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Activity-dependent regulation of Arc and protein kinase D in neurons

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Activity-dependent Regulation of *Arc* and Protein Kinase D in Neurons

by

Vikram Ramnath Rao

DISSERTATION

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For my parents

Acknowledgments

My journey through graduate school was a rocky one, with many more emotional valleys than peaks. Perhaps this is common. The tremendous support network of family, friends, and colleagues I have enjoyed throughout is, I'm sure, more rare. For this, I feel extremely fortunate, and I would like to acknowledge some of those people here.

First, I thank my advisor, Steve Finkbeiner, one of the most remarkable individuals I have ever had the good fortune of working with. It would be easy to gush about the qualities that make Steve a great advisor: his enormous breadth of knowledge, his approachability and accessibility, his efficiency and productivity, his commitment to teaching and mentoring, his scientific vision and creativity, his uncanny sense of how to design simple experiments that yield significant information. But probably the best thing I can say about Steve is that I have learned as much from him about how to be a good person as how to be a good scientist. Steve's patience, enthusiasm, relentless optimism, sense of humor, commitment to his family, and genuine interest in his lab members' well-being—these have been some of my greatest lessons in graduate school. Steve's famous list of 'Strategies for Success at the Bench'—which are really strategies for success in life—will be posted on the wall of every office I ever have; I will probably be quoting him for the rest of my life. I am also grateful to the other members of my thesis committee, Robert Edwards and Fen-Biao Gao, for their support and thoughtful comments about the work in this dissertation. Some of this work was supported by a pre-doctoral fellowship from the Epilepsy Foundation.

During my five years in graduate school, I relied on many members of the Finkbeiner lab for scientific advice, assistance with experiments, commiseration over negative results, and frivolous distractions from the daily grind. Several individuals have made my tenure in the lab particularly enjoyable, and many thanks go to them: John Bradley, for his friendship and incorrigibly crass British humor; Siddhartha Mitra, for his microscopy expertise, Haiku prowess, and sardonic wit; Sean Pintchovski, for teaching me real-time PCR and making me want my own RNA monkey; Carol Peebles, for her motherly nature and love of useless toys from vendors; and Jason Miller, for just being the smart, funny, interesting person that he is.

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Contributions of Others to the Presented Work

Chapter 1 reviews the history of research on learning and memory and provides specific background on Arc and Protein Kinase D (PKD), the molecules which are investigated in the dissertation. This chapter is an original composition and no portion of it has been submitted for publication.

Chapter 2 is based on a manuscript currently in press at *Nature Neuroscience*. Shyamal Kapadia developed primers and reaction conditions for detecting *Arc* mRNA levels by real-time PCR. Sean Pintchovski made some of the original observations of stimulus-induced changes in *Arc* mRNA levels, and Carol Peebles performed the *in situ* hybridization and immunocytochemistry in Figure 2.1e. Siddhartha Mitra performed the imaging experiments with GFP^u (Figure 2.12b), and Jeannie Chin prepared the organotypic brain slices (Figure 2.5e). Dr. Steven Finkbeiner supervised the research and wrote portions of the text, and Dr. Lennart Mucke helped revise the Abstract and Discussion sections.

The experiments described in Chapter 3 are aimed at elucidating the function and regulation of PKD in neurons. Sarah Carter performed the live-cell imaging and quantification of stimulus-induced Venus-PKD translocation (Figure 3.1c,d, Figure 3.2b,c). Shyamal Kapadia made the original observation of depolarization-induced HDAC5 nuclear extrusion (Figure 3.4). Maribel Parra assisted with preliminary experiments that examined PKD activation in neurons by Western blot analysis. Dr. Steven Finkbeiner supervised the research. No portion of this chapter has been submitted for publication.

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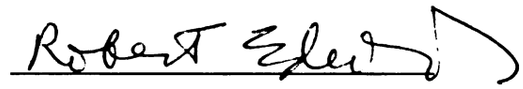

Robert H. Edwards

Committee Chair

Abstract

The remarkable ability of the brain to convert transient experiences into enduring memories has long been attributed to activity-dependent changes in synaptic strength. Long-lasting changes in synaptic strength essential for learning and memory require neuronal gene expression, but the underlying mechanisms are unclear. In particular, the mechanisms by which synaptic activity triggers neuronal gene expression, and by which gene products act specifically at synapses that triggered their expression, are poorly understood. To gain insight into these mechanisms, we investigated the activity-dependent regulation of *Arc*, an immediate-early gene essential for synaptic plasticity. We found that neurons regulate *Arc* expression at multiple levels, and that pathways that control *Arc* transcription integrate signals from NMDA and AMPA receptors. A role for AMPA receptors in regulating *Arc* expression is particularly surprising in light of the prevailing view that AMPA receptors

mediate fast excitatory synaptic transmission and effect short-term plasticity, but do not directly regulate neuronal gene expression. We examined the mechanism by which AMPA receptors control *Arc* transcription and identified a role for pertussis toxin–sensitive G-proteins. This finding adds to a growing body of evidence that AMPA receptors are cell-surface signal transducers, not just passive conduits for current flux. We also provide preliminary evidence that another molecule, protein kinase D (PKD), may play a critical role in activity-dependent neuronal gene expression. PKD regulates histone deacetylase (HDAC)-mediated gene expression in cardiomyocytes and lymphocytes, but virtually nothing is known about its role in neurons. We found that NMDA receptor stimulation induces PKD activation and dendritic translocation. NMDA receptors also regulate the nucleocytoplasmic distribution of HDACs, suggesting that PKD may mediate a novel synapse-to-nucleus signal transduction pathway. Indeed, protein microarray experiments identified neuronal substrates of PKD that are known to regulate synaptic function. Thus, our investigation of *Arc* and PKD uncovered novel mechanisms by which neuronal activity couples to gene expression. The diversity of mechanisms that regulate *Arc* and PKD likely reflects the complexity of neuronal adaptive responses to synaptic activity.

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Chapter 1:

From Molecules to Memories

Learning and memory

Encoding and storage of information by the nervous system has captivated more intellectual disciplines—philosophy and psychology, anatomy and physiology, biochemistry and molecular biology, cell biology and computational neuroscience—than virtually any other area of biology. Learning—the ability to acquire new information from experience—and memory—the retention of this information over time—are essential for human activity and integral to our sense of what it means to be human. With unprecedented clarity, modern neuroscience peers into the inner workings of how we learn and remember. In some cases, the ultimate reductionist fantasy has been fulfilled: transgenic mice engineered to have a single amino-acid substitution at a crucial kinase autophosphorylation site show no spatial memory in a water maze test[1]. At the same time, as our appreciation of the brain's complexity grows, an alarming possibility gnaws at us ever more: that our efforts are akin to trying to explain how a door works by molecular analysis of the hinge[2]. Of course, molecular analysis has powered the great triumphs of biology over the past 50 years, but can molecules explain memory[3]? Only time will tell, and so we proceed. For inspiration and optimism, it is worth considering the remarkable successes of those who came before us.

Historical perspective

Attempts to explain the biological basis of learning and memory go back at least to the ancient Greek philosophers. Plato likened the mind to a ball of wax that becomes impressed with grooves (knowledge) as we learn and recall information over the same pathways.

Aristotle proposed that the heart was the source of memory and that the brain served to cool the blood; Herophilius later challenged this notion, identifying the brain as the seat of intelligence.

Theories pertaining to learning and memory remained the province of philosophy until the nineteenth century. In 1893, Italian anatomist Eugenio Tanzi advanced the idea that contact points between cells in the brain were the locus of change that encodes experience[4]. Santiago Ramón y Cajal, the brilliant Spanish anatomist and foremost advocate of the neuron doctrine, seized upon this idea and integrated it with his observations of neuronal numbers and patterns of connectivity. Cajal knew that in most regions of the mammalian brain no additional neurons are generated in postembryonic development, and that the patterns of connectivity that are laid down during development are, of necessity, highly specific[5]. With typical clairvoyance, Cajal proposed a possible solution to these problems in his 1894 Croonian Lecture to the Royal Society[6]:

> "These observations ... have suggested to us an hypothesis which will enable us to understand ... intelligence acquired by good mental training ....
>
> Mental training cannot better the organization of the brain by adding to the number of cells; we know that nervous elements have lost the property of multiplication past embryonic life; but it is possible to imagine that mental exercise facilitates a greater development of the protoplasmic apparatus and of the nervous collaterals in the part of the brain in use. In this way, pre-existing connections between groups of cells could be reinforced...."

Cajal thus anticipated that biochemical and structural changes at synapses might underlie memory formation. Some fifty years later, Donald Hebb formalized this notion, postulating that synaptic efficacy may be changed as a result of activity, and provided a specific neuronal basis for such changes[7]:

> "When an axon of cell A ... excite[s] cell B and repeatedly and persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells so that A's

efficiency as one of the cells firing B is increased."

The idea that synapses could undergo plastic, activity-dependent alterations in strength that persisted over time was hugely influential[8], as it immediately suggested a cellular basis for memory storage. Experimental validation of this idea, however, awaited the discovery of long-term potentiation.

Long-term potentiation

In the early 1970s, Tim Bliss and Terje Lomo recorded extracellular field potentials in the hippocampus evoked by stimulation of the perforant path of anesthetized rabbits. They found that repetitive stimulation resulted in a persistent increase in synaptic efficacy[9,10] (Figure 1.1), a phenomenon which came to be known as long-term potentiation (LTP).

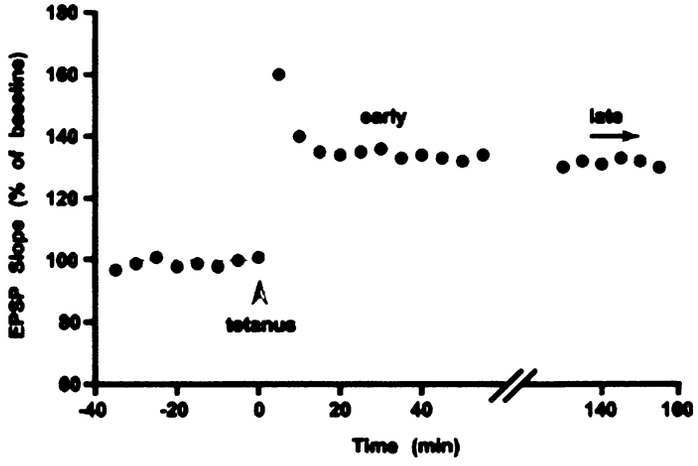


Figure 1.1 Long-term potentation of synaptic transmission.

Illustration showing that the efficacy of synaptic transmission, as measured by the slope of the excitatory postsynaptic potential (EPSP), is persistently enhanced following a short, high-frequency volley of stimuli, called a tetanus. Early and late phases are indicated. Adapted from Martin *et al.* (2000)[11].

Over the next 30 years, LTP emerged as an almost ubiquitous property of excitatory

synapses in the mammalian brain[12]. It is now widely accepted that reversible physiological changes in synaptic transmission, like LTP, occur during learning, and that these changes must be stabilized or consolidated in order for memory to persist[13]. A correlate of this idea is that the engram, or memory trace, is encoded by the distribution of synaptic weights in a neural network. Elucidation of the mechanisms underlying LTP is a major goal of efforts to understand the cellular basis of learning and memory[14]. Importantly, little evidence exists to indicate that synaptic plasticity is sufficient for memory formation, and the notion that 'LTP equals memory' is almost certainly too simplistic[11,15]. Nevertheless, LTP has captured the imagination of many scientists, as evidenced by the exponential increase in number of publications dealing with LTP over the past 30 years[3,12] (Figure 1.2).

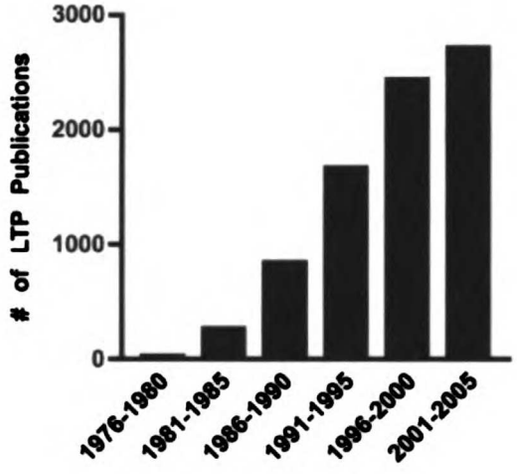


Figure 1.2 Exponential increase in papers published on LTP.

Results of a PubMed search using the search term 'LTP or long-term potentiation' for specific years. Adapted from Malenka (2003)[12].

Several physiological properties of LTP have contributed to its establishment as the likely cellular correlate of learning and memory[16,17]. First, LTP occurs on a time-scale long enough

to be potentially useful for information storage[3,17]. Second, the expression of LTP is synapse-specific, such that groups of synapses converging on a single neuron can be potentiated independently[18], within certain limits[19]. In principle, this specificity confers upon a neuron the ability to process and store thousands of bits of information[20]. Third, LTP has the Hebbian property believed to underlie associative learning: presynaptic activity must be temporally coincident with postsynaptic depolarization for synaptic strengthening to occur. Fourth, LTP can be induced by physiological patterns of neural activity, such as the hippocampal theta rhythm[11]. Fifth, like memory, synaptic plasticity occurs in both short- and long-lasting forms. These two forms of plasticity are mechanistically separable, with short-term changes in synaptic strength involving the covalent modification of pre-existing proteins, and long-lasting changes requiring gene transcription and mRNA translation[21-23] (but see Fonseca *et al.* (2006)[24]). The late, protein synthesis–dependent phase of LTP is of particular interest as a candidate mechanism of information storage. Since molecular changes are transient, it is generally believed that structural changes in synaptic morphology are also required for long-term memory[25].

Synapse-specific plasticity

Transcription of mRNA from genomic DNA is necessarily a nuclear event, but LTP can be induced at synapses in distal regions of the dendritic arbor, often several hundred microns from the neuronal cell body. The large spatial separation between synapse and nucleus creates a cell biological problem for the neuron[20]: how do the products of transcription and/or

translation selectively reach synaptic sites whose activation history merits a change in synaptic strength? At least three models have been advanced to explain how gene expression–dependent synaptic plasticity can occur in a synapse-specific manner (Figure 1.3)[20]. In the first model, called 'selective transport,' synaptic activity initiates a signal that travels to the nucleus[26], triggers gene expression, and targets newly synthesized proteins back to the activated synapses[27]. According to a second model, called 'selective capture,' the products of gene expression are delivered throughout the neuron, but modify only those synapses that have been 'tagged' in an activity-dependent manner[28-30]. A third model, called 'local production,' which has only recently gained experimental support, involves local, activity-dependent translation of dendritic mRNAs near active synapses[31-34]. In this view, the extreme functional and morphological polarity of neurons necessitates decentralized protein production. Recent reports that RNA splicing can occur in dendrites[35] and that the ubiquitin-proteasome system operates in both pre- and postsynaptic compartments[36] suggest that synapses may be more autonomous with regard to gene expression than previously thought. Synapse-specific modifications dependent on new proteins, therefore, could be achieved through the micromanagement of gene expression in postsynaptic compartments[37].

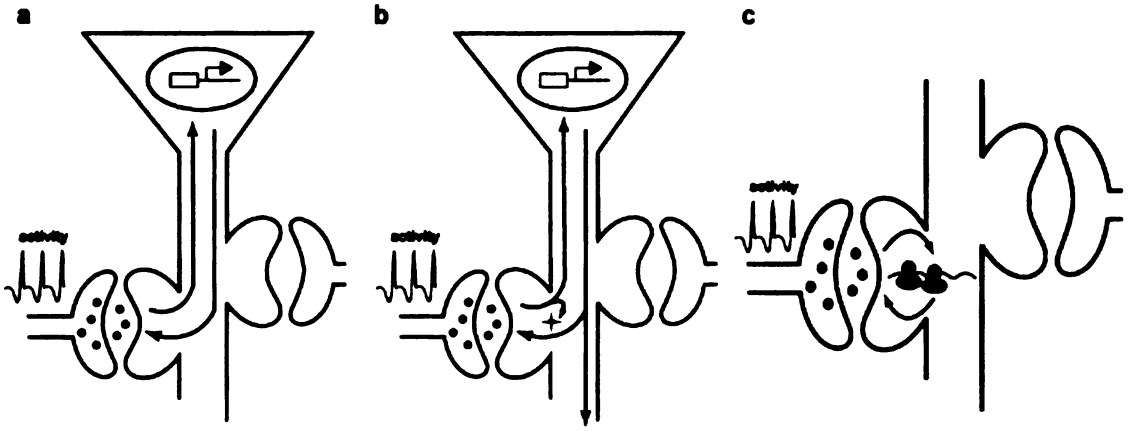


Figure 1.3 Models for synapse-specificity during long-term information storage.

(a) Selective transport. A synapse-to-nucleus signal triggers gene expression, and gene products are specifically targeted to appropriate synapses. (b) Selective capture. Synaptic activity sets up a local tag that serves to capture gene products delivered throughout the cell. (c) Local production. Synaptic activity regulates dendritic mRNA translation to produce proteins locally. Adapted from Schuman (1997)[20].

These models, which are not mutually exclusive, all leave a fundamental question unanswered: what are the gene products whose synthesis is required for late-phase LTP? At the time the research in this dissertation was begun, over 100 molecules had been suggested to be causally associated with LTP in the hippocampus alone[3]. Many more have been added since, but focus is gradually shifting away from implicating additional molecules[2] and moving toward understanding which molecules are most critical[38]. In addition, how synaptic activity triggers gene expression[39] and how gene products act specifically at synapses that triggered their expression[20] are questions of great current interest. One molecule that may provide insight into these questions is the activity-regulated cytoskeleton-associated (Arc) protein[40,41].

The immediate-early gene *Arc*

In 1995, *Arc* (also called *arg3.1*) was independently isolated by two groups who performed differential screening to identify seizure-induced immediate-early genes (IEGs) in the rat hippocampus[42,43]. IEGs are the first genes induced in response to robust synaptic activity, and *de novo* protein synthesis is not required for their expression[44,45]. *Arc* is unique among IEGs in that nascent *Arc* transcripts are rapidly trafficked into neuronal dendrites[42,43], possibly owing to dendritic targeting elements in the mRNA 3′-untranslated region[46]. Traveling at ~300 μm/h, *Arc* transcripts reach the distal ends of most dendrites in less than 1 h[47]. Once in dendrites, *Arc* mRNA selectively localizes near activated synapses[48]. This remarkable property was demonstrated by stimulating select groups of perforant path fibers, which terminate topographically in the hippocampus on dendrites of dentate gyrus granule cells, and showing that *Arc* mRNA in the granule cells localizes to the stimulated dendritic laminae (Figure 1.4)[48]. NMDA receptor activation, which is required for most[49,50], but not all[51], forms of LTP, is necessary and sufficient for *Arc* induction and synaptic targeting[52]. Large amounts of Arc protein are synthesized, possibly in dendrites[53-55], in the first few hours after induction[41]—approximately the same time period in which protein synthesis–dependent synaptic modifications are occurring[21,41]—and, like the mRNA, Arc protein becomes enriched at recently activated synapses[48,56]. Here, Arc protein may integrate into postsynaptic signaling assemblies, such as the NMDA receptor multiprotein complex[57], and may regulate synaptic strength by modulating AMPA receptor trafficking[58-61]. Correlative data suggest that Arc may play a role in synaptogenesis[62], but the precise function of Arc remains unclear.

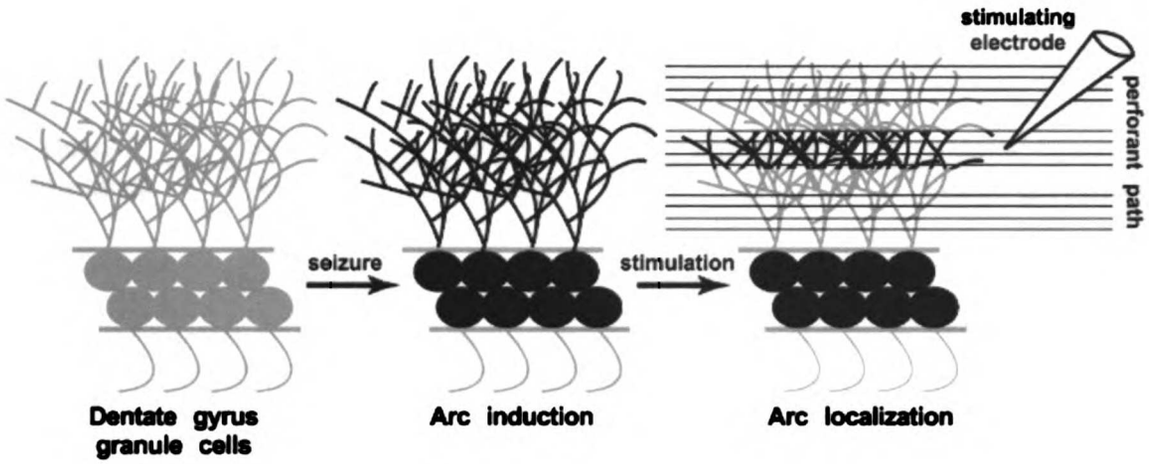


Figure 1.4 *Arc* localizes to activated postsynaptic sites on dendrites.

Arc mRNA induced by seizure fills granule cell dendrites; stimulation of a select layer of topographically-projecting perforant path afferents induces *Arc* relocalization to the corresponding dendritic lamina. Based on Steward *et al.* (1998)[48].

The reported *Arc* knockout mouse fails to gastrulate and dies during embryonic development[63], so the contribution of *Arc* to synaptic plasticity has not been explored in genetic models. However, several lines of evidence indicate that *Arc* is critical for learning and memory. Antisense-mediated knockdown approaches in adult animals have revealed that *Arc* is required for maintenance of synaptic potentiation and long-term consolidation of memory[55,64]. Neural activity associated with LTP[65] and learning[66-69] induces *Arc* expression, and hippocampal *Arc* expression levels correlate with performance in a spatial learning paradigm[67]. Exposure to a novel environment induces *Arc* expression[70,71] in neuronal ensembles that are activated specifically in that environment[72], suggesting a role for *Arc* in a network mechanism for encoding spatial and contextual information[73]. Taken together, *Arc* is an attractive candidate to mediate synapse-specific long-term plasticity and at least some forms of learning and memory (Figure 1.5).

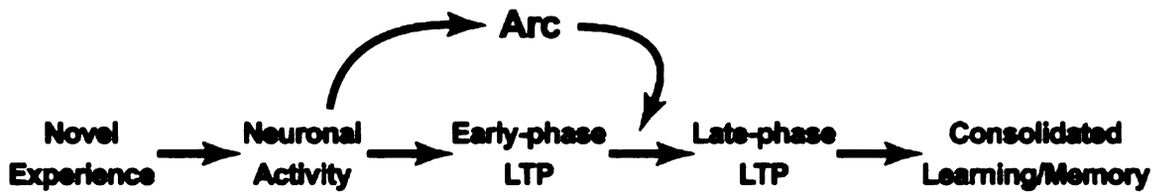


Figure 1.5 A model for the role of *Arc* in learning and memory.

Experience-dependent synaptic activity induces *Arc* expression and early-phase LTP. Arc facilitates establishment of late-phase LTP, which underlies learning and memory.

The close relationship between neural activity and *Arc* expression has been exploited to dissect the role of different brain regions in processing behavioral experience[74-79] and in spatial memory recall[80], as well as to monitor the recovery of polysynaptic circuits after trauma[81]. *Arc* is also used as a histological marker of neuronal activation in studies on subjects as diverse as Alzheimer's disease[82-85], stroke[86], odor processing in the olfactory bulb[87-89], neural processing in avian species[90-92], circadian rhythms[93], spermiogenesis[94], the efficacy of antidepressant therapies[95-98], and the effects of drugs of abuse[99-103] and other neurotoxic insults[104,105]. Understanding how synaptic activity couples to *Arc* expression is critical to interpret the use of *Arc* as a marker. For example, *Arc* expression levels can be modified by recent behavioral history: animals repeatedly exposed to the same environment exhibit similar patterns of neural activity but decreasing *Arc* induction upon each exposure[106]. This phenomenon, a form of metaplasticity[106], suggests that the coupling between synaptic activity and *Arc* transcription is plastic and behavioral state–dependent. Therefore, elucidating the molecular mechanisms that regulate *Arc* expression may provide insight into how *Arc* reports neuronal activity, and may help bridge the chasm between intracellular signal transduction and whole-animal behavior.

