Title
The Role of Arc in Regulating Spine Morphology and Neural Network Stability In Vivo

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Publication Date
2009

Peer reviewed|Thesis/dissertation

The Role of Arc in Regulating Spine Morphology and Neural Network Stability *In Vivo*

by

Carol Lee Peebles

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

For my family

Acknowledgements

My time in graduate school has been surprisingly wonderful. While there have been the expected bumps along the way and multiple meetings with failure, looking back on it all, I have thoroughly enjoyed the journey. From day one, the Finkbeiner lab has been a supportive, collaborative and just plain fun place to work. And for this, I am extremely grateful.

First, I thank Steve Finkbeiner for being such an inspiring and caring mentor. I am very fortunate to have trained with someone who not only is passionate about science but also cares deeply about his lab members' success and happiness. For Steve, every moment is a teachable moment - no matter how busy he is, or how small the lesson. Because of this, I am a better scientist and a better person. Thank you.

The lab for much of my graduate school was a second home. And despite the long hours, the people in the Finkbeiner lab made every day enjoyable. There a few people in particular I would like to thank. Gaia – who found her way from Swansea to be my biggest cheerleader in lab and in life. Don't worry, let us have fun! Your support, optimism, good qi and friendship means the world to me. Jason– I could not have asked for a better cubicle buddy or friend. You are brilliant and have a humongous heart (it may even be bigger than your stomach!). Thanks for sharing some of it with me. To Monste, Punita and Hong Joo – being a scientist is hard, but being a mommy scientist has got to take the cake. It has been inspiring and reassuring to see how well you have balanced it all. To Vikram and Sean, the original Arc team: thank you for showing me the way.

I have received much help from members outside our lab – thanks especially to Jorge Palop, Alice Thwin, Luisa Stamm, and Brad Zuchero who all spent much of their free time to teach me new techniques.

Thanks must also go to my thesis committee: Grae Davis, Rob Edwards, Fen Biao Gao and Lu Chen. Also Gary Howard for his efficient editing prowess. And Kelley Nelson, for being a stellar administrative assistant, and for all the good laughs and distractions. Finally, but most importantly, I want to thank my family. My success is the result of their love, support, inspiration and encouragement.

Contribution of others to the presented work

Chapter 1 provides background synaptic plasticity and Arc. Portions of the section, *Arc and RNA regulation*, were published as a News and Views in *Nature Neuroscience* (September 2007).

Chapter 2 is based on manuscript currently in review at *Nature Neuroscience*. Jong Yoo and Jeffrey Noebels performed EEG studies and analysis. Alice Thwin and Jorge Palop assisted in kainic acid studies and immunohistochemistry.

In Chapter 3, Renatta Knox cloned the GST-Arc construct. Jason Breck designed, cloned and tested the Wave3 shRNA. All other experiments and analyses described in Chapters 2, 3 and 4 were performed by C.L.P. Dr. Steven Finkbeiner supervised all research and provided direction.

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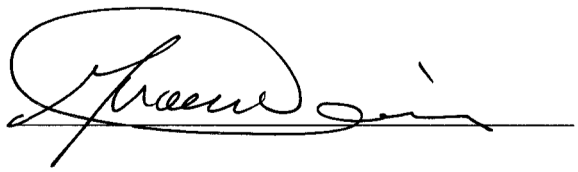

Graeme Davis

Committee Chair

Abstract

The understanding of human memory is one of the greatest challenges facing neuroscience today. The brain's extraordinary ability to integrate information and appropriately adapt in response to various stimuli is at the core of learning and memory. At a cellular and molecular level, learning and memory relies on a neuron's ability to facilitate activity-dependent changes in synaptic efficacy. In order to better understand the mechanism behind such changes, we investigate the function of Arc, an immediate-early gene essential for both long-term and homeostatic plasticity. We find that through AMPA receptor endocytosis, Arc expression modulates spine morphology to favor more plastic thin spines and filopodia. Thus, Arc expression simultaneously reduces synaptic efficacy through AMPA receptor endocytosis while increasing structural plasticity by favoring thin spines. Supporting this, we

find that loss of *Arc in vivo* leads to a decrease in the proportion of thin spines as well as neural network hyperexcitability. Given Arc's role in spine morphology we also investigate possible actin-regulating Arc-binding partners. We find that Arc directly binds to Wave3, an actin-nucleating factor, in neurons. We further demonstrate that reduction of Wave3 expression leads a marked decrease in primary dendrite length. In mature neurons, reduction of Wave3 results in decreased spine density and increased filopodia. Finally, Arc expression partially rescued these reductions in primary dendrite length and spine density, supporting a functional role for the Arc-Wave3 interaction. Thus, our investigations of Arc and Wave3 have contributed to the understanding of synaptic plasticity, and suggest new links between synaptic efficacy, structural plasticity, homeostasis and memory.

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Chapter 1

Synaptic Plasticity and Memory Formation

Memory is a way of holding onto the things you love, the things you are, the things you never want to lose. **"**Kevin Arnold" from The Wonder Years

On March 29th, 1985, Clive Wearing lost his ability to remember. At the time he was in his mid-forties and an accomplished musician living in London. Clive contracted a common virus (Herpes simplex) and the infection tragically spread to his brain, destroying the region central to memory formation: *the hippocampus.* Not only was Clive left with a memory span of only a few seconds, he also lost the vast majority of his past memories. Experiences, historical events, the faces of his children all were now gone. It is hard to imagine what such an existence would be like. In Clive's words: "It's like being dead. I came to the conclusion that I was dead."

Memories not only allow us to function in a constantly changing world, they are the foundation of who we are. It is no wonder that humans have attempted to understand the basis of memory since ancient times. However it wasn't until the end of the 19th century that the Spanish neuropathologist, Santiago Ramón y Cajal, suggested that chemical and structural changes at sites of neuronal connections could be the foundation of memory. Since then, generations of neuroscientists have continued to validate and expand on Cajal's hypothesis of synaptic plasticity.

Synaptic Plasticity

Our ability to learn and remember is based on our brain's ability to biochemically and structurally modulate its neuronal connections in response to experience. This statement seems relatively simple at first glance. But when one considers the complexity of daily experience and multiplies this by a lifetime, it is mind-boggling: how can an organ as small as the brain integrate and store such massive amounts of information over so many years? While we still do not fully understand how the brain conquers this herculean task, much progress has been made since Cajal first theorized the plastic synapse.

The most commonly studied cellular and molecular models of memory are long-term potentation (LTP), and long-term depression (LTD). LTP is defined as the persistent increase in synaptic efficacy resulting from repetitive stimulation[1,2]. LTP has several key features which make it attractive as a possible mechanism for memory formation. First, LTP is synapse-specific, such that it is selectively expressed at synapses that have experienced a particular pattern of activity[3]. Second, inhibition of LTP impairs learning and memory in spatial memory tasks[4]. Third, late-LTP, like memory consolidation, occurs over two phases: an early phase independent of new gene expression and a late phase lasting for hours which requires new gene transcription and protein synthesis.

LTD, or the long-term weakening of a synapse, is divided into several forms. Homosynaptic LTD describes synaptic depression following repetitive low-frequency stimulation[5]. This form of LTD, like LTP, is long lasting, input specific, and protein synthesis-dependent. Heterosynaptic LTD refers to depression at inactivated synapses that neighbor recently stimulated synapses[6,7]. Finally, mGluR-LTD requires both mGluR and

NMDA receptors, and is distinguished by rapid protein synthesis within minutes of mGluR stimulation[8].

Over the last two decades, much research has focused on understanding the molecular mechanism behind long-term plasticity. As a result, well over 100 molecules have been implicated in LTP[9]! Why is it *so* complicated? Central to models of LTP and LTD is the idea of synapse-specific plasticity. It is believed that a neuron, with thousands of synaptic connections, can and must modulate each individual synapse based on the input it receives. However, neurons are some of the most complicated cells in our body – heavily compartmentalized, with arbors that can extend inches and even feet away from their cell body. For synapse-specific plasticity to occur, a neuron must choreograph numerous cellular mechanisms to ensure that specific proteins are present at the right place and time.

There are several leading hypotheses to explain how long-lasting synaptic plasticity can occur in a synapse-specific fashion. In one model synaptic activity induces a signal that is sent back to the nucleus, triggering gene expression and the targeting of newly synthesized protein to the activated synapses. In this scenario proteins can either be directly transported to activated synapses, or transported throughout the dendrite but sequestered at activated synapses by a "synaptic tag"[10,11]. A second model involves the translation of dendritically localized mRNA in an activity-dependent manner. Both of these models require signaling to the nucleus for the production of new mRNAs either to be directly transported, or translated and then transported as protein to the activated synapse.

How do these new proteins ultimately lead to long-term synaptic strengthening? One possibility is that newly synthesized targeted proteins are involved in altering synaptic morphology. LTP has been shown to increase both spine density and size, which in theory

should allow for larger and stronger synapses[12,13]. LTP has been shown to increase F-actin content in spines in an actin-polymerization dependent manner[14]. Furthermore, inhibition of actin-polymerization specifically blocks late-LTP without affecting induction or early maintenance of LTP, indicating that alteration in the actin cytoskeleton is carefully regulated and critical specifically for the maintenance of late-LTP[14].

Activity-Regulated Cytoskeleton-Associated Protein (Arc)

The immediate early gene (IEG) *Arc* is a unique and fascinating molecule crucial for several forms of synaptic plasticity and memory consolidation. The gene was originally discovered during a screen for hippocampal genes upregulated by seizure[15]. It was quickly learned that *Arc*'s induction and localization are tightly regulated by synaptic activity. *Arc* mRNA is induced within 2 minutes of activity and is trafficked to dendrites, where it can be synthesized into protein. Steward, Worely and colleagues showed that *Arc* mRNA not only localizes to dendrites upon stimulation, but also accumulates specifically in activated dendritic regions[16-18]. Targeting of the *Arc* mRNA to the dendrites is independent of protein synthesis, suggesting that localization is regulated by the mRNA itself[19]. In these studies, Steward *et al.* took advantage of the organized neuroanatomy of the dentate gyrus and entorhinal cortex (EC). Projections from two different layers of the EC (medial and lateral) terminate at two separate regions of the dentate gyrus (middle molecular layer, and outer molecular layer respectively). By stimulating projections from the medial EC, Steward observed *Arc* mRNA selectively accumulate (via *in situ* hybridization) at the middle molecular layer, and not the outer molecular layer, of the dentate gyrus.

Arc's synaptic localization in response to activity makes it a prime candidate for playing a role in synaptic plasticity. This has been confirmed in several studies demonstrating that Arc is required for late-LTP, mGluR-LTD and homeostatic plasticity.

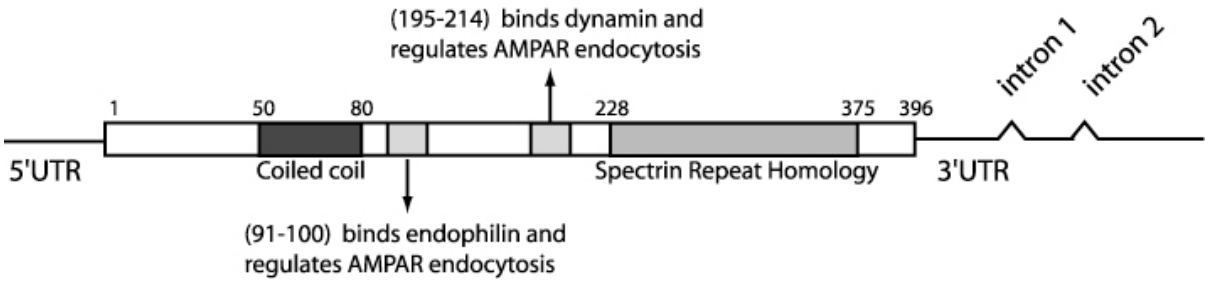

Figure 1.1: Activity-Regulated Cytoskeleton-Associated Protein
The rat *Arc* mRNA is 3.2kb in length with two introns located within the 3'UTR. The Arc ORF produces a 396aa protein of 55kDa. The protein has only a few recognizable domains: a coiled coil domain at the N-terminus and a spectrin repeat homology domain at the C-terminus[15]. Arc has been shown to interact with endophilin 2 or 3 and dynamin 2 at 91-100aa and 195-214aa respectively[20].

Features of the Arc gene and protein

The *Arc* gene is located on mouse chromosome 15 and rat chromosome 7. It produces an mRNA transcript ~3.2kb in length with a large 3'UTR which includes the transcript's only two introns (Figure 1.1). Exon 1 contains the entire coding sequence which produces a protein of 396 amino acids with a molecular weight of 55kDa. The C-terminus has a spectrin repeat homology domain that is 22% identical (77% homologous) to the 21st and 22nd repeats of α-spectrin. The N-terminal half of *Arc* has no significant homology to other proteins but does contain a coiled coil domain.

Arc has been shown to directly interact with endocytic proteins endophilin 3 and dynamin 2, and through this interaction mediate AMPA receptor (AMPAR) endocytosis

(Figure 1.2). Overexpression of Arc in hippocampal neurons leads to a decrease in AMPAR surface expression[20] as well as reduced AMPAR mediated currents[21]. Arc deletion constructs which abrogate binding to endophilin 3 or dynamin 2 fail to reduce AMPAR surface expression or AMPAR mediated currents.

Arc and LTP consolidation

Several in vivo studies have demonstrated that Arc is required for late-LTP and memory consolidation. In 2002, work by John Guzowski, using antisense oligonucleotides (ODNs) against Arc infused into the hippocampus, first demonstrated that Arc is required for late-LTP but not LTP induction[22]. Guzowksi also found that reduction of Arc impaired long-term memory but not learning, using the hippocampus-dependent spatial version of the Morris water maze task. In 2006, Arc's role in late-LTP and memory consolidation was confirmed using Arc knockout mice.[23] Plath et al. observed that early-phase LTP is enhanced and late-phase LTP is blocked in both the dentate gyrus and CA1 of knockout animals. Furthermore, the mice were impaired in a variety of memory tasks including fear conditioning, novel object recognition, conditioned taste aversion, and the Morris water maze. Finally, in 2007 Messoudi et al. demonstrated temporal requirements for Arc expression in facilitating LTP. Specifically, antisense-mediated reduction of Arc 15 minutes after high frequency stimulation (HFS) of the medial perforant path transiently blocked LTP expression, paralleling the transient knockdown of Arc protein expression[24]. Antisense blockade of Arc 2 hours, but not 4 hours, after HFS led to rapid and permanent reversal of LTP. Furthermore the authors observed that reduction of Arc blocked LTP-induced F-actin

stabilization and cofilin phosphorylation, indicating that Arc may also play a role in LTP-induced cytoskeletal changes at spines.

Arc and mGluR-LTD

Two recent studies have demonstrated that Arc is also essential for mGluR-LTD[25,26]. Both groups found that stimulation of hippocampal cultures by the mGluR agonist dihydroxphenylglycine (DHPG) led to a long-term reduction of surface AMPARs that was dependent on the rapid de novo protein synthesis of Arc (Figure 1.2). Furthermore, mGluR-LTD was impaired in slices from Arc knockout mice and after Arc reduction using antisense ODNs. The rapid protein synthesis of Arc after mGluR activation requires eukaryotic elongation factor 2 kinase (eEF2K). eEF2K binds mGluR and, upon activation, is released and phosphorylates eukaryotic elongation factor (eEF2). Park et al. demonstrated that while eEF2 inhibits general protein synthesis, it increases Arc translation[26]. Interestingly, Arc is not essential for NMDAR-dependent LTD which does require rapid protein synthesis but does have a late-phase that is translation, but not transcription, dependent.[27-29].

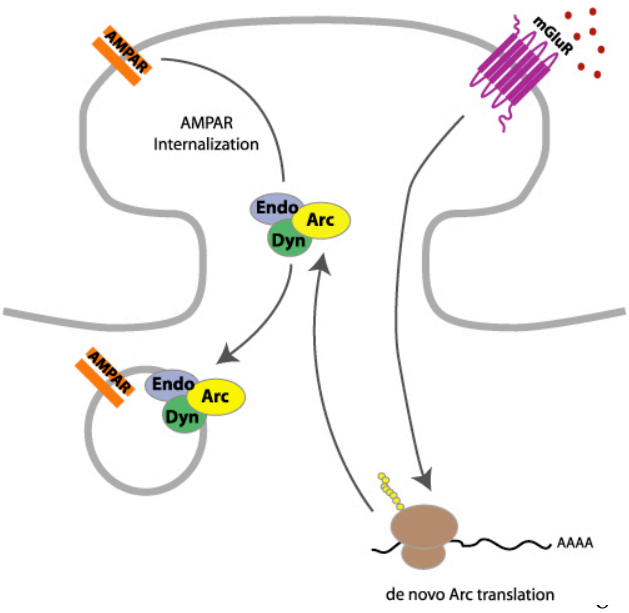

Figure 1.2: Arc mediated AMPAR endocytosis: Through its interaction with Endophilin3 and Dynamin2, Arc mediates endocytosis of AMPARs. mGluR-LTD leads to rapid de novo synthesis of Arc protein and subsequent AMPAR internalization. Arc-mediated endocytosis of AMPARs is required for mGluR-LTD as well as homeostatic plasticity. Figure adapted from Bramham et al. 2008[30]

Arc and RNA regulation

The rapid translation of Arc also indicates that a significant amount of untranslated Arc mRNA is sequestered in dendrites or spines under basal conditions. Recent work has established that degradation of Arc mRNA is regulated by non-sense mediated decay (NMD). NMD is an mRNA surveillance mechanism that identifies and degrades truncated mRNAs produced by nonsense and frameshift mutations or improper pre-mRNA splicing[31]. The pathway recognizes premature stop codons based on their upstream proximity to splice sites. Since most normal stop codons lie downstream of any splice sites, stop codons upstream of a splice site are usually aberrant. During mRNA splicing a complex of proteins called the exon-junction complex (EJC) is deposited at each splice site[32] (Figure 1.3). When the translational machinery reaches a stop codon, UPF1, a critical NMD protein, is recruited to the site by peptide-release factors[33]. Interaction of UPF1 with a downstream EJC leads to rapid degradation of the mRNA (Figure 1.4a).

