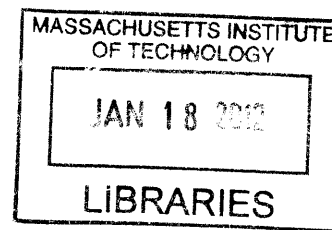Regulation of Mammalian Neuronal Circuit Development by CPG15

by

Jennifer H. Leslie

B.S Biology
B.S. Chemistry
University of Virginia, 2005

SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL
FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOLOGY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

February 2012

Signature of Author: ___

Department of Biology
January 17, 2012

Certified by: _____

Dr. Elly Nedivi
Associate Professor of Brain and Cognitive Sciences and Biology
Thesis Supervisor

Accepted by: ___

Dr. Stephen Bell
Professor of Biology
Co-Chair Graduate Committee

Regulation of Mammalian Neuronal Circuit Development by CPG15

by

Jennifer H. Leslie

**Submitted to the Department of Biology
January 17, 2012 in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy in Biology**

ABSTRACT

The orderly assembly of neuronal circuits is specified by developmental programs of gene expression, however, the final stage in circuit development, maturation and refinement of specific synaptic connections, is strongly influenced by neuronal activity. It is thus not surprising that the products of many activity-regulated genes have been implicated in synapse development and plasticity. The extracellular signaling protein CPG15 is one such activity-regulated gene product that promotes the maturation and growth of synapses, dendrites, and axonal arbors during development. Expression of *cpg15* mRNA is spatiotemporally correlated with periods of synapse maturation and refinement, posing it to play a central role in the wiring of developing brain circuits. Here we utilize a mouse mutant, which is null for the *cpg15* gene (*cpg15* KO), to elucidate the mechanism of CPG15 function in the developing brain. Analysis of the *cpg15* KO mouse suggests that CPG15 signaling leads to the selection and stabilization of synapses in the developing brain, as well as in the adult. Loss of CPG15 results in reduced synapse numbers and synapse maturation with a corresponding reduction in the complexity and growth of neuronal arbors. This is most pronounced during early periods of promiscuous synapse formation and arbor growth that provide a physical substrate upon which subsequent experience-dependent processes act to sculpt mature patterns of neuronal connectivity. Consequently, *cpg15* KO mice do not appear to undergo the same extensive refinement as their wild type (WT) counterparts. *cpg15* KO mice are also slow learners, requiring repeated training in learning tasks to perform at WT levels. These results led us to propose that the selection and stabilization of synapses by CPG15 mediates optimal wiring of developing neuronal circuits important for brain function throughout life. To test this possibility we investigated the function of CPG15 in the developing thalamocortical circuit in the visual system. The thalamus is the major hub for sensory information flow (minus olfaction) en route from the periphery to the cortex. As CPG15 is expressed in both input and target structures in this circuit we compared cortical synapse development in the global *cpg15* KO mouse which lacks CPG15 expression in both the cortex and thalamus to a cortex-specific *cpg15* KO mouse which retains thalamic expression of CPG15. Previous work has shown the importance of cortically-derived signaling factors in the maturation of thalamocortical circuits, however, we were surprised to find that CPG15 signaling by the thalamus has a stronger contribution to cortical synapse development than cortical CPG15. This work reveals a novel function for thalamic signaling in the maturation of cortical circuits.

Thesis Supervisor: Dr. Elly Nedivi
Title: Associate Professor of Brain and Cognitive Sciences and Biology

Acknowledgements

First and foremost I need to thank my advisor Dr. Elly Nedivi. Over the past five and some odd years Elly has provided unlimited opportunities for my scientific and professional growth. She has always supported me in my endeavors, and she has shared with me her most frank, honest, and useful advice and insights. Most astonishingly she encouraged me to learn electrophysiology, a technique that no one in the lab had experience with, and about which I had only a very hazy understanding. However, I found that the best way to learn electrophysiology was to do electrophysiology. And for that I must also thank Dr. Yael Amitai from Ben-Gurion University in Israel. She spent two separate semesters teaching me how to record from neurons. I appreciate her sharing not only her expertise but also her personal experiences in the field. To my undergraduate advisor, Dr. Lucy Pemberton at the University of Virginia, and the Pemberton lab, thank you for introducing me to the world of academic science and inspiring this journey.

Thank you to the members of my thesis committee, Dr. Troy Littleton, Dr. Martha Constantine-Paton, and Dr. Weifeng Xu. Troy for having assembled the absolute best group of 3rd floor neighbors, Martha for generously allowing me the use of her lab's extensive resources, and Weifeng for sharing her electrophysiology expertise and teaching me her hippocampal prep, and all of you for your guidance and support. Thank you to many of the whole-cell patch clamp electrophysiologists of the Picower and McGovern who at some time or another I have probably bugged for advice.

I need to thank the members of the Nedivi lab for always being there to help out, especially Tad Fujino for making the *cpg15* KO mouse and leaving behind the most comprehensive set of resources that I continue to mine to the day. I would also like to specially thank Sara Trowbridge without whom I would not have made nearly as much progress towards understanding CPG15.

I'd like to thank the Biology Class of 2005 for the most fantastic first year experience, and all the friends that I have made while here at MIT. Most especially I want to thank Robin, Leah, Steph, Aline, and Cindy for providing so many diversions outside the lab. Additionally, my MIT experience would not have been the same without the 2006 to 2010 residents of Burton 4, thank you for all the memories. And thank you to Dr. Roe Smith and Bronwyn Mellquist, the Burton-Conner housemasters, and all the Burton-Conner GRTs, most especially the Hugs.

Thank you to my parents, Mary and Terry, for their unfailing love and encouragement, and to my siblings, Brian and Michelle, for making me so proud with their own pursuits of science. Finally I need to thank my fiancé Sebastian who has seen me through all the ups and downs of grad school and has been my constant support for the past five years.

TABLE OF CONTENTS

Chapter 1: Activity-Regulated Genes and Synaptic Plasticity

Jennifer H. Leslie and Elly Nedivi

1.1 INTRODUCTION

There are an estimated 0.15 quadrillion synapses in the adult human cortex with billions of neurons each forming thousands of synapses onto each other (Pakkenberg et al., 2003). It would be nearly impossible to encode into the genome the directions for wiring each and every unique connection in this massive circuit. Instead, the developing nervous system has evolved mechanisms that guide the formation of efficient connections through an adaptive approach in which synapses are selected based on their use in response to salient patterns of activity. These mechanisms are collectively termed synaptic plasticity, and they allow fine tuning of nervous system structure and function during development as well as during day-to-day activity in the adult.

1.2 THE ROLE OF ACTIVITY IN CIRCUIT FORMATION

Development of precise neuronal connections occurs in several stages. First, axonal projections grow towards and into their appropriate target area. Axonal and dendritic arbors then elaborate, staking out prospective innervation domains and receptive fields. These processes are controlled by a combination of long-range and local guidance cues in the form of both secreted and cell surface molecules, acting as attractants and/or repellants (Plachez and Richards, 2005). Next, there is a period when neural activity directs the precision of synaptic connections through selective synapse stabilization or elimination, concomitant with dendritic and axonal arbor elaboration or pruning (Constantine-Paton et al., 1990, Goodman and Shatz, 1993, Katz and Shatz, 1996). Sensory, cognitive, and motor experience, all play important roles during this developmental period when activity has its most profound effect on circuit wiring (Greer and Greenberg, 2008).

In pioneering studies of the developing kitten and monkey visual system, Hubel and Wiesel were the first to demonstrate how sensory experience could influence the structure and function of brain circuits. They showed that occluding vision through one eye during early postnatal development caused thalamocortical afferents from the spared eye to commandeer cortical territory normally innervated by the deprived eye (Hubel et al., 1977). Consistent with this altered wiring of the visual cortex, cortical neurons shifted their responsiveness towards the spared eye (Wiesel and Hubel, 1963, Hubel et al., 1977). These structural changes and functional ocular dominance shifts took place in response to manipulations of the visual environment only during a specific developmental time window, which they termed the critical period (Wiesel, 1982). The critical period, during which experience provides patterns of activity that direct circuit refinement, turns out to be a common feature of sensory systems across many species (Hensch, 2004) and is likely relevant to cognitive and social development as well (Blakemore, 2010).

While axon guidance, target selection, and synapse formation were once thought to be activity-independent processes, occurring prior to activity-dependent neuronal arbor growth and refinement, the dividing line between these two stages is no longer clear. Below, I review the activity-dependent aspects of circuit development and refinement that are tied to the selection of synapses for strengthening and stabilization or for weakening and elimination. I will also discuss activity-regulated genes, which provide the mechanistic link between patterned activity and synaptic modification.

1.2.1 Synapse formation as an instructive force in shaping neuronal arbors

During neuronal arbor development, individual branches perform an intricate dance as they gradually increase in size and complexity. Through a dynamic process of branch extension and retraction neurons sample the local environment in search of correct synaptic partners (Rajan et al., 1999, O'Rourke and Fraser, 1990, Wu and Cline, 1998, Wu et al., 1999, Wong and Ghosh, 2002, Wong and Wong, 2000, Sin et al., 2002, Niell et al., 2004, Cline and Haas, 2008). Most branches last only minutes, while a select few are stabilized, persisting hours or longer (Rajan and Cline, 1998, Wu and Cline, 1998, Ruthazer et al., 2006, Cline and Haas, 2008). Dendrites of most mammalian excitatory projection neurons are studded with small protrusions, the site of most excitatory synapses in the central nervous system (Harris and Kater, 1994). Early in development these protrusions take the form of highly motile, long, thin projections called filopodia, which are replaced by more stable dendritic spines over the course of development (Ziv and Smith, 1996, Fischer et al., 1998). The development of synapses, spines, and dendritic and axonal arbors occurs in concert and shares common signaling pathways (Wu and Cline, 1998, Zou and Cline, 1999, Wong et al., 2000, Cline, 2001, Bozdagi et al., 2004, Van Aelst and Cline, 2004). This is consistent with the idea that persistent branches and spines are likely those that have successfully found an appropriate partner, and are stabilized by synaptic contacts (Ziv and Smith, 1996, Rajan and Cline, 1998, Cline, 2001, Wong and Ghosh, 2002, Portera-Cailliau et al., 2003, Hua and Smith, 2004, Konur and Yuste, 2004, Niell et al., 2004, Hua et al., 2005, Ruthazer et al., 2006, Cline and Haas, 2008).

In 1989, James E. Vaughn proposed the synaptotrophic hypothesis, which states that synapse formation acts to stabilize growing dendritic arbors (Vaughn, 1989). Using electron microscopy, Vaughn discovered that synapses are present on dendritic and axonal growth cones,

leading him to infer that synapse formation may be a stabilizing force on nascent branches. He suggested that this required communication between the axon and dendrite by what he termed 'epigenetic factors' to signal the creation of synaptic contacts. These 'epigenetic factors' are now thought to be proteins such as cell-surface adhesion molecules and secreted neurotrophins (Hua and Smith, 2004, Cline and Haas, 2008). We also now know that it is not the synaptic contact, but rather activity across the synapse that is important for regulating dendritic and axonal branch growth as well as maintenance. Disruption of neuronal activity can lead to profound defects in neuronal arbor development. In the vertebrate visual system, blockade of glutamatergic transmission though ionotropic glutamate receptors alters branch dynamics of both axons and dendrites (Rajan and Cline, 1998, Cohen-Cory, 1999, Rajan et al., 1999, Wong and Wong, 2000, Haas et al., 2006, Sin et al., 2002, Hua and Smith, 2004, Cline and Haas, 2008, McAllister et al., 1996). Dendritic spines also appear or disappear in an activity-dependent manner, suggesting excitatory synapses on those spines also turn over in response to activity (Engert and Bonhoeffer, 1999, Lendvai et al., 2000, Nagerl et al., 2004). In the cat visual system, blockade of retinal action potentials leads to the formation of abnormal retinogeniculate axonal arbors. Instead of projecting to restricted eye-specific layers, arbors are unrefined and projections from the two eyes overlap extensively (Shatz and Stryker, 1988, Sretavan et al., 1988).

Consistent with the synaptotrophic hypothesis, activity-dependent arbor refinement occurs in concert with synapse selection (Hua and Smith, 2004). There are many examples in the nervous system demonstrating activity-dependent pruning and selection of functionally appropriate pre- and postsynaptic partners by specific synapse remodeling. A classic example is the vertebrate neuromuscular junction where initially multiple inputs innervate individual muscle fibers. During early postnatal development, these inputs are pruned down to a single axon. The

remaining axon's inputs are strengthened by addition of new synaptic contacts with the muscle fiber. Blocking transmission at neuromuscular synapses with the acetylcholine receptor blocker α-bungarotoxin prevents this developmental pruning. Synchronizing fiber activity similarly prevents refinement, and blocking only some of the inputs to a given muscle fiber will result in selective elimination of the blocked inputs, suggesting that selective synapse elimination is dependent on relative activity levels of competing inputs. (Lichtman and Colman, 2000). This process of activity-based synapse selection is also seen in the central nervous system. In the visual system prior to eye opening, lateral geniculate nucleus (LGN) neurons initially receive weak inputs from 20 or more different retinogeniculate axons. After eye opening, synapses are eliminated until contacts from only 1-3 axons remain per cell. As is observed at the neuromuscular junction, the remaining inputs are strengthened by increases in synaptic strength and formation of new synapses (Chen and Regehr, 2000). Activity is critical in partner selection and maintenance of these connections once they are established (Hooks and Chen, 2006). A third example is found in the developing cerebellum, where cerebellar climbing fiber synapses are similarly refined. Multiple climbing fibers innervating a purkinje cell are pruned down to a single climbing fiber connection early in postnatal development. Prior to elimination, inputs that will be lost are weakened, while those to be kept are strengthened (Hashimoto and Kano, 2003, Hashimoto and Kano, 2005).

Functionally discernable weakening of inputs that will be lost and strengthening of those that will be maintained is commonly seen prior to activity-based synapse elimination and stabilization. Long-term potentiation (LTP), and long-term depression (LTD) are mechanisms thought to underlie the activity-dependent strengthening and weakening of synapses, respectively (Feldman, 2009). In the developing brain, these mechanisms can serve to alter synaptic strength

prior to selective synapse stabilization or elimination and consequent arbor rearrangements. Correlated activity induces synapse strengthening by LTP, and also drives the stabilization and subsequent addition of new axonal branches. Conversely, uncorrelated activity leads to LTD and drives selective elimination of axonal branches (Ruthazer et al., 2003, Feldman, 2009).

1.3 SIGNALING FROM THE SYNAPSE TO THE NUCLEUS

Activity plays an important role in the refinement of neural circuits. How does neurotransmitter release into the synaptic cleft and its binding to a postsynaptic receptor lead to dramatic and specific growth and restructuring of arbors and synapses? What are the mechanistic links between neural activity and synapse remodeling?

1.3.1 Synaptic glutamate receptors

In the CNS the neuronal activity that modulates developmental plasticity is primarily derived from the activation of ionotropic glutamatergic receptors at the synapse. There are two families of these receptors, NMDA and AMPA receptors.

The NMDA receptor is a heterotetramer generally composed of a combination of three families of subunits. GluN1, GluN2A-D, and GluN3A-B (Paoltetti, P. (2011)). Receptors containing GluN1, a constitutive component of all NMDA receptors, and GluN2A and/or GluN2B subunits have been studied most extensively. The NMDA receptor is permeable to Ca^{2+} as well as Na^+ and K^+. Permeability to Ca^{2+} varies, depending on subunit composition. GluN2B containing receptors have a higher affinity for glutamate and a longer channel open time allowing them to conduct more Ca^{2+} than GluN2A containing receptors (Feldmeyer and Cull-Candy, 1996). Early in development, GluN1/GluN2B NMDA receptors are preferentially

13

expressed. Later, with postnatal onset of GluN2A expression, GluN1/GluN2B/GluN2A and GluN1/GluN2A containing NMDA receptors predominate (Monyer et al., 1994, Sheng et al., 1994). This subunit switch can be regulated by activity and occurs over the course of postnatal development (Scheetz and Constantine-Paton, 1994). While it has been speculated that the developmental switch in subunit composition from more Ca^{2+}-permeable to less permeable subunits may regulate the leveling off of plasticity that occurs towards the end of postnatal development, this view is likely too simplistic (Perez-Otano and Ehlers, 2004, van Zundert et al., 2004). More likely, the precise GluN2A/GluN2B ratio may determine the type and extent of NMDA receptor-dependent plasticity during development (Yashiro and Philpot, 2008).

At the resting potential of the cell, the NMDA receptor is blocked by Mg^{2+} ions. This Mg^{2+} block is removed upon depolarization, allowing ions to pass through the NMDA receptor upon glutamate binding (Mori and Mishina, 1995, Ozawa et al., 1998). This endows the NMDA receptor with a unique ability to detect coincident activity, since it can be activated only after the postsynaptic membrane has been depolarized by two closely timed inputs. The NMDA receptor is therefore a critical mediator of many forms of LTP and LTD in response to differing patterns of activity (Yashiro and Philpot, 2008).

Early in development, many nascent synapses contain only NMDA type glutamate receptors (Durand et al., 1996, Isaac et al., 1997, Rao and Craig, 1997, Nusser et al., 1998, Petralia et al., 1999, Takumi et al., 1999)(Wu et al., 1996, Liao et al., 1995). These synapses are functionally 'silent', as they cannot be activated at resting membrane potential due to the Mg^{2+} block of NMDA receptors. As synapses mature, AMPA-type glutamate receptors are inserted into synapses alongside NMDA receptors, converting 'silent synapses' into functional ones and strengthening the synaptic response. This insertion can be driven by synaptic activity

(Constantine-Paton and Cline, 1998, Carroll et al., 1999, Liao et al., 1995, Shi et al., 1999). Some forms of hippocampal LTP work by converting 'silent synapses' into functional AMPA receptor containing synapses, and generally LTP and LTD can be mediated by AMPA receptor insertion and removal at the synapse. However, this form of LTP is attenuated with developmental age as the incidence of 'silent synapses' decreases (Kerchner and Nicoll, 2008).

AMPA receptor subunit expression is also developmentally regulated. AMPA receptors are heteromers of four subunits, GluA1-4 (Hollmann and Heinemann, 1994, Dingledine et al., 1999). Only those AMPA receptors not containing the RNA-edited GluA2 subunit can flux Ca^{2+} (Seeburg et al., 1998). Ca^{2+}-permeable AMPA receptors are expressed preferentially during early development, while Ca^{2+}-impermeable, GluA2 containing AMPA receptors are more common in mature neurons (Kumar et al., 2002).

1.3.2 Second messenger cascades and transcription factor activation

Neurons maintain gradients of many ions across their membranes. Most, such as Na^+, K^+, and Cl^-, are important regulators of membrane excitability. Ca^{2+} however, is a potent activator of intracellular signaling cascades. For this reason, it is normally maintained at very low concentrations within the cytoplasm. At glutamatergic synapses, activity can lead to Ca^{2+} influx into the postsynaptic cell via activation of Ca^{2+}-permeable AMPA and NMDA type glutamate receptors, as well as through voltage sensitive Ca^{2+} channels (VSCCs), particularly L-type VSCCs (Catterall, 1995, Rosen et al., 1995). Ca^{2+} acts as a second messenger to trigger signaling cascades that activate nuclear transcription factors, which in turn induce distinct patterns of gene expression. The various routes of Ca^{2+} entry into the cell activate different, but overlapping cellular responses, providing an additional level of control to activity-dependent gene regulation

(Greer and Greenberg, 2008). In this way, synaptic activity, in lieu of an intrinsic genetic blueprint, acts as the master controller over when and which pathways and genetic programs are activated in order to implement synaptic and neuronal arbor rearrangements.

Linking Ca^{2+} entry at the cell surface to gene expression in the nucleus are kinase pathways activated by Ca^{2+}-sensitive factors, such as the high affinity Ca^{2+} binding protein, calmodulin. After binding Ca^{2+} near its site of entry, Ca^{2+}-calmodulin can signal to the nucleus through multiple pathways. Ca^{2+}-calmodulin can bind and activate Ca^{2+}-calmodulin-dependent protein kinases (CaMKs) in the cytoplasm. Activated CaMKs may work locally near the synapse, or they can translocate into the nucleus and phosphorylate transcription factors (Ghosh and Greenberg, 1995). Ca^{2+}-calmodulin itself can also rapidly translocate to the nucleus in response to synaptic activation. In the nucleus, Ca^{2+}-calmodulin binding activates the nuclear kinase CaMKIV, and perhaps other CaMKs. Activated CaMKIV is then free to phosphorylate transcription factors that initiate new gene expression (Deisseroth et al., 1998). CaMK pathways are known to regulate activity-dependent growth of dendritic arbors (Wu and Cline, 1998, Wayman et al., 2008), LTP induction (Kirkwood et al., 1997), and experience-dependent plasticity in the developing visual cortex (Taha and Stryker, 2005).

Another way in which Ca^{2+} entry signals to the nucleus is through the Ras-dependent kinase signaling cascade. Although the mechanism is unclear, Ca^{2+} entry can activate the small GTP-binding protein Ras, which activates the kinase Raf. Raf then triggers the MAP kinase cascade: MEK-1 (MAPK kinase) activates the ERKs (also called MAP kinases), which activate the ribosomal S6 kinases (RSKs) (Bading and Greenberg, 1991, Rosen et al., 1994, Rusanescu et al., 1995). RSKs are able to translocate to the nucleus and phosphorylate transcription factors (Impey et al., 1998). The Ras signaling cascade can mediate neurite outgrowth in PC12 cells

16

(Rusanescu et al., 1995). ERK1/2 are also important for regulating LTP as well as experience-dependent development and plasticity of the visual system (Di Cristo et al., 2001, Naska et al., 2004).

Once Ca^{2+}-activated signaling pathways converge on the nucleus they target transcriptional activators that can initiate gene expression. One major nuclear target of activity-dependent signaling pathways is the transcription factor CREB which initiates transcription of genes containing Ca^{2+}/cAMP response elements (CREs) sites within their promoter (Brindle and Montminy, 1992, Sassone-Corsi, 1995). Sensory manipulations, such as exposure to light after dark adaptation, and monocular deprivation, induce CRE-mediated gene transcription during the critical period for visual cortex development (Pham et al., 1999, Cancedda et al., 2003). Studies expressing a dominant-negative form of CREB have demonstrated that CREB function is essential for ocular dominance plasticity in the visual cortex (Mower et al., 2002). In keeping with its role in circuit plasticity, CREB is also an important regulator of activity-dependent dendritic arbor growth (Redmond et al., 2002, Wayman et al., 2006), as well as refinement of retinogeniculate projections (Pham et al., 2001).

1.4 ACTIVITY-DEPENDENT GENE EXPRESSION

Activation of transcription factors such as CREB by rapid phosphorylation cascades initiates the first phase of a bi-phasic transcriptional program. The first phase is comprised of rapid response genes, termed immediate early genes (IEGs) that do not require protein synthesis for their expression. Many IEGs encode transcription factors that in turn activate a second phase of the activity-dependent transcriptional program by inducing expression of another gene set, the delayed early genes.

17

The first IEG shown to be a transcription factor, c-*fos*, was also the first gene discovered to be regulated by neuronal activity. Depolarizing stimuli and Ca^{2+} influx through VSCCs could elicit c-*fos* induction in cultured PC12 cells (Morgan and Curran, 1986). This led to the examination of c-*fos* expression, as well as that of other transcription factor IEGs such as c-*jun* and *zif268*, in the brain (Morgan et al., 1987, Saffen et al., 1988). All were found to be robustly activated in seizure paradigms, as well as by more natural, physiological levels of stimulation (Loebrich and Nedivi, 2009). *zif268* can also be induced by stimuli that induce LTP, in an NMDA receptor-dependent manner (Cole et al., 1989, Wisden et al., 1990). Mutant mice lacking Zif/268 protein show a defect in long-lasting LTP indicating that *zif268* expression is not simply coincident with LTP induction, but is also important for maintenance of LTP (Jones et al., 2001). Studies of IEGs like c-*fos*, c-*jun*, and *zif268* were critical to the realization that gene expression is a normal downstream response to neuronal depolarization. Moreover, elucidation of signaling pathways that couple other extracellular cues to transcriptional activation has led to a better understanding of neuronal responses to activity.

The initiation of gene expression programs in response to synaptic activity is analogous in many ways to the cellular response program to other extracellular stimuli such as growth factors, mitogens, and phorbol esters (Loebrich and Nedivi, 2009). Both types of responses begin at the cell membrane where extrinsic stimuli activate cell surface receptors which in turn induce intracellular signaling cascades to the nucleus. Neuronal activity, like other types of stimuli, induces a bi-phasic transcriptional response where early induction of transcription factors then activates further changes in gene expression. Neuronal activity also utilizes much of the same intracellular signaling machinery that has been described for other cellular stimuli. However, in neurons the site of activation for these pathways is spatially discrete. Unlike growth factors

whose receptors are distributed throughout the cell membrane, the bulk of signaling by neuronal activity occurs through synaptically localized receptors. This spatial restriction in combination with the unique properties of NMDA receptors at these sites allows the de-coding of temporally and spatially distinct activity patterns and their translation into diverse cellular responses.

Many of the IEGs first studied in the context of activity-regulated gene expression in neurons were initially identified as responsive to other extracellular cues such as growth factors and mitogens. Since they also encode ubiquitous transcription factors that are expressed in multiple cell types, their characterization did little to reveal the cellular processes recruited to implement activity-dependent genetic programs in neurons. In the 1990's several groups performed large-scale screens to directly identify genes regulated in the brain by neuronal activity (Nedivi et al., 1993, Qian et al., 1993, Yamagata et al., 1993). Using a conceptually similar approach, these groups utilized a combination of subtractive hybridization and differential screening methods to select for seizure-induced transcripts in the rat cerebral cortex, or more specifically the hippocampus. Based on these screens the number of activity-regulated genes in the brain was estimated at 500-1,000 (Nedivi et al., 1993). Many of the activity-regulated genes identified in these screens were later shown to be induced in the adult brain by physiological stimuli, such as vision and by LTP-inducing protocols (Lanahan and Worley, 1998, Nedivi, 1999). Some of the same genes were also found to be developmentally regulated in correlation with critical periods for activity-dependent circuit remodeling. This suggests that, at least in part, the activity-dependent genetic program is common to periods of synapse and branch selection during development and activity-dependent synaptic plasticity in the adult (Nedivi, 1999, Bailey and Kandel, 1993).

While later studies have added to the list of genes regulated by activity, these first screens identified a significant number of the activity-regulated genes that have been functionally characterized in the context of synaptic plasticity to date. They also afforded the first view of the cellular mechanisms likely to take part in activity-dependent plasticity. Some of the most abundantly represented functional categories of activity-regulated genes include transcription factors, signal transduction proteins, trophic factors, structural, and synaptic proteins. The number and diversity of activity-regulated genes indicate that neurons activate a complex, multi-faceted response to input activity (Nedivi et al., 1993, Lanahan and Worley, 1998, Nedivi, 1999). Yet ultimately the products of these genes work to effect changes in synaptic connectivity in response to changing levels and patterns of activity.

1.5 CANDIDATE PLASTICITY GENE 15 IN DEVELOPMENTAL PLASICITY

Candidate plasticity gene 15 (CPG15), also known as Neuritin, has striking effects on axonal, dendritic, and synaptic growth and maturation. *cpg15* mRNA is expressed at high levels in developing vertebrate brains including the frog *Xenopus laevis*, rodents, and felines (Nedivi et al., 2001, Corriveau et al., 1999, Lee and Nedivi, 2002, Nedivi et al., 1996). In the visual system, this expression temporally coincides with periods of synapse formation and refinement, beginning first in the retina, then progressing to the LGN, and finally to the cortex, as these various structures sequentially mature (Corriveau et al., 1999). In the visual cortex, expression peaks during the height of the critical period for ocular dominance plasticity, when this area of the brain is most susceptible to manipulations of visual activity (Corriveau et al., 1999, Lee and Nedivi, 2002). Adult expression of *cpg15* is regulated by physiological forms of activity such as exposure to light in visual cortex and single whisker experience in barrel cortex (Harwell et al.,

2005, Lee and Nedivi, 2002, Nedivi et al., 1996). *cpg15* is an IEG whose expression is induced by classical activity-dependent signaling pathways involving Ca^{2+} influx through NMDA receptors and L-type VSCCs, activation of CaMK and MAPK signaling cascades, and induction of transcription by CREB (Fujino et al., 2003).

CPG15 protein is approximately 142 amino acids and is very highly conserved across vertebrate species with 97% identity between mouse and human, and 63% identity, 90% homology between mouse and *Xenopus laevis* (Fig. 1.1). CPG15 contains an N-terminal signal sequence marking it for secretion. In the mature form, the C-terminal tail of CPG15 is modified with a glyosyl-phosphatidylinositol (GPI) anchor linking it to the cell membrane. This GPI-link can be cleaved to release CPG15 from the membrane (Putz et al., 2005, Naeve et al., 1997). The soluble form of CPG15 can be harvested from the media of CPG15-expressing HEK cells, and is active in promoting cell survival, however it is not known which form, cell-attached or soluble, is important for the function of CPG15 *in vivo*. CPG15 also contains six conserved cysteine residues (Fig. 1.1). Interestingly, neurotrophins all have six conserved cysteine residues which mediate intermolecular disulfide bonds within homodimers (Lewin and Barde, 1996), however the positions of the cysteine residues in CPG15 do not correspond to those of neurotrophins. CPG15 protein has one known homolog, CPG15-2, which is also activity-regulated and functions in neurite outgrowth and cell survival (Fujino et al., 2003).

```
                            ☆                    ☆
Mus musculus    MGLKLNGRYISLILAVQIAYLVQAVRAAGKCDAVFKGFSDCLLKLGDSMA 50
Homo sapiens    MGLKLNGRYISLILAVQIAYLVQAVRAAGKCDAVFKGFSDCLLKLGDSMA 50
Xenopus laevis  MGLKLSGRYIFLVLAVHLAYLLQAVKATGKCDAVFKGLSDCMLTLGDKVA 50
                *****.**** *:***:.***:***:*:***********:***:*.***.:*

                             ☆           ☆          ☆
Mus musculus    NYPQGLDDKTNIKTVCTYWEDFHSCTVTALTDCQEGAKDMWDKLRKESKN 100
Homo sapiens    NYPQGLDDKTNIKTVCTYWEDFHSCTVTALTDCQEGAKDMWDKLRKESKN 100
Xenopus laevis  NYPQDLEEKKNLDTICSYWDDFHVCTVTALADCQEGAADIWEKLKRQSKN 100
                ****.*::*.*:.*:*:**:*** ******:****** *:*:**:::***

                         ☆
Mus musculus    LNIQGSLFELCGSSNGAAGS--LLPALSVLLVSLSAALATWFSF 142
Homo sapiens    LNIQGSLFELCGSGNGAAGS--LLPAFPVLLVSLSAALATWLSF 142
Xenopus laevis  LNIQGSLFELCPGSAGAPGQRLLFPAFLPLLMVFLSTLFILVLQ 144
                ********** .. **.*.  *:**:  **: : ::*   .
```

Figure 1.1 Conservation of CPG15 protein. Alignments of CPG15 protein sequences between *Mus musculus*, *Homo sapiens*, and *Xenopus laevis*. Blue indicates signal sequence, magenta is putative GPI-linked reside, yellow indicates GPI-link sequence that is removed in the mature form of the protein. Six conserved cysteine residues are indicated by red stars. Asterisk denotes identity between all sequences, colon denotes a conserved substitution, period denotes a semi-conserved substitution.