Protein kinase D

After a decade of investigation, *Arc* is now firmly entrenched as a molecule that is critical for learning and memory. Interest in *Arc* as a plasticity-related molecule originally stemmed from its unique subcellular distribution following induction by synaptic activity—*Arc* localizes near activated synapses, so it is in the right place at the right time to mediate synapse-specific plasticity. Another molecule whose dynamic intracellular localization suggests a role in synaptic function is protein kinase D (PKD). Though it receives considerably less attention than *Arc* in neurobiology circles, PKD is well-suited to temporally and spatially disseminate signals from the plasma membrane, suggesting a potential role in synapse-to-nucleus signaling and synaptic plasticity.

PKD is a serine/threonine kinase that was independently cloned by two laboratories in 1994[107-109]. Subsequently, two additional kinases sharing extensive overall homology to PKD were identified, termed PKD2[110] and PKD3[111]. Although PKDs were originally classified as atypical PKC isoforms—PKD and PKD3 were previously termed PKCμ and PKCν, respectively—the domain structure (Figure 1.6) and functional properties of the PKDs indicate that they represent a distinct kinase family[112]. First, although PKDs have two N-terminal cysteine-rich zinc-finger domains (CRDs) homologous to the diacylglycerol (DAG)-binding C1 domains of PKCs, they lack the C2 domain responsible for the Ca^{2+} sensitivity of PKCs[113]; thus, PKDs are considered Ca^{2+}-independent enzymes. Second, PKDs have a pleckstrin homology (PH) domain reminiscent of protein kinase B (PKB)/Akt[114] and not

found in any PKC[115]. Third, the PKD catalytic domain is unlike that of the PKCs and is instead distantly related to the Ca^{2+}/calmodulin-dependent kinases (CaMKs)[107,116]. Fourth, PKDs are insensitive to PKC inhibitors[117] and show a unique substrate specificity[118], strongly preferring serine sites with leucine in the –5 position over sites flanked by basic residues, as favored by PKCs. Fifth, although the CRD and PH domains of PKD are inhibitory to kinase activity[119,120], PKDs lack the autoinhibitory pseudosubstrate region found in PKCs[113]. Thus, PKDs represent a novel family of second-messenger-stimulated protein kinases in the AGC superfamily, joining protein kinase A (PKA), PKB/Akt, PKC, and the CaMKs.

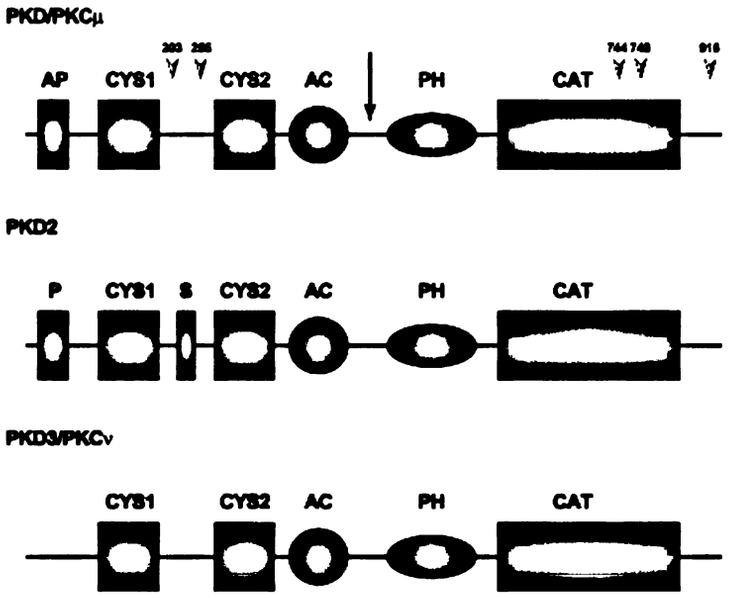


Figure 1.6 Domain structure of members of the protein kinase D family.

Abbreviations: AP, alanine- and proline-rich domain; P, proline-rich domain; S, serine-rich domain; CYS1 and 2, cysteine-rich zinc-finger domains (also called C1a and C1b, respectively); AC, acidic domain; PH, pleckstrin-homology domain; CAT, kinase catalytic domain. Arrow indicates caspase-cleavage site. Arrowheads and numbers indicate location of phosphorylated serine residues in PKD. Figure not drawn to scale. Adapted from Van Lint *et al.* (2002)[112].

PKD activation can occur through at least four general mechanisms[112]. In the first,

receptor tyrosine kinase (RTK) activation or G-protein coupled receptor (GPCR) stimulation leads to activation of phospholipase C (PLC) and production of DAG. DAG activates 'novel' PKCs (δ, ϵ, η, and θ)[113] that then activate PKD by phosphorylation of serines 744 and 748 in its activation loop[121,122] (Figure 1.6). A plethora of stimuli can activate PKD through the activation of PKCs, including phorbol esters and DAG analogs[123], neuropeptides and growth factors[124,125], bryostatin[126], reactive oxygen species[127-130], and agents that induce antigen receptor crosslinking[131-135]. In a second mechanism, G-protein $\beta\gamma$ subunits can activate PKD by directly binding to its PH domain[136], which may relieve the inhibitory effect normally exerted by the PH domain on kinase activity. A third mechanism of activation involves caspase-mediated cleavage of PKD[137], an event that liberates the PH and catalytic domains from the rest of the molecule (Figure 1.6). Finally, a fourth mechanism has been proposed in which 14-3-3 proteins activate PKD by binding to phosphorylated serines in the linker region between the N-terminal CRDs[112,138] (Figure 1.6). The variety of activation mechanisms suggests that PKD can integrate multiple signals to coordinate diverse cellular processes.

A striking feature of PKD activation is that it involves a dynamic series of translocations between intracellular compartments[112]. Each translocation step is mediated by a particular domain within PKD[109]. PKD is found predominantly in the cytoplasm of unstimulated cells but rapidly translocates to the plasma membrane in response to receptor activation[139]. PKD then returns to the cytoplasm[125], with kinase activity sustained[131], and subsequently translocates to the nucleus[140]. Finally, PKD completes the cycle, returning to the cytoplasm by CRM1-mediated nuclear export[140]. Thus, PKD transduces a transient signal at the

membrane into a sustained signal that travels to distant regions of the cell.

The signal-integration capacity and intracellular mobility of PKD are critical for many fundamental biological processes, including immune cell regulation[131-135], the oxidative stress response[127-130], mitogen-activated protein kinase (MAPK) signaling[138,141-143], and secretory vesicle trafficking[136,144-147], as well as cell survival[128], migration[148], differentiation[149,150], and proliferation[151,152]. Many of these functions were originally inferred from PKD localization to specific subcellular compartments, such as the trans-Golgi network[153], mitochondrial membranes[127], or the invadopodia of metastasizing cancer cells[148]. Indeed, the prevailing model holds that PKD is targeted to various intracellular destinations where it recruits different effector proteins that determine its function locally[112]. In this view, the nucleocytoplasmic shuttling[140] of PKD implies a function in the nucleus—regulating DNA synthesis, for example[112]—but, until recently, the role of nuclear PKD was unknown. Several reports now suggest that nuclear translocation of PKD may be functionally relevant for coupling extracellular cues to chromatin modifications that determine patterns of gene expression[133,134,154,155]. This coupling is achieved by PKD-mediated regulation of histone deacetylases (HDACs), chromatin-modifying enzymes that function as transcriptional repressors.

Covalent modification of nucleosomal histones has emerged as a central mechanism in the control of gene transcription[156-158]. Histone acetylation by histone acetyltransferases promotes transcription by relaxing chromatin structure, whereas histone deacetylation by HDACs reverses this process, resulting in transcriptional repression. Class IIa HDACs

(HDAC4, 5, 7, and 9) display restricted tissue expression patterns and contain an N-terminal extension that mediates interactions with transcription factors, like MEF-2, and confers responsiveness to upstream signaling pathways[159]. Phosphorylation of the N-termini of class IIa HDACs creates docking sites for 14-3-3 proteins, which promote HDAC nuclear export through a CRM1-mediated mechanism[159]. PKD directly phosphorylates and induces nuclear export of HDAC5 in cardiomyocytes[154], HDAC7 in T cells[133,134], and both HDAC5 and HDAC7 in B cells[155]. Nuclear export functionally inhibits HDACs, disrupting HDAC-MEF2 interactions and allowing MEF2-dependent gene transcription to occur. PKD/HDAC-mediated transcriptional derepression induces cardiac hypertrophy in cardiomyocytes and apoptosis in T cells. Thus, by regulating HDACs, PKD can control large sets of genes and effect profound cellular changes.

The cellular changes induced by PKD have been demonstrated in a wide variety of cell types, including fibroblasts[126,143], intestinal[130,160] and kidney[125] epithelial cells, smooth muscle cells[161], cardiomyocytes[154,162], osteoblasts[163], B and T lymphocytes[133-135,155,164], mast cells[135], platelets[165], and neoplastic cells[148,166]. Remarkably, only two reports have investigated the role of PKD in neuronal cells[167,168], and only a single neuron-specific PKD substrate is known[169]. Thus, whereas the importance of PKD in other cell types has been firmly established, virtually nothing is known about the role of PKD in neurons. For example, although HDACs mediate activity-dependent gene expression in neurons[170,171], the role of neuronal PKD in this process is unknown. Similarly, neurons express several A-kinase anchoring proteins (AKAPs)[172,173], large molecules which form multiprotein

complexes that facilitate PKD activation[174], but the relationship between AKAPs and PKD in neurons has not been explored. A role for PKD in the nervous system is suggested by the fact that PKD was identified in a screen for genes involved in synaptic function in *Caenorhabditis elegans*[175]. A particularly tantalizing prospect is that PKD plays a role in gene expression–dependent forms of synaptic plasticity. The stimulus-dependent translocation of PKD from cytoplasm to membrane to nucleus seems well-suited to translate synaptic activity into a nuclear response. Such a mechanism would obviously require that PKD translocates to synaptic sites and is activated by synaptic stimuli.

Elucidation of the function of PKD in neurons would be facilitated by knowledge of specific PKD substrates which mediate downstream responses[176]. Relatively few substrates of PKD are known in any cell type, and only a subset of these are expressed in the nervous system: kinase D-interacting substrate of 220 kDa (Kidins220)[167], a lipid raft–associated integral membrane protein; Ras and Rab interactor 1 (RIN1)[142], a Ras effector; HDAC5[154,155] and HDAC7[133,134,155]; vanilloid receptor 1 (VR1)[168], a polymodal nociceptor; and E-cadherin[166], a cell adhesion molecule. Given the large number of cellular responses attributed to PKD, it seems likely that numerous other neuronal substrates exist but have yet to be discovered. PKD substrates that are localized to synapses or that function in dendritic spines would be of particular interest.

Main findings of the dissertation

Despite widespread use of *Arc* as a marker of neural circuits, the activity-dependent

mechanisms that regulate *Arc* expression are poorly understood. For example, *Arc* induction and synaptic targeting require NMDA receptor activation[52], but potential regulatory roles of other glutamate receptors are virtually unexplored. Additionally, although several extracellular stimuli are capable of inducing *Arc* expression[82,177-182], the mechanisms involved are unknown. In part, this is because previous work has been performed either *in vivo*[96,181,183,184], where mechanistic studies are challenging, or in cell lines[178,180,182] and synaptoneurosomes[53,179], where neuronal regulatory mechanisms may be absent. In experiments described in Chapter 2, we established a primary neuronal culture system that recapitulates essential features of *Arc* regulation *in vivo*. Using this system, we uncovered a novel role for AMPA receptors in regulating *Arc* transcription. Additionally, we identified a signaling mechanism that involves a pertussis toxin–sensitive G-protein. These findings, which provide insights into the activity-dependent mechanisms of *Arc* expression, are surprising in light of the prevailing view on the role of AMPA receptors in synaptic plasticity: changes in AMPA receptor surface expression effect short-term plasticity, but AMPA receptors do not directly regulate genes required for long-term plasticity.

The experiments described in Chapter 3 focus on PKD and provide preliminary evidence for its role in neuronal function. We imaged live neurons expressing fluorescent protein–fused versions of PKD and found that phorbol ester treatment induces CRD-dependent membrane translocation of PKD, as in other cell types[139]. Remarkably, NMDA receptor stimulation induced PKD activation and translocation into dendrites. NMDA receptor–dependent PKD activation occurred with rapid kinetics, distinct from PKD activation by

other calcium channels, and may involve tethering of PKD to the NMDA receptor by an AKAP, Yotiao[172]. Consistent with a previous report, we found that NMDA receptor stimulation induced nuclear export of HDAC5, suggesting the existence of a NMDA receptor–PKD–HDAC signaling pathway that regulates neuronal gene expression. Finally, we used a protein microarray to identify novel PKD substrates, including a number of proteins involved in dendritic spine morphology and synaptic function.

Chapter 2:

AMPA Receptors Regulate *Arc* Transcription

Abstract

Learning and memory depend critically on long-term synaptic plasticity, which requires neuronal gene expression. In the prevailing view, AMPA receptors mediate fast excitatory synaptic transmission and effect short-term plasticity, but do not directly regulate neuronal gene expression. By studying regulation of *Arc*, a gene required for long-term plasticity, we uncovered a new role for AMPA receptors in neuronal gene expression. Spontaneous synaptic activity or activity induced by brain-derived neurotrophic factor (BDNF) elicited *Arc* expression in cultures of primary neurons and organotypic brain slices. Surprisingly, inhibiting AMPA receptors markedly potentiated activity-dependent *Arc* expression. Moreover, we found that AMPA receptors negatively regulate *Arc* transcription, but not translation or stability, through a mechanism involving a pertussis toxin–sensitive G-protein. These results provide insights into the activity-dependent mechanisms of *Arc* expression and suggest that, in addition to effecting short-term plasticity, AMPA receptors may regulate genes involved in long-term plasticity.

Introduction

Activity-dependent changes in synaptic strength are likely required for the brain to form enduring memories of transient experiences[11,13]. Neurons achieve short-term changes in synaptic strength through the reciprocal actions of two types of glutamate-gated ion channels, AMPA receptors and NMDA receptors. During bouts of synaptic activity, AMPA receptors depolarize the postsynaptic membrane, facilitating activation of NMDA receptors. NMDA receptors, in turn, initiate signaling pathways that modulate AMPA receptor conductance and surface expression to produce short-term changes in synaptic strength[49]. Long-term consolidation of short-term changes triggered by synaptic activity requires new gene expression[21,185]. How activity induces gene expression and how gene products act specifically at synapses that triggered their expression are questions of great current interest[20,27].

One molecule that may provide insight into these questions is the activity-regulated cytoskeleton-associated (Arc) protein, an immediate early gene (IEG) product[41]. After induction by synaptic activity, *Arc* mRNA is rapidly trafficked into dendrites[42,43,47]. Elegant work from Steward and Worley showed that *Arc* mRNA selectively localizes to activated dendritic regions[48], and Arc protein is enriched at recently activated synapses[48,56]. The precise function of Arc at synaptic sites is unknown, but Arc may regulate synaptic strength by modulating AMPA receptor trafficking[58-61]. Neural activity associated with synaptic potentiation[65] and learning induces *Arc* expression[66-68], and *Arc* is required for maintenance of synaptic potentiation and long-term consolidation of memory[55,64].

The close relationship between neuronal activity and *Arc* expression has made *Arc* a useful marker of neural circuits[72,73,79,81]. However, the activity-dependent mechanisms that regulate *Arc* expression are poorly understood. For example, Steward and Worley convincingly demonstrated that *Arc* induction and synaptic targeting require NMDA receptor activation[52], but potential regulatory roles of other glutamate receptors are virtually unexplored. In part, this is because previous work has been performed either *in vivo*[96,181,183,184], where mechanistic studies are challenging, or in cell lines[178,180,182] and synaptoneurosomes[53,179], where neuronal regulatory mechanisms may be absent.

To overcome these limitations, we investigated *Arc* expression in primary neuronal cultures that recapitulate many features of *Arc* regulation *in vivo*. Our findings provide insight into the activity-dependent mechanisms of *Arc* expression and reveal an unexpected role for AMPA receptors in regulating neuronal gene expression.

Results

An *in vitro* model for elucidating mechanisms of *Arc* regulation

To study mechanisms of *Arc* regulation, we developed an *in vitro* model that recapitulates key features of *Arc* regulation discovered *in vivo*: (1) *Arc* is induced by synaptic activity in an NMDA receptor–dependent manner[42,43,52], (2) *Arc* mRNA is trafficked into dendrites[42,43,72], and (3) *Arc* is induced by BDNF in a manner that depends on mitogen-activated protein kinase (MAPK)[184].

We induced synaptic activity in cultured cortical neurons with two protocols and examined the effects on *Arc* expression. In the first protocol, synaptic activity was suppressed for 2 d with tetrodotoxin (TTX), a sodium channel antagonist. Then, neurons were washed into drug-free medium, producing a rebound of enhanced synaptic activity[186-188]. *Arc* mRNA levels increased ~200-fold over control (Figure 2.1a), and Arc protein expression was also strongly induced (Figure 2.1b). Re-addition of TTX completely suppressed rebound activity (Figure 2.2) and *Arc* expression (Figure 2.1a,b).

In the second protocol, *Arc* mRNA and protein were strongly induced with bicuculline, a $GABA_A$ receptor antagonist that disinhibits and thereby enhances ambient synaptic activity[189] (Figure 2.1c; data not shown). *Arc* induction by synaptic activity with either protocol was blocked by AP5 (Figure 2.1a–c), indicating that NMDA receptor activation was required, as observed *in vivo*.

To localize *Arc* mRNA subcellularly, we used fluorescence *in situ* hybridization (FISH). In unstimulated neurons, *Arc* mRNA was undetectable. After application of BDNF, *Arc*

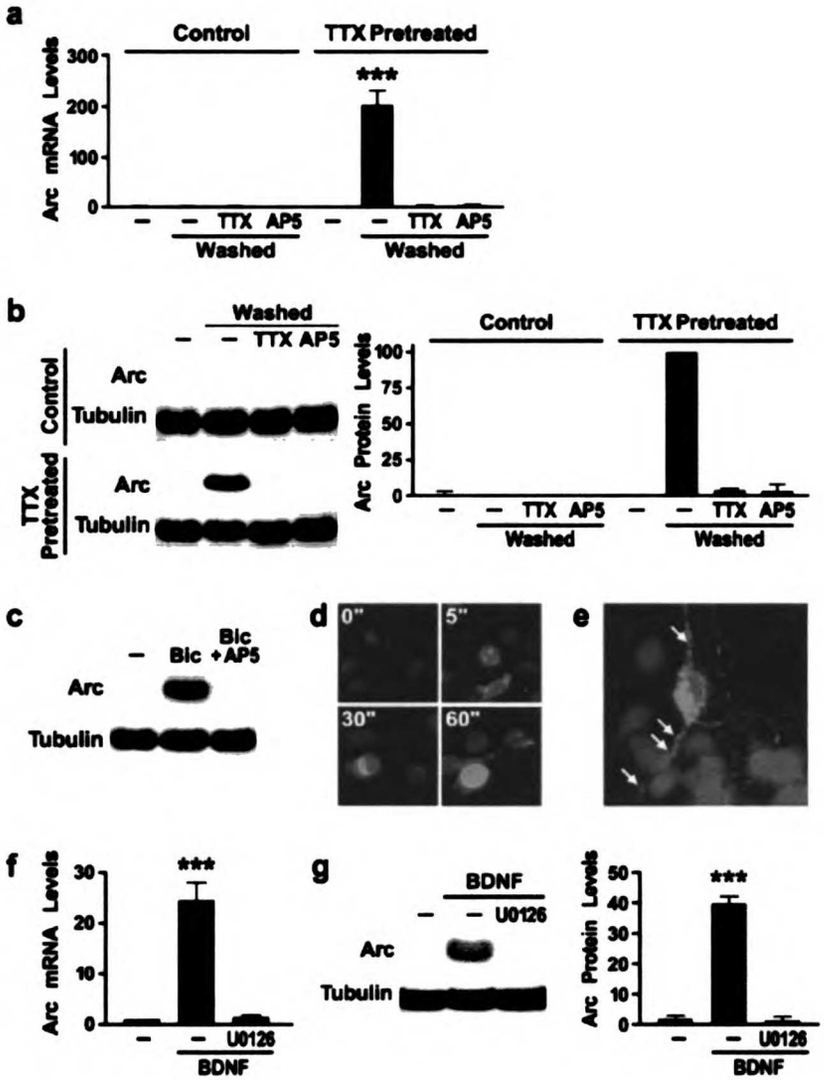


Figure 2.1 Primary cultured neurons recapitulate essential features of *Arc* regulation.

(**a**) *Arc* mRNA is strongly induced by washout of TTX (2 μM, 48 h; TTX Pretreated) but not vehicle (Control). *Arc* induction, measured 8 h after washout, was inhibited by TTX (1 μM) or AP5 (100 μM) added after washout. Data are plotted as fold inductions relative to unwashed controls (n=4 independent preparations of cells). (**b**) (left) Western blots showing that Arc protein is induced by TTX washout and inhibited by TTX or AP5 that was added subsequently. In this and subsequent Western blots, membranes were stripped and re-probed for tubulin to verify protein loading. (right) Signal intensities of blots as percent of maximum (n=4). (**c**) Arc protein expression induced by bicuculline (40 μM, 8 h) is inhibited by AP5. (**d**) FISH reveals progressive movement of *Arc* mRNA (red) from nucleus (blue) to cytoplasm over 60 min after stimulation with BDNF (100 ng/ml). (**e**) FISH combined with immunostaining for MAP2 (green) reveals *Arc* mRNA granules (red) in dendrites (arrows) of cultured neurons. (**f, g**) *Arc* mRNA (**f**; data plotted as fold relative to controls) and protein (**g**) levels induced by treatment with BDNF are inhibited by U0126 (20 μM) (n=4). Quantitative analysis of Arc protein expression (**g**, right) in arbitrary units. Error bars in this and subsequent figures represent standard deviations, unless otherwise stated. ***, p<0.001 versus control.

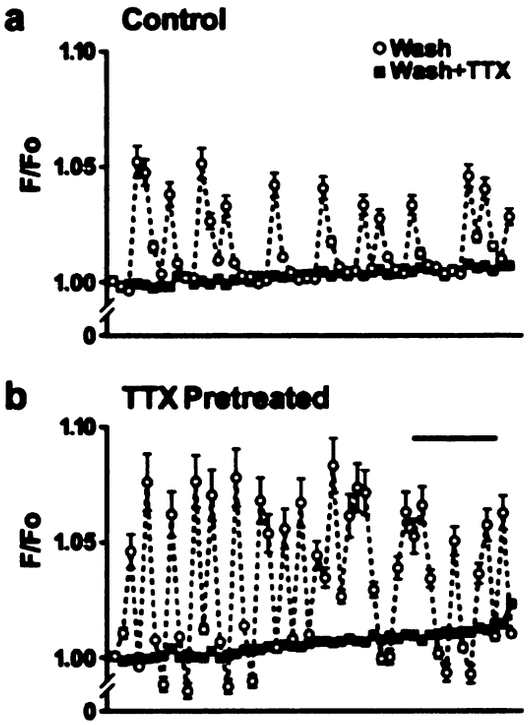


Figure 2.2 Activity induced by TTX washout enhances calcium oscillations in cultured neurons.

