Title

Arc modulates the rapid endocytosis of GluA1-containing AMPA receptors through a functional interaction with the E3 ligase Nedd4-1

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Arc modulates the rapid endocytosis of GluA1-containing AMPA receptors through a functional interaction with the E3 ligase Nedd4-1

by

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DISSERTATION

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Acknowledgements

Graduate school has been formative for me, both in my development as a scientist and as a person. I am glad to have this opportunity to acknowledge and thank the many people who made this such a unique and rewarding experience.

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A few weeks before I moved to California, I was seated next to a couple from New Jersey at an MIT alumni event. When they found out that I was about to begin medical school, they told me I must seek out Carol Peebles, the wonderful Princeton student who had cared for their special-needs son and was now at UCSF. Not only did Carol (now Wilkinson) turn out to be the first friend I made in San Francisco, but, without her, this research project would not exist. As a graduate student in the Finkbeiner Lab, she performed the screen that first identified the interaction between Arc and Nedd4-1. I am forever grateful to her for that crucial finding and for her continuing friendship.

I would never have known about Carol's result if it weren't for another graduate student in the lab, Erica Korb. Over a beer in our backyard, Erica made the connection between the work I had been doing on receptor trafficking and Carol's discovery of Nedd4-1. At the time that Carol had run the screen, nothing was known about the function of Nedd4-1 in AMPA receptor trafficking, but since then, work from other groups had implicated it in the endocytosis of GluA1. Erica had been following up on some of the results from Carol's screen and realized that I might be interested in Nedd4-1. This came at a time when I was struggling to find a direction for my project, and I can't thank Erica enough for pointing me down the path that ultimately lead to this dissertation. Erica is one of the most impressive people I know, and I look forward to her inevitable future triumphs.

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Speaking of friends, there are two in particular who I would like to mention. Sara Haug, Carina Marquez and I began medical school together in 2004. They have since gone on to become doctors, survive residency, and begin fellowships. Thankfully, they stayed in San Francisco through all of it and our countless dinners in the Mission and nights spent raiding Sara's wine cellar have sustained me through the difficult times and buoyed me higher through the good.

Finally, I want to thank my family, both old and new. My parents somehow managed to set an exceedingly high bar with their own educational achievements while simultaneously allowing me complete freedom to follow whatever path I chose. I would

like to dedicate this dissertation to my mother, who taught me to think creatively and to fight fiercely for my beliefs.

As I started by saying, graduate school has been formative for me. The most important thing to happen to me in the last seven years was being invited to a dinner party on June 17th, 2010. That was the night that I met my now husband, Brian Paul Overland. Brian has seen me through the most challenging and the most exhilarating moments of this journey, and I can't wait to begin the next stage in our careers side-by-side.

Arc modulates the rapid endocytosis of GluA1-containing AMPA receptors through a functional interaction with the E3 ligase Nedd4-1

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Abstract

The activity-regulated cytoskeleton-associated protein, Arc, is known to promote the internalization of surface AMPA-type glutamate receptors in neurons and is thought to do so via physical interactions with the endocytic machinery. However, the specific molecular pathway linking Arc to the trafficking of AMPA receptors remains unknown. In this study, we find that Arc knockdown in cultured rat cortical neurons slows the rate of endocytosis of pHluorin-tagged GluA1 AMPA receptor subunits in response to direct AMPA receptor activation but has no effect on the endocytosis of pHluorin-tagged GluA2 in response to either AMPA or NMDA stimulation. We further demonstrate that this effect is specific to the AMPA-induced endocytosis of homomeric GluA1 receptors and that Arc knockdown has no effect on the rapid endocytosis of heteromeric AMPA receptors containing both GluA1 and GluA2. We report a novel binding interaction between Arc and the E3 ubiquitin ligase Nedd4-1 as identified in an unbiased yeast two-hybrid screen and show that the effect of reduced Arc expression on GluA1 endocytosis is occluded both by Nedd4-1 knockdown and by a variant of GluA1 that can not be ubiquitinated by Nedd4-1. Together, our results demonstrate a subtype-specific role for

Arc in the endocytic response to direct AMPA receptor stimulation in cultured cortical neurons and suggest that this specificity may be conferred by the substrate preference of Nedd4-1.

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Chapter 1. Introduction

AMPA-type Glutamate Receptors

AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) receptors mediate the fast transmission of electrical signals between neurons at excitatory synapses in the central nervous system. AMPA receptors are tetrameric, most often heteromers of GluA1-4 subunits. The inclusion of one or more GluA2 subunits determines the biophysical properties of the receptor, including its permeability to divalent cations (Cull-Candy et al. 2006; Isaac et al. 2007; Jonas 2000). The trafficking of AMPA receptors is also determined by subunit composition and different subunits are dominant in heteromeric receptors, depending upon the initiating stimulus (Bredt and Nicoll 2003; Malinow and Malenka 2002; Shepherd and Huganir 2007).

AMPA receptors are trafficked to and from the surface pool by tightly regulated exocytosis and endocytosis. A major point of regulation of this process is post-translational modification of the intracellular c-terminal tail of the receptor subunits. For example, it is well established that phosphorylation of the S845 residue of the GluA1 tail results in receptor exocytosis while dephosphorylation at the same residue targets AMPA receptors containing GluA1 for endocytosis in response to specific stimuli. Recent work identified the ubiquitination of the K868 residue of GluA1 as another such post-translational modification that can regulate AMPA receptor trafficking. Ubiquitination at

this locus by the Nedd4-1 E3 ligase is required for the endocytosis of GluA1-containing receptors in response to direct AMPA receptor stimulation (Lin et al. 2011; Schwarz et al. 2010).

Postsynaptic plasticity at most excitatory synapses in the cortex and hippocampus is ultimately expressed as a modification in the number of available AMPA receptors. The number of AMPA receptors at a given synapse is determined by an interplay between the total available surface receptor pool and the mean dwell time of these receptors in the postsynaptic density (Petrini et al. 2009). Thus, processes that regulate the number of total surface receptors also contribute to the modulation of synaptic strength.

The Activity-Regulated Cytoskeleton-Associated Protein, Arc

The activity-regulated cytoskeleton-associated protein, Arc (Lyford et al. 1995) also termed Arg3.1 (Link et al. 1995), is required for long-term memory formation in multiple learning paradigms (Guzowski et al. 2000; Holloway and McIntyre 2011; Plath et al. 2006; Ploski et al. 2008). Arc is a highly regulated protein found in the dendrites, postsynaptic terminals, and nuclei of glutamatergic neurons in the brain (Bloomer et al. 2007; Moga et al. 2004; Rodriguez et al. 2005).

Arc was identified in a screen for genes upregulated by seizure activity and coprecipitates with crude F-actin. Soon after its identification, it was shown that Arc mRNA induced by seizure localized to activated dendritic segments in an NMDA receptor-dependent manner and that Arc protein too was enriched at the site of local activity (Steward et al. 1998). Arc expression was found to be regulated by many plasticity-related processes, including synaptic activity, NMDA and AMPA receptor activation, brain derived neurotrophic factor (BDNF) stimulation, and cyclic adenosine monophosphate (cAMP) levels (Lyford et al. 1995; Rao et al. 2006; Steward and Worley 2001; Waltereit and Weller 2003; Yin et al. 2002). In behaving animals exposed to a novel environment, Arc expression increased in hippocampal areas CA1, CA3, and dentate gyrus (Guzowski et al. 2000; Guzowski et al. 1999). Arc appeared to be in the right places at the right times to play a key role in plasticity and, indeed, Arc was next shown to be required for long-term memory formation in vivo (Guzowski et al. 2000).

The expression and localization of Arc transcript and protein were very carefully characterized over the first decade after its discovery, but the mechanisms of Arc's role in memory formation remained a matter of speculation until 2006 when a series of papers demonstrated that Arc binds Endophilin 3 and Dynamin 2 (two proteins involved in receptor endocytosis), colocalizes with endosomal markers, reduces surface and synaptic AMPA receptor levels, and is necessary for the internalization of AMPA receptors in response to prolonged increases in synaptic activity (Chowdhury et al. 2006; Plath et al. 2006; Rial Verde et al. 2006; Shepherd et al. 2006). This led to a hypothesized role for Arc in homeostatic scaling and it was suggested that the observed defects in long-term

memory formation followed from the inability of neurons to appropriately adapt to increased activity (Shepherd et al. 2006).

Concurrent work showed that the acute RNAi-mediated knock-down of Arc immediately before or after high frequency stimulation results in a transient loss of potentiation, though consolidation still occurs, and that knockdown two hours after induction abolishes both late long term potentiation and layer-specific actin polymerization in vivo. Arc expression is required to maintain phosphorylation-dependent inhibition of cofilin, a promoter of actin depolymerization, and the application of jasplakinolide, an F-actin stabilizing drug, blocks the reversal of LTP by Arc knockdown (Messaoudi et al. 2007). In combination with the evidence for an association of Arc with the actin cytoskeleton, this new data led to a competing hypothesis that Arc plays a direct role in actin-mediated synaptic consolidation (Zhou et al. 2001).

Arc knockout animals show an enhanced early phase of hippocampal long term potentiation, followed by an immediate and steady decline to baseline. Low frequency stimulation in CA1 *in vitro* results in a decreased induction of long term depression in Arc knockout mice, also followed by a decline to baseline (Plath et al. 2006). Most recently, it was shown that mGluR- but not NMDA-mediated long term depression requires the rapid dendritic translation of Arc (Waung et al. 2008). In behavioral tests, the knockout animals demonstrate impaired long-term memory formation in both spatial memory and novel object recognition tasks (Plath et al. 2006).

In summary, the transcription, translation, half-life, and subcellular localization of Arc are all tightly controlled by neuronal activity (Shepherd and Bear 2011), leading to a precise spatio-temporal pattern of expression within each cell. Arc is necessary for many different forms of synaptic plasticity but efforts to understand the mechanistic role that Arc plays in each process have been complicated by the numerous neuronal functions that are attributed to this single protein (Korb and Finkbeiner 2011), including a role in regulating the trafficking of AMPA-type glutamate receptors (Chowdhury et al. 2006).

Arc and AMPA Receptor Trafficking

Arc expression is required for changes in surface and synaptic AMPA receptor numbers in response to acute stimuli on a time scale of minutes to hours (Messaoudi et al. 2007; Park et al. 2008; Waung et al. 2008) and is also required for the homeostatic scaling of AMPA receptors in response to prolonged changes in synaptic activity on a scale of hours to days (Shepherd et al. 2006). On this longer time scale, Arc acts in the nucleus to modulate the transcription of AMPA receptor subunits (Korb et al. 2013) and mediates long-term structural changes at the synapse via interactions with the actin cytoskeleton (Messaoudi et al. 2007; Peebles et al. 2010), thus regulating the total number of available AMPA receptors in the cell and influencing synaptic strength. In its more immediate role in the dendrites, Arc is believed to directly regulate surface receptor numbers by promoting the endocytosis of AMPA receptors.

Arc binds the endosome-associated proteins Endophilin 3 and Dynamin 2 and, when overexpressed in neurons, these complexes colocalize to AMPA receptor-containing vesicles. Overexpression of Arc leads to a reduction in numbers of both total surface and postsynaptic AMPA receptors. Deletion of the Endophilin-binding domain in Arc blocks this effect but overexpression of Endophilin 3 alone does not change surface AMPA receptor levels (Chowdhury et al. 2006). Together, these observations have led to the prevailing hypothesis that Arc mediates AMPA receptor endocytosis via an interaction with Endophilin 3.

References

Bloomer, W. A., H. M. VanDongen, and A. M. VanDongen. 2007. Activity-regulated cytoskeleton-associated protein Arc/Arg3.1 binds to spectrin and associates with nuclear promyelocytic leukemia (PML) bodies. *Brain Res* 1153: 20-33.

Bredt, D. S., and R. A. Nicoll. 2003. AMPA receptor trafficking at excitatory synapses. *Neuron* 40 (2): 361-79.

Chowdhury, Shoaib, Jason D. Shepherd, Hiroyuki Okuno, Gregory Lyford, Ronald S. Petralia, Niels Plath, Dietmar Kuhl, Richard L. Huganir, and Paul F. Worley. 2006. Arc/Arg3.1 Interacts with the Endocytic Machinery to Regulate AMPA Receptor Trafficking. *Neuron* 52 (3): 445-459.

Cull-Candy, S., L. Kelly, and M. Farrant. 2006. Regulation of Ca2+-permeable AMPA receptors: synaptic plasticity and beyond. *Curr Opin Neurobiol* 16 (3): 288-97.

Guzowski, J. F., G. L. Lyford, G. D. Stevenson, F. P. Houston, J. L. McGaugh, P. F. Worley, and C. A. Barnes. 2000. Inhibition of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory. *J Neurosci* 20 (11): 3993-4001.

Guzowski, J. F., B. L. McNaughton, C. A. Barnes, and P. F. Worley. 1999. Environment-specific expression of the immediate-early gene Arc in hippocampal neuronal ensembles. *Nat Neurosci* 2 (12): 1120-4.

Holloway, C. M., and C. K. McIntyre. 2011. Post-training disruption of Arc protein expression in the anterior cingulate cortex impairs long-term memory for inhibitory avoidance training. *Neurobiol Learn Mem* 95 (4): 425-32.

Isaac, J. T., M. C. Ashby, and C. J. McBain. 2007. The role of the GluR2 subunit in AMPA receptor function and synaptic plasticity. *Neuron* 54 (6): 859-71.

Jonas, P. 2000. The Time Course of Signaling at Central Glutamatergic Synapses. *News Physiol Sci* 15: 83-89.

Korb, E., and S. Finkbeiner. 2011. Arc in synaptic plasticity: from gene to behavior. *Trends Neurosci* 34 (11): 591-8.