1.5.1 CPG15 in arbor growth, synapse maturation, and neuronal progenitor survival

CPG15 overexpression in the developing *Xenopus* optic tectum has profound effects on the maturation of both synapses and arbors. CPG15 overexpression results in increased dendritic and axonal arbor size and complexity (Fig. 1.2) (Nedivi et al., 1998, Cantallops et al., 2000). Time-lapse imaging of retinotectal axons reveals that CPG15 stabilizes growing branches and reduces the rate of branch retractions (Cantallops et al., 2000). At the same time, CPG15 promotes synapse maturation through the insertion of AMPA-type glutamate receptors into NMDA receptor containing-only "silent" synapses (Cantallops et al., 2000). This results in increased AMPA to NMDA ratios, and increased frequencies of AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs) (Fig. 1.3). On *Xenopus* motor neuron axons, CPG15 increases synapse density, as well as axon branching and size (Javaherian and Cline, 2005). CPG15 can be transported down axons and exocytosed in response to neuronal activity, suggesting multiple modes of regulating activity-dependent signaling by CPG15 (Cantallops and Cline, 2008).

In addition to its role in neuron structural and functional maturation, CPG15 also works as a survival factor during embyronic development (Putz et al., 2005). RNAi-mediated knockdown of CPG15 in the subventricular zone of embryonic rats leads to decreased survival of cortical progenitors whereas overexpression has the opposite effect. In this manner CPG15 functions similarly to neurotrophins such as NGF and BDNF, playing a pleiotropic role in cell survival, growth, and synapse development (Schuman, 1999, Lewin and Barde, 1996). Unlike what has been proposed for neurotrophins, the effects of CPG15 on synapse and arbor development are likely both downstream results from a specific role for CPG15 in the stabilization of newly forming synapses.
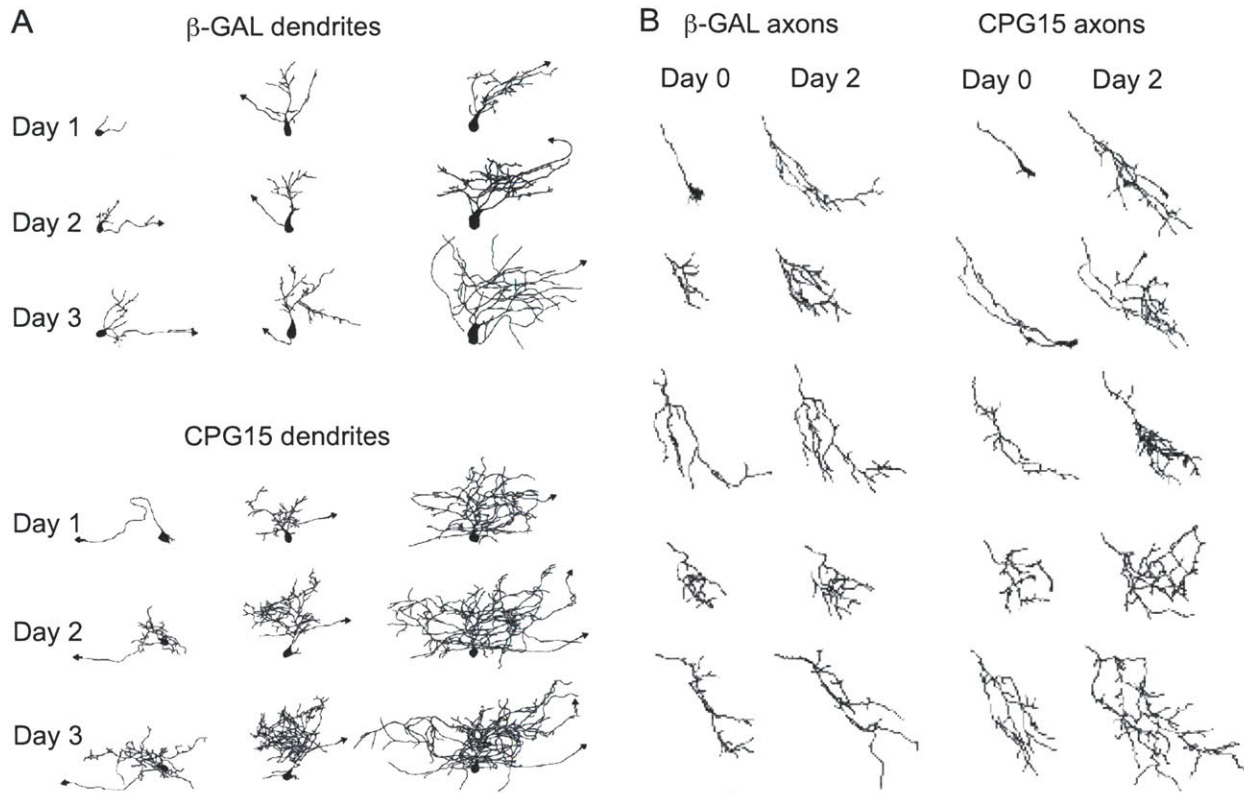
Figure 1.2 CPG15 enhances growth and complexity of dendritic and axonal arbors. Overexpression of CPG15 greatly increases the growth and branching of dendrites (A) and axons (B) in immature *Xenopus* optic tectum compared with β-GAL protein controls. Figures adapted from Nedivi et al., 1998 (A), and Cantallops et al., 2000 (B).
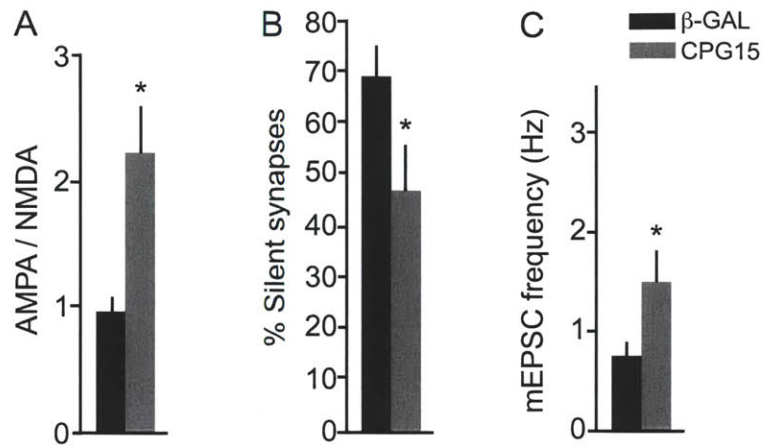
Figure 1.3 CPG15 increases synapse maturation. Overexpression of CPG15 in immature *Xenopus* optic tectum increases AMPA/NMDA ratios (A), leading to a reduction in the number of "silent" synpases (B), and increases AMPA mediated mEPSC frequencies (C). Figures adapted from Cantallops et al., 2000.

1.5.2 Model of CPG15 function

As discussed earlier, neuronal development proceeds through a dynamic process of branch extensions and retractions as neurons seek appropriate synaptic partners. The synaptotrophic hypothesis proposes that these growing branches can be stabilized through the formation of stable synaptic contacts (Vaughn, 1989). Neuronal activity plays an important role in this selection of synaptic partners (Hua and Smith, 2004), however little is known about the molecular signals downstream of activity that select certain synapses for stabilization, while others are eliminated. Work to date strongly implicates CPG15 as an activity-regulated signal for synapse stabilization.

Signaling by CPG15 stabilizes activated synapses allowing them to mature further, for example by inserting AMPA receptors into NMDA receptor-only "silent" synapses (Fig. 1.4). The formation of stable synaptic contacts in turn stabilizes growing axonal and dendritic branches. This decreases branch retractions and promotes further growth and elaboration of neuronal arbors. The selection of activated synapses by CPG15 may be an important mechanism for the experience-dependent sculpting of developing neural circuits, a process that is critical for brain function throughout life.

Along with developing *Xenopus* neural circuits, CPG15 also plays a role in neuron growth and development in mammals. *In vitro* neurite outgrowth and branching of rat hippocampal neurons increase in the presence of exogenous CPG15 protein (Fujino et al., 2008, Naeve et al., 1997). These effects on neurite maturation likely have corresponding effects on synapses formation and maturation; however, a role for CPG15 in mammalian synapse maturation has yet to be established.
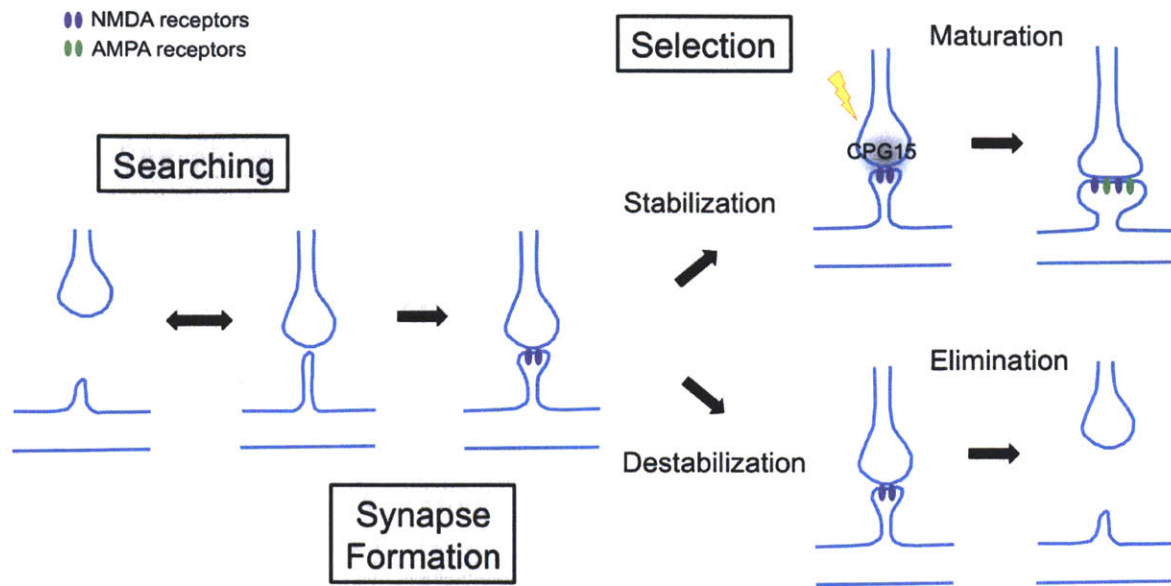
Figure 1.4 CPG15 stabilizes nascent synapses. Neuronal processes explore their environment in search of synaptic partners. Once synapses form they can be selected for either stabilization and subsequent maturation, or destabilization and elimination. Neuronal activity across a synapse may lead to the externalization of CPG15, stabilizing newly formed synapses, allowing for further synapse maturation.

1.6 CONCLUSIONS

To create efficiently wired networks, developing neurons make and break synaptic connections depending on their use in the circuit. Patterned activity selectively strengthens and stabilizes some connections, while weakening and pruning others. Activity-dependent modulation of synaptic connectivity is also a critical regulator of dendritic and axonal arbor morphology. Synapse formation and strengthening stabilizes branches, allowing for further elaboration. In contrast, synapse pruning can lead to arbor loss and retraction. Intracellular signaling pathways mediate between neuronal activity and structural remodeling by integrating neuronal activity and initiating transcription of activity-regulated genes that act to modulate synaptic strength, number, and arbor morphology. While synapse formation and arbor growth can proceed without activity-regulated gene products, activity-regulated genes are essential mediators of the selective processes by which activity sculpts optimally functioning circuits.

1.7 REFERENCES

Bading, H. & Greenberg, M. E. 1991. Stimulation of protein tyrosine phosphorylation by NMDA receptor activation. *Science*, 253, 912-4.

Bailey, C. H. & Kandel, E. R. 1993. Structural changes accompanying memory storage. *Annu Rev Physiol*, 55, 397-426.

Blakemore, S. J. 2010. The developing social brain: implications for education. *Neuron*, 65, 744-7.

Bozdagi, O., Valcin, M., Poskanzer, K., Tanaka, H. & Benson, D. L. 2004. Temporally distinct demands for classic cadherins in synapse formation and maturation. *Mol Cell Neurosci*, 27, 509-21.

Brindle, P. K. & Montminy, M. R. 1992. The CREB family of transcription activators. *Curr Opin Genet Dev*, 2, 199-204.

Cancedda, L., Putignano, E., Impey, S., Maffei, L., Ratto, G. M. & Pizzorusso, T. 2003. Patterned vision causes CRE-mediated gene expression in the visual cortex through PKA and ERK. *J Neurosci*, 23, 7012-20.

Cantallops, I. & Cline, H. T. 2008. Rapid activity-dependent delivery of the neurotrophic protein CPG15 to the axon surface of neurons in intact Xenopus tadpoles. *Dev Neurobiol*, 68, 744-59.

Cantallops, I., Haas, K. & Cline, H. T. 2000. Postsynaptic CPG15 promotes synaptic maturation and presynaptic axon arbor elaboration in vivo. *Nat Neurosci*, 3, 1004-11.

Carroll, R. C., Lissin, D. V., Von Zastrow, M., Nicoll, R. A. & Malenka, R. C. 1999. Rapid redistribution of glutamate receptors contributes to long-term depression in hippocampal cultures. *Nat Neurosci*, 2, 454-60.

Catterall, W. A. 1995. Structure and function of voltage-gated ion channels. *Annu Rev Biochem*, 64, 493-531.

Chen, C. & Regehr, W. G. 2000. Developmental remodeling of the retinogeniculate synapse. *Neuron*, 28, 955-66.

Cline, H. & Haas, K. 2008. The regulation of dendritic arbor development and plasticity by glutamatergic synaptic input: a review of the synaptotrophic hypothesis. *J Physiol*, 586, 1509-17.

Cline, H. T. 2001. Dendritic arbor development and synaptogenesis. *Curr Opin Neurobiol*, 11, 118-26.

Cohen-Cory, S. 1999. BDNF modulates, but does not mediate, activity-dependent branching and remodeling of optic axon arbors in vivo. *J Neurosci*, 19, 9996-10003.

Cole, A. J., Saffen, D. W., Baraban, J. M. & Worley, P. F. 1989. Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. *Nature*, 340, 474-6.

Constantine-Paton, M. & Cline, H. T. 1998. LTP and activity-dependent synaptogenesis: the more alike they are, the more different they become. *Curr Opin Neurobiol*, 8, 139-48.

Constantine-Paton, M., Cline, H. T. & Debski, E. 1990. Patterned activity, synaptic convergence, and the NMDA receptor in developing visual pathways. *Annu Rev Neurosci*, 13, 129-54.

Corriveau, R. A., Shatz, C. J. & Nedivi, E. 1999. Dynamic regulation of cpg15 during activity-dependent synaptic development in the mammalian visual system. *J Neurosci*, 19, 7999-8008.

Deisseroth, K., Heist, E. K. & Tsien, R. W. 1998. Translocation of calmodulin to the nucleus supports CREB phosphorylation in hippocampal neurons. *Nature,* 392, 198-202.

Di Cristo, G., Berardi, N., Cancedda, L., Pizzorusso, T., Putignano, E., Ratto, G. M. & Maffei, L. 2001. Requirement of ERK activation for visual cortical plasticity. *Science,* 292, 2337-40.

Dingledine, R., Borges, K., Bowie, D. & Traynelis, S. F. 1999. The glutamate receptor ion channels. *Pharmacol Rev,* 51, 7-61.

Durand, G. M., Kovalchuk, Y. & Konnerth, A. 1996. Long-term potentiation and functional synapse induction in developing hippocampus. *Nature,* 381, 71-5.

Engert, F. & Bonhoeffer, T. 1999. Dendritic spine changes associated with hippocampal long-term synaptic plasticity. *Nature,* 399, 66-70.

Feldman, D. E. 2009. Synaptic mechanisms for plasticity in neocortex. *Annu Rev Neurosci,* 32, 33-55.

Feldmeyer, D. & Cull-Candy, S. 1996. Functional consequences of changes in NMDA receptor subunit expression during development. *J Neurocytol,* 25, 857-67.

Fischer, M., Kaech, S., Knutti, D. & Matus, A. 1998. Rapid actin-based plasticity in dendritic spines. *Neuron,* 20, 847-54.

Fujino, T., Lee, W. C. & Nedivi, E. 2003. Regulation of cpg15 by signaling pathways that mediate synaptic plasticity. *Mol Cell Neurosci,* 24, 538-54.

Fujino, T., Wu, Z., Lin, W. C., Phillips, M. A. & Nedivi, E. 2008. cpg15 and cpg15-2 constitute a family of activity-regulated ligands expressed differentially in the nervous system to promote neurite growth and neuronal survival. *J Comp Neurol,* 507, 1831-45.

Ghosh, A. & Greenberg, M. E. 1995. Calcium signaling in neurons: molecular mechanisms and cellular consequences. *Science,* 268, 239-47.

Goodman, C. S. & Shatz, C. J. 1993. Developmental mechanisms that generate precise patterns of neuronal connectivity. *Cell,* 72 Suppl, 77-98.

Greer, P. L. & Greenberg, M. E. 2008. From synapse to nucleus: calcium-dependent gene transcription in the control of synapse development and function. *Neuron,* 59, 846-60.

Haas, K., Li, J. & Cline, H. T. 2006. AMPA receptors regulate experience-dependent dendritic arbor growth in vivo. *Proc Natl Acad Sci U S A,* 103, 12127-31.

Harris, K. M. & Kater, S. B. 1994. Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function. *Annu Rev Neurosci,* 17, 341-71.

Harwell, C., Burbach, B., Svoboda, K. & Nedivi, E. 2005. Regulation of cpg15 expression during single whisker experience in the barrel cortex of adult mice. *J Neurobiol,* 65, 85-96.

Hashimoto, K. & Kano, M. 2003. Functional differentiation of multiple climbing fiber inputs during synapse elimination in the developing cerebellum. *Neuron,* 38, 785-96.

Hashimoto, K. & Kano, M. 2005. Postnatal development and synapse elimination of climbing fiber to Purkinje cell projection in the cerebellum. *Neurosci Res,* 53, 221-8.

Hensch, T. K. 2004. Critical period regulation. *Annu Rev Neurosci,* 27, 549-79.

Hollmann, M. & Heinemann, S. 1994. Cloned glutamate receptors. *Annu Rev Neurosci,* 17, 31-108.

Hooks, B. M. & Chen, C. 2006. Distinct roles for spontaneous and visual activity in remodeling of the retinogeniculate synapse. *Neuron,* 52, 281-91.

Hua, J. Y., Smear, M. C., Baier, H. & Smith, S. J. 2005. Regulation of axon growth in vivo by activity-based competition. *Nature,* 434, 1022-6.

Hua, J. Y. & Smith, S. J. 2004. Neural activity and the dynamics of central nervous system development. *Nat Neurosci,* 7, 327-32.

Hubel, D. H., Wiesel, T. N. & Levay, S. 1977. Plasticity of ocular dominance columns in monkey striate cortex. *Philos Trans R Soc Lond B Biol Sci,* 278, 377-409.

Impey, S., Obrietan, K., Wong, S. T., Poser, S., Yano, S., Wayman, G., Deloulme, J. C., Chan, G. & Storm, D. R. 1998. Cross talk between ERK and PKA is required for Ca2+ stimulation of CREB-dependent transcription and ERK nuclear translocation. *Neuron,* 21, 869-83.

Isaac, J. T., Crair, M. C., Nicoll, R. A. & Malenka, R. C. 1997. Silent synapses during development of thalamocortical inputs. *Neuron,* 18, 269-80.

Javaherian, A. & Cline, H. T. 2005. Coordinated motor neuron axon growth and neuromuscular synaptogenesis are promoted by CPG15 in vivo. *Neuron,* 45, 505-12.

Jones, M. W., Errington, M. L., French, P. J., Fine, A., Bliss, T. V., Garel, S., Charnay, P., Bozon, B., Laroche, S. & Davis, S. 2001. A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. *Nat Neurosci,* 4, 289-96.

Katz, L. C. & Shatz, C. J. 1996. Synaptic activity and the construction of cortical circuits. *Science,* 274, 1133-8.

Kerchner, G. A. & Nicoll, R. A. 2008. Silent synapses and the emergence of a postsynaptic mechanism for LTP. *Nat Rev Neurosci,* 9, 813-25.

Kirkwood, A., Silva, A. & Bear, M. F. 1997. Age-dependent decrease of synaptic plasticity in the neocortex of alphaCaMKII mutant mice. *Proc Natl Acad Sci U S A,* 94, 3380-3.

Konur, S. & Yuste, R. 2004. Developmental regulation of spine and filopodial motility in primary visual cortex: reduced effects of activity and sensory deprivation. *J Neurobiol,* 59, 236-46.

Kumar, S. S., Bacci, A., Kharazia, V. & Huguenard, J. R. 2002. A developmental switch of AMPA receptor subunits in neocortical pyramidal neurons. *J Neurosci,* 22, 3005-15.

Lanahan, A. & Worley, P. 1998. Immediate-early genes and synaptic function. *Neurobiol Learn Mem,* 70, 37-43.

Lee, W. C. & Nedivi, E. 2002. Extended plasticity of visual cortex in dark-reared animals may result from prolonged expression of cpg15-like genes. *J Neurosci,* 22, 1807-15.

Lendvai, B., Stern, E. A., Chen, B. & Svoboda, K. 2000. Experience-dependent plasticity of dendritic spines in the developing rat barrel cortex in vivo. *Nature,* 404, 876-81.

Lewin, G. R. & Barde, Y. A. 1996. Physiology of the neurotrophins. *Annu Rev Neurosci,* 19, 289-317.

Liao, D., Hessler, N. A. & Malinow, R. 1995. Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. *Nature,* 375, 400-4.

Lichtman, J. W. & Colman, H. 2000. Synapse elimination and indelible memory. *Neuron,* 25, 269-78.

Loebrich, S. & Nedivi, E. 2009. The function of activity-regulated genes in the nervous system. *Physiol Rev,* 89, 1079-103.

Mcallister, A. K., Katz, L. C. & Lo, D. C. 1996. Neurotrophin regulation of cortical dendritic growth requires activity. *Neuron,* 17, 1057-64.

Monyer, H., Burnashev, N., Laurie, D. J., Sakmann, B. & Seeburg, P. H. 1994. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron,* 12, 529-40.

Morgan, J. I., Cohen, D. R., Hempstead, J. L. & Curran, T. 1987. Mapping patterns of c-fos expression in the central nervous system after seizure. *Science,* 237, 192-7.

Morgan, J. I. & Curran, T. 1986. Role of ion flux in the control of c-fos expression. *Nature,* 322, 552-5.

Mori, H. & Mishina, M. 1995. Structure and function of the NMDA receptor channel. *Neuropharmacology,* 34, 1219-37.

Mower, A. F., Liao, D. S., Nestler, E. J., Neve, R. L. & Ramoa, A. S. 2002. cAMP/Ca2+ response element-binding protein function is essential for ocular dominance plasticity. *J Neurosci,* 22, 2237-45.

Naeve, G. S., Ramakrishnan, M., Kramer, R., Hevroni, D., Citri, Y. & Theill, L. E. 1997. Neuritin: a gene induced by neural activity and neurotrophins that promotes neuritogenesis. *Proc Natl Acad Sci U S A,* 94, 2648-53.

Nagerl, U. V., Eberhorn, N., Cambridge, S. B. & Bonhoeffer, T. 2004. Bidirectional activity-dependent morphological plasticity in hippocampal neurons. *Neuron,* 44, 759-67.

Naska, S., Cenni, M. C., Menna, E. & Maffei, L. 2004. ERK signaling is required for eye-specific retino-geniculate segregation. *Development,* 131, 3559-70.

Nedivi, E. 1999. Molecular analysis of developmental plasticity in neocortex. *J Neurobiol,* 41, 135-47.

Nedivi, E., Fieldust, S., Theill, L. E. & Hevron, D. 1996. A set of genes expressed in response to light in the adult cerebral cortex and regulated during development. *Proc Natl Acad Sci U S A,* 93, 2048-53.

Nedivi, E., Hevroni, D., Naot, D., Israeli, D. & Citri, Y. 1993. Numerous candidate plasticity-related genes revealed by differential cDNA cloning. *Nature,* 363, 718-22.

Nedivi, E., Javaherian, A., Cantallops, I. & Cline, H. T. 2001. Developmental regulation of CPG15 expression in Xenopus. *J Comp Neurol,* 435, 464-73.

Nedivi, E., Wu, G. Y. & Cline, H. T. 1998. Promotion of dendritic growth by CPG15, an activity-induced signaling molecule. *Science,* 281, 1863-6.

Niell, C. M., Meyer, M. P. & Smith, S. J. 2004. In vivo imaging of synapse formation on a growing dendritic arbor. *Nat Neurosci,* 7, 254-60.

Nusser, Z., Lujan, R., Laube, G., Roberts, J. D., Molnar, E. & Somogyi, P. 1998. Cell type and pathway dependence of synaptic AMPA receptor number and variability in the hippocampus. *Neuron,* 21, 545-59.

O'rourke, N. A. & Fraser, S. E. 1990. Dynamic changes in optic fiber terminal arbors lead to retinotopic map formation: an in vivo confocal microscopic study. *Neuron,* 5, 159-71.

Ozawa, S., Kamiya, H. & Tsuzuki, K. 1998. Glutamate receptors in the mammalian central nervous system. *Prog Neurobiol,* 54, 581-618.

Pakkenberg, B., Pelvig, D., Marner, L., Bundgaard, M. J., Gundersen, H. J., Nyengaard, J. R. & Regeur, L. 2003. Aging and the human neocortex. *Exp Gerontol,* 38, 95-9.

Perez-Otano, I. & Ehlers, M. D. 2004. Learning from NMDA receptor trafficking: clues to the development and maturation of glutamatergic synapses. *Neurosignals,* 13, 175-89.

Petralia, R. S., Esteban, J. A., Wang, Y. X., Partridge, J. G., Zhao, H. M., Wenthold, R. J. & Malinow, R. 1999. Selective acquisition of AMPA receptors over postnatal development suggests a molecular basis for silent synapses. *Nat Neurosci,* 2, 31-6.

Pham, T. A., Impey, S., Storm, D. R. & Stryker, M. P. 1999. CRE-mediated gene transcription in neocortical neuronal plasticity during the developmental critical period. *Neuron,* 22, 63-72.

Pham, T. A., Rubenstein, J. L., Silva, A. J., Storm, D. R. & Stryker, M. P. 2001. The CRE/CREB pathway is transiently expressed in thalamic circuit development and contributes to refinement of retinogeniculate axons. *Neuron,* 31, 409-20.

Plachez, C. & Richards, L. J. 2005. Mechanisms of axon guidance in the developing nervous system. *Curr Top Dev Biol,* 69, 267-346.

Portera-Cailliau, C., Pan, D. T. & Yuste, R. 2003. Activity-regulated dynamic behavior of early dendritic protrusions: evidence for different types of dendritic filopodia. *J Neurosci,* 23, 7129-42.

Putz, U., Harwell, C. & Nedivi, E. 2005. Soluble CPG15 expressed during early development rescues cortical progenitors from apoptosis. *Nat Neurosci,* 8, 322-31.

Qian, Z., Gilbert, M. E., Colicos, M. A., Kandel, E. R. & Kuhl, D. 1993. Tissue-plasminogen activator is induced as an immediate-early gene during seizure, kindling and long-term potentiation. *Nature,* 361, 453-7.

Rajan, I. & Cline, H. T. 1998. Glutamate receptor activity is required for normal development of tectal cell dendrites in vivo. *J Neurosci,* 18, 7836-46.

Rajan, I., Witte, S. & Cline, H. T. 1999. NMDA receptor activity stabilizes presynaptic retinotectal axons and postsynaptic optic tectal cell dendrites in vivo. *J Neurobiol,* 38, 357-68.

Rao, A. & Craig, A. M. 1997. Activity regulates the synaptic localization of the NMDA receptor in hippocampal neurons. *Neuron,* 19, 801-12.

Redmond, L., Kashani, A. H. & Ghosh, A. 2002. Calcium regulation of dendritic growth via CaM kinase IV and CREB-mediated transcription. *Neuron,* 34, 999-1010.

Rosen, L. B., Ginty, D. D. & Greenberg, M. E. 1995. Calcium regulation of gene expression. *Adv Second Messenger Phosphoprotein Res,* 30, 225-53.

Rosen, L. B., Ginty, D. D., Weber, M. J. & Greenberg, M. E. 1994. Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. *Neuron,* 12, 1207-21.

Rusanescu, G., Qi, H., Thomas, S. M., Brugge, J. S. & Halegoua, S. 1995. Calcium influx induces neurite growth through a Src-Ras signaling cassette. *Neuron,* 15, 1415-25.

Ruthazer, E. S., Akerman, C. J. & Cline, H. T. 2003. Control of axon branch dynamics by correlated activity in vivo. *Science,* 301, 66-70.

Ruthazer, E. S., Li, J. & Cline, H. T. 2006. Stabilization of axon branch dynamics by synaptic maturation. *J Neurosci,* 26, 3594-603.

Saffen, D. W., Cole, A. J., Worley, P. F., Christy, B. A., Ryder, K. & Baraban, J. M. 1988. Convulsant-induced increase in transcription factor messenger RNAs in rat brain. *Proc Natl Acad Sci U S A,* 85, 7795-9.