Intracellular calcium oscillations (dashed lines) in cells after TTX washout (lower graph) were enhanced relative to control cells (upper graph), indicating increased synaptic activity[190]. Calcium oscillations in both conditions were inhibited by TTX (1 μM; solid lines) applied just before imaging. Scale bar, 1 min. Data are plotted as mean ± s.e.m. (n=50 cells per timepoint).

mRNA appeared in discrete intranuclear foci within 5 min and had translocated into the cytoplasm by 30 min (Figure 2.1d), as reported[72]. By 60 min, *Arc* mRNA granules had co-localized with MAP2-positive dendrites (Figure 2.1e). BDNF induced *Arc* mRNA to levels ~25-fold greater than controls (Figure 2.1f) and also strongly upregulated Arc protein levels (Figure 2.1g). BDNF-induced increases in *Arc* mRNA and protein levels were blocked by two inhibitors of the MAPK pathway, U0126 (Figure 2.1f,g) and PD 98059 (data not shown).

Arc protein can be expressed by glia[65] but was undetectable in pure glial cultures

stimulated with BDNF (Figure 2.3). Cultures maintained in medium that promotes a nearly pure neuronal population[191] showed robust *Arc* expression in response to BDNF (Figure 2.3). Thus, neurons, not glia, are responsible for BDNF-induced *Arc* expression. Taken together, we conclude that this *in vitro* model recapitulates critical features of *Arc* expression observed *in vivo* and is useful for elucidating molecular mechanisms that regulate *Arc* in neurons.

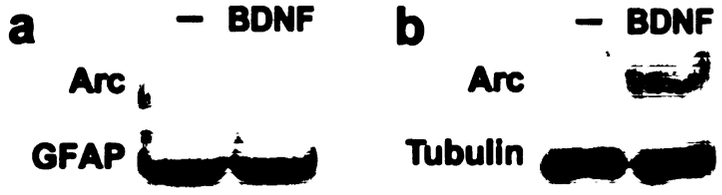


Figure 2.3 BDNF induces Arc protein expression in neurons, not glia.

(**a**) Western blot showing that Arc expression is undetectable in pure glial cultures stimulated with BDNF (100 ng/ml, 8 h). Blots were re-probed for GFAP, a glial-specific protein, to verify protein loading. (**b**) Western blot showing that BDNF induces Arc expression in cultures maintained in Neurobasal medium, which promotes a nearly pure neuronal population[191]. Western blots are representative of results from at least three independent experiments.

Full BDNF-induced *Arc* expression requires synaptic activity

BDNF and synaptic activity are tightly intertwined[192]: BDNF promotes synaptic activity[193] and synaptic activity promotes BDNF release[194]. Since both potently induce *Arc* in our system (Figure 2.1), we determined if the mechanism of *Arc* induction is either by synaptic activity–induced BDNF release or by BDNF-induced synaptic activity.

We blocked endogenous BDNF signaling with TrkB-IgG, a soluble form of the BDNF receptor[195], which competes with endogenous TrkB for binding to BDNF[196]. TrkB-IgG (10 μg/ml) completely blocked Arc protein induction by exogenous BDNF (100 ng/ml; data not

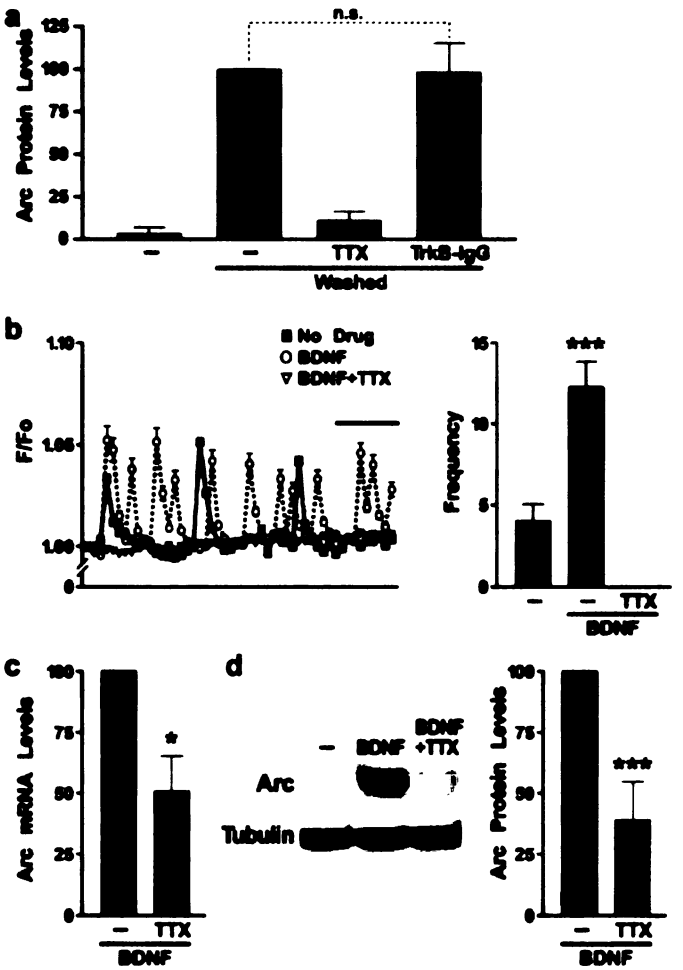


Figure 2.4 Synaptic activity is required for a component of BDNF-induced *Arc* expression.

(**a**) TrkB-IgG (10 μg/ml) does not inhibit Arc protein expression induced by TTX washout (see **Fig. 1a,b**). Signal intensities of Western blots as percent of maximum (n=4). (**b**) (left) Calcium oscillations recorded in the presence of BDNF (100 ng/ml) are inhibited by TTX (1 μM). The average response (F/F_o; see Methods) of 50 randomly selected cells is plotted at each timepoint (mean ± s.e.m.), so oscillations above baseline indicate synchronous responses. Scale bar, 1 min. (right) Quantitative analysis of calcium oscillations showing frequency per 5 min (n=7 imaging runs using cells from three independent preparations). (**c**, **d**) TTX partially inhibits BDNF-induced increases in *Arc* mRNA (**c**) and protein (**d**) levels (n=4). Data in **c** and **d** are plotted as percent of *Arc* expression induced by BDNF alone to highlight the ~50% inhibition by TTX. n.s. = not statistically significant; *, $p<0.05$; ***, $p<0.001$ versus control.

shown) but had no effect on *Arc* induction after TTX washout (Figure 2.4a). In contrast, inhibiting BDNF-induced synaptic activity (Figure 2.4b) with TTX reduced BDNF-induced

increases in *Arc* mRNA (Figure 2.4c) and protein (Figure 2.4d) levels by ~50%. Thus, endogenous BDNF is not required for synaptic activity to induce *Arc* expression but a component of BDNF-induced *Arc* expression requires synaptic activity.

Bidirectional regulation of *Arc* by NMDA and AMPA receptors

Synaptic activity produces currents and intracellular calcium rises that depend on postsynaptic glutamate receptors. To determine how synaptic activity couples to *Arc* expression, we pharmacologically dissected synaptic activity with specific antagonists of glutamate receptors.

We stimulated neurons with BDNF in the presence of AP5 and measured *Arc* mRNA and protein levels. Like TTX (Figure 2.4c,d), AP5 inhibited BDNF-induced increases in *Arc* mRNA and protein by ~50% (Figure 2.5a,b) and occluded the effects of TTX (data not shown), suggesting that NMDA receptors are essential only for the activity-dependent component of BDNF-induced *Arc* expression. MCPG, a potent mGluR antagonist, had no effect on BDNF-induced *Arc* expression (Figure 2.5a,b).

To determine the role of AMPA receptors, we used NBQX and GYKI, highly selective AMPA receptor antagonists that are structurally and mechanistically distinct[197,198]. Unexpectedly, both drugs strongly *potentiated* BDNF-induced increases in *Arc* mRNA and protein levels (Figure 2.5a,b). That two different AMPA receptor antagonists affect BDNF-induced *Arc* expression so similarly is strong evidence against non-specific effects of these drugs. Additionally, AMPA receptor antagonists have no effect on neurotrophin-induced *Arc*

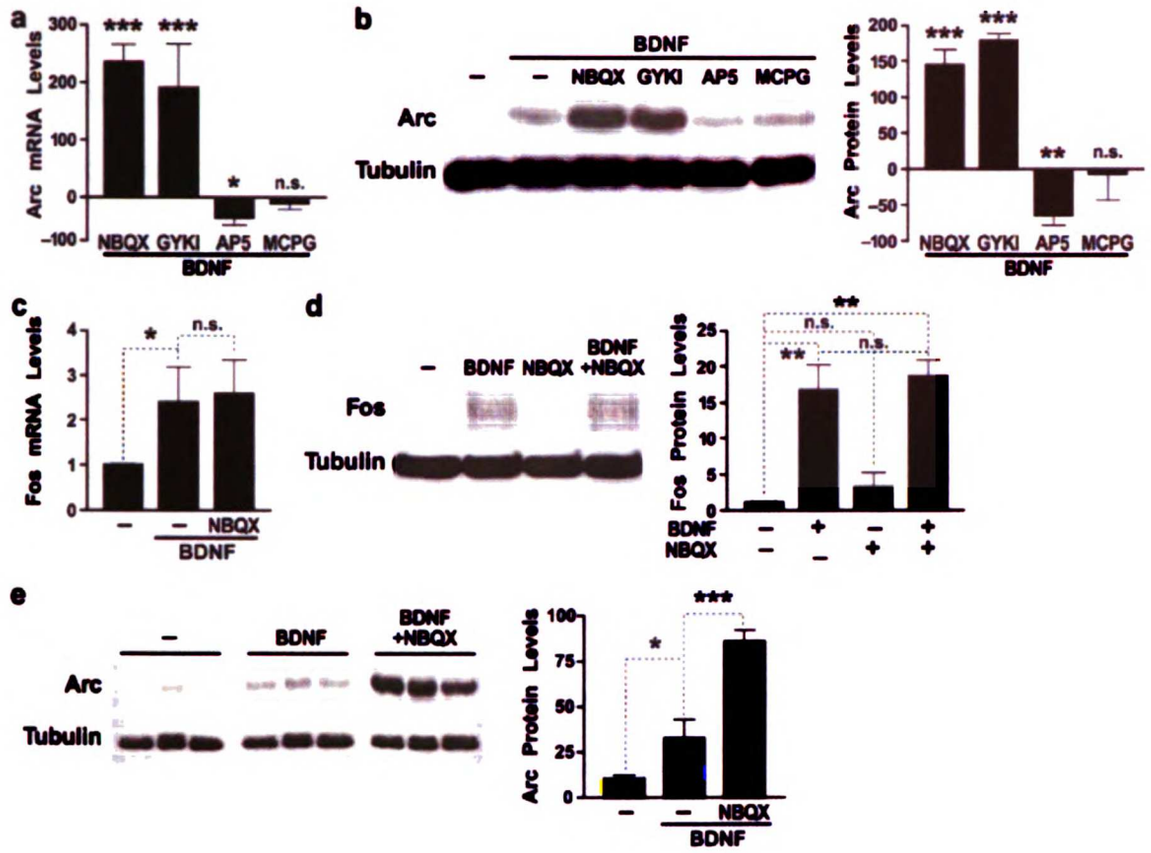


Figure 2.5 AMPA receptor inhibition selectively potentiates BDNF-induced *Arc* expression in cultured neurons and brain slices.

(**a**, **b**) Effects of glutamate receptor antagonists (in μM: 10 NBQX, 15 GYKI, 100 AP5, 100 MCPG) on BDNF-induced *Arc* mRNA (**a**) and protein (**b**) levels. Data in **a** and **b** are plotted as percent difference from BDNF alone to highlight the bidirectional effects of AMPA and NMDA receptor antagonists (n=8). (**c**, **d**) BDNF-induced expression of *Fos* mRNA (**c**; data plotted as fold relative to controls) and protein (**d**) is not potentiated by NBQX, indicating that AMPA receptor inhibition does not non-specifically increase gene expression (n=4). Quantitative analysis of Fos protein expression (**d**, right) in arbitrary units. (**e**) (left) Western blot showing that NBQX potentiates BDNF-induced Arc protein expression in organotypic brain slices (three representative slices per condition shown). (right) Quantitative analysis of Arc protein expression in arbitrary units (n = three different animals, six slices per stimulation condition). n.s. = not statistically significant; *, p<0.05; **, p<0.01; ***, p<0.001 versus control.

expression in undifferentiated PC12 cells (data not shown), which only express low levels of GluR2 subunits[199] and therefore essentially lack functional AMPA receptors[200]. Potentiation of BDNF responses by NBQX was blocked by TTX, confirming that only the activity-

dependent component of BDNF-induced *Arc* expression was regulated by AMPA receptors (data not shown). Additionally, NBQX did not affect BDNF-induced expression of *Fos* (Figure 2.5c,d), an IEG used as a control for *Arc*-inducing stimuli[55,76], indicating that gene expression was not globally upregulated.

Although our primary neuronal culture system recapitulates many key features of *Arc* regulation *in vivo* (Figure 2.1), we wondered whether AMPA receptor inhibition would potentiate *Arc* expression in a more physiological context. To test this, we stimulated organotypic brain slice cultures[201] with BDNF in the presence or absence of NBQX. BDNF upregulated Arc protein expression in cultured slices by threefold (Figure 2.5e), consistent with *in vivo* findings[183,184]. As in primary neurons, NBQX strongly potentiated BDNF-induced increases in Arc protein levels (Figure 2.5e).

Specific patterns of activity-dependent gene expression are often determined by calcium signaling[27,202]. AMPA receptor antagonists should limit postsynaptic depolarization, inhibiting voltage-sensitive membrane events such as activation of L-type voltage-sensitive Ca^{2+} channels (L-VSCCs). Since L-VSCCs can couple synaptic activity to neuronal gene expression[203-205] and since the loss of Ca^{2+} entry through L-VSCCs can influence gene expression[206], we determined whether L-VSCC inhibition mimics the effect of AMPA receptor inhibition. Nimodipine, an L-VSCC antagonist, did not significantly affect Arc protein expression induced by BDNF (Figure 2.6). Although dihydropyridines like nimodipine are poor blockers of L-VSCCs under some conditions[207], nimodipine inhibited Arc protein expression induced by direct activation of L-VSCCs with high KCl (Figure 2.6).

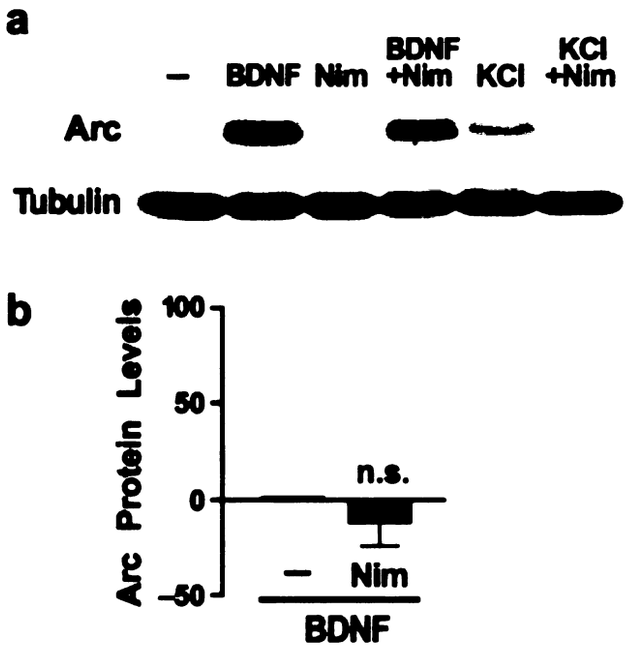


Figure 2.6 An L-VSCC antagonist does not potentiate BDNF-induced Arc protein expression.

(**a**) Western blots showing that L-VSCC inhibition with nimodipine (Nim) blocks Arc protein expression induced by high KCl (55 mM, 8 h) but does not mimic the effect of AMPA receptor inhibition on BDNF-induced Arc expression (compare to **Fig. 3b**). (right) Signal intensities of blots plotted as percent difference from BDNF alone (n=3). n.s. = not statistically significant compared to control.

In addition to regulating calcium entry through L-VSCCs, a subset of AMPA receptors are themselves calcium-permeable and can signal to the nucleus[208]. Approximately 15% of neurons in cortical cultures possess calcium-permeable AMPA receptors[209,210]. To test whether blockade of these AMPA receptors potentiates BDNF-induced Arc protein levels, we used Joro spider toxin (JSTX), a specific antagonist of calcium-permeable AMPA receptors[211,212]. JSTX inhibited calcium-permeable AMPA receptors in heterologous cells, but did not potentiate BDNF-induced Arc protein expression in neurons (Figure 2.7). Thus, calcium-impermeable AMPA receptors are the ones that regulate BDNF-induced Arc

expression.

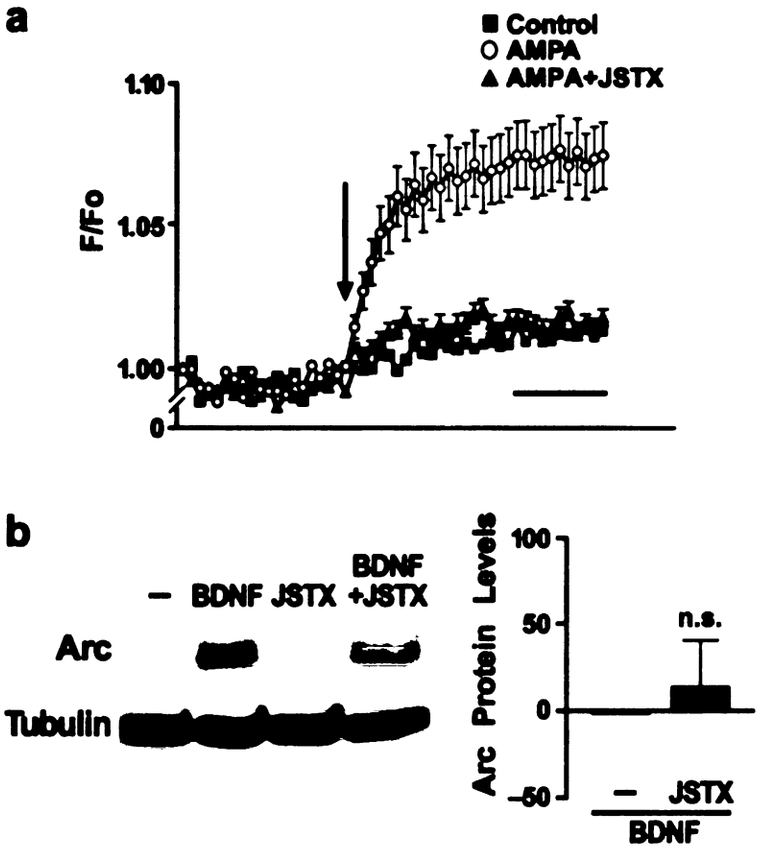


Figure 2.7 A specific antagonist of calcium-permeable AMPA receptors does not potentiate BDNF-induced Arc protein expression.

(**a**) HEK293T cells expressing homomeric, calcium-permeable AMPA receptors were produced by transient transfection of GluR1. Joro spider toxin (JSTX; 1 μM) inhibits calcium responses induced in transfected cells by AMPA (50 μM; added at time point indicated by arrow). The average response (F/F_o; see Methods) of 50 transfected cells is plotted at each time point (mean ± s.e.m.). AMPA did not induce calcium responses in untransfected cells (data not shown). (**b**) (left) Western blot showing that JSTX does not affect BDNF-induced Arc protein expression in neurons. (right) Signal intensities of blots plotted as percent difference from BDNF alone (n=4). n.s. = not statistically significant compared to control.

The NMDA:AMPA ratio determines *Arc* expression

Given the opposing effects of NMDA and AMPA receptor antagonists, we hypothesized that activity-dependent *Arc* expression is determined by the balance between these receptor

activities.

BDNF-induced Arc protein levels were unaffected by simultaneous addition of AP5 and NBQX (Figure 2.8a,b), a striking contrast to the effects of each antagonist singly applied (Figure 2.5b and Figure 2.8a,b). We stimulated neurons with the endogenous neurotransmitter glutamate, predicting that net *Arc* expression after simultaneous activation of both NMDA and AMPA receptors would be low. Indeed, glutamate produced only modest increases in *Arc* mRNA and protein levels (Figure 2.8c; data not shown). However, AP5 inhibited and NBQX potentiated glutamate-induced *Arc* expression (Figure 2.8c; data not shown).

We next determined the effect of blocking NMDA or AMPA receptors on *Arc* expression under more physiological conditions. Spontaneous synaptic activity can trigger nuclear gene expression[213] and may be critical for the establishment of functional neuronal circuits *in vivo*[214]. TTX inhibited the low basal expression of *Arc* mRNA in our cultures (Figure 2.8d), indicating that spontaneous synaptic activity induces ongoing *Arc* expression. *Arc* mRNA levels induced by ambient activity were increased by NBQX and decreased by AP5, but were unaffected by simultaneous addition of both drugs (Figure 2.8d). These data suggest that the relative extent of NMDA and AMPA receptor activation is a critical determinant of *Arc* expression.

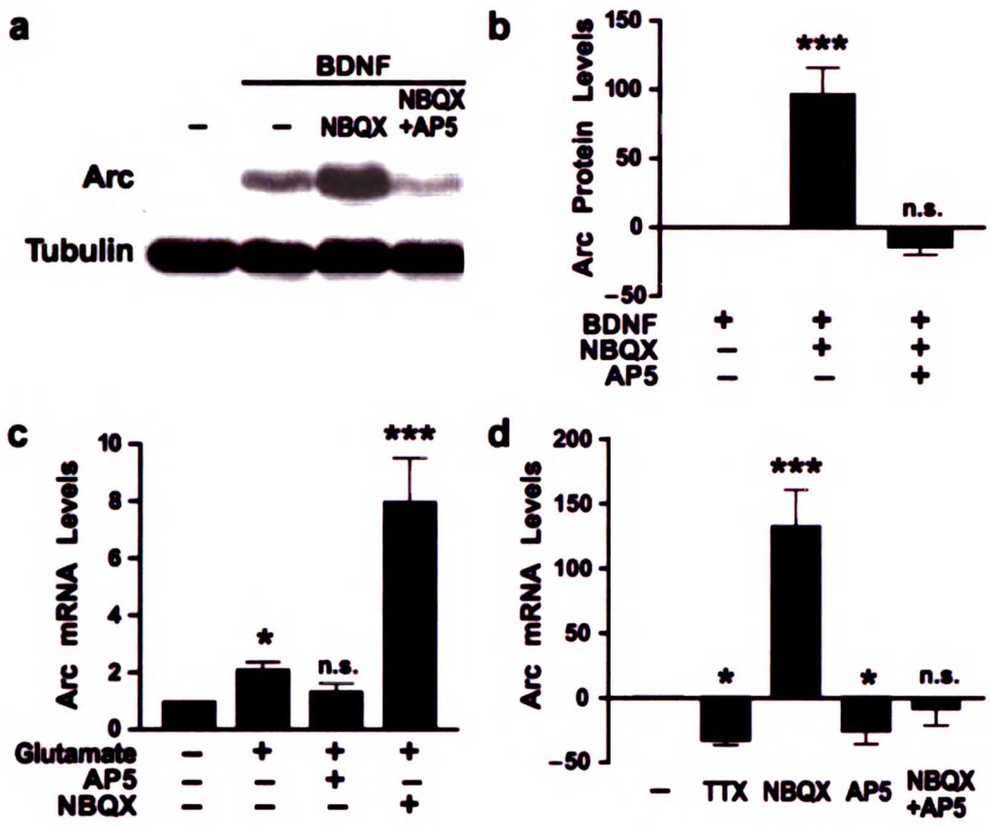


Figure 2.8 The relative extent of NMDA and AMPA receptor activation determines *Arc* expression.

(**a**) Western blots showing that simultaneous blockade of NMDA and AMPA receptors with NBQX+AP5 has no effect on BDNF-induced Arc protein expression. Effect of NBQX shown for comparison. (**b**) Signal intensities from blots in **a** plotted as percent difference from BDNF alone (n=4). (**c**) *Arc* mRNA levels induced by simultaneous activation of NMDA and AMPA receptors with glutamate (30 μM, 6 h). Glutamate-induced *Arc* levels are increased by NBQX and decreased by AP5. Data are plotted as fold induction relative to unstimulated controls (n=4). (**d**) NMDA and AMPA receptors bidirectionally regulate *Arc* mRNA levels in the absence of exogenous BDNF or glutamate. Effect of TTX indicates that spontaneous synaptic activity in the culture induces ongoing *Arc* expression. *Arc* levels induced by ambient activity are increased or decreased by NBQX or AP5, respectively. Data are plotted as percent difference from control (n=4). n.s. = not statistically significant; *, $p<0.05$; ***, $p<0.001$ versus control.