Korb, E., C.L. Wilkinson, R.N. Delgado, K.L Lovero, and S. Finkbeiner. 2013. Arc in the nucleus regulates PML dependent GluA1 transcription and homeostatic plasticity. *Nat Neurosci*: In Press.

Lin, A., Q. Hou, L. Jarzylo, S. Amato, J. Gilbert, F. Shang, and H. Y. Man. 2011. Nedd4-mediated AMPA receptor ubiquitination regulates receptor turnover and trafficking. *J Neurochem* 119 (1): 27-39.

Link, W., U. Konietzko, G. Kauselmann, M. Krug, B. Schwanke, U. Frey, and D. Kuhl. 1995. Somatodendritic expression of an immediate early gene is regulated by synaptic activity. *Proc Natl Acad Sci U S A* 92 (12): 5734-8.

Lyford, G. L., K. Yamagata, W. E. Kaufmann, C. A. Barnes, L. K. Sanders, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, A. A. Lanahan, and P. F. Worley. 1995. Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. *Neuron* 14 (2): 433-45.

Malinow, R., and R. C. Malenka. 2002. AMPA receptor trafficking and synaptic plasticity. *Annu Rev Neurosci* 25: 103-26.

Messaoudi, E., T. Kanhema, J. Soule, A. Tiron, G. Dagyte, B. da Silva, and C. R. Bramham. 2007. Sustained Arc/Arg3.1 synthesis controls long-term potentiation consolidation through regulation of local actin polymerization in the dentate gyrus in vivo. *J Neurosci* 27 (39): 10445-55.

Moga, D. E., M. E. Calhoun, A. Chowdhury, P. Worley, J. H. Morrison, and M. L. Shapiro. 2004. Activity-regulated cytoskeletal-associated protein is localized to recently activated excitatory synapses. *Neuroscience* 125 (1): 7-11.

Park, Sungjin, Joo Min Park, Sangmok Kim, Jin-Ah Kim, Jason D. Shepherd, Constance L. Smith-Hicks, Shoaib Chowdhury, Walter Kaufmann, Dietmar Kuhl, and Alexey G. Ryazanov. 2008. Elongation Factor 2 and Fragile X Mental

Retardation Protein Control the Dynamic Translation of Arc/Arg3.1 Essential for mGluR-LTD. *Neuron* 59 (1): 70-83.

Peebles, C. L., J. Yoo, M. T. Thwin, J. J. Palop, J. L. Noebels, and S. Finkbeiner. 2010. Arc regulates spine morphology and maintains network stability in vivo. *Proc Natl Acad Sci U S A* 107 (42): 18173-8.

Petrini, E. M., J. Lu, L. Cognet, B. Lounis, M. D. Ehlers, and D. Choquet. 2009. Endocytic trafficking and recycling maintain a pool of mobile surface AMPA receptors required for synaptic potentiation. *Neuron* 63 (1): 92-105.

Plath, N., O. Ohana, B. Dammermann, M. L. Errington, D. Schmitz, C. Gross, X. Mao, A. Engelsberg, C. Mahlke, H. Welzl, U. Kobalz, A. Stawrakakis, E. Fernandez, R. Waltereit, A. Bick-Sander, E. Therstappen, S. F. Cooke, V. Blanquet, W. Wurst, B. Salmen, M. R. Bosl, H. P. Lipp, S. G. Grant, T. V. Bliss, D. P. Wolfer, and D. Kuhl. 2006. Arc/Arg3.1 is essential for the consolidation of synaptic plasticity and memories. *Neuron* 52 (3): 437-44.

Ploski, J. E., V. J. Pierre, J. Smucny, K. Park, M. S. Monsey, K. A. Overeem, and G. E. Schafe. 2008. The activity-regulated cytoskeletal-associated protein (Arc/Arg3.1) is required for memory consolidation of pavlovian fear conditioning in the lateral amygdala. *J Neurosci* 28 (47): 12383-95.

Rao, V. R., S. A. Pintchovski, J. Chin, C. L. Peebles, S. Mitra, and S. Finkbeiner. 2006. AMPA receptors regulate transcription of the plasticity-related immediate-early gene Arc. *Nat Neurosci* 9 (7): 887-95.

Rial Verde, Emiliano M., Jane Lee-Osbourne, Paul F Worley, Roberto Malinow, and Hollis T Cline. 2006. Increased Expression of the Immediate-Early Gene

Arc/Arg3.1 Reduces AMPA Receptor-Mediated Synaptic Transmission. *Neuron* 52 (3): 461-474.

Rodriguez, J. J., H. A. Davies, A. T. Silva, I. E. J. De Souza, C. J. Peddie, F. M. Colyer, C. L. Lancashire, A. Fine, M. L. Errington, T. V. P. Bliss, and M. G. Stewart. 2005. Long-term potentiation in the rat dentate gyrus is associated with enhanced Arc/Arg3.1 protein expression in spines, dendrites and glia (vol 21, pg 2384, 2005). *European Journal of Neuroscience* 22 (5): 1264-1264.

Schwarz, L. A., B. J. Hall, and G. N. Patrick. 2010. Activity-dependent ubiquitination of GluA1 mediates a distinct AMPA receptor endocytosis and sorting pathway. *J Neurosci* 30 (49): 16718-29.

Shepherd, J. D., and M. F. Bear. 2011. New views of Arc, a master regulator of synaptic plasticity. *Nat Neurosci* 14 (3): 279-84.

Shepherd, J. D., and R. L. Huganir. 2007. The cell biology of synaptic plasticity: AMPA receptor trafficking. *Annu Rev Cell Dev Biol* 23: 613-43.

Shepherd, Jason D., Gavin Rumbaugh, Jing Wu, Shoaib Chowdhury, Niels Plath, Dietmar Kuhl, Richard L. Huganir, and Paul F. Worley. 2006. Arc/Arg3.1 Mediates Homeostatic Synaptic Scaling of AMPA Receptors. *Neuron* 52 (3): 475-484.

Steward, O., C. S. Wallace, G. L. Lyford, and P. F. Worley. 1998. Synaptic activation causes the mRNA for the IEG Arc to localize selectively near activated postsynaptic sites on dendrites. *Neuron* 21 (4): 741-51.

Steward, O., and P. F. Worley. 2001. Selective targeting of newly synthesized Arc mRNA to active synapses requires NMDA receptor activation. *Neuron* 30 (1): 227-40.

Waltereit, R., and M. Weller. 2003. Signaling from cAMP/PKA to MAPK and synaptic plasticity. *Mol Neurobiol* 27 (1): 99-106.

Waung, M. W., B. E. Pfeiffer, E. D. Nosyreva, J. A. Ronesi, and K. M. Huber. 2008. Rapid translation of Arc/Arg3.1 selectively mediates mGluR-dependent LTD through persistent increases in AMPAR endocytosis rate. *Neuron* 59 (1): 84-97.

Yin, Y., G. M. Edelman, and P. W. Vanderklish. 2002. The brain-derived neurotrophic factor enhances synthesis of Arc in synaptoneurosomes. *Proc Natl Acad Sci U S A* 99 (4): 2368-73.

Zhou, Q., M. Xiao, and R. A. Nicoll. 2001. Contribution of cytoskeleton to the internalization of AMPA receptors. *Proc Natl Acad Sci U S A* 98 (3): 1261-6.

Chapter 2. Arc regulates the rapid endocytosis of homomeric GluA1 receptors in response to direct AMPA stimulation

Background

Though it is generally accepted that Arc is required for AMPA receptor endocytosis, the only published experiments directly testing this hypothesis did so by assaying the fraction of total surface receptors internalized over a single extended time interval (Chowdhury et al. 2006; Waung et al. 2008), which represents the net effect of receptor endocytosis, exocytosis, synthesis, and degradation integrated across the entirety of this period. In addition, reports conflict as to whether Arc mediates the endocytosis of GluA1-containing or GluA2-containing AMPA receptors. One study demonstrated that basal GluA1 endocytosis is decreased in neurons cultured from Arc knockout mice as measured by a live antibody uptake assay over a 30 minute epoch (Chowdhury et al. 2006). A second group published findings demonstrating that Arc decreases surface and synaptic expression of GluA2 but not GluA1 by a mechanism that requires both an interaction between Arc and Endophilin 3 and an interaction between the GluA2 c-terminus and the adaptor protein complex AP2, a component of the clathrin-coated vesicle endocytosis pathway (Rial Verde et al. 2006).

To address this apparent contradiction and to obtain a more dynamic measure of AMPA receptor trafficking, we began by quantifying the rate of endocytosis of

homomeric AMPA receptors containing only GluA1 or GluA2 while manipulating the levels of available Arc protein in individual cortical neurons. By studying these homomeric receptors, we were able to parse out the differential effect of Arc expression on the trafficking of each subunit type independently. Tagging these subunits with an environment-sensitive fluor enabled us to discern their subcellular localization in real time and to directly quantify the temporal dynamics of bulk receptor endocytosis in response to acute pharmacological stimulation, demonstrating a role for Arc in AMPA receptor trafficking on a significantly shorter time scale than observed in previous studies.

Results

Arc is required for the rapid endocytosis of GluA1 but not GluA2 in response to direct AMPA stimulation

To elucidate the role of Arc in the AMPA receptor trafficking response to acute stimulation, we created a simplified culture system in which we could assess the dynamics of homomeric AMPA receptor trafficking in real time. Because Arc is both regulated by (Bloomer et al. 2008; Park et al. 2008; Rao et al. 2006; Wang et al. 2009; Waung et al. 2008; Yilmaz-Rastoder et al. 2010) and is a determinant of (Rial Verde et al. 2006; Shepherd et al. 2006) synaptic activity, we opted to focus on cell-

autonomous effects by manipulating Arc expression in individual cells rather than in the culture at large. This way, we could be certain that any effects we observed were due to altered Arc expression in the cell in question and not because of changes in presynaptic input.

In order to knock down endogenous Arc expression, we generated a short-hairpin RNA (shRNA) targeting bp 426 to 444 of the Arc coding sequence and a corresponding scrambled shRNA control. Efficacy of the knockdown was assessed by co-expressing rat Arc cDNA with either anti-Arc or control shRNA in HEK293 cells and probing the cell lysates for Arc protein on western blot. The anti-Arc shRNA resulted in a significant decrease in Arc protein expression as compared to the scrambled control. In order to rescue Arc expression in these cells, we introduced 5 silent point mutations in the targeted region of the Arc cDNA construct, rendering it insensitive to the shRNA-mediated knockdown (Fig 1a-c).

To quantify AMPA receptor endocytosis rates, we co-transfected cortical neurons with the shRNA constructs and either GluA1 or GluA2 tagged at its N-terminal extracellular domain with super ecliptic pHluorin (SEP) (Lin and Huganir 2007; Yudowski et al. 2007). The pH-sensitive SEP fluoresces at the cell surface but is quenched in the acidic environment of endosomes (Miesenbock et al. 1998). Using fluorescence time-lapse imaging, we quantified changes in the steady-state population of tagged receptors at the cell surface and extrapolated the endocytosis time constant (τ) in response to bath drug application. By studying homomeric receptors, we eliminated the

confounding effects inherent in studying endogenous AMPA receptors, which primarily exist as heteromers (Lu et al. 2009).

After establishing a baseline level of fluorescence in each cell, we stimulated with 100 μM AMPA and imaged the decline in SEP signal. Direct AMPA receptor stimulation has been shown to induce dynamin-dependent, clathrin-mediated endocytosis of both GluA1 and GluA2 (Lin et al. 2000). In our system, bath application of AMPA resulted in a rapid endocytosis of SEP-GluA1 in 14 - 15 DIV cortical neurons transfected with the scrambled shRNA ($\tau = 0.99 \pm 0.27$ min) while, in cells transfected with the anti-Arc shRNA, SEP-GluA1 endocytosis in response to AMPA was significantly slowed ($\tau = 1.95 \pm 0.73$ min) (Fig 2a,b). In contrast, there was no difference between knockdown and control neurons in the time constant of AMPA-induced SEP-GluA2 endocytosis (Fig 2c,d).