Sassone-Corsi, P. 1995. Transcription factors responsive to cAMP. *Annu Rev Cell Dev Biol,* 11, 355-77.

Scheetz, A. J. & Constantine-Paton, M. 1994. Modulation of NMDA receptor function: implications for vertebrate neural development. *FASEB J,* 8, 745-52.

Schuman, E. M. 1999. Neurotrophin regulation of synaptic transmission. *Curr Opin Neurobiol,* 9, 105-9.

Seeburg, P. H., Higuchi, M. & Sprengel, R. 1998. RNA editing of brain glutamate receptor channels: mechanism and physiology. *Brain Res Brain Res Rev,* 26, 217-29.

Shatz, C. J. & Stryker, M. P. 1988. Prenatal tetrodotoxin infusion blocks segregation of retinogeniculate afferents. *Science,* 242, 87-9.

Sheng, M., Cummings, J., Roldan, L. A., Jan, Y. N. & Jan, L. Y. 1994. Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. *Nature,* 368, 144-7.

Shi, S. H., Hayashi, Y., Petralia, R. S., Zaman, S. H., Wenthold, R. J., Svoboda, K. & Malinow, R. 1999. Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. *Science,* 284, 1811-6.

Sin, W. C., Haas, K., Ruthazer, E. S. & Cline, H. T. 2002. Dendrite growth increased by visual activity requires NMDA receptor and Rho GTPases. *Nature,* 419, 475-80.

Sretavan, D. W., Shatz, C. J. & Stryker, M. P. 1988. Modification of retinal ganglion cell axon morphology by prenatal infusion of tetrodotoxin. *Nature,* 336, 468-71.

Taha, S. A. & Stryker, M. P. 2005. Ocular dominance plasticity is stably maintained in the absence of alpha calcium calmodulin kinase II (alphaCaMKII) autophosphorylation. *Proc Natl Acad Sci U S A,* 102, 16438-42.

Takumi, Y., Ramirez-Leon, V., Laake, P., Rinvik, E. & Ottersen, O. P. 1999. Different modes of expression of AMPA and NMDA receptors in hippocampal synapses. *Nat Neurosci,* 2, 618-24.

Van Aelst, L. & Cline, H. T. 2004. Rho GTPases and activity-dependent dendrite development. *Curr Opin Neurobiol,* 14, 297-304.

Van Zundert, B., Yoshii, A. & Constantine-Paton, M. 2004. Receptor compartmentalization and trafficking at glutamate synapses: a developmental proposal. *Trends Neurosci,* 27, 428-37.

Vaughn, J. E. 1989. Fine structure of synaptogenesis in the vertebrate central nervous system. *Synapse,* 3, 255-85.

Wayman, G. A., Impey, S., Marks, D., Saneyoshi, T., Grant, W. F., Derkach, V. & Soderling, T. R. 2006. Activity-dependent dendritic arborization mediated by CaM-kinase I activation and enhanced CREB-dependent transcription of Wnt-2. *Neuron,* 50, 897-909.

Wayman, G. A., Lee, Y. S., Tokumitsu, H., Silva, A. J. & Soderling, T. R. 2008. Calmodulin-kinases: modulators of neuronal development and plasticity. *Neuron,* 59, 914-31.

Wiesel, T. N. 1982. Postnatal development of the visual cortex and the influence of environment. *Nature,* 299, 583-91.

Wiesel, T. N. & Hubel, D. H. 1963. Single-Cell Responses in Striate Cortex of Kittens Deprived of Vision in One Eye. *J Neurophysiol,* 26, 1003-17.

Wisden, W., Errington, M. L., Williams, S., Dunnett, S. B., Waters, C., Hitchcock, D., Evan, G., Bliss, T. V. & Hunt, S. P. 1990. Differential expression of immediate early genes in the hippocampus and spinal cord. *Neuron,* 4, 603-14.

Wong, R. O. & Ghosh, A. 2002. Activity-dependent regulation of dendritic growth and patterning. *Nat Rev Neurosci,* 3, 803-12.

Wong, W. T., Faulkner-Jones, B. E., Sanes, J. R. & Wong, R. O. 2000. Rapid dendritic remodeling in the developing retina: dependence on neurotransmission and reciprocal regulation by Rac and Rho. *J Neurosci,* 20, 5024-36.

Wong, W. T. & Wong, R. O. 2000. Rapid dendritic movements during synapse formation and rearrangement. *Curr Opin Neurobiol,* 10, 118-24.

Wu, G., Malinow, R. & Cline, H. T. 1996. Maturation of a central glutamatergic synapse. *Science,* 274, 972-6.

Wu, G. Y. & Cline, H. T. 1998. Stabilization of dendritic arbor structure in vivo by CaMKII. *Science,* 279, 222-6.

Wu, G. Y., Zou, D. J., Rajan, I. & Cline, H. 1999. Dendritic dynamics in vivo change during neuronal maturation. *J Neurosci,* 19, 4472-83.

Yamagata, K., Andreasson, K. I., Kaufmann, W. E., Barnes, C. A. & Worley, P. F. 1993. Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids. *Neuron,* 11, 371-86.

Yashiro, K. & Philpot, B. D. 2008. Regulation of NMDA receptor subunit expression and its implications for LTD, LTP, and metaplasticity. *Neuropharmacology,* 55, 1081-94.

Ziv, N. E. & Smith, S. J. 1996. Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. *Neuron,* 17, 91-102.

Zou, D. J. & Cline, H. T. 1999. Postsynaptic calcium/calmodulin-dependent protein kinase II is required to limit elaboration of presynaptic and postsynaptic neuronal arbors. *J Neurosci,* 19, 8909-18.

Chapter 2: CPG15 Regulates Synapse Stability in the Developing and Adult Brain

Tadahiro Fujino, Jennifer H. Leslie, Ronen Eavri, Jerry L. Chen, Walter C. Lin, Genevieve H. Flanders, Erzsebet Borok, Tamas L. Horvath, and Elly Nedivi

J.H.L performed diolistic-labeling experiments at P15 and electrophysiology in figures 2.3 and S2.2. J.H.L. wrote sections of the manuscript pertinent to these figures and J.H.L. revised the manuscript with E.N.

2.1 INTRODUCTION

During development, neuronal processes extend and retract as they explore their environment to identify appropriate synaptic partners. The establishment of pre and post-synaptic contact is an early event in an ordered progression that can lead to formation of stable mature synapses. It has been proposed that synapse formation consequently acts as a stabilizing force on growing axonal and dendritic processes (Ruthazer et al., 2006, Meyer and Smith, 2006). While studies have delineated some aspects of synaptogenesis and synaptic maturation (McAllister, 2007), the signals at the contact point of axon and dendrite that determine whether a synapse will stabilize and persist have not been fully elucidated.

Although excitatory synaptogenesis can occur in the absence of neural activity (Verhage et al., 2000, Gomperts et al., 2000), strong evidence suggests that experience plays a critical role in biasing the formation and stabilization of synapses that transmit appropriately patterned activity (Hua and Smith, 2004), and that NMDA type glutamate receptors mediate this activity-dependent synapse selection (Gomperts et al., 2000). Activation of NMDA receptors allows Ca^{+2} influx into the postsynaptic cell, turning on kinase signaling cascades, which in turn initiate transcription factor activation and new gene expression (reviewed in (Flavell and Greenberg, 2008, Loebrich and Nedivi, 2009). Yet how this set of events leads to local implementation of synaptic stabilization is not clear. In fact, little is known about the molecular mechanisms regulating selective synapse and dendrite stabilization in response to activity.

cpg15 (also termed *neuritin*) was first identified in a screen for activity-regulated genes in rat (Nedivi et al., 1993, Hevroni et al., 1998) and is a downstream target of the classic synaptic-plasticity signaling cascade, involving the NMDA receptor, MAPK, CaMK, and CREB (Fujino et al., 2003). During development, *cpg15* expression is induced in presynaptic neurons upon contact with their target (Diaz et al., 2002), and is spatially and temporally correlated with

37

synapse formation and activity-dependent plasticity (Nedivi et al., 1996, Lee and Nedivi, 2002, Corriveau et al., 1999). *cpg15* encodes a small extracellular protein anchored to the cell surface via a glycosyl-phosphoinositide (GPI) link (Naeve et al., 1997). CPG15 overexpression in the developing *Xenopus* enhances dendritic and axonal elaboration in a non cell-autonomous manner, as well as synapse formation and maturation (Nedivi et al., 1998, Cantallops et al., 2000, Javaherian and Cline, 2005).

To investigate the requirement for CPG15 in a mammalian system, we generated a knockout mouse for *cpg15* (*cpg15* KO). We find that in the *cpg15* KO, there is a developmental delay in axonal and dendritic arborization and maturation of excitatory synapses. In the dentate gyrus of the hippocampus as many as 30% of spines initially lack synapses. Chronic *in vivo* imaging of cortical pyramidal neurons through a cranial window shows that while dendritic spine dynamics in *cpg15* KO mice are comparable to controls, fewer events in these mice are stabilized, and thus favor persistent spine loss. These results suggest that the *in vivo* developmental deficits in the *cpg15* KO mouse derive from lack of a synaptic stabilization signal, perhaps supplied in an activity-dependent manner. While adult circuits appear normal, they are functionally suboptimal, leading to poor performance in learning tasks. These findings establish a role for CPG15 in efficient circuit formation and function, and provide a potential molecular mechanism for selective synapse stabilization.

2.2 RESULTS

2.2.1 Generation of a *cpg15* knockout mouse

To investigate the *in vivo* role of *cpg15*, we generated a mouse lacking *cpg15* using a conditional knockout approach based on the Cre-*loxP* system. We first made a "floxed" *cpg15* mouse with *loxP* sites flanking *cpg15* exons two and three (Fig. 2.1A). Exons two and three contain the entire coding sequence for the mature form of CPG15 and excision of the sequence between the *loxP* sites by Cre recombinase would generate a null mutation. Floxed *cpg15* mice were crossed to a global Cre-deleter line that expresses Cre recombinase in all tissues including the germ line (Lakso et al., 1996b) to obtain general *cpg15* null mice (*cpg15* KO mice). Southern blotting showed successful homologous recombination in the floxed *cpg15* mouse and Cre excision in the *cpg15* null mouse (Fig. 2.1B). Northern blotting and Western blotting confirmed absence of *cpg15* mRNA and CPG15 protein in the brains of *cpg15* KO mice (Fig. 2.1C, D).

Homozygous *cpg15* KO mice were born at a Mendelian ratio and showed no overt behavioral abnormalities. Their muscle strength and motor coordination were also normal (Fig. 2.S1A, B). *cpg15* KO mice were leaner than wild-type (WT) littermates, with a body length 3% shorter, and on average weighing 20-30% less (Table 2.S1). *cpg15* KO brains were similar in weight and size to WT brains, although the cerebellum tended to be slightly smaller (Table 2.S1). The anatomy of Nissl-stained *cpg15* KO brains at 8 weeks of age appeared normal (Fig. 2.1E). Neocortical volume, cell density, and total cell number were similar between WT and *cpg15* KO mice (Table 2.S1).
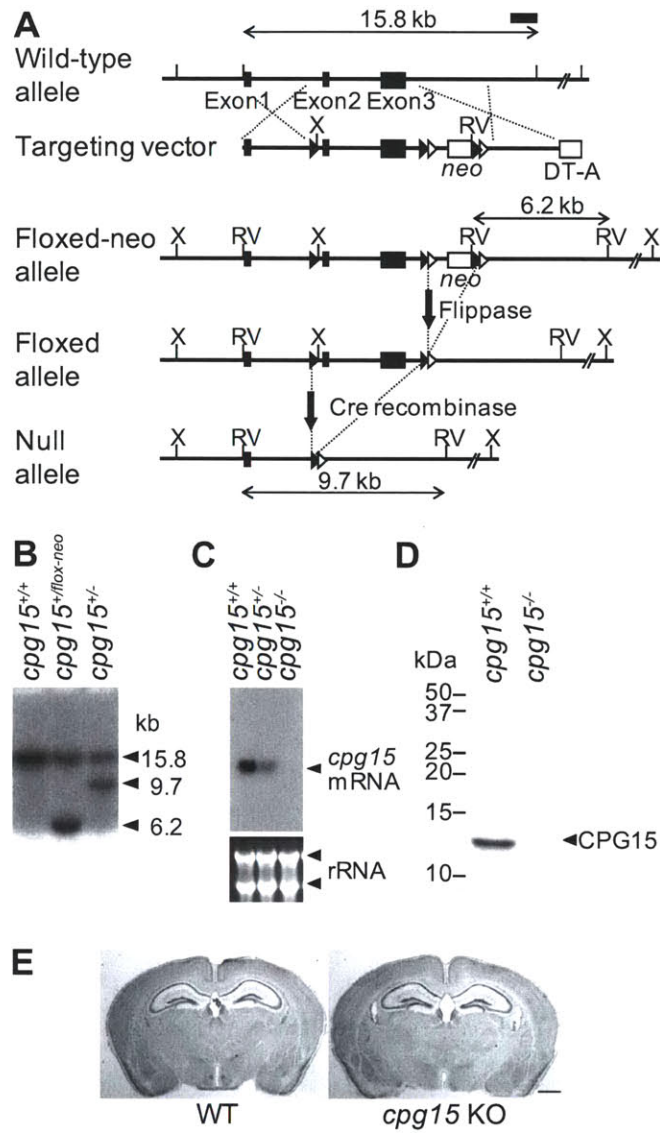
Figure 2.1 Generation of the *cpg15* KO mouse. (A) Schematic drawing of the WT *cpg15* allele, the targeting vector, the floxed allele with the neomycin resistant gene (*neo*), the floxed allele without *neo*, and the null allele. Indicated are: three *cpg15* exons (closed boxes), the *neo* and the diphtheria toxin A gene (DT-A) serving as positive and negative selection markers respectively (open boxes), *loxP* sites (closed triangles), *FRT* sites (open triangles), and *Xho*I (X) and *Eco*RV (RV) restriction sites. Homologous recombination between the WT allele and the targeting vector generated a floxed-neo allele, and eliminated the DT-A gene. The *neo* gene flanked by two *FRT* sites was deleted by injection of flippase RNA into eggs harvested from the

floxed-neo mice. Mice after Flippase recombination were crossed with a Cre-deleter line to generate the *cpg15* null allele. Positions of the 3' probe used for Southern blot analysis and expected band sizes are indicated. (B) Southern blot analysis of WT (*cpg15$^{+/+}$*), heterozygous floxed (*cpg15$^{+/flox-neo}$*), and heterozygous null mouse (*cpg15$^{+/-}$*). Genomic DNA was digested with *Eco*RV and probed with the 3' probe shown in (A). In addition to the WT 15.8-kb band, the floxed-neo mouse shows a 6.2-kb band and the null mouse shows a 9.7-kb band as expected. (C) Northern blot analysis of brain RNA from WT, heterozygous, and homozygous-null mouse probed with *cpg15* cDNA. Ribosomal RNA (rRNA) is shown as a loading control. (D) Western blot analysis of brain extracts from WT and homozygous-null mouse probed with an anti-CPG15 antibody. (E) Nissl-stained coronal sections from WT and *cpg15* KO mice. Scale bar: 1 mm

2.2.2 Delayed axonal and dendritic arbor development in *cpg15* KO mice

In the developing *Xenopus*, CPG15 overexpression promotes arborization of retinal ganglion cell axonal projections in the tectum by reducing branch retractions (Cantallops et al., 2000). To test for deficits in axon arbor development in *cpg15* KO mice, we bulk-labeled retinal ganglion cell projections as these axons were growing and elaborating their arbors in the lateral geniculate nucleus (LGN) of the thalamus by injecting each eye with an anterograde tracer conjugated to a different fluorophore. In WT mice, the total area of LGN covered by projections from both eyes increased dramatically between postnatal day (P) 4 and P21 (Fig. 2.2A, B). Since neuronal proliferation in the LGN occurs during embryogenesis (Brückner et al., 1976), the postnatal increase in LGN volume is largely due to axon ingrowth, and elaboration of both the axonal and dendritic neuropil. In the *cpg15* KO mice, the retinogeniculate projection area is similar in size to that of WT mice at P4, but the projection area does not grow to the same extent as controls by P9 (Fig. 2.2A, B). LGN cell count and density in the *cpg15* KO mice at P9 were comparable to controls (Table 2.S2), suggesting that the difference in projection areas at this age is not due to a decrease in LGN cell numbers, but rather due to delayed development of the neuropil including retinal ganglion cell axons and LGN cell dendrites. By P90, the retinogeniculate projection area was indistinguishable between genotypes, with similar levels of segregation of ipsilateral and contralateral arbors as measured by the degree of overlap (Fig. 2.2C). While the delay in retinogeniculate projection development in the LGN of *cpg15* KO mice indicates that there may be deficits in axonal and perhaps dendritic elaboration, the normal appearance of the LGN at P90 suggests that these deficits are overcome with age.

CPG15 overexpression studies showing enhanced dendritic arborization (Nedivi et al., 1998) and the potential delay in LGN neuropil development seen in *cpg15* KO mice, led us to
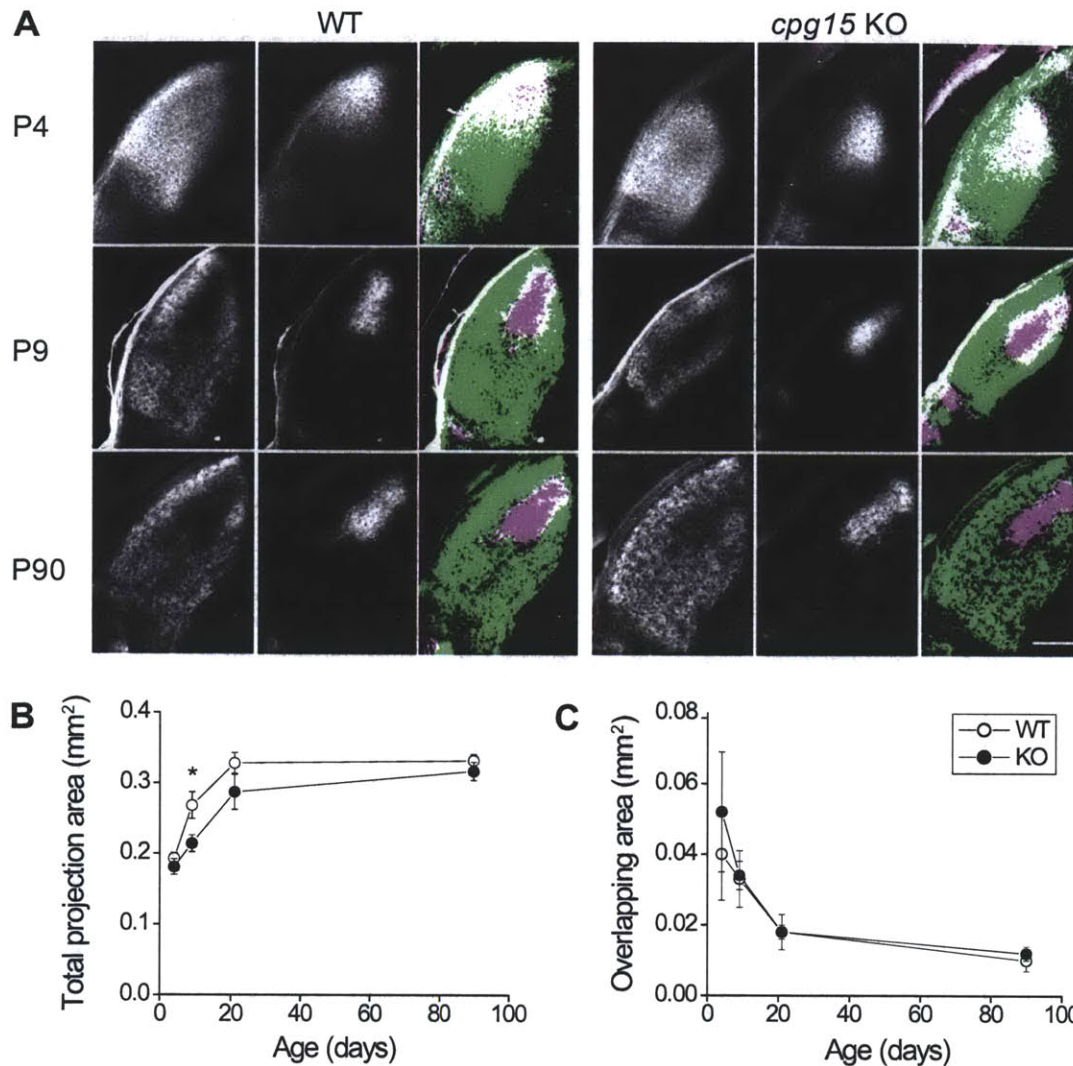
Figure 2.2 Delayed axon arbor development in the LGN of *cpg15* KO mice. (A) Representative images of the dorsal LGN at different developmental times from WT and *cpg15* KO mice injected in each eye with wheat germ agglutinin conjugated to different fluorophores. Projections from the contralateral eye (left), ipsilateral eye (middle), and the merged image from both eyes (right) are shown. For merged images, all pixels above background were pseudo-colored in green for contralateral or magenta for ipsilateral eye. White indicates overlap. Notice similar overlap but different size of labeled LGN at P9. (B) Age-dependent change in total area of LGN covered by projections from both ipsilateral and contralateral eyes ($n = 3$ for P4, P9,

P21, $n = 5$ for P90). (*) P-value < 0.05. (C) Age-dependent change in the area of overlap between projections from ipsilateral and contralateral eyes did not differ between genotypes. Scale bar: 200 mm.

examine whether dendritic arbor development was also delayed in *cpg15* KO neurons. Using diolistic labeling (Grutzendler et al., 2003) we visualized neurons in the granule cell layer of the dentate gyrus (DG) of the hippocampus (Fig. 2.3A), a region that normally shows high *cpg15* expression throughout development, and has been extensively characterized in the context of synaptic plasticity and paradigms of learning and memory.

Granule cell dendritic arbors in the hippocampal DG predominantly develop in two stages (Rahimi and Claiborne, 2007). The bulk of neurogenesis occurs embryonically through P14, peaking during the second postnatal week, with the majority of cells born postnatally. During this period granule cell dendritic trees are initially established. The second phase begins at the end of the second postnatal week and lasts until about 2 months of age. It is during this time that the dendritic trees are sculpted and refined. At the onset of this second phase, granule cell dendritic trees have many segments including short terminal branches, which are largely pruned by 2 months. The remaining branches increase in length over this period resulting in a conservation of total dendrite length but a reduction in segments and terminal ends. To assess the contribution of CPG15 at these two phases we compared granule cell dendritic morphology in the *cpg15* KO and WT littermates at P15 and P60. At P15, DG granule cells in the *cpg15* KO showed significantly fewer branch tips per neuron as compared to WT, suggesting an initial lack of complexity due to reduced numbers of short terminal branches. Between P15 and P60 we observed branch tip pruning that was more pronounced in the WT as compared to KO animals, such that the difference between them was diminished by 2 months of age and dendritic arbors appeared normal (Fig. 2.3B). This is similar to our findings regarding neuropil development in the LGN.
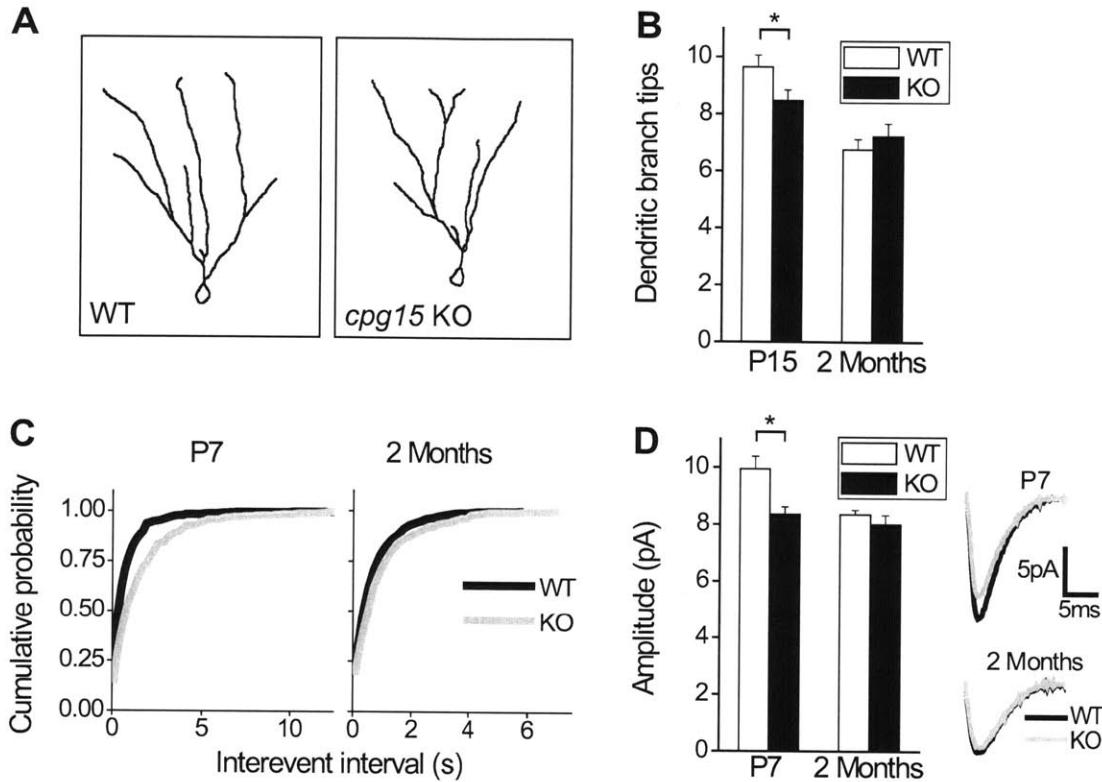
Figure 2.3 Delayed dendritic arbor and synapse development in DG of *cpg15* KO mice. (A) Representative traces of reconstructed DG granule cells from the WT, left, and *cpg15* KO, right, at 2 months of age. (B) Average number of dendritic branch tips per cell at P15, left, ($n = 39$ WT cells, $n = 32$ KO cells) and 2 months, right ($n = 32$ cells for WT and KO). (*) P-value < 0.05. (C) Cumulative probability plots of interevent intervals of mEPSCs of DG granule cells at ages P7, left ($n = 9$ WT cells, $n = 7$ KO cells) P-value < 0.001 by K-S test, and 2 months, right ($n = 13$ WT cells, $n = 7$ KO cells) P-value = 0.003 by K-S test. (D) Average mEPSC amplitudes of DG granule cells at ages P7, left ($n = 9$ WT cells, $n = 7$ KO cells), and 2 months, right ($n = 13$ WT cells, $n = 7$ KO cells). Averaged mEPSCs are plotted for both ages (right inset). (*) P-value = 0.01.

2.2.3 Delayed synaptic development and maturation in *cpg15* KO mice

Next, we tested for deficits in the *cpg15* KO that might derive from the effects of CPG15 on synapse formation and maturation. We examined formation of functional synapses by performing whole cell patch clamp recordings in the granule cell layer of the DG in acute hippocampal slices and recorded spontaneous miniature post-synaptic currents (mEPSCs) (Fig. 2.3C). We found that in the DG of *cpg15* KO mice at P7, mEPSCs occur with lower frequency. This deficit persisted at 2 months of age, but was less significant (Fig. 2.3C). In the P7 DG of *cpg15* KO mice, mEPSC amplitudes were also reduced, however this deficit was no longer observed by 2 months of age (Fig. 2.3D). These results suggest that during development *cpg15* KO mice have fewer and less mature functional synapses.