Metabotropic signaling by AMPA receptors regulates *Arc* expression

NMDA receptors are well-known mediators of gene expression[215,216], but a prevailing view holds that AMPA receptors are effectors, not mediators, of activity-dependent plasticity[217].

Therefore, the ability of AMPA receptor inhibition to potentiate *Arc* expression was unexpected, and we focused on elucidating the mechanisms by which AMPA receptors regulate *Arc*.

A growing body of evidence indicates that AMPA receptors are cell-surface signal transducers, not just passive conduits for current flux[208,218-221]. We hypothesized that AMPA receptor inhibition potentiates *Arc* expression by limiting activation of signaling molecules which associate with the channel, including a Src-family tyrosine kinase, Lyn[219,222], and a G-protein[223,224]. Therefore, we determined whether specific inhibition of these effector molecules mimics the effect of AMPA receptor inhibition on *Arc* expression. PP2, a Src-family tyrosine kinase inhibitor, had no effect on BDNF-induced Arc protein expression (Figure 2.9a), suggesting that Lyn may not be required for AMPA receptor–mediated control of *Arc* expression. However, pertussis toxin (PTX), which inhibits a G_i-protein activated by AMPA receptors in cortical neurons[223], potentiated basal- and BDNF-induced *Arc* expression (Figure 2.9b,c) and occluded the effect of NBQX (Figure 2.9b,c). Cholera toxin (CTX), which has a catalytic activity similar to that of PTX but activates G_s-proteins, had no effect on basal- or BDNF-induced *Arc* expression (Figure 2.9b,c). PTX-induced increases in basal *Arc* expression were inhibited by TTX (Figure 2.9d), suggesting that PTX-sensitive G-proteins, but not Src-family kinases, mediate an activity-dependent signaling mechanism linking AMPA receptors and *Arc* expression.

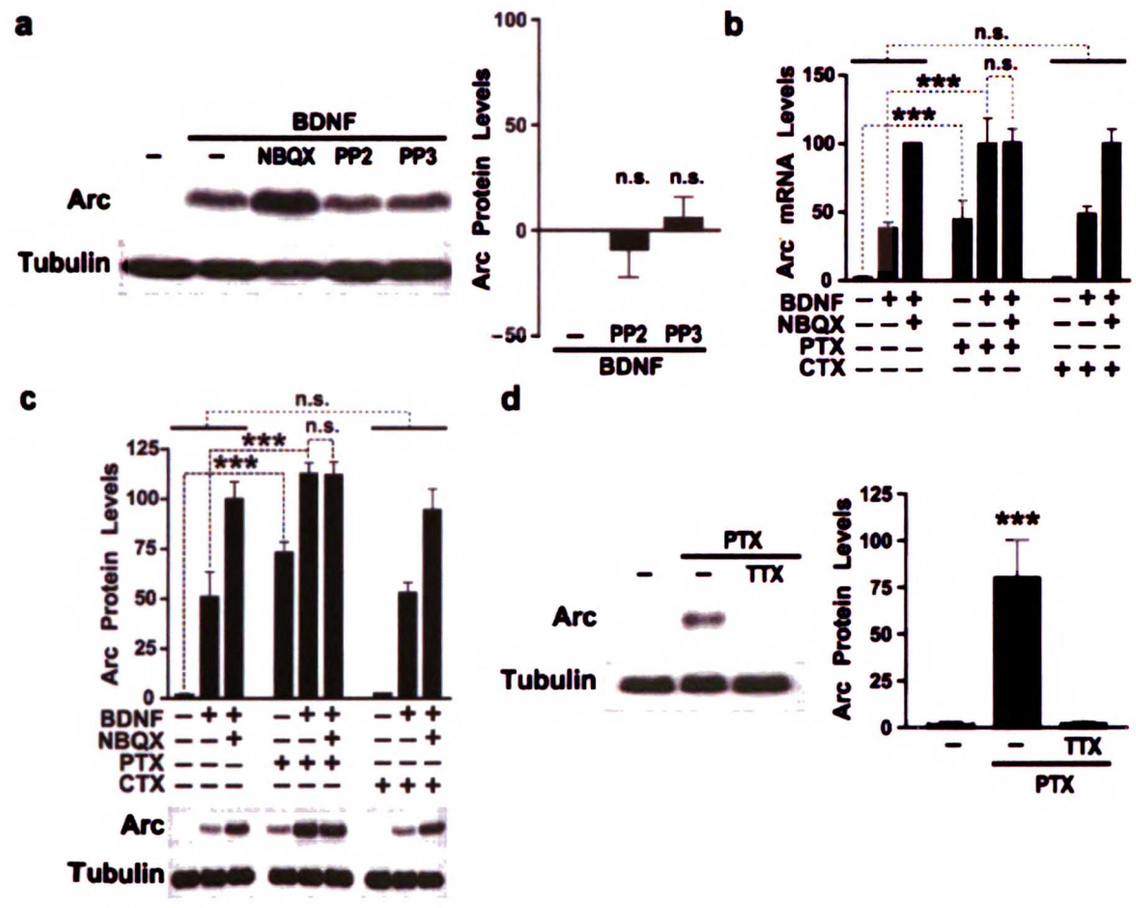


Figure 2.9 Pertussis toxin-sensitive G-proteins, but not Src-family kinases, may mediate regulation of *Arc* expression by AMPA receptors.

(**a**) (left) Western blot showing that neither PP2 (1 μM), a Src-family kinase inhibitor, nor its inactive analog, PP3 (1 μM), affect Arc protein levels induced by BDNF. (right) Quantitative analysis of Arc protein expression plotted as percent difference from BDNF alone (n=6). (**b**, **c**) Pertussis toxin (PTX; 200 ng/ml, 20 h pre-incubation), but not cholera toxin (CTX; 1 μg/ml, 20 h pre-incubation), potentiates basal- and BDNF-induced *Arc* mRNA (**b**) and protein (**c**) expression. PTX occludes the effect of NBQX on BDNF-induced *Arc* expression. Data are plotted as percent of response to BDNF (**b**; n=7) or in arbitrary units (**c**; n=6). Lanes of Western blot shown in (**c**) align with symbols above indicating drug combinations. (**d**) (left) Western blot showing that Arc protein levels induced by PTX are inhibited by TTX (1 μM) (n=3). (right) Quantitative analysis of Arc protein expression in arbitrary units. n.s. = not statistically significant; ***, p<0.001 versus control.

AMPA receptors do not regulate major TrkB signaling pathways

We next sought to determine the level at which AMPA receptor signaling regulates BDNF-

induced expression of *Arc*. TrkB is a receptor tyrosine kinase that binds BDNF and then initiates downstream signaling, including phosphatidylinositol 3-kinase (PI3K), phospholipase Cγ (PLCγ), and MAPK pathways[225]. TrkB localizes to the postsynaptic density of glutamatergic synapses[226]. Here, surface expression and activation of TrkB are regulated by synaptic activity[227-229], and TrkB signaling can be modulated by G-proteins[230]. Thus, one possibility is that AMPA receptor inhibition potentiates BDNF-induced *Arc* expression by affecting the activation of TrkB or downstream signaling molecules.

Levels of phosphorylated, active Trk receptor increased rapidly after treatment with BDNF (Figure 2.10). Trk phosphorylation was blocked by K252a, a Trk kinase inhibitor, but unaffected by NBQX (Figure 2.10). Levels of phosphorylated extracellular signal-regulated kinase (ERK) also increased and were inhibited by U0126, a MAPK/ERK-kinase inhibitor (Figure 2.10). NBQX inhibited ERK phosphorylation induced by AMPA but not BDNF (Figure 2.10). Tyrosine phosphorylation of immunoprecipitated PLCγ1 was similar in neurons stimulated with BDNF or BDNF+NBQX (Figure 2.10), indicating similar levels of activation. PI3K pathway activation was determined at the level of a downstream effector, Akt. BDNF-induced Akt phosphorylation was sensitive to LY 294002, a PI3K inhibitor, but unaffected by NBQX (Figure 2.10).

An effect of AMPA receptor inhibition on TrkB signal transduction in the previous experiments might have gone unnoticed because of subtle differences in the activation time course of the signaling pathways we tested. To address this issue, we transfected neurons with a plasmid carrying the *Photinus* luciferase gene driven by the serum response element

(SRE), a MAPK pathway target sequence upstream of many activity-dependent genes[231-233]. BDNF was applied to transfected cells for several hours, and TrkB signaling was measured as cumulative luciferase expression, a sensitive downstream endpoint that integrates changes in upstream signaling pathways over time. BDNF-induced luciferase expression was inhibited by U0126 but not significantly affected by NBQX (Figure 2.10). Thus, AMPA receptors do not detectably regulate major TrkB signaling pathways and instead may directly regulate *Arc* synthesis and/or turnover.

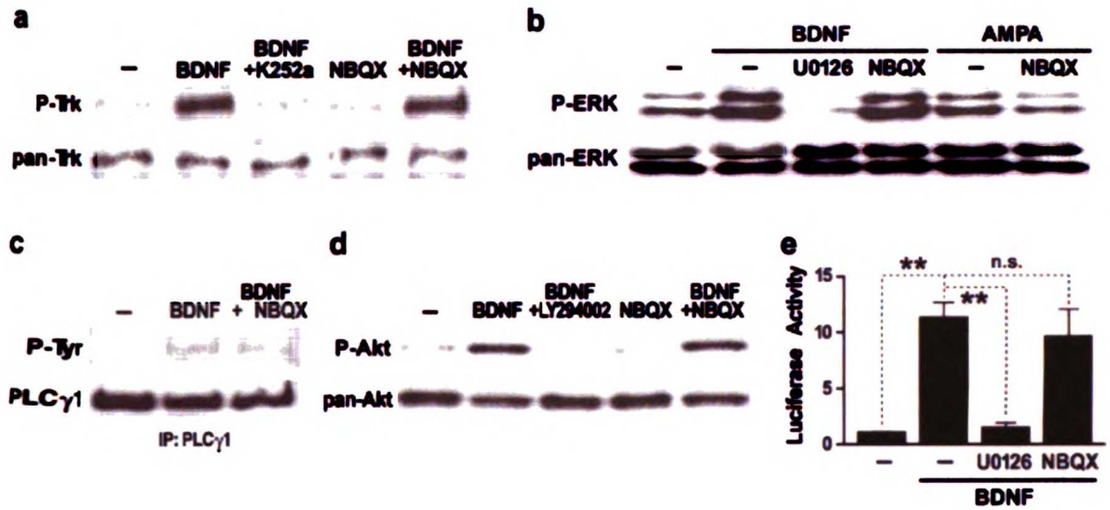


Figure 2.10 AMPA receptors do not regulate signals sent by TrkB.

(**a**) Western blots showing that Trk phosphorylation (P-Trk) induced by BDNF (100 ng/ml, 10 min) is blocked by K252a (400 nM) but unaffected by NBQX (10 μM). (**b**) Western blots showing that phosphorylation of ERK1/2 (P-ERK) induced by 5 min of stimulation with BDNF is blocked by U0126 (20 μM) but unaffected by NBQX. Verifying its efficacy, NBQX inhibited ERK phosphorylation induced by AMPA (1 μM, 5 min). (**c**) Western blots showing that tyrosine phosphorylation (P-Tyr) of immunoprecipitated PLCγ1 is similar in cells stimulated with BDNF (100 ng/ml, 1 h) or BDNF+NBQX. (**d**) Western blots showing that phosphorylation of Akt (P-Akt) induced by 10 min of stimulation with BDNF is blocked by LY 294002 (10 μM) but unaffected by NBQX. (**e**) BDNF-induced expression of a SRE reporter gene construct is inhibited by U0126 but unaffected by NBQX. Normalized luciferase activity (see Methods) is plotted as fold induction relative to unstimulated controls (n=5). Western blots in **a**–**d** are representative of results from at least three independent experiments and were reprobed for total (pan) levels of TrkB, ERK, PLCγ1, or Akt to verify protein loading. n.s. = not statistically significant; **, p<0.01 versus control.

AMPA receptors are not major regulators of *Arc* translation

Translational control has emerged as a critical mechanism in long-term synaptic plasticity[37,234-236], but the evidence for translational regulation of *Arc* is mixed: some studies suggest that *Arc* may be translated near synaptic sites[53,55], while another failed to detect *Arc* in a screen for mRNAs that associate with polysomes in response to BDNF[237]. Given these conflicting reports, we tested directly whether AMPA receptors regulate *Arc* translation

Cis-acting regulatory elements mediating translational control are often found in the untranslated regions (UTRs) of mRNA transcripts[238], and these elements can be targeted by signaling cascades involved in plasticity processes[239]. Translational control mediated by these elements is commonly studied using plasmids in which the 5' and/or 3' UTRs from the gene of interest are fused to a reporter gene[240-242]. We synthesized an *Arc* reporter mRNA comprising the *Photinus pyralis* luciferase coding sequence flanked by the 5' and 3' UTRs from the rat *Arc* gene (Figure 2.11a). Using an RNA-based translation reporter avoids confounding effects from stimulus-induced transcription. We controlled for global effects of BDNF on translation[243,244] by co-transfection of a *Renilla reniformis* luciferase mRNA lacking UTRs (Figure 2.11a). Neurons co-transfected with both mRNAs were stimulated and assayed for *Photinus* and *Renilla* luciferase expression. The *Arc* UTRs enhanced BDNF-induced translation (Figure 2.11b), providing direct evidence for post-transcriptional regulation of *Arc* by BDNF. NBQX alone induced a slight increase in *Arc* translation (Figure 2.11b), possibly reflecting enhanced dendritic protein synthesis resulting from blockade of

miniature excitatory synaptic events (minis)[245]. However, BDNF-dependent translation was not potentiated by NBQX (Figure 2.11b).

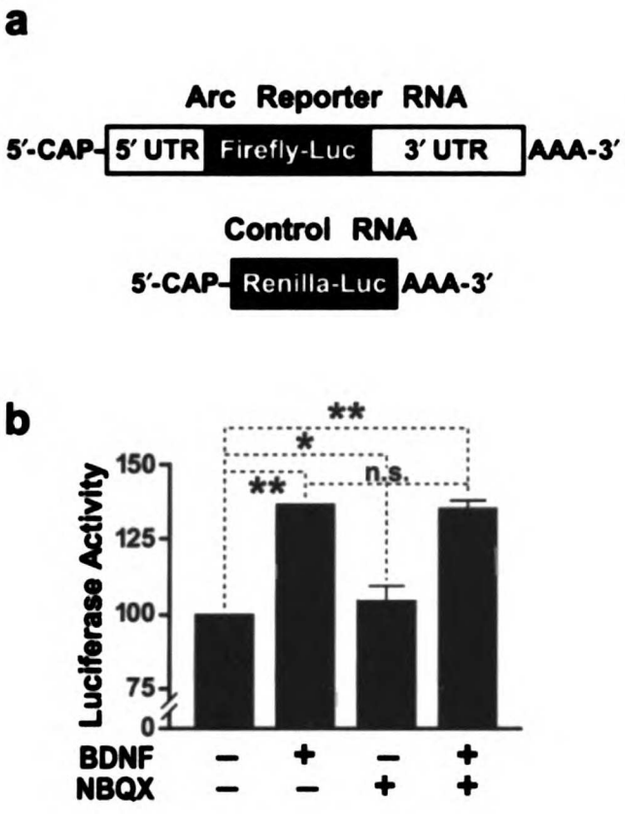


Figure 2.11 AMPA receptor inhibition does not affect BDNF-induced *Arc* translation.

(**a**) A schematic (not drawn to scale) illustrates the design of RNA reporters used for assaying translation mediated by *Arc* UTRs. (**b**) BDNF-induced translation of an *Arc* reporter RNA is not significantly affected by NBQX. NBQX alone induces a slight increase in *Arc* reporter expression. Normalized luciferase activity is plotted as percent of unstimulated controls (n=4). n.s. = not statistically significant; *, p<0.05; **, p<0.01 versus control.

AMPA receptors do not regulate Arc protein turnover

Activity-dependent and brain region–specific changes in *Arc* stability have been proposed as mechanisms for regulation of *Arc* expression[52,77]. In neurons, most regulated protein turnover is handled by the ubiquitin-proteasome system (UPS)[246,247]. AMPA receptors can regulate

UPS-dependent protein degradation[248], but the endogenous substrates have not been identified[249]. We examined whether AMPA receptors inhibit proteasome-dependent Arc degradation, thereby potentiating the levels of Arc that accumulate after induction by BDNF.

BDNF-induced Arc protein levels were potentiated by two proteasome inhibitors, MG-132 (Figure 2.12a) and ALLN (data not shown), suggesting that Arc protein is a proteasome substrate. To test whether AMPA receptors regulate proteasome-dependent degradation, we used a fluorescent protein reporter of the UPS, GFP^{u}[250]. Consisting of a degron peptide fused to green fluorescent protein, GFP^{u} undergoes rapid turnover by the UPS ($t_{1/2}$ = 20–30 min)[250], allowing inhibition of proteasome function to be visualized in live transfected cells by increases in green fluorescence. We imaged the same population of GFP^{u}-expressing neurons before and after treatment with MG-132, AMPA receptor antagonists, or vehicle control. All treatments included cycloheximide (CHX), a protein synthesis inhibitor, to isolate effects on the pre-existing intracellular pool of GFP^{u}. GFP^{u} fluorescence was stable in the presence of MG-132 but not AMPA receptor antagonists (Figure 2.12b), indicating that AMPA receptor inhibition does not lead to general inhibition of the UPS.

AMPA receptor inhibition might still reduce Arc protein turnover by acting on some upstream component of the UPS. For example, a putative kinase or E3 ubiquitin ligase could modify Arc and target it for degradation without globally affecting UPS function. To test whether AMPA receptor inhibition blocks degradation of endogenous Arc protein, we first induced Arc expression by stimulating neurons with BDNF or BDNF+NBQX. Then, either MG-132 or NBQX was added to the medium, along with CHX to prevent additional Arc

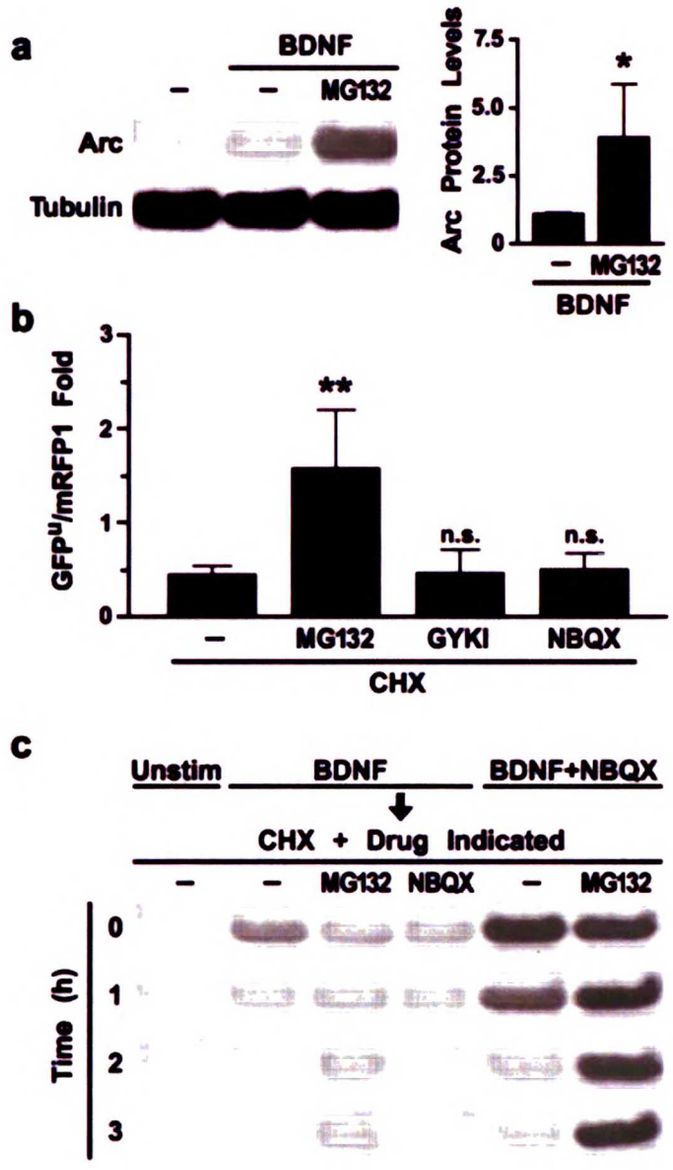


Figure 2.12 AMPA receptor inhibition does not stabilize Arc protein.

(**a**) (left) Western blot showing that BDNF-induced Arc protein expression is potentiated by MG-132 (50 μM), a proteasome inhibitor. (right) Signal intensities of blots plotted as fold induction relative to BDNF alone (n=3). (**b**) GFP^u (normalized by mRFP1; see Methods) is stabilized in the presence of MG-132 but not AMPA receptor antagonists (n=208 cells). In cells treated with MG-132, fluorescence fold changes greater than 1 likely reflect maturation of GFP^u molecules that were translated but incompletely folded at the time of CHX addition. (**c**) Arc protein induced during a 5-h pre-treatment (represented by arrow) with BDNF or BDNF+NBQX is stabilized by MG-132 but not AMPA receptor antagonists after addition of CHX. n.s. = not statistically significant; *, $p<0.05$; **, $p<0.01$ versus control.

synthesis, and Arc protein levels were measured at times thereafter. If AMPA receptor blockade inhibits the turnover of Arc protein, Arc levels should decline more slowly in NBQX-treated cells than in control cells. Arc levels were stable in MG-132-treated cells, but in NBQX-treated cells, as in control cells, Arc levels declined to baseline over the course of 3 h (Figure 2.12c). Thus, although the UPS rapidly degrades Arc protein, changes in UPS function do not mediate AMPA receptor regulation of Arc protein levels.

AMPA receptors regulate *Arc* transcription, but not mRNA stability

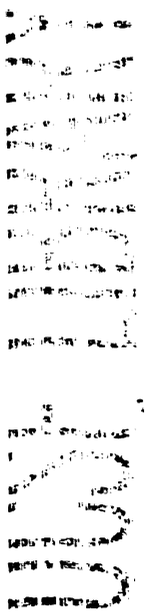
Increased Arc protein expression could result from a primary effect of AMPA receptor inhibition on *Arc* mRNA levels. IEG expression in neurons is tightly controlled and mRNAs transcribed from these genes are often short-lived. For example, *Fos* mRNA has an intracellular half-life of 10-15 min[44], and transcript stability is one mechanism by which activity regulates *Fos* expression[251]. Therefore, we determined whether AMPA receptors potentiate levels of *Arc* mRNA by stabilizing *Arc* transcripts.

First, *Arc* mRNA was induced in cultured neurons by treatment with BDNF. Then, either NBQX or vehicle was added along with actinomycin D (Act D), a potent transcriptional inhibitor that prevents synthesis of additional *Arc* mRNA. At various times, total *Arc* mRNA levels were determined by qfRT-PCR (Figure 2.13a) and Northern blotting (data not shown). By fitting these data to single-exponential decay equations, we estimated an intracellular half-life for *Arc* mRNA of 47 min, considerably shorter than a previous estimate of 1.26 h for *Arc* mRNA induced by KCl depolarization[252]. Importantly, NBQX did not significantly affect

the decay kinetics of *Arc* mRNA (Figure 2.13a; data not shown).

Although AMPA receptor inhibition produced no overall effects on *Arc* mRNA stability, we worried that we might have missed important effects on the stability of subcellular pools of *Arc*. For example, local enhancement of *Arc* transcript stability has been proposed to underlie the enrichment of *Arc* at activated synaptic sites[40,52]. Therefore, we assessed *Arc* transcript stability by FISH, a method that can reveal effects on mRNA stability in different subcellular compartments[253].