However, the NMD pathway is not just for quality control purposes. It is now recognized that NMD regulates the expression of a number of normal transcripts and, in turn, could have major affects on cellular function. Normal transcripts with introns in the 3'UTR and functional isoforms created from alternative splicing can also have termination sites upstream of splice sites and thus will mimic a premature stop codon[34]. Arc is one such gene[23,35]. Arc's normal stop codon lies upstream of its two introns, both located in the 3'UTR.

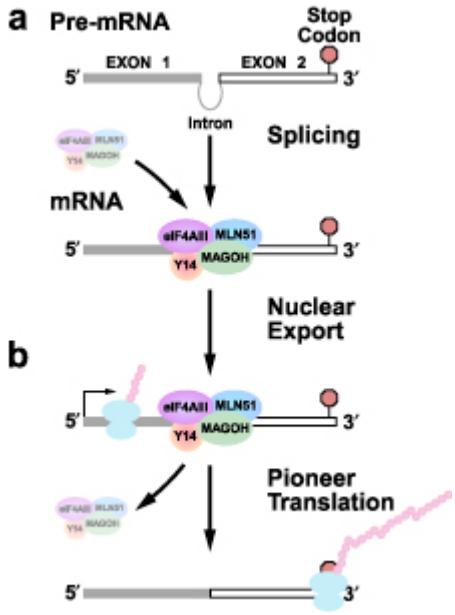

Figure 1.3: Exon-Junction Complex

(a) During pre-mRNA splicing the exon-junction complex (EJC) is deposited 20-24 nucleotides upstream of every splice junction. The EJC is composed of an RNA binding tetrameric core (eIF4AIII, MAGOH, MLN51, Y14) that travels with the mRNA as it is transported from the nucleus to the cytoplasm. Core components eIF4AIII, a DEAD box helicase, and MLN51 bind directly to the mRNA and stabilize the core complex. The EJC core complex serves as a binding platform for other transiently bound factors and can also function to recruit ribosomes for translation. (b) During the first ("pioneer") round of translation the EJC core complexes are displaced and bulk rounds of translation can proceed.

Giorgi et al. observed that NMD components are present in dendrites and colocalized with RNA granules and Arc mRNA[36]. Inhibition of NMD by RNAi-mediated knockdown of eiF4AIII or UPF1 in PC12 cells led to a twofold increase in Arc mRNA levels and a fourfold increase in protein levels. NMD could be a potent form of RNA degradation that results in immediate mRNA decay after a single round of translation. At 100% efficiency NMD could limit protein levels to the number of RNA molecules produced, facilitating extreme temporal and spatial restriction of the protein. It is also likely that Arc mRNA is protected from degradation prior to translation within the RNA granule.

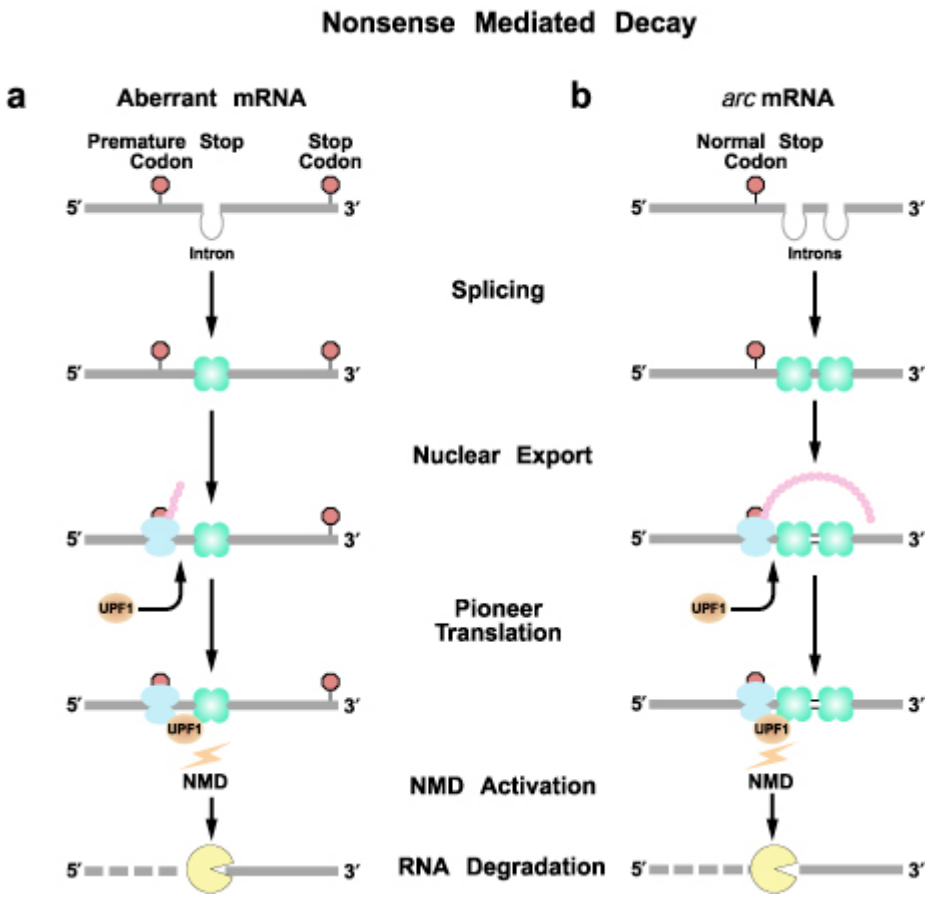

Figure 1.4: Nonsense Mediated Decay

(a) The nonsense mediated decay pathway is an RNA degradation pathway that detects premature stop codons and signals for subsequent degradation. As the majority of terminal stop codons are located within the final exon, detection of a premature stop codon is based on its location more than 50 nucleotides upstream of a splice site. When a ribosome reaches a stop codon, termination factors recruit UPF1 to the ribosome. If the stop codon is upstream of a splice site, UPF1 will interact with the bound EJC complex. This interaction leads to rapid mRNA degradation by exoribonucleases. (b) mRNAs with introns in their 3'UTR, such as arc, are natural targets for NMD. Arc's normal stop codon is located upstream of its two introns and NMD occurs after the full length protein is produced.

Arc, a marker of neuronal activity in response to behavior.

Arc's temporally tight association with neuronal activation has made it an ideal marker for neuronal activity in response to behavior. Many behavioral studies show that Arc is induced in various brain regions after information processing such as: novel environment

exposure[37], spatial learning paradigms[38] and fear conditioning[39]. The temporal induction and localization of Arc mRNA also led to the development of a gene-imaging approach called catFISH (cellular compartment analysis of temporal activity by fluorescence in situ hybridization)[40]. Because Arc mRNA is transported out into dendrites after induction (Figure 1.5), different neuronal populations induced at different times can be distinguished by identifying neurons with Arc in their nucleus or in their dendrites. Using catFISH, studies have demonstrated that Arc is upregulated in separate hippocampal neuronal ensembles in response to exposure to distinct environments – and thus may be a genetic read out of hippocampal place cell activation[37].

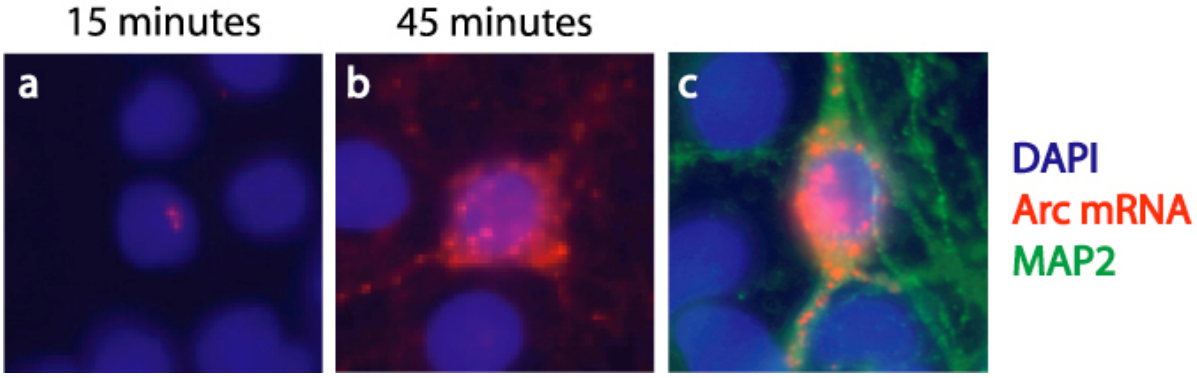

Figure 1.5: Temporal localization of Arc mRNA after BDNF stimulation
(a and b). catFISH detection of Arc mRNA 15 and 45 minutes after BDNF (50ng/µl) stimulation. Arc mRNA is first detected as two foci within the nucleus and then travels out to the cell body and dendrites. (c) 90 minutes after stimulation Arc mRNA is localized within MAP2 labeled dendrites.

Studying Arc induction and expression may also give insight into the function of different brain regions associated with learning and memory. For example, the temporal expression of Arc in the hippocampus is quite different between regions. Activated CA1 neurons express Arc up to 2 hours after novel environment exposure, while neurons in the dentate gyrus express Arc for over 8 hours. Also, CA3 and parietal neurons that express Arc

immediately after spatial exploration undergo a second wave of Arc induction 8-24hrs later, indicating that neurons in these regions are reactivated, perhaps facilitating memory consolidation[41].

The tight coupling of Arc transcription to neuronal activity has made the Arc promoter of high interest to the field. Using DNaseI hypersensitivity assays our lab has demonstrated that Arc has two activity-dependent enhancer regions ~6.5 kb and ~1.4kb upstream of the start codon. These regions contain a target for serum response factor as well as a "zeste-like" response element that may bind a novel transcription factor[42].

The Arc promoter has also been used to create live reporters of neuronal activity. Arc-d2EGFP knock-in mice have a GFP coding sequence inserted at the Arc locus, leading to GFP expression when Arc is induced. Researchers have used the Arc-positive GFP heterozygous mice to demonstrate that Arc is induced in vivo by visual stimuli. In this paradigm, GFP expression under the Arc promoter can be detected using two-photon imaging in live animals over multiple days after repeated stimulus presentations[43]. Wang et al. observed that repeated presentation of the same visual stimulus led to the reactivation of a smaller, but more reliable, populations of GFP positive neurons. Interestingly, the lack of Arc did not affect this adaptive response but did lead to a greater proportion of neurons with low orientation specificity and a broader spike-tuning curve.

Main findings of the dissertation

Arc is one of the mostly tightly regulated genes in the nervous system. Its transcription, translation, localization and degradation are all finely regulated and often linked to activity. The protein's function is clearly important for synaptic plasticity as it is

required for multiple forms of plasticity including LTP, LTD and homeostatic plasticity. Despite its common usage as a neuronal marker for activity, Arc's molecular function is still unclear.

In the experiments presented in Chapter 2, we demonstrate that Arc regulates spine morphology through endocytosis of AMPA receptors. We propose that by integrating AMPAR endocytosis with spine size, Arc balances downscaling of synapses with increased morphological plasticity. Furthermore, we find that loss of Arc *in vivo* leads to epileptic-like network hyperexcitability.

Given Arc's indirect association with the actin cytoskeleton and its involvement in regulating spine morphology, we hypothesized that Arc interacts with actin-binding proteins. The experiments presented in Chapter 3 demonstrate that Arc interacts with an actin-nucleating protein, Wave3. We find that Arc colocalizes with Wave3 in dendritic spines and that loss of Wave3 leads to dramatic reductions in both primary dendrite length and spine density.

Finally, Chapter 4 is a collection of odds and ends – interesting pieces of data, such as hits from an Arc yeast-two hybrid screen, that didn't fit well into publication form. Hopefully these will be useful to current and future members of the lab.

Chapter 2

Arc increases thin spines and maintains network stability in vivo

Abstract

Long-term memory relies on modulation of synaptic connections in response to experience. This plasticity involves trafficking of AMPA receptors and alteration of spine morphology. *Arc*, a gene induced by synaptic activity, mediates the endocytosis of AMPA receptors and is required for both long-term and homeostatic plasticity. We found Arc regulates spine morphology by decreasing spine width and increasing the proportion of thin "learning" spines. Arc specifically reduces surface GluR1 puncta at thin spines. Furthermore, Arc's effect on spine morphology depends on its interaction with endophilin3, a component of the endocytosis machinery, suggesting that Arc-mediated AMPA receptor endocytosis facilitates alterations in spine morphology. Thus, by linking spine morphology with AMPA receptor endocytosis, Arc balances synaptic downscaling with increased structural plasticity. Supporting this, loss of Arc *in vivo* leads to a significant decrease in the proportion of thin spines and an epileptic-like network hyperexcitability.

Introduction

Formation of long-term memory is based on a neuron's ability to modulate its synaptic connections in response to the input it receives. This plasticity requires coordinated activity-dependent synthesis of specific mRNAs and proteins that facilitate molecular and structural changes at the synapse. Excitatory synapses and their molecular components are located at dendritic spines that receive glutamatergic presynaptic inputs[44]. Spines exist in a variety of shapes and sizes that correlate with their synaptic strength, motility, and structural plasticity[45,46]. For example, thin spines are motile and likely to change shape in response to activity, while stubby and mushroom spines are less motile and more stable[45]. Furthermore, spine shape, motility, and turnover can be modulated by neuronal activity. Long-term potentiation (LTP) in the dentate gyrus (DG) leads to a decrease in stubby spines and an increase in plastic thin spines[47]. In the barrel cortex, novel sensory input leads to stabilization of new thin spines and destabilization of larger, more persistent spines[48,49].

While spines seem to be important in plasticity and synaptic efficacy, how a neuron regulates spine morphology in response to activity is still unclear. Spines are filled with actin, and remodeling the cytoskeleton is critical for spine shape plasticity. Recycling endosomes have also been implicated in regulating spine size[50], and exocytosis of GluR1 after LTP is required for maintaining spine enlargement[51]. Furthermore, long-term depression (LTD) leads to AMPA receptor (AMPAR) internalization and reductions in spine size and density[52-54], suggesting that activity-dependent exo- and endocytosis of receptors may be tightly linked to alterations in spine morphology.

Arc, activity-regulated cytoskeleton-associated protein, is an ideal candidate for regulating spine morphology in response to synaptic inputs. Its expression is tightly regulated by neuronal activity[42,55], and its RNA and protein are both localized to dendrites and spines after activity[15,16,56-58]. Furthermore, Arc induction is required for late-LTP and memory consolidation[23,24,35], as well as LTP-induced cofilin phosphorylation and F-actin stabilization[24]. Finally, Arc facilitates endocytosis of AMPARs through its interaction with endocytic proteins endophilin 3 and dynamin 2[20,21] and, in doing so, is critical for homeostatic plasticity[59] and LTD[25,26].

In this study, we investigated the role of Arc in regulating spine morphology. We report that Arc significantly decreases spine width, leading to an increase in the proportion of thin "plastic" spines. Furthermore, we find that Arc-mediated decreases in GluR1 surface expression are specific to thin spines, indicating that AMPAR endocytosis may facilitate the alterations in spine morphology. Indeed, an Arc mutant unable to interact with the endocytosis machinery had no effect on spine morphology. These findings suggest that Arc plays a role linking activity-dependent receptor endocytosis with spine morphology. This coordinate mechanism ultimately increases the potential for plasticity through addition of thin spines, while decreasing synaptic efficacy by reducing surface GluR1. Such changes in spine morphology fit well with the net affect of homeostatic plasticity: stabilization of activity while maintaining relative changes in synaptic strength. Interestingly, while overexpression of Arc in culture had no significant affect on spine density, analysis of hippocampal Golgi-Cox staining from *Arc*$^{-/-}$ mice revealed significant loss of spines in the dentate gyrus (DG) granule cells and CA1 layer pyramidal neurons. Given Arc's role in

homeostatic plasticity and downward scaling, we hypothesized that loss of Arc could lead to unrestrained network activity and subsequent spine loss. In support of this downstream remodeling, we find that mice lacking (even one copy of) Arc have alterations in neuropeptide Y (NPY) and calbindin expression, as well as aberrant spontaneous cortical network discharge activity that are highly associated with epileptic mouse models.

Results

Arc expression alters spine morphology to favor thin spines and filopodia

Arc mRNA is virtually undetectable under basal conditions, but it dramatically increases over 60-fold in a subset of neurons after appropriate synaptic activity[60]. It can remain upregulated for more than 8 hours in parts of the hippocampus[41]. To determine if Arc is sufficient to alter spine density and/or morphology, we sought to mimic the strong induction of Arc after activity by exogenously expressing it in mature (18–19 days in vitro (DIV)) primary hippocampal cultures. In this neuronal culture system, exogenously expressed Arc localizes to dendritic spines and colocalizes with actin enriched in spines (Figure 2.1a), similar to Arc *in vivo*[56,57]. Changes in spine morphology and density were visualized using co-transfected GFP as a morphology marker, and fixed neurons transfected with Arc or a control vector were imaged by confocal microscopy (Figure 2.1b).

Neurons overexpressing Arc showed no change in spine density (Figure 2.1c), but the spines were significantly thinner than those of neurons transfected with GFP alone (Figure 2.1d). No change in spine length was observed. To determine if Arc expression altered the distribution of spine type, we categorized spines into stubby, thin, mushroom and filopodia (see Methods) and calculated the percentage of each spine type per dendrite. We found that Arc overexpression increased the percentage of thin spines and filopodia and decreased the percentage of stubby spines (Figure 2.1e). The percentage of mushroom spines was unaffected.