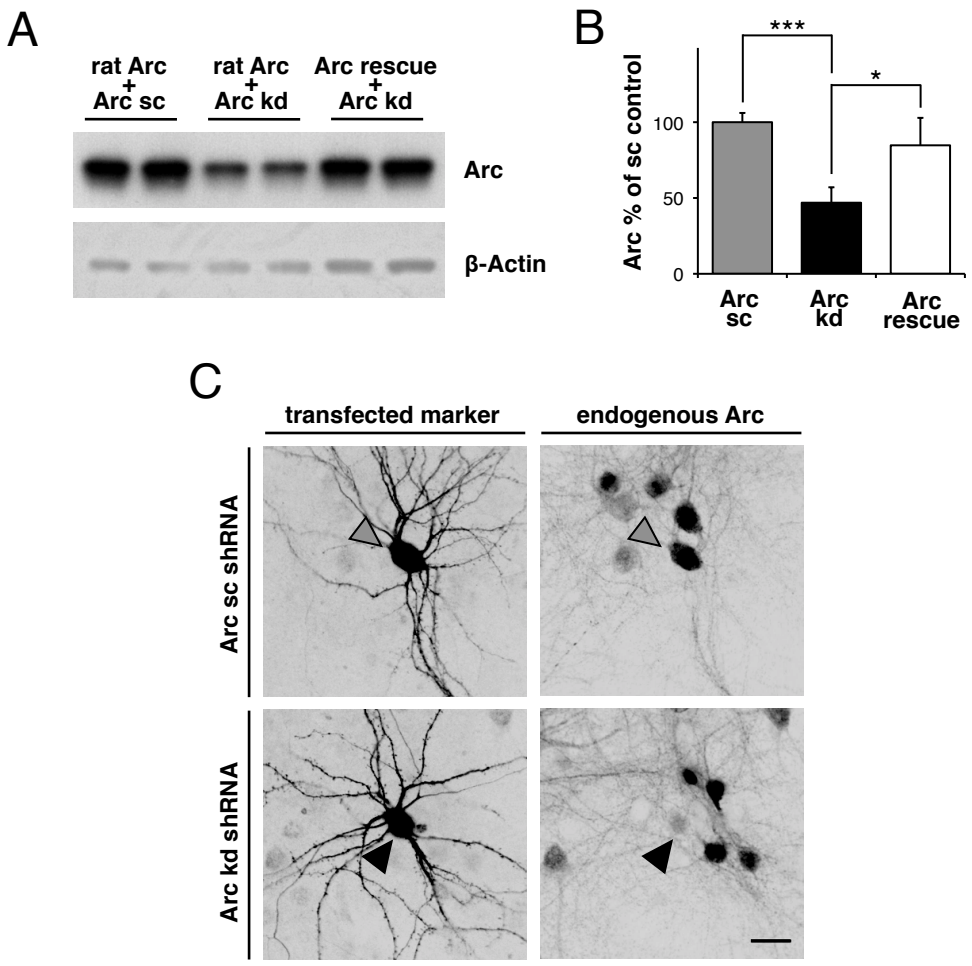


Figure 1. shRNA-mediated knockdown of Arc protein expression in individual rat cortical neurons

(A) Representative western blot and **(B)** densitometric quantification demonstrating efficacy of the Arc knockdown shRNA (Arc kd) as compared to the scrambled control shRNA (Arc sc) and successful expression of the shRNA-resistant Arc (Arc rescue) in HEK293 cells expressing rat Arc protein (n=4 replicates, * $p < 0.017$, *** $p < 0.0003$, $p \geq 0.017$ is non-significant). **(C)** Representative images showing knockdown of endogenous Arc protein in individual 14 DIV rat cortical neurons as assessed by immunostaining. Arrows indicate transfected cells and the scale bar corresponds to 20 μm. P values were calculated using an unpaired two-tailed t-test with the Bonferroni correction for multiple comparisons after a one-way ANOVA. Error bars represent S.D.

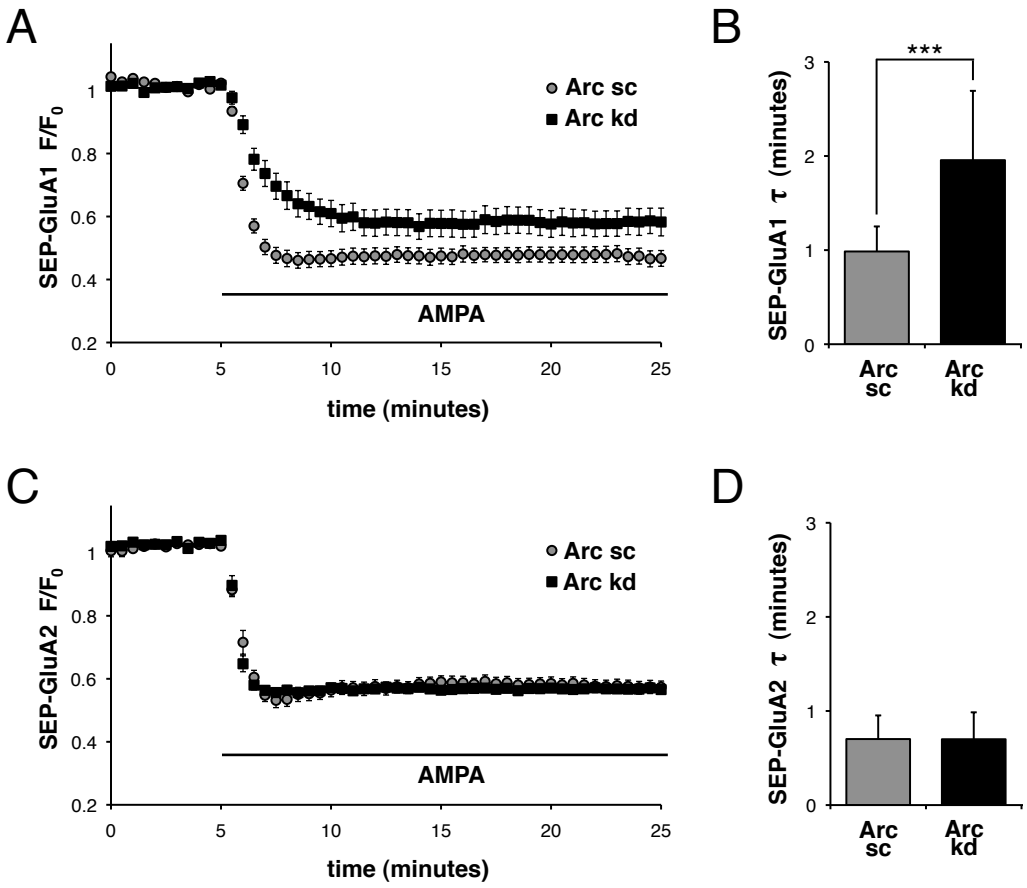


Figure 2. Arc is required for the rapid endocytosis of GluA1 but not GluA2 in response to direct AMPA receptor stimulation

(A) Normalized fluorescence traces showing endocytosis of SEP-GluA1 in response to 100 μM AMPA application in 14-15 DIV neurons expressing either the Arc knockdown shRNA (Arc kd) or the scrambled control shRNA (Arc sc) (error bars are S.E.M.) and **(B)** time constant (τ) derived from an exponential fit to the curve from each neuron represented in A (n=14 cells per condition, *** $p < 0.001$, error bars are S.D.). **(C)** Normalized fluorescence traces showing endocytosis of SEP-GluA2 in response to 100 μM AMPA application in 14-15 DIV neurons (error bars are S.E.M.) and **(D)** time constant (τ) derived from an exponential fit to the curve from each neuron represented in C (n=14 cells per condition, $p \geq 0.05$ is non-significant, error bars are S.D.). P values were calculated using an unpaired two-tailed t-test.

The rate of NMDA-induced AMPA receptor endocytosis is independent of Arc protein levels

Arc has been shown to be involved in the endocytosis of GluA1 in response to mGluR receptor stimulation (Waung et al. 2008) and we have demonstrated it to be necessary for the rapid endocytic response to AMPA. Stimulation of NMDA-type glutamate receptors is also known to induce the rapid endocytosis of AMPA receptors (Lin et al. 2000) and so we next asked whether Arc regulates AMPA receptor endocytosis in response to NMDA stimulation. We tested the effect of Arc knockdown on the rate of GluA1 and GluA2 endocytosis in response to bath application of 40 μM NMDA in low magnesium Tyrodes with 10 μM glycine as a co-agonist. To ensure the absence of drug-induced toxicity, we verified that the neurons demonstrated a complete recovery to baseline fluorescence after NMDA washout. Interestingly, we observed no significant difference between Arc knockdown and scrambled control in the time constants of the resultant SEP-GluA1 or SEP-GluA2 endocytosis (Fig 3a-d). This indicated that Arc is dispensable for the initial endocytic response to NMDA stimulation even though it does modulate the rapid response to direct AMPA receptor stimulation.

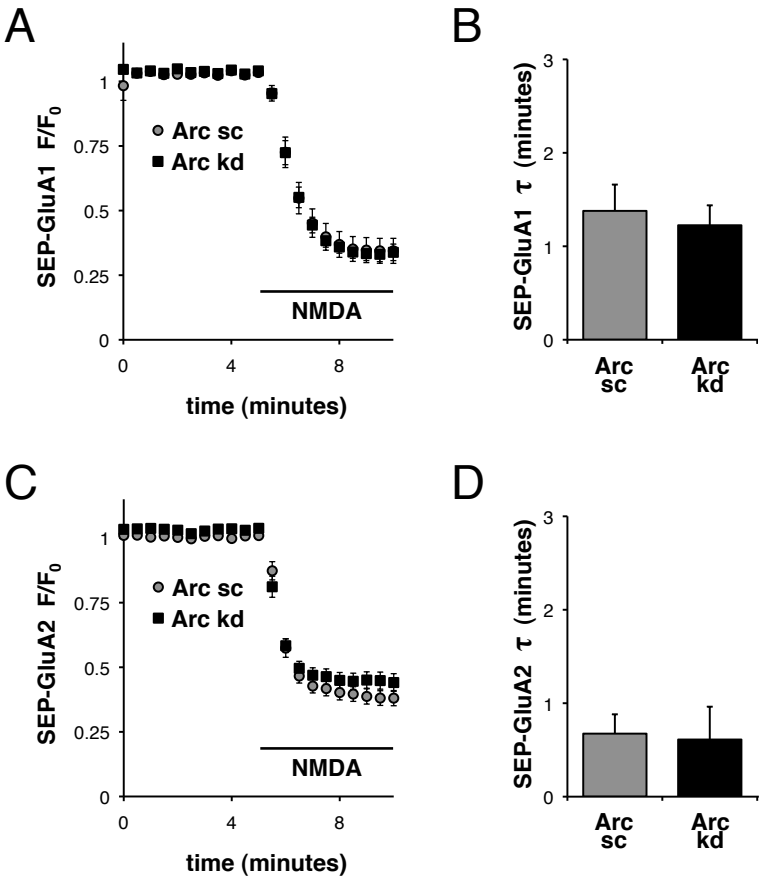


Figure 3. Arc is not required for the rapid endocytosis of GluA1 or GluA2 in response to NMDA receptor stimulation

(A) Normalized fluorescence traces showing endocytosis of SEP-GluA1 in response to 40 μM NMDA + 10 μM glycine application in 14-15 DIV neurons expressing either the Arc knockdown shRNA (Arc kd) or the scrambled control shRNA (Arc sc) (error bars are S.E.M.) and **(B)** time constant (τ) derived from an exponential fit to the curve from each neuron represented in A (n=14 cells per condition, error bars are S.D.). **(C)** Normalized fluorescence traces showing endocytosis of SEP-GluA2 in response to 40 μM NMDA + 10 μM glycine application in 14-15 DIV neurons (error bars are S.E.M.) and **(D)** time constant (τ) derived from an exponential fit to the curve from each neuron represented in C (n=14 cells per condition, error bars are S.D.). P values were calculated using an unpaired two-tailed t-test ($p \geq 0.05$ is non-significant).

Arc knockdown slows AMPA-induced GluA1 endocytosis in 7 - 8 DIV cortical neurons

To further characterize the role of Arc in regulating the endocytosis of GluA1 in response to direct stimulation, we tested the effect in younger (7 - 8 DIV) cortical neurons and again found that Arc knockdown slows endocytosis of GluA1 ($\tau = 2.26 \pm 0.46$ min) as compared to control ($\tau = 1.50 \pm 0.39$ min) in response to 100 μM AMPA. To verify that the effects observed were due to the knockdown of Arc and not due to non-specific effects of the transfected shRNA, we reintroduced shRNA-resistant Arc driven by a constitutive promoter and found that it rescued the rate of AMPA-induced GluA1 endocytosis to near control ($\tau = 1.43 \pm 0.53$ min) (Fig 4a,b).

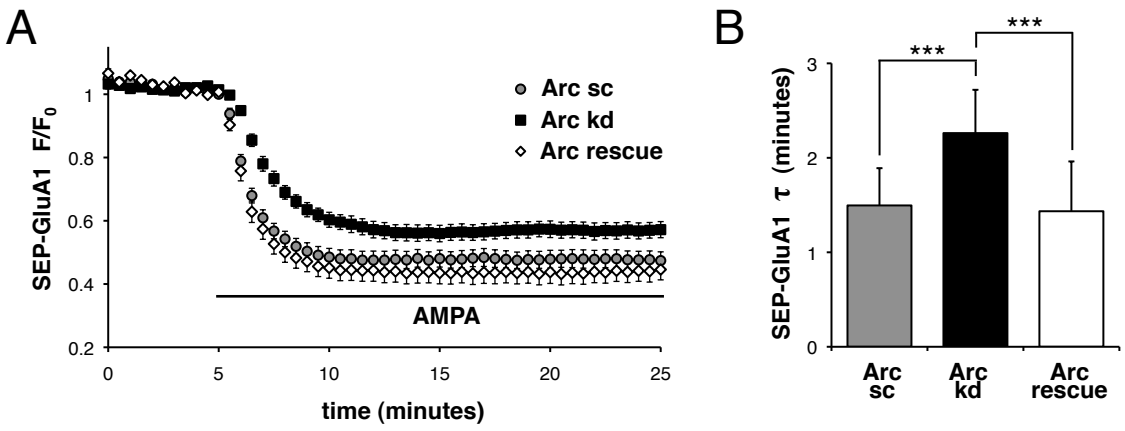


Figure 4. shRNA-resistant Arc rescues AMPA-induced GluA1 endocytosis after Arc knockdown

(A) Normalized fluorescence traces showing endocytosis of SEP-GluA1 in response to 100 μM AMPA application in 7-8 DIV neurons (error bars are S.E.M.) and **(B)** time constant (τ) derived from an exponential fit to the curve from each neuron represented in **A** (n=16 cells per condition, *** $p < 0.0002$, $p > 0.0083$ is non-significant, error bars are S.D.). Sample populations were compared using an unpaired two-tailed t-test with the Bonferroni correction for multiple comparisons after a one-way ANOVA.