Cells were treated as before (Figure 2.13a) and fixed at various times after addition of Act D. As observed by qfRT-PCR (Figure 2.13a), total *Arc* mRNA levels returned to baseline by 2 h (Figure 2.13b). Imaging cells at higher magnification failed to reveal preferential stabilization of *Arc* mRNA in any subcellular region (data not shown), confirming that AMPA receptors do not regulate *Arc* mRNA stability. Thus, AMPA receptor–mediated regulation of *Arc* mRNA expression may occur principally by a transcriptional mechanism.



Transcription of some IEG's, like *Fos*, is known to be highly regulated through multiple mechanisms[44,254]. By contrast, the mechanisms controlling *Arc* transcription have only begun to be explored[180,233].

The rat *Arc* gene has two short introns, located within the 3'UTR, which are presumably spliced out during transcript maturation. We sought to monitor *Arc* transcription by measuring levels of nascent, unspliced *Arc* transcript, an approach that compares favorably with nuclear run-on assays for assessment of rapid changes in neuronal gene transcription[255].

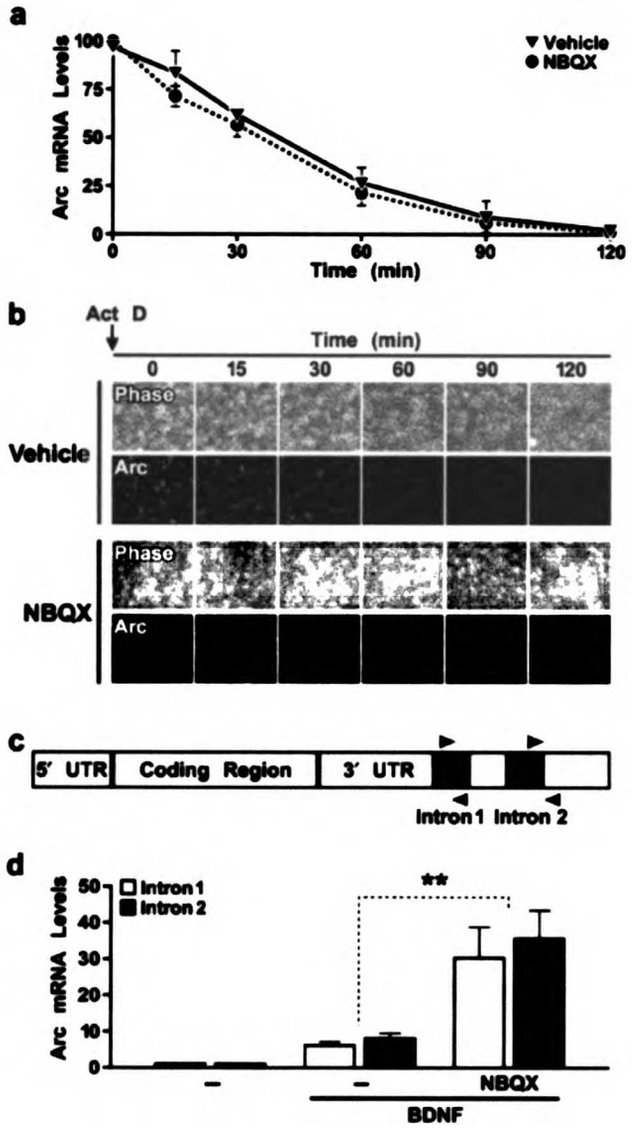


Figure 2.13 AMPA receptors regulate *Arc* expression at the level of transcription, but not mRNA stability.

(**a**) Relative to vehicle control, NBQX does not stabilize BDNF-induced *Arc* mRNA. Data are plotted as percent of *Arc* mRNA levels immediately after addition of Act D (1 μM). Decay curves were not significantly different and, when fit to single-exponential decay equations, indicated a half-life for *Arc* mRNA of 47 min. (**b**) FISH reveals that NBQX does not stabilize BDNF-induced *Arc* mRNA in any subcellular region after addition of Act D. Epifluorescence images of *Arc* mRNA signal are shown along with corresponding phase contrast images from 20× fields of cells. (**c**) Schematic (not drawn to scale) illustrating the putative organization of nascent *Arc* transcripts. Intron 1 (235 bases) and intron 2 (179 bases) are located within the ~1.6-kb 3'UTR. Arrowheads indicate positions of qfRT-PCR primers. (**d**) qfRT-PCR with intronic primer pairs reveals that NBQX potentiates BDNF-induced increases in nascent *Arc* transcript levels, indicating an effect of AMPA receptor inhibition on *Arc* transcription. Nascent transcript levels are plotted as fold induction over unstimulated controls (n=5). n.s. = not statistically significant; **, p<0.01 versus control.

To do this, we designed two qfRT-PCR primer pairs, which anneal either entirely within intron 1 or, to ensure detection of introns before they are spliced out, across an intron 2/exon boundary (Figure 2.13c). We stimulated neurons with BDNF with and without NBQX. NBQX potentiated BDNF-induced increases in nascent *Arc* transcript levels, with both intronic primer pairs producing similar results (Figure 2.13d), indicating that AMPA receptors regulate *Arc* transcription. The lower-fold induction of BDNF-induced *Arc* transcript levels measured using intronic primers as opposed to exonic primers, which detect all *Arc* transcripts (compare Figure 2.1f and Figure 2.13d), may reflect rapid splicing of nascent *Arc* transcripts. Comparable potentiation of BDNF-induced *Arc* transcript levels by NBQX was observed with both intron and exon primers (~threefold; compare Figure 2.5a and Figure 2.13d).

Taken together, these results demonstrate that although *Arc* mRNA is rapidly turned over within the cell, AMPA receptors regulate *Arc* levels primarily through a transcriptional mechanism.

Discussion

Here we investigated the activity-dependent regulation of *Arc* expression. *Arc* is critical for long-term plasticity and memory consolidation[55,64], and its expression marks neuronal circuits activated during learning[66,72]. This suggests that *Arc* expression is tightly regulated by neuronal activity, but the underlying mechanisms are unclear. In our investigation, we unexpectedly found that AMPA receptors regulate *Arc*. Inhibiting AMPA receptors strongly increased activity-dependent *Arc* expression in cultures of primary neurons and organotypic brain slices. We found that AMPA receptors negatively regulate *Arc* transcription, but not translation or stability, through a mechanism involving G-protein signaling. These findings, which provide insights into the activity-dependent mechanisms of *Arc* expression, are surprising in light of the prevailing view on the role of AMPA receptors in synaptic plasticity: changes in AMPA receptor surface expression effect short-term plasticity, but AMPA receptors do not directly regulate genes required for long-term plasticity.

Some recent results have hinted at the possibility that AMPA receptors may play additional roles regulating synaptic plasticity, beyond those required for short-term plasticity. For example, chronic suppression of neuronal activity with TTX increases synaptic AMPA receptor expression[256-258] and the amplitude of minis[257-259], suggesting that AMPA receptors can undergo long-term homeostatic changes that counteract global changes in activity.

In fact, neurons co-regulate AMPA and NMDA receptors at synapses during changes in synaptic strength so that the ratio of currents through these channels remains relatively fixed[260,261]. Homeostatic regulation of the NMDA:AMPA ratio may help preserve the

information content of synaptic transmission[262]. Another possibility, not mutually exclusive, is that the NMDA:AMPA ratio is critical for coupling synaptic activity to long-term adaptive responses that require gene expression. Consistent with this possibility, we found that *Arc* expression is determined by the relative extent to which NMDA and AMPA receptors are activated. This suggests a novel mechanism in which the pathways that control *Arc* transcription integrate signals from NMDA and AMPA receptors. One speculative explanation for the existence of this mechanism is that it allows negative feedback control of *Arc* expression: First, synaptic activity triggers *Arc* expression and induces AMPA receptor surface delivery. Then, the decreased NMDA:AMPA ratio inhibits excessive *Arc* transcription. Finally, a delayed NMDA receptor potentiation[261] resets the ratio and allows subsequent *Arc* expression and synaptic plasticity. Thus, Arc may regulate surface expression of AMPA receptors[58-61] and AMPA receptors, together with NMDA receptors, may regulate expression of *Arc*.

Our findings may help address an unresolved paradox in the *Arc* literature: NMDA receptor antagonists block *Arc* expression induced by synaptic stimulation *in vivo*, but AMPA receptor antagonists are relatively ineffective[52]. This observation was attributed[52] to poor diffusion of the AMPA receptor antagonist, CNQX, a less specific drug than either NBQX or GYKI 52466 that partially inhibits NMDA receptors[263]. We suggest that the apparent insensitivity of *Arc* expression to CNQX may represent the net result of potentiation from AMPA receptor blockade and inhibition from NMDA receptor blockade.

How might AMPA and NMDA receptors cooperate to regulate *Arc* expression? We

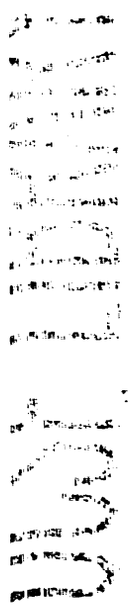
found that a pertussis toxin–sensitive G-protein may mediate the effects of AMPA receptor inhibition on *Arc* expression. AMPA receptor–mediated G_i-protein signaling results in prolonged activation of MAPK[264]. By contrast, NMDA receptor stimulation leads to rapid but transient MAPK activation[264,265]. Since the MAPK pathway is essential for *Arc* expression[184,233] (Figure 2.1f,g), one possibility is that differences in the extent or time course of MAPK activation, determined by the relative activity of AMPA and NMDA receptors, produce corresponding differences in *Arc* expression.

Another possibility is suggested by the regulation of the CaMKII holoenzyme, a heteromeric complex composed primarily of α and β subunits[266,267]. Expression of *αCaMKII* is inhibited by NMDA receptor blockade, while expression of *βCaMKII* is potentiated by AMPA receptor blockade[268]. NMDA receptors are known to regulate *αCaMKII* expression at the level of dendritic translation[241,269,270], and it has been proposed[268], but not directly shown, that AMPA receptors regulate *βCaMKII* expression at the level of nuclear transcription. Based on our results, it is tempting to speculate that *Arc* expression is regulated through similar mechanisms (though some differences must exist, since NMDA receptor blockade affects levels of both *Arc* mRNA and protein). Alternatively, *Arc* expression could be regulated by CaMKII itself, which, by virtue of changes in its subunit composition, reads-out the relative activity of NMDA and AMPA receptors. Supporting this possibility, the subunit composition of CaMKII determines its functional properties[271], CaMKII interacts directly with Arc[272], and *Arc* is only expressed in neurons that also express *CaMKII*[273]. More work is needed to elucidate what role, if any, CaMKII plays in regulating *Arc* expression.

AMPA receptors may also regulate synaptic plasticity through non-homeostatic mechanisms. Tsien and colleagues[206,268] found that chronic (~24 h) blockade of AMPA receptors with NBQX induces pre- and postsynaptic changes that alter the rules governing plasticity, a phenomenon known as metaplasticity. Importantly, these changes are NMDA receptor–independent and are mimicked and occluded by L-VSCC blockade[206]. The idea that AMPA receptor–mediated depolarization can trigger adaptive responses mediated by L-VSCCs is not new: calcium entry through L-VSCCs regulates the expression of genes required for neuronal growth, survival, and plasticity[202]. By contrast, our findings that AMPA receptors regulate gene transcription on a short timescale (~6 h) and by mechanisms that are NMDA receptor–dependent and not mimicked by either TTX or L-VSCC antagonists are unexpected. That AMPA receptors utilize this pathway to regulate transcription of *Arc*, a gene implicated in maintenance of synaptic plasticity[64], further supports a new role for AMPA receptors as mediators of long-term, activity-dependent synaptic changes. However, as we have not yet determined whether AMPA receptors regulate *Arc* expression *in vivo*, it will be important for future studies to test this in intact animals.

Finally, our study shows that primary cultured neurons are a powerful model system for mechanistic investigations of *Arc* biology. Much of the previous work on *Arc* was performed either *in vivo*, where mechanistic studies are challenging, or in cell lines and synaptoneurosomes, where neuronal regulatory mechanisms may be absent. Our cell-culture system addresses this technical gap, recapitulating critical features of *Arc* expression observed *in vivo*. Using this system, we found that *Arc* mRNA and protein are short-lived in

neurons. Rapid turnover is a common feature of intracellular molecules with key regulatory functions, such as IEGs. We showed that Arc protein, like Fos[274], is subject to proteasome-dependent degradation, possibly owing to its carboxy-terminal PEST sequence (residues 351–392; PESTfind Analysis Webtool: https://emb1.bcc.univie.ac.at/content/view/21/45/). In contrast, *Arc* but not *Fos* is regulated by AMPA receptor inhibition (Figure 2.5c,d). That these and other IEGs are controlled by multiple mechanisms, which are only partly overlapping, expands the complexity of the adaptive responses that their expression can produce in response to synaptic activity.



Materials and Methods

Plasmids

GFP^{u250} and *mRFP1*^{275} were subcloned into plasmid *pGW1-CMV* (British Biotechnology; Oxford, UK) for higher expression in neurons. Plasmid *pBSII(KS)-5'-luciferase-3'* was constructed as follows. First, PCR was used to amplify the *Arc* 5'UTR (primers: 5'-tattataagcttagtgctctggcgagtagtcctc-3' and 5'-tattatgaattctcttctaccggtctgctcgccggggttacggttc-3') and 3'UTR (primers: 5'-aggggccagcccagg-3' and 5'-atttatgctagccgtaatacgactcactatagggcgaa-3') from oligo-dT-primed rat cDNA. *Photinus* luciferase was excised from plasmid *pGL3-Basic* (Promega; Madison, WI). These three fragments were assembled in plasmid pBSII(KS) (Stratagene; Cedar Creek, TX). Plasmid *pSRE-Luc* was from Stratagene. A 'promoter-less' *Renilla* luciferase plasmid (*pRL0-Renilla*) was constructed by removing SV40 promoter elements from *pRL-SV40* (Promega). Enzymes used for cloning were from New England Biolabs (Beverly, MA).

Cell culture

Cortical neurons from P0 rat pups were dissociated and cultured using a modified version of a previously described protocol[276]. Pups were sacrificed by CO_2 inhalation, cerebral cortices were rapidly isolated, and meninges were removed. Dissection was performed with tissue submerged in ice-cold HEPES-buffered dissociation medium (in mM: 81.8 Na_2SO_4, 30 K_2SO_4, 15.2 $MgCl_2$, 0.25 $CaCl_2$, 1 HEPES, pH 7.4, 20 glucose, and 0.001% phenol red)

supplemented with kynurenic acid (1 mM; Sigma; St. Louis, MO). Tissue was treated with papain (10 U/ml; Worthington Biochemical; Lakewood, NJ) for 30 min and then with trypsin inhibitor (10 mg/ml; Sigma) for 15 min. After trituration in OptiMEM (Invitrogen; Carlsbad, CA) supplemented with glucose (20 mM), suspensions of isolated neurons were plated on plastic tissue culture plates (3.4×10^5 cells/cm^2) or 12-mm glass coverslips (6.8×10^5 cells/cm^2) coated with laminin and poly-D-lysine (BD Biosciences; Bedford, MA). After 2 h, cells were transferred into a Basal Media Eagle's (BME; Invitrogen)-based neuronal culture medium (NCM; per 100 ml: 91 ml BME, 5 ml bovine calf serum (Hyclone; Logan, UT), 1.4 ml of 2.5M glucose, 0.5 ml of 0.2M L-glutamine, 0.5 ml of penicillin/streptomycin, 0.45 ml of Stable Vitamin Mix (3 mg/ml L-proline, 3 mg/ml L-cystine, 1 mg/ml *p*-aminobenzoic acid, 0.4 mg/ml vitamin B-12, 2 mg/ml *myo*-inositol, 2 mg/ml choline chloride, 5 mg/ml fumaric acid, 80 μg/ml coenzyme A, 0.4 μg/ml D-biotin, 0.1 mg/ml DL-6,8,-thioctic acid), 50 μl of ITS (5 mg/ml insulin, 5 mg/ml human transferrin, 5 μg/ml sodium selenite), 0.5 ml of 1.6 mg/ml putrescine, 0.5 ml of 5 mg/ml transferrin, and 12 μl of 1 mM progesterone; all reagents from Sigma unless otherwise indicated). Cells were fed every 3 days by transfer into conditioned NCM supplemented 1:1 with fresh NCM. Neurons were used for experiments at 12 days *in vitro* (DIV), unless otherwise stated.

Glial cultures were obtained by maintaining neuronal cultures until neurons died out. For RNA transfection experiments, neurons were grown in Neurobasal-A with B27 (Invitrogen). Human embryonic kidney cells (HEK293T) were grown in Dulbecco's Modified Eagle's medium with 10% calf serum (Invitrogen).

Organotypic slice culture

Organotypic slices were prepared as described[201]. Briefly, a McIlwain Tissue Chopper (Campden Instruments; Lafayette, IN) was used to cut 400 μm–thick coronal brain slices from P7 rat pups. Slices were isolated in ice-cold Gey's balanced salt solution (in mM: 136.9 NaCl, 1.5 $CaCl_2$, 5 KCl, 0.2 KH_2PO_4, 11.1 $MgCl_2$, 0.2 $MgSO_4$, 2.7 $NaHCO_3$, 0.8 Na_2HPO_4, 33.6 glucose, and 20 HEPES, pH 7.2) and grown on semipermeable filter inserts (Millipore; Bedford, MA) in six-well plates containing culture medium (50% Minimum Essential medium, 25% Hank's balanced salt solution, and 25% heat-inactivated horse serum, with (in mM) 36 glucose, 1 GlutaMAX-I, and 25 HEPES, pH 7.2; all from Invitrogen).

Antibodies and drugs

Rabbit polyclonal antibodies (Cell Signaling) were phospho-Trk(Tyr490), phospho-ERK1/2(Thr202/Tyr204), pan-ERK, phospho-Akt(Ser473), pan-Akt, and PLCγ1. Other rabbit polyclonal antibodies used were Arc (H-300; Santa Cruz Biotechnology; Santa Cruz, CA; this antibody was used until we obtained another Arc antibody[42] that produced qualitatively similar results, but with greater sensitivity and specificity), Fos (Calbiochem; La Jolla, CA), pan-Trk (Santa Cruz Biotechnology), and glial fibrillary acidic protein (GFAP; DakoCytomation; Carpinteria, CA). Mouse monoclonal antibodies used were tubulin (Sigma), microtubule-associated protein 2 (MAP2; Chemicon; Temecula, CA), and phosphotyrosine (4G10; Upstate Biotechnology; Lake Placid, NY). All secondary antibodies

were from Jackson ImmunoResearch (West Grove, PA). 1,2,3,4-Tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBQX), 1-(4-aminopropyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine (GYKI 52466; further abbreviated in text as GYKI), D-2-amino-5-phosphonovaleric acid (AP5), (S)-α-methyl-4-carboxyphenylglycine (MCPG), (S)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), nimodipine, U0126, and LY 294002 were from Tocris (Ellisville, MO). Tetrodotoxin (TTX), pertussis toxin (PTX), cholera toxin (CTX), PP2, PP3, and K252a were from Calbiochem. TrkB-IgG was from R&D Systems (Minneapolis, MN). All other chemicals were from Sigma.

Western blots

Cortical neurons (10 DIV) or slice cultures (4 DIV) were switched to serum-free NCM. Two days later, drugs were applied for 8 h in BME, and tissue was harvested in ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, 1% NP-40, 0.5% deoxycholate, and 0.1% SDS) containing protease inhibitors (Roche; Indianapolis, IN). For slice cultures, cortical tissue was dissected away from the rest of the slice, homogenized with a motorized pestle, and sonicated on ice. Protein concentrations were determined by Bradford assay[277], and all lysates were diluted to the same final protein concentration (2 $\mu g/\mu l$) in 2x Laemmli sample buffer (4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol (added just before use), 0.02% (w/v) bromophenol blue, and 100 mM Tris, pH 6.8). Equal quantities of total protein (30 μg/lane) were separated by 8% SDS-PAGE and transferred to nitrocellulose (Amersham Biosciences; Piscataway, NJ). Membranes were blocked with 5% (w/v) nonfat

milk (Bio-Rad; Hercules, CA) in TBS containing 0.1% (v/v) Tween-20 (TBS/T) and probed with primary antibodies and peroxidase-conjugated secondary antibodies. Blots were visualized using enhanced chemiluminescence reagents (Perkin Elmer; Boston, MA) and Kodak BioMax MR film (Fisher Scientific; Hampton, NH). For quantitative analysis, film was scanned and band intensities were analyzed using MetaMorph software (Universal Imaging; Downingtown, PA). Blots were stripped by incubation in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris, pH 6.7) for 50 min at 55°C. Membranes were washed in TBS/T, blocked, and re-probed with antibodies.

Immunoprecipitation

For PLCγ1 immunoprecipitation (IP) experiments, neurons were stimulated with drugs and harvested in non-denaturing IP buffer (in mM: 20 Tris, pH 7.5, 150 NaCl, 1 EDTA, 1 EGTA, 1% Triton X-100, 2.5 sodium pyrophosphate, 1 β-glycerophosphate, 1 $NaVO_4$, 50 NaF, 0.01 leupeptin, 0.01 aprotinin, and 1 PMSF). Lysates were sonicated (4 × 5 sec) and centrifuged at 20,000 × *g* for 10 min at 4°C. Rabbit anti-PLCγ1 antibody was added to the supernatants, and samples were incubated overnight at 4°C with end-over-end rotation. Immune complexes were pulled down with Protein A beads (Roche) for 2 h at 4°C. Beads were washed with IP buffer and boiled in 2× sample buffer. Supernatants were loaded on SDS-PAGE.

Ca^{2+} imaging

Cells were loaded for 1 h at 37°C in HEPES-buffered saline (in mM: 119 NaCl, 2.5 KCl, 2

$MgCl_2$, 2 $CaCl_2$, 25 HEPES, pH 7.4, 30 glucose, and 0.01 glycine) containing Fura-2AM (4 μM; Molecular Probes; Eugene, OR) and pluronic F-127 (0.01% w/v; Sigma). Cells were imaged using an inverted epifluorescence microscope (Nikon; Melville, NY) equipped with a digital cooled CCD camera (Hammatsu; Bridgewater, NJ). MetaMorph controlled an excitation filter wheel for alternate 340 nm and 380 nm excitation and emitted light was collected through a Fura-2 dichroic block (Chroma; Rockingham, VT). Equal-sized regions of interest were applied to pairs of 340/380-nm images for 50 randomly selected cells per 20× field, and ratios of average pixel intensities were calculated with MetaMorph. Calcium responses for each cell were normalized to the first image (F/F_0) and averaged across all selected cells at each timepoint.

Single-cell GFP^u imaging

Cortical neurons (11 DIV) were co-transfected with plasmids *pGW1-GFP^u* and *pGW1-mRFP1*. At 24 h after transfection, pre-stimulation images of randomly selected transfected cells – mRFP1 signal was used to select cells for imaging so as to eliminate bias based on initial GFP^u fluorescence – were captured using appropriate filters for red and green fluorescence. The positions of these cells relative to an internal fiduciary mark on the tissue culture plate were recorded. Cells were switched to medium containing either drug or vehicle, with cycloheximide (10 μg/ml), and returned to an incubator for 12 h. Recorded coordinates were used to capture post-stimulation images of previously identified cells. Post/pre-stimulation fold changes in fluorescence for individual cells were calculated using

average pixel intensities of regions of interest drawn over the cell soma. Data were plotted as mean fold change of GFP^u/mRFP1 fluorescence across all cells measured for each condition.

In situ hybridization and immunocytochemistry

FISH was performed using a described protocol[72] adapted for neurons grown on coverslips. Immunostaining was performed at room temperature immediately after *in situ* hybridization. Cells were blocked for 2 h with 5% (v/v) normal goat serum (NGS) in PBS with 0.1% Triton X-100 (PBS/T). MAP2 antibody was diluted in PBS/T containing 3% (w/v) BSA and 1% NGS and applied to cells for 2 h. Cells were washed with PBS/T and incubated with a Cy2-conjugated secondary antibody (1:250 dilution) for 1 h. Finally, cells were washed and mounted in glycerol gelatin (Sigma) for imaging.