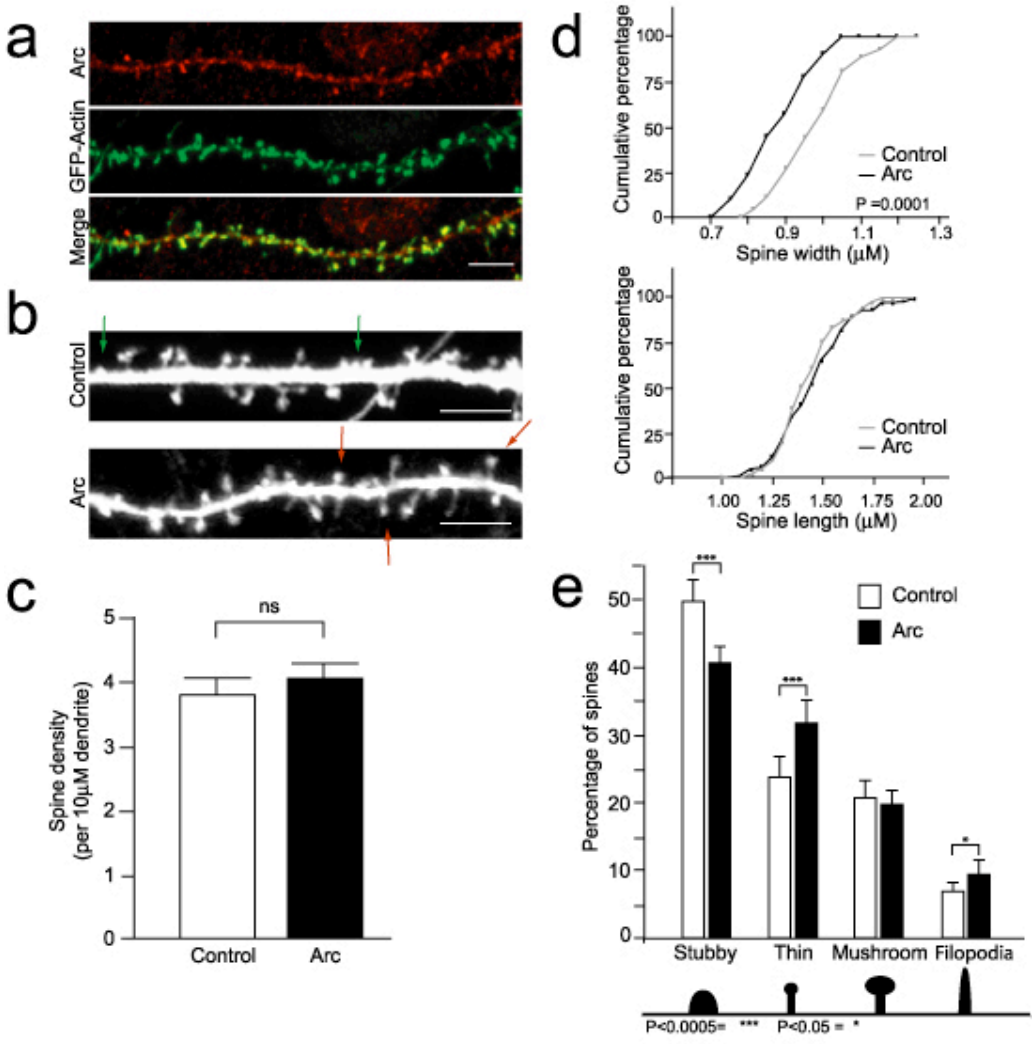

Figure 2.1: Arc expression increases spine density and alters spine morphology. (a) Arc (red) localizes to dendritic spines and colocalizes with GFP-Actin (green). Scale bar = 5µm. (b–e) Hippocampal neurons at 18–19DIV were transfected with GFP and Arc or an empty vector and imaged 36–48hr post transfection. Examples of thin and stubby spines are marked by red and green arrows respectively. Scale bars = 5µm (c) Arc expression does not affect spine density. (d) Cumulative frequency plots of spine width and length. Arc significantly decreases spine width but does not affect length. Komogorov-Smirnov test: $p<0.0001$. (e) Arc significantly increases the percentage of thin spines (red arrows in b) and filopodia, and decreases the percentage of stubby spines (green arrows in b). t-test ***$p<0.0005$, *$p<0.05$. Error bars represent 95% CI. Over 2500 spines from 64–65 dendrites over 12–18 neurons from three separate experiments were analyzed per condition. Measurements were averaged per dendrite.

Arc specifically alters Glur1 surface localization at thin spines.

Synaptic insertion of GluR1 and exocytosis of recycling endosomes is required for activity-induced spine enlargement[50,51]. Since Arc regulates AMPA receptor endocytosis[20,21], we hypothesized that Arc could mediate spine thinning through the endocytosis of AMPARs at specific spines. To test this, we utilized a mutant of Arc: Arc Δ91–100. Amino acids 91–100 of Arc interact with endophilin 3, a component of the clathrin-coated vesicle endocytosis machinery, and Arc Δ91–100 fails to induce AMPAR endocytosis[20]. If Arc-mediated AMPAR endocytosis is required for the alterations in spine morphology, overexpression of Arc Δ91–100 should have no affect on spine morphology. Indeed, unlike Arc, overexpression of Arc Δ91–100 had the same distribution of spine morphologies as control (Figure 2.2).

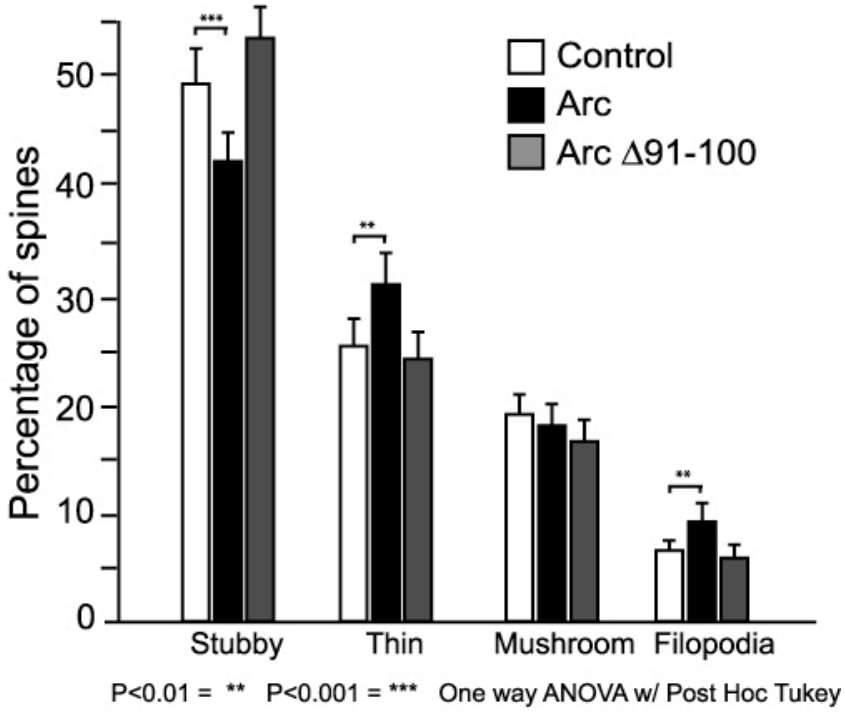

Figure 2.2: ArcΔ91–100 expression has no affect on spine morphology.
Over 1500 spines from 40 dendrites over three experiments per condition were analyzed. Error bars represent 95% CI.

If Arc links surface expression of AMPARs with spine morphology, we hypothesized that Arc expression would specifically reduce surface AMPARs at thin spines. To test this, we performed surface staining of GluR1 on cultured hippocampal neurons transfected with Arc or a control vector, and used GFP for spine visualization (Figure 2.3a). Each spine was

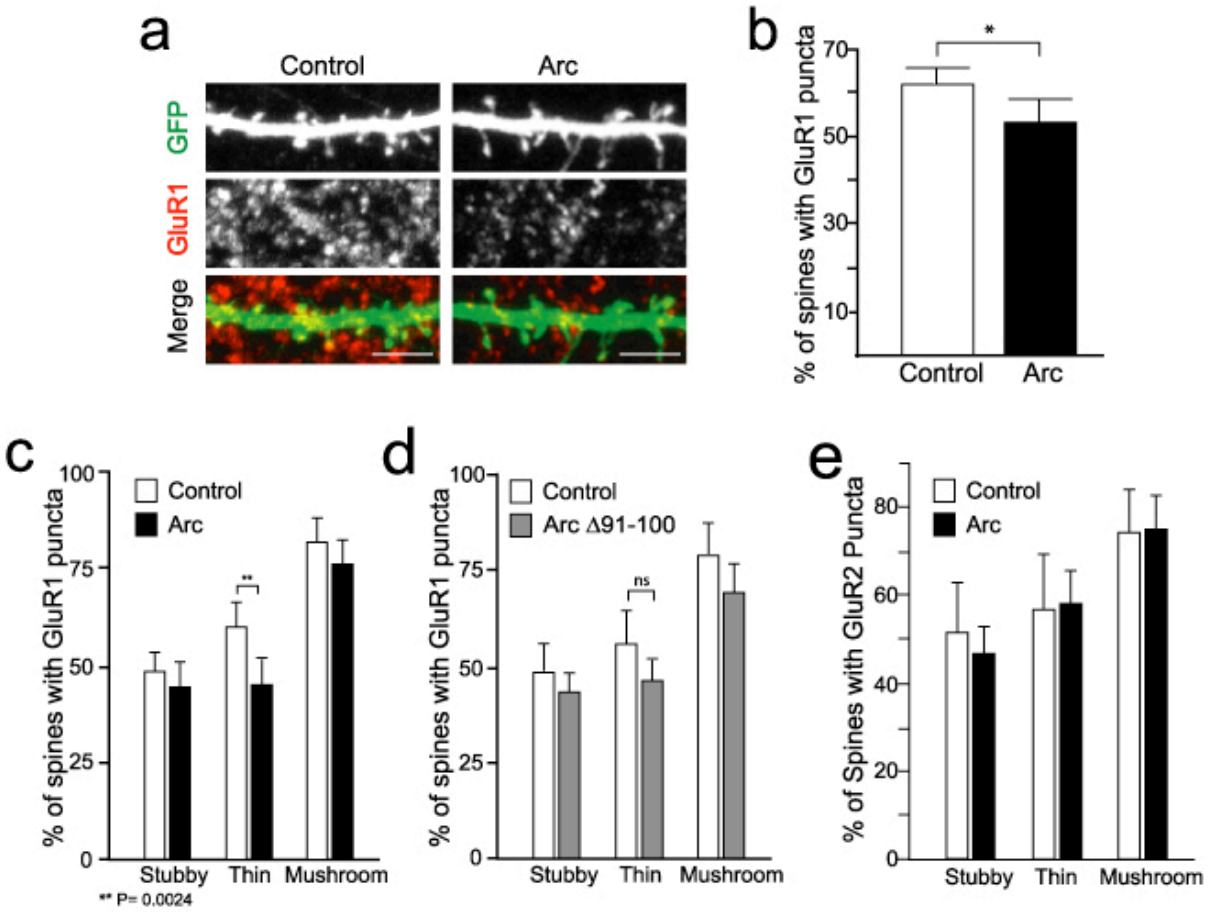

Figure 2.3: Arc-mediated GluR1 endocytosis is required for alterations in spine morphology.

(a) Hippocampal neurons at 18–19DIV were transfected with GFP and Arc or an empty vector. 36–48h post transfection, neurons were incubated with GluR1 N-terminal antibody for 45 minutes to stain surface GluR1. Scale bar = 5μm. (b) Arc expression decreases the percentage of spines with surface GluR1. (c) Surface GluR1 is specifically decreased in thin spines. (d) ArcΔ91–100 expression does not reduce GluR1 surface expression at thin spines. (e) Arc expression did not alter surface GluR2. For AMPAR staining, over 3500 spines from 26-27 cells per condition over five experiments were analyzed. Error bars represent 95% CI.

analyzed for colocalization with GluR1 puncta and classified morphologically. Arc overexpression reduced the percentage of spines with surface GluR1 puncta (Figure 2.3b), and this reduction in GluR1 puncta was specific to thin spines (Figure 2.3c). By contrast, a version of Arc incapable of interacting with endophilin 3 (ArcΔ91-100) did not alter GluR1 surface expression at thin spines (Figure 2.3d). Surface staining of GluR2 was not reduced in any spine type (Figure 2.3e). Such specificity for thin spines could be explained by either lack of surface AMPARs in newly formed thin spines or by endocytosis of AMPARs from larger spines leading to reduced spine size.

To further confirm that Arc-mediated increases in thin spines act through AMPAR endocytosis, we tested a second Arc deletion construct (Arc Δ195-214) that does not bind dynamin and also fails to mediate AMPAR endocytosis. Overexpression of Arc Δ195-214

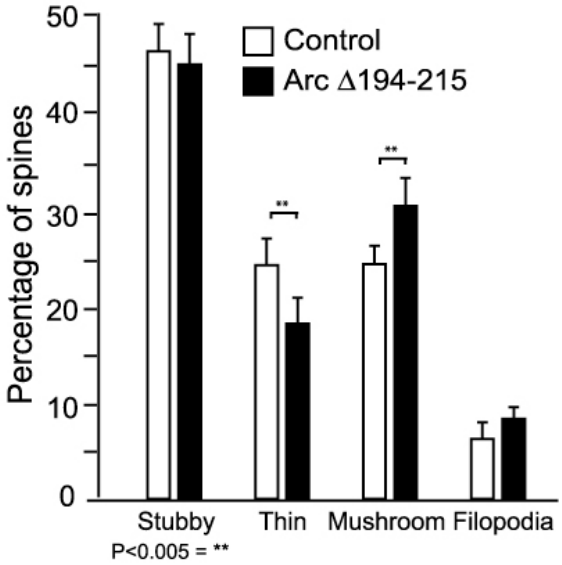

Figure 2.4: Loss of Arc-Dynamin binding decreases thin spines
Expression of Arc Δ194-215 that does not bind dynamin 2 decreased the percentage of thin spines and increased the percentage of mushroom spines compared to control. Over 2000 spines from 45 dendrites over two experiments per condition were analyzed. Error bars represent 95% CI.

significantly decreased the percentage of thin spines and increased the percentage of mushroom spines compared to control (Figure 2.4), suggesting this mutant acts as a dominant negative by blocking the effects of endogenous Arc in maintaining thin spines. Together, these data support a role for Arc in regulating spine morphology through AMPAR endocytosis.

Arc decreases synapse density

To determine if Arc also alters synapse number, we stained transfected neuronal cultures with anti-synapsin I antibody (Figure 2.5a). Confocal images were acquired, and spines were analyzed for length, width and colocalization with synapsin I puncta. While Arc expression did not affect spine density (Figure 2.1c), it did decrease synapsin I density (Figure 2.5b). These data suggest that (1) the majority of new thin spines are not synaptic, and (2) some old spines have lost synaptic contacts.

To further determine if specific spine types were more prone to loss of synaptic contacts, we specifically analyzed the distribution of synapsin I labeled puncta with respect to spine type. We found no significant change in the density of thin spine synapses (Figure 2.5c) despite an increase in their spine type (Figure 2.1e), suggesting that new thin spines are not yet synaptic. Synapsin I density on stubby spines did significantly decrease (Figure 2.5c). To determine if this decrease was simply due to decreased stubby spine density or a selective loss of synapses on stubby spines, we calculated the percentage of stubby spines that showed synapsin I staining (Figure 2.5d). Indeed, the percentage of stubby spines with synapsin I puncta was also significantly lower, indicating that Arc overexpression leads to a specific loss of synaptic contacts on stubby spines.

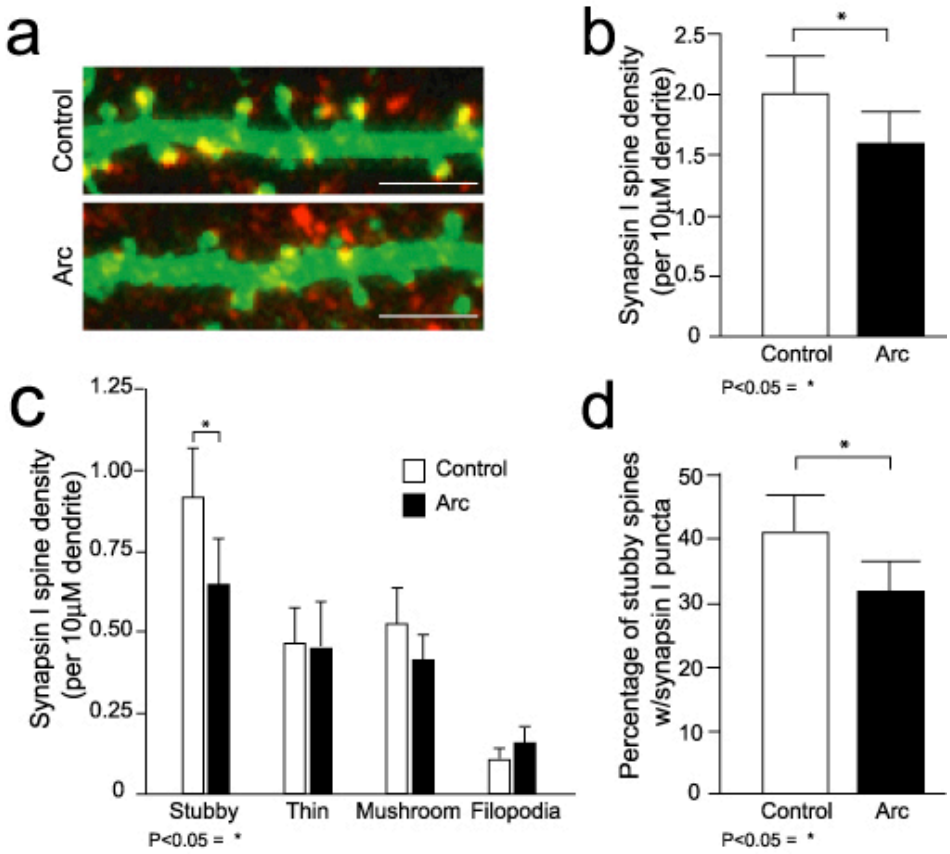

Figure 2.5: Arc expression decreases synapse number

(a) Hippocampal neurons at 18DIV transfected with GFP (green) and Arc or an empty vector were fixed and stained for the presynaptic marker synapsin I (red). Scale bars = 5 μm (b) Arc expression significantly reduces spine-associated synapsin I density. (c) Reduction of synapsin I is specific to stubby spines. (d) The percentage of stubby spines associated with synapsin I puncta is also significantly decreased. Over 2200 spines from 35 dendrites over three experiments per condition were analyzed. Error bars represent 95% CI.