[N.B. Scramble and knockdown traces in this figure are the same as shown in Chapter 2, Figure 6 and the Bonferroni correction was calculated assuming 4 sample conditions]

Arc is required for the rapid endocytosis of GluA1/1 homomers but not GluA1/2 heteromers in response to direct AMPA stimulation

Given that the majority of AMPA receptors in the adult brain are not homomeric, we next asked whether our results would extend to heteromeric receptors containing GluA1. Exogenous expression of a tagged AMPA receptor subunit has been shown to result in the assembly of these subunits into functional homomeric AMPA receptors and co-transfection of a cell with two exogenous subunits leads to the formation of functional heteromers (Lee et al. 2004; Shi et al. 2001). To study the effect of Arc knockdown on heteromeric GluA1/2 receptors, we co-transfected cells with SEP-GluA1 and either HA-GluA1 or HA-GluA2 along with Arc knockdown or control shRNA. To further ensure that the majority of SEP-tagged receptors that we visualized contained both subunits, we transfected the HA-tagged subunits at a molar ratio of 2:1 as compared to SEP-GluA1.

As expected, Arc knockdown slowed the endocytosis of SEP-GluA1/HA-GluA1 ($\tau = 1.86 \pm 0.67$ min) as compared to control ($\tau = 1.31 \pm 0.45$ min). Interestingly, Arc knockdown had no significant effect on the rate of endocytosis of SEP-GluA1/HA-GluA2 ($\tau = 1.50 \pm 0.42$ min) as compared to the control shRNA ($\tau = 1.37 \pm 0.52$ min). This led us to conclude that the Arc-independent mechanism that mediates GluA2 endocytosis in response to direct AMPA stimulation is dominant in determining the rate of AMPA-induced endocytosis of GluA1/2 heteromers in cultured cortical neurons (Fig 5).

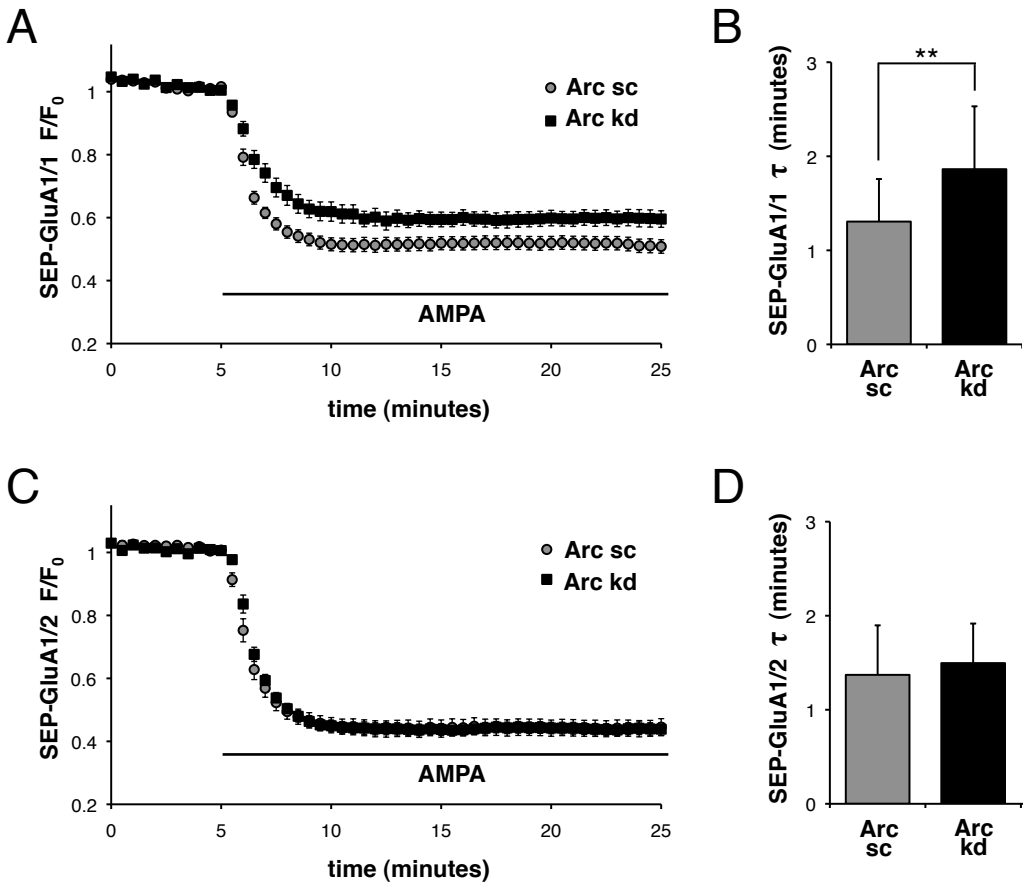


Figure 5. Arc is required for the rapid endocytosis of GluA1/1 homomers but not GluA1/2 heteromers in response to direct AMPA receptor stimulation
(A) Normalized fluorescence traces showing endocytosis of SEP-GluA1/HA-GluA1 in response to 100 μM AMPA application in 7-8 DIV neurons (error bars are S.E.M.) and **(B)** time constant (τ) derived from an exponential fit to the curve from each neuron represented in **A** (n=16 cells per condition). **(C)** Normalized fluorescence traces showing endocytosis of SEP-GluA1/HA-GluA2 in response to 100 μM AMPA application in 7-8 DIV neurons (error bars are S.E.M.) and **(D)** time constant (τ) derived from an exponential fit to the curve from each neuron represented in **C** (n=16 cells per condition). Sample populations were compared using an unpaired two-tailed t-test (** $p < 0.01$, $p \geq 0.05$ is non-significant). Error bars represent S.D.

Discussion

The studies that first identified Arc's role in AMPA receptor trafficking disagreed as to whether the effects were limited to GluA1- or GluA2-containing receptors. Interpretation of these results is complicated by the fact that most AMPA receptors in the rodent cortex are heterotetramers and therefore observed changes in surface levels of one subunit can be driven by processes regulating the trafficking of the other subunit. Experiments studying changes in endogenous AMPA receptor distributions are important for their physiological relevance but are also limited in their ability to dissect the mechanisms underlying these changes. By creating a simplified system in which we were able to study the effect of Arc protein levels on the acute trafficking response of homomeric AMPA receptors, we demonstrated that Arc regulates the rapid endocytosis of GluA1 homomers but not GluA2-containing receptors in response to direct AMPA receptor activation in cultured cortical neurons (Fig 1,3).

How do we reconcile our result with the previously published work showing that Arc regulates the endocytosis of GluA2 (Rial Verde et al. 2006)? We know that Arc regulates the available pool of AMPA receptors at multiple levels, including through regulating the transcription and total availability of AMPA receptor subunits. While our results point to a GluA1-specific role for Arc in the rapid response to direct AMPA stimulation, we cannot rule out the possibility that Arc may also regulate the trafficking of GluA2 by one of these other mechanisms in response to alternative stimuli.

In our experiments, the GluA2 subunit appears to be dominant in the trafficking of GluA1/2 heteromers, rendering the rate of AMPA-induced endocytosis of GluA1/2 receptors independent of Arc (Fig 4). While it is intriguing to speculate on a role for Arc specific to GluA2-lacking calcium-permeable receptors, it is also important to remember that we have only characterized the endocytosis of GluA1/2 in response to bath AMPA application. There may well exist other physiological circumstances in which the GluA2 subunit is not targeted for internalization and instead the Arc-regulated endocytosis of GluA1 becomes the rate-determining factor for the heteromer.

Methods

Transfection of primary neuronal cultures: Neurons were dissociated from E20-21 rat embryonic cortex and cultured in Neurobasal media supplemented with serum-free B-27, L-glutamine and penicillin/streptomycin antibiotics. All animal procedures were approved by the University of California, San Francisco Institutional Animal Care and Use Committee. Cells were transfected with lipid-DNA complexes at 6 - 7 or 13 - 14 days in vitro and were imaged, harvested or fixed 24 - 48 hours post transfection. Short hairpin RNA sequences driven by the pSilencer2.0 promoter were cloned into a FUGW expression vector, which also contained mCherry under an independent promoter. All other proteins were expressed using pGW1 and pCaggs CMV and β-actin based expression vectors.

Immunocytochemistry: Cells were fixed in 4% PFA with 4% sucrose, permeabilized with 0.1% Triton X-100 in PBS for 10 min, and blocked in PBS with 2% donkey serum, 3% BSA, and 0.1% Triton for 1 hour. Cells were incubated in a polyclonal rabbit anti-Arc antibody (Peebles et al. 2010) overnight at 4°C and fluorescent secondary antibodies were applied for 1 hour at room temperature. Images were acquired with a Zeiss LSM confocal microscope. Background subtraction and intensity measurements were performed in ImageJ.

pHluorin imaging: Healthy neurons with numerous dendritic spines were selected based upon the diffuse mCherry morphology marker. Images were acquired every 30 seconds in the green and red channels using a 1.45 NA, 60X objective. Cells were perfused at a rate of 1mL/min with Tyrodes solution (120mM NaCl, 5mM KCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 25mM HEPES, 30mM glucose, pH 7.35) with 1µM Tetrodotoxin Citrate (TTX) to block action potentials. After establishing a steady baseline, cells were stimulated with either 40µM *N*-Methyl-D-aspartic acid (NMDA) and 10µM glycine in low magnesium (0.5mM) Tyrodes + 1µM TTX or with 100µM (*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) in standard Tyrodes + 1µM TTX. Rapid solution changes were automated using the Warner VT-8 valve timer. To minimize drift of the focal plane, all imaging and perfusion equipment was housed within an environmental chamber held at 37 degrees and Nikon's infrared Perfect Focus System was engaged to

counteract any remaining z-axis drift. Images were aligned in the red channel using the MultiStackReg plugin for NIH ImageJ and dendritic regions of interest were defined manually. Endocytic time constants (τ) were estimated by fitting a single exponential to the normalized intensity data averaged over 3 dendritic segments from each cell in the green channel using the NonlinearModelFit least-squares function in Wolfram Mathematica. (See Appendix I for more detail).

Western Blots: Transfected HEK293 cells were lysed in phospho-buffered saline supplemented with 1mM EDTA, 0.1% SDS, 1% Triton-X and protease inhibitors. Proteins were eluted by boiling in 2x Laemlli buffer and were separated on an SDS-PAGE gel. Western blots were probed using polyclonal rabbit anti-Arc (Peebles et al. 2010) and monoclonal mouse anti-β-actin (Sigma clone AC-15) primary with HRP-conjugated secondary antibodies (Dako). Band intensities were calculated using NIH ImageJ densitometry and normalized to the mean intensity of the corresponding internal controls within each experimental replicate.

References

Bloomer, W. A., H. M. VanDongen, and A. M. VanDongen. 2008. Arc/Arg3.1 translation is controlled by convergent N-methyl-D-aspartate and Gs-coupled receptor signaling pathways. *J Biol Chem* 283 (1): 582-92.

Chowdhury, Shoaib, Jason D. Shepherd, Hiroyuki Okuno, Gregory Lyford, Ronald S. Petralia, Niels Plath, Dietmar Kuhl, Richard L. Huganir, and Paul F. Worley. 2006. Arc/Arg3.1 Interacts with the Endocytic Machinery to Regulate AMPA Receptor Trafficking. *Neuron* 52 (3): 445-459.

Lee, S. H., A. Simonetta, and M. Sheng. 2004. Subunit rules governing the sorting of internalized AMPA receptors in hippocampal neurons. *Neuron* 43 (2): 221-36.

Lin, D. T., and R. L. Huganir. 2007. PICK1 and phosphorylation of the glutamate receptor 2 (GluR2) AMPA receptor subunit regulates GluR2 recycling after NMDA receptor-induced internalization. *J Neurosci* 27 (50): 13903-8.

Lin, J. W., W. Ju, K. Foster, S. H. Lee, G. Ahmadian, M. Wyszynski, Y. T. Wang, and M. Sheng. 2000. Distinct molecular mechanisms and divergent endocytotic pathways of AMPA receptor internalization. *Nat Neurosci* 3 (12): 1282-90.

Lu, W., Y. Shi, A. C. Jackson, K. Bjorgan, M. J. During, R. Sprengel, P. H. Seeburg, and R. A. Nicoll. 2009. Subunit composition of synaptic AMPA receptors revealed by a single-cell genetic approach. *Neuron* 62 (2): 254-68.

Miesenbock, G., D. A. De Angelis, and J. E. Rothman. 1998. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* 394 (6689): 192-5.

Park, Sungjin, Joo Min Park, Sangmok Kim, Jin-Ah Kim, Jason D. Shepherd, Constance L. Smith-Hicks, Shoaib Chowdhury, Walter Kaufmann, Dietmar Kuhl, and Alexey G. Ryazanov. 2008. Elongation Factor 2 and Fragile X Mental Retardation Protein Control the Dynamic Translation of Arc/Arg3.1 Essential for mGluR-LTD. *Neuron* 59 (1): 70-83.

Peebles, C. L., J. Yoo, M. T. Thwin, J. J. Palop, J. L. Noebels, and S. Finkbeiner. 2010. Arc regulates spine morphology and maintains network stability in vivo. *Proc Natl Acad Sci U S A* 107 (42): 18173-8.

Rao, V. R., S. A. Pintchovski, J. Chin, C. L. Peebles, S. Mitra, and S. Finkbeiner. 2006. AMPA receptors regulate transcription of the plasticity-related immediate-early gene Arc. *Nat Neurosci* 9 (7): 887-95.

Rial Verde, Emiliano M., Jane Lee-Osbourne, Paul F Worley, Roberto Malinow, and Hollis T Cline. 2006. Increased Expression of the Immediate-Early Gene Arc/Arg3.1 Reduces AMPA Receptor-Mediated Synaptic Transmission. *Neuron* 52 (3): 461-474.

