It will be interesting to examine whether TKO mice show a broad range of deficits in memory formation.

MEF2 regulates sleep

Sleep is a fundamental biological process conserved from worms to mammals. Since the first report in 1992 showing a sleep disorder is caused by a genetic mutation (Medori et al., 1992), several genes have been shown to affect sleep across species. These genes can be grouped into four major functional categories, ion channels, circadian genes, neurotransmitters, and hormones. Despite this significant progress, little is known about the transcription factors involved in the

regulation of sleep except in a very few cases, such as circadian transcription factors, CLOCK and BMAL1 (Cirelli, 2009). Here we showed that MEF2 transcription factors regulate sleep duration. TKO mice showed decreased REM sleep duration and concomitant increased wakefulness duration without affecting non-rapid eye movement (NREM) sleep. Currently, it is unknown whether MEF2 affects circadian or homeostatic regulation of sleep.

Although the molecular mechanisms by which MEF2 regulates sleep are unknown, several findings suggest that MEF2 may regulate sleep through either the overall regulation of neuronal excitability or the regulation of circadian rhythmicity. Recent genetic studies showed that mutations that can significantly change the balance between inhibitory and excitatory neurotransmission and the overall neuronal excitability of large sets of neurons affects the quantity and quality of sleep as exemplified in *Shaker*, *sleepless* and *Kv3.1* or *Kv3.3* mutants (Douglas et al., 2007; Espinosa et al., 2008). Since MEF2 negatively regulates excitatory synapse numbers so as to affect the balance between inhibitory and excitatory neurotransmission or the overall neuronal excitability, this might be the mechanism by which MEF2 regulates sleep. Among MEF2 targets we found, F-box and leucine-rich repeat protein 3 (Fbxl3) is particular interesting with regard to sleep regulation by MEF2. Fbxl3 is a component of the SKP1-CIL1-F-box-protein (SCF) E3 ubiquitin ligase complex and known to modulate mammalian circadian rhythmicity by regulating the circadian clock protein, Cryptochrome

proteins (Godinho et al., 2007; Siepka et al., 2007). Mice bearing a mutation in Fbxl3 display an increased circadian periodicity. Since circadian genes are one of the major functional categories that affect sleep, it will be interesting to test whether MEF2 regulates sleep through the regulation of circadian rhythmicity. Although it is not known whether Fbxl3 KO mice have defects in sleep, double KO mice of their targets, Cryptochrome 1 and 2 showed increased sleep duration, suggesting the possibility that MEF2 may regulate sleep by modulating the levels of Cryptochrome 1 and 2 proteins through Fbxl3-dependent degradation (Godinho et al., 2007). It will be interesting to determine whether MEF2 TKO mice show any circadian abnormalities.

Potential roles of MEF2 in synaptic homeostasis during sleep

Recent genetic studies suggested that sleep may be involved in maintaining synaptic homeostasis altered by waking activities. The researchers showed that the levels of several synaptic proteins, as well as synapse numbers, increase during wakefulness and decrease during sleep (Donlea et al., 2009; Gilestro et al., 2009). They also found that sleep deprivation prevents the decrease in several synaptic proteins and synapse numbers. Taken together, these findings suggest that sleep decreases synaptic proteins and synapse number to maintain synaptic homeostasis. However, the underlying molecular mechanisms remain elusive. Interestingly, it has been reported that calcium/calmodulin-dependent protein

kinase 4 (CamKIV) and calcineurin, well-known positive regulators of MEF2 activity, are upregulated during sleep (Cirelli et al., 2004). This finding, together with the fact that MEF2 negatively regulates several genes involved in synapse development and synapse numbers, leads to the intriguing possibility that MEF2 decreases several synaptic proteins and synapse numbers during sleep. It will be interesting to examine if TKO mice have defects in decreasing synaptic proteins and synapse numbers after sleep.

Materials and Method

Generation of MEF2A conditional mice

Genomic regions of the *Mef2a* locus were isolated from 129SvEv genomic DNA by high-fidelity PCR (Takara LA taq PCR system) and cloned into pGKneoF2L2DTA vector, which contains a neomycin resistance gene, flanked by FRT and *loxP* sites, and a diphtheria toxin gene cassette. A 1.7-kb genomic sequence (5' arm) upstream of *Mef2a* exon 2 was cloned upstream of 5' loxP sequence in pGKneoF2L2DTA vector. A 1.1-kb fragment (knockout arm) harboring exon2 was cloned between the 5' loxP sequence and 5' FRT sequence. A 3.7-kb fragment (3' arm) downstream of *Mef2a* exon 2 was cloned downstream of 3' loxP sequence. The targeting vector was linearized with PvuI and electroporated into 129SvEv-derived ES cells. One-thousand ES clones were isolated and analyzed for homologous recombination by PCR. One clone with a properly targeted *Mef2a* allele was injected into 3.5-d C57BL/6 blastocysts, and the resulting chimera were crossed to C57BL/6 mice to achieve germline transmission.

EEG/EMG recording

TKO (n=4) and control mice (n=4) were anesthetized and surgically implanted for long-term EEG/EMG monitoring as previously described (Chemelli et al., 1999).

EEG/EMG records were visually scored into 20 second epochs of Awake, REM, and NREM sleep according to standard criteria of rodent sleep.

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