Arc$^{-/-}$ mice have increased spine width and decreased spine density

Given Arc's affect on spine morphology and homeostatic plasticity in culture, we next wondered if loss of Arc *in vivo* would lead to alterations in spine morphology. To test this, we performed Golgi-Cox staining on brains from adult mice lacking Arc[43] (Figure 2.6a). Morphometric analysis confirmed that both CA1 pyramidal neurons and DG cells lacking

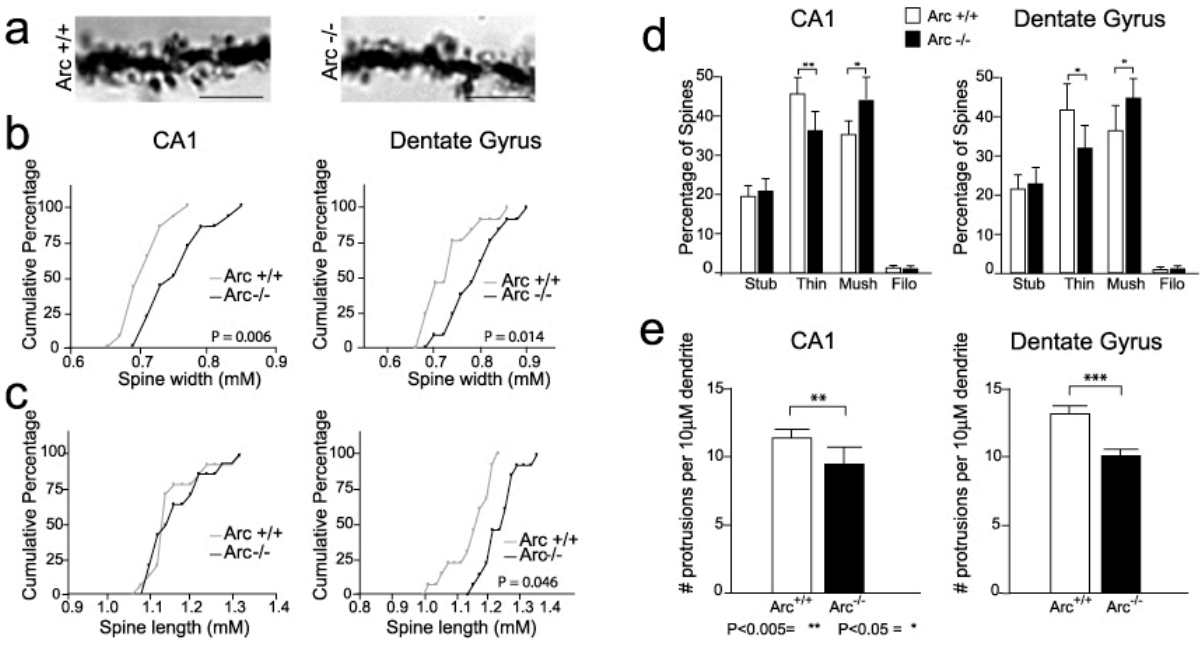

Figure 2.6: *Arc* $^{-/-}$ mice have decreased spine density and increased spine width
(a) Typical example of dentate granule cell Golgi staining from 3 month old *Arc* $^{+/+}$ and *Arc* $^{-/-}$ mice. Scale bars = 5 µm (b) Loss of Arc significantly decreases spine density in CA1 pyramidal cells of the hippocampus. (c and d) Cumulative frequency plots of spine width and length. *Arc*$^{-/-}$ mice have increased spine width and no change in length. P values determined using Komogorov-Smirnov test. (e) Loss of Arc *in vivo* decreases the percentage of thin spines and increases the percentage of mushroom spines. 14 dendrites from three animals per genotype were analyzed. Error bars represent 95% CI.

Arc had increased spine width, leading to a decrease in the proportion of thin spines (Figure 2.6b,d). These data are consistent with our primary culture data, in which Arc overexpression leads to an increase in thin spines, and suggest that Arc plays a major role in homeostatic control of spine morphology. However, loss of Arc *in vivo* did not alter the proportion of stubby spines, as was observed following overexpression of Arc, but rather increased the percentage of mushroom spines (Figure 2.6d). This discrepancy could reflect the different spine distribution patterns observed *in vivo* and in cultured hippocampal neurons, or differences in neuronal activity between the two systems. Hippocampal neurons *in vivo* displayed more mushroom spines, and fewer stubby spines than in culture (Figure 2.1e and

Figure 2.6d). Also, while *Arc*$^{-/-}$ CA1 neurons exhibited no change in spine length, DG cells did show increased spine length (Figure 2.6c).

Surprisingly, Arc$^{-/-}$ mice also had significantly lower spine densities in both CA1 and DG cells (Figure 2.6e). Since Arc expression did not alter spine density in culture, the decrease observed *in vivo* may reflect an important difference between these two systems. For example, neurons *in vivo* have higher synaptic densities and therefore might need to adapt more strongly to mitigate the possibility of overexcitation as a result of too little Arc and inadequate scaling. We also cannot exclude a possible compensatory change in spine density during development due to loss of Arc since birth.

Arc$^{-/-}$ mice exhibit aberrant NPY expression consistent with network hyperexcitability.

Arc is thought to mediate homeostatic plasticity through endocytosis of AMPARs. Specifically, Arc induction after strong bouts of synaptic activity facilitates downward scaling of synapses by reducing surface GluR1[59]. Our data support this model by demonstrating that Arc expression reduces the number of stubby synapses and the size of dendritic spines. Homeostatic plasticity is believed to be important for regulating network activity in response to excessive neuronal discharge, such as a seizure. Loss of such a negative feedback loop could lead to an epileptic-like state and associated spine loss[61].

To examine this hypothesis, we asked if mice lacking Arc were more susceptible to convulsant drugs. Mice were given an intraperitoneal injection of either kainate or saline to induce seizure activity and chronic epilepsy. In both human and animal models of chronic hippocampal epilepsy, neuropeptide Y (NPY) expression is transiently increased and

ectopically expressed in mossy fibers, the axons of DG cells[62,63]. To our surprise, both kainate- and saline-injected *Arc*$^{-/-}$ animals showed aberrant expression of NPY (Figure 2.7a), suggesting that hippocampal networks in *Arc*$^{-/-}$ mice are hyperexcitable even under

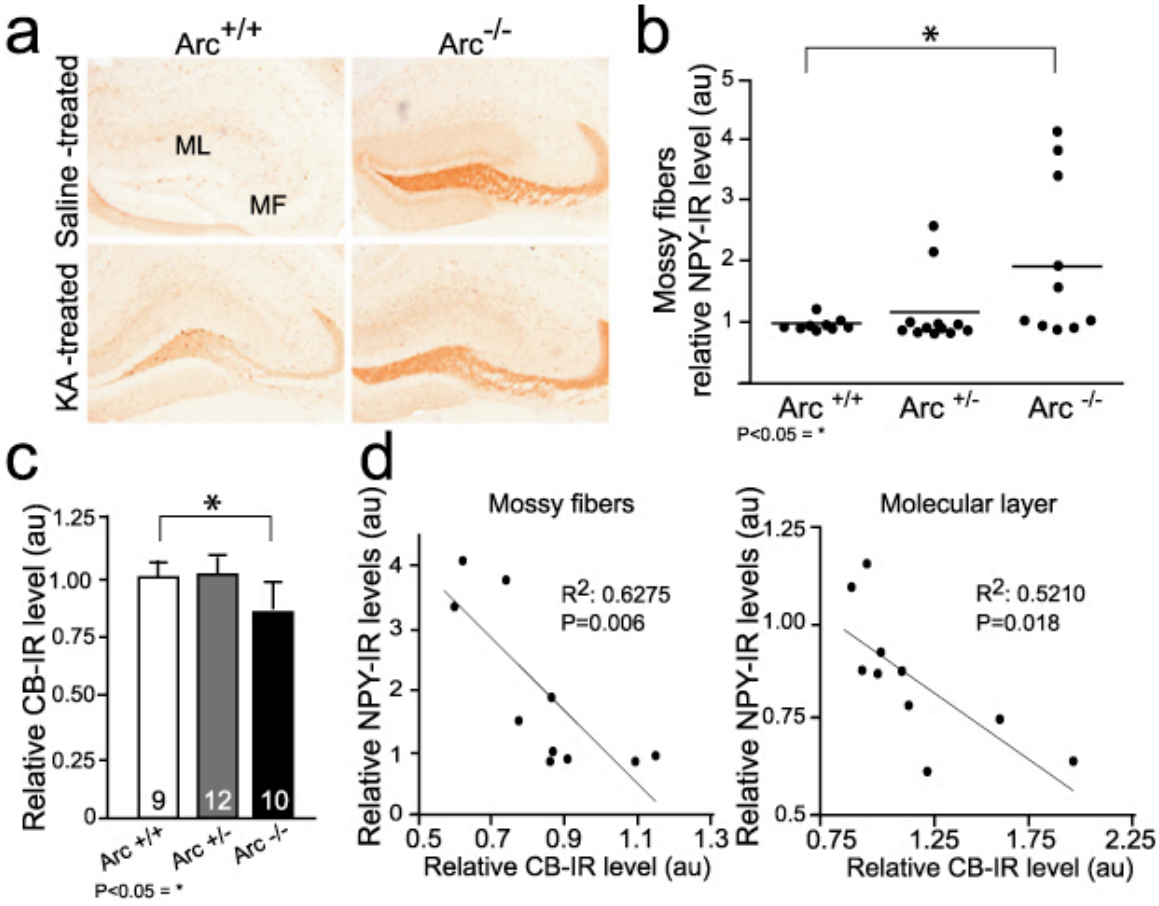

Figure 2.7: Alterations in NPY and Calbindin in the dentate gyrus of *Arc*$^{-/-}$ mice. (a) 4–6 month old mice were injected intraperitoneally with saline or kainate (KA) (17mg/kg) and analyzed 5 days later. Brain sections were immunoperoxidase-stained for NPY. Wildtype *Arc*$^{+/+}$ and *Arc*$^{-/-}$ mice injected with KA exhibit expected aberrant NPY expression in mossy fibers. Some saline treated *Arc*$^{-/-}$ mice also exhibited increased NPY expression in the DG molecular layer (ML) and mossy fibers (MF). (b) Quantification of mossy fiber NPY levels in untreated animals. au = arbitrary units. (c) *Arc*$^{-/-}$ mice show decreased levels of calbindin (CB) in the dentate gyrus molecular layer. (d) Level of calbindin depletion correlates with NPY increases in the mossy fibers and molecular layer in *Arc*$^{-/-}$ mice. For panels b and c, P values were determined by one-way ANOVA followed by post-hoc Tukey tests.

normal conditions. Five of 10 *Arc*$^{-/-}$ animals showed aberrant NPY expression, presumably due to intermittent seizures, and interestingly two of 12 *Arc* $^{+/-}$ mice also showed aberrant NPY expression (Figure 2.7b). Similarly, epileptic activity in human and rodent hippocampus has also been associated with alterations in calcium homeostasis and the proteins that buffer calcium. Levels of calbindin-D28K, a major calcium binding protein in the brain, are lower in the hippocampus of epileptic rats[64] and humans[65,66]. *Arc*$^{-/-}$ mice also show lower dentate gyrus calbindin levels than controls (Figure 2.7c). Furthermore, levels of calbindin negatively correlate with levels of NPY in the mossy fibers and molecular layer of the DG (Figure 2.7d). These data suggest that loss of Arc expression *in vivo* leads to significant alterations in protein expression indicative of recurrent seizure activity.

To determine whether *Arc*$^{-/-}$ mice display aberrant neuronal activity, we performed prolonged monitoring of 9 adult (aged 7-8 months) animals (5 *Arc*$^{-/-}$, 2 *Arc*$^{+/-}$, and 2 *Arc*$^{+/+}$ mice) over a 1 month period with a digital video-EEG system. The background cortical

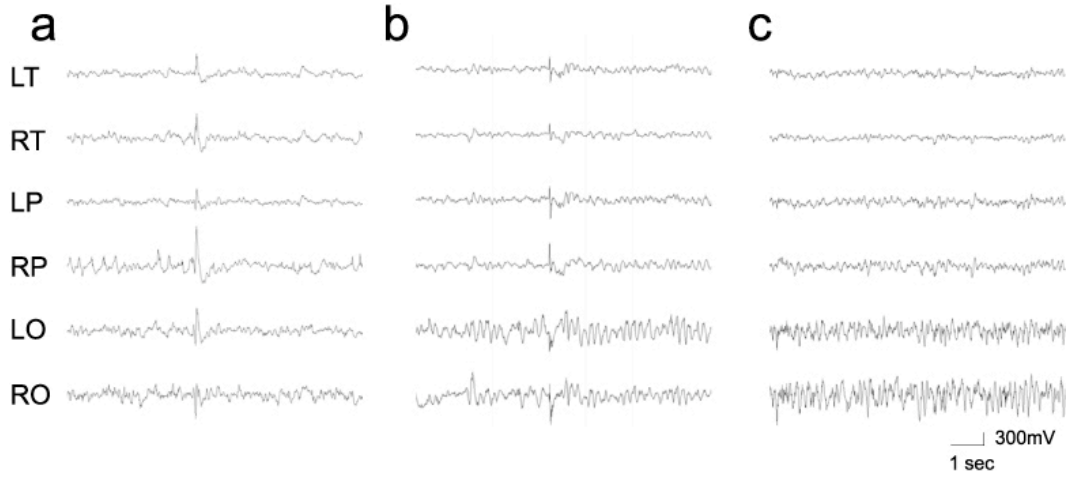

Figure 2.8: Chronic EEG recordings reveal frequent generalized cortical interictal spike discharges in *Arc*$^{-/-}$ mice

Example EEG recordings from (a) *Arc*$^{-/-}$, (b) *Arc*$^{+/-}$ and (c) wt mice. Calibration 1 sec, 300mV, electrode montage LT: left temporal; RT: right temporal; LP: left parietal; RP: right parietal; LO: left occipital; RO: right occipital.

activity recorded in freely behaving *Arc*$^{+/-}$ and *Arc*$^{-/-}$ showed a frequent generalized pattern of (0-25/hour) sharp, synchronous epileptiform discharges in all cortical electrodes with no concurrent behavioral manifestation (Figure 2.8a,b) that were never seen in wildtype littermates (Figure 2.8c**)**. Despite the abundant abnormal cortical hyperexcitability no spontaneous cortical seizures were witnessed during the recording period.

Dose-dependent effect of Arc on long-term spatial memory

The observed alterations of NPY and calbindin expression in a portion of the heterozygous as well as homozygous mice was unexpected and indicate that the amount of Arc expression is important for homeostatic regulation of network activity. We next wondered whether the Arc dose was also important for long-term memory. In Arc knockout mice, loss of Arc affects spatial memory in the Morris water maze[23], however behavioral testing of heterozygous mice has not been reported. To determine if *Arc*$^{+/-}$ mice have deficits in spatial learning, we compared *Arc*$^{+/-}$ and *Arc*$^{-/-}$ mice to wildtype controls in the Morris water maze. Animals were tested over 5 days (two sessions per day) for their ability to locate a submerged platform in a pool of opaque liquid. The swim distance for each trial was determined as a measure of their spatial memory. While all three groups showed an overall decline in swim distance during acquisition, indicating that learning was occurring, *Arc*$^{-/-}$ and *Arc*$^{+/-}$ mice showed significantly longer swim paths compared to wildtype (Figure 2.9a,b). There was also clear gene dose-dependence in that *Arc* $^{+/-}$ mice performed better than *Arc*$^{-/-}$ but worse than wildtype mice. Interestingly, after the second day, the *Arc*$^{-/-}$ mice showed a marked decrease in performance the first session of each day, but were able to improve by the following session 2 hours later,

indicating that their long-term, but not short-term, memory is specifically impaired. After 5 days of training, mice were given two probe tests, 24 and 72 hours after the 10th session. In these tests, the platform was removed and the mice were given 1 min to explore the pool. At the 72 hour probe test the *Arc*$^{-/-}$ mice showed no significant preference for the location of the trained platform, indicating long-term spatial memory deficits (Figure 2.9c). While *Arc*$^{+/-}$ mice demonstrated a preference for the trained platform, their preference was again weaker

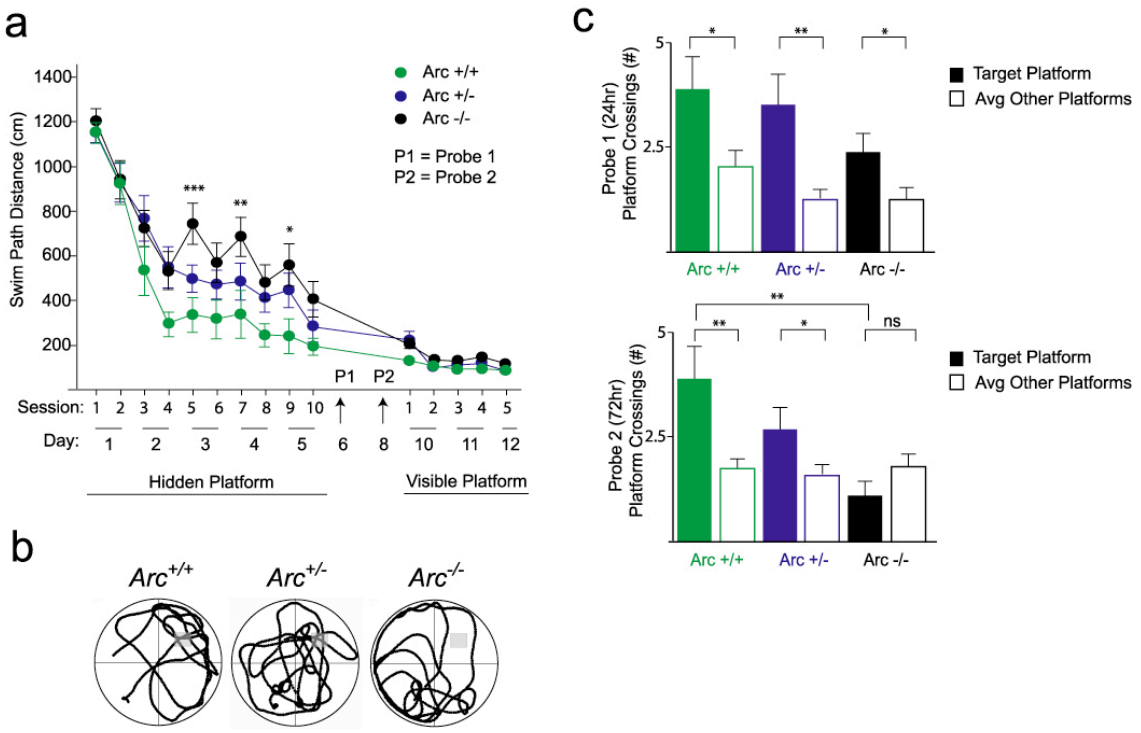

Figure 2.9: Gene dose-dependent water maze deficits in *Arc*$^{+/-}$ and *Arc*$^{-/-}$ mice

(n = 8-12 male mice/genotype, 5-7 months of age). (a) Hidden platform learning curves differed by genotype (repeated-measures ANOVA: $p<0.0001$). In Tukey-Kramer post hoc comparisons, *Arc*$^{+/+}$ differed from *Arc*$^{+/-}$ and *Arc*$^{-/-}$ mice ($p<0.01$ and $p<0.001$ respectively). In 2-way ANOVA comparisons of individual sessions *Arc*$^{+/+}$ differed from *Arc*$^{-/-}$ in Sessions 5, 7, and 9 (***$p<0.001$, **$p<0.01$, *$p<0.05$). No differences in swim path were observed when the platform was visible. (b and c) Probe trial 24 and 72 hours after hidden platform sessions. (c) Representative path tracings of 72h probe trials. (d) Number of target platform crossings differed by genotype at the 72h probe trial (One-way ANOVA of target crossings for *Arc*$^{+/+}$and *Arc*$^{-/-}$ $p<0.01$). All genotypes except *Arc*$^{-/-}$ exhibited preference for the target location at the 72h probe trial (**$p<0.01$, *$p<0.05$).

than wildtype controls. No differences in swim path were observed when the platform was visible and swim velocities were identical between genotypes (data not shown), indicating normal vision and sensory-motor function.