Shepherd, Jason D., Gavin Rumbaugh, Jing Wu, Shoaib Chowdhury, Niels Plath, Dietmar Kuhl, Richard L. Huganir, and Paul F. Worley. 2006. Arc/Arg3.1 Mediates Homeostatic Synaptic Scaling of AMPA Receptors. *Neuron* 52 (3): 475-484.

Shi, S., Y. Hayashi, J. A. Esteban, and R. Malinow. 2001. Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. *Cell* 105 (3): 331-43.

Wang, Y., F. Zheng, X. Zhou, Z. Sun, and H. Wang. 2009. Converging signal on ERK1/2 activity regulates group I mGluR-mediated Arc transcription. *Neurosci Lett* 460 (1): 36-40.

Waung, M. W., B. E. Pfeiffer, E. D. Nosyreva, J. A. Ronesi, and K. M. Huber. 2008. Rapid translation of Arc/Arg3.1 selectively mediates mGluR-dependent LTD through persistent increases in AMPAR endocytosis rate. *Neuron* 59 (1): 84-97.

Yilmaz-Rastoder, E., T. Miyamae, A. E. Braun, and E. Thiels. 2010. LTP- and LTD-inducing stimulations cause opposite changes in arc/arg3.1 mRNA level in hippocampal area CA1 in vivo. *Hippocampus*.

Yudowski, G. A., M. A. Puthenveedu, D. Leonoudakis, S. Panicker, K. S. Thorn, E. C. Beattie, and M. von Zastrow. 2007. Real-time imaging of discrete exocytic events mediating surface delivery of AMPA receptors. *J Neurosci* 27 (41): 11112-21.

Chapter 3. Arc interacts with the E3 Ubiquitin Ligase, Nedd4-1 to modulate the rapid endocytosis of GluA1

Background

In the previous chapter, we showed that Arc modulates the rate of GluA1 endocytosis in response to direct AMPA receptor stimulation. This finding fits with previous studies demonstrating roles for Arc in increasing the rate of GluA1 endocytosis after the induction of long term depression (LTD) (Waung et al. 2008) and in the constitutive endocytosis of AMPA receptors in unstimulated neurons (Chowdhury et al. 2006). The constitutive endocytosis of GluA1 was found to be dependent upon the binding interaction between Arc and Endophilin 3 and so we next tested whether this interaction is also required for the rapid endocytosis of GluA1 in response to direct AMPA stimulation.

We found that Arc missing the Endophilin 3 binding domain was capable of fully rescuing AMPA-induced GluA1 endocytosis. In combination with the preceding findings, this demonstrated that Arc modulates the rapid endocytosis of GluA1 homomers in response to AMPA but not NMDA stimulation via a pathway that does not require a binding interaction with endophilin. Since no such pathway has been reported, we hypothesized that the mechanism involved a yet-to-be discovered interaction between Arc and a novel binding partner. We performed a yeast two-hybrid screen to identify

proteins from a Sprague-Dawley rat brain cDNA library that directly interact with full length Arc.* One of the candidates identified was the HECT family E3 ubiquitin ligase, Nedd4-1. Nedd4-1 has been shown to ubiquitinate the c-terminus of GluA1 but not GluA2 in response to AMPA but not NMDA receptor activation and this ubiquitination is required for the subsequent endocytosis of GluA1 (Lin et al. 2011; Schwarz et al. 2010). Given the specificity for AMPA-induced GluA1 endocytosis on the part of both Nedd4-1 and Arc, we hypothesized that some part of Arc's role in modulating surface AMPA receptor numbers might be effected through an interaction with Nedd4-1.

Results

Rapid AMPA-induced GluA1 endocytosis is independent of the Arc-Endophilin 3 interaction

We deleted amino acids 91-100, the known Endophilin binding domain (Chowdhury et al. 2006), from our shRNA-insensitive Arc construct. Surprisingly, we found that this Arc deletion mutant fully rescued the rate of GluA1 endocytosis in response to 100 μM AMPA after Arc knockdown ($\tau = 1.35 \pm 0.44$ min), leading us to conclude that Arc's role in regulating rapid AMPA-induced GluA1 endocytosis is independent of its ability to bind Endophilin 3 and that Arc must be acting in some other

* Work done by Carol Lee Wilkinson

pathway in response to direct AMPA stimulation (Fig 6a,b).

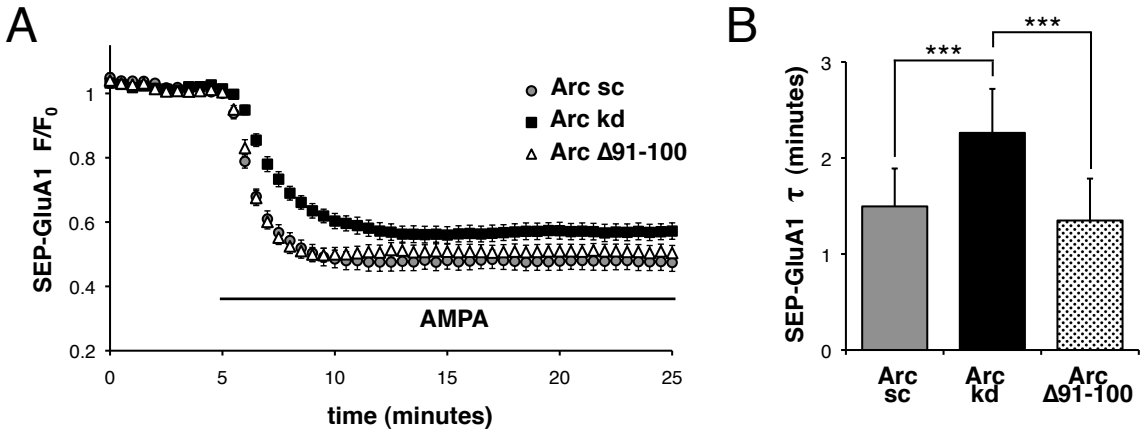


Figure 6. GluA1 endocytosis in response to direct AMPA receptor stimulation is independent of the binding interaction between Arc and Endophilin 3
(A) Normalized fluorescence traces showing endocytosis of SEP-GluA1 in response to 100 μM AMPA application in 7-8 DIV neurons (error bars are S.E.M.) and **(B)** time constant (τ) derived from an exponential fit to the curve from each neuron represented in **A** (n=16 cells per condition, *** $p < 0.0002$, $p > 0.0083$ is non-significant, error bars are S.D.). Sample populations were compared using an unpaired two-tailed t-test with the Bonferroni correction for multiple comparisons after a one-way ANOVA.

[N.B. Scramble and knockdown traces in this figure are the same as shown in Chapter 1, Figure 4 and the Bonferroni correction was calculated assuming 4 sample conditions]

Arc binds the E3 ubiquitin ligase Nedd4-1

To verify the binding interaction between Arc and Nedd4-1, we transfected HEK293 cells with untagged rat Arc and Nedd4-1 expression constructs and immunoprecipitated Arc from whole cell lysate using rabbit Arc antibody and IgG as a negative control. We separated the precipitated proteins on an SDS-PAGE gel and probed for Arc and Nedd4-1. We found that Nedd4-1 co-precipitated with Arc but was absent in

the IgG-only control lanes, confirming that Arc and Nedd4-1 are present in the same protein complexes (Fig 7a,b). This result, in combination with the yeast two-hybrid screen, indicates that Arc binds directly to Nedd4-1.

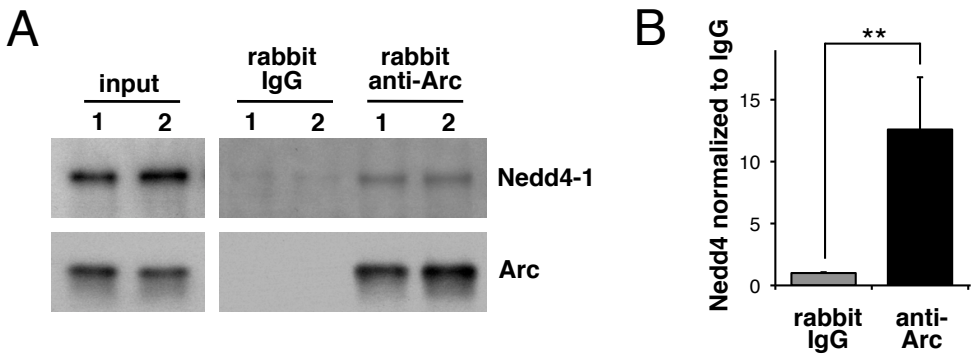


Figure 7. Arc binds the E3 Ubiquitin Ligase Nedd4-1
(A) Representative co-immunoprecipitation experiment showing two independent replicates (1 & 2) and **(B)** densitometric quantification of Nedd4-1 co-precipitation with rabbit anti-Arc antibody as compared to the IgG control (n=4 replicates). P values were calculated using an unpaired two-tailed t-test (** $p < 0.01$, error bars represent S.D.).

Nedd4-1 knockdown occludes the effect of Arc knockdown on AMPA-induced GluA1 endocytosis

Nedd4-1 knockdown has been shown to block AMPA-induced reduction in surface GluA1 in cultured hippocampal neurons (Schwarz et al. 2010). To manipulate Nedd4-1 expression in our system, we made an shRNA expression construct targeting the same region (bp 646 to 664) of the rat Nedd4-1 coding sequence and the corresponding scrambled shRNA control (Schwarz et al. 2010). Efficacy of the knockdown was

assessed by co-expressing rat Nedd4-1 cDNA with either anti-Nedd4-1 or control shRNA in HEK293 cells and probing the cell lysates for Nedd4-1 protein on western blot. The anti-Nedd4-1 shRNA resulted in a significant decrease in Nedd4-1 protein expression as compared to the scrambled control (Fig 8a,b).

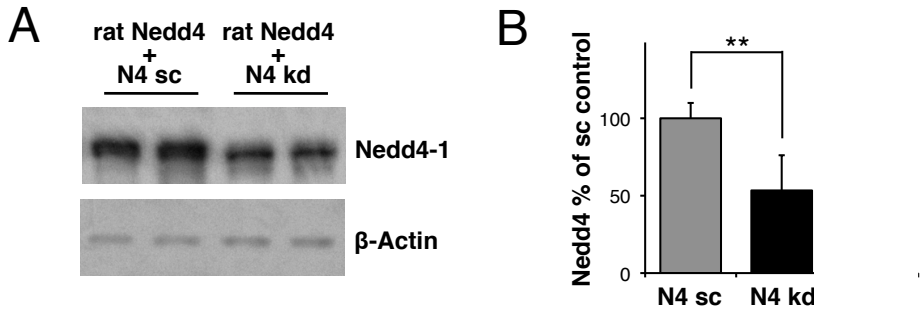


Figure 8. shRNA-mediated knockdown of Nedd4-1 protein expression in individual rat cortical neurons
(A) Representative western blot and **(B)** densitometric quantification demonstrating efficacy of the Nedd4-1 knockdown shRNA (N4 kd) as compared to the scrambled control shRNA (N4 sc) in HEK293 cells expressing rat Nedd4-1 protein. P values were calculated using an unpaired two-tailed t-test (n=4 replicates, ** $p < 0.01$, error bars represent S.D.).

Previous work has shown that Nedd4-1 expression regulates the internalization of surface GluA1 over a 10-15 minute epoch in response to direct AMPA receptor stimulation (Schwarz et al. 2010). To confirm that Nedd4-1 knockdown slows the rate of AMPA-induced GluA1 endocytosis in our system, we co-transfected cells with SEP-GluA1 and either anti-Nedd4-1 shRNA or the scrambled control. As expected, Nedd4-1 knockdown slowed AMPA-induced GluA1 endocytosis ($\tau = 1.88 \pm 0.48$ min) as compared to control ($\tau = 1.41 \pm 0.43$ min) (Fig 9a).

To test whether Arc's effect on rapid AMPA-induced GluA1 endocytosis requires endogenous levels of Nedd4-1 expression, we next co-transfected cells with SEP-GluA1, anti-Nedd4-1 shRNA and either anti-Arc shRNA or the scrambled control. We found that Arc knockdown does not significantly slow AMPA-induced GluA1 endocytosis further in the context of Nedd4-1 knockdown (Fig 9b), leading us to infer that Arc modulates AMPA-induced GluA1 endocytosis via a molecular pathway that includes Nedd4-1.

The rate of AMPA-induced GluA1(K868R) endocytosis is independent of Arc protein levels

To test whether this Arc-Nedd4-1 mechanism involves the ubiquitination of GluA1, we generated a point mutant of the SEP-GluA1 construct in which we replaced the lysine residue known to be ubiquitinated by Nedd4-1 (Lin et al. 2011) with an arginine. Consistent with previously published work showing that altered Nedd4-1 expression had no effect on the internalization of the GluA1 lysine mutant (Lin et al. 2011; Schwarz et al. 2010), Nedd4-1 knockdown in our system had no effect on the rate of AMPA-induced endocytosis of SEP-GluA1(K868R) (Fig 9c). We next tested whether manipulating Arc protein levels would have an effect on the endocytosis of this GluA1 mutant. Neither Arc knockdown nor subsequent rescue had a discernible effect on AMPA-induced GluA1(K868R) endocytosis (Fig 9d), suggesting that preventing

modification of the GluA1 subunit at K868 may block Arc-dependent AMPA receptor endocytosis in our system.