Transfections

RNA transfections: 5'-capped reporter mRNAs were synthesized *in vitro* from plasmid *pBSII(SK)-5'-luciferase-3'* using the T3 mMessage mMachine kit (Ambion). Cortical neurons (7 DIV) were transfected with reporter mRNAs with the TransMessenger transfection reagent (Qiagen; Valencia, CA) according to the manufacturer's instructions (0.25 μg RNA per 1.9 cm^2 well, 3:1 (v/w) lipid:RNA ratio). Drugs were applied during the 3-h transfection. Immediately after the transfection/stimulation period, cells were lysed and assayed for luciferase.

DNA transfections: Cells were transfected with plasmid DNA using the Lipofectamine 2000

transfection reagent (Invitrogen) according to the manufacturer's instructions (0.9 μg of DNA and 2 μl of lipid per 1.9 cm^2 well).

Reporter gene assays

Neurons (11 DIV) were co-transfected with *pSRE-Luc* and *pRL0-Renilla*. *Renilla* luciferase expression was not significantly affected by stimuli used in this study (data not shown) and was used to normalize for differences in transfection efficiency and sample handling. At 20–24 h after transfection, neurons were stimulated with drugs for 8 h, harvested, and assayed for *Photinus* and *Renilla* luciferase using a Dual Luciferase Assay Kit (Promega) and a Luminoskan Ascent luminometer (Thermo Electron; Waltham, MA).

qfRT-PCR

Cortical neurons were stimulated with drugs for 6 h, and total RNA was isolated with the RNeasy Mini kit (Qiagen). Random hexamer-primed first-strand cDNA was synthesized with 150 ng of total RNA and TaqMan reverse transcription reagents (Applied Biosystems; Foster City, CA). Quantitative fluorogenic reverse-transcription PCR (qfRT-PCR) was performed using the ABI Prism 7700 sequence detector (Applied Biosystems) with SYBR green PCR core reagents (Applied Biosystems). *Arc* mRNA levels were normalized to an internal control gene, *GAPDH*. The following gene-specific primers were used: 5'-cctgagccacctggaagagta-3' and 5'-ggcccattcatgtggttctg-3' for *Arc*, 5'-aatccgaagggaaaggaataaga-3' and 5'-cgcttggagcgtatctgtca-3' for *Fos*, 5'-catcaagaaggtggtgaagca-3' and 5'-

ctgttgaagtcacaggagaca-3' for *GAPDH*. *Arc* results were confirmed by using the same cDNA samples with a second *Arc*-specific primer pair: 5'-agaacaacttggacggctatgt-3' and 5'-acaggccttgatggacttcttc-3'. In all cases, virtually identical results were obtained with both *Arc* primer pairs (data not shown). For detection of nascent *Arc* transcripts, primer pairs used were: intron 1 (both primers anneal within intron 1): 5'-ccctgctccatgtatcttagagttg-3' and 5'-tccacccttgcagctaatctg-3'; intron 2 (primers flank an intron-exon boundary): 5'-taacctggtgtccctcctagatc-3' and 5'-ggaaagacttctcagcagcttga-3'.

Statistical analysis

Multiple comparisons were performed with Prism software (GraphPad Software; San Diego, CA) using one-way ANOVA and post-hoc Tukey *t* tests.

Chapter 3:

Regulation and Function of Protein Kinase D in Neurons

Abstract

The serine/threonine protein kinase D (PKD) is a critical regulator of cell survival, migration, proliferation, and differentiation. PKD effects changes in cellular function in part by regulating the nucleocytoplasmic distribution of histone deacetylases (HDACs), a family of transcriptional repressors. Despite a wealth of information about PKD in cardiomyocytes, lymphocytes, and fibroblasts, virtually nothing is known about its function in neurons. Here, we provide preliminary evidence that neuronal PKD may play a critical role in synapse-to-nucleus signaling and synaptic function. In cultures of primary cortical neurons, NMDA receptor stimulation induced PKD activation and translocation into dendrites, suggesting a role for PKD at synapses. NMDA receptor stimulation activated PKD with rapid kinetics, distinct from PKD activation by other calcium channels, and promoted nuclear export of HDAC5. These findings suggest the existence of a NMDA receptor–PKD–HDAC signaling pathway that mediates activity-dependent neuronal gene expression. Finally, protein microarray experiments identified several novel PKD substrates, including proteins that regulate synaptic function. Thus, PKD may be a novel mediator of multiple neuronal processes underlying synaptic plasticity.

Introduction

Long-lasting changes in synaptic strength are essential for learning and memory[11] and require neuronal gene expression[21-23]. However, the mechanisms by which synaptic activity triggers neuronal gene expression are poorly understood. Several mechanisms have been proposed to explain how different patterns of synaptic activity can be translated into distinct nuclear responses[27,39]. These mechanisms generally involve synapse-to-nucleus movement of various mediators—calcium ions[278,279], action potentials[26], or proteins[280-283]—that encode information by virtue of their amplitude, frequency, or pattern of activation.

Upon arrival at the nucleus, these mediators must relay their signal to the nuclear gene expression machinery. Recently, covalent modification of nucleosomal histones has emerged as a central mechanism in the control of neuronal gene transcription[156-158]. Histone acetylation by histone acetyltransferases (HATs) promotes transcription by relaxing chromatin structure, whereas histone deacetylases (HDACs) reverse this process, resulting in transcriptional repression. HDACs are principally regulated by phosphorylation-dependent changes in their nucleocytoplasmic distribution[159]. In the nucleus, HDACs interact with transcription factors, like MEF-2, and negatively regulate the expression of large sets of genes. Nuclear export functionally inhibits HDACs, disrupting HDAC-MEF2 interactions and allowing MEF2-dependent gene transcription to occur. There is considerable interest in identifying synaptic activity–dependent proteins that regulate the nucleocytoplasmic distribution of HDACs[170].

Nuclear export of HDACs in cardiomyocytes and lymphocytes is regulated by a

serine/threonine kinase called protein kinase D (PKD)[133,134,154,155]. Originally classified as an atypical protein kinase C (PKC) isoform[107-109], PKD in fact belongs to a distinct kinase family, owing to its unique domain structure and substrate specificity[284]. Since its discovery, PKD has been implicated in processes as diverse as cell proliferation, apoptosis, immune cell regulation, tumor cell invasion and regulation of Golgi vesicle fission[112]. PKD is activated by upstream PKCs, which phosphorylate PKD in its activation loop on serines 744 and 748[121,122]. A striking feature of PKD activation is that it involves a dynamic series of translocations between intracellular compartments, with each translocation step mediated by a particular domain within PKD[109]. PKD is cytoplasmic in unstimulated cells but rapidly translocates to the plasma membrane in response to receptor activation[139]. PKD then returns to the cytoplasm[125], with kinase activity sustained[131], and subsequently translocates to the nucleus[140]. Here, PKD can phosphorylate HDACs[133,134,154,155], inducing their nuclear export and promoting gene transcription. Finally, PKD completes the cycle, returning to the cytoplasm by CRM1-mediated nuclear export[140].

The stimulus-dependent translocation cycle of PKD seems well-suited to relay information about synaptic activity to nuclear effectors, like HDACs. However, virtually nothing is known about the regulation or function of PKD in neurons. Here, we provide evidence that neuronal PKD may play a critical role in synapse-to-nucleus signaling and synaptic function. Our results suggest that PKD may mediate signaling from NMDA receptors to nuclear HDACs and may directly regulate proteins that control synaptic function.

Results

PMA-induced PKD activation and membrane translocation in neurons

To investigate the role of PKD in neurons, we first developed an *in vitro* model that recapitulates essential features of PKD regulation discovered in other systems: (1) phorbol ester treatment activates PKD[285,286], (2) phorbol ester induces PKD translocation to the plasma membrane[139], and (3) membrane translocation requires the two N-terminal cysteine-rich zinc-finger domains (CRDs) of PKD[139].

We stimulated cultures of cortical neurons with phorbol 12-myristate 13-acetate (PMA), a phorbol ester, and examined levels of activated PKD by Western blot. PMA induced robust activation of endogenous PKD (Figure 3.1a). PMA-induced PKD activation, which is mediated by PKCs[285,286], was inhibited by the PKC inhibitor GF109203X (data not shown).

To visualize the subcellular distribution of PKD, we expressed fluorescent protein–fused versions of PKD in neurons. In unstimulated cells, GFP-PKD[139] was predominantly cytoplasmic and excluded from the nucleus (Figure 3.1b), as in other cell types[131,139,160]. We did not observe preferential localization of GFP-PKD to Golgi[136,147,153,287] or mitochondrial[127,288] membranes (data not shown). Treatment with PMA induced a dramatic redistribution of GFP-PKD to the plasma membrane (Figure 3.1b)[139]. A single amino-acid substitution in the second CRD of PKD (P287G), which prevents phorbol ester–induced membrane translocation in fibroblasts and mast cells[139], did not prevent membrane translocation in neurons (data not shown). However, deletion of the entire CRD prevented PMA-induced membrane translocation (Figure 3.1b)[139].

Since most of the plasma membrane surface area of neurons is found in the dendrites[289], we imaged dendrites of transfected neurons before and after stimulation with PMA. To obtain sufficient fluorescence signal from dendrites, we fused PKD to Venus[290], a variant of yellow fluorescent protein that is considerably brighter than GFP. Like GFP-PKD, Venus-PKD was excluded from the nucleus of unstimulated cells. Venus-PKD signal was enhanced in dendrites and dendritic spines following stimulation with PMA (Figure 3.1c). Dendritic signal enhancement was accompanied by decreased cell body fluorescence (Figure 3.1d), suggesting redistribution of existing Venus-PKD molecules rather than effects on their synthesis or stability. By contrast, fluorescence of a co-transfected red fluorescent protein, mCherry[291], was unaffected by PMA in both dendrites and the cell body (data not shown).

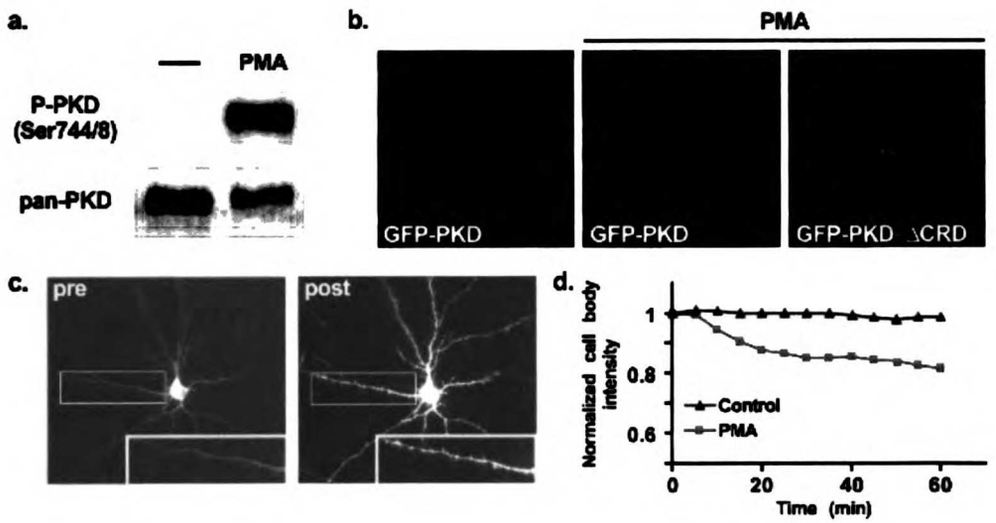


Figure 3.1 PMA induces PKD activation and translocation in neurons.

(**a**) Western blots showing that PMA (1 h, 100 nM) activates endogenous PKD in primary rat cortical neurons. In this and subsequent blots, levels of activated PKD were determined using an antibody that recognizes phosphorylated activation loop serines 744 and 748; membranes were stripped and reprobed for total levels of PKD ('pan-PKD') to confirm equal protein loading across lanes. (**b**) Confocal images of neurons showing that PMA-induced membrane translocation of GFP-PKD (green) requires the cysteine-rich domain (CRD). Hoechst-stained nuclei are shown in blue. (**c**) Epifluorescence images showing enhanced dendritic localization of a

Venus-PKD fusion protein following treatment with PMA. Insets show boxed dendrite at higher magnification. Note that dendritic spines are more visible after PMA treatment. (**d**) PMA-induced dendritic localization of Venus-PKD is associated with decreased cell body fluorescence.

NMDA induces PKD activation and dendritic translocation

The stimulus-induced translocation of Venus-PKD to dendrites and dendritic spines prompted us to consider whether PKD might be regulated by synaptic activity. Although PKD lacks the C2 domains responsible for the Ca^{2+}-sensitivity of PKCs[113], PKD can be activated by PKC isoforms that are Ca^{2+}-responsive[292,293]. Therefore, we examined the effect of stimulating neuronal calcium channels on PKD activation and translocation.

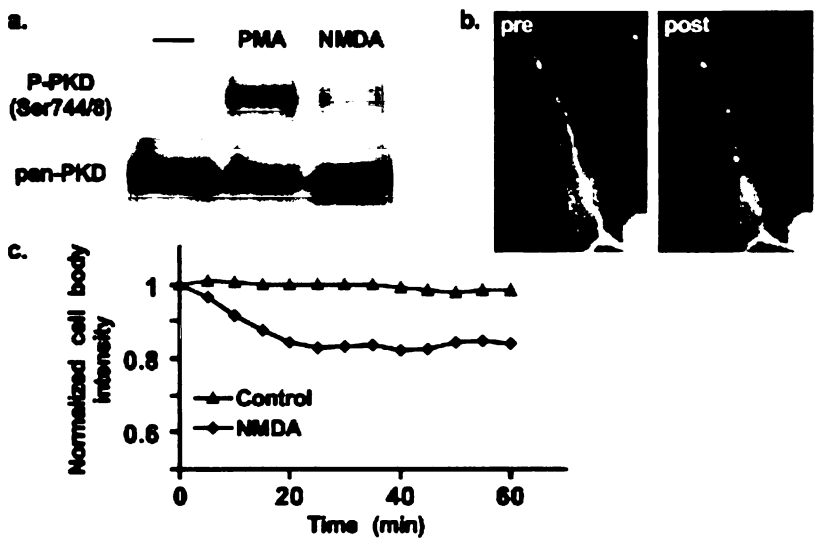


Figure 3.2 NMDA induces PKD activation and dendritic translocation.

(**a**) Western blots showing that NMDA (30 μM, 5 min; stimulation solution included glycine (10 μM), a co-agonist at NMDA receptors) induces activation of endogenous PKD in primary rat cortical neurons. PMA-induced PKD activation shown for comparison. (**b**) NMDA induces Venus-PKD dendritic translocation and puncta formation. Images of the same neuron are shown before and 5 min after application of NMDA. (**c**) NMDA-induced dendritic translocation of Venus-PKD is evidence by decreased cell body fluorescence. Primary mouse cortical neurons were used in **b** and **c**.

The *N*-methyl-D-aspartate (NMDA) receptor is a glutamate-gated calcium channel essential for learning and memory[294]. Stimulation of the NMDA receptor induced rapid activation of PKD in neurons (Figure 3.2a). To determine whether the subcellular localization of PKD was also affected, we imaged neurons transfected with Venus-PKD before and after treatment with NMDA. Remarkably, treatment with NMDA induced the formation of Venus-PKD puncta in dendrites (Figure 3.2b). Although we did not test for co-localization with synaptic markers, the puncta we observed were strikingly similar to the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) puncta that form near synaptic sites in response to NMDA receptor stimulation[215,295]. PKD binding to Yotiao, an A-kinase anchoring protein (AKAP) that associates with NMDA receptors[172], may be responsible for this synaptic localization (S.R. Carter and S. Finkbeiner, unpublished observations). Dendritic puncta formation was associated with decreased Venus-PKD fluorescence in cell bodies (Figure 3.2c), consistent with dendritic translocation in response to NMDA receptor activation. As with PMA, NMDA did not affect mCherry fluorescence in either dendrites or the cell body (data not shown).

NMDA receptors and L-VSCCs activate PKD with distinct kinetics

Having found that NMDA receptor–mediated calcium influx induces PKD activation and translocation, we next determined whether PKD can be regulated by calcium entry through other channels. L-type voltage-sensitive calcium channels (L-VSCCs) couple membrane depolarization in neurons to numerous processes including gene expression, synaptic

efficacy, and cell survival[296]. Stimulation of L-VSCCs with high KCl gradually induced moderate levels of PKD activation over the course of 90 min (Figure 3.3). By contrast, NMDA receptor–mediated PKD activation was rapid (<5 min), robust, and sustained over a 90 min timecourse (Figure 3.3).

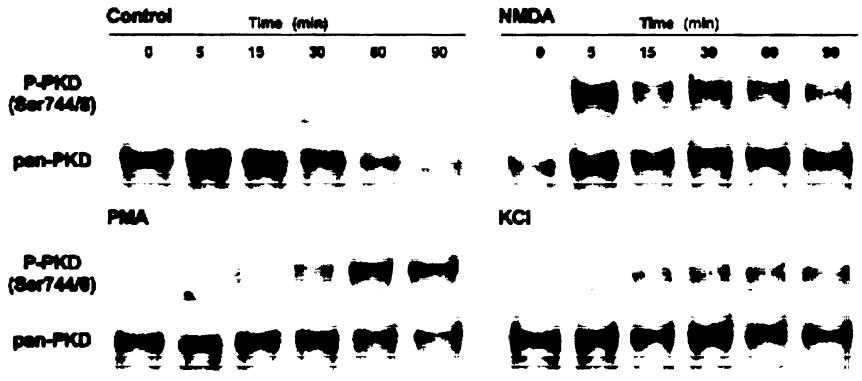


Figure 3.3 Rapid kinetics of NMDA-induced PKD activation.

Western blots showing timecourse of endogenous PKD activation in neurons stimulated with PMA (100 nM), NMDA (30 μM), or KCl (55 mM). KCl-depolarization, which activates L-type voltage-sensitive calcium channels, induces PKD activation with a markedly different timecourse than NMDA stimulation. For NMDA stimulation, TTX (1 μM; action potential blocker), verapamil (100 μM; L-VSCC antagonist), and NBQX (10 μM; AMPA receptor antagonist) were present. For stimulation with KCl, TTX, NBQX, and AP5 (100 μM; NMDA receptor antagonist) were present.

The distinct temporal profiles of PKD activation induced by NMDA receptors and L-VSCCs suggest that these channels may activate PKD through distinct mechanisms[112], consistent with the notion that the route of calcium entry into the cell is a critical determinant of neuronal signal transduction[297]. We next examined whether L-VSCC activation can induce dendritic translocation of Venus-PKD. After 1 h stimulation with high KCl, transfected neurons showed no change in the intracellular distribution of Venus-PKD (data not shown), suggesting that stimulation of NMDA receptors and L-VSCCs triggers distinct signaling

events that differentially affect activation and translocation of PKD.

NMDA receptors regulate the nucleocytoplasmic distribution of HDAC5

PKD activation is necessary and sufficient for nuclear export of HDACs[133,134,154,155]. In neurons, HDACs undergo nucleocytoplasmic shuttling that is dynamically regulated by synaptic activity[170], but the molecular mediators that link synaptic activity to HDACs are not known. The ability of NMDA receptors to regulate activation of PKD (Figure 3.2 and Figure 3.3) suggests that PKD may be one such mediator.

To test this, we expressed HDAC5 in neurons and examined changes in its subcellular distribution induced by NMDA receptor stimulation. In unstimulated neurons, HDAC5 immunoreactivity was exclusively nuclear (Figure 3.4), consistent with a previous report that spontaneous synaptic activity is not sufficient to induce nuclear export of HDAC5[170]. Brief stimulation of transfected cells with NMDA induced redistribution of HDAC5 to the cytoplasm. Residual signal from HDAC5 was still detectable in the nucleus (Figure 3.4), possibly owing to the fact that bath application of NMDA activates some extrasynaptic NMDA receptors[216], which oppose HDAC5 nuclear export[170], in addition to synaptic NMDA receptors. By contrast, stimulation of L-VSCCs with high KCl induced virtually complete nuclear export of HDAC5, with no detectable signal remaining in the nucleus (Figure 3.4). Thus, the differential activation of PKD by NMDA receptors and L-VSCCs is paralleled by the differential effects of these channels on HDAC5 subcellular localization. One possibility is that the slower activation of PKD induced by L-VSCCs somehow leads to more efficient

nuclear extrusion of HDAC5. Alternatively, L-VSCCs may utilize distinct signaling pathways—for example, pathways mediated by CaMKs, which can phosphorylate HDACs[298] and regulate their interactions with other factors[299]—to regulate the nucleocytoplasmic distribution of HDACs in neurons[300].

Taken together, these results provide preliminary evidence that a PKD–HDAC5 signaling pathway in neurons may regulate activity-dependent gene expression.

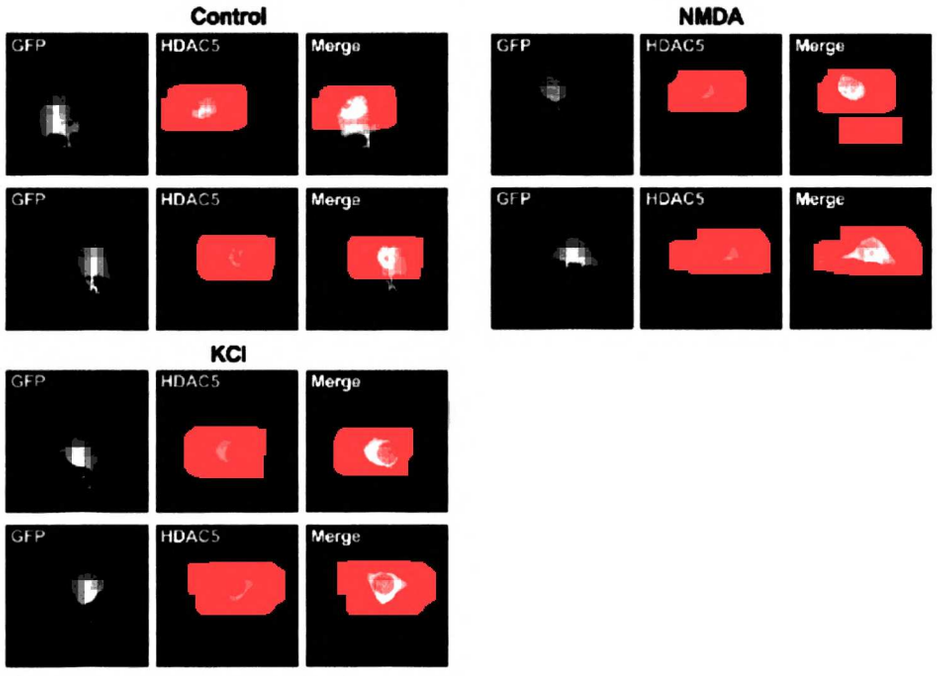


Figure 3.4 NMDA and KCl induce nuclear extrusion of HDAC5.

Neurons transfected with HDAC5 and GFP (a cell marker) were stimulated, fixed, immunostained for HDAC5, and imaged by confocal microscopy. HDAC5 is exclusively nuclear under control conditions, but becomes predominantly cytoplasmic upon stimulation with NMDA (30 μM, 30 min) or KCl (55 mM, 90 min). Two representative cells per stimulus condition are shown.

PKD substrates regulate synaptic morphology and function

Having identified a potential role for PKD in activity-dependent neuronal gene expression,

we next considered the possibility that PKD might also have more direct effects on neuronal physiology by phosphorylating effector proteins that regulate synaptic function. A large-scale genetic screen in *Caenorhabditis elegans* recently identified PKD as a protein that regulates synaptic function[175], however only a single PKD substrate in neurons is known[169]. To identify novel neuronal substrates of PKD, we used a protein microarray with about 5000 full-length human proteins spotted in duplicate[301,302]. Arrays were incubated with recombinant hexahistidine- or GST-tagged PKD in the presence of γ-^{33}P-ATP to allow phosphorylation of target proteins. To minimize the number of false positives, arrays were then washed in dilute SDS to denature ATP-binding proteins, and 'no kinase' control arrays were run in parallel to identify autophosphorylated proteins. Arrays were exposed to film and signals were analyzed to determine statistically significant hits, defined as those proteins having a Z-score greater than 3.0 on all four arrays that were tested (Table 3.1).