Discussion

Here we report that Arc plays an important role in regulating dendritic spine morphology. Furthermore, our data suggest that Arc, a protein whose expression and localization is highly associated with synaptic activity, links structural and functional plasticity by altering spine morphology through AMPAR endocytosis. Arc expression critically balances reduction in synaptic strength[21] with increased structural plasticity by increasing the proportion of thin spines. Consistent with this dynamic regulation, we show that loss of Arc *in vivo* leads to aberrant network hyperexcitability, further supporting Arc's role in homeostatic plasticity and demonstrating the importance of homeostatic plasticity in preventing aberrant network signaling patterns such as epilepsy.

Arc modulates spine morphology

Dendritic spine size is a critical determinant of activity-dependent plasticity. Specifically, thinner spines have been nicknamed "learning spines." They are more motile, more transient, and have greater capacity to enlarge and stabilize after LTP. Large spines, nicknamed "memory spines," are stable and are less likely to change structure in response to activity[49,67,68]. By increasing the proportion of these learning spines, Arc expression may enhance the ability of the neuron to form new synaptic connections and respond to changes in activity. Recent studies in slices and *in vivo* demonstrate that synaptic activity leads to selective spine turnover by stabilizing active spines and replacing inactive spines with new ones[69]. Similar to what we have observed with Arc overexpression, these new spines tend to be thin, suggesting that Arc expression after activity may facilitate this selective turnover.

While synaptic activity does not affect Arc-mediated AMPAR endocytosis[21], activity could regulate the location of Arc's affects. Thus, future investigation of Arc-mediated changes in spine morphology in the presence or absence of synaptic activity would be useful.

Synaptic activity has also been shown to affect synapses. LTP through the DG perforant path decreases the number of stubby spine synapses[47] but not mushroom spine synapses. Interestingly, we find that Arc expression also specifically reduces stubby spine synapses.

We show that Arc-mediated alterations in spine morphology depend on Arc's ability to endocytose AMPARs through its interaction with endophilin 3 and dynamin 2. Surface expression of AMPARs is tightly linked to spine size; large spines contain many AMPARs and thin spines contain few AMPARs. Local exocytosis of recycling endosomes has been implicated as a source for activity-dependent spine enlargement[50], and insertion of GluR1 into synapses is required for stable spine enlargement after LTP[51]. We found that Arc-mediated endocytosis of GluR1 is required for decreasing spine head size, indicating that endosomal recycling of receptors can bidirectionally affect spine morphology and synaptic strength. Furthermore, it highlights Arc as a co-regulator of spine morphology and synaptic transmission. While deletion of Arc's endophilin binding domain (Arc Δ91–100) blocked Arc mediated increases in thin spines, deletion of the dynamin binding domain (Arc Δ195-214) further decreased thin spines and increased mushroom spines compared to control. In the Arc-endophilin-dynamin compex, dynamin is thought to interact with endophilin's SH3 domain[20]. One possible explanation for the dominant-negative affects of Arc Δ195-214 is that removal of dynamin frees the endophilin SH3 domain to bind other proteins. One intriguing endophilin SH3 binding partner is the actin-nucleating factor N-WASP which could enlarge spines through Arp2/3 activation.

Arc has been shown to reduce surface expression of both GluR1 and GluR2[20,21]. However in our system reduction of GluR2 surface expression was not specific to thin spines, suggesting that Arc-mediated endocytosis of GluR1, not GluR2 regulates spine morphology, at least in culture

Arc and synaptic plasticity

Arc has been implicated in LTD and LTP plasticity paradigms as well as homeostatic plasticity[23,25,26,59]. How Arc facilitates such opposing forms of plasticity has largely remained unanswered. Our data support Arc's role in LTD through endocytosis of AMPARs and reduction of spine size. However, it is still unclear how Arc facilitates LTP maintenance. Later phases of LTP are associated with new spine formation[69]. Messaoudi et al.[24] showed that knockdown of Arc 2 hours after high-frequency stimulation blocks LTP induced F-actin polymerization and induces dephosphorylation of hyperphosphorylated cofilin, a regulator of actin polymerization, indicating that Arc helps facilitate actin reorganization at spines. While we did not observe an effect of Arc on spine density in culture, decreases in spine density were clearly observed in specific neuron types of *Arc*$^{-/-}$ mice, suggesting that Arc may play a role in spine formation. However, it is difficult to determine if the changes in spine density *in vivo* were the result of cell autonomous loss of Arc or of intermittent seizure activity. Lower spine densities have been observed in both status epilepticus and multiple models of epilepsy[70-72]. Interestingly, dephosphorylation of cofilin by calcineurin has also been linked to loss of spines from seizures[73]. The lack of Arc during seizure activity could further promote dephosphorylation of cofilin and subsequent spine loss.

Our observation of decreased spine density in *Arc*$^{-/-}$ mice conflicts with a previous study by Plath et al.[23] which reported no change in spine density due to Arc loss. In our study, spines were imaged after Golgi staining of whole brains, while Plath *et al.* imaged 3 biocytin injected CA1 pyramidal neurons from knockout hippocampal slices. Since spine density can rapidly change during slicing and is strongly dependent on temperature[74], these methodological differences might explain the apparent discrepancy.

Arc's role in homeostatic plasticity may also be critical for LTP expression and memory consolidation. Arc-mediated changes in spine morphology and receptor content could act to prevent saturation of LTP. In support of this, unrestrained epileptiform activity can prevent LTP expression[75] and interfere with memory consolidation[76-78]. However, transient reduction of Arc in wildtype animals via antisense oligonucleotide injections into the hippocampus blocks late-LTP and memory consolidation. This indicates that the deficits in late-LTP and memory consolidation observed in the *Arc*$^{-/-}$ mice are likely directly due to loss of Arc rather than compensatory network alterations occurring over time.

Is Arc-mediated homeostasis antiepileptogenic?

Seizure activity is characterized by highly synchronized and high-frequency activation of neurons caused by the imbalance of excitatory and inhibitory circuits. These abnormally high levels of activity often result in long-lasting synaptic changes and excitotoxicity that may lead to increased hyperexcitability of the system and development of the recurrent seizure activity that defines epilepsy. Seizures alter the expression of a number of genes whose downstream products likely change neuronal function and synaptic efficacy. In fact, *Arc* was originally discovered as a gene highly induced in response to seizure induction[15]. Genes

upregulated after seizure may also act in a negative feedback loop, preventing further activity and inhibiting the formation of epileptogenesis.

We found that genetic disruption of Arc expression leads to histological alterations observed in epileptic models. We suggest that loss of Arc leads to strengthening of synaptic contacts without restraint, leading to network hyperexcitability and ultimately epilepsy. However, only 50% of *Arc*$^{-/-}$ mice in our study exhibited the molecular alterations characteristic of severe epilepsy. The likely explanation for this is that seizures are infrequent in the mutant mice. In fact, despite the presence of relatively abundant hypersynchronous discharge activity, we were unable to witness any spontaneous seizures during the prolonged monitoring period.

Intriguingly, some mice with only one copy of Arc also develop aberrant NPY expression. This observation suggests that Arc related genetic variants may produce dominant excitability phenotypes. Furthermore, less than a 50% knockdown of Arc by antisense oligonucleotides is sufficient to block LTP maintenance *in vivo*[24], and we found that Morris water maze testing demonstrated a clear gene dose-dependent affect of Arc on long-term spatial memory. Such sensitivity to Arc levels emphasizes the importance of Arc in plasticity and suggests that its function cannot be easily compensated by related proteins.

Recent work in Alzheimer's mouse models shows that hAPP-J20 mice also have aberrant excitatory neuronal activity and exhibit similar alterations in NPY and calbindin expression[79]. Furthermore, hAPP-J20 mice display significantly lower levels of Arc in the DG than non-transgenics[80], and decreased levels of immediate early genes, such as c-fos and Arc, are tightly coupled to cognitive deficits[81]. In these mice, levels of Arc also correlate extremely well with NPY alterations, calbindin expression, and seizure severity[79].

Such correlations raise the provocative possibility that decreased basal Arc expression in hAPP-J20 mice plays a critical role mediating their epileptic activity and memory deficits. Like Arc$^{-/-}$ mice, Alzheimer's mouse models have decreased spine density, impaired LTP and LTD[31,82], and memory deficits.

In conclusion, we show that Arc is critical in regulating spine and synapse morphology. By integrating AMPAR endocytosis with spine size, Arc balances the downscaling of synapses with increased morphological plasticity. We suggest that this dual role allows Arc to facilitate both homeostatic and Hebbian plasticity. Understanding how hypersynchronous network activity affects Hebbian plasticity and memory consolidation in *Arc*$^{-/-}$ mice should give additional insight into the relationship between homeostatic scaling, LTP and LTD.

Methods

Mice

We studied 4-9 month old Arc-d2EGFP knockin mice[43] (C57/BL6 strain) which contain d2EGFP followed by a Neo cassette inserted right after the ATG start codon in the Arc gene.

Plasmids.

GW1-full length *Arc* was constructed as follows: rArc coding sequence was amplified from pBSKII(+) rArc[15] and the 5'UTR and 3'UTRs were PCR amplified from oligo-dT-primed rat cDNA. Arc deletion construct ArcΔ91–100 and ArcΔ195-214 were made using a QuickChange Site-Directed Mutagenesis Kit (Stratagene).

Cell culture and transfection

E20–21 rat hippocampi were dissected and treated with papain (10 units ml^{-1}, 15 min; Worthington Biochemical) and then with a trypsin inhibitor (10 mg ml^{-1}, 15 min; Sigma). After trituration, dissociated hippocampal neurons were plated on coverslips (2 x 10^5 cells/coverslip) coated with laminin (BD Biosciences) and poly-D-lysine (Chemicon). After 1 h plating, neurons were transferred into neurobasal media with B27 (Gibco). Primary hippocampal cultures at 18–19DIV were co-transfected with GW1-Arc and GW1-GFP with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions (1.5:1 molar ratio, 1.5μg of total DNA and 2μl of lipid per 1.9 cm^2 well).

Immunostaining

For surface AMPAR staining, transfected neurons were incubated with N-terminal GluR1 antibody (rabbit, Calbiochem; 1:40) or N-terminal GluR2 antibody (mouse, Chemicon;

1:300) for 45 min and then fixed with 4% PFA/4% sucrose. Coverslips were then blocked in PBS with 3% donkey serum, 3% BSA for 1h and then incubated with fluorescent secondary antibody (Alexa donkey 647, Invitrogen, 1:200) for 1 hour. For synapsin I (Chemicon; 1:3000) staining, neurons were fixed and permeabilized in 0.1% Triton X-100 in PBS for 15 min. Cells were then blocked as above, and incubated with primary antibody overnight at 4^{o}C, followed by secondary antibody incubation and mounting.

Golgi-Cox staining

To examine hippocampal morphology in Arc wildtype and mutant mice, brains from 3.5 month animals were impregnated in Golgi solution using the FD Rapid Golgi staining kit (FD Neurotechnologies, Baltimore, MD) according to the manufacturers instructions. Coronal sections of 100 μm were made on a cryostat and spine morphology was examined after staining.

Microscopy and image analysis

Images of primary hippocampal cultures were acquired with a LSM510 confocal microscope system (Zeiss, Oberkochen, Germany) and a 63× oil immersion lens (1024 × 1024 pixels). Each image consisted of a stack of images taken through the *z*-plane of the cell. Confocal microscope settings were kept the same for all scans in each experiment. Healthy, pyramidal-like neurons were chosen randomly for quantification from three coverslips from 3–4 independent experiments for each construct. For spine size, the maximal length and head width were measured manually using Metamorph (Universal Imaging). Each spine was categorized as having or not having a neck. Spines with necks were separated into thin and mushroom spines based on head width. Spines with heads less than the average width (1 μm

for GFP images, 0.75 μm for Golgi staining) were categorized as thin, and those with heads greater than the average width were categorized as mushroom. Filopodia were protrusions greater than 1.5 μm in length without a neck. The investigator was "blind" to experimental condition during both image acquisition and morphometric analysis. Golgi stained sections were imaged under brightfield using a 60× oil immersion objective on a Nikon Eclipse TE2000-E microscope. Z stacks were collected, and spine length and width were measured as above using Metamorph.

Immunohistochemistry

Tissue preparation and immunohistochemisty were performed as described[79,80] . Primary antibodies used included rabbit anti-neuropeptide Y (1:8000; ImmunoStar), rabbit anti-calbindin (1:40,000; Swant). Primary antibodies were detected with biotinylated goat anti-rabbit (1:200 Vector Laboratories).

Chronic EEG recordings

Adult (aged 7-9 months) $Arc^{-/-}$, $Arc^{+/-}$ and $Arc^{+/+}$ wt littermate mice were implanted for chronic video-EEG monitoring. Mice were anesthetized with Avertin (1.25% tribromoethanol/amyl alcohol solution, injected i.p. at 0.02 ml/g). Teflon-coated silver wire electrodes (0.005 inch diameter) attached to a microminiature connector were implanted bilaterally into the subdural space over temporal, parietal, and occipital cortices. Digital EEG activity was monitored 2-24 hours daily over 1 month (Stellate Systems, Harmonie software version6.1c). Recordings of similar durations among genotypes were reviewed by an investigator unaware of the genotypes of the mice. A video camera was used to monitor

behavior during EEG recording periods. All recordings were carried out at least 24 hr after surgery on mice freely moving in the test cage.

Morris Water Maze

Experimenters were blind to the genotypes of the mice. Only male mice were used for behavioral assessment. The water maze consisted of a pool (122 cm in diameter) containing opaque water (20°C ± 1°C) and a platform (14 cm in diameter) submerged 1.5 cm under the water. Mice were first given four pretrainings (90 s/trial, day 0) in which they had to swim down a channel (15 × 122 cm) and mount a platform hidden 1.5 cm below the water surface at the middle of the channel. Hidden-platform training (days 1–5) consisted of ten sessions (two per day, 2 hr apart), each with three trials. The platform location remained constant in the hidden-platform sessions, and the entry points were changed semirandomly between trials. The maximum trial time was 60 s. Mice that failed to find the platform were led to it and placed on it for 15 s. 24 and 72h after the last hidden-platform training session, a probe trial was conducted by removing the platform and allowing mice to search in the pool for 60 s. For cued training sessions (days 9–11), the platform was marked with a visible beacon, and the mice were trained to locate the platform over five sessions (two per day for the first 2 days, 4 hr apart; one for the last day), each with two trials. The platform location was changed for each session. Time to reach the platform, time in target quadrant, platform crossings, path length, and swim speed were recorded with an EthoVision video tracking system (Noldus, Netherlands).

Statistical Analysis

Statistical significance was evaluated by t-test, one, or two-way ANOVA and post-hoc Tukey *t* test unless otherwise noted. Statistical significance was set at $p<0.05$.

Chapter 3

Wave3 interacts with Arc and regulates dendrite and spine morphology

Abstract

Long-term potentiation and memory formation requires activity dependent, long lasting structural changes at the synapse. The activity-dependent and synaptically localized protein, Arc, is required for late-LTP and indirectly interacts with the actin-cytoskeleton. Here we demonstrate that Arc directly interacts with Wave3, an actin-nucleating factor. Reduction of Wave3 expression in immature neurons led to a marked reduction in primary dendrite length. In mature neurons, reduction of Wave3 resulted in reduced spine density and alterations in spine morphology. Reduction in primary dendrite length and spine density was partially rescued by expression of Arc.

Introduction

Arc, activity-regulated cytoskeleton-associated protein, is required for multiple forms of long-term plasticity, including late-LTP, mGluR-LTD and homeostatic plasticity. Arc is uniquely suited for affecting synapse-specific plasticity in that its expression and localization is highly regulated by neuronal activity. As its name indicates, Arc indirectly associates with the actin cytoskeleton and the protein localizes to actin-rich dendritic spines. Reduction of Arc expression via antisense oligonucleotides reverses LTP, prevents F-actin stabilization and dephosphorylates hyperphosphorylated cofilin in response to LTP induction in the dentate gyrus[24]. Furthermore, stabilization of the actin cytoskeleton blocks Arc antisense reversal of LTP, indicating that Arc may function to stabilize the cytoskeleton after LTP. Despite Arc's strong association with the actin cytoskeleton and its regulation of actin dynamics, it is still unclear how Arc interacts with the cytoskeleton.