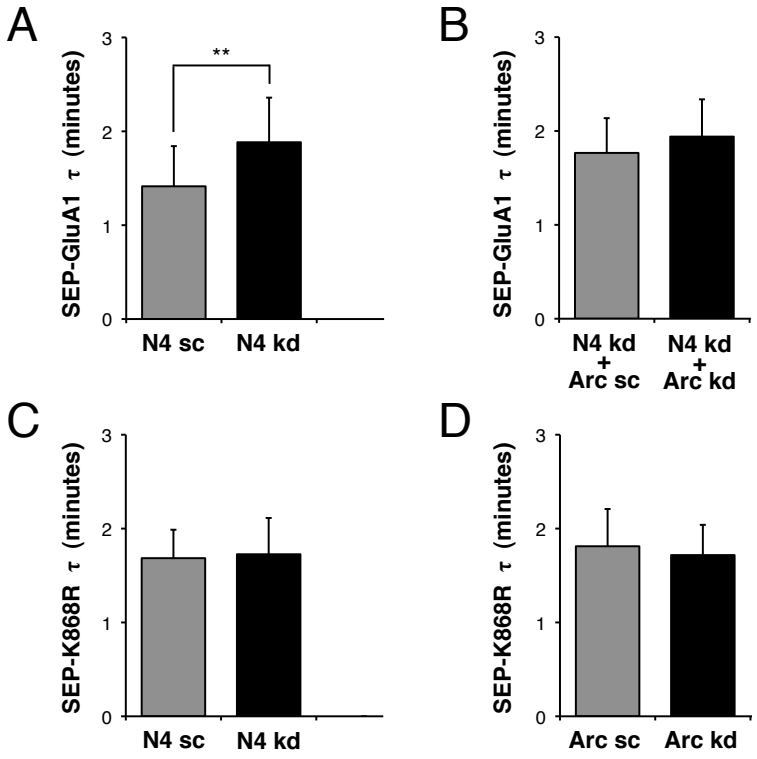


Figure 9. The effect of Arc knockdown on GluA1 endocytosis is occluded by both Nedd4-1 knockdown and by a GluA1 variant that can not be ubiquitinated by Nedd4-1 (A,B) Time constant (τ) for endocytosis of SEP-GluA1 in response to 100 μM AMPA in 7-8 DIV neurons (n=16 cells per condition). **(C,D)** Time constant (τ) for endocytosis of SEP-GluA1 K868R mutant in response to 100 μM AMPA in 7-8 DIV neurons (n=16 cells per condition). Sample populations were compared using an unpaired two-tailed t-test (** $p < 0.01$, $p \geq 0.05$ is non-significant). Error bars represent S.D.

Discussion

We showed that rapid GluA1 endocytosis in response to AMPA does not require a binding interaction between Arc and Endophilin 3 (Fig 1). While our finding does not contradict the hypothesis that Arc modulates the trafficking of GluA1 or GluA2-containing AMPA receptors via an interaction with Endophilin 3 in response to stimuli other than direct AMPA receptor stimulation, it did prompt us to look for an alternative mechanism by which Arc might regulate trafficking in this specific circumstance. In doing so, we discovered a novel direct binding interaction between Arc and the E3 ubiquitin ligase, Nedd4-1 (Fig 5).

AMPA receptors are trafficked to and from the surface pool by tightly regulated exocytosis and endocytosis. A major point of regulation of this process is post-translational modification of the intracellular c-terminal tail of the receptor subunits. For example, it is well established that phosphorylation of the S845 residue of the GluA1 tail results in receptor exocytosis while dephosphorylation at the same residue targets a GluA1-containing receptor for endocytosis in response to specific stimuli. Recent work identified the ubiquitination of the K868 residue of GluA1 as another such post-translational modification that can regulate AMPA receptor trafficking. Ubiquitination at this locus by the Nedd4-1 E3 ligase is required for the endocytosis of GluA1-containing receptors in response to direct AMPA receptor stimulation (Lin et al. 2011; Schwarz et al. 2010).

We hypothesized that Arc's role in GluA1 endocytosis might be mediated through the activity of Nedd4-1 and showed that reducing Arc expression had no discernable effect on GluA1 endocytosis in the context of either Nedd4-1 knockdown or a variant of GluA1 in which K868 has been mutated to an arginine (Fig 5). Although we did not directly test whether this is mediated by the binding interaction that we discovered between Arc and Nedd4-1 or whether Arc modulates the ubiquitination of GluA1, our experiments do provide epistatic evidence for a functional interaction between Arc and Nedd4-1 involving lysine 868 on the GluA1 c-terminal tail.

The previous studies that implicated Arc in AMPA receptor endocytosis found that Arc localizes to the same endocytic vesicles that contain AMPA receptors and that manipulating Arc levels changes the proportion of surface GluA1 internalized over a fixed interval of time. Here we further this hypothesis by providing a potential mechanistic link between Arc and a modification of the GluA1 c-terminus known to promote endocytosis. That GluA1 but not GluA2 is a substrate for Nedd4-1 ubiquitination (Schwarz et al. 2010) may well explain the GluA1-specific phenotype that we observed in response to direct AMPA receptor stimulation.

Methods

Transfection of primary neuronal cultures: Neurons were dissociated from E20-21 rat embryonic cortex and cultured in Neurobasal media supplemented with serum-free B-27, L-glutamine and penicillin/streptomycin antibiotics. All animal procedures were approved by the University of California, San Francisco Institutional Animal Care and Use Committee. Cells were transfected with lipid-DNA complexes at 6 - 7 or 13 - 14 days in vitro and were imaged, harvested or fixed 24 - 48 hours post transfection. Short hairpin RNA sequences driven by the pSilencer2.0 promoter were cloned into a FUGW expression vector, which also contained mCherry under an independent promoter. All other proteins were expressed using pGW1 and pCaggs CMV and β-actin based expression vectors.

pHluorin imaging: Healthy neurons with numerous dendritic spines were selected based upon the diffuse mCherry morphology marker. Images were acquired every 30 seconds in the green and red channels using a 1.45 NA, 60X objective. Cells were perfused at a rate of 1mL/min with Tyrodes solution (120mM NaCl, 5mM KCl, 2mM $CaCl_2$, 2mM $MgCl_2$, 25mM HEPES, 30mM glucose, pH 7.35) with 1μM Tetrodotoxin Citrate (TTX) to block action potentials. After establishing a steady baseline, cells were stimulated with either 40μM *N*-Methyl-D-aspartic acid (NMDA) and 10μM glycine in low magnesium (0.5mM) Tyrodes + 1μM TTX or with 100μM (*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) in standard Tyrodes + 1μM TTX. Rapid solution

changes were automated using the Warner VT-8 valve timer. To minimize drift of the focal plane, all imaging and perfusion equipment was housed within an environmental chamber held at 37 degrees and Nikon's infrared Perfect Focus System was engaged to counteract any remaining z-axis drift. Images were aligned in the red channel using the MultiStackReg plugin for NIH ImageJ and dendritic regions of interest were defined manually. Endocytic time constants (τ) were estimated by fitting a single exponential to the normalized intensity data averaged over 3 dendritic segments from each cell in the green channel using the NonlinearModelFit least-squares function in Wolfram Mathematica. (See Appendix I for more detail).

Yeast Two-Hybrid Screening: Screening was performed with the Clontech Matchmaker GAL4 kit according to the manufacturer's protocols. Briefly, the full-length Arc 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. 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 *Escherichia coli*, and sequenced. Hits were retransformed with empty pGBKT7 vector to eliminate nonspecific interactions with the vector.

Immunoprecipitations and Western Blots: Transfected HEK293 cells were lysed in

phospho-buffered saline supplemented with 1mM EDTA, 0.1% SDS, 1% Triton-X and protease inhibitors. For immunoprecipitation experiments, cleared lysates were incubated at 4°C overnight with primary antibody and Protein A magnetic beads (Pierce). Precipitated proteins were eluted by boiling in 2x Laemlli buffer and were separated on an SDS-PAGE gel. Western blots were probed using polyclonal rabbit anti-Arc (Peebles et al. 2010), polyclonal rabbit anti-Nedd4-1 (Abcam), and monoclonal mouse anti-β-actin (Sigma clone AC-15) primary and HRP-conjugated secondary antibodies (Dako). Band intensities were calculated using NIH ImageJ densitometry and normalized to the mean intensity of the corresponding internal controls within each experimental replicate.

References

Chowdhury, Shoaib, Jason D. Shepherd, Hiroyuki Okuno, Gregory Lyford, Ronald S. Petralia, Niels Plath, Dietmar Kuhl, Richard L. Huganir, and Paul F. Worley. 2006. Arc/Arg3.1 Interacts with the Endocytic Machinery to Regulate AMPA Receptor Trafficking. *Neuron* 52 (3): 445-459.

Lin, A., Q. Hou, L. Jarzylo, S. Amato, J. Gilbert, F. Shang, and H. Y. Man. 2011. Nedd4-mediated AMPA receptor ubiquitination regulates receptor turnover and trafficking. *J Neurochem* 119 (1): 27-39.

Peebles, C. L., J. Yoo, M. T. Thwin, J. J. Palop, J. L. Noebels, and S. Finkbeiner. 2010. Arc regulates spine morphology and maintains network stability in vivo. *Proc Natl Acad Sci U S A* 107 (42): 18173-8.

Schwarz, L. A., B. J. Hall, and G. N. Patrick. 2010. Activity-dependent ubiquitination of GluA1 mediates a distinct AMPA receptor endocytosis and sorting pathway. *J Neurosci* 30 (49): 16718-29.

Waung, M. W., B. E. Pfeiffer, E. D. Nosyreva, J. A. Ronesi, and K. M. Huber. 2008. Rapid translation of Arc/Arg3.1 selectively mediates mGluR-dependent LTD through persistent increases in AMPAR endocytosis rate. *Neuron* 59 (1): 84-97.

Chapter 4. Discussion

Given the evident importance of Arc in neuroplasticity, it is not surprising that its molecular function has been the subject of several studies in the past decade. While much has been learned about Arc, contradictions between the findings of these studies have also raised new questions. Arc is required for both increases and decreases in synaptic AMPA receptor numbers, depending upon the learning paradigm (Shepherd and Bear 2011). Arc is required for the homeostatic scaling of AMPA receptors in neuronal cultures experiencing global changes in activity (Shepherd et al. 2006) as well as for the scaling of receptor numbers at chronically stimulated individual synapses (Beique et al. 2011). At a structural level, Arc has been shown to interact with the actin cytoskeleton (Link et al. 1995; Lyford et al. 1995; Messaoudi et al. 2007) and to regulate dendritic spine morphology (Peebles et al. 2010). In addition to its dendritic functions, Arc is present in the nucleus where it likely regulates the transcription of plasticity-related genes, including GluA1 (Bloomer et al. 2007; Korb et al. 2013).

The complexity and interdependency of the processes that modulate synaptic strength makes them both fascinating and uniquely challenging to study. In order to unravel the specific mechanisms by which Arc regulates AMPA receptor availability in these various plasticity paradigms, we must carefully design studies that allow us to isolate the various aspects of Arc function using unambiguous functional readouts. Here, we attempted to address the basic question of whether Arc is involved in the rapid AMPA receptor trafficking response to acute pharmacological stimulation and to elucidate the

molecular mechanisms that functionally connect Arc to GluA1-specific AMPA receptor trafficking.

Summary of Findings

We found that Arc knockdown in cultured rat cortical neurons slows the rate of endocytosis of pHluorin-tagged GluA1 AMPA receptor subunits in response to direct AMPA receptor activation but has no effect on the endocytosis of pHluorin-tagged GluA2 in response to either AMPA or NMDA stimulation. We further demonstrated that this effect is specific to the AMPA-induced endocytosis of homomeric GluA1 receptors and that Arc knockdown has no effect on the rapid endocytosis of heteromeric AMPA receptors containing both GluA1 and GluA2. We reported a novel binding interaction between Arc and the E3 ubiquitin ligase Nedd4-1 as identified in an unbiased yeast two-hybrid screen and showed that the effect of reduced Arc expression on GluA1 endocytosis is occluded both by Nedd4-1 knockdown and by a variant of GluA1 that can not be ubiquitinated by Nedd4-1. Together, our results demonstrate a subtype-specific role for Arc in the endocytic response to direct AMPA receptor stimulation in cultured cortical neurons and suggest that this specificity may be conferred by the substrate preference of Nedd4-1.

Implications for neuroplasticity

Though ours is the first demonstration of a direct interaction between Arc and Nedd4-1, a study that looked for genes upregulated after chem-LTD induction identified the Nedd4 WW domain binding protein, *N4WBP4*, as one of nine genes that showed a significant increase in the same temporal pattern as Arc (Wibrand et al. 2006). While it is well established that Arc is crucial to long-term memory formation, Nedd4-1 has only recently begun to be studied in the context of synaptic plasticity. In addition to its function in regulating GluA1 endocytosis (Lin et al. 2011; Schwarz et al. 2010), Nedd4-1 is upregulated in response to sensory deprivation in the rodent barrel cortex (Butko et al. 2013) and has been shown to be necessary for synapse-specific scaling (Hou et al. 2011), as is Arc (Beique et al. 2011).

A study looking at receptor endocytosis in LTD found that Arc knockdown blocks the increase in GluA1 endocytosis observed by single epoch measurement 1 hour after mGluR-LTD induction (Waung et al. 2008). Our finding that Arc knockdown does not impede NMDA-induced AMPA receptor endocytosis (Fig 2) agrees with their data showing that Arc is not required for NMDA-mediated LTD. Given our results demonstrating a role for Nedd4-1 in the Arc-regulated endocytosis of GluA1 in response to direct AMPA stimulation, it would be worthwhile to test whether Nedd4-1 activity is also required for the increase in GluA1 endocytosis in mGluR-LTD. It would also be interesting to examine whether the interaction between Arc and Nedd4-1 is required for successful synapse-specific scaling and in other plasticity paradigms which depend upon

Arc-regulated GluA1 endocytosis.