To examine the synaptic structural correlates of reduced mEPSC frequency and amplitude in the developing hippocampus we performed electron microscopy (EM) in different subfields of the hippocampal formation. No apparent qualitative differences in synaptic structure were seen in any of these areas, as assessed by the presence of presynaptic terminal zones with vesicles and apposed post-synaptic densities (PSDs) (Fig. 2.4A). However, when the density of asymmetric synapses on dendritic spines was measured by unbiased stereology, we found that *cpg15* KO mice had spine synapse densities in the DG that were 26% lower than WT controls at 2 months of age (Fig. 2.4B). This change appeared specific to the DG and was not observed in CA1 at this age. The decrease in DG spine density correlates with our findings of decreased mEPSC frequency in the *cpg15* KO DG (Fig. 2.3C) but not in CA1 (Fig. 2.S2) at 2 months of age, and may be due to late development of the DG as compared to other hippocampal regions. Interestingly, when we measured the density of spines and spine-like protrusions at 2 months
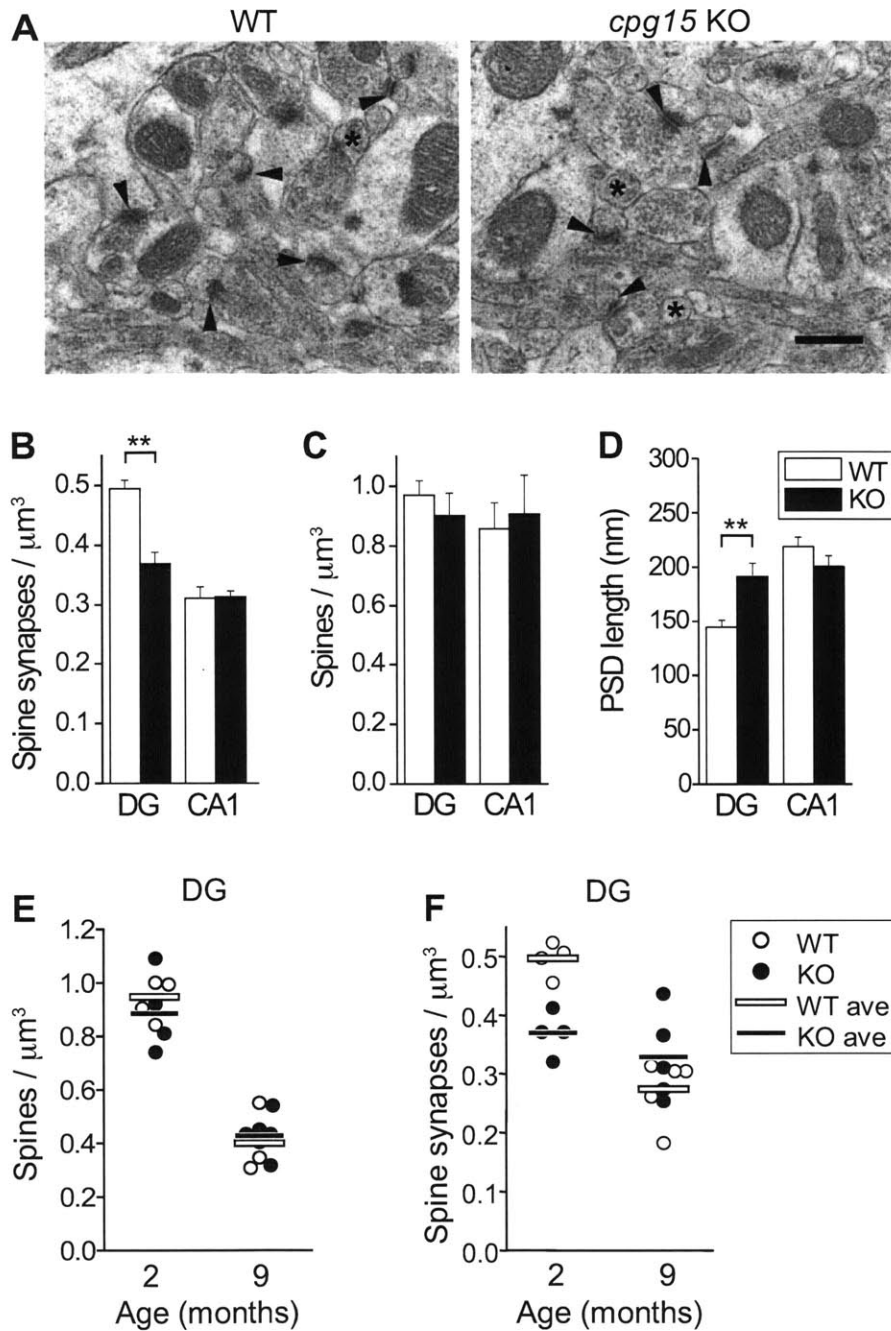
Figure 2.4 Delayed spine synapse development and lack of age-dependent synaptic pruning in *cpg15* KO mice. (A) Representative EM images from the molecular layer of the DG of WT and *cpg15* KO mice. Arrowheads indicate spine synapses and asterisks mark spine or spine-like protrusions. (B) Spine synapse density, (C) spine or spine-like protrusion density, and (D) PSD length of indicated regions ($n = 4$ mice for WT and KO). (**) P-value < 0.05. (E) Age-dependent

change in spine density of DG region. Spine density per mouse is represented by circles, and average for each genotype is indicated by a horizontal line ($n = 4$ mice each for WT and KO at 2 months, $n = 4$ mice for WT and 5 mice for KO at 9 months). P-value < 0.001 for 2 months vs. 9 months for WT and KO DG. (F) Age-dependent change in spine-synapse density in the DG region. Spine synapse density per mouse is represented by circles, and average for each genotype is indicated by a horizontal line ($n = 4$ mice for 2 months, $n = 5$ mice for 9 months for WT and KO). P-value < 0.001 for 2 months vs. 9 months for WT, n.s. for KO. Scale bar: 1 mm.

regardless of whether they contained a synaptic specialization, there were no significant differences between *cpg15* KO mice and WT controls even in the DG (Fig. 2.4C). These results suggest that in the developing DG of the *cpg15* KO a large fraction of spine-like protrusions lack a synaptic structure. EM examination of synapse size at 2 months, as measured by PSD length, showed that PSDs of *cpg15* KO mice were 33% larger than those of WT mice in the DG but not in CA1 (Fig. 2.4D). Perhaps the increased synapse size is a compensatory response to the decrease in spine synapse numbers in the DG of *cpg15* KO mice.

In the retinogeniculate pathway, as well as the hippocampus, neuropil development in the *cpg15* KO is initially delayed but eventually reaches WT levels. We examined whether the deficit in dendritic spine synapse number also recovers with age by comparing spine and spine synapse densities between 2- and 9-month-old *cpg15* KO mice and WT littermates. Between 2 and 9 months both *cpg15* KO mice and WT controls prune spines in a similar manner (Fig. 2.4E). WT controls also prune spine synapses over this period consistent with ongoing synapse remodeling and refinement (Markus and Petit, 1987). However, *cpg15* KO synapse density, which starts out lower than WT at 2 months, is not further reduced by 9 months of age and remains relatively constant during this period (Fig. 2.4F). Thus, we see that neurons in the late developing DG of *cpg15* KO mice initially form fewer spine synapses than WT neurons. Over time, these synapses are less likely to be pruned, so that by 9 months, synapse numbers are similar in WT and KO.

2.2.4 Increased loss of persistent spines in *cpg15* KO mice

In vivo imaging studies followed by EM have demonstrated that spine sprouting and retraction are associated with synapse formation and elimination (Trachtenberg et al., 2002,

Knott et al., 2006) and that synaptogenesis is inversely correlated with spine motility (Konur and Yuste, 2004). In light of the unusually large fraction of dendritic spines lacking synapses in the developing DG of *cpg15* KO mice we asked whether CPG15 depletion also affects dendritic spine dynamics. To this purpose we performed *in vivo* imaging of neurons in the visual cortices of adult *cpg15* KO mice. The cortex was chosen to assay spine dynamics because it is optically accessible via implantation of cranial windows, allowing chronic monitoring of spine dynamics. In addition, previous studies have shown that even in the adult cortex a significant fraction of dendritic spines remain dynamic, with the majority of events being either transient spine additions or reversible eliminations (Holtmaat et al., 2005, Holtmaat et al., 2006). Persistent dynamic events, including spines that emerge and persist (new-persistent spines) as well as spines that disappear and do not re-emerge (lost-persistent spines) likely best represent events that correspond to synapse formation and elimination (Holtmaat et al., 2005, Holtmaat et al., 2006).

We generated *cpg15* KO mice expressing GFP in a random subset of neurons sparsely distributed within the neocortical layers, by crossing the *thy1*-GFP-S line that expresses GFP in a random subset of neocortical neurons (Feng et al., 2000, Lee et al., 2006) to the *cpg15* KO (see methods). Adult *thy1*-GFP/ *cpg15* KO mice (homozygous for *thy1*-GFP and *cpg15*$^{-/-}$) and *thy1*-GFP littermate controls were surgically implanted with bilateral cranial windows over the visual cortices. Following 3 weeks of recovery, layer 5 (L5) pyramidal neurons were identified and a two-photon imaging volume encompassing their apical tufts in L1 was acquired at 4-day intervals (Fig. 2.5A). We found that the total rate of dynamic events, including both spine gain and loss was not significantly different between WT and *cpg15* KO mice (Fig. 2.5B). However, in the *cpg15* KO the percentage of dynamic events that persisted was significantly higher than in
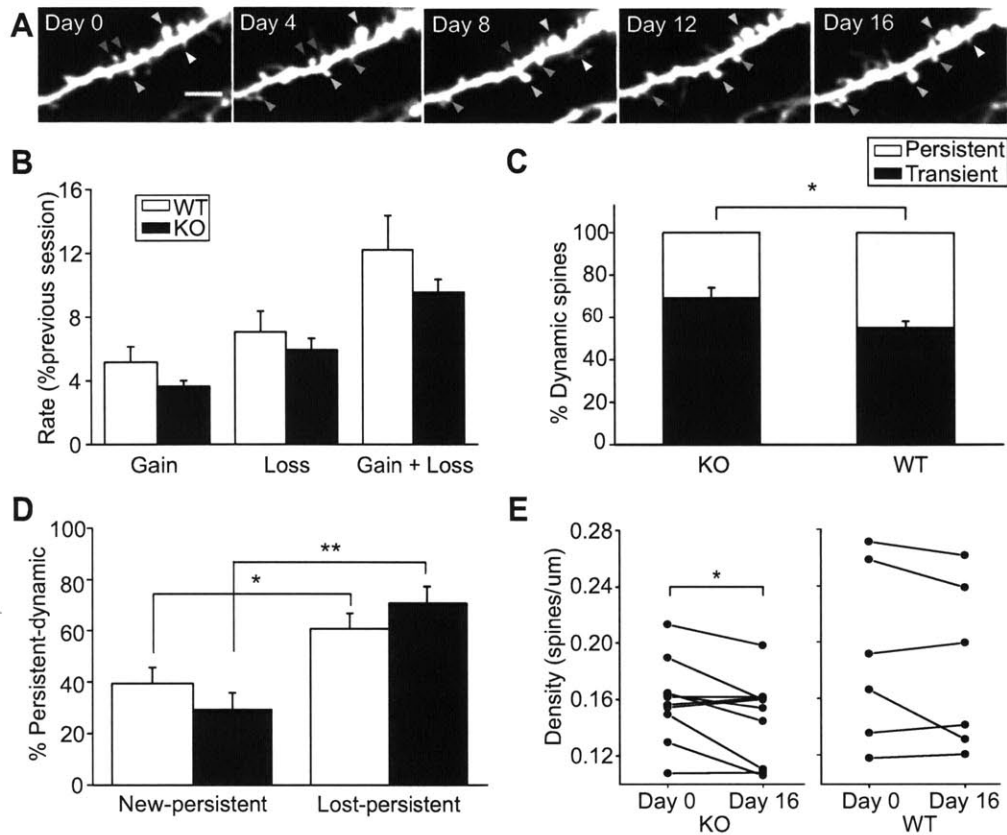
Figure 2.5 Repeated imaging of apical L5 cell dendrites in visual cortex of *cpg15* KO and WT mice show that dendritic spine dynamics in *cpg15* KO neurons are weighed toward spine loss. (A) Representative example of a dendrite stretch showing persistent spines (yellow arrows), new-persistent spines (green arrows), lost-persistent spines (orange arrows) and transient spines (white arrows). (B) Averaged rates of spine gain and spine loss for all sessions (*n* = 9 KO mice, 13 cells, 794 spines, *n* = 8 WT mice, 16 cells, 847 spines). (C) Percent of persistent-dynamic and transient spines out of total dynamic spines. Persistent-dynamic includes lost-persistent and new-persistent spines. (*n* = 9 KO mice, 13 cells, 794 spines, *n* = 6 WT mice, 11 cells, 609 spines). (*) *P*-value <0.05. (D) Percent of new-persistent and lost-persistent spines out of total persistent-dynamic spines. (*) *P*-value <0.05, (**) *P*-value < 0.001. (E) Spine densities of individual animals between the first and fifth imaging sessions. Student's paired t-test (*) *P*-value <0.05. Scale bar: 5 μm.

the WT. Conversely, the percentage of transient events was significantly lower (persistent-dynamic 69.23% for KO, 55.06% for WT; transient 30.76% for KO, 44.94% for WT; P-value < 0.05) (Fig. 2.5C). For both the WT and *cpg15* KO dynamic-persistent events that favor spine loss (lost-persistent) were higher than dynamic-persistent events that favor spine gain (new-persistent). However, this difference was more pronounced for the *cpg15* KO (lost-persistent 70.79% for KO, 60.64% for WT; new-persistent 29.21% for KO, 39.36% for WT; P-value < 0.001 for KO, P-value < 0.05 for WT) (Fig. 2.5D). Since the *cpg15* KO has a greater percentage of dynamic-persistent events overall, and persistent events favor loss, we observe a significant reduction in spine densities for individual *cpg15* KO neurons over the course of the imaging period, which was not seen in WT neurons (Fig. 2.5E). Overall, these results suggest that loss of *cpg15* leads to a decrease in spine stabilization, resulting in reduced maintenance of both newly formed and existing spines.

2.2.5 Inefficient learning in *cpg15* KO mice

Given the contribution of activity-regulated genes to plasticity (Leslie and Nedivi, 2011), we next examined whether the deficits in cellular development and spine stabilization seen in *cpg15* KO mice impacted behavioral plasticity in the adult, such as learning and memory. *cpg15* KO mice were subjected to a fear conditioning test, a form of classical conditioning. Context-dependent fear conditioning is a hippocampal-dependent paradigm. We rationalized that due to cellular defects observed in the hippocampus that *cpg15* KO mice may not perform as well as WT counterparts in this task. Mice were given a paired shock and tone in a conditioning chamber. Afterwards, they were returned to the same chamber to test for contextual memory, or presented with the same tone in a different context to test for tone-dependent memory.

Surprisingly, *cpg15* KO mice exhibited less freezing than WT controls in response to both context and tone (Fig. 2.6A), suggesting deficits in both contextual and tone-dependent memory. Interestingly, *cpg15* KO mice showed little freezing in response to context even 1 hour after training, suggesting impaired short-term memory. In this test it was impossible to discriminate whether there was a true deficit in long-term memory or if it was secondary to the short-term memory deficit. To address this question, mice were trained using repeated conditioning sessions on days 1, 3, 5, 7 and tested 1 day after each session on days 2, 4, 6, 8. For tone-dependent memory, WT mice showed a robust freezing response after the first conditioning trial with an additional small increase after the second trial (Fig. 2.6B). *cpg15* KO mice showed little freezing after the first conditioning trial, despite a slightly lower threshold for pain and higher anxiety that WT controls (Fig. 2.S1C, D) but showed a large increase after the second trial before reaching a similar plateau as WT controls by the third trial. To see if stronger conditioning would improve learning, in the fourth conditioning session three tone-shock pairs were given instead of one. This fourth conditioning session did not elicit increased freezing in either genotype, indicating saturation of the response. The repeated training experiment demonstrates that while *cpg15* KO mice are slow to learn the task, they do have the basic sensory and motor functions to perform it and are able to form and retrieve long-term memories once they learn the task.

To see if memory was stable on a longer time scale, we tested the mice 2 weeks after conditioning by repeated training. Memory retention, calculated as the ratio of freezing at 2 weeks to 1 day after completion of the repeated training trials, showed that *cpg15* KO mice retained memory similarly to WT controls (Fig. 2.6C). In summary, *cpg15* KO mice showed inefficient learning of fear memories. However, this impairment could be overcome by repeated training, and memory was stable once acquired.
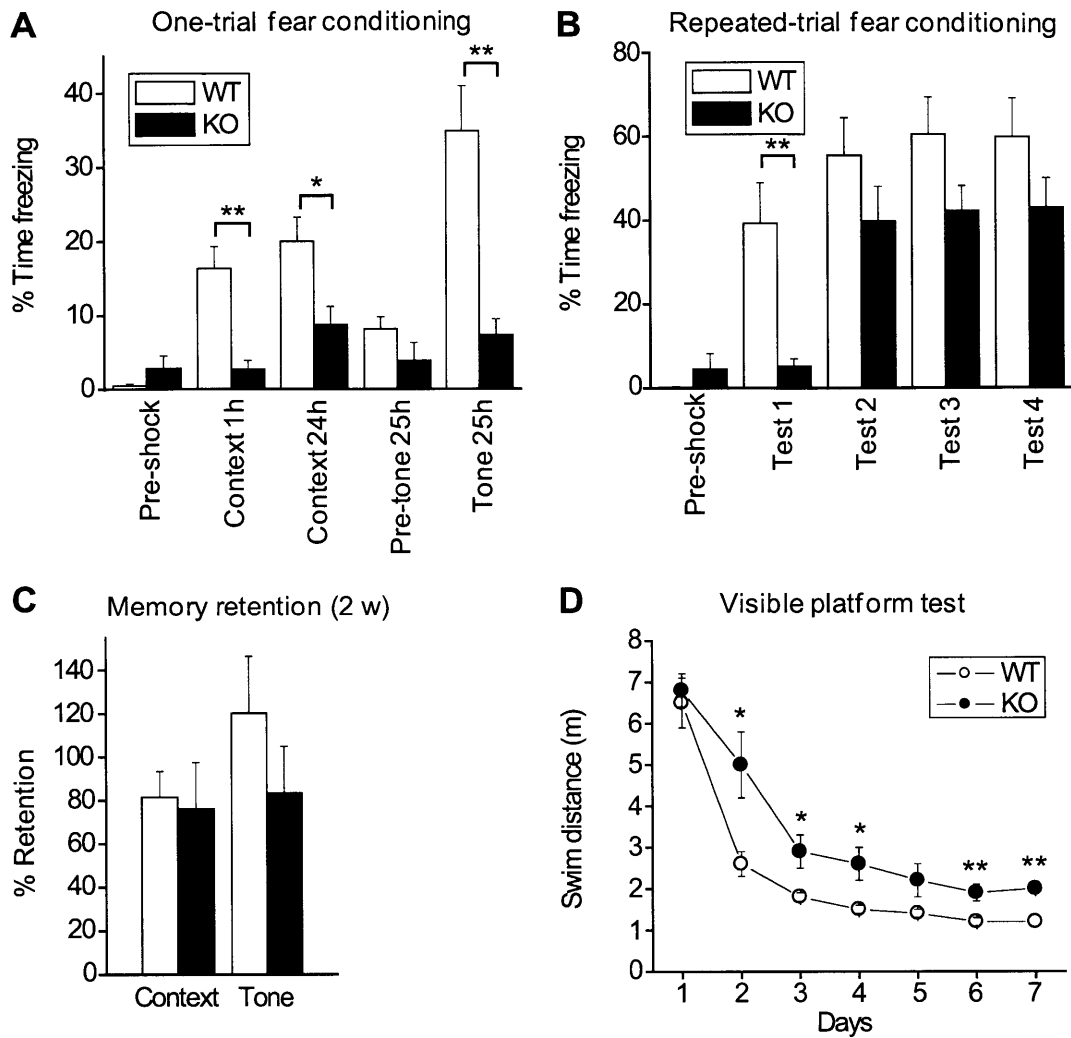
Figure 2.6 *cpg15* **KO mice show slow learning in fear conditioning and visible platform tests.** (A) Mice were given a paired tone and shock, then tested for contextual memory 1h and 24 h later, and for tone-dependent memory at 25 h after the shock. Fear memory is measured as percent time spent freezing ($n = 23$ for WT, $n = 27$ for KO). (*) P-value < 0.05, (**) P-value < 0.01. (B) Freezing response to tone after repeated training. Mice were given tone-shock pairings every other day, and were tested 1 day after each training session ($n = 10$ for WT and KO). (**) P-value < 0.01. (C) Fear conditioning is stable for at least 2 weeks. Mice were tested at 1 day and 2 weeks after the repeated training shown in (B). Percent retention was calculated as the ratio in

freezing time at 2 weeks to 1 day after training, and averaged for each genotype. Some mice showed longer freezing times at 2 weeks, resulting in some retention scores above 100% ($n = 10$ for WT and KO). (D) Swim distance with repeated training in the Morris water maze visible platform test ($n = 12$ for WT and KO). (*) P-value < 0.05, (**) P-value < 0.001.

To confirm that the learning deficit in *cpg15* KO mice was not specific to the fear conditioning task, we also tested learning in the Morris water maze. The Morris water maze requires mice to locate a platform submerged in a pool using only visual or spatial cues. In the visible platform version of the Morris water maze, a visual cue is placed on the submerged platform so that mice can locate the platform based on this cue. This task does not require spatial memory, but does require mice to make certain associations such as those between the visual cue and the platform, in addition to basic visual and motor abilities to perform the task.

During the seven days of training, swim distance to reach the platform decreased for both WT and *cpg15* KO mice indicating that they were able to learn the task (Fig. 2.6D). However, *cpg15* KO mice showed slower acquisition when compared to WT controls. The difference was most obvious on the second day of the training, when WT controls were close to their peak performance but many of the *cpg15* KO mice showed no improvement. Despite these deficits, improvement in performance and the directed swimming towards the platform after training suggests that *cpg15* KO mice were able to recognize the visual cue. Thus in the visible platform test, *cpg15* KO mice showed slower acquisition of the task but improved with repeated training, similar to what we observed in the fear conditioning test.

2.3 DISCUSSION

Here we show that a KO mouse lacking *cpg15* exhibits delayed axonal, dendritic, and synaptic development. Adult *cpg15* KO mice display reduced spine maintenance leading to gradual spine loss. Loss of *cpg15* also has behavioral consequences, manifested as poor performance in learning tasks.

The finding that adult *cpg15* KO mice have an apparently normal neocortex with regards to size and cell number was initially surprising, since acute RNA interference (RNAi)-mediated knockdown in embryonic rat brains showed a requirement for CPG15 in cortical progenitor-cell survival (Putz et al., 2005). We were also surprised that despite the robust effect of CPG15 overexpression on dendritic and axonal arbor growth in the developing *Xenopus* retinotectal system (Nedivi et al., 1998, Cantallops et al., 2000), the effects of CPG15 deletion on both axonal and dendritic arborization are more subtle and are fully compensated for in the *cpg15* KO mice by 2 months of age. There are precedents for *in vivo* RNAi-mediated interventions and overexpression studies during development resulting in deficits not observed in knockout animals. For example, in the case of the doublecortin (*DCX*) gene, acute knockdown early in development results in severe cortical lamination deficits but in the knockout mouse cortical lamination is normal (Bai et al., 2003). Even in the case of neurotrophins and their receptors, mutant mouse studies repeatedly show less overt phenotypes than might be expected for molecules considered critical for both neuronal survival and differentiation (Conover and Yancopoulos, 1997). Molecules such as neuroligins that exhibit robust synaptogenic activity in co-culture assays (Scheiffele et al., 2000), when completely eliminated in KO mice fail to affect synapse numbers *in vivo* (Varoqueaux et al., 2006). Rather, neuroligin KO mice show subtle synaptic deficits that are likely associated with selection of specific synapse types. When a gene product is missing from the outset, compensatory or redundant molecules and mechanisms may be brought into play before developmental programs are significantly compromised. This is likely to be particularly true for molecules involved in synapse formation and maturation due to the multiplicity of players involved (Brose, 2009). This does not mean that the function of these molecules is completely interchangeable, rather combinations of these molecules likely instruct

specificity and diversity of synaptogenesis. In the *cpg15* KO other molecules can apparently replace the trophic function of CPG15, resulting in normal cell number and structure. *cpg15* KO neurons can form functional synapses with normal ultrastructure indicating that CPG15 is also dispensable for synaptogenesis and synaptic growth. However, the delay in arbor growth and synapse development and the reduced stability of dendritic spines in the adult suggest that CPG15 plays a critical regulatory role in determining which synaptogenic events are stabilized and maintained.

2.3.1 A biphasic role for CPG15 in arbor and synapse development

During development, increased rate of growth is often equated with maturation, but this interpretation may be too simplistic. There are many examples throughout the developing brain where maturation proceeds first through the initial establishment of exuberant and/or promiscuous dendritic and axonal branches and synapses, followed by a period of dynamic refinement and sculpting during which inappropriate contacts and branches are eliminated and appropriate ones are stabilized and elaborated (Kano and Hashimoto, 2009). The *cpg15* KO mouse exhibits a lack of initial exuberance in dendritic branch elaboration and synapse formation in areas of the developing brain investigated in this study. This results in some cases in the superficial appearance of "mature" looking branches from an early age, however these arbors have not matured in the sense that they have not undergone the same extensive remodeling as their WT counterparts. We would not interpret this as a precocious maturation, since a true precocious maturation would go through the same sequence of events as a normally developing animal, except earlier. Consistently our data shows that this in not the case for the *cpg15* KO mouse. Instead we show that the *cpg15* KO never reaches the phase in development where

processes and synapses become exuberant enough for large-scale sculpting and refinement to be observed.

This interpretation is also consistent with the developmental regulation of *cpg15* expression. In the visual system early in development *cpg15* expression is activity independent (Corriveau et al., 1999, Lee and Nedivi, 2002). Promiscuous early expression of CPG15 may result in the establishment of large arbors and many synapses, effective substrates upon which activity-dependent mechanisms can later work to sculpt circuits by choosing which synapses and branches to keep and which to prune. During these later periods *cpg15* expression becomes activity-dependent, perhaps allowing CPG15 to function in the stabilization of appropriate activity-selected synaptic partners and branches.

2.3.2 CPG15 as a synapse stabilization factor

Despite lower spine synapse density in the hippocampal DG of 2-month-old *cpg15* KO mice as compared to WT controls, spine counts in this region were normal. This suggests that *cpg15* KO neurons have a larger percentage of spines without well-defined synaptic structures. Previous serial EM reconstruction studies have found little evidence of spines without synaptic specializations in adult animals (Harris et al., 1989, Harris and Kater, 1994), and developmental studies in dissociated and organotypic slice cultures suggest that synaptic molecules can cluster at pre and postsynaptic sites shortly after contact (Vardinon Friedman et al., 2000, Okabe et al., 2001). Imaging studies combined with retrospective EM show that the latency to formation of synaptic structures on nascent spines varies from several hours up to 4 days (Knott et al., 2006, Nägerl et al., 2007, Zito et al., 2009). Thus, spine formation can lead to rapid synapse recruitment and stabilization, but does not necessarily do so. The choice between synapse

stabilization and elimination is likely guided by specific signaling molecules that ensure the selection of optimal synaptic partners and efficient circuit wiring. Reduction in spine synapse density in the *cpg15* KO mice without a change in spine number may reflect an increase in spine-like protrusions that fail to stabilize nascent synapses. Indeed, when spine and spine synapse densities are analyzed at a later age, *cpg15* KO mice prune spines, but not spine synapses suggesting selective pruning of unstabilized, synapse-less spines. Further, *in vivo* time-lapse imaging of dendritic spines on cortical layer 5 neurons of wild-type and *cpg15* KO mice show that while overall spine dynamics on *cpg15* KO neurons is normal, there is a bias towards increased spine loss, suggesting that spines are less well maintained. The fact that this can be observed even in the adult on an individual cell basis further suggests an acute requirement for CPG15 in spine and perhaps synapse stabilization.

During development, synapses have been shown to act as anchoring points for growing dendritic and axonal branches, facilitating branch extension and giving pause to branch retractions, so that arbor dynamics are strongly influenced by synapse stabilization (Ruthazer et al., 2006, Meyer and Smith, 2006). It is therefore likely that delayed axon and dendrite development in the *cpg15* KO is directly linked to the delay in functional synapse formation and maturation. This interpretation is consistent with findings from previous overexpression studies in the developing *Xenopus* which showed that CPG15 coordinately promotes synaptic maturation and arbor growth (Cantallops et al., 2000, Javaherian and Cline, 2005), and that increased arbor growth is due to fewer branch retractions (Cantallops et al., 2000).

In rodents, exposure to an enriched environment improves performance in learning and memory tasks and increases dendritic spine density (van Praag et al., 2000), suggesting that changes in connectivity may be a cellular correlate of improved performance. *In vivo* imaging

studies show that dendritic spines retain dynamic qualities even in the adult brain (Grutzendler et al., 2002, Trachtenberg et al., 2002), and that activity appears to play an instructive role in this remodeling. Spines appear and disappear in hippocampal slices after electrical stimulation as well as *in vivo* in response to experience (reviewed in Bhatt et al., 2009). Thus, it seems that the dynamic nature of dendritic spines provides the capacity for local circuit restructuring in response to normal day-to-day levels of activity. In the same way that synapse formation on growing axons and dendrites provides an anchor for arbor stabilization during development, synapse formation on dynamic spines could serve to consolidate nascent connections in a mature circuit. This suggests that CPG15 could continue to play a role as a synaptic stabilizing factor extending past developmental circuit wiring and into optimization of adult circuitry. *cpg15* KO neurons may be unable to stabilize connections in the adult, and thus may have to rely on an initial set of synapses stochastically formed during development.

Based on our analysis of the *cpg15* KO mouse we propose that CPG15 acts to stabilize nascent synapses on dendritic spines resulting in spine and arbor stabilization and synaptic maturation. Neuroligins were recently proposed to act as a signal for the validation of excitatory versus inhibitory synapses. Rather than mediating synapse formation per se, neuroligins signal whether a transiently initiated connection stays or goes (Chubykin et al., 2007). It is intriguing to consider that since CPG15 is expressed and can be externalized in response to activity (Cantallops and Cline, 2008), it could provide a saliency signal for selective stabilization of active synapses. While synaptic connections can form without CPG15, they are slower to form and are not optimized by activity patterns that are required for efficient learning. *cpg15* KO mice are capable of learning with increased trials and their sensory and motor functions appear to be within the normal range, suggesting that many behaviors required for survival are hard wired

through highly overlapping and redundant mechanisms. Only when peak performance is desired does the role of activity-dependent tuning mediated by molecules such as CPG15 become starkly evident.

The subtle deficit we observed in relation to synapse stabilization allowed us to probe the consequences of CPG15 functional deletion on circuit properties and behavior, without the confounding aspects of complex phenotypes commonly found in general knockout mice. Our results lead us to speculate that the inefficient learning seen in *cpg15* KO mice derives from a diminished capacity for selective synapse stabilization.