In this study we demonstrate that Arc interacts with the actin-nucleating factor, Wave3 (Wiskott-Aldrich syndrome protein (WASP)--family verprolin homologous protein 3) in neurons. Wave3 is a key regulator of the actin-related protein (Arp2/3) complex and like Arc, is abundant in dendritic spines[83,84]. Wave proteins have an N-terminal Scar homology domain (SHD), a proline-rich region, and a C-terminal Verprolin-Cofilin-Acidic (VCA) domain[85]. The VCA domain functions to bind the Arp2/3 complex and facilitate actin nucleation. The three Wave isoforms have different expression patterns with Wave1 being primarily expressed at extending portions of lammelipodia, and Wave2 and 3 expressed at the tips of filopodia[86]. Wave1 and Wave3 are primarily expressed in the brain, while Wave2 has strong expression in the peripheral blood[83].

Several studies have demonstrated that Wave proteins regulate spine density and morphology. Neurons from Wave1 knockout mice have altered spine morphology, favoring filopodia[87], and reduced spine density[88]. However, less is known about the role of Wave3 in neuronal morphology. Given its interaction with Arc, we investigated its role in dendrite and spine morphology. We found that knockdown of Wave3 in young primary hippocampal cultures leads to a dramatic decrease in primary dendrite length. Knockdown of Wave3 in mature hippocampal neurons reduced spine density and increased the proportion of filopodia. Interestingly, this phenotype was partially rescued by Arc expression.

Results

Arc directly interacts with Wave3

Since Arc does not directly interact with the cytoskeleton, we hypothesized that it likely affects actin stabilization by interacting with other actin-related proteins. To investigate this, we performed a yeast-two hybrid screen using a Sprague Dawley rat brain cDNA library to search for proteins that interact with full-length Arc. One clone, confirmed to interact with Arc in yeast, was Wave3 (aa36-327), a key regulator of the actin cytoskeleton (Figure 3.1a). Yeast growth is specific to the Arc-Wave3 interaction as no growth was observed when Arc or Wave3 was cotransformed with control vectors GADT7-T or GBK-53 respectively. To confirm that Wave3 and Arc interact in neurons we expressed fluorescently tagged Wave3 and Arc constructs in mature primary hippocampal neurons (Figure 3.1b). Both proteins are abundant in dendritic spines and strongly colocalize. GFP-Wave3 also colocalizes with

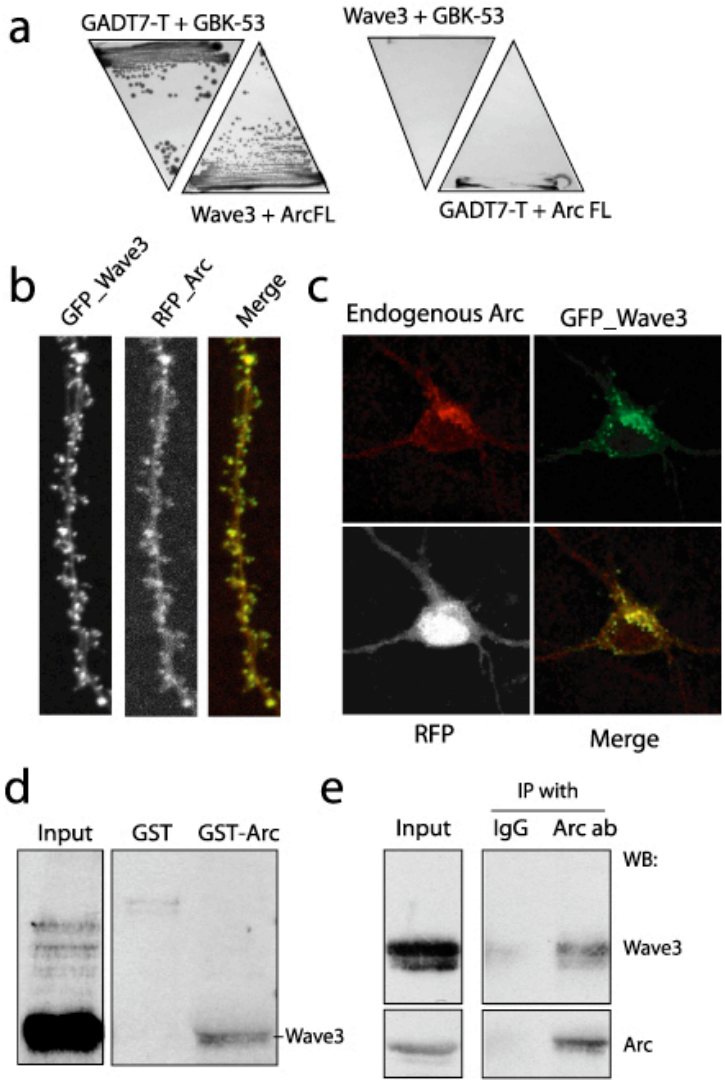

Figure 3.1: Arc directly interacts with Wave3 in neurons

(a) To discover new binding partners of Arc, we performed a yeast-two hybrid screen. We found that Arc directly interacts with amino acids 36-327 of Wave3. Positive control interaction between T-antigen (GADT7-T) and p53 (GBK-53) show yeast growth equivalent to Arc and Wave3 co-tranformation. No growth was observed between Wave3 and bait plasmid expressing p53, or between Arc and prey plasmid expressing T-antigen. (b) GFP_Wave3 expressed in hippocampal neurons localizes to dendritic spines and strongly colocalizes with RFP_Arc. (c) GFP_Wave3 colocalizes with endogenous Arc in hippocampal neurons. (d) GST-Arc pulls down Wave3 from rat cortical neuron extracts. (e) Wave3 co-immunoprecipitates with Arc pulled down from crude synaptosomal fractions of rat brain lysate.

endogenous Arc protein in hippocampal neurons (Figure 3.1c). To independently confirm that Wave3 interacts with Arc we performed glutathione-S-transferase (GST) pull-down assays. Full-length GST-Arc was purified and used to selectively pull-down endogenous Wave3 from extracts of rat cortical neurons (Figure 3.1d). To confirm that Arc and Wave3 interact *in vivo*, crude synaptosomal fractions from rat brain were precipitated with Arc antibody. Wave3 co-immunoprecipitates with Arc but not with control IgG (Figure 3.1e).

Wave3 plays a role in development of dendrites and spines

The strong localization of Wave3 in spine heads suggested that it may play a role in regulating dendrite and spine morphology. To determine the effect of Wave3 on dendrite and spine morphology we expressed a shRNA against rat Wave3 in primary hippocampal neurons (Figure 3.2a). Reduced expression of Wave3 in immature neurons (transfected 7DIV, fixed 13DIV) led to a dramatic reduction in the length of primary dendrites (Figure 3.2b,c). The affect on primary dendrite length was rescued by co-expression of mouse Wave3 that is resistant to the rWave3 shRNA (Figure 3.2b,c). However, the dendrites of these neurons were not completely normal in that they were often "kinked." Since we have found that Wave3 and Arc interact directly, we also tested whether expression of Arc could rescue neurite growth. Indeed, expression of Arc partially rescued the phenotype in that primary dendrite length was significantly increased compared to neurons expressing rWave3 shRNA alone.

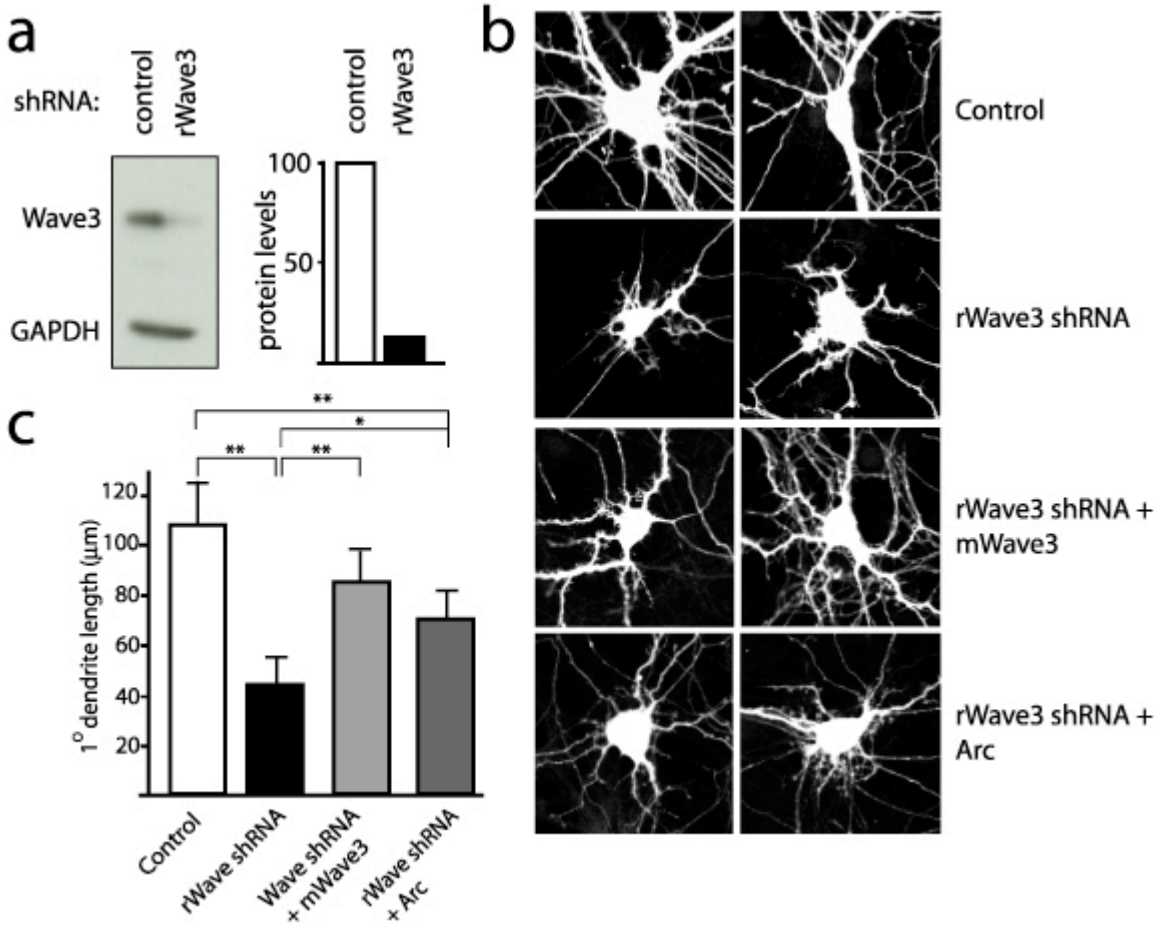

Figure 3.2: Reduction of Wave3 expression reduces primary dendrite length
(a) shRNA against Wave3 reduces expression around 80%. Cortical neurons were infected with a lentvirus expressing shRNA against Wave3 or a control shRNA at 5DIV. 5d after infection, lysates were collected and proteins levels quantified by western blot. (b-c) Reduction of Wave3 expression reduces primary dendrites of immature hippocampal neurons. Neurons were transfected with the shRNA and GFP at 7DIV and fixed 6d post-transfection. Expression of shRNA resistant mouse Wave3 rescued the reduction in primary dendrite length. $P<0.05$= *, $P<0.001$ = ** by one-wave ANOVA and post-hoc Tukey test.

To determine whether Wave3 also plays a role in regulating spine density or morphology we waited until the neurons were 14DIV, with established dendrites, before introducing the shRNA. Spine density and morphology were analyzed at 20DIV (Figure 3.3a). Reduction of Wave3 decreased spine density (Figure 3.3b), decreased spine width and increased spine

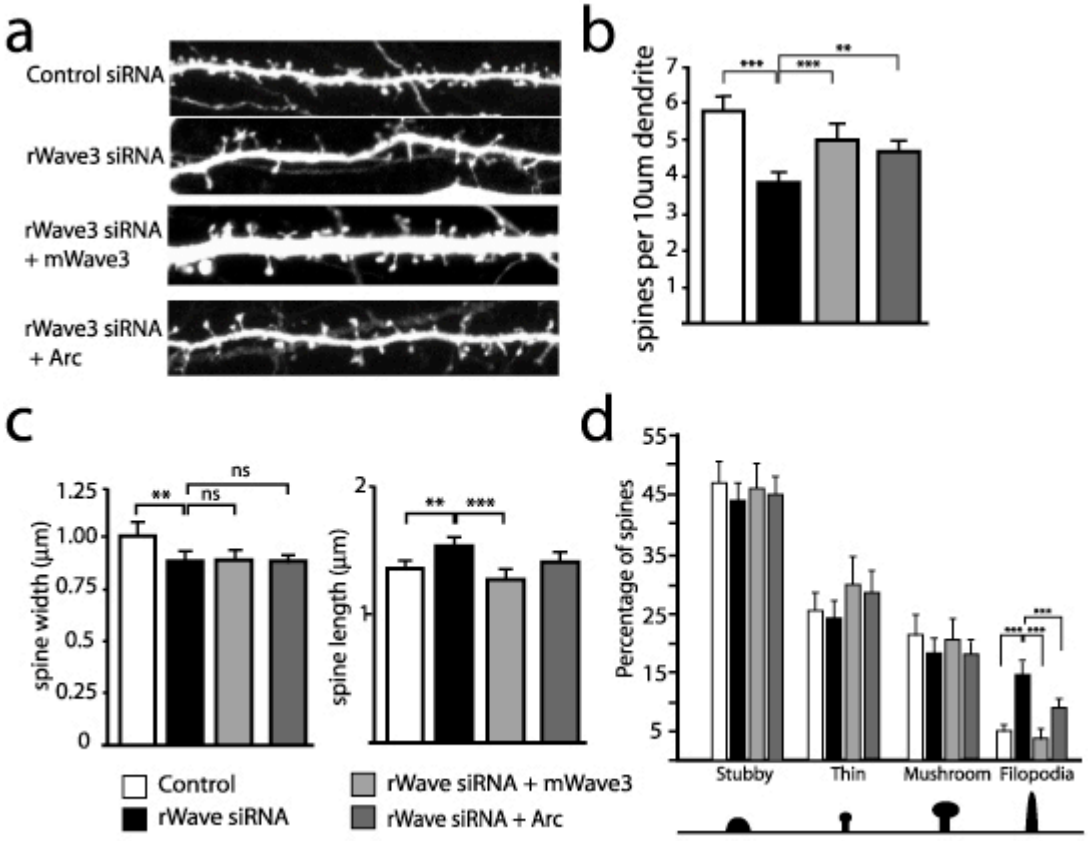

Figure 3.3: Reduction of Wave3 reduces spine density and increases filopodia
(a-b) shRNA against Wave3 reduces spine density. Hippocampal neurons were transfected with shRNA against Wave3 or a control at 14DIV and fixed 6d after transfection. Co-transfection with GFP allowed for quantification of spine density and spine morphology. Co-transfection with either shRNA resistant mouse Wave3 or Arc partially rescued the spine density reduction. (c) Reduction of Wave3 expression increased spine length but did not affect spine width. (d) The increase in spine length was likely due to a significant increase in the percent of filopodia in neurons with reduced Wave3. This was also rescued by mWave3 expression and partially rescued by Arc expression. $P<0.05$ = *, $P<0.01$ = **, $P<0.001$ = *** by one-way ANOVA post-hoc Tukey test

length (Figure 3.3c). This decrease in width and increase in length is likely attributable to an increase in the percentage of filopodia (Figure 3.3d). The alterations in spine density, spine length and morphology were rescued by exogenous expression of mouse Wave3, indicating these effects were specific to Wave3 reduction and not off- target effects. However,

mWave3 overexpression did not rescue the reduction in spine width, possibly because it increased thin spines while reducing filopodia, thus maintaining an overall decrease in spine width. Interestingly, expression of Arc also partially rescued the effect of Wave3 shRNA on spine and filopodia density (Figure 3.3b,d).

Discussion

Maintenance of long-term potentiation involves lasting structural synaptic changes including postsynaptic density expansion and enlargement of dendritic spines. Such structural changes require modulation of the actin cytoskeleton. Furthermore, inhibition of actin polymerization prevents LTP maintenance, and actin stabilization after high frequency stimulation (HFS) is synapse-specific[14]. Several studies have suggested that Arc regulates activity-induced actin polymerization and is required for F-actin stabilization after HFS[24]. In this study we demonstrate that Arc interacts with Wave3, a key regulator of the actin cytoskeleton, and that Wave3 plays a critical role in dendrite development and spine morphology. Finally, Arc expression partially rescues spine loss associated with reduction of Wave3 expression.

Our work, along with studies focused on Wave1, support a role for the Wave proteins in regulating dendrite and spine morphology. However, Wave1 and 3 appear to play distinct roles in dendrite and spine development. For example, we observe a strong affect of Wave3 on neurite development. However, a Wave1 specific knockout mouse produced by genetrap surprisingly has no alterations in neurite development *in vitro* or *in vivo*[89]. In support of this, Wave1 and 3 have different localization patterns in growth cones: Wave1 is localized along the leading edge and Wave3 is localized both at the leading edge, *and* the tips of filopodia[86].

Furthermore, we find that reduction of Wave3 leads to loss of spines, while Wave1 reduction leads to alterations in spine morphology but not significant spine loss[87]. Interestingly, a second Wave1 knockout mouse does have reductions in neurite development and spine density. However, this mouse also has unexplained loss of Wave2 and Wave3 expression, suggesting that the observed affects on neurite and spine development could be due to Wave3, not Wave1, reduction. Also, in contrast to our work, a previous study observed that overexpression of Wave3 decreased spine density[84]. This suggests that the overexpressed protein may act as a dominant negative.

Activation of Wave1 and 3 are also regulated by different kinases. Wave1's effect on spine morphology is dependent on Cdk5 phosphorylation[87], while Wave3 is phosphorylated by c-abl tyrosine kinase[87]. Inhibition of Abl kinases disrupts dendrogenesis if administered prior to 7DIV and specifically affects dendrogenesis of actively developing dendrites[87,90]. This suggests that Wave3-mediated effects on dendrite and spine morphology depend on Abl kinase activation. Future experiments investigating the role of synaptic activity on Wave3 activation and subsequent spine alterations will give added insight into the connection between activity and spine morphology.