Even though GluA2-lacking receptors represent only a small subpopulation of the AMPA receptors in adult cortical neurons, there is an increasing body of evidence demonstrating a role for these receptors in various forms of synaptic plasticity (Lee 2012; Man 2011; Shepherd 2012; Wiltgen et al. 2010). Although there is no significant change in the AMPA receptor I-V rectification (a measure of the relative number of GluA2-lacking AMPA receptors present in synapses) at baseline in neurons from Arc knockout mice (Plath et al. 2006), it is possible that there is a larger extrasynaptic surface pool of calcium-permeable receptors in the knockouts available for recruitment into the synapse. In fact, this may explain the enhanced early phase of long term potentiation observed in hippocampal slices from Arc knockout mice (Plath et al. 2006), which has been shown to be dependent upon recruitment from the extrasynaptic pool of receptors (Petrini et al. 2009) and to involve the insertion of GluA2-lacking AMPA receptors in certain systems (Man 2011).

Caveats

It is important to remember, however, that there are bound to be differences between any *in vitro* model system and its physiological *in vivo* correlate. There may be discrepancies due to subtle differences in the expression and trafficking of endogenous

vs. tagged receptor subunits or age-dependent changes in the subtypes of receptors expressed and the trafficking mechanisms employed by neurons across their postnatal development (Kumar et al. 2002). The simplified system that we employed allowed us parse out the subunit specificity of Arc-modulated AMPA receptor endocytosis with a clarity that would have been difficult to achieve otherwise but likely does not replicate all the nuances of endogenous AMPA receptor trafficking.

Proposed Model

Bearing in mind these caveats, we propose a model in which Arc facilitates an interaction between Nedd4-1 and GluA1, promoting the ubiquitination of the GluA1 c-terminus. We hypothesize that the tightly regulated spatio-temporal distribution of Arc protein may act to focus the process of Nedd4-1 mediated ubiquitination and subsequent GluA1 endocytosis to appropriate dendritic regions according to patterns of synaptic activity, providing a mechanistic link between complex neuronal activity and the highly regulated available AMPA receptor surface pool. More work is needed to fully characterize and probe the physical and functional interactions between Arc, Nedd4-1 and GluA1 and we look forward to future studies elucidating the relationship between these three determinants of synaptic plasticity.

References

Beique, J. C., Y. Na, D. Kuhl, P. F. Worley, and R. L. Huganir. 2011. Arc-dependent synapse-specific homeostatic plasticity. *Proc Natl Acad Sci U S A* 108 (2): 816-21.

Bloomer, W. A., H. M. VanDongen, and A. M. VanDongen. 2007. Activity-regulated cytoskeleton-associated protein Arc/Arg3.1 binds to spectrin and associates with nuclear promyelocytic leukemia (PML) bodies. *Brain Res* 1153: 20-33.

Butko, M. T., J. N. Savas, B. Friedman, C. Delahunty, F. Ebner, J. R. Yates, 3rd, and R. Y. Tsien. 2013. In vivo quantitative proteomics of somatosensory cortical synapses shows which protein levels are modulated by sensory deprivation. *Proc Natl Acad Sci U S A* 110 (8): E726-35.

Hou, Q., J. Gilbert, and H. Y. Man. 2011. Homeostatic regulation of AMPA receptor trafficking and degradation by light-controlled single-synaptic activation. *Neuron* 72 (5): 806-18.

Korb, E., C.L. Wilkinson, R.N. Delgado, K.L Lovero, and S. Finkbeiner. 2013. Arc in the nucleus regulates PML dependent GluA1 transcription and homeostatic plasticity. *Nat Neurosci*: In Press.

Kumar, S. S., A. Bacci, V. Kharazia, and J. R. Huguenard. 2002. A developmental switch of AMPA receptor subunits in neocortical pyramidal neurons. *J Neurosci* 22 (8): 3005-15.

Lee, H. K. 2012. Ca-permeable AMPA receptors in homeostatic synaptic plasticity. *Front Mol Neurosci* 5: 17.

Lin, A., Q. Hou, L. Jarzylo, S. Amato, J. Gilbert, F. Shang, and H. Y. Man. 2011. Nedd4-mediated AMPA receptor ubiquitination regulates receptor turnover and trafficking. *J Neurochem* 119 (1): 27-39.

Link, W., U. Konietzko, G. Kauselmann, M. Krug, B. Schwanke, U. Frey, and D. Kuhl. 1995. Somatodendritic expression of an immediate early gene is regulated by synaptic activity. *Proc Natl Acad Sci U S A* 92 (12): 5734-8.

Lyford, G. L., K. Yamagata, W. E. Kaufmann, C. A. Barnes, L. K. Sanders, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, A. A. Lanahan, and P. F. Worley. 1995. Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. *Neuron* 14 (2): 433-45.

Man, H. Y. 2011. GluA2-lacking, calcium-permeable AMPA receptors--inducers of plasticity? *Curr Opin Neurobiol* 21 (2): 291-8.

Messaoudi, E., T. Kanhema, J. Soule, A. Tiron, G. Dagyte, B. da Silva, and C. R. Bramham. 2007. Sustained Arc/Arg3.1 synthesis controls long-term potentiation consolidation through regulation of local actin polymerization in the dentate gyrus in vivo. *J Neurosci* 27 (39): 10445-55.

Peebles, C. L., J. Yoo, M. T. Thwin, J. J. Palop, J. L. Noebels, and S. Finkbeiner. 2010. Arc regulates spine morphology and maintains network stability in vivo. *Proc Natl Acad Sci U S A* 107 (42): 18173-8.

Petrini, E. M., J. Lu, L. Cognet, B. Lounis, M. D. Ehlers, and D. Choquet. 2009. Endocytic trafficking and recycling maintain a pool of mobile surface AMPA receptors required for synaptic potentiation. *Neuron* 63 (1): 92-105.

Plath, N., O. Ohana, B. Dammermann, M. L. Errington, D. Schmitz, C. Gross, X. Mao, A. Engelsberg, C. Mahlke, H. Welzl, U. Kobalz, A. Stawrakakis, E. Fernandez, R. Waltereit, A. Bick-Sander, E. Therstappen, S. F. Cooke, V. Blanquet, W. Wurst, B. Salmen, M. R. Bosl, H. P. Lipp, S. G. Grant, T. V. Bliss, D. P. Wolfer, and D. Kuhl. 2006. Arc/Arg3.1 is essential for the consolidation of synaptic plasticity and memories. *Neuron* 52 (3): 437-44.

Schwarz, L. A., B. J. Hall, and G. N. Patrick. 2010. Activity-dependent ubiquitination of GluA1 mediates a distinct AMPA receptor endocytosis and sorting pathway. *J Neurosci* 30 (49): 16718-29.

Shepherd, J. D. 2012. Memory, plasticity and sleep - A role for calcium permeable AMPA receptors? *Front Mol Neurosci* 5: 49.

Shepherd, J. D., and M. F. Bear. 2011. New views of Arc, a master regulator of synaptic plasticity. *Nat Neurosci* 14 (3): 279-84.

Shepherd, Jason D., Gavin Rumbaugh, Jing Wu, Shoaib Chowdhury, Niels Plath, Dietmar Kuhl, Richard L. Huganir, and Paul F. Worley. 2006. Arc/Arg3.1 Mediates Homeostatic Synaptic Scaling of AMPA Receptors. *Neuron* 52 (3): 475-484.

Waung, M. W., B. E. Pfeiffer, E. D. Nosyreva, J. A. Ronesi, and K. M. Huber. 2008. Rapid translation of Arc/Arg3.1 selectively mediates mGluR-dependent LTD through persistent increases in AMPAR endocytosis rate. *Neuron* 59 (1): 84-97.

Wibrand, K., E. Messaoudi, B. Havik, V. Steenslid, R. Lovlie, V. M. Steen, and C. R. Bramham. 2006. Identification of genes co-upregulated with Arc during BDNF-

induced long-term potentiation in adult rat dentate gyrus in vivo. *Eur J Neurosci* 23 (6): 1501-11.

Wiltgen, B. J., G. A. Royle, E. E. Gray, A. Abdipranoto, N. Thangthaeng, N. Jacobs, F. Saab, S. Tonegawa, S. F. Heinemann, T. J. O'Dell, M. S. Fanselow, and B. Vissel. 2010. A role for calcium-permeable AMPA receptors in synaptic plasticity and learning. *PLoS One* 5 (9).

Appendix I. pHluorin Imaging Protocol

Dendritic pHluorin Signal

Although total internal reflection fluorescence microscopy (TIRFM) is the gold standard for isolating fluorescence signal from the cell membrane, we were able to achieve high signal specificity from dendritic SEP-GluA1 and SEP-GluA2 using standard epifluorescence microscopy with a 60X 1.45 NA objective. In agreement with previous studies (Khiroug et al. 2009), somatic fluorescence was incompletely quenched by a brief wash with low pH Tyrodes solution. However, greater than 80% of dendritic SEP-GluA1 and 90% of SEP-GluA2 signal was reversibly quenched by pH 5.0 Tyrodes (Fig A1), reducing levels to near that of the autofluorescence observed in untransfected cells.

Image Acquisition

Cells were plated on 35 mm coverslip glass bottom dishes from Mattek. 10 mm cell culture cylinders were placed on the coverslip bottom to create an appropriate volume for neuronal culture. After transfection, the cylinders were removed and a 35 mm perfusion insert from Warner was used to convert the dish into a flow-through chamber. All solutions were stored within the microscope environmental chamber to maintain a constant temperature throughout the experiment. Rapid solution exchange was achieved

using gravity flow and a vacuum aspirator within the perfusion chamber. Solution exchange was automated using the Warner VT-8 valve controller software.

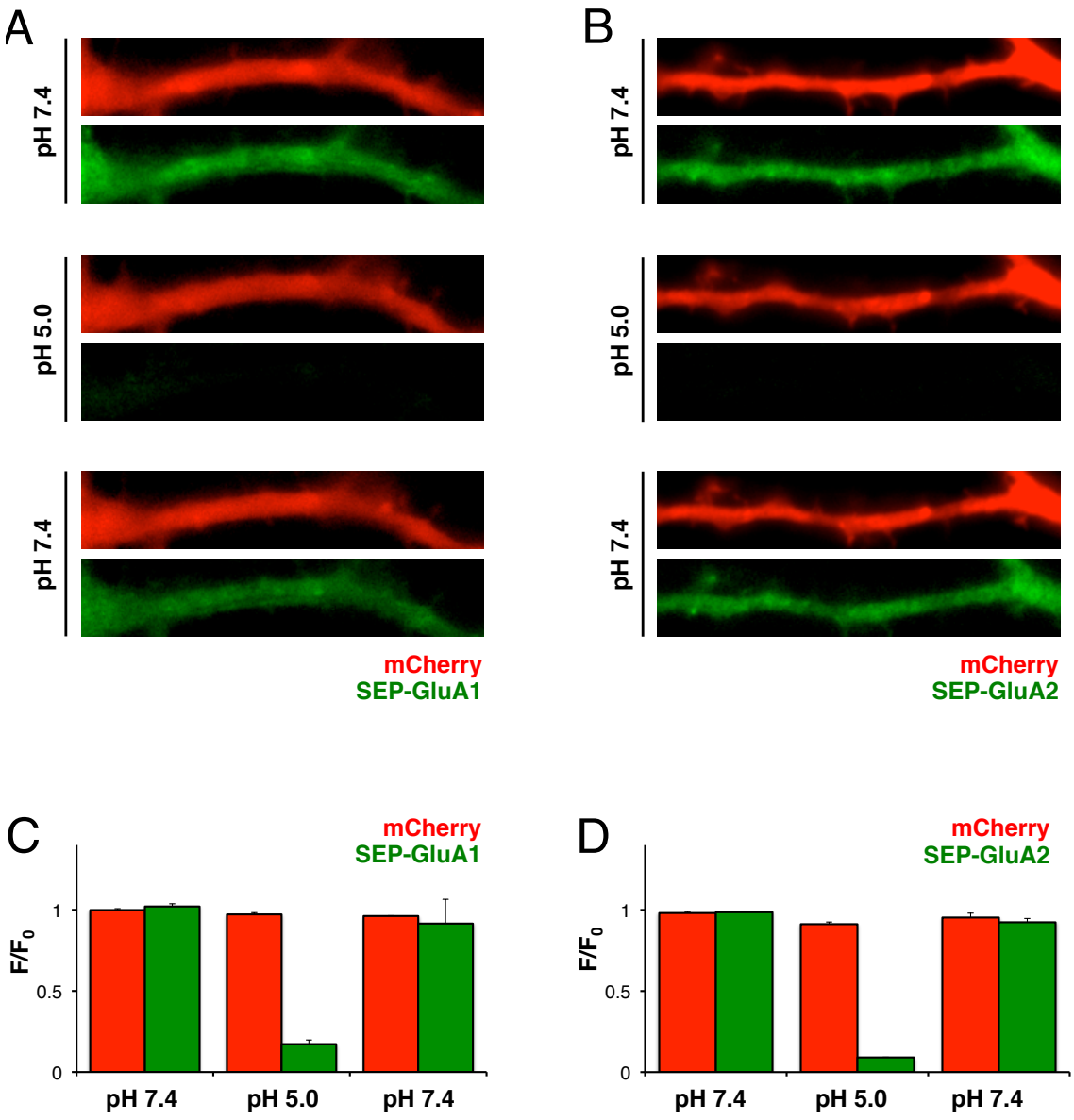


Figure A1. Dendritic SEP-GluA1 and SEP-GluA2 fluorescence is reversibly quenched by a low pH wash
(A,B) mCherry and SEP fluorescence in dendrites from 7 DIV rat cortical neurons at alternating physiological and low pH and **(C,D)** normalized fluorescence averaged over 3 dendrites from each of the sample cells from **A** and **B**. Error bars represent S.D.