2.4 MATERIALS AND METHODS

2.4.1 Generation of the *cpg15* KO mice

All animal work was approved by the Massachusetts Institute of Technology Committee on Animal Care and conforms to NIH guidelines for the use and care of vertebrate animals. To allow conditional deletion of the *cpg15* gene using the Cre-*loxP* system, we generated floxed *cpg15* mice. The targeting vector was constructed starting with a 13.7-kb genomic fragment spanning a region of the *cpg15* gene from the *Eco*RV site 0.2-kb upstream of exon one to the *Afl*II site 4.7-kb downstream of exon three, cloned from a C57BL/6 mouse BAC library (Genomic Systems). Exons two and three were floxed by replacing a 19-bp sequence between the *Sac*I and *Kpn*I sites 0.5-kb upstream of exon two with a 40-bp fragment containing *loxP* and *Xho*I sites, and inserting a 2.9-kb LFNT-TK cassette (gift of Kazu Nakazawa), consisting of a neomycin-resistance (*neo*) gene flanked by two *loxP* sites and two *FRT* sites, into the *Sac*I site 0.9-kb downstream of exon three. The diphtheria toxin A gene from pMCI DT-A (Yagi et al., 1990)was inserted at the end of the targeting vector for

negative selection. The linearized targeting vector was electroporated into a C57BL/6 ES cell line. G418-resistant ES cell clones were tested for homologous recombination by Southern blot analysis using an internal probe and 5'- and 3'-external probes. ES clones showing the expected patterns were injected into blastocysts from BALB/c mice to obtain chimeric mice. Chimeras were bred with C57BL/6 mice to generate mice carrying the floxed-*cpg15* allele and *neo* gene (*cpg15*$^{flox-neo/+}$). The *neo* gene was then removed by injecting *flippase* cRNA synthesized *in vitro* from pOG-Flpe6 (Buchholz et al., 1998) into fertilized eggs from *cpg15*$^{flox-neo/+}$ mice, thus generating floxed-*cpg15* mice without the *neo* gene in pure C57BL/6 background (*cpg15*$^{flox/+}$). To generate general *cpg15* null mice, *cpg15*$^{flox/+}$ mice were crossed to an adenovirus EIIa promoter-driven Cre transgenic line in C57BL/6 background (Jackson Laboratory, B6.FVB-TgN(EIIa-cre)C5379Lmgd) (Lakso et al., 1996a). Progeny of this cross that were heterozygous for the *cpg15* null allele (*cpg15*$^{+/-}$) were intercrossed to obtain homozygous *cpg15* null mice (*cpg15*$^{-/-}$). The absence of floxed-*cpg15* genomic sequence, *cpg15* mRNA, and CPG15 protein was confirmed by Southern blots of tail genomic DNA (Sambrook et al., 1989), Northern blots on brain RNA (Fujino et al., 2003), and Western blots, respectively. For Western blots, membrane protein enriched fractions were prepared from the cerebral cortex and hippocampus of adult mice using Mem-PER kit (Pierce) and PAGEprep advance kit (Pierce). Forty micrograms of protein were resolved by 15% SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with rabbit anti-CPG15 (1:100), then with HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, 1:50,000), and visualized by chemiluminescence (Pierce). Antibodies against CPG15 were generated by immunizing rabbits with peptides corresponding to amino acids 28-40 and 99-116, and affinity purified with the immunizing peptides (Open Biosystems, Huntsville, AL).

cpg15 KO lines were maintained as *cpg15*$^{+/-}$ x *cpg15*$^{+/-}$ crosses. Genotyping was done by tail-DNA PCR. Wild-type forward primer (5'-CGCAGCCCAATCTGCATTC-3', 0.13 pmol/ml), *cpg15* null forward primer (5'-GTTGTGGTCTTCCAAAGACC-3', 0.5 pmol/ml), and common reverse primer (5'-GGAGCAGCGAGATCTCCTT-3', 0.5 pmol/ml) were used to amplify a 230-bp wild-type band and a 350-bp *cpg15* null band. Mice were housed under a 12-h light/dark cycle with *ad libitum* access to food and water.

2.4.2 Data collection and analysis

All quantification comparing WT and *cpg15* KO mice were done blind to genotype. Statistical analysis was done using StatView software (SAS Institute, Cary, NC). Unless otherwise stated, Student's unpaired t-test was used for two-group comparisons, and analysis of variance (ANOVA) and Student-Newman-Keuls *post hoc* analysis were used for comparisons involving more than two groups. Error bars are standard error of the mean unless otherwise stated.

2.4.3 Brain measurements

Age-matched males were perfused with phosphate-buffered saline (PBS) then with 4% paraformaldehyde in PBS. Brains were dissected out, weighed, and measured with vernier calipers. After overnight post-fixation at 4°C, brains were cryoprotected in 30% sucrose in PBS, frozen in powdered dry ice, sectioned coronally at 40 mm with a cryostat (Leica). Every sixth section was stained with cresyl violet. For volume measurements, the area on each section was measured using the point-counting method, and the total volume estimated based on Cavalieri's

rule (Rosen and Williams, 2003). Cell density was measured by the three-dimensional counting method (Williams and Rakic, 1988).

2.4.4 Labeling retinal ganglion projections in the LGN

Mice younger than P9 were anesthetized by hypothermia or with 2.5% Avertin (250 mg /kg i.p.) at later ages. Animals received an intravitreal injection of wheat germ agglutinin (WGA) conjugated to Alexa Fluor 555 (1 mg/ml, Molecular Probes, Eugene, OR) in the left eye and WGA-Alexa Fluor 488 in the right eye. For injections prior to natural eye opening, fused eyelids were separated or cut to expose the temporal region of the eye. After 24 h, animals were perfused with 4% paraformaldehyde in PBS. Brains were removed and postfixed overnight, then coronally sectioned at 75 mm using a vibratome.

LGN images were acquired with an epi-fluorescence microscope (Nikon, Tokyo, Japan) using a 10×/N.A. 0.3 objective lens (Nikon). Four successive sections, representing the middle third of the LGN, were selected. Background fluorescence was subtracted and grayscale images were normalized (0–255) using ImageJ (http://rsb.info.nih.gov/ij/). Grayscale images were converted into binary high-contrast black and white images by employing a threshold procedure that distinguishes signal from residual background fluorescence (Muir-Robinson et al., 2002, Torborg and Feller, 2004) Contralateral (pseudo-colored green) and ipsilateral signals (pseudo-colored magenta) were superimposed. An outline of the entire LGN was drawn, and the area measured. The extent of overlapping projections was determined by counting pixels that contained both green and magenta signal, represented as white.

2.4.5 Diolisitic labeling in the hippocampus

Brain sections from P7, P15, and 2-month-old mice were processed for diolistic labeling as described (Grutzendler et al., 2003) with the following modifications. After rapidly perfusing P15 and 2-month-old mice with 4% paraformaldehyde in PBS (40 ml in 2 min), brains were dissected out and postfixed for 10 min at room temperature. P7 brains were removed without perfusion and then fixed for 10min in 4% PFA at room temperature. All brains were coronally sectioned at 100 μm with a vibratome, and stored in 30% sucrose in PBS. Bullets were prepared by coating tungsten particles (1.7 μm, Biorad) with DiI (Molecular Probes), then loaded into Tefzel tubing (Biorad) pretreated with polyvinylpyrrolidone (1 mg/ml in isopropanol, Sigma), and dried with N_2. Brain sections were covered with a tissue culture insert with a 3-μm pore size (Greiner), and then shot with DiI-coated particles using a Helios gene gun system (Biorad) set at 160 psi. Sections were postfixed in 4% paraformaldehyde / 30% sucrose in PBS overnight and mounted on glass slides with Fluoromount-G (Sourthern Biotech, Birmingham, AL). Neurons were imaged with a Nikon PCM 2000 confocal microscopy system controlled by the Simple PCI software (Compix Inc. Image system, Cranberry Township, PA) or an Olympus (Melville, NY, USA) FluoView 300 laser-scanning confocal microscope and FluoView 500 acquisition software. Stacks of 26 images at 2-μm interval, spanning 50 μm in thickness, were obtained with a 20x objective lens. Images were analyzed using Object-Image software (http://simon.bio.uva.nl/object-image.html) with Morphometry Macros (Ruthazer and Cline, 2002) or with Neurolucida and Neurolucida Explorer software (MBF Bioscience). Total dendritic branch length and branch tip number within the imaging volume were quantified for dendritic arbors of DG granule cells.

2.4.6 Electrophysiology

Hippocampi of P7 or 2-month-old mice were isolated and 300um slices were prepared with a vibratome (World Precision Instruments) in ice-cold cutting solution containing (in mM) 238 Sucrose, 26 $NaHCO_3$, 10 Glucose, 2.5 KCl, 1 NaH_2PO_4, 3 $MgSO_4$ and 1 $CaCl_2$ constantly bubbled with 95% O_2/5% CO_2. The slices were transferred to a holding chamber filled with artificial cerebrospinal fluid (ACSF) containing (in mM) 119 NaCl, 26 $NaHCO_3$, 10 Glucose, 2.5 KCl, 1 NaH_2PO_4, 1.3 $MgSO_4$ and 2.5 $CaCl_2$ constantly bubbled with 95% O_2/5% CO_2 and recovered at 32°C for half an hour, then at room temperature for half an hour. Slices were placed in a recording chamber continuously perfused with 32°C ACSF bubbled with 95% O_2/5% CO_2, plus 100uM picrotoxin and 1uM tetrodotoxin to isolate mEPSCs. Whole cell recordings were performed with patch pipettes (5-7MΩ), containing (in mM) 130 K-gluconate, 4 KCl, 2 NaCl, 10 Hepes, 0.2 EGTA, 4 ATP-Mg, 0.3 GTP-Tris, 7 phosphocreatine-Tris and 10 sucrose, pH 7.25, 290mOsm. Neurons in CA1 or dentate were patched under visual guidance with a Nikon microscope equipped with IR/DIC optics using a black and white CCD camera (CCD-300IFG, Dage-MTI). Current traces were collected using a AxoPatch 2B amplifier (Axon), digitized at 5kHz by a Digidata 1322A (Axon), and analyzed offline using Clampfit software (Axon). At least 150 mEPSCs, thresholded at 6pA, were recorded at -70mV from each cell. 20uM 6,7-Dinitroquinoxaline-2,3-dione was applied during some recordings to verify AMPAR mEPSCs.

2.4.7 Electron microscopy

Two- and 9-month-old male littermates were perfused, and brains fixed as described (Diano et al., 2006). Ultrathin sections were prepared as described (Diano et al., 2006) from stratum radiatum of CA1 and CA3, and molecular layer of dentate gyrus. Spine and spine

synapse density was calculated by unbiased stereological methods based on the dissector technique (Diano et al., 2006). PSD length was measured from 100 to 150 spine synapses in 10 non-overlapping sections and averaged for each animal.

2.4.8 Two-photon imaging

Cranial window implantation and multi photon imaging were done essentially as described (Lee et al., 2008). Mice (6-8 weeks of age) were implanted with cranial windows and imaged 2-3 weeks after surgery at 4 day intervals. For imaging mice were anesthetized with 1.25% avertin (250 mg per kg body weight, (i.p.)). Anesthesia was monitored by breathing rate and foot-pinch reflex, and additional doses of anesthetic were administered during the imaging session as needed. Two-photon imaging was performed using a custom-built microscope modified for *in vivo* imaging by inclusion of a custom-made stereotaxic restraint affixed to a stage insert and custom acquisition software. The light source for two-photon excitation was a commercial Mai Tai HP titanium:sapphire laser (Spectra-Physics) pumped by a 14-W solid-state laser delivering 100-fs pulses at a rate of 80 MHz with the power delivered to the objective ranging from approximately 37 to 50 mW depending on imaging depth. z resolution was obtained with a piezo actuator positioning system (Piezosystem Jena) mounted to the objective. The excitation wavelength was set to 950 nm, with the excitation signal passing through a 20×, 1.0–numerical aperture water-immersion objective (Plan-Apochromat, Zeiss) and collected after a barrier filter by a photomultiplier tube.

Given the sparse density of GFP expression in the *thy1*-GFP-S line, typically a dendritic segment from one L5 pyramidal neuron (and a maximum of two) was imaged per animal. Cells mapped to visual cortex were then identified as L5 pyramidal neurons based on their depth from

the pial surface and morphology. Blood vessel pattern maps were used to locate the cells and dendritic branches throughout the imaging sessions. Low-resolution image stacks (512 x 512 pixels, 1mm/ pixel X-Y resolution, 5 μm z-step size) were used to identify cell type and depth. High-resolution image stacks (768 x 768 pixels, 0.2mm/ pixel X-Y resolution, 0.7 μm z-step size) were used to capture spine dynamics on L5 dendrites in L1.

2.4.9 Dendritic spine analysis

Image stacks were aligned such that the presence or absence of an individual spine at a given location on the dendrite could be determined in each session. Spines were scored only if they protruded greater than 0.4 microns from the dendritic shaft, and were projecting into the XY plane. Long thin spines without bulbous heads were excluded from analysis since they were always transient and were not significantly different in number or dynamics between genotypes. The rate of spine gain and spine loss was defined as the percentage of spines appearing (spine gain) or disappearing (spine loss) in a session compared to the total number of spines in the previous session. Dynamic spines were sorted into the following categories: Transient spines were those that appeared for no more than one or two imaging sessions then disappeared, or spines that disappeared and reappeared through the imaging period; New-persistent spines were defined as those which were not present in the first session and subsequently gained for at least the last two sessions; Lost-persistent spines were defined as those that were present in at least the first two sessions then lost for the remaining sessions. Analysis was performed using V3D software (Peng et al., 2010) by an observer blind to genotype.

2.4.10 Behavioral tests

Male littermates between 3 to 6 months of age were used for behavioral experiments, unless otherwise noted.

Grip strength test: Mice were allowed to hold on to a wire attached to a spring scale (Pesola) and pulled horizontally by their tail. Maximal pulling force before releasing the wire was recorded. Five trials were done for each mouse, and the average was calculated for best three.

Rotarod test: Mice were placed on an accelerating rotarod and latency to falling from the rod was recorded. Each trial lasted for a maximum of 8 min, during which the rotation speed increased linearly from 2.5 rpm to 40 rpm. Three trials were done per day for two consecutive days.

Open field test: Mice were placed in the center of an open field (40 x 40 cm) and allowed to move freely for 30 min. Their activity was monitored by a Digiscan system in 1-min bins and analyzed by Versa Max software (AccuScan Instruments). Data was averaged for 30 min.

Hotplate test: Mice were placed on a hotplate preheated to 55°C. Latency to paw lifting or sudden movement was recorded. All mice responded within 15 s.

Fear conditioning: One-trial fear conditioning was done as described (Zeng et al., 2001) with modifications described in the supplemental information. Memory was tested after 1 h and 24 h of the conditioning trial for contextual memory and after 25 h for tone-dependent memory. For repeated training, conditioning sessions with one tone-shock pairing were given on days 1, 3, 5, and a session with three tone-shock pairings (each pair starting at 1, 2, and 3 min after placement in chamber) was given on day 7. Context and tone tests were done 24 h and 25 h, respectively, after each conditioning session. For one-trial fear conditioning, 4- to 9-month-old male mice were used.

<u>Morris water maze visible platform test</u>: The visible platform test was done essentially as described (Zeng et al., 2001). The pool was 150 cm in diameter, with water at room temperature (21-22°C). The platform was 10 cm in diameter. Each mouse was trained three trials per day with intertrial intervals of approximately 30 min. The platform position was changed for each trial. In each trial the mouse was allowed to swim until it found the platform or until 60 s had elapsed at which point the mouse was guided to the platform. The mouse was allowed to sit on the platform for 30 s before being picked up.

2.5 SUPPLEMENTARY DATA

Motor, sensory, & behavioral characterization of *cpg15* KO mice

To evaluate potential motor and sensory deficits, *cpg15* KO mice were subjected to a series of behavioral tests. Assessing forepaw muscle strength using the grip-strength test indicated that general muscle strength in *cpg15* KO mice was similar to that of WT littermates (Fig. S1A). In the rotarod test, when placed on a rotating rod that gradually increased in speed, *cpg15* KO mice showed similar latency to falling as WT mice (Fig. S1B), indicating they have comparable balance and coordination. Furthermore, *cpg15* KO mice showed progressively longer fall latency with more trials similar to WT controls, demonstrating normal motor learning. Pain sensitivity was intact and even slightly enhanced in *cpg15* KO mice when tested on the hotplate test (Fig. S1C).

In the open field test, *cpg15* KO mice showed similar horizontal travel distance, vertical movements (rearing), and time spent moving as WT controls (Fig. S1D), indicating they are neither hyper- nor hypo-active. However, time spent in the center of the arena was shorter in *cpg15* KO mice (Fig. S1D), suggesting they may be more anxious than WT controls.

Normal progenitor cell survival in *cpg15* KO mice

Previous studies have identified cellular effects of CPG15 in promoting progenitor cell survival in the embryonic brain (Putz et al. 2005). We examined progenitor survival in the *cpg15* KO mice, although normal cell numbers and density in the *cpg15* KO brain (Table S1) suggest that there is compensation for CPG15 in promoting progenitor survival when it is absent from the outset. Pregnant mothers were injected with BrdU to label proliferating cells in embryos. At embryonic day (E) E15.5 when neural progenitors are rapidly proliferating, WT and *cpg15* KO

embryos showed similar cell density in each layer of the cerebral cortex (Figure S3A, B). The proportion of proliferating cells assessed by the BrdU labeling index in the progenitor layers was similar between genotypes (Figure S3C). These results are consistent with normal cell numbers in the adult brain and indicate that absence of CPG15 during embryonic development does not affect the size of the cortical progenitor pool.
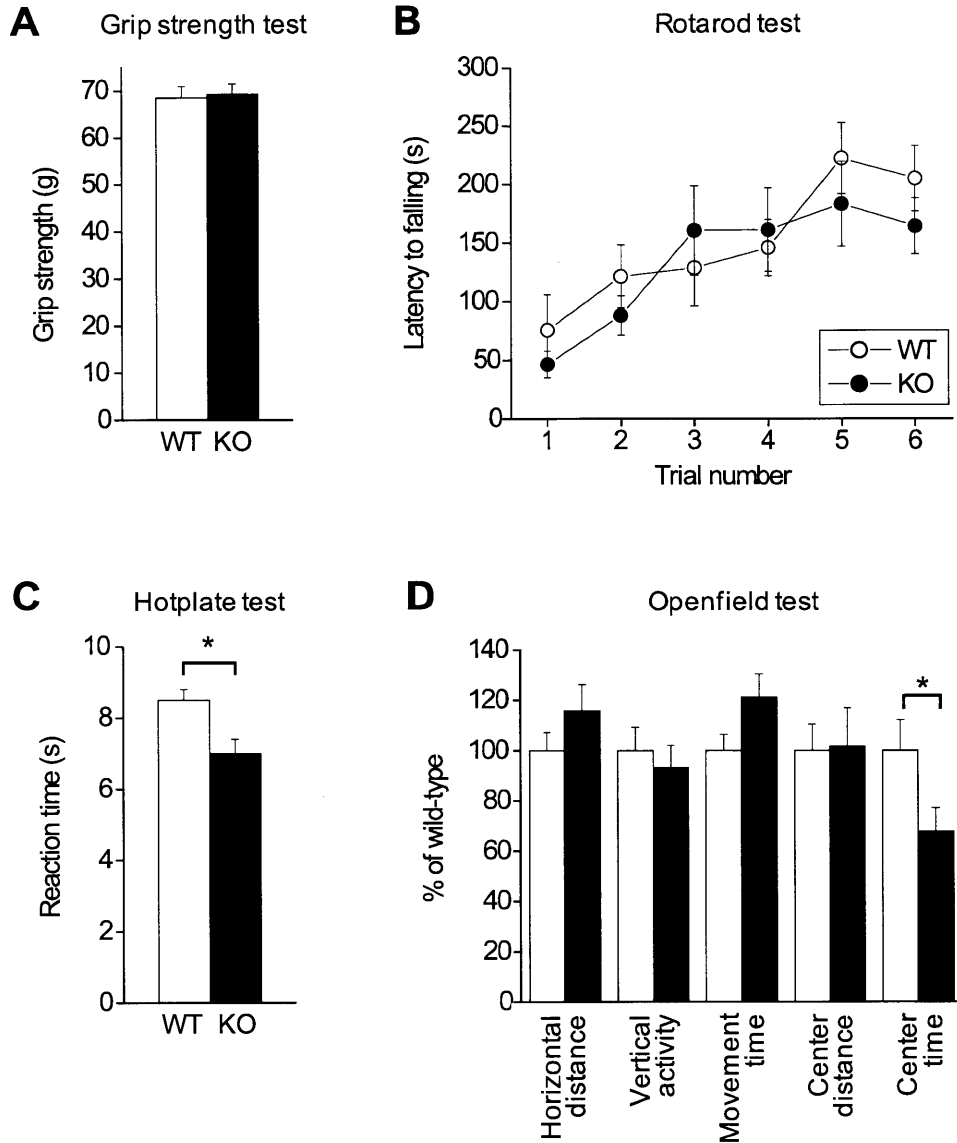
Figure S2.1 *cpg15* **KO mice basic motor and sensory functions are essentially normal.** (A) Normal forepaw grip strength in *cpg15* KO mice ($n = 10$). (B) Accelerating rotarod test showing similar latencies to falling in both genotypes. Three trials were done on two consecutive days ($n = 10$). (C) Slightly enhanced pain sensitivity in *cpg15* KO mice. Hotplate test, measuring reaction times after being placed on a 55°C plate, shows faster reaction times in *cpg15* KO mice ($n = 10$). (*) P-value < 0.05. (D) Open field test showing similar spontaneous movement, but less time spent in the center of the field. Parameters are normalized to WT values ($n = 22$). (*) P-value < 0.05.
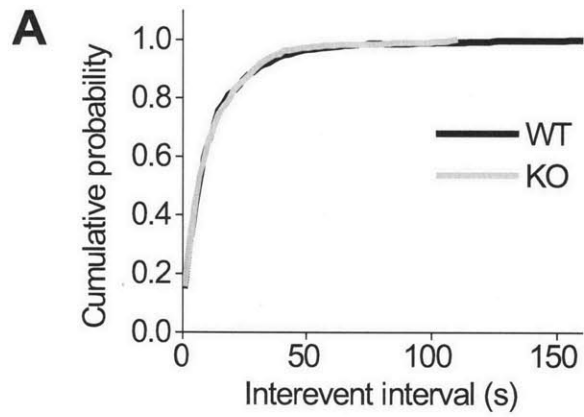
Figure S2.2 Normal mEPSCs in *cpg15* KO CA1 at 2 months. (A) Cumulative probability plot of interevent intervals of mEPSCs in the CA1 of *cpg15* KO and WT mice at 2 months ($n = 13$ WT cells, $n = 9$ KO cells). *P-value* = n.s. by K-S test.
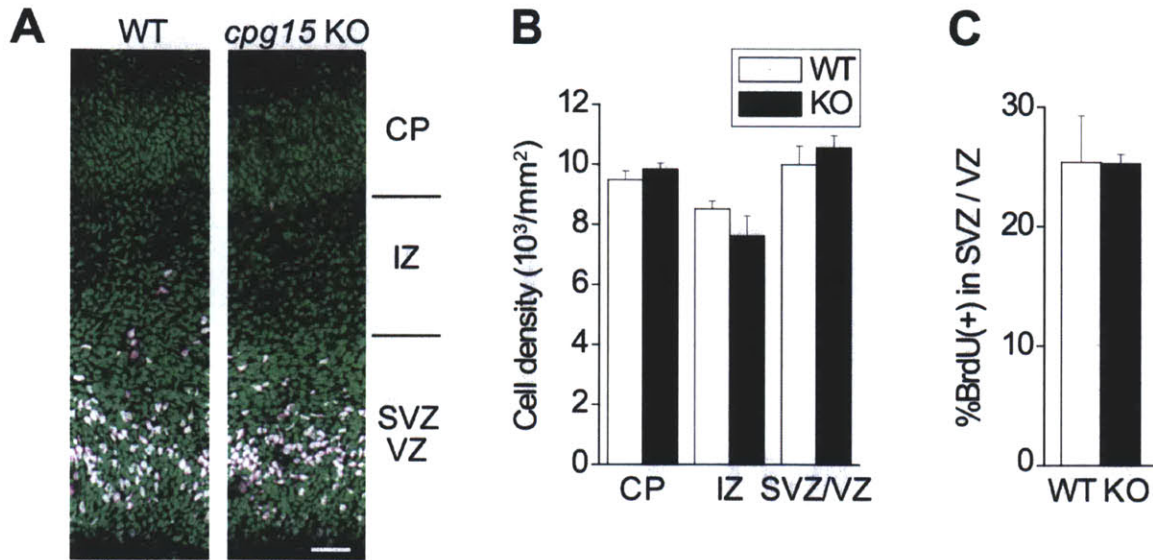
Figure S2.3 Normal embryonic progenitor numbers in *cpg15* KO mice. (A) Cerebral cortex of E15.5 embryos stained with anti-BrdU (magenta) and fluorescent Nissl (green). White indicates overlap. (B) Nissl-stained cell densities in cortical plate (CP), intermediate zone (IZ), and subventricular/ventricular zones (SVZ/VZ). Data are represented as mean ± standard error of the mean. (C) BrdU labeling index in SVZ/VZ ($n = 3$). Scale bar: 50 mm.

Parameter	WT	*cpg15* KO	t-test[d]
Body length (cm)[a]	10.6 ± 0.0	10.2 ± 0.1	***
Body weight (g)[a]	45.0 ± 1.6	31.4 ± 0.9	***
Brain weight (mg)[b]	433 ± 6	428 ± 6	
Brain size (mm)[b]			
Cortex (antero-posterior length)	0.90 ± 0.01	0.90 ± 0.02	
Cortex (lateral width)	1.04 ± 0.00	1.03 ± 0.01	
Cerebellum (antero-posterior length)	0.30 ± 0.01	0.27 ± 0.01	*
Cerebellum (lateral width)	0.80 ± 0.01	0.78 ± 0.01	*
Regional volume (mm^3)[c]			
Neocortex	85.3 ± 1.0	86.4 ± 3.2	
Hippocampal formation	17.7 ± 0.8	17.6 ± 0.3	
Neuronal density (x10^3/mm^3)[c]			
Neocortex	93 ± 5	98 ± 4	
Amygdala basolateral nucleus	77 ± 3	77 ± 5	

Mean ± standard error of mean is shown for each parameter.
[a] n = 8 mice, 7-8 months
[b] n = 10 mice, 6-7 months
[c] n = 3 mice, 6-7 months
[d] * $p < 0.05$, *** $p < 0.001$

Table S2.1 Comparison measurements of *cpg15* KO mice and WT controls.

Parameter	WT	*cpg15* KO	t-test[e]
Retinal ganglion cell axon projection area $(mm^2)^a$	0.27 ± 0.02	0.21 ± 0.01	*
LGN area $(mm^2)^b$	0.26 ± 0.01	0.22 ± 0.01	
Percentage of LGN area covered by axonal projection $(\%)^c$	105 ± 9	96 ± 6	
LGN cell density $(10^3$ cells/$mm^2)$	1.9 ± 0.2	2.1 ± 0.1	
Number of cells per section $(cells)^d$	483 ± 54	461 ± 21	

Mean ± standard error of mean is shown for each parameter.

[a] Area determined by WGA-conjugated dye labeling.

[b] Area determined by fluorescent Nissl staining.

[c] Percentage of axonal projection area per Nissl stained LGN area

[d] Cell density x LGN area

[e] * $p < 0.05$ ($n = 3$)

Table S2.2 Decreased axonal projection area but similar cell counts in the LGN of *cpg15* KO mice at P9.

2.6 REFERENCES

Bai, J., Ramos, R. L., Ackman, J. B., Thomas, A. M., Lee, R. V. & Loturco, J. J. 2003. RNAi reveals doublecortin is required for radial migration in rat neocortex. *Nat. Neurosci.*, 6, 1277-1282.

Bhatt, D. H., Zhang, S. & Gan, W. B. 2009. Dendritic spine dynamics. *Annu Rev Physiol*, 71, 261-82.

Brose, N. 2009. Synaptogenic proteins and synaptic organizers: "many hands make light work". *Neuron*, 61, 650-2.

Brückner, G., Mares, V. & Biesold, D. 1976. Neurogenesis in the visual system of the rat. An autoradiographic investigation. *J. Comp. Neurol.*, 166, 245-255.

Buchholz, F., Angrand, P. O. & Stewart, A. F. 1998. Improved properties of FLP recombinase evolved by cycling mutagenesis. *Nat Biotechnol*, 16, 657-62.

Cantallops, I. & Cline, H. T. 2008. Rapid activity-dependent delivery of the neurotrophic protein CPG15 to the axon surface of neurons in intact Xenopus tadpoles. *Dev. Neurobiol.*, 68, 744-759.

Cantallops, I., Haas, K. & Cline, H. T. 2000. Postsynaptic CPG15 promotes synaptic maturation and presynaptic axon arbor elaboration *in vivo*. *Nat. Neurosci.*, 3, 1004-1011.

Chubykin, A. A., Atasoy, D., Etherton, M. R., Brose, N., Kavalali, E. T., Gibson, J. R. & Südhof, T. C. 2007. Activity-dependent validation of excitatory versus inhibitory synapses by neuroligin-1 versus neuroligin-2. *Neuron*, 54, 919-931.

Conover, J. C. & Yancopoulos, G. D. 1997. Neurotrophin regulation of the developing nervous system: analyses of knockout mice. *Rev. Neurosci.*, 8, 13-27.

Corriveau, R., Shatz, C. J. & Nedivi, E. 1999. Dynamic regulation of *cpg15* during activity-dependent synaptic development in the mammalian visual system. *J. Neurosci.*, 19, 7999-8008.

Diano, S., Farr, S. A., Benoit, S. C., Mcnay, E. C., Da Silva, I., Horvath, B., Gaskin, F. S., Nonaka, N., Jaeger, L. B., Banks, W. A., Morley, J. E., Pinto, S., Sherwin, R. S., Xu, L., Yamada, K. A., Sleeman, M. W., Tschop, M. H. & Horvath, T. L. 2006. Ghrelin controls hippocampal spine synapse density and memory performance. *Nat Neurosci*, 9, 381-8.

Diaz, E., Ge, Y., Yang, Y. H., Loh, K. C., Serafini, T. A., Okazaki, Y., Hayashizaki, Y., Speed, T. P., Ngai, J. & Scheiffele, P. 2002. Molecular analysis of gene expression in the developing pontocerebellar projection system. *Neuron*, 36, 417-34.

Feng, G., Mellor, R. H., Bernstein, M., Keller-Peck, C., Nguyen, Q. T., Wallace, M., Nerbonne, J. M., Lichtman, J. W. & Sanes, J. R. 2000. Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. *Neuron*, 28, 41-45.

Flavell, S. W. & Greenberg, M. E. 2008. Signaling mechanisms linking neuronal activity to gene expression and plasticity of the nervous system. *Annu. Rev. Neurosci.*, 31, 563-590.

Fujino, T., Lee, W. A. & Nedivi, E. 2003. Regulation of *cpg15* by signaling pathways that mediate synaptic plasticity. *Mol. Cell Neurosci.*, 24, 538-554.

Gomperts, S. N., Carroll, R., Malenka, R. C. & Nicoll, R. A. 2000. Distinct roles for ionotropic and metabotropic glutamate receptors in the maturation of excitatory synapses. *J. Neurosci.*, 20, 2229-2237.

Grutzendler, J., Kasthuri, N. & Gan, W.-B. 2002. Long-term dendritic spine stability in the adult cortex. *Nature*, 420, 812-816.

Grutzendler, J., Tsai, J. & Gan, W. B. 2003. Rapid labeling of neuronal populations by ballistic delivery of fluorescent dyes. *Methods,* 30, 79-85.

Harris, K. M., Jensen, F. E. & Tsao, B. H. 1989. Ultrastructure, developement and plasticity of dendritic spine synapses in area CA1 of the rat hippocampus: extending our vision with serial electron microscopy and three-dimensional analyses. *In:* CHAN-PALAY, V. & KOHLER, C. (eds.) *The hippocampus:new vistas.* New York: Liss.