Expression of Arc partially rescued deficits in spine density and morphology caused by reduction of Wave3 expression. This finding suggests that Arc may be acting downstream of Wave3 to facilitate spine formation. It is also possible that by expressing Arc, the residual Wave3 present in the neuron is properly localized to facilitate spine formation in dendritic regions experiencing increased synaptic activity. At the synapse Arc is known to facilitate AMPAR endocytosis through its interaction with endophilin. Interestingly, N-WASP and Wave family proteins have also been implicated in endocytosis

through interactions with SH3 containing proteins such as endophilin. Furthermore, endophilin A has been shown to enhance N-WASP induced Arp2/3 complex activation. In Chapter 2 we presented data demonstrating that Arc modulates spine morphology through endocytosis of AMPARs. Cooperative binding of Wave3, endophilin and Arc may facilitate coordinated AMPAR endocytosis and spine thinning.

Methods

Plasmids.

mWave3 cDNA was purchased from Open Biosystems and cloned into GW1 or GW1-GFP to create the N-terminally tagged GFP-mWave3 construct. The pSicoR/U6 lentiviral vector was used to make the Wave3 shRNA. The target sequence used was: 5'-GGAGGGGAAAGTTTAACAA-3' (nucleotides 2436-44). An ineffective target sequence: 5'-GGACCCCTCTTACTTCTTT-3' was used as the control shRNA.

Yeast-Two Hybrid Screening

The Matchmaker GAL4 kit (Clontech) was used to perform the screen, according to the manufacturer's protocols. Briefly, the full length *Arc/Arg3.1* open reading frame was subcloned into the bait vector, pGBKT7. Yeast (AH109) was co-transformed with the bait plasmid and plasmids encoding an adult rat brain cDNA library in the prey vector GADT7[91]. The positive colonies that grew on plates lacking Ade, His, Leu and Trp were selected and confirmed by β-galactosidase filter assay. Plasmid DNA was isolated from the yeast, transformed into *E. coli*, and sequenced. Hits were retransformed with the empty pGBKT7 vector to eliminate non-specific interactions with the vector.

GST-Pull downs

Full-length Arc was cloned into the pGEX-4T expression plasmid and batch purified from bacterial lysate with GST beads and glutathione elution. For pull-down assays 12DIV primary cortical neurons were lysed in TGEK (50mM TrisHCl [pH 7.5], 10% vol glycerin, 1 mM EDTA, 100 mM KCl, and 0.5% NP40) and centrifuged at 14,000rpm for 10 minutes at

4ºC. The lysates were precleared with GST and glutathione beads for 2 hours at 4 ºC, and then incubated for 2 hours with glutathione beads bound to GST-Arc or GST alone. Bound complexes were washed, separated by SDS-PAGE, and blotted with anti-Wave3 antibody (Rabbit, Upstate 1:400).

Preparation of Crude Synaptosomal Fraction from Brain

A Long Evans rat was placed into two sequential novel environments for 30 minutes per environment to induce Arc expression. Rat brains were homogenenized in 10 volumes of buffered sucrose (0.32 M sucrose, 4 mM HEPES/NaOH [pH 7.4], 1 mM EDTA, 1 mM EGTA, PMSF and protease inhibitor cocktail) with a glass-teflon homeogenizer, centrifuged 800 x g for 15 minutes. The supernatant was subsequently centrifuged for 9,000 x g for 15 minutes and the pellet collected as crude synaptosomal fraction P2.

Coimmunoprecipitation

P2 was resuspended in TGEK with protease inhibitors and PMSF. The homogenate was centrifuged at 100,000 x g for 20 minutes at 4ºC, and supernatants were preincubated with BSA (3%) coated magnetic protein G beads for 1.5 hours. Supernatants and beads were separated and supernatants were incubated with 4μg of Arc antibody or 4μg of mouse IgG for 2 hours at 4ºC. BSA coated Magnetic Protein G beads were added and incubated for 1 hour. The beads were washed 3 times with 1% Triton/PBS and eluted with SDS loading buffer and boiled for 5 minutes.

Cell culture and transfection

E20–21 rat hippocampi were dissected and treated with papain (10 units ml^{-1}, 15 min; Worthington Biochemical) and then with a trypsin inhibitor (10 mg ml^{-1}, 15 min; Sigma). After trituration, dissociated hippocampal neurons were plated on coverslips (2 x 10^5 cells/coverslip) coated with laminin (BD Biosciences) and poly-D-lysine (Chemicon). One hour after plating, neurons were transferred into neurobasal media with B27 (Gibco). Primary hippocampal cultures were transfected at 7-8DIV via calcium phosphate methods or at 13-14DIV using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Immunocytochemistry and Microscopy

Six days after transfection, neurons were fixed in 4% PFA/4% sucrose. Coverslips were then permealized in 0.1% Triton X-100 in PBS for 15 minutes, blocked in PBS with 3% donkey serum, 3% BSA for 1 hour, and then incubated with primary Wave3 antibody (Millipore, 1:400) overnight at 4^{o}C.

Microscopy and Image Analysis

Images of primary hippocampal cultures were acquired with a LSM510 confocal microscope system (Zeiss, Oberkochen, Germany) and a 25x or 63× oil immersion lens (1024 × 1024 pixels). For spine analysis each image consisted of a stack of images taken through the *z*-plane of the cell. Confocal microscope settings were kept the same for all scans in each experiment. Healthy, pyramidal-like neurons with significant Wave3 reduction were selected

for quantification from three coverslips from 3–4 independent experiments for each construct. Dendrites were traced and lengths quantified using NeuronJ[92]. For spine size, the maximal length and head width were measured manually using Metamorph (Universal Imaging). Each spine was categorized as having or not having a neck. Spines with necks were separated into thin and mushroom spines based on head width. Spines with heads less than 1μm were categorized as thin, and those with heads greater than 1μm were categorized as mushroom. Filopodia were protrusions greater than 1.5 μm in length without a neck.

Chapter 4

Extra Tidbits and Concluding Remarks

Arc overexpression does not alter survival

Many of the experiments performed during this project involved overexpression of Arc in primary neuronal cultures. Since we are analyzing the affects of Arc expression on neuronal morphology it is critical that Arc overexpression does not also increase toxicity. To test this, we transfected neurons with GFP and Arc or a control vector and followed their survival over time using GFP as a viability marker[93]. There was no difference in survival between neurons transfected with Arc or the control vector (Figure 4.1).

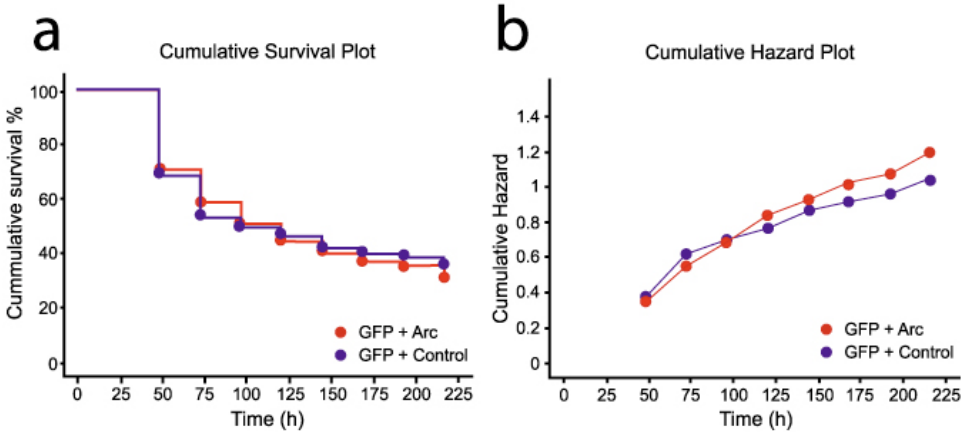

Figure 4.1: Arc does not affect neuronal survival
Cortical neurons cotransfected with GFP and Arc or control constructs were followed overtime using our robotic microscope. Individual survival times were calculated for 262 neurons transfected with GFP and Arc, and 289 neurons transfected with GFP and a control vector. Kaplan-Meir analysis was used to estimate survival and hazard functions.

Arc expression increases dendritic branch dynamics

In Chapter 2 we show that Arc expression regulates spine morphology in mature hippocampal cultures and *in vivo*. In Chapter 3, we demonstrate that Wave3 interacts with Arc and regulates both spine morphology in mature neurons *and* dendrite morphology in immature neurons. Here we ask: Does Arc affect dendrite morphology in immature neurons?

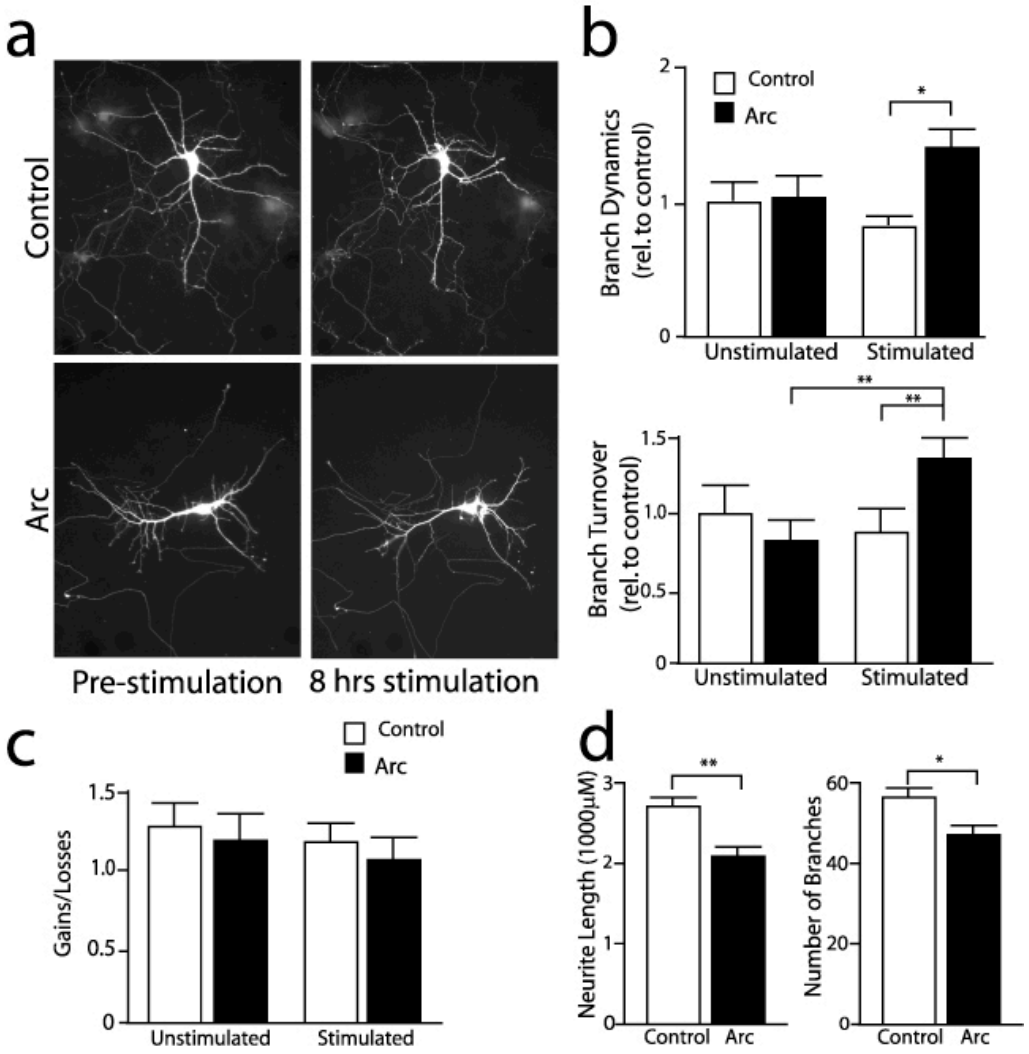

Figure 4.2: Arc increases dendritic branch dynamics

(a) Longitudinal analysis of neurons expressing Arc or a control vector. Neurons were stimulated with 40μM bicuculline for 8 hours. (b) Under stimulated conditions Arc expression increased dendritic branch dynamics defined as: Σ(length gained, length lost)/starting length. Arc expression also increased branch turnover defined as: Σ(# of branches gained and lost)/starting # of branches. * = $p < 0.05$ using one-way ANOVA and post-hoc Tukey tests. ** = $p < 0.05$ using one way ANOVA and post-hoc Fisher's PLSD. (c) Increases in branch dynamics did not lead to a significant gain or loss of dendritic length. (d) Arc expression reduced the average neurite starting length and the average number of branches per neuron. * = $p<0.05$; ** = $p<0.001$ using t-test.

To monitor activity-dependent changes in neuron morphology it is best to follow single cells over time. Our laboratory has developed and validated a new experimental

system for longitudinal analysis of single cells[93]. To determine whether Arc regulates dendritic branch plasticity, hippocampal neurons were co-transfected with GFP and either an *Arc* expression plasmid or an empty vector control. Then the neurons were imaged before stimulation and several times over an 8 hour period of bicuculline bath stimulation (Figure 4.2a). Dendritic arbors from cells over several timepoints were then traced and measured for changes in branch length and turnover. Overexpression of Arc led to increased branch dynamics (defined as the Σ(length gained, length lost)/starting length) and branch turnover (defined as the Σ(# branches gained and lost)/starting # of branches) only when neurons were stimulated (Figure 4.2b). In other words, neurons with increased Arc expression exhibited more plasticity in dendrite length and dendrite turnover during stimulation. This however, did not affect the overall length of the dendrites before and after stimulation. The amount of dendrite length gained was similar to the amount lost (Figure 4.2c) over the 8 hour stimulation period. However, the average neurite length and the average number of branches per neuron prior to stimulation were decreased in neurons expressing Arc (Figure 4.2d).

Interestingly, Arc's affect on dendritic branch plasticity is similar to what we observe in mature neurons (see Chapter 2). Expression of Arc in 20DIV hippocampal neurons increases the proportion of thin spines and filopodia, which are more plastic and more likely to lengthen, retract, and enlarge. While no exogenous stimulation was required to observe these changes in spine morphology, the mature hippocampal cultures have much more basal activity compared to the low density immature hippocampal cultures analyzed in the above dendritic branching experiments. Overall, we find that Arc expression, in both immature and mature neurons, increases structural plasticity.

Arc in the nucleus

The majority of the research on Arc is focused on its role at the synapse. However, a significant amount, if not the majority, of Arc protein is actually localized to the nucleus. Its function within the nucleus is unknown. Its localization to the nucleus is regulated by the

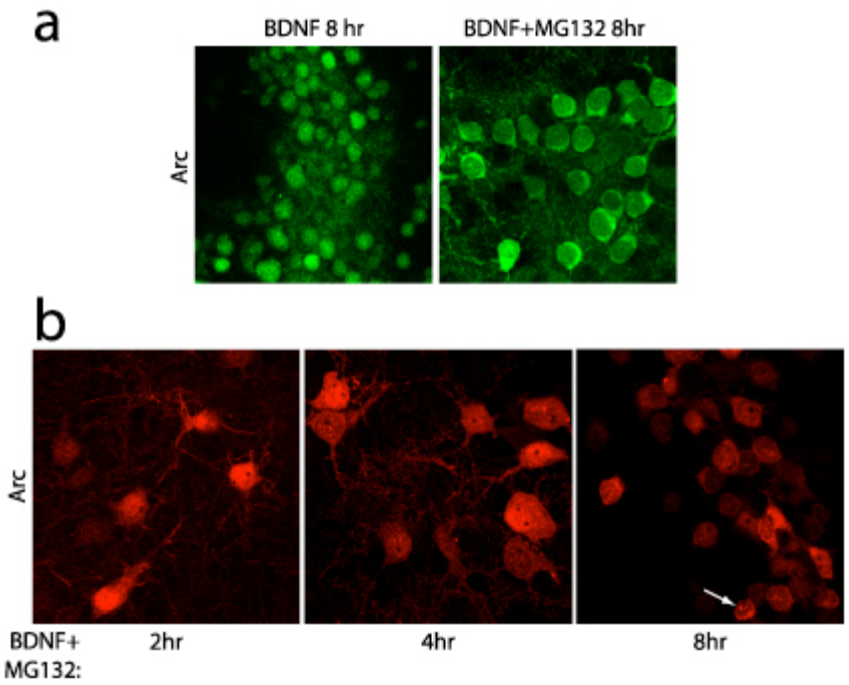

Figure 4.3: Arc nuclear localization is regulated by the proteasome
(a) BDNF (50ng/ul) bath stimulation of cortical neurons robustly induces Arc expression in the nucleus. Addition of the proteasome inhibitor MG132 leads to Arc expression predominantly outside of the nucleus. (b) Timecourse of BDNF +MG132. At 2hr Arc is expressed in the nucleus. After 4hr of stimulation roughly half the neurons have los significant expression within the nucleus. By 8hr stimulation the majority of neurons have predominantly cytoplasmic Arc expression. Arc puncta are now observed in the nucleus (arrow).

proteasome as stimulation of neurons with BDNF (50ng/ul) *and* a proteasome inhibitor, MG132, leads loss of diffuse Arc from the nucleus (Figure 4.3a). A timecourse of Arc expression after BDNF and MG132 bath stimulation shows that Arc nuclear expression is

lost over time (Figure 4.3b). After 2 hours of BDNF/MG132 stimulation the majority of neurons express Arc in the nucleus. By 4 hours, half the neurons have reduced nuclear Arc expression. And at 8 hours all the neurons have a predominantly cytoplasmic Arc distribution. This suggests that Arc is shuttled in and out of the nucleus and that this shuttling may be regulated by the proteasome. Proteasome inhibitor incubation also leads to Arc puncta formation within the nucleus (Figure 4.3b). These puncta colocalize with promyelocytic leukemia (PML) bodies (unpublished data from E. Korb).