Protein Name	GenBank#	Z-score (mean)	SD
Liprin β2	NM_003621.1	+6.98	1.19
Jak and microtubule interacting protein (JAKMIP) 2	NM_014790.3	+6.41	1.09
WEE1 homolog	NM_003390.2	+6.16	1.16
Rho GTPase activating protein (RhoGAP) 15	BC038976.1	+5.69	0.9
WD repeat domain 5, transcript variant 1	NM_017588.2	+5.6	0.71
RAB3A interacting protein (rabin3)-like 1	NM_013401.2	+5.57	0.44

Table 3.1 Putative PKD substrates identified by a protein microarray screen.

Table lists the six top-ranked human proteins that were scored as hits (Z>3.0) on each of four independent arrays. The Z-Score for a protein indicates how far and in what direction the signal from that protein deviates from the mean of the distribution of signals on the array, expressed in units of the distribution's standard deviation. Z-scores shown are the means of four independent arrays, and standard deviations (SD) of these means are provided in the adjacent column.

Although the arrays we used were not enriched for neuronal proteins, we identified a

number of putative PKD substrates that have known roles in the nervous system. For example, liprin β2 belongs to a family of proteins that interact with LAR transmembrane protein tyrosine phosphatases[303] and have roles in synapse morphogenesis[304,305], synaptic transmission[306], and AMPA receptor expression[307]. JAKMIP2, also known as marlin-1[308], is a RNA-binding protein expressed in brain that regulates GABA receptor expression[309]. RhoGAPs have many links to neuronal function—including axonal outgrowth, dendritic morphogenesis, and synaptic regulation[310]—primarily through effects on the actin cytoskeleton[311]. Rabin3-like 1 interacts with the GTPase Rab3a[312], the most abundant Rab protein in the brain, and may be involved in synaptic vesicle exocytosis[313]. The human homolog of WEE1, a nuclear tyrosine kinase involved in cell cycle regulation, is constitutively active in postmitotic neurons and may play a role in the pathogenesis of Alzheimer's Disease[314]. A neuronal function for WD repeat domain 5 is less clear, but members of the WD repeat family are involved in a variety of cellular processes, including signal transduction and gene regulation[315].

Thus, we identified putative PKD substrates that regulate diverse aspects of neuronal function. Future work will be needed to determine which of these phosphorylation events are functionally relevant *in vivo*.

Discussion

Regulated changes in chromatin structure are essential for neuronal gene expression, synaptic plasticity, and long-term memory[171]. Nuclear export of HDACs leads to chromatin relaxation and transcriptional derepression[159], but the activity-dependent mechanisms that regulate the nucleocytoplasmic distribution of HDACs in neurons are poorly understood. In cardiomyocytes and lymphocytes, the serine/threonine kinase PKD is necessary and sufficient for nuclear export of HDACs[133,134,154,155], but virtually nothing is known about the function of PKD in the nervous system. Here we examined the activity-dependent regulation of PKD in cultures of primary cortical neurons, a system which is conducive to mechanistic investigations of neuronal signal transduction (Chapter 2).

We validated this system by demonstrating phorbol ester–induced activation and CRD-dependent membrane translocation of PKD, as observed in other cell types. We found that NMDA receptor stimulation induced PKD activation, dendritic translocation, and puncta formation, suggesting recruitment to synaptic sites. By contrast, calcium influx through L-VSCCs activated PKD with distinct kinetics and did not induce PKD translocation or puncta formation. However, HDAC nuclear export was induced more effectively by activation of L-VSCCs than by activation of NMDA receptors, suggesting differential involvement of PKD in signaling downstream of these channels. These results support a role for PKD in synapse-to-nucleus signaling and regulation of neuronal gene expression. Our discovery of putative PKD substrates that regulate synaptic function suggests that PKD may also regulate neuronal physiology through mechanisms not involving nuclear HDACs.

How might NMDA receptors activate PKD? PKD activation can occur through at least four general mechanisms[112]. In the first, plasma membrane receptor activation leads to activation of phospholipase C (PLC), production of diacylglycerol (DAG), and activation of PKCs, which in turn activate PKD. In a second mechanism, G-protein $\beta\gamma$ subunits can directly bind to and activate PKD, presumably through an allosteric mechanism. A third mechanism of activation involves caspase-mediated cleavage of PKD[137], and a fourth mechanism involves binding of 14-3-3 proteins to PKD[112,138]. Several studies have implicated PKCs in NMDA receptor signaling[316-318], and PKCs are found in the NMDA receptor signaling complex[57]. Furthermore, NMDA receptor activation can induce rapid, PKC-dependent translocation of proteins to synaptic sites[319]. Thus, it seems likely NMDA receptors activate PKD through a PKC-dependent mechanism.

The distinct temporal profiles of PKD phosphorylation induced by NMDA receptors and L-VSCCs suggest that PKD is activated by different mechanisms depending on the route of calcium entry into the cell. Recent evidence suggests that activation of intracellular signaling pathways by specific types of calcium channels depends on localization of signaling molecules close to the channel mouth[204,215,320]. NMDA receptors, for example, interact with scaffolding proteins in the postsynaptic density[57] that serve to localize signaling molecules with Ca^{2+} influx and facilitate activation of downstream signaling pathways. One such scaffolding protein is Yotiao, an A-kinase anchoring protein (AKAP) that interacts with specific splice variants of the NMDA receptor subunit NR1[172]. The only known function of Yotiao is to physically link type I protein phosphatase (PP1) and protein kinase A (PKA) to

NMDA receptors to regulate channel activity[321]. More recently, Yotiao was found to also interact with PKD (S.R. Carter and S. Finkbeiner, unpublished observations). Although the functional significance of this interaction is unclear, an intriguing possibility is suggested by recent work on an AKAP called AKAP-Lbc, which was found to nucleate a PKD activation scaffold in fibroblasts[174]. Thus, Yotiao may localize PKD near NMDA receptors, thereby accounting for the rapid activation of PKD following NMDA receptor stimulation (Figure 3.2 and Figure 3.3). This model predicts that PKD should be activated less effectively by NMDA receptors containing NR1 splice variants that do not bind Yotiao. An elegant system for studying splice variant–specific signaling by NMDA receptors has been described[215] and will be useful in testing this prediction.

NMDA receptors and L-VSCCs are critical for neuronal gene expression and adaptive responses to synaptic activity[202]. Calcium influx through these channels differentially activates intracellular signaling pathways, suggesting that the route by which calcium enters cells can determine its downstream effects[297]. We observed that stimulation of NMDA receptors and L-VSCCs elicited differential cellular responses at the levels of HDAC nuclear export and PKD phosphorylation. HDAC5 was completely cytoplasmic in neurons stimulated with high KCl to activate L-VSCCs, consistent with the cytoplasmic localization of HDAC5 in cerebellar granule cells cultured in depolarizing medium[300]. NMDA receptor stimulation induced HDAC5 nuclear export to a slightly lesser extent. However, NMDA receptor stimulation led to more rapid and robust PKD phosphorylation than did activation of L-VSCCs. We suggest that L-VSCCs signal through CaMKs to induce HDAC5 nuclear

export[300], whereas NMDA receptor–dependent HDAC5 nuclear export is mediated by PKD. Thus, signals from NMDA receptors and L-VSCCs may be relayed by distinct HDAC kinases, PKD and CaMKs, respectively, to elicit transcriptional responses in the nucleus.

The stimulus-dependent translocation of PKD from cytoplasm to membrane to nucleus[112] seems well-suited to translate synaptic activity into a nuclear response. However, the diversity of cellular responses attributed to PKD in other cell types[109] suggests that PKD may have other functions in neurons as well. Indeed, the activity-dependent dendritic puncta formation we observed with Venus-PKD suggests a role in regulating synaptic function. Since relatively few substrates of PKD are known in any cell type, we employed protein microarrays in a screen to identify novel PKD substrates. Remarkably, virtually all of the significant hits that came out of our screen have known roles in the nervous system (Table 3.1 and Results). Some of these hits—liprins, WD repeat proteins, regulators of Rho and Rab GTPases—and PKD itself were previously identified in a *C. elegans* screen for genes that regulate synaptic transmission[175]. Thus, it seems likely that at least some of these molecules are downstream effectors of PKD that regulate the structure and function of synapses. It is tempting to arrange these proteins into putative synaptic signaling pathways based on their known functions—for example, the fact that PKD phosphorylates a RhoGAP (Table 3.1) and interacts with AKAPs that have RhoGEF activity[174] suggests the existence of a regulatory loop. However, such speculation is premature until substrates have been validated by solution-based assays or other methods; in a recent report, 80% of kinase substrates identified on a protein microarray were subsequently confirmed[301]. Ultimately, the functional

significance of substrate phosphorylation events will have to be determined *in vivo*.

Prior to the work described here, only two published studies investigated the role of PKD in neuronal cells[167,168]. Our results provide preliminary evidence that PKD mediates multiple cellular processes in neurons, including activity-dependent changes in gene expression and synaptic function, but many questions remain. Most importantly, it remains to be determined whether PKD activity induces HDAC nuclear export in neurons as in cardiomyocytes and lymphocytes[133,134,154,155]. PKD mutants that have glutamates in place of serines 744 and 748 are constitutively-active[121] and should be useful for testing this. Another important question concerns the role of the AKAP Yotiao in PKD function and regulation in neurons. Yotiao's binding partners include NMDA receptors, PKA, PP1, and PKD, suggesting that, like AKAP-Lbc[174], Yotiao could orchestrate the activation of multiple enzymes to facilitate information flow to downstream effectors. If the binding interaction between Yotiao and PKD is regulated by NMDA receptor activity, Yotiao molecules localized to synapses could also be responsible for PKD puncta formation in dendrites. Although we observed stimulus-dependent translocation of PKD into dendrites, we did not determine whether PKD subsequently translocates to the nucleus, as in other cell types[140]. Direct visualization of synapse-to-nucleus movement would provide strong evidence that PKD, like NF-κB[281], transduces synaptic signals into altered patterns of gene expression. Finally, characterization of the endogenous genes that are regulated by PKD and HDACs in neurons would help clarify the biological significance of this signaling axis. Thus, the neuronal function of PKD promises to be a fruitful area for future investigation.

Materials and Methods

Plasmids

HDAC5 cDNA[322] in expression plasmid *pcDNA3.1*[298] (Invitrogen, Carlsbad, CA) was a gift from Dr. T. A. McKinsey (University of Texas Southwestern Medical Center, Dallas, TX). The cDNA for *mCherry*[291], a gift from Dr. R. Y. Tsien (Howard Hughes Medical Institute, University of California, San Diego, CA), was subcloned into plasmid *pGW1-CMV* (British Biotechnology; Oxford, UK) for higher expression in neurons. Plasmid *pGW1-GFP* was a gift from Dr. D. B. Arnold (University of Southern California, Los Angeles). GFP-tagged versions of PKD[139] were provided by Dr. Enrique Rozengurt (University of California, Los Angeles, School of Medicine). *Venus* cDNA[290] was a gift from Dr. A. Miyawaki (Brain Science Institute, RIKEN, Japan). A Venus-PKD expression plasmid was constructed in three steps: First, *Venus* cDNA lacking a stop codon was subcloned into the KpnI/XbaI sites of *pGW1-CMV* (performed by S. Mitra). Next, the *PKD* coding region and N-terminal linker from plasmid *pEF-plink2-GFP$_{C3}$-PKD*[139] were excised using EcoRI and inserted downstream of *Venus* in *pGW1-CMV*. Finally, this construct was linearized with BglII, treated with mung bean nuclease, and re-ligated to get *Venus* and *PKD* coding regions in frame, separated by a 46 amino-acid linker. Enzymes used for cloning were from New England Biolabs (Beverly, MA).

Cell culture

Cortical neurons from E20 rat pups or E18-20 mouse pups were dissociated and cultured

using a modified version of a previously described protocol[276]. Pups were sacrificed by CO_2 inhalation, cerebral cortices were rapidly isolated, and meninges were removed. Dissection was performed with tissue submerged in ice-cold HEPES-buffered dissociation medium (in mM: 81.8 Na_2SO_4, 30 K_2SO_4, 15.2 $MgCl_2$, 0.25 $CaCl_2$, 1 HEPES, pH 7.4, 20 glucose, and 0.001% phenol red) supplemented with kynurenic acid (1 mM; Sigma; St. Louis, MO). Cells were dissociated and plated as described previously (see Chapter 2 Methods and Bradley *et al.* (2006)[215]) and grown in neuronal culture medium[276] (NCM; for recipe, see Chapter 2 Methods). Cells were fed every 3 days by transfer into conditioned NCM supplemented 1:1 with fresh NCM. Neurons were used for experiments at 12 days *in vitro* (DIV). For stimulation of L-type voltage sensitive calcium channels (L-VSCCs), cells were incubated in a 1:1 mixture of Basal Media Eagle's (Invitrogen) and depolarization solution (in mM: 5.36 NaCl, 110 KCl, 1.8 $CaCl_2$, 0.925 NaH_2PO_4, 0.39 $MgSO_4$, 1 HEPES, pH 7.4) such that the final concentration of KCl was 55 mM.

Transfections

Cells were transfected with plasmid DNA using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions (0.9 μg of DNA and 2 μl of lipid per 1.9 cm^2 well).

Antibodies and drugs

Rabbit polyclonal antibodies that recognize phosphorylated PKD (Ser744/8) and HDAC5

were from Cell Signaling (Beverly, MA). A rabbit polyclonal antibody that recognizes PKCμ (PKD) was from Santa Cruz Biotechnology (C-20; Santa Cruz, CA). Peroxidase- and fluorophore-conjugated secondary antibodies were from Jackson Immunoresearch (West Grove, PA). Phorbol 12-myristate 13-acetate (PMA), *N*-methyl-D-aspartate (NMDA), and all other chemicals were from Sigma.

Western blots

Cortical neurons (10 DIV) were switched to serum-free NCM. Two days later, cells were washed twice with Basal Media Eagle's before being stimulated with drugs for various durations. Stimulations were terminated by aspiration of drug-containing media and addition of boiling 2x Laemmli sample buffer (4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol (added just before use), 0.02% (w/v) bromophenol blue, and 100 mM Tris, pH 6.8). Lysates were scraped into eppendorfs, boiled for 10 min, and cooled on ice. Proteins were separated by 8% SDS-PAGE and transferred to nitrocellulose (Amersham Biosciences; Piscataway, NJ). Membranes were blocked with 5% (w/v) nonfat milk (Bio-Rad; Hercules, CA) or 5% (w/v) bovine serum albumin (BSA; USB Corp.; Cleveland, OH) in TBS containing 0.1% (v/v) Tween-20 (TBS/T) and probed with primary antibodies and peroxidase-conjugated secondary antibodies. Blots were visualized using enhanced chemiluminescence reagents (Perkin Elmer; Boston, MA) and Kodak BioMax MR film (Fisher Scientific; Hampton, NH). Blots were stripped by incubation in stripping buffer (100 mM 2-mercaptoethanol, 2% (w/v) SDS, and 62.5 mM Tris, pH 6.7) for 50 min at 55°C.

Membranes were washed in TBS/T, blocked, and re-probed with antibodies.

Immunocytochemistry

Stimulated cells were fixed in warm PBS containing 4% (w/v) paraformaldehyde (Electron Microscopy Sciences; Hatfield, PA) for 15 min at room temperature. Fixed cells were blocked for 2 h with 5% (v/v) normal goat serum (NGS) in PBS with 0.1% Triton X-100 (PBS/T). Rabbit anti-HDAC5 antibody was diluted 1:500 in PBS/T containing 3% (w/v) BSA and 1% NGS and applied to cells for 2 h. Cells were washed with PBS/T and incubated with a Cy5-conjugated goat anti-rabbit secondary antibody (1:250 dilution) for 1 h. Nuclei were stained with Hoechst 33342 (2.5 μg/ml; Tocris; Ellisville, MO) for 10 min and cells were mounted in glycerol gelatin (Sigma) for imaging.

Microscopy and image analysis

Epifluorescence images were captured using an inverted microscope (Nikon; Melville, NY) equipped with a digital cooled CCD camera (Hammatsu; Bridgewater, NJ) and appropriate excitation and emission filters for GFP, Venus, and mCherry. Confocal images were acquired with a LSM 510 Meta system (Zeiss; Thornwood, NY) equipped with laser lines to excite GFP (488 nm) and Cy5 (633 nm). Hoechst 33342 fluorescence was visualized by two-photon excitation with a Mai Tai Ti:sapphire laser (Spectra-Physics; Mountain View, CA). Image analysis was performed off-line using MetaMorph software (Universal Imaging; Downingtown, PA).

Protein microarrays

Human protein microarrays provided in the ProtoArray Kinase Substrate Identification kit (Invitrogen) were probed with recombinant PKD using a modified version of a previously described protocol[301]. Arrays stored at -20°C were allowed to equilibrate at 4°C for 1 h before being blocked with 1% BSA in PBS for 3 h at 4°C. Recombinant PKDs from different vendors and with different epitope tags were used on separate arrays to increase the probability of identifying true PKD substrates. N-terminal GST-tagged PKD (Invitrogen) or hexahistidine-tagged PKD (Upstate Biotechnology; Lake Placid, NY) was diluted to 50 nM in kinase buffer (Invitrogen) containing 1 μM dithiothreitol. After γ-^{33}P-ATP (10 μCi/μl; Amersham Biosciences; Piscataway, NJ) was added to kinase solutions (33.3 nM final concentration), solutions were immediately overlaid on arrays, covered with a coverslip, and placed in a humified chamber at 30°C for 1 h. An additional array was incubated with kinase buffer and γ-^{33}P-ATP in the absence of kinase and served as an autophosphorylation reference. Arrays were washed three times with 0.5% SDS and three times with distilled water before being spun dry and exposed to Kodak BioMax MR film for 13 h. Film was scanned at 2400 dots per inch and digitized array images were cropped, re-sized, and contrast-inverted using Adobe Photoshop software (Adobe Systems Inc.; San Jose, CA). Images were then analyzed using GenePix Pro 6.0 software (Molecular Devices; Sunnyvale, CA) and lot-specific array information (Invitrogen). Finally, ProtoArray Prospector software (Invitrogen) was used to determine Z-scores for all proteins spotted on the arrays. The Z-

score for a protein indicates how far and in what direction the signal from that protein deviates from the mean of the distribution of signals on the array, expressed in units of the distribution's standard deviation. Proteins with Z>3.0 were scored as hits for each array. A total of four arrays from two different manufacturing lots were probed with PKD, and only proteins which scored as hits on all four arrays were considered significant.

Chapter 4:

Concluding Remarks

Summary of findings

> "... cognitive activity ... is almost certainly accompanied by molecular changes in neurons, as well as by very complex changes in relationships between neurons. Therefore, to understand cognitive activity, it will be necessary to understand these molecular and connectional changes .... Our knowledge is far from complete. While waiting for chemistry, cell biology, and histology to help achieve this goal, which will take a very long time, we must be content with hypotheses that occasionally lead to the discovery of a useful observation or formulate a more precise concept."
>
> –Santiago Ramón y Cajal[323]

Cajal's remarkable prediction, that "changes in relationships between neurons"—now known to involve long-lasting changes in synaptic strength, like LTP—are essential for learning and memory, has gained considerable experimental support[11]. That these changes will be "very complex" and that fully understanding them will "take a very long time" are predictions even more likely to be true.

Forms of LTP that may be useful for memory storage are long-lasting and require neuronal gene expression. However, the mechanisms by which synaptic activity triggers neuronal gene expression, and by which gene products act specifically at synapses that triggered their expression, are poorly understood. The work in this dissertation begins to describe the activity-dependent regulation of Arc and PKD, molecules that may be critical for these mechanisms. We found that AMPA receptors regulate transcription of *Arc* (Chapter 2) and that NMDA receptors regulate activation and translocation of PKD (Chapter 3). These results provide insight into the coupling between neuronal activity and gene expression and expand the complexity of the roles played by glutamate receptors in synaptic plasticity.

A model for activity-dependent regulation of *Arc* expression

The complex regulation of *Arc* affords a unique opportunity to probe neuronal mechanisms of gene expression-dependent plasticity. The results in Chapter 2 support a model in which opposing signals from NMDA and AMPA receptors are integrated to determine the extent of *Arc* transcription (Figure 4.1). For many of our experiments, we used BDNF, which robustly induces *Arc* expression through two distinct pathways: (1) an activity-independent pathway that may involve BDNF binding to postsynaptic TrkB receptors, and (2) an activity-dependent pathway involving TTX-sensitive enhancement of synaptic glutamate release. These pathways contribute equally to BDNF-induced *Arc* expression and both require the MAPK pathway. Using antagonists of synaptic glutamate receptors, we found that activity-dependent *Arc* expression is specified by the relative extent of NMDA and AMPA receptor activity, with a high NMDA:AMPA receptor activity ratio leading to more *Arc* expression and vice versa. G_i-proteins that associate with AMPA receptors may be involved in reading out the NMDA:AMPA ratio. Finally, we determined that NMDA and AMPA receptors regulate *Arc* expression primarily at the level of transcription, though *Arc* translation and stability can also be regulated through other mechanisms.

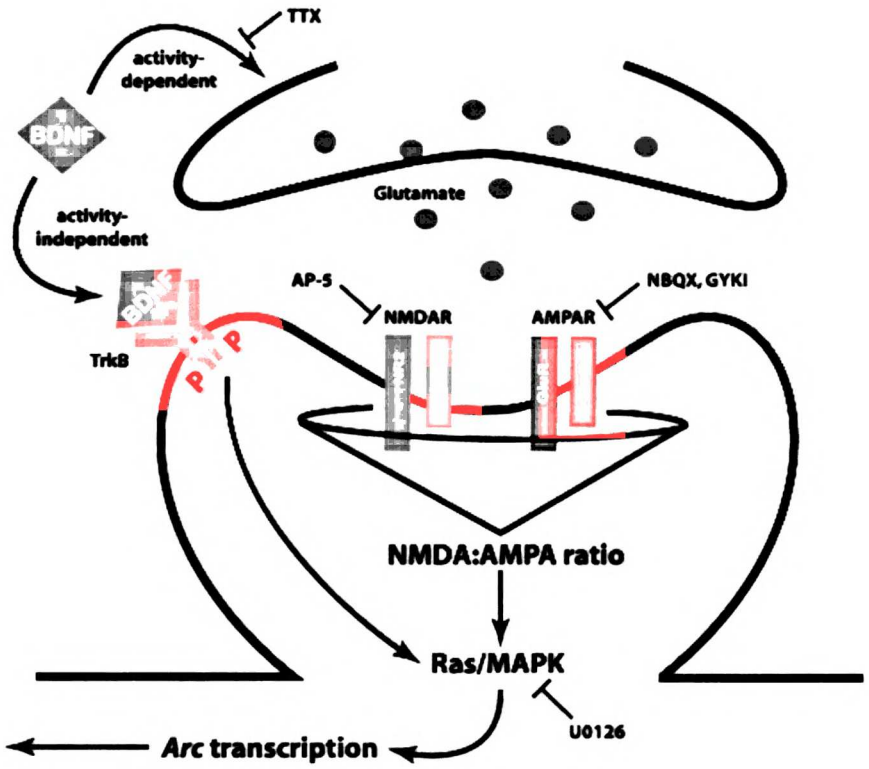


Figure 4.1 A model for activity-dependent regulation of *Arc* expression.