Harris, K. M. & Kater, S. B. 1994. Dendritic spines: cellular specializations imparting both stability and flexibility to synaptic function. *Ann. Rev. Neurosci.,* 17, 341-71.

Hevroni, D., Rattner, A., Bundman, M., Lederfein, D., Gbarah, A., Mangelus, M., Silverman, M., Kedar, H., Naor, C., Kornuc, M., Hanoch, T., Seger, R., Theill, L., Nedivi, E., Richter Levin, G. & Citri, Y. 1998. Hippocampal plasticity involves extensive gene induction and multiple cellular mechanisms. *J. Mol. Neurosci.,* 10, 75-98.

Holtmaat, A., Wilbrecht, L., Knott, G. W., Welker, E. & Svoboda, K. 2006. Experience-dependent and cell-type-specific spine growth in the neocortex. *Nature,* 441, 979-983.

Holtmaat, A. J., Trachtenberg, J. T., Wilbrecht, L., Shepherd, G. M., Zhang, X., Knott, G. W. & Svoboda, K. 2005. Transient and persistent dendritic spines in the neocortex in vivo. *Neuron,* 45, 279-91.

Hua, J. Y. & Smith, S. J. 2004. Neural activity and the dynamics of central nervous system development. *Nat. Neurosci.,* 7, 327-332.

Javaherian, A. & Cline, H. T. 2005. Coordinated motor neuron axon growth and neuromuscular synaptogenesis are promoted by CPG15 in vivo. *Neuron,* 45, 505-512.

Kano, M. & Hashimoto, K. 2009. Synapse elimination in the central nervous system. *Curr. Opin. Neurobiol.,* 19, 154-161.

Knott, G. W., Holtmaat, A., Wilbrecht, L., Welker, E. & Svoboda, K. 2006. Spine growth precedes synapse formation in the adult neocortex in vivo. *Nat. Neurosci.,* 9, 1117-1124.

Konur, S. & Yuste, R. 2004. Imaging the motility of dendritic protrusions and axon terminals: roles in axon sampling and synaptic competition. *Mol. Cell. Neurosci.,* 27, 427-440.

Lakso, M., Pichel, J. G., Gorman, J. R., Sauer, B., Okamoto, Y., Lee, E., Alt, F. W. & Westphal, H. 1996a. Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. *Proc Natl Acad Sci U S A,* 93, 5860-5.

Lakso, M., Pichel, J. G., Gorman, J. R., Sauer, B., Okamoto, Y., Lee, E., Alt, F. W. & Westphal, H. 1996b. Efficient in vivo manipulation of mouse genomice sequences at the zygote stage. *Proc. Natl. Acad. Sci. U S A,* 93, 5860-5865.

Lee, W. C. A., Chen, J. L., Huang, H., Leslie, J. H., Amitai, Y., So, P. T. & Nedivi, E. 2008. A dynamic zone defines interneurons remodeling in the adult neocortex. *Proc. Natl. Acad. Sci. U S A,* 105, 19968-19973.

Lee, W. C. A., Huang, H., Feng, G., Sanes, J. R., Brown, E. N., So, P. T. & Nedivi, E. 2006. Dynamic remodeling of dendritic arbors in GABAergic interneurons of adult visual cortex. *PLoS Biol.,* 4, e29.

Lee, W. C. A. & Nedivi, E. 2002. Extended plasticity of visual cortex in dark-reared animals may result from prolonged expression of genes like *cpg15. J. Neurosci.,* 22, 1807-1815.

Leslie, J. H. & Nedivi, E. 2011. Activity-regulated genes as mediators of neural circuit plasticity. *Prog. Neurobiol.,* 94, 223-237.

Loebrich, S. & Nedivi, E. 2009. The function of activity-regulated genes in the nervous system. *Physiol. Rev.,* 89, 1079-1103.

Markus, E. J. & Petit, T. L. 1987. Neocortical synaptogenesis, aging, and behavior: lifespan development in the motor-sensory system of the rat. *Exp. Neurol.,* 96, 262-278.

Mcallister, K. A. 2007. Dynamic aspects of CNS synapse formation. *Annu. Rev. Neurosci.,* 30, 425-450.

Meyer, M. P. & Smith, S. J. 2006. Evidence from in vivo imaging that synaptogenesis guides the growth and branching of axonal arbors by two distinct mechanisms. *J. Neurosci.,* 26, 3604-3614.

Muir-Robinson, G., Hwang, B. J. & Feller, M. B. 2002. Retinogeniculate axons undergo eye-specific segregation in the absence of eye-specific layers. *J Neurosci,* 22, 5259-64.

Naeve, G. S., Ramakrishnan, M., Rainer, K., Hevroni, D., Citri, Y. & Theill, L. E. 1997. Neuritin: a gene induced by neural activity and neurotrophins that promotes neuritogenesis. *Proc. Natl. Acad. Sci. USA,* 94, 2648-2653.

Nägerl, U. V., Köstinger, G., Anderson, J. C., Martin, K. A. & Bonhoeffer, T. 2007. Protracted synaptogenesis after activity-dependent spinogenesis in hippocampal neurons. *J. Neurosci.,* 27, 8149-8156.

Nedivi, E., Fieldust, S., Theill, L. E. & Hevron, D. 1996. A set of genes expressed in response to light in the adult cerebral cortex and regulated during development. *Proc. Natl. Acad. Sci. U S A,* 93, 2048-2053.

Nedivi, E., Hevroni, D., Naot, D., Israeli, D. & Citri, Y. 1993. Numerous candidate plasticity-related genes revealed by differential cDNA cloning. *Nature,* 363, 718-722.

Nedivi, E., Wu, G. Y. & Cline, H. T. 1998. Promotion of dendritic growth by CPG15, an activity-induced signaling molecule. *Science,* 281, 1863-1866.

Okabe, S., Miwa, A. & Okado, H. 2001. Spine formation and correlated assembly of presynaptic and postsynaptic molecules. *J. Neurosci.,* 21, 6105-6114.

Peng, H., Ruan, Z., Long, F., Simpson, J. H. & Myers, E. W. 2010. V3D enables real-time 3D visualization and quantitative analysis of large-scale biological image data sets. *Nat Biotechnol,* 28, 348-353.

Putz, U., Harwell, C. & Nedivi, E. 2005. Soluble CPG15 expressed during early development rescues cortical progenitors from apoptosis. *Nature Neurosci.,* 8, 322-331.

Rahimi, O. & Claiborne, B. J. 2007. Morphological development and maturation of granule neuron dendrites in the rat dentate gyrus. *Prog Brain Res,* 163, 167-81.

Rosen, G. & Williams, R. 2003. Estimation of regional volume from serial sections using point counting and Cavalieri's rule. *In:* AUSUBEL, F. M., BRENT, R., KINGSTON, R. E., MOORE, D. D., SEIDMAN, G. J., SMITH, J. A. & STRUHL, K. (eds.) *Current Protocols in Neuroscience Online.* John Wiley & Sons, Inc.

Ruthazer, E. S. & Cline, H. T. 2002. Multiphoton imaging of neurons in living tissue: acquisition and analysis of time-lapse morphological data. *Real-Time Imag.,* 8, 175-188.

Ruthazer, E. S., Li, J. & Cline, H. T. 2006. Stabilization of axon branch dynamics by synaptic maturation. *J. Neurosci.,* 26, 3594-3603.

Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989. Molecular cloning: a laboratory manual. Second Edition ed. New York: Cold Spring Harbor Laboratory Press.

Scheiffele, P., Fan, J., Choih, J., Fetter, R. & Serafini, T. 2000. Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell,* 101, 657-69.

Torborg, C. L. & Feller, M. B. 2004. Unbiased analysis of bulk axonal segregation patterns. *J Neurosci Methods,* 135, 17-26.

Trachtenberg, J. T., Chen, B. E., Knott, G. W., Feng, G., Sanes, J. R., Welker, E. & Svoboda, K. 2002. Long-term *in vivo* imaging of experience-dependent synaptic plasticity in adult cortex. *Nature,* 420, 788-794.

Van Praag, H., Kempermann, G. & Gage, F. H. 2000. Neural consequences of environmental enrichment. *Nat Rev Neurosci,* 1, 191-8.

Vardinon Friedman, H., Bresler, T., Garner, C. C. & Ziv, N. E. 2000. Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment. *Neuron,* 27, 57-69.

Varoqueaux, F., Aramuni, G., Rawson, R. L., Mohrmann, R., Missler, M., Gottmann, K., Zhang, W., Sudhof, T. C. & Brose, N. 2006. Neuroligins determine synapse maturation and function. *Neuron,* 51, 741-54.

Verhage, M., Maia, A. S., Plomp, J. J., Brussaard, A. B., Heeroma, J. H., Vermeer, H., Toonen, R. F., Hammer, R. E., Van Den Berg, T. K., Missler, M., Geuze, H. J. & Sudhof, T. C. 2000. Synaptic assembly of the brain in the absence of neurotransmitter secretion. *Science,* 287, 864-9.

Williams, R. W. & Rakic, P. 1988. Three-dimensional counting: an accurate and direct method to estimate numbers of cells in sectioned material. *J Comp Neurol,* 278, 344-52.

Yagi, T., Ikawa, Y., Yoshida, K., Shigetani, Y., Takeda, N., Mabuchi, I., Yamamoto, T. & Aizawa, S. 1990. Homologous recombination at c-fyn locus of mouse embryonic stem cells with use of diphtheria toxin A-fragment gene in negative selection. *Proc Natl Acad Sci U S A,* 87, 9918-22.

Zeng, H., Chattarji, S., Barbarosie, M., Rondi-Reig, L., Philpot, B. D., Miyakawa, T., Bear, M. F. & Tonegawa, S. 2001. Forebrain-specific calcineurin knockout selectively impairs bidirectional synaptic plasticity and working/episodic-like memory. *Cell,* 107, 617-29.

Zito, K., Scheuss, V., Knott, G., Hill, T. & Svoboda, K. 2009. Rapid functional maturation of nascent dendritic spines. *Neuron,* 61, 247-258.

Chapter 3: Thalamically-Derived CPG15 Regulates the Maturation of Cortical Circuits

Jennifer H. Leslie and Elly Nedivi

3.1 INTRODUCTION

The thalamocortical circuit is central to mammalian brain function. The thalamus is the major hub of sensory information flow to the cerebral cortex and an important modulatory site for information en route from the periphery to the cortex and of cortical feedback loops (Saalmann and Kastner, 2011). In the visual system, the primary visual cortex receives projections from the dorsal lateral geniculate nucleus (dLGN) of the thalamus. Activity in developing thalamocortical and corticocortical networks work together to sculpt mature patterns of connectivity in the cortex (Lopez-Bendito and Molnar, 2003). Synapse maturation in the visual cortex occurs postnatally. The first synapses are detected in the cortical plate in the days after birth (Blue and Parnavelas, 1983). A mature distribution of synapses in the cortex does not arise until postnatal day 14 (P14), and reaches adult levels of synapse density by P20. Like many of the excitatory synapses in the brain, immature thalamocortical and corticocortical synapses are thought to be postsynaptically "silent" containing only NMDA receptors which are then converted into AMPA receptor-containing functionally mature synapses in an activity-dependent manner (Kerchner and Nicoll, 2008). In the barrel cortex where thalamocortical synapses can be directly interrogated in acute slices in which the thalamocortical tract is preserved (Agmon and Connors, 1991) silent synapses are present only until P8 (Isaac et al., 1997, Feldman et al., 1999). However, in the visual cortex, silent synapses are found much later in development and are regulated differentially depending on the cortical layer (Rumpel et al., 2004). In layer II/III silent synapses peak at P14 and then decline over the next week and a half.

The orderly assembly of neuronal circuits is specified by developmental programs of gene expression, however, the final stage in circuit development, maturation and refinement of specific synaptic connections, is strongly influenced by neuronal activity (Greer and Greenberg,

2008, Lopez-Bendito and Molnar, 2003). It is thus not surprising that the products of many activity-regulated genes have been implicated in synapse development and plasticity (Leslie and Nedivi, 2011). In the case of the thalamocortical circuit, little is know of the cellular and molecular mechanisms underlying specific synapse selection and maturation.

The activity-regulated gene product CPG15 has been shown to enhance synapse maturation by promoting the incorporation of AMPA receptors into silent synapses in the developing *Xenopus* retinotectal system coincident with the promotion of dendritic and axonal arbor growth and elaboration (Cantallops et al., 2000, Nedivi et al., 1998). In the mammalian brain, expression of *cpg15* mRNA in presynaptic structures is spatiotemporally correlated with periods of synapse formation and maturation in target regions (Nedivi et al., 1996, Corriveau et al., 1999, Nedivi et al., 2001, Lee and Nedivi, 2002). For example, *cpg15* expression in thalamus is high during development of thalamocortical synapses. In addition, *cpg15* expression in presynaptic structures may be regulated by contact with postsynaptic targets (Diaz et al., 2002). CPG15 protein is detected in retinotecal fiber tracts in developing *Xenpus* optic tectum suggesting is can also be transported along afferent inputs into target brain regions (Nedivi et al., 1998). CPG15 is thought to act as a selective synaptic stabilizer, providing a signal for further synapse and arbor maturation (see Chapter 2)(Fujino et al., 2011).

The function and expression patterns of CPG15 make it a promising candidate for regulating the final stages of thalamocortical circuit assembly. To test this, we examined the early postnatal development of excitatory synapses and neurons in the visual cortex of a mouse with a global deletion of *cpg15* (see Chapter 2)(Fujino et al., 2011). Further, to differentiate whether the source of CPG15 critical for cortical synapse maturation was thalamic or cortical,

we utilized the Cre-*loxP* system to specifically delete *cpg15* in the cortex and then analyzed excitatory synapse maturation in the visual cortex in the absence of cortically-derived CPG15.

3.2 RESULTS

3.2.1 Deficient excitatory synapse development in the visual cortex of global *cpg15* KO mice

In the visual cortex, excitatory synapse development has been extensively studied in the context of activity-dependent circuit formation and maturation, and can be associated with onset of visual experience. During normal development, visual experience leads to a gradual reduction in mEPSC amplitudes triggered by eye opening around P12 thought to be a homeostatic tuning of the neuron in response to increasing levels of presynaptic activity (Desai et al., 2002). There is also a dramatic increase in mEPSC frequencies likely due to large increases in synapse density (Blue and Parnavelas, 1983) as well as the conversion of postsynaptically silent synapses into functionally mature AMPA receptor-containing synapses (Rumpel et al., 2004). CPG15 has been shown to play a role in excitatory synapse stabilization (see Chapter 2)(Fujino et al., 2011), and has also been implicated in conversion of silent synapses to mature AMPA receptor-containing synapses in the *Xenopus* optic tectum (Cantallops et al., 2000). To investigate the role of CPG15 in excitatory synapse development during this period when visual input conveyed by developing thalamocortical synapses has a profound effect on visual cortex development, we used whole-cell patch clamp to record spontaneous miniature excitatory postsynaptic currents (mEPSCs) from layer II/III pyramidal neurons in acute slices prepared from the primary visual cortex of developing *cpg15* KO mice and WT littermate controls (Fig 3.1A). We analyzed both mEPSC amplitudes as a correlate of synapse strength, and mEPSC frequency as a correlate of functional, AMPA receptor-containing synapse number. Three ages were chosen for this study based on

visual development milestones and the *cpg15* expression profile; P10 prior to eye opening and the onset of *cpg15* expression in visual cortex, P14 shortly after eye opening and coincident with early onset of *cpg15* expression, and P28 the peak of *cpg15* expression (Lee and Nedivi, 2002) which also corresponds to the height of the critical period for eye specific preference in the binocular zone of the primary visual cortex (Gordon and Stryker, 1996).

In our WT controls we observed a gradual decrease in mEPSC amplitude consistent with previous studies (Desai et al., 2002). At P10, we found no difference in mEPSC amplitudes between pyramidal neurons in *cpg15* KO and WT littermate control mice. However, by P14 *cpg15* KO mice displayed a significant reduction in mEPSC amplitudes compared to controls, which was even more significant by P28 (Fig. 3.1B, C) indicating that *cpg15* KO neurons receive weaker synaptic input at P14 and P28. This amplitude deficit is closely correlated with the normal onset of *cpg15* expression in the cortex.

Similarly, no difference was observed in mEPSC frequency early in development at P10 (Fig. 3.1D, E). Between P10 and P14, both *cpg15* KO and WT neurons undergo a similar increase in mEPSC frequency consistent with a dramatic developmental increase in synapse density (Blue and Parnavelas, 1983). WT mice exhibit another large increase in mEPSC frequency between P14 and P28 that likely corresponds to another jump in synapse density is known to occur between P14 and P16 (Blue and Parnavelas, 1983). In contrast, *cpg15* KO mice do not show any further increase in frequency by P28, instead remaining at P14 levels, significantly lower than WT controls (Fig. 3.1D, E). Silent synapses, which peak in layer II/III around P14, are largely converted to functionally mature AMPA receptor-containing synapses between P14 to P28 (Rumpel et al., 2004). Consistent with synapse maturation occurring at this time, presynaptic vesicle numbers also increase (Blue and Parnavelas, 1983). The observations
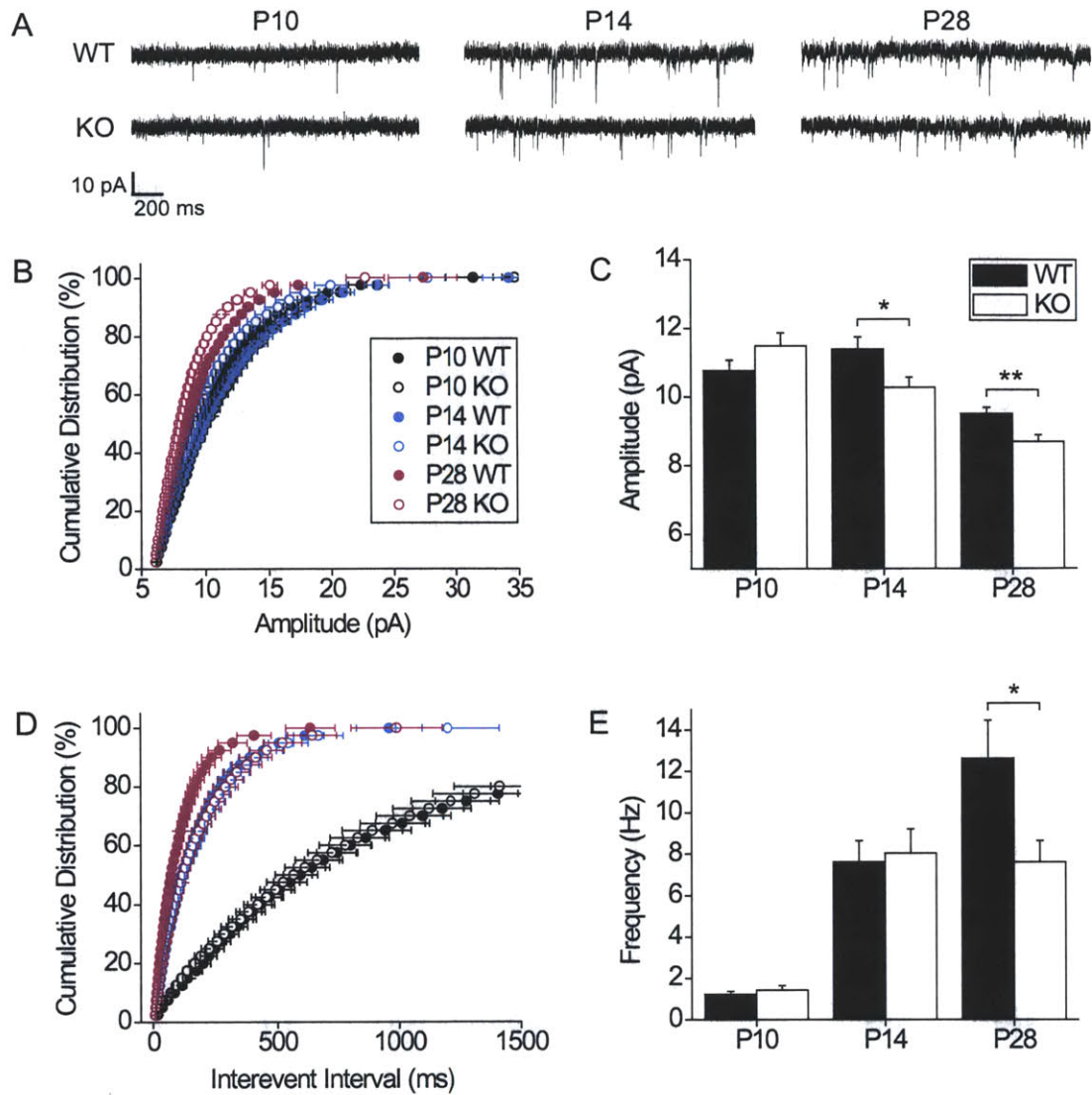
Figure 3.1 Reduction of mEPSC amplitudes and frequencies in layer II/III pyramidal neurons in the visual cortex of cpg15 KO mice. (A) Representative traces of whole-cell patch clamp spontaneous mEPSC recordings from layer II/III pyramidal neurons in the visual cortex of *cpg15* KO and WT littermates at P10, P14, and P28. (B) Cumulative distribution of mEPSC amplitudes. (C) Average mEPSC amplitudies from. (D) Cumulative distribution of mEPSC interevent intervals. E, Average mEPSC frequencies. (P10 n = 14 WT, n = 15 KO cells; P14 n = 17 WT, KO cells; P28 n = 13 WT, n = 14 KO cells; * p < 0.05, **p < 0.01).

that there is no deficit in mEPSC frequency between P10 and P14 in the *cpg15* KO mouse despite the large amount of synaptogenesis that normally occurs during this period, and weaker synapses in the *cpg15* KO mouse at both P14 and P28, are consistent with previous studies showing that CPG15 is not required for synapse formation but is instead important for synapse maturation. We conclude that the deficit we observe in frequency and amplitude is most likely due to a reduction in the insertion of AMPA receptors into developing synapses.

3.2.2 Reduced dendrite growth and complexity in the visual cortex of global *cpg15* KO mice

The synaptotrophic hypothesis (Vaughn, 1989) postulates that the formation and maturation of synapses promotes the growth and stabilization of neuronal arbors, most demonstratively axons (Meyer and Smith, 2006, Ruthazer et al., 2006). Overexpression of CPG15 results in enhanced synapse maturation concomitant with enhanced arbor development (Nedivi et al., 1998, Cantallops et al., 2000, Javaherian and Cline, 2005), and loss of CPG15 in hippocampus results in delayed synapse formation and arbor maturation (see Chapter 2)(Fujino et al., 2011). We therefore investigated whether the deficient synapse development seen in visual cortex layer II/III pyramidal neurons is accompanied by delays in dendritic arbor formation. Full dendritic trees of neurons were sparsely labeled in fixed slices using diolistics (Grutzendler et al., 2003), then imaged with confocal microscopy, and reconstructed in 2 dimensions (Fig 3.2A). Basal dendrites of pyramidal neurons in *cpg15* KO mice and WT littermate controls were examined at the same ages as synapse development, P10, P14, and P28. Apical dendrite analysis was not included as the variance in apical dendrite length in the imaged slices precluded analyses of subtle differences in growth and complexity.

Our observations on the growth of WT cortical pyramidal neuron basal dendrites over time (Fig. 3.2B, C) are in agreement with previous studies showing that in layer II/III of the rodent visual cortex the number of primary basal dendrites is established by P10, higher order branches continue to be added throughout the second postnatal week, and branches and segments grow in size, reaching maturity around P30 (Juraska and Fifkova, 1979, Miller, 1981, Juraska, 1982). In *cpg15* KO neurons we found no significant difference in total dendrite length or primary branches number as compared to WT neurons at the ages tested. However, when we examined the difference in length between ages, while WT dendrites significantly increase in length between both time intervals, *cpg15* KO dendrites only increase in length between P10 and P14, showing no statistically significant change in length between P14 and P28 (Fig. 3.2B), suggesting an impairment in arbor growth rate coincident with impaired synaptic maturation observed in recording of mEPSCs.

We also examined parameters of dendritic complexity. While WT neurons establish the proper number of primary basal dendrites by P10, *cpg15* KO neurons show a significant increase in the primary dendrite numbers between P10 and P14 and a trend towards pruning primary dendrites between P14 and P28 (Fig. 3.2C). This aberrant change in complexity suggests that there may be less structural consolidation of *cpg15* KO arbors at this age in development. It is possible that reduced synapse stabilization resulting from a lack of CPG15 destabilizes what normally is a well established primary structure of the basal dendritic arbor, allowing a greater scale of morphological plasticity in the *cpg15* KO mouse at this stage of development.

Scholl analysis was used as an additional measure of basal dendrite complexity, revealing that at P10 *cpg15* KO dendrites are less complex than WT with a significantly lower peak number of intersections (Fig. 3.2D). At P14 *cpg15* KO dendrites overshoot WT in complexity at
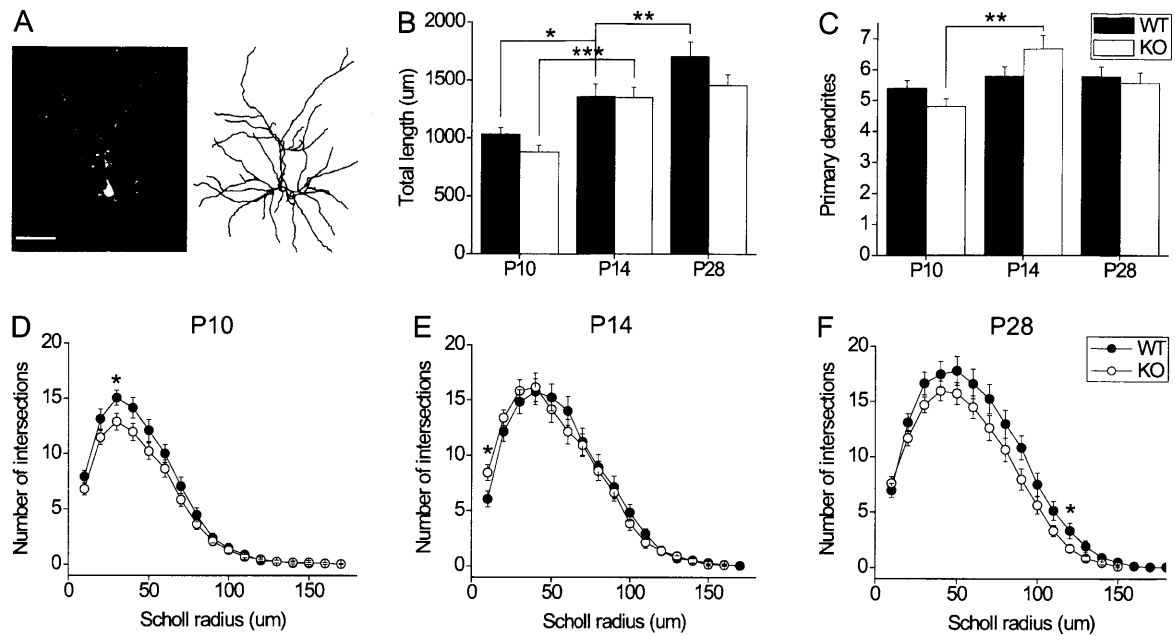
Figure 3.2 Delayed growth and complexity of basal dendrites of layer II/III pyramidal neurons in *cpg15* KO mice. (A) Example of a diolistically labeled layer II/III pyramidal neuron in slice (left) and after tracing (right) from P14 mouse. Scale bar is 50um. (B) Total length of basal dendrites in *cpg15* KO and WT littermates at P10, P14, and P28. (C) Average number of primary dendrites per neuron. (D) Scholl analysis of basal dendrites at P10, E, P14, and F, P28. (P10 n = 24 WT, KO cells; P14 n = 23 WT, n = 21 KO cells; P18 n = 22 WT, n = 25 KO cells; *p < 0.05, **p < 0.01, ***p < 0.001).

the radius closest to the cell body, a reflection of the addition of primary branches (Fig. 3.2C, E). However, by P28 *cpg15* KO dendrites again lag behind WT dendrites in complexity most significantly at a radius far from the cell body reflecting the reduced rate of growth of *cpg15* KO neurons (Fig. 3.2F).

The emergence of a deficit in dendrite complexity as early at P10 in *cpg15* KO neurons was surprising in that we did not expect to see differences between *cpg15* KO mice and WT controls at a time prior to the normal onset of *cpg15* expression in the cortex. At this time however, *cpg15* mRNA is expressed in the dLGN of the thalamus (Nedivi et al., 1996, Corriveau et al., 1999). There is also evidence from studies in the developing *Xenopus* retinotectal system that CPG15 can be transported along axons into brain regions far from the cell body (Nedivi et al., 1998, Cantallops and Cline, 2008). This led us to speculate that thalamocortical projections from the dLGN may provide a significant source of CPG15 protein during cortical development.

3.2.3 Transient deficit in synapse maturation in the visual cortex of conditional *cpg15* KO mice

To differentiate between possible contributions of CPG15 from thalamus and cortex during the development of cortical neurons we generated a cortex-specific knockout of *cpg15*. A "floxed" *cpg15* mouse containing *loxP* sites flanking exons 2 and 3 of the *cpg15* gene (see Chapter 2)(Fujino et al., 2011) was crossed with a knockin mouse expressing Cre recombinase under control of the endogenous *emx1* gene locus (Gorski et al., 2002). In these mice *emx1* gene expression is not disrupted and Cre recombinase is expressed throughout the cortex in precursors of excitatory neurons and glia starting by embryonic day 10, with no expression in the retina and only very sparse expression in the thalamus. The conditional *cpg15* KO mice resulting from this

cross retain *cpg15* expression in the thalamus but show significantly reduced expression in the cortex as compared to floxed littermate controls (Fig. 3.3A).