Healthy cells with numerous visible dendritic spines were selected by eye in the red channel. Nikon PerfectFocus was engaged and images were acquired in the green and red channels every 30 seconds using the following routine in Media Cybernetics ImagePro*:

```
Sub green_red_timelapse

    length=30 'define length of experiment in minutes
    inter=30 'define interval for timelapse in sec
    greenexp=500 'define green channel exposure in msec
    redexp=500 'define red channel exposure in msec
    pathfolder="D:\maya\06062013\s3a1c2" 'define path for saved files
    greenname="06062013_s3a1c2_100uM_AMPA_7div_g" 'define base names for gfp images
    redname="06062013_s3a1c2_100uM_AMPA_7div_r" 'define base names for red images

    ret = IpAcqControl(813, 0, IPNULL) 'turn off file save prompt

    timepoints = (length*60)/inter + 1 ' includes t=0
    starttime=Timer

    For I = 1 To (timepoints)

    While Timer < (starttime+(I*inter))

            Wait((starttime+(I*inter)-Timer))
```

* Because the time-lapse script uses the internal computer clock to calculate intervals, it will halt mid-acquisition at midnight and is not suitable for an overnight run

```
            ret = IpScopeSettings("D:\IPWIN70\ScpConfig\mrc gfp.scp", SCP_LOAD)
            ipDVal = greenexp
            ret = IpAcqControl(84, 0, ipDVal)

            Debug.Print Time
            ret = IpScopeAcquire(ACQ_CURRENT)
            greensave = pathfolder + "\" + greenname + Format(CStr(I),"000") +".tif"
            ret = IpWsSaveAs(greensave,"TIF")
            Debug.Print greensave

            ret = IpScopeSettings("D:\IPWIN70\ScpConfig\mrc rfp.scp", SCP_LOAD)
            ipDVal = redexp
            ret = IpAcqControl(84, 0, ipDVal)

            ret = IpScopeAcquire(ACQ_CURRENT)
            redsave = pathfolder + "\" + redname + Format(CStr(I),"000") +".tif"
            ret = IpWsSaveAs(redsave,"TIF")

    Wend
    Next I
End Sub
```

Image Analysis

Image sequences were imported into FIJI (ImageJ) using the LOCI > Bio-Formats Importer plugin and the red stack was aligned using the translational alignment command in the MultiStackReg plugin. The alignment file was saved and applied to the green stack. Three dendritic regions of interest (ROIs) were hand drawn for each neuron in the red channel (Fig A2). The ROIs were then transferred to the aligned green stack and, after a rolling-ball background subtraction was performed, the Multi-Measure command was used to obtain an average intensity for each ROI at each time point.

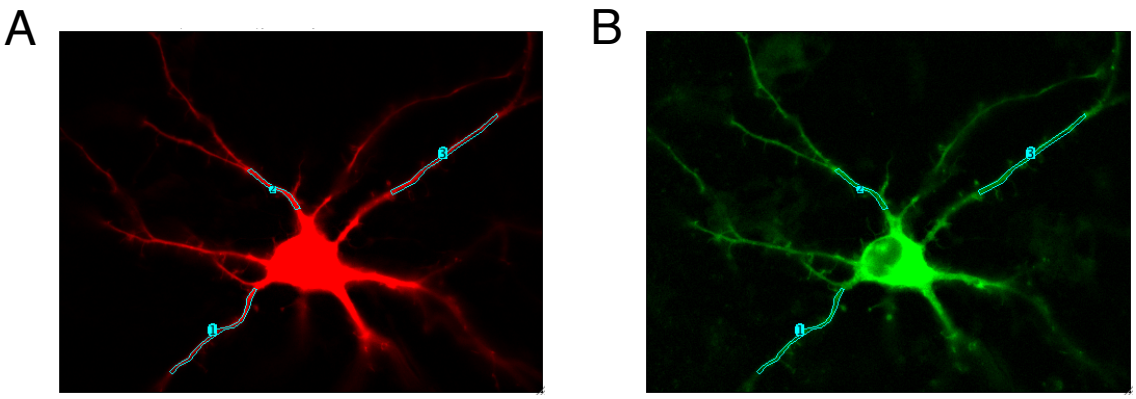


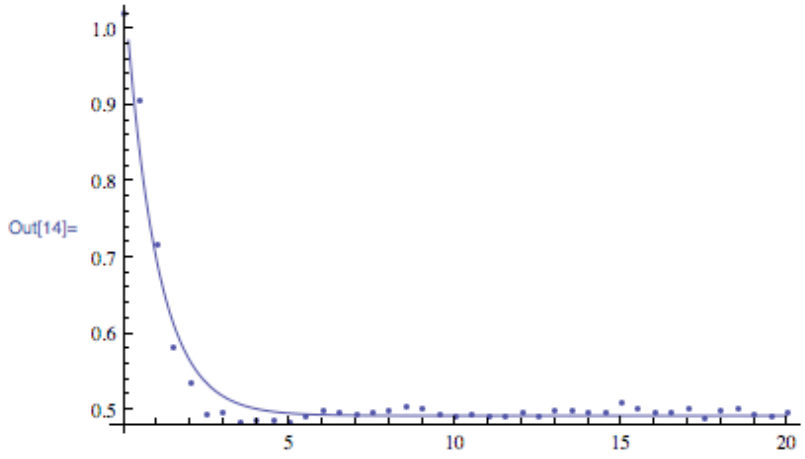
Figure A2. Dendritic regions of interest are defined based upon the morphology marker
(A) Three dendritic regions of interest (ROIs) from each cell are traced using the diffuse mCherry morphology marker and **(B)** are transferred to the image stack in the green channel

The average of the normalized intensity from the three dendritic segments was plotted at each time point and the time constant of endocytosis, tau, was calculated by fitting an exponential curve to the trace from each cell using the following script in Wolfram Mathematica:

```
In[12]:= nlm = NonlinearModelFit[paired, (c + a * Exp[-b * x]), {a, b, c}, x]
Out[12]= FittedModel[ 0.492558 + 0.565663 e^(-1.03656 x) ]

In[13]:=

In[14]:= Show[ListPlot[paired], Plot[nlm[x], {x, -3, 20}], PlotRange → All]
```



Out[14]=

```
In[15]:=

In[16]:= FindFit[paired, (c + a * Exp[-x / tau]), {a, c, tau}, x]
Out[16]= {a → 0.565663, c → 0.492558, tau → 0.96473}
```

References

Khiroug, S. S., E. Pryazhnikov, S. K. Coleman, A. Jeromin, K. Keinanen, and L. Khiroug. 2009. Dynamic visualization of membrane-inserted fraction of pHluorin-tagged channels using repetitive acidification technique. *BMC Neurosci* 10: 141.

Appendix II. Preliminary observations

Arc knockdown slows the recycling of SEP-GluA2 but not SEP-GluA1 after NMDA washout

After NMDA-induced endocytosis, surface levels of GluA1 and GluA2 rapidly return to baseline. In the case of GluA2, this recovery has been shown to be due to the recycling of recently endocytosed receptors (Lin and Huganir 2007). We tested whether Arc knockdown affects the rate of recovery of SEP-GluA1 and SEP-GluA2 fluorescence after NMDA stimulation in a subset of the 14 – 15 DIV cortical neurons represented in Chapter 2, Figure 3. After establishing a baseline, we induced endocytosis by perfusion with 40 μM NMDA in low magnesium Tyrodes with 10 μM glycine as a co-agonist for 5 minutes followed by washout with Tyrodes (2mM $MgCl_2$).

We observed no difference between knockdown and control neurons in the time constant of SEP-GluA1 recovery (Fig A3). However, in our preliminary analysis, recovery of SEP-GluA2 fluorescence after NMDA washout was significantly slowed (Fig A4), indicating that Arc may be involved in regulating the rate of GluA2 recycling in cortical neurons.

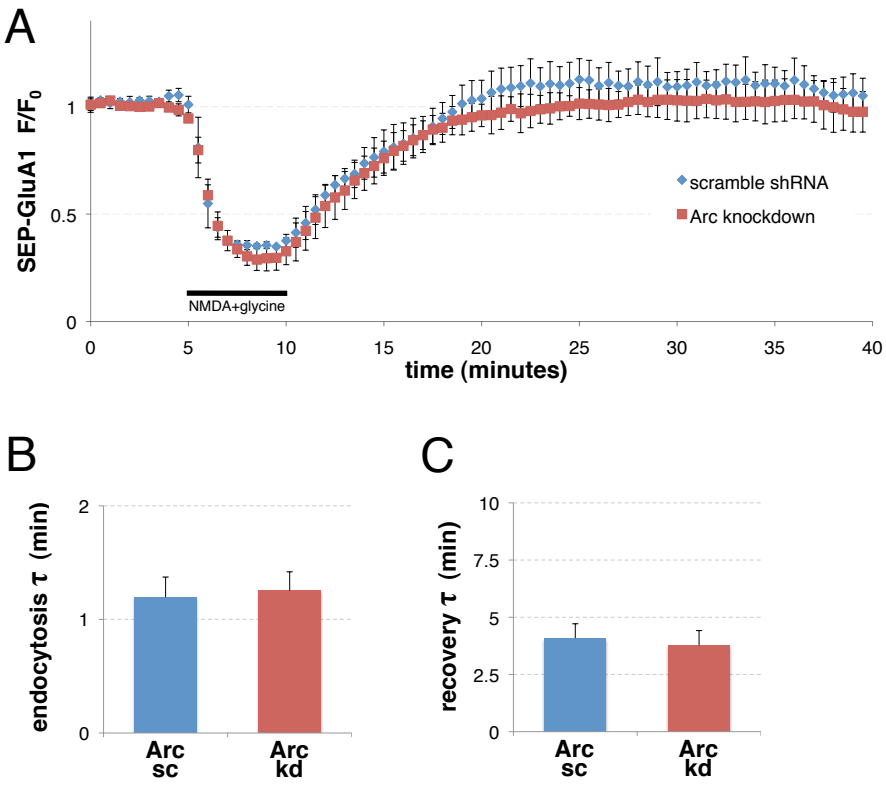


Figure A3. Arc is not required for the recovery of surface GluA1 after NMDA washout **(A)** Normalized fluorescence traces showing endocytosis and subsequent recovery of SEP-GluA1 in response to 40 μM NMDA + 10 μM glycine application in 14-15 DIV neurons expressing either the Arc knockdown shRNA (Arc kd) or the scrambled control shRNA (Arc sc) and **(B,C)** time constant (τ) derived from an exponential fit to the endocytosis and recovery curves from each neuron represented in A (n=8 cells per condition). P values were calculated using an unpaired two-tailed t-test ($p \geq 0.05$ is non-significant, error bars are S.E.M.).

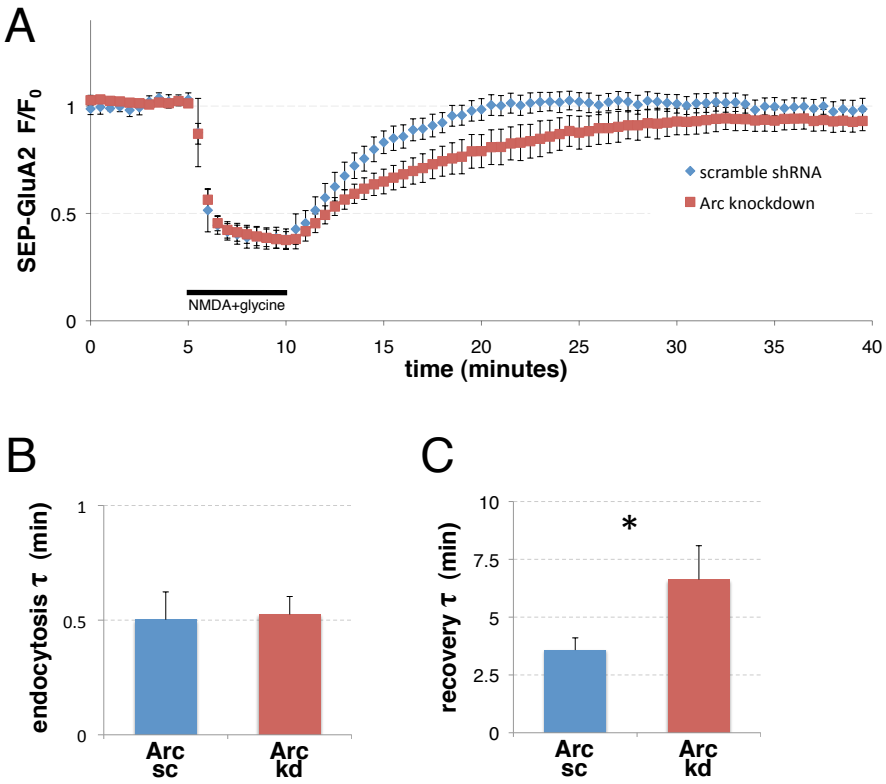


Figure A4. Arc knockdown slows the recovery of surface GluA2 after NMDA washout
(A) Normalized fluorescence traces showing endocytosis and subsequent recovery of SEP-GluA2 in response to 40 μM NMDA + 10 μM glycine application in 14-15 DIV neurons expressing either the Arc knockdown shRNA (Arc kd) or the scrambled control shRNA (Arc sc) and **(B,C)** time constant (τ) derived from an exponential fit to the endocytosis and recovery curves from each neuron represented in A (n=8 cells per condition). P values were calculated using an unpaired two-tailed t-test (* $p < 0.05$, $p \geq 0.05$ is non-significant, error bars are S.E.M.).

References

Lin, D. T., and R. L. Huganir. 2007. PICK1 and phosphorylation of the glutamate receptor 2 (GluR2) AMPA receptor subunit regulates GluR2 recycling after NMDA receptor-induced internalization. *J Neurosci* 27 (50): 13903-8.

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