Significance of results on *Arc* regulation

Several features of our findings make them novel. (1) To our knowledge, this is the first study to show that a reduction in AMPA receptor activity can increase neuronal gene transcription. (2) We show that this effect is independent of Ca^{2+} influx directly through AMPA receptors (Figure 2.7) or indirectly through L-VSCCs (Figure 2.6). Previously, neuronal gene expression was generally linked to Ca^{2+}-permeable AMPA receptors[208] or to well-known secondary effects of AMPA receptor–induced depolarization (e.g., L-VSCC activation)[324-326]. (3) We provide novel data regarding the mechanisms by which AMPA receptors regulate gene expression. For example, previous studies showing that AMPA

receptor activation upregulates BDNF mRNA levels did not examine mRNA stability[219,324-328], as we did for *Arc* (Figure 2.13a,b), despite evidence that rapid increases in AMPA-induced BDNF mRNA levels may be due to stabilization of pre-formed mRNA[327]. Indeed, none of these studies[219,324-328] directly assayed for transcription, as we did with *Arc* using intronic reverse-transcription PCR (Figure 2.13d). Additionally, we provide mechanistic insight into the signaling pathways linking AMPA receptors and *Arc* expression (Figure 2.9). (4) We show that AMPA receptors regulate *Arc* expression through a mechanism that depends critically on NMDA receptor activity. In previous studies, AMPA receptor–mediated increases in BDNF mRNA levels were unaffected by NMDA receptor antagonists[324,325,328]. By contrast, we found that NMDA and AMPA receptor antagonists have opposing effects on *Arc* expression (Figure 2.8), suggesting a novel mechanism in which pathways that control *Arc* transcription integrate signals from NMDA and AMPA receptors. The ability of the NMDA:AMPA ratio to specify patterns of gene expression provides a possible explanation for the observation that this ratio is under tight homeostatic control in neurons[260,261].

A role for AMPA receptors in regulating *Arc* expression is surprising in light of the prevailing view that AMPA receptors mediate fast excitatory synaptic transmission and effect short-term plasticity[49], but do not directly regulate neuronal gene expression. Our findings are consistent with recent evidence indicating that AMPA receptors are cell-surface signal transducers, not just passive conduits for current flux[208,218-220]. For example, AMPA/kainate receptors can associate with and signal through pertussis toxin–sensitive G-

proteins[223,224,329,330]. Thus, our finding that AMPA receptors may regulate *Arc* transcription through a metabotropic mechanism (Figure 2.9) integrates our results with previous work on the signaling capacity of AMPA receptors[221].

Arc has been implicated in mechanisms of synaptic plasticity based on its pattern of induction[41-43,48,56], the effects of inhibiting its expression[55,64], and correlative studies linking *Arc* expression and learning[66-68]. However, despite several preliminary reports suggesting that Arc may have a role in synaptic AMPA receptor trafficking[58-61], the precise molecular function of Arc is unknown, making it difficult to do more than speculate about the functional significance of our findings. However, given that *Arc* mRNA levels *in vivo* are tightly correlated with acquisition and performance of learning tasks[67,68], regulatory mechanisms that control *Arc* levels are likely to be of high biological significance.

Despite the paucity of information about its function, *Arc* has been widely employed as a marker of activated neuronal circuits[71-74,79,81]. How circuit activity regulates *Arc* expression is critical to interpret the use of *Arc* as a marker. However, little is known about the activity-dependent mechanisms which regulate *Arc* expression. Thus, we assert that the significance of our work lies in the elucidation of regulatory mechanisms for a widely-used, plasticity-related gene and in the discovery of a novel role for AMPA receptors in this mechanism.

AMPA receptors may regulate a program of IEG expression

In our experiments, NBQX did not affect BDNF-induced expression of *Fos* (Figure 2.5), an IEG used as a control for *Arc*-inducing stimuli[55,76], indicating that gene expression was not

globally upregulated by AMPA receptor blockade. However, it remained possible that *Arc* belongs to a subset of IEGs whose expression is regulated by AMPA receptors. To test this, we examined the effect of AMPA receptor antagonists on BDNF-induced expression of other IEGs. BDNF-induced expression of *zif268*, an IEG essential for late-phase LTP and memory[331], is potentiated by AMPA receptor antagonists to a similar extent as *Arc* expression (Figure 4.2). Expression of *nur77* shows a more dramatic increase, whereas expression levels of *Krox20* and *JunB* are affected to a lesser extent. Thus, AMPA receptors may regulate a program of gene expression that includes a specific subset of IEGs. More work is needed to determine which promoter elements are critical for transcriptional regulation of these IEGs by AMPA receptors. Detailed cross-species analysis of the *Arc* promoter identified multiple conserved regions containing putative transcription factor binding sites[332]. A number of transcription factors have been implicated in regulating *Arc* expression, including Egr[333], CREB[184], SRF[232], and MEF2[334], suggesting that transcriptional control of *Arc* may be highly complex. Future studies will determine the mechanisms by which synaptic activity orchestrates transcription factors and signaling molecules to precisely regulate intracellular *Arc* mRNA levels.

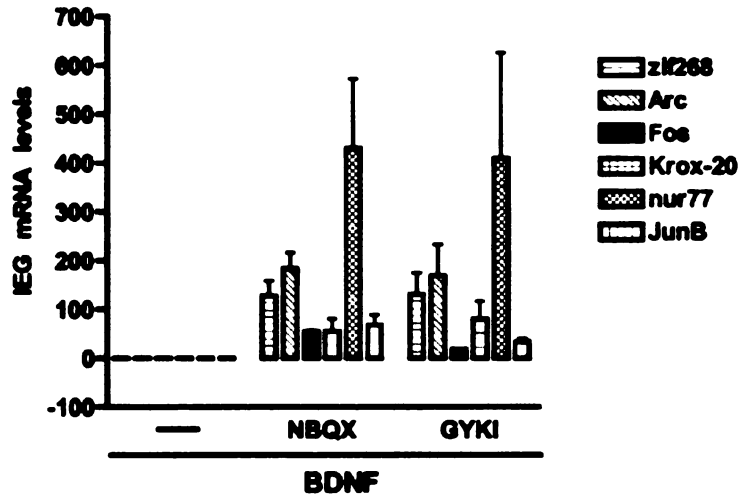


Figure 4.2 AMPA receptors regulate a subset of IEGs.

AMPA receptor antagonists NBQX (10 μM) and GYKI 52466 (15 μM) differentially affect BDNF-induced expression levels of six IEG mRNAs, determined by qfRT-PCR. *Arc* and *Fos* primers are described in Chapter 2. Other primers used were: *zif268*: 5'- gactatctgtttccacaacaacag-3' and 5'- ctttgatagtggatagtggagtga-3'; *Krox20*: 5'- caggatccttcagcattcttatc-3' and 5'- ggagatggaaaaaatccaggatag-3'; *nur77*: 5'- gtgttgatgttcctgcctttg-3' and 5'- tgttccttcagacagctagca-3'; *JunB*: 5'- cctggacgacctgcacaa-3' and 5'- gtgcagaggctggagagtaa-3'. Data are plotted as percent difference from BDNF alone (n=5).

AMPA receptors may regulate *Arc* in networks of neurons

The qfRT-PCR and Western blot experiments in Chapter 2 involve determining levels of *Arc* mRNA and protein, respectively, in lysates of cells collected *en masse*. As such, these methods cannot determine whether stimulus-induced changes in *Arc* levels reflect changes in a fixed group of responding cells or changes in the number of responding cells. Using FISH and immunocytochemistry, we determined that NBQX dramatically increases the number of cells expressing *Arc*, with only a small effect on per-cell *Arc* expression (Figure 4.3). Thus, AMPA receptor activity controls the population size of *Arc*-expressing neurons. The fact that per-cell *Arc* expression changes only minimally suggests that *Arc* may be regulated as a binary switch with all-or-none expression determined by AMPA receptor activity. Since *Arc*

expression defines neural circuits involved in information processing[72,73,79], this regulatory mechanism might have important implications *in vivo*.

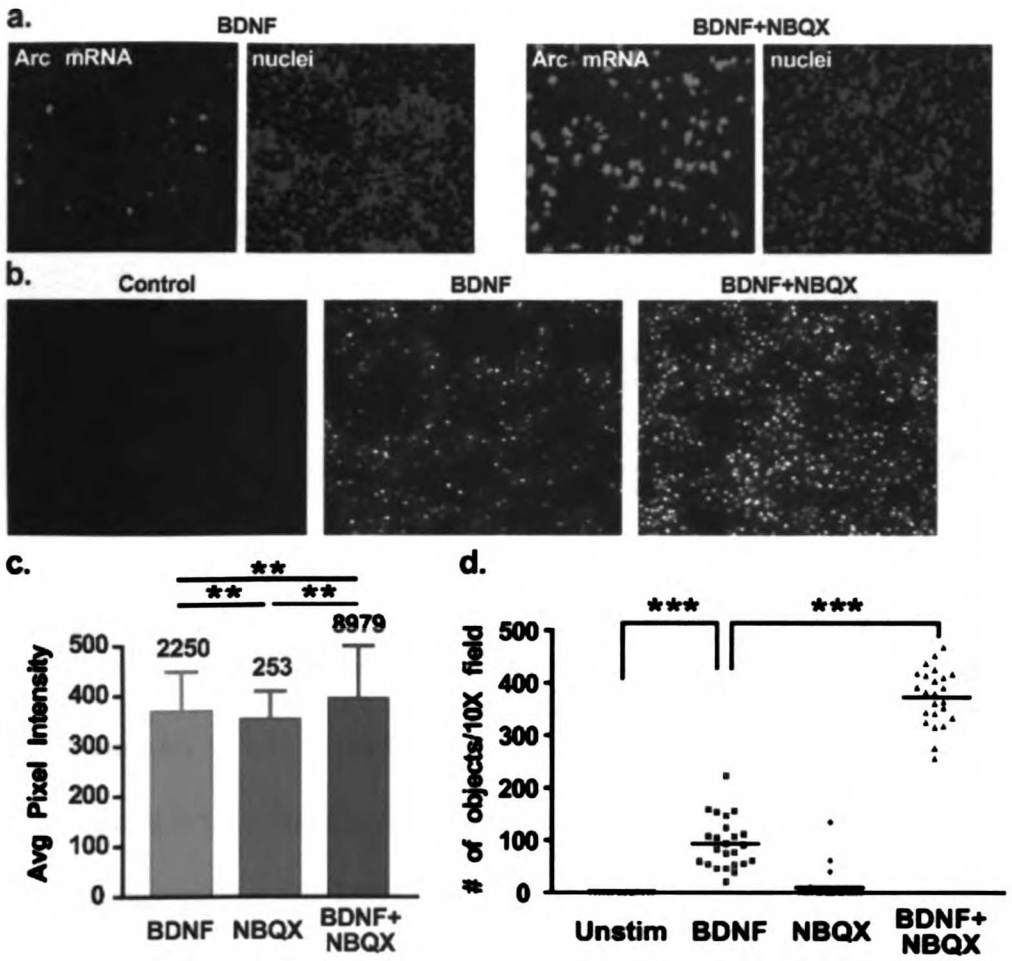


Figure 4.3 AMPA receptors regulate the number of *Arc*-expressing cells.

FISH (**a**) and immunocytochemistry (**b**) reveal that cultures stimulated with BDNF (100 ng/mL) in the presence of NBQX (10 μM) have a greater fraction of *Arc*-expressing neurons than cultures stimulated with BDNF alone. 20× images are shown in **a**, and 10× images are shown in **b**. (**c**) Quantitative analysis of per-cell Arc protein expression reveals small, but statistically significant, differences between treatment conditions. Bars show average pixel intensities of *Arc*-expressing cells in images as shown in **b**. Numbers above bars indicate how many cells were analyzed for each condition. (**d**) Number of *Arc*-expressing cells per 10× field as shown in **b**. Cultures treated with BDNF+NBQX have approximately threefold more *Arc*-expressing neurons than cultures treated with BDNF alone, comparable to the increases in *Arc* mRNA and protein expression observed by qfRT-PCR and Western blot (compare Figure 2.5). **, $p<0.01$; ***, $p<0.001$ versus control (one-way ANOVA and post-hoc Tukey *t* tests).

Arc and the nucleus

We (Figure 4.4) and others[40] have observed that Arc protein is heavily enriched in neuronal nuclei—does Arc have a nuclear function?

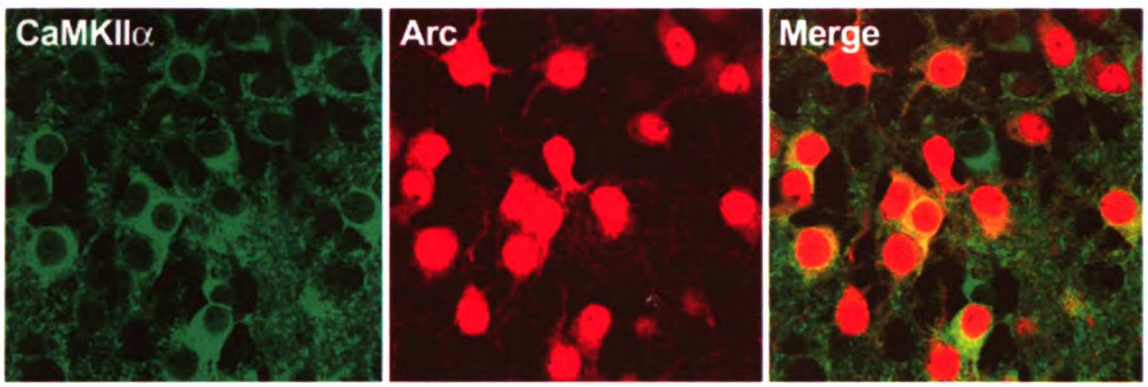


Figure 4.4 Arc protein is found in the nucleus.

Confocal images of neurons immunostained for both Arc and CaMKIIα, a marker of excitatory neurons that is excluded from the nucleus, reveal that Arc protein is enriched in neuronal nuclei.

Domain analysis of Arc protein reveals no canonical transcription factor motifs, but preliminary results from a yeast two-hybrid screen suggest that Arc may interact with nuclear proteins, including mRNA splicing factors and several RNA-binding proteins (C. L. Peebles and S. Finkbeiner, unpublished observations). Nuclear localization of Arc may be regulated by proteasome-dependent protein degradation: Arc protein redistributes to the cytoplasm in cells stimulated with BDNF in the presence of a proteasome inhibitor (Figure 4.5). This suggests the existence of a proteasome substrate that regulates nuclear import and/or export of Arc, reminiscent of NF-κB regulation by I-κB[335]. Thus, proteasome-dependent mechanisms regulate both the steady-state level of Arc protein (Figure 2.12) and its

nucleocytoplasmic distribution (Figure 4.5).

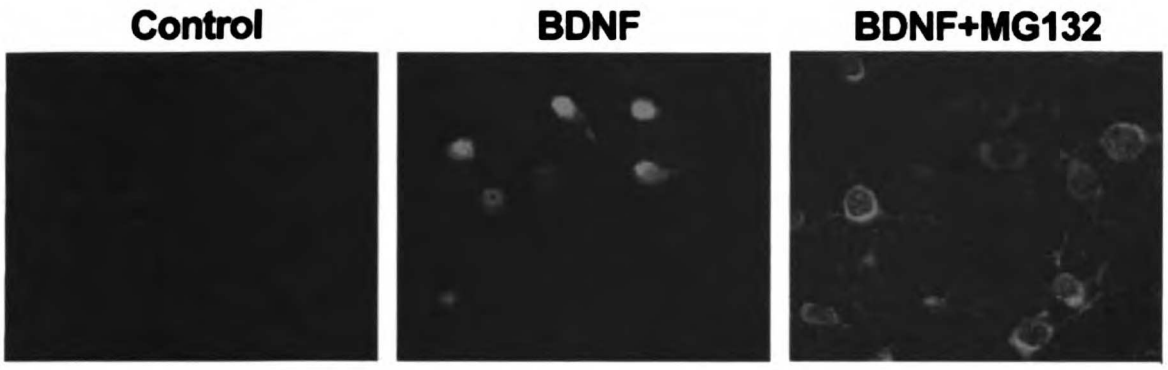


Figure 4.5 Proteasome activity regulates the nucleocytoplasmic distribution of Arc.

20× immunofluorescence images of cortical neurons (12 DIV) showing that endogenous Arc protein is predominantly nuclear when induced by BDNF (100 ng/mL; 8 h) and predominantly cytoplasmic when MG-132 (50 μM), a proteasome inhibitor, is present. Neurons were stained with rabbit polyclonal Arc antisera[42] and an Alexa647-coupled goat anti-rabbit secondary antibody.

Neuronal functions of Arc and PKD

The elucidation of protein function is greatly facilitated by knowledge of the molecules with which it interacts. For example, the PKD substrates we identified using protein microarrays (Table 3.1) suggest a role for PKD in cytoskeletal remodeling, possibly in dendritic spines. Interestingly, Arc may also interact with proteins that regulate cytoskeletal dynamics in dendritic spines (C. L. Peebles and S. Finkbeiner, unpublished observations), consistent with its original characterization as a cytoskeleton-associated protein[42,43]. Other labs have taken a variety of approaches, including yeast two-hybrid screens[61,336], biochemical pull-downs[272], and immunocytochemisty[337], to identify putative Arc binding partners (Table 4.1). The next step will be to determine the functional significance of these and other interactions. Additionally, since PKD has been implicated in a wide array of cellular processes in other

tissues[109,112]—cardiomyocytes, lymphocytes, osteoblasts, fibroblasts, and epithelial cells—it will be important to determine whether PKD plays similar roles in neurons.

Arc Binding Partner	Cellular Function	References
CaMKII	Synaptic plasticity	Donai et al. (2003)[272]
MAP2/microtubules	Cytoskeleton, active transport	Fujimoto et al. (2004)[337]
Amida	Cell survival/apoptosis?	Irie et al. (2000)[336]
Endophilin	Receptor endocytosis	Shepherd et al. (2004)[61]

Table 4.1 Putative Arc binding partners.

Our work on *Arc* and PKD shows that primary cultured neurons may be a powerful model system for future studies. Much of the previous work on *Arc* and PKD was performed either *in vivo*[96,181,183,184], where mechanistic studies are challenging, or in cell lines[125,126,130,143,160,178,180,182], where neuronal regulatory mechanisms may be absent. Our primary neuronal culture system addresses this technical gap, recapitulating critical features of *Arc* and PKD biology observed *in vivo* (Figure 2.1 and Figure 3.1). Using this system, a number of interesting questions can be addressed: Does PKD link synaptic receptors to nuclear HDACs, and what genes are regulated by this signaling pathway? What are the mechanisms by which *Arc* localizes near activated synaptic sites, and how does *Arc* contribute to the maintenance of synaptic potentiation? Do *Arc* and PKD mediate activity-dependent cytoskeletal remodeling, and, if so, what are the physiological implications? The answers to these questions will provide insight into the mechanisms of activity-dependent plasticity and will enhance our understanding of the neural basis of learning and memory.

Chapter 5: References

1. Giese, K.P., Fedorov, N.B., Filipkowski, R.K. & Silva, A.J. Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. *Science* **279**, 870-873 (1998).
2. Lisman, J., Lichtman, J.W. & Sanes, J.R. LTP: perils and progress. *Nat. Rev. Neurosci.* **4**, 926-929 (2003).
3. Sanes, J.R. & Lichtman, J.W. Can molecules explain long-term potentiation? *Nat. Neurosci.* **2**, 597-604 (1999).
4. Tanzi, E. I fatti i le induzione nell'odierna istologia del sisterna nervoso. *Riv. Sper. Freniatr.* **19**, 419-472 (1893).
5. Cowan, W.M. & Kandel, E.R. A Brief History of Synapses and Synaptic Transmission. in *Synapses* (ed. W.M. Cowan, T.C. Sudhof & C.F. Stevens) 1-87 (The Johns Hopkins University Press, Baltimore, MD, 2001).
6. Cajal, S.R.y. The Croonian Lecture: La fine structure des centres nerveux. *Proc. R. Soc. London Ser. B* **55**, 444-467 (1894).
7. Hebb, D.O. *The Organization of Behavior: a Neuropsychological Theory* (Wiley, New York, 1949).
8. Kandel, E.R. Cellular Mechanisms of Learning and the Biological Basis of Individuality. in *Principles of Neural Science* (ed. E.R. Kandel, J.H. Schwartz & T.M. Jessell) 1247-1279 (Elsevier, New York, 2000).

9. Bliss, T.V. & Lomo, T. Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J. Physiol.* **232**, 331-356 (1973).

10. Bliss, T.V. & Gardner-Medwin, A.R. Long-lasting potentiation of synaptic transmission in the dentate area of the unanaestetized rabbit following stimulation of the perforant path. *J. Physiol.* **232**, 357-374 (1973).

11. Martin, S.J., Grimwood, P.D. & Morris, R.G. Synaptic plasticity and memory: an evaluation of the hypothesis. *Annu. Rev. Neurosci.* **23**, 649-711 (2000).

12. Malenka, R.C. The long-term potential of LTP. *Nat. Rev. Neurosci.* **4**, 923-926 (2003).

13. Miller, S. & Mayford, M. Cellular and molecular mechanisms of memory: the LTP connection. *Curr. Opin. Genet. Dev.* **9**, 333-337 (1999).

14. Lisman, J.E. & Harris, K.M. Quantal analysis and synaptic anatomy--integrating two views of hippocampal plasticity. *Trends Neurosci.* **16**, 141-147 (1993).

15. Stevens, C.F. A million dollar question: does LTP = memory? *Neuron* **20**, 1-2 (1998).

16. Bliss, T.V. & Collingridge, G.L. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**, 31-39 (1993).

17. Abraham, W.C. & Williams, J.M. Properties and mechanisms of LTP maintenance. *Neuroscientist* **9**, 463-474 (2003).

18. Andersen, P., Sundberg, S.H., Sveen, O. & Wigstrom, H. Specific long-lasting

potentiation of synaptic transmission in hippocampal slices. *Nature* **266**, 736-737 (1977).

19. Engert, F. & Bonhoeffer, T. Synapse specificity of long-term potentiation breaks down at short distances. *Nature* **388**, 279-284 (1997).

20. Schuman, E.M. Synapse specificity and long-term information storage. *Neuron* **18**, 339-342 (1997).

21. Goelet, P., Castellucci, V.F., Schacher, S. & Kandel, E.R. The long and the short of long-term memory--a molecular framework. *Nature* **322**, 419-422 (1986).

22. Davis, H.P. & Squire, L.R. Protein synthesis and memory: a review. *Psychol. Bull.* **96**, 518-559 (1984).

23. Kandel, E.R. The molecular biology of memory storage: a dialogue between genes and synapses. *Science* **294**, 1030-1038 (2001).

24. Fonseca, R., Nagerl, U.V. & Bonhoeffer, T. Neuronal activity determines the protein synthesis dependence of long-term potentiation. *Nat. Neurosci.* **9**, 478-480 (2006).

25. Lamprecht, R. & LeDoux, J. Structural plasticity and memory. *Nat. Rev. Neurosci.* **5**, 45-54 (2004).

26. Adams, J.P. & Dudek, S.M. Late-phase long-term potentiation: getting to the nucleus. *Nat. Rev. Neurosci.* **6**, 737-743 (2005).

27. Bradley, J. & Finkbeiner, S. An evaluation of specificity in activity-dependent gene expression in neurons. *Prog. Neurobiol.* **67**, 469 (2002).

28. Frey, U. & Morris, R.G. Synaptic tagging and long-term potentiation. *Nature* **385**, 533-536 (1997).

29. Frey, U. & Morris, R.G. Synaptic tagging: implications for late maintenance of hippocampal long-term potentiation. *Trends Neurosci.* **21**, 181-188 (1998).

30. Sajikumar, S., Navakkode, S., Sacktor, T.C. & Frey, J.U. Synaptic tagging and cross-tagging: the role of protein kinase Mzeta in maintaining long-term potentiation but not long-term depression. *J. Neurosci.* **25**, 5750-5756 (2005).

31. Martin, K.C., Barad, M. & Kandel, E.R. Local protein synthesis and its role in synapse-specific plasticity. *Curr. Opin. Neurobiol.* **10**, 587-592 (2000).

32. Steward, O. & Schuman, E.M. Protein synthesis at synaptic sites on dendrites. *Ann. Rev. Neurosci.* **24**, 299-325 (2001).

33. Steward, O. & Schuman, E.M. Compartmentalized synthesis and degradation of proteins in neurons. *Neuron* **