To assess the role of cortically expressed CPG15 on synapse development we performed whole-cell patch-clamp recording of mEPSCs from layer 2/3 pyramidal neurons in acute slices of visual cortex from conditional *cpg15* KO mice and floxed littermate controls at the same developmental ages assayed in the global *cpg15* KO line. Like global *cpg15* KO mice, conditional *cpg15* KO mice displayed no difference in mEPSC amplitudes at P10 when compared to controls. At P14, during the period of normal onset of *cpg15* expression in the visual cortex, mEPSCs recorded from conditional *cpg15* KO mice showed significantly reduced amplitudes when compared to floxed littermates. However, unlike global *cpg15* KO mice, in conditional *cpg15* KO mice this deficit was transient and no deficit was observed in mEPSC amplitudes at P28 (Fig. 3.3B, C). No significant difference was observed in mEPSC frequencies between conditional *cpg15* KO mice and floxed littermate controls at any age (Fig. 3.3D, E). These results suggest that cortically-derived CPG15 might only have a very limited role in the development of excitatory synapses on layer II/III neurons. Instead thalamic afferents might provide a critical presynaptic supply of CPG15 or at the very least are able to compensate for the loss of cortical CPG15 essential for synaptic maturation.

3.2.4 Rescue of the synaptic deficit in conditional *cpg15* KO mice

To determine whether acute delivery of CPG15 could rescue the synaptic deficit observed in the conditional *cpg15* KO mEPSC amplitudes at P14, we utilized a lentiviral-mediated expression strategy. A lentivirus expressing CPG15 and co-expressing GFP, or a control lentivirus expressing GFP alone, was injected into the visual cortex of conditional *cpg15* KO and
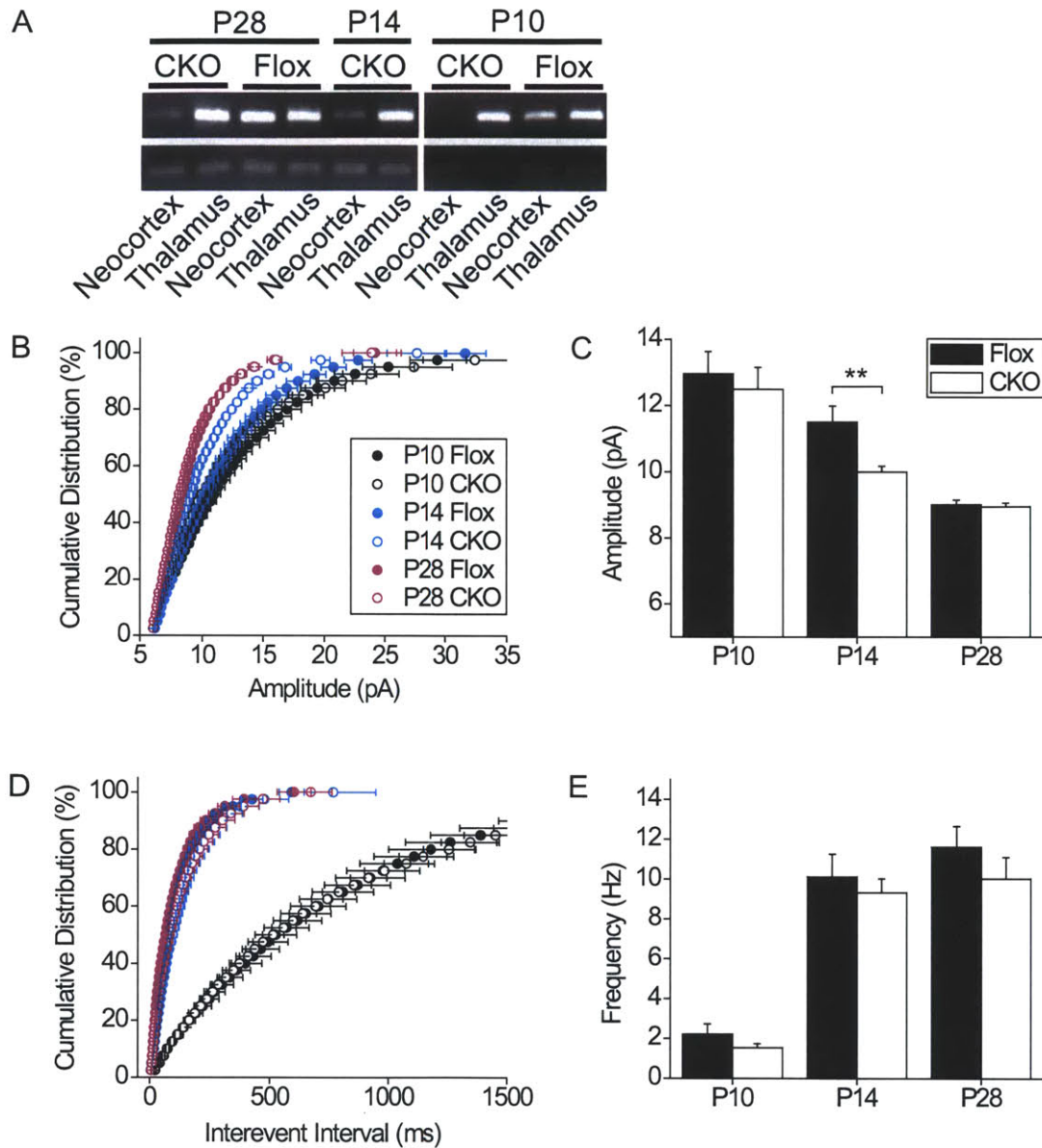
Figure 3.3 Transient deficit in mEPSC amplitude in a cortex-specific *cpg15* KO mice. (A) RT-PCR of *cpg15* mRNA (top) and control *gapdh* mRNA (bottom) from neocortex and thalamus of cortex-specific *cpg15* KO and floxed littermates (Flox) at P28, P14, and P10. (B) Cumulative distribution of mEPSC amplitudes from cortex-specific *cpg15* KO and floxed littermates at P10, P14, and P28. (C) Average mEPSC amplitudies. (D) Cumulative distribution of mEPSC

interevent intervals. (E) Average mEPSC frequencies. (P10 n = 15 Flox, CKO cells; P14 n = 13

Flox, n = 14 CKO cells; P28 n = 15 Flox, CKO cells; *p < 0.01).

floxed littermates at P4 (Fig. 3.4A). At P14 acute slices of visual cortex were prepared from injected mice and whole-cell patch-clamp recording of mEPSCs were performed on cells within injection sites visualized by GFP protein expression (Fig. 3.4A, B). As we hypothesized that CPG15 protein might be supplied at synapses from presynaptic infected neurons, we targeted cells that were not expressing GFP, yet were near GFP-expressing cells. The intracellular recording solution contained biocytin allowing for posthoc staining with a streptavidin-conjugated fluorophore to verify that recorded neurons did not express GFP yet were located within infection sites (Fig. 3.4B). Conditional *cpg15* KO mice injected with control GFP expressing lentivirus has smaller mEPSC amplitudes compared to injected floxed littermate controls at P14 as was observed in uninjected animals. Conditional *cpg15* KO mice injected with CPG15 expressing lentivirus no longer displayed a difference in mEPSC amplitude when compared to controls suggesting that an acute delivery of exogenous CPG15 can rescue the mESPC deficit (Fig. 3.4C) indicating that this deficit was not secondary to larger scale wiring deficits results from early loss of CPG15 expression.

We observed no significant difference in mEPSC frequency between the conditional *cpg15* KO mice and floxed controls with GFP expression alone. However, with CPG15 expression we observed a significant difference in mEPSC frequency between conditional *cpg15* KO mice and floxed littermates (Fig. 3.4D). This appears to be due to a CPG15 overexpression-mediated increase in mEPSC frequency in floxed mice. This result is similar to experiments in developing *Xenopus* optic tectum where even on a WT background CPG15 overexpression increases mEPSC frequencies in immature tectal neurons (Cantallops et al., 2000).

As we only recorded from uninfected neurons within injection sites, we were able to find
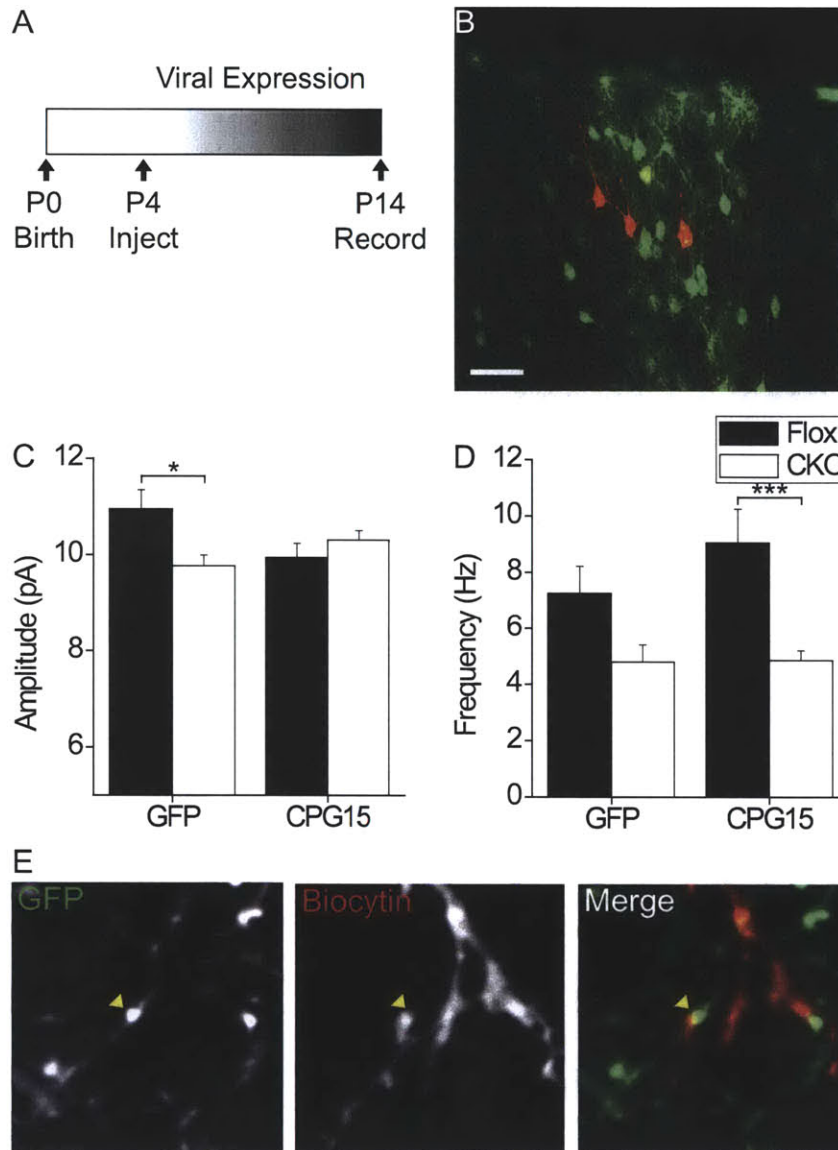
Figure 3.4 Lentivirus-mediated rescue of deficit in mEPSC amplitude in cortex-specifc _cpg15_ KO mice. (A) Cortical injections of lentivirus expressing CPG15 and GFP, or control lentivirus expressing GFP alone were injected into primary visual cortex of cortex-specific _cpg15_ KO and floxed littermates at P4. Recording were taken from acute slices at P14. (B) Slice stained for GFP (green), marking infected cells expressing CPG15, and recorded neurons filled with biocytin and stained with streptavidin conjugated to AlexaFluor555 (red). Scale bar is 50um. (C) Average mEPSC amplitudes at P14 from mice injected with virus expressing GFP alone, or

coexpressing CPG15. (D) Average mEPSC frequencies. (E) Putative synapse (yellow arrowhead) between an axonal bouton on a GFP labeled infected neuron (left panel), and a spine on a biocytin filled neuron (middle panel), merged (right). (GFP virus n = 11 Flox, n = 9 CKO cells; CPG15 virus n = 7 Flox, n = 14 CKO cells; *p < 0.05; ***p < 0.001).

putative synaptic contacts where GFP labeled axons with buoton swellings were positioned in very close apposition to spines on filled recorded neurons (Fig. 3.4D). The existence of synaptic contacts between infected and recorded neurons allows for the possibility that CPG15 could be supplied presynaptically to mediate its effects on synapse maturation.

3.3 DISCUSSION

Here we describe a novel mechanism by which thalamocortical inputs can regulate maturation of cortical synapses through signaling by the secreted protein CPG15. Immature thalamocortical synapses are postsynaptically silent, containing only NMDA receptors (Isaac et al., 1997, Feldman et al., 1999). Throughout postnatal development AMPA receptors are trafficked into synapses in an activity-dependent manner. Synapse maturation has been shown to be critical for the proper establishment of thalamocortical circuits. In a mouse mutant lacking type-1 adenylyl cyclase (AC1) activity, AMPA receptor content in synapses is greatly reduced (Lu et al., 2003). This mouse mutant is known as *barrelless* as it has no barrel formation in the somatosensory cortex demonstrating the critical need for synapse maturation in the establishment of mature thalamocortical cytoarchitecture despite the proper targeting of thalamic axons to the cortex in these mice. Presynaptic deficits in synapse function, such as found in the GAP-43 knockout and the RIM1α knockout mouse, also correlate with defective thalamocortical patterning in somatosensory cortex (Lu et al., 2006, Albright et al., 2007).

Molecular signals expressed by the cortex have been shown to be important in regulating innervation and maturation of thalamic inputs (Lopez-Bendito and Molnar, 2003). In the visual cortex, Neurotrophin-3, expressed in cortical layer IV, acts as a local trophic signal for thalamic axons to invade and establish synapses in the cortical plate (Ma et al., 2002). In mouse

somatosensory cortex BDNF expression regulates the segregation of thalamocortical axons into barrels by signaling through TrkB receptors localized on thalamic axons (Lush et al., 2005). It seems that for neurotrophins, expression and release from cortical neurons drives the maturation of thalamic afferents in the cortex by signaling through receptors expressed on thalamic axons. Evidence is also emerging that cell-attached molecules such as ephrins are also important for the establishment and collateralization of thalamocortical projections. Ephrin-A5, which is expressed in layer IV of somatosensory cortex, can regulate the arborization of thalamic axon terminals (Mann et al., 2002). A variety of ephrins-As have also been implicated in guiding retinotopic map development in the visual cortex (Cang et al., 2005). However, CPG15 is the first described signaling protein in which this role seems to be reversed. Our results suggest that expression and release of CPG15 from thalamic axons mediates CPG15's effects on cortical synapse maturation.

There is evidence suggesting that thalamic signaling through a diffusible factor regulates the proliferation of cortical precursors in the subventricular zone during embryonic development (Dehay et al., 2001), as well as their radial migration into the cortex (Edgar and Price, 2001). These studies implicate a role for thalamic signaling in very early cortical development, while our study extends this result suggesting even a late stage role for thalamic signaling during cortical synapse maturation. CPG15 signaling also plays a role during embryonic corticogenesis by promoting survival of cortical progenitors (Putz et al., 2005). It is interesting to speculate that embryonic effects of CPG15 on neuronal precursors might also be mediated by thalamic secretion of CPG15.

Our results reveal a role for thalamic signaling in late stages of cortical circuit development during the maturation of excitatory synaptic connections. CPG15 may be released from thalamic afferents in an activity-dependent manner leading to the stabilization and thus

maturation of immature cortical synapses. While the relay of peripheral sensory activity to the cortex by the thalamus has long been known to be important for thalamocortical circuit development, we propose that it is not solely the conveyance of neuronal activity, but additionally the secretion of CPG15 by thalamic inputs that aids in the establishment of mature synaptic connections between the thalamus and the cortex.

3.4 MATERIALS AND METHODS

3.4.1 Animals

All animal work was approved by the Massachusetts Institute of Technology Committee on Animal Care and conforms to NIH guidelines for the use and care of vertebrate animals. Generation of *cpg15* KO and *cpg15* floxed mice is previously described (Fujino et al., 2011). To generate cortex-specific *cpg15* KO mice, *cpg15* floxed male mice were crossed with female B6.129S2-*Emx1$^{tm1(cre)Krj}$*/J mice (Gorski et al., 2002). PCR genotyping was used to identify *cpg15$^{flox/flox}$;Emx1-Cre* mice and *cpg15$^{flox/flox}$* littermate controls.

3.4.2 Electrophysiology

Acute 300um coronal slices of visual cortex from P10, P14, and P28 day old mice were prepared with a vibratome (World Precision Instruments) in ice-cold cutting solution containing (in mM) 238 Sucrose, 26 $NaHCO_3$, 10 Glucose, 2.5 KCl, 1 NaH_2PO_4, 3 $MgSO_4$ and 1 $CaCl_2$ constantly bubbled with 95% O_2/5% CO_2. The slices were transferred to a holding chamber filled with artificial cerebrospinal fluid (ACSF) containing (in mM) 119 NaCl, 26 $NaHCO_3$, 10 Glucose, 2.5 KCl, 1 NaH_2PO_4, 1.3 $MgSO_4$ and 2.5 $CaCl_2$ constantly bubbled with 95% O_2/5%

CO_2 and recovered at 32°C for half an hour, then at room temperature for half an hour. Slices were placed in a recording chamber continuously perfused with 32°C ACSF bubbled with 95% O_2/5% CO_2, plus 100uM pictroxin and 1uM tetrodotoxin to isolate mEPSCs. Whole cell recordings were performed with patch pipettes (5-7MΩ), containing (in mM) 130 K-gluconate, 4 KCl, 2 NaCl, 10 Hepes, 0.2 EGTA, 4 ATP-Mg, 0.3 GTP-Tris, 7 phosphocreatine-Tris and 10 sucrose, pH 7.25, 290mOsm. For some recordings 0.2% biocytin (Sigma) was also included in the patch pipette. Layer 2/3 pyramidal neurons were patched under visual guidance with a Nikon microscope equipped with IR/DIC optics using a black and white CCD camera (CCD-300IFG, Dage-MTI). Current traces were collected using an AxoPatch 2B amplifier (Axon), digitized at 5kHz by a Digidata 1322A (Axon), and analyzed offline using Clampfit software (Axon). At least 200 mEPSCs, thresholded at 6pA, were recorded at -70mV from each cell.

3.4.3 Diolisitic labeling in the visual cortex

Brain sections from P10, P14, P28, and 3-month-old mice were processed for diolistic labeling as described (Grutzendler et al., 2003) with the following modifications. After rapidly perfusing mice with 4% paraformaldehyde in PBS (40 ml in 2 min), brains were dissected out and postfixed for 10 min at room temperature. All brains were coronally sectioned at 150 µm with a vibratome, and stored in 30% sucrose in PBS. Bullets were prepared by coating tungsten particles (1.7 µm, Biorad) with DiI (Molecular Probes), then loaded into Tefzel tubing (Biorad) pretreated with polyvinylpyrrolidone (1 mg/ml in isopropanol, Sigma), and dried with N2. Brain sections were covered with a tissue culture insert with a 3µm pore size (Greiner), and then shot with DiI-coated particles using a Helios gene gun system (Biorad) set at 160 psi. Sections were

postfixed in 4% paraformaldehyde / 30% sucrose in PBS overnight and mounted on glass slides with Fluoromount-G (Sourthern Biotech, Birmingham, AL). Neurons were imaged with an Olympus (Melville, NY, USA) FluoView 300 laser-scanning confocal microscope and FluoView 500 acquisition software. For dendrite analysis, stacks 50 um thick stacked were acquired at 2 μm intervals using with a 20x objective lens. Dendritic trees were traced and analyzed with Neurolucida and Neurolucida Explorer software (MBF Bioscience). For spine analysis, dendrite segments that were relatively level within the XY plane of the slice were collected in 8 um stacks with 0.3 um imaging intervals using a 60x lens. Spine images were analyzed with ImageJ software (http://rsb.info.nih.gov/ij/).

3.4.4 RT-PCR

Total RNA was extracted from neocortex and thalamus dissected from P10, P14, and P28 condition *cpg15* KO and floxed littermate brains using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 1ug RNA using the AffinityScript Multiple Temperature cDNA Synthesis Kit (Stratagene) with random hexamers according to the manufacturer's instructions. PCR reactions were performed using HotstarTaq DNA Polymerase (Qiagen) and contained 1x Hotstar Taq buffer, 1mM dNTPs, and 1uM each primer. For *cpg15* amplification primers 5'-GGGCTTTTCAGACTGTTTGC-3' for sense and 5'-GCTAAAGCTGCCGAGAGAGA-3' for antisense were used with an expected product size of 308 base pairs. For *gapdh* amplification sense primer 5'-TGACGTGCCGCCTGGAGAAA-3' and antisense primer 5'-AGTGTAGCCCAAGATGCCCTTCAG-3' were used with an expected product size of 98 base pairs. PCR conditions were 95°C for 15 minutes, followed by 40 cycles

of 94°C for 40 seconds, 56°C for 40 seconds, and 72°C for 1 min, followed by 10 minutes at 72°C. PCR reactions were run out on a 2% agarose gel.

3.4.5 Cortical injections of lentivirus

A *pFUGW* vector (Lois et al., 2002) containing *CPG15-FLAG-IRES-EGFP* subcloned downstream of the ubiquitin promoter was used as for *in vivo* CPG15 expression (Putz et al., 2005). pFUGW lentivirus was used as the EGFP control. Lentivirus production, concentration and titer determination were done as described (Lois et al., 2002). Typical titers for *in vivo* injections were greater than 1×10^6 pfu/ul.

P4 conditional *cpg15* KO mice and floxed littermates were anesthetized with isoflurane anesthesia. A small incision was made down the midline of the head then a small hole was drilled bilaterally over the visual cortex, ~2mm lateral to the lambda fissure, using a 1.4mm carbon steel burr (Fine Science Tools). A pipette puller (Sutter) was used to pull glass capillary needles (Drummond) with 40 to 50um tips. Five injections of 27.5nL virus were made at depth of 350um from the pial surface, then 5 more at 250um using a Nanoject II (Drummond Scientific) for a total of approximately 600nL virus injected in each hemisphere. Incisions were sutured with size 6-0 nylon monofilament (Hannah Pharmaceutical Supply). Pups were allowed to recover at 37°C then placed with the dam.

3.4.6 Immunohistochemistry and biocytin staining

Recorded slices were postfixed overnight in 4% paraformaldehyde in PBS at 4°C, then placed in 30% sucrose in PBS overnight at 4°C for cryoprotection. Slices were frozen in

powdered dry ice for 5 minutes then thawed at room temperature to aid in permeabilization. Slices were incubated overnight with Streptavidin-conjugated AlexaFluor 555 (Invitrogen) diluted 1:500 in PBS containing 0.1% Triton X-100. They were washed in PBS then blocked for 1-2 hours room temperature in 10% normal goat serum, 1% Triton X-100 in PBS. Slices were incubated with rabbit anti-GFP (Abcam) diluted 1:3000 overnight at 4°C, washed 3 times in PBS, then incubated for 2 hours room temperature with anti-rabbit AlexFluor 488 (Invitrogen) diluted 1:400. All antibodies were diluted in 5% normal goat serum, 0.1% Triton X-100 in PBS. Slices were also DAPI stained to visualize cortical layers and mounted with Fluoromount-G (Southern Biotech). Biocytin-filled and infected neurons were imaged using an Olympus (Melville, NY, USA) FluoView 300 laser-scanning confocal microscope with FluoView 500 acquisition software.

3.5 REFERENCES

Agmon, A. & Connors, B. W. 1991. Thalamocortical responses of mouse somatosensory (barrel) cortex in vitro. *Neuroscience,* 41, 365-79.

Albright, M. J., Weston, M. C., Inan, M., Rosenmund, C. & Crair, M. C. 2007. Increased thalamocortical synaptic response and decreased layer IV innervation in GAP-43 knockout mice. *J Neurophysiol,* 98, 1610-25.

Blue, M. E. & Parnavelas, J. G. 1983. The formation and maturation of synapses in the visual cortex of the rat. II. Quantitative analysis. *J Neurocytol,* 12, 697-712.

Cang, J., Kaneko, M., Yamada, J., Woods, G., Stryker, M. P. & Feldheim, D. A. 2005. Ephrin-as guide the formation of functional maps in the visual cortex. *Neuron,* 48, 577-89.

Cantallops, I. & Cline, H. T. 2008. Rapid activity-dependent delivery of the neurotrophic protein CPG15 to the axon surface of neurons in intact Xenopus tadpoles. *Dev Neurobiol,* 68, 744-59.

Cantallops, I., Haas, K. & Cline, H. T. 2000. Postsynaptic CPG15 promotes synaptic maturation and presynaptic axon arbor elaboration in vivo. *Nat Neurosci,* 3, 1004-11.

Corriveau, R. A., Shatz, C. J. & Nedivi, E. 1999. Dynamic regulation of cpg15 during activity-dependent synaptic development in the mammalian visual system. *J Neurosci,* 19, 7999-8008.

Dehay, C., Savatier, P., Cortay, V. & Kennedy, H. 2001. Cell-cycle kinetics of neocortical precursors are influenced by embryonic thalamic axons. *J Neurosci,* 21, 201-14.

Desai, N. S., Cudmore, R. H., Nelson, S. B. & Turrigiano, G. G. 2002. Critical periods for experience-dependent synaptic scaling in visual cortex. *Nat Neurosci,* 5, 783-9.

Diaz, E., Ge, Y., Yang, Y. H., Loh, K. C., Serafini, T. A., Okazaki, Y., Hayashizaki, Y., Speed, T. P., Ngai, J. & Scheiffele, P. 2002. Molecular analysis of gene expression in the developing pontocerebellar projection system. *Neuron,* 36, 417-34.

Edgar, J. M. & Price, D. J. 2001. Radial migration in the cerebral cortex is enhanced by signals from thalamus. *Eur J Neurosci,* 13, 1745-54.

Feldman, D. E., Nicoll, R. A. & Malenka, R. C. 1999. Synaptic plasticity at thalamocortical synapses in developing rat somatosensory cortex: LTP, LTD, and silent synapses. *J Neurobiol,* 41, 92-101.

Fujino, T., Leslie, J. H., Eavri, R., Chen, J. L., Lin, W. C., Flanders, G. H., Borok, E., Horvath, T. L. & Nedivi, E. 2011. CPG15 regulates synapse stability in the developing and adult brain. *Genes Dev,* 25.

Gordon, J. A. & Stryker, M. P. 1996. Experience-dependent plasticity of binocular responses in the primary visual cortex of the mouse. *J Neurosci,* 16, 3274-86.

Gorski, J. A., Talley, T., Qiu, M., Puelles, L., Rubenstein, J. L. & Jones, K. R. 2002. Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage. *J Neurosci,* 22, 6309-14.

Greer, P. L. & Greenberg, M. E. 2008. From synapse to nucleus: calcium-dependent gene transcription in the control of synapse development and function. *Neuron,* 59, 846-60.

Grutzendler, J., Tsai, J. & Gan, W. B. 2003. Rapid labeling of neuronal populations by ballistic delivery of fluorescent dyes. *Methods,* 30, 79-85.

Isaac, J. T., Crair, M. C., Nicoll, R. A. & Malenka, R. C. 1997. Silent synapses during development of thalamocortical inputs. *Neuron,* 18, 269-80.

Javaherian, A. & Cline, H. T. 2005. Coordinated motor neuron axon growth and neuromuscular synaptogenesis are promoted by CPG15 in vivo. *Neuron,* 45, 505-12.

Juraska, J. M. 1982. The development of pyramidal neurons after eye opening in the visual cortex of hooded rats: a quantitative study. *J Comp Neurol,* 212, 208-13.

Juraska, J. M. & Fifkova, E. 1979. A Golgi study of the early postnatal development of the visual cortex of the hooded rat. *J Comp Neurol,* 183, 247-56.

Kerchner, G. A. & Nicoll, R. A. 2008. Silent synapses and the emergence of a postsynaptic mechanism for LTP. *Nat Rev Neurosci,* 9, 813-25.

Lee, W. C. & Nedivi, E. 2002. Extended plasticity of visual cortex in dark-reared animals may result from prolonged expression of cpg15-like genes. *J Neurosci,* 22, 1807-15.

Leslie, J. H. & Nedivi, E. 2011. Activity-regulated genes as mediators of neural circuit plasticity. *Prog Neurobiol,* 94, 223-37.

Lois, C., Hong, E. J., Pease, S., Brown, E. J. & Baltimore, D. 2002. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science,* 295, 868-72.

Lopez-Bendito, G. & Molnar, Z. 2003. Thalamocortical development: how are we going to get there? *Nat Rev Neurosci,* 4, 276-89.

Lu, H. C., Butts, D. A., Kaeser, P. S., She, W. C., Janz, R. & Crair, M. C. 2006. Role of efficient neurotransmitter release in barrel map development. *J Neurosci,* 26, 2692-703.

Lu, H. C., She, W. C., Plas, D. T., Neumann, P. E., Janz, R. & Crair, M. C. 2003. Adenylyl cyclase I regulates AMPA receptor trafficking during mouse cortical 'barrel' map development. *Nat Neurosci,* 6, 939-47.

Lush, M. E., Ma, L. & Parada, L. F. 2005. TrkB signaling regulates the developmental maturation of the somatosensory cortex. *Int J Dev Neurosci,* 23, 523-36.

Ma, L., Harada, T., Harada, C., Romero, M., Hebert, J. M., Mcconnell, S. K. & Parada, L. F. 2002. Neurotrophin-3 is required for appropriate establishment of thalamocortical connections. *Neuron,* 36, 623-34.

Mann, F., Peuckert, C., Dehner, F., Zhou, R. & Bolz, J. 2002. Ephrins regulate the formation of terminal axonal arbors during the development of thalamocortical projections. *Development,* 129, 3945-55.

Meyer, M. P. & Smith, S. J. 2006. Evidence from in vivo imaging that synaptogenesis guides the growth and branching of axonal arbors by two distinct mechanisms. *J Neurosci,* 26, 3604-14.

Miller, M. 1981. Maturation of rat visual cortex. I. A quantitative study of Golgi-impregnated pyramidal neurons. *J Neurocytol,* 10, 859-78.

Nedivi, E., Fieldust, S., Theill, L. E. & Hevron, D. 1996. A set of genes expressed in response to light in the adult cerebral cortex and regulated during development. *Proc Natl Acad Sci U S A,* 93, 2048-53.

Nedivi, E., Javaherian, A., Cantallops, I. & Cline, H. T. 2001. Developmental regulation of CPG15 expression in Xenopus. *J Comp Neurol,* 435, 464-73.

Nedivi, E., Wu, G. Y. & Cline, H. T. 1998. Promotion of dendritic growth by CPG15, an activity-induced signaling molecule. *Science,* 281, 1863-6.

Putz, U., Harwell, C. & Nedivi, E. 2005. Soluble CPG15 expressed during early development rescues cortical progenitors from apoptosis. *Nat Neurosci,* 8, 322-31.