Given Arc's nuclear localization and the tight temporal regulation of its expression, we wondered whether Arc negatively regulates its own expression. To test this we utilized a new Arc luciferase reporter construct that incorporates novel distal and proximal enhancer regions[42]. Cortical neurons were co-transfected with the luciferase reporter, a renilla reporter to control for transfection efficiency, and Arc or a control vector. The neurons were then stimulated to induce *Arc* transcription. To our surprise we found that the Arc reporter's

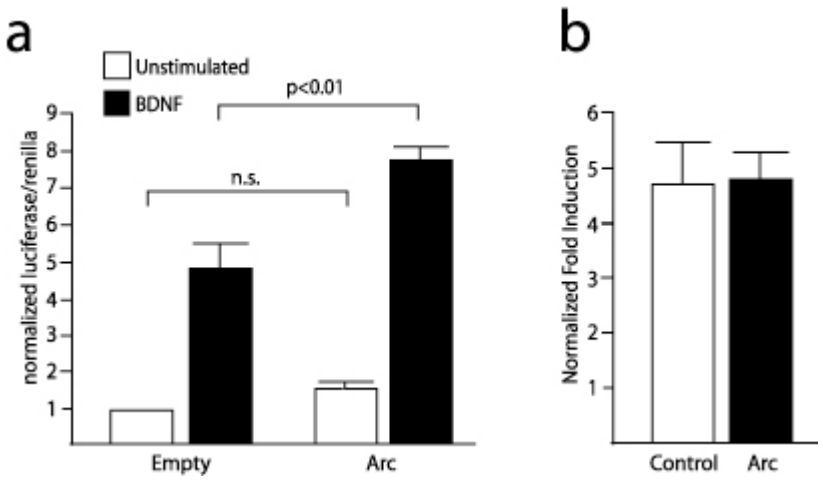

Figure 4.4: Arc overexpression positively regulates its own transcription
Cortical neurons were transfected with an Arc promoter luciferase reporter construct[42], and Arc or a control vector. (a) Expression of Arc increased luciferase expression under the Arc promoter when neurons were stimulated with BDNF. (b) Arc expression did not increase the BDNF fold induction.

response to BDNF was enhanced in neurons overexpressing Arc (Figure 4.4a). This suggests that Arc expression acts a positive, not negative, feedback agent. However, the BDNF fold induction over unstimulated neurons was not affected by Arc expression (Figure 4.4b). While not significant, Arc expression also increased reporter activity in unstimulated neurons, thus maintaining the same fold induction with BDNF stimulation.

What is the significance of Arc's enhancement of its own transcription? Several studies have demonstrated that place cells active during the day are reactivated during sleep as possible mechanism of memory consolidation. Perhaps the initial induction of Arc facilitates future reactivation of the neuron by promoting a second round of Arc transcription. Alternatively, Arc's enhancement of its own transcription may also act to compensate for its functional homeostatic downscaling of synaptic efficacy. Arc expression could simultaneously reduce the neuron's excitability by AMPAR endocytosis and reduce the activity threshold for Arc transcription.

Possible tertiary complex between Arc, Wave3 and Endophilin

To further investigate the interaction between Arc and Wave3, we sought to determine the Wave3 binding domain on Arc. We created several Arc deletion constructs and tested their binding to Wave3 using the yeast-two hybrid system. Deletions of amino acids 90-100, 101-120 and 121-140 blocked Arc-Wave3 binding (Figure 4.5a). The Arc-Wave3 binding domain (aa90-140) significantly overlaps with the Arc-Endophilin3 binding domain (aa80-120) (Figure 4.5b).

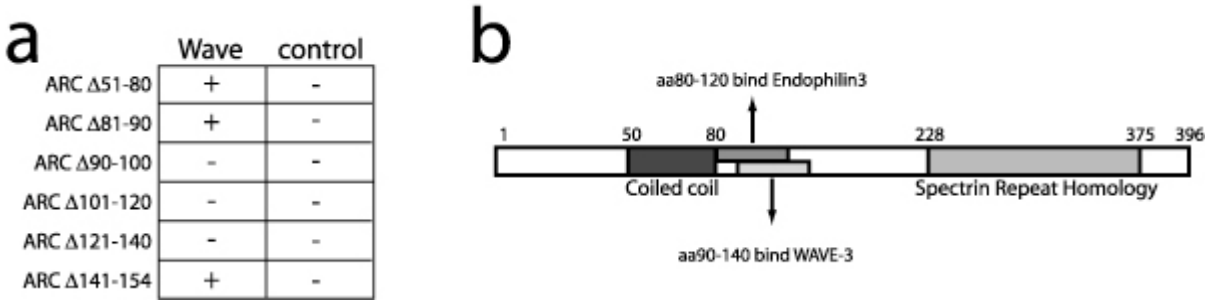

Figure 4.5: Arc interacts with Wave3 at Endophilin3 binding domain
(a) Arc deletion yeast-two hybrid constructs were cotransformed with pGBK-Wave3 (aa36-327) into yeast and tested for growth. Arc deletions 90-100, 101-120 and 121-140 failed to grow. (b) The interaction domain of Wave3 overlaps with the interaction domain of Endophilin3.

Endophilin3 is a BAR domain protein that facilitates endocytosis and its interaction with Arc is thought to be required for Arc-mediated AMPAR receptor endocytosis. In Chapter 2 we demonstrated that Arc's interaction with Endophilin3 is required its effects on spine morphology. However, given Wave3's shared binding domain with Endophilin3, it is possible that Wave3 also plays a role in both Arc mediated AMPAR endocytosis and spine plasticity.

To address this possibility, we have begun to investigate whether Arc binds Wave3 and Endophilin3 separately or in a tertiary complex. First we asked whether it was possible that Wave3 and Endophilin3 interact with each other, independent of Arc. We co-expressed myc- Endophilin3 and GFP_Wave3 constructs in HeLa cells to determine if the two proteins colocalize (Figure 4.6a). Indeed, both GFP_Wave3 and Endophilin3 formed cytoplasmic puncta and these puncta colocalized. GFP_Wave3 also formed nuclear puncta that did not colocalize with Endophilin3 (Figure 4.6a upper panel). The function of these nuclear puncta is still unclear. GFP_Wave3 and Endophilin3 also showed strong colocalization in puncta located at the tips of filopodia (Figure 4.6a lower panel), suggesting that Endophilin3 and

Wave3 may work together to modulate filopodia or spine morphology. Indeed, in hippocampal neurons, co-expressed Arc, GFP_Wave3 and Myc-Endophilin3 showed strong colocalization in spines (Figure 4.6b).

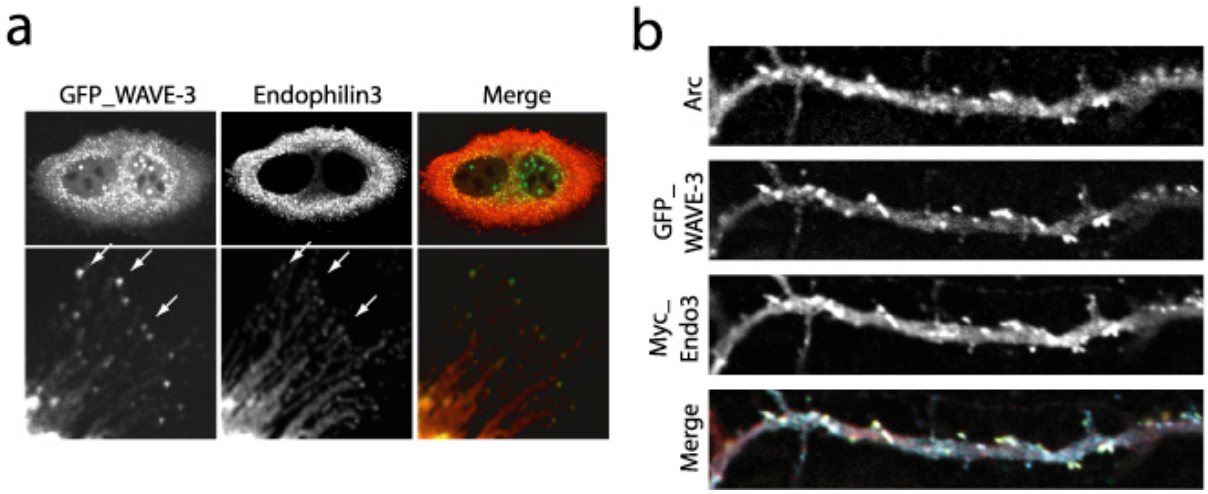

Figure 4.6: Wave3, Endophilin and Arc colocalize
(a) GFP_WAVE3 and Endophilin3 colocalize in HEK cells. Lower panel: close up of filopodia extending from HEK cell body. GFP_Wave3 and Endophilin3 colocalize at the tips of the filopodia. (b) Arc, GFP_Wave3 and Myc Endophilin3 were co-transfected into hippocampal neurons. All three proteins show strong colocalization in spine.

The colocalization of Arc, GFP_Wave3 and Myc-Endophilin3 suggests that these proteins exist in a tertiary complex and may cooperatively function to regulate AMPAR endocytosis and spine morphology. Future biochemical experiments from brain extracts will give needed confirmation and insight into the regulation of this complex. It would be particular interesting to know whether synaptic activity regulates Arc interaction with Wave3 and Endophilin3 and how this may alter the function of the complex.

Interestingly, there is a considerable body of work demonstrating a role for WASP family proteins in endocytosis[94]. In fact, Endophilin1 has been shown to interact with N-WASP and enhance N-WASP induced Arp2/3 complex activation[95]. To test whether Wave3 plays a role in endocytosis, we transfected GFP_Wave3 into HeLa cells and assayed uptake

of Alexa 555-labeled transferrin (Figure 4.7a). Strikingly, expression of GFP_Wave3 completely blocked transferrin uptake in 50% of transfected cells, supporting a role in endocytosis (Figure 4.7b). It is possible that overexpression of GFP_Wave3 in this system is acting as a dominant negative. Experiments studying the effects of Wave3 knockdown on endocytosis should help to clarify the role of Wave3 in transferrin uptake.

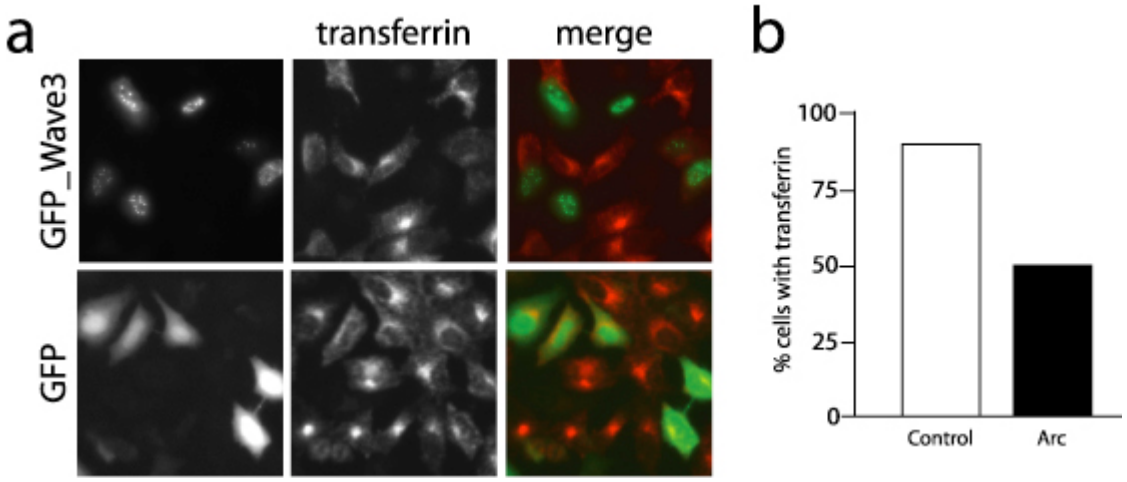

Figure 4.7: Expression of GFP_Wave3 blocks transferrin uptake
(a) HeLa cells were transfected with GFP_Wave3 or GFP. One day post-transfection, HeLa cells were incubated with Alexa 555-labeled transferrin for 15 minutes at 37ºC and then fixed. (b) Quantification of transferrin uptake.

Hits from Arc yeast-two hybrid screen

In order to discover new binding partners of Arc, we performed a yeast-two hybrid screen using full-length Arc as bait against a rat brain cDNA library (gift from David Bredt). From this screen, 16 candidate proteins were confirmed to interact with Arc in yeast (Table 4.1). The candidates can be divided into four categories: RNA binding proteins, metabolic proteins, the ubiquitin system, cytoskeletal and associated proteins, and signaling proteins.

Arc protein is strongly expressed in the nucleus after induction. However, very little work has been published regarding its nuclear function. Its possible interactions with RNA binding proteins like DEAD box5/p68, indicate that it may be involved in RNA splicing. Within the nucleus Arc colocalizes with promyelocytic leukemia (PML) bodies which have been associated with a variety of nuclear functions including transcriptional regulation and DNA repair. Interestingly, the PML protein, which is found in all PML bodies, has a TRIM/RBCC motif which is common to one of the most frequent hits from our screen: Tripartite motif (TRIM) 37. Little is known about TRIM37's functions in neurons, although a recent study showed Alzheimer's patients have altered expression of TRIM37[96].

Given Arc's localization to both the nucleus and the dendrites, it is tempting to hypothesize that Arc is involved in RNA nuclear export and dendritic trafficking. Several candidate proteins are also associated with RNA trafficking: Elongation initiation factors, such as EIF4γ2 are often found within RNA transport granules, and these granules are transported along microtubules via motor proteins such as another candidate: dynein. Finally, the candidate protein RICS is intriguing given its recent connection to neurite outgrowth[97]. RICS was originally identified as a novel RhoGAP that interacts with β-catenin. It has GAP activity for Cdc42 and Rac1. It is noteworthy that Wave3 is activated downstream of Rac. RICS also been shown to associate with N-cadherin, N-methyl-D-aspartate, and the postsynaptic density, placing it in the right location to also interact with Arc.

Table 4.1: Hits from Yeast-Two Hybrid Screen

Candidates	Accession #	Insert	# hits	Published Functions
RNA Binding Proteins				
R3hdm1	NM_001134867	2844-3570	50	Binds single stranded nucleic acids[98]
DEAD box polypeptide 5, p68	BC009142	956-1646	2	RNA Helicase, RNA splicing, transcriptional co-activator, miRNA and rRNA processessing[99]
DEAH 34	NM_027883	3346-4081	1	RNA Helicase
EIF4γ2	NM_013507.3	871-1564	N/A	Translational repressor
Metabolism				
GAPDH	BC059110.1	1130-2036	17	Glycolysis pathway, multiple non-metabolic functions: endocytosis, translational control, RNA export, apoptosis[100]
Phospholipid Scramblase3	NM_001012139.1	317-898	2	Mitochondrial respiration, morphology, apoptosis response[101]
Catalase	NM_012520.1	31-1442	1	Hydrogen Peroxide reductase
Glutamine Synthetase	X07921.1	538-1169	N/A	Glutamate and Nitrogen metabolism
Ubiquitin System				
Tripartite motif 37	NM 197987.1	630-1800	46	E3 ubiquitin ligase, mutations associated with Mulibrey Nanism[102]
Ubiquitin conjugating enzyme E2B	BC070946	223-1295	1	Ubiquitin Conjugation
Nedd4L	BC08371.1	627-1787	N/A	E3 Ubiquitin Ligase[103]
Cytoskeleton				
Tubulin beta 5	NM_173102.1	416-1014	2	Cytoskeleton, Microtubules
Tubulin beta 4	NM_009451.3,	221-918	2	Cytoskeleton, Microtubules
Dynein light chain LC8-type 1	NM_053319.2	58-652	3	Component of dynein motor protein, myosin V, neuronal nitric oxide synthase[104]
Wave3	NM_145155.1	539-1414	1	Actin-nucleation, endoctyosis?[105]
Signaling				
RICS (RhoGAP)	XM_236020.4	4130-5026	N/A	RhoGAP for Cdc42 and Rac1. Involved in neurite outgrowth and NMDA signaling[97,106]

Concluding Remarks

"If the human brain were so simple that we could understand it, we would be so simple that we couldn't." - Emerson M. Pugh

The brain is an exquisite organ. So exquisite that I even have trouble calling it an "organ." It seems so much more impressive than other organs like the heart or liver. Those organs are closer to machines that move liquid or sift out toxins. The brain is not so simple and certainly not replaceable or transplantable! It is our brain that connects us to the world around us and internalizes our personal past. We are perhaps born with innate individual personalities, but they are modified and layered with new dimensions by the experiences we have and the abilities we learn. It is the complexity of the brain combined with its elegant plasticity that allow us as humans to transform from crying dependent babies to opinionated, emotional, creative, and independent adults.

Summary of Findings

The work presented in this thesis adds several major findings to the field of synaptic plasticity. First we found that Arc plays a role in increasing the structural plasticity of neurons by increasing the proportion of thin "plastic" spines (Chapter 2) and by increasing dendritic branch dynamics (Chapter 4). At the same time Arc reduces synaptic efficacy through endocytosis of AMPARs. We hypothesize that this balance of increased structural plasticity and homeostatic downscaling is required for the maintenance of a dynamic network. Second, we found that loss of *Arc in vivo* leads to aberrant neuronal activity and impaired long-term spatial memory. Notably, we observed that mice with only one copy of Arc also have deficits in long-term spatial memory and aberrant neural network activity.

This finding suggests that the *amount* of Arc made in response to activity is critical for its proper function. Finally, we found that Arc interacts with the actin-nucleating protein Wave3 (Chapter 3). Wave3 expression is critical for both dendritic branch development as well as spine density and morphology. We hypothesize that through its interaction with Wave3 Arc may facilitate new spine formation and/or AMPAR endocytosis.

What is next for the Arc field? After studying Arc for the last five years I am left with an even stronger sense of amazement - and I am sure we have only touched the surface! There are many questions still unanswered. We know that Arc expression is tightly linked to synaptic activity, but is Arc protein function also regulated by activity? Our data indicate that activity is required for Arc to increase dendritic branch dynamics, but what about its effects on spine morphology or AMPAR endocytosis? Does Arc preferentially facilitate AMPAR endocytosis and spine thinning at unactivated synapses? Also, what would happen if Arc expression was no longer linked to activity. For example, would LTP and memory be impaired if Arc was constitutively expressed?

What is Arc's function in the nucleus? Is it sequestered there simply to limit its dendritic function, or does it regulate transcription, RNA splicing, or RNA transport as some of the yeast-two hybrid hits would indicate?

Finally, our data indicates that Arc expression plays an important role in regulating network activity. Long-term loss of Arc causes epileptic-like network hyperexcitability. However, it is still unclear whether Arc's homeostatic function plays a role in LTP maintenance and memory consolidation. Does seizure activity, or increased interictal spiking further impair spatial memory in knockout mice? A conditional-knockout mouse may prove particularly useful in separating short-term and long-term consequences of Arc expression.

I look forward to following research on Arc in the years to come. I have no doubt that through understanding the function and regulation of this unique protein we will glean new and exciting knowledge of how our brain functions on both a molecular and network level.

Chapter 5

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