Rumpel, S., Kattenstroth, G. & Gottmann, K. 2004. Silent synapses in the immature visual cortex: layer-specific developmental regulation. *J Neurophysiol,* 91, 1097-101.

Ruthazer, E. S., Li, J. & Cline, H. T. 2006. Stabilization of axon branch dynamics by synaptic maturation. *J Neurosci,* 26, 3594-603.

Saalmann, Y. B. & Kastner, S. 2011. Cognitive and perceptual functions of the visual thalamus. *Neuron,* 71, 209-23.

Vaughn, J. E. 1989. Fine structure of synaptogenesis in the vertebrate central nervous system. *Synapse,* 3, 255-85.

Chapter 4: Conclusions and Future Directions

Jennifer H. Leslie

4.1 SUMMARY

Protein products of many activity-regulated genes act as important intermediaries between neuronal activity and synaptic plasticity. Induction of new gene expression is required for the long-term consolidation of structural and functional changes in neuronal circuits during development as well as throughout life (Leslie and Nedivi, 2011). The immediate early gene *candidate plasticity gene 15* (*cpg15*) is one such gene whose product functions to directly effect synaptic change in response to activity. Previous work characterizing CPG15 function in the nervous system has largely been performed using an overexpression paradigm in developing *Xenopus* tadpoles (Nedivi et al., 1998, Cantallops et al., 2000, Javaherian and Cline, 2005). These studies revealed profound effects of CPG15 on enhancing synapse maturation and dendritic and arbor growth and complexity, most dramatically observed in immature regions of the developing *Xenopus* optic tectum (Nedivi et al., 1998, Cantallops et al., 2000). While these studies have yielded tremendous insight into the role of CPG15 in neuronal synapse and arbor maturation, overexpression experiments have some limitations. They are able to reveal what uncharacterized proteins may be capable of doing in the system, however they are unable to definitively answer what functions novel proteins actually do perform. To form a more unambiguous picture of how CPG15 works in the vertebrate brain, our lab turned to a loss-of-function approach by generating a mutant mouse with a genetic ablation of the *cpg15* gene (*cpg15* KO; see Chapter 2)(Fujino et al., 2011).

4.1.1 CPG15 stabilizes synapses to promote synapse and arbor maturation

To understand the mechanism by which CPG15 regulates the maturation of synapses and arbors in the developing mammalian brain, we began by assaying axon, dendrite, and synapse

development in *cpg15* KO mice compared to WT littermate controls. We found that the retinogeniculate projection showed delayed growth and elaboration in the dLGN of the thalamus. In addition in the dentate gyrus of the hippocampus, during development granule cell dendrites are less complex and synapses are fewer in number and weaker than in WT animals. Interestingly, these phenotypes are all largely attenuated at later, more mature time points. However, *cpg15* KO neurons did not simply "catch-up" to WT controls later in life. Instead, WT controls seemed to exhibit initial promiscuous growth followed by extensive pruning and remodeling. *cpg15* KO neurons on the other hand showed little evidence of such refinement and did not greatly change over time, resulting in later time points in which the *cpg15* KO no longer appeared different than WT. These results indicate that CPG15 is particularly important for the establishment of exuberant synapses and arbors during early development. This initial promiscuous growth provides a physical substrate on which experience-dependent refinement can then act to sculpt precisely wired brain circuits.

Further analysis using chronic *in vivo* imaging of GFP expressing neurons in the visual cortex of adult animals revealed that spines, the major site of excitatory synaptic contacts, are less stable in *cpg15* KO mice compared to WT, suggesting a role for CPG15 in stabilizing synaptic contacts. Behavioral tests show that adult *cpg15* KO mice are slow learners, requiring repeated training in learning tasks to perform at WT levels. Analysis of developmental and adult phenotypes in the *cpg15* KO led us to propose that the selection and stabilization of synaptic contacts by CPG15 signaling is critical for the establishment of efficiently wired neuronal networks.

4.1.2 Input-derived CPG15 may regulate target synapse development

To test the role of CPG15 in circuit development, I turned to the developing thalamocortical circuit in the visual system. Primary visual cortex receives visual information through inputs from the dorsal lateral geniculate nucleus (dLGN) of the thalamus (Saalmann and Kastner, 2011). The developmental expression pattern of *cpg15* mRNA is spatiotemporally correlated with periods of synapse formation and refinement (Nedivi et al., 1996, Corriveau et al., 1999, Lee and Nedivi, 2002). In the thalamocortical circuit CPG15 is expressed first in the input structure, the thalamus, then following thalamic innervation during periods of thalamocortical afferent remodeling, in the cortex, the target. CPG15 is therefore poised to act as an important mediator of circuit formation during periods when inputs converge on their targets.

To dissect the role of input- versus target-derived CPG15 signaling in the development of visual thalamocortical circuits, I used the Cre-*loxP* system to genetically disrupt CPG15 expression specifically in the cortex. Comparing synapse development in layer II/III pyramidal neurons in the visual cortex of the previously generated global *cpg15* KO mice with cortex-specific *cpg15* KO mice, I found a markedly more pronounced reduction in synapse development when CPG15 was lost throughout the thalamocortical circuit rather than just lost in the cortex. These results indicate the CPG15 signaling by thalamic afferents in the cortex critically regulates cortical synapse maturation. While many cortically expressed cell-attached and diffusible signaling molecules have been shown to be important for thalamocortical circuit development (Lopez-Bendito and Molnar, 2003), this reveals a novel mechanism by which inputs from the thalamus may signal to regulate the development of circuits in the cortex.

4.1.3 CPG15 in the nervous system

Throughout the mammalian brain CPG15 promotes the maturation of synapses as well as dendritic and axonal arbors (see Chaper 2 and 3)(Fujino et al., 2011). Results to date suggest that the primary function of CPG15 signaling is in the selection and stabilization of newly forming synapses. Synapse stabilization allows for subsequent synapse maturation and also reduces the retraction of growing neuronal branches at points of stabilized synaptic contacts (Javaherian and Cline, 2005, Meyer and Smith, 2006). Signaling by CPG15 can likely be regulated by neuronal activity (Cantallops and Cline, 2008), acting as a positive synapse selector during experience-dependent remodeling of neuronal circuits. Spatiotemporal expression patterns of CPG15 in the developing brain correlate well with developmental periods of synapse formation and refinement (Nedivi et al., 1996, Corriveau et al., 1999, Lee and Nedivi, 2002). In thalamocortical circuit development CPG15 signaling by thalamic afferents in the cortex may help establish mature patterns of connectivity (see Chapter 3). However, CPG15's role is not limited to developing circuits, CPG15 likely remains important into adulthood enabling circuit modifications that underlie lifelong processes including learning and memory (see Chapter 2)(Fujino et al., 2011).

4.2 FUTURE DIRECTIONS

4.2.1 Mechanism through which CPG15 promotes thalamocortical synapse maturation

Immature excitatory synapses are often postsynaptically silent, containing only NMDA receptors (Kerchner and Nicoll, 2008). During development, the activity dependent trafficking of AMPA receptors converts synapses from silent into mature functional synapses. Thalamocortical synapse maturation is known to proceed in this manner (Isaac et al., 1997, Feldman et al., 1999) and synaptic maturation and function has been shown to be critical for the development of thalamocortical networks (Lu et al., 2003, Lu et al., 2006, Albright et al., 2007).

In Chapter 3, I utilized recordings of spontaneous mESPCs to assess synapse maturation in both global *cpg15* KO mice, lacking CPG15 expression throughout the thalamocortical circuit, and cortex-specific *cpg15* KO mice, lacking CPG15 expression only in the target for thalamic axons, the cortex. Average mEPSC amplitude provides a correlate for AMPA receptor containing synapse strength, while frequency can be correlated with synapse number. In these experiments I found that average mEPSC amplitudes and frequencies were reduced in the global *cpg15* KO (Fig. 3.1, Chapter 3), however only a transient deficit in mEPSC amplitudes was observed in the cortex-specific KO (Fig. 3.3, Chapter 3). CPG15 overexpression has previously been shown to enhance synapse maturation through the incorporation of AMPA receptors into silent synapses, thus increasing mESPC frequency (Cantallops et al., 2000), therefore these data strongly suggest that in the without thalamic CPG15 signaling, AMPA receptors trafficking into immature cortical synapses is greatly reduced. However, reductions in mEPSC amplitude and frequency alone cannot prove reduced AMPA receptor synaptic content and the presence of immature silent synapses.

To more rigorously test the mechanism by which CPG15 promotes mammalian excitatory synapse maturation, I plan to do two experiments. First, to test if there are indeed synapses that do not contain AMPA receptors in the global *cpg15* KO I will record depolarize neurons to record NMDA receptor-mediated mESPCs as well as AMPA receptor-mediated mESPCs at resting potentials and compare their frequencies. I will perform this experiment at P28, the time point when I initially observed the deficit in mEPSC frequency. A ratio of NMDA receptor-mediated mEPSC frequency to AMPA receptor-mediated mESPC frequency above one is strongly indicative of the presence of silent synapses (Rumpel et al., 2004). I will compare these ratios between *cpg15* KO mice and WT littermates. Second, I will also measure AMPA/NMDA ratios by stimulating white matter and recording evoked responses in layer II/III pyramidal neurons. By comparing AMPA/NMDA ratios between *cpg15* KO and WT mice I can determine whether synaptic insertion of AMPA receptors is deficient in the absence of CPG15 signaling. This experiment will also give higher spatial resolution by specifically interrogating synapses from extracortical inputs, which include thalamocortical afferents. It is possible that I will find no difference between *cpg15* KO mice and WT littermate controls in these experiments. If so, then is it likely that the reduction in mEPSC frequency observed in the absence of CPG15 may be due to a reduction in overall synapse numbers and not in deficient synapse maturation.

These proposed experiments will shed light of the role of CPG15 in excitatory synapse maturation in the mammalian brain and will hopefully confirm previous results from overexpression studies the developing *Xenopus* retinotecal system (Cantallops et al., 2000). They will help resolve ambiguity in interpreting the analysis of mESPCs in the global *cpg15* KO (see Chapter 3), and will additionally address whether defects in the cortex arise specifically at synapses from extracortical inputs such as the thalamus.

4.2.2 Rescue of thalamic afferent CPG15 signaling in the cortex

Previously I described a rescue of synaptic deficits by restoring CPG15 expression in the cortex of cortex-specific *cpg15* KO mice using a lentiviral mediated expression system (see Chapter 3). To prove that CPG15 signaling from thalamic afferents in the cortex can promote cortical synapse maturation, I plan to employ a similar strategy to rescue CPG15 expression in the thalamus of global *cpg15* KO mice. By targeting injections of lentivirus expressing CPG15, or GFP as a control, to the dLGN of global *cpg15* KO mice I can restore thalamic signaling by CPG15. After allowing sufficient time for expression, I will record from layer II/III pyramidal neurons at P28 in acute slices of visual cortex from injected *cpg15* KO mice and WT littermates. This experiment should hopefully attenuate the synaptic phenotypes in mEPSC frequency and amplitude that occur at P28 in the *cpg15* KO thus showing conclusively that thalamic expression of CPG15 can provide a signal for synapse maturation in the cortex.

4.2.3 Functional consequences of CPG15 signaling in thalamocortical circuits

Much of the work to understand the function of molecules in late stages of patterning of thalamocortical connectivity has been performed in the somatosensory barrel cortex of rodents. In this region thalamocortical afferents in layer IV are anatomically arranged into a somatosensory map of distinct barrel-like structures each corresponding to an individual whisker. Change in the patterning of the cytoarchitecture of the somatosensory barrel cortex serves as a useful indicator of thalamocortical connectivity deficits (Lopez-Bendito and Molnar, 2003). In cats and primates thalamocortical afferents in the primary visual cortical areas develop similarly distinct anatomical organizations known as ocular dominance columns in which inputs from each eye are segregated into alternating stripes (Hubel and Wiesel, 1962, Hubel and Wiesel, 1969).

However, the mouse visual cortex has no detectable columnar arrangement of thalamocortical inputs, although it is likely that there is some level of columnar organization that cannot be discerned anatomically (Shatz, 1992).

The segregation of ocular columns can be manipulated by alterations in visual experience (Hubel et al., 1977), as well as pharmacologically through the application of drugs that affect neuronal activity (Shaw and Cynader, 1984, Reiter et al., 1986), and even the cortical infusion of neurotrophins such as BNDF and NGF (Cabelli et al., 1995). Ocular dominance plasticity has functional correlates whereby the strength of connectivity of inputs from each eye, measured using *in vivo* electrophysiological recordings, can be altered by manipulations in visual experience (Wiesel and Hubel, 1963, Hubel et al., 1977). This functional ocular dominance plasticity has also been observed in the mouse visual cortex (Gordon and Stryker, 1996). Monocular deprivation, or the closing of one eye, leads to a depression in cortical responses to the deprived eye and a potential of responses to the spared eye (Frenkel and Bear, 2004). Therefore despite the lack of anatomical correlates for thalamocortical connectivity in mouse, functional paradigms exist through which the development of this circuit can be probed. For example, BDNF and its cognate receptor TrkB, known to be important for the establishment of somatosensory barrels in rodents (Lush et al., 2005), as well as ocular dominance columns in cats (Cabelli et al., 1995), are also important for functional ocular dominance plasticity in mouse visual cortex (Hanover et al., 1999, Huang et al., 1999).

To establish a definitive role for CPG15 in the development and refinement of thalamocortical circuitry in the mouse visual cortex, it will be important to test the role of CPG15 in ocular dominance plasticity. Optical imaging of intrinsic signals has recently been developed as a method to examine retinotopic maps as well as to measure ocular dominance

plasticity in the visual cortex of mice (Kalatsky and Stryker, 2003, Cang et al., 2005). Our lab has recently acquired an optical imaging system and would be able to perform such experiments. It can be hypothesized that the global *cpg15* KO mouse would display reduced cortical plasticity due to a failure to stabilize synapse and mature new connections forming between the spared eye and cortical neurons. However, if thalamic-mediated CPG15 signaling is important for the organization of thalamocortical connectivity, then it is likely that the cortex-specific *cpg15* KO mouse will not show deficits in ocular dominance plasticity. This would provide more definitive evidence for CPG15 signaling by thalamic inputs guiding the wiring of thalamocortical circuits during development.

4.3 REFERENCES

Albright, M. J., Weston, M. C., Inan, M., Rosenmund, C. & Crair, M. C. 2007. Increased thalamocortical synaptic response and decreased layer IV innervation in GAP-43 knockout mice. *J Neurophysiol*, 98, 1610-25.

Cabelli, R. J., Hohn, A. & Shatz, C. J. 1995. Inhibition of ocular dominance column formation by infusion of NT-4/5 or BDNF. *Science*, 267, 1662-6.

Cang, J., Kalatsky, V. A., Lowel, S. & Stryker, M. P. 2005. Optical imaging of the intrinsic signal as a measure of cortical plasticity in the mouse. *Vis Neurosci*, 22, 685-91.

Cantallops, I. & Cline, H. T. 2008. Rapid activity-dependent delivery of the neurotrophic protein CPG15 to the axon surface of neurons in intact Xenopus tadpoles. *Dev Neurobiol*, 68, 744-59.

Cantallops, I., Haas, K. & Cline, H. T. 2000. Postsynaptic CPG15 promotes synaptic maturation and presynaptic axon arbor elaboration in vivo. *Nat Neurosci*, 3, 1004-11.

Corriveau, R. A., Shatz, C. J. & Nedivi, E. 1999. Dynamic regulation of cpg15 during activity-dependent synaptic development in the mammalian visual system. *J Neurosci*, 19, 7999-8008.

Feldman, D. E., Nicoll, R. A. & Malenka, R. C. 1999. Synaptic plasticity at thalamocortical synapses in developing rat somatosensory cortex: LTP, LTD, and silent synapses. *J Neurobiol*, 41, 92-101.

Frenkel, M. Y. & Bear, M. F. 2004. How monocular deprivation shifts ocular dominance in visual cortex of young mice. *Neuron*, 44, 917-23.

Fujino, T., Leslie, J. H., Eavri, R., Chen, J. L., Lin, W. C., Flanders, G. H., Borok, E., Horvath, T. L. & Nedivi, E. 2011. CPG15 regulates synapse stability in the developing and adult brain. *Genes Dev*, 25.

Gordon, J. A. & Stryker, M. P. 1996. Experience-dependent plasticity of binocular responses in the primary visual cortex of the mouse. *J Neurosci*, 16, 3274-86.

Hanover, J. L., Huang, Z. J., Tonegawa, S. & Stryker, M. P. 1999. Brain-derived neurotrophic factor overexpression induces precocious critical period in mouse visual cortex. *J Neurosci*, 19, RC40.

Huang, Z. J., Kirkwood, A., Pizzorusso, T., Porciatti, V., Morales, B., Bear, M. F., Maffei, L. & Tonegawa, S. 1999. BDNF regulates the maturation of inhibition and the critical period of plasticity in mouse visual cortex. *Cell*, 98, 739-55.

Hubel, D. H. & Wiesel, T. N. 1962. Receptive fields, binocular interaction and functional architecture in the cat's visual cortex. *J Physiol*, 160, 106-54.

Hubel, D. H. & Wiesel, T. N. 1969. Anatomical demonstration of columns in the monkey striate cortex. *Nature*, 221, 747-50.

Hubel, D. H., Wiesel, T. N. & Levay, S. 1977. Plasticity of ocular dominance columns in monkey striate cortex. *Philos Trans R Soc Lond B Biol Sci*, 278, 377-409.

Isaac, J. T., Crair, M. C., Nicoll, R. A. & Malenka, R. C. 1997. Silent synapses during development of thalamocortical inputs. *Neuron*, 18, 269-80.

Javaherian, A. & Cline, H. T. 2005. Coordinated motor neuron axon growth and neuromuscular synaptogenesis are promoted by CPG15 in vivo. *Neuron*, 45, 505-12.

Kalatsky, V. A. & Stryker, M. P. 2003. New paradigm for optical imaging: temporally encoded maps of intrinsic signal. *Neuron*, 38, 529-45.

Kerchner, G. A. & Nicoll, R. A. 2008. Silent synapses and the emergence of a postsynaptic mechanism for LTP. *Nat Rev Neurosci,* 9, 813-25.

Lee, W. C. & Nedivi, E. 2002. Extended plasticity of visual cortex in dark-reared animals may result from prolonged expression of cpg15-like genes. *J Neurosci,* 22, 1807-15.

Leslie, J. H. & Nedivi, E. 2011. Activity-regulated genes as mediators of neural circuit plasticity. *Prog Neurobiol,* 94, 223-37.

Lopez-Bendito, G. & Molnar, Z. 2003. Thalamocortical development: how are we going to get there? *Nat Rev Neurosci,* 4, 276-89.

Lu, H. C., Butts, D. A., Kaeser, P. S., She, W. C., Janz, R. & Crair, M. C. 2006. Role of efficient neurotransmitter release in barrel map development. *J Neurosci,* 26, 2692-703.

Lu, H. C., She, W. C., Plas, D. T., Neumann, P. E., Janz, R. & Crair, M. C. 2003. Adenylyl cyclase I regulates AMPA receptor trafficking during mouse cortical 'barrel' map development. *Nat Neurosci,* 6, 939-47.

Lush, M. E., Ma, L. & Parada, L. F. 2005. TrkB signaling regulates the developmental maturation of the somatosensory cortex. *Int J Dev Neurosci,* 23, 523-36.

Meyer, M. P. & Smith, S. J. 2006. Evidence from in vivo imaging that synaptogenesis guides the growth and branching of axonal arbors by two distinct mechanisms. *J Neurosci,* 26, 3604-14.

Nedivi, E., Fieldust, S., Theill, L. E. & Hevron, D. 1996. A set of genes expressed in response to light in the adult cerebral cortex and regulated during development. *Proc Natl Acad Sci U S A,* 93, 2048-53.

Nedivi, E., Wu, G. Y. & Cline, H. T. 1998. Promotion of dendritic growth by CPG15, an activity-induced signaling molecule. *Science,* 281, 1863-6.

Reiter, H. O., Waitzman, D. M. & Stryker, M. P. 1986. Cortical activity blockade prevents ocular dominance plasticity in the kitten visual cortex. *Exp Brain Res,* 65, 182-8.

Rumpel, S., Kattenstroth, G. & Gottmann, K. 2004. Silent synapses in the immature visual cortex: layer-specific developmental regulation. *J Neurophysiol,* 91, 1097-101.

Saalmann, Y. B. & Kastner, S. 2011. Cognitive and perceptual functions of the visual thalamus. *Neuron,* 71, 209-23.

Shatz, C. J. 1992. How are specific connections formed between thalamus and cortex? *Curr Opin Neurobiol,* 2, 78-82.

Shaw, C. & Cynader, M. 1984. Disruption of cortical activity prevents ocular dominance changes in monocularly deprived kittens. *Nature,* 308, 731-4.

Wiesel, T. N. & Hubel, D. H. 1963. Single-Cell Responses in Striate Cortex of Kittens Deprived of Vision in One Eye. *J Neurophysiol,* 26, 1003-17.

Appendix: Additional Analyses of Global and Conditional *cpg15* KO Mice

Jennifer H. Leslie

A.1 RESULTS

A.1.1 Spine density in the visual cortex of *cpg15* KO mice

The frequency of spontaneous miniature excitatory postsynaptic currents (mEPSCs) recorded from layer II/III pyramidal neurons in primary visual cortex of *cpg15* KO mice is lower than WT littermates at postnatal day 28 (P28) (see Chapter 3). This suggests that *cpg15* KO neurons may have fewer functional, AMPA receptor-containing synapses than controls, or possibly fewer synapses in general, (AMPA receptor-containing, as well as "silent" NMDA receptor-only synapses). Spines are often used as a correlate of excitatory synapses. Therefore we measured the density of spines on layer II/III pyramidal neuron basal dendrites using diolistic labeling of neurons in slices. Three ages were assessed, P10, P28, and 3 months. Normally, spine density in the visual cortex greatly increases after P10 in correlation with a large amount of synaptogenesis reaching mature levels around the second week postnatal (Miller, 1981). There was no difference in spine density between *cpg15* KO mice and WT littermates at any age (Fig. A.1). This result supports a role for CPG15 that is downstream of spinogenesis. Previously we have shown using electron microscopy that in the dentate gyrus (DG) of the hippocampus while there is no difference in the density of spines and spine-like protrusions, there is a 26% reduction in the density of spine synapses in *cpg15* KO mice compared to controls (see Chapter 2)(Fujino et al., 2011). This suggests that while spines may normally be a useful approximation of excitatory synapses, this may not be the case when examining synaptic phenotypes involving proteins such as CPG15 that likely play a role in synapse biogenesis downstream of spinogenesis. Further work is needed to determine whether the reduction in mESPC frequencies
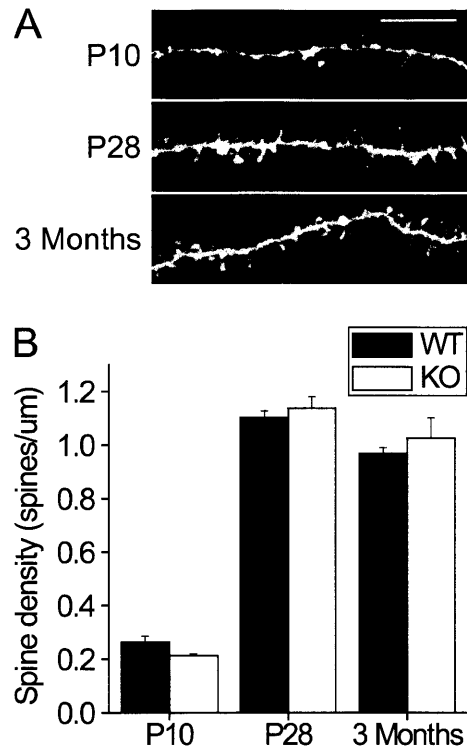
Figure A.1 Spine density on basal dendrites of layer II/III pyramidal neurons in *cpg15* KO mice. (A) Representative examples of diolistic labeling of spines on segments of dendrites at ages P10, P28, and 3 months. Scale bar is 10um. (B) Quantification of spine densities for *cpg15* KO mice and WT littermates. (P10 n = 8 mice, 625 spines for WT, n = 4 mice, 462 spines for KO; P28 n = 7 mice, 2303 spines for WT, n = 7 mice, 2247 spines for KO; 3 months n = 4 animals, 1163 spines for WT, n = 5 animals, 1075 spines for KO).

in the visual cortex of *cpg15* KO mice is due to a reduction in overall synapse density or in functional AMPA receptor-containing synapses.

A.1.2 Other electrophysiological measurements of pyramidal neurons in the visual cortex

In addition to analysis of mEPSCs recorded from layer II/III pyramidal neurons in the primary visual cortex of global and conditional *cpg15* KO mice, we also assessed other parameters that are known to change as pyramidal neurons mature. The resting potential of pyramidal neurons gradually becomes more hyperpolarized during postnatal development, and as the cells add additional ionotropic channels, the membrane resistance also decreases (Etherington and Williams, 2011, Maravall et al., 2004). We measured both of these parameters in global and conditional *cpg15* KO mice at the same ages that mEPSC analysis was performed, P10, P14, and P28. We found no significant differences between KO and WT littermate controls at any ages for either parameter except at P14 conditional *cpg15* KO mice show slightly lower (more hyperpolarized) resting membrane potentials than their WT counterparts (Fig. A.2). Interestingly this is the same age at which the only mEPSC phenotype was observed in the conditional *cpg15* KO line. We found decreased mEPSC amplitudes at P14 (see Chapter 3). All mEPSCs were recorded from cells clamped at -70mV to ensure that membrane potential did not affect mESPC amplitudes. While changes in membrane resistance could affect mEPSC amplitudes this difference was not observed (Fig. A.2).
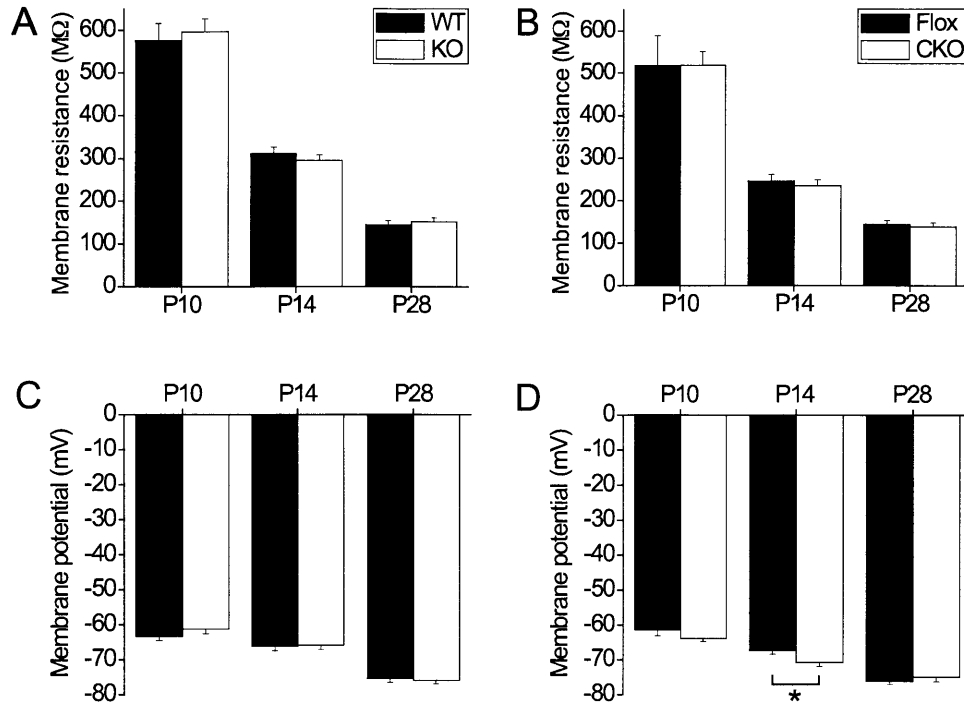
Figure A.2 Membrane resistance and membrane potential in layer II/III pryamidal neurons of global and conditional *cpg15* **KO mice.** Membrane resistance in layer 2/3 pyramidal neurons in the primary visual cortex of global *cpg15* KO mice compared to WT controls, (A) and conditional *cpg15* KO mice compared to floxed controls (B). Membrane potentials from the same neurons in global *cpg15* KO mice and WT controls, (C), and conditional *cpg15* KO mice and controls, (D). (*$p < 0.05$).

A.2 MATERIALS AND METHODS

A.2.1 Diolistic labeling and counting of spine density ·

Please see Chapter 3 Materials and Methods for a description of diolistic labeling of layer 2/3 pyramidal neurons in the primary visual cortex. Segment of basal dendrites from labeled neurons were imaged with an Olympus (Melville, NY, USA) FluoView 300 laser-scanning confocal microscope and FluoView 500 acquisition software. For spine analysis, dendrite segments that were relatively level within the XY plane of the slice were collected in 8 um stacks with 0.3 um imaging intervals using a 60x lens. Spine images were analyzed with ImageJ software (http://rsb.info.nih.gov/ij/). Only spine densities on secondary and tertiary branch segments were included in the analysis.

A.2.2 Electrophysiology

Please see Chapter 3 Materials and Methods for a description of whole-cell patch clamp electrophysiology. Membrane potentials were recorded immediately after cells were successfully patched. Membrane resistance was recorded from cells held at -70mV prior to the start of mEPSC recordings using the "Membrane Test" function in pCLAMP 9 (Axon Instruments).

A.3 REFERENCES

Etherington, S. J. & Williams, S. R. 2011. Postnatal development of intrinsic and synaptic properties transforms signaling in the layer 5 excitatory neural network of the visual cortex. *J Neurosci,* 31, 9526-37.

Fujino, T., Leslie, J. H., Eavri, R., Chen, J. L., Lin, W. C., Flanders, G. H., Borok, E., Horvath, T. L. & Nedivi, E. 2011. CPG15 regulates synapse stability in the developing and adult brain. *Genes Dev,* 25.

Maravall, M., Stern, E. A. & Svoboda, K. 2004. Development of intrinsic properties and excitability of layer 2/3 pyramidal neurons during a critical period for sensory maps in rat barrel cortex. *J Neurophysiol,* 92, 144-56.

Miller, M. 1981. Maturation of rat visual cortex. I. A quantitative study of Golgi-impregnated pyramidal neurons. *J Neurocytol,* 10, 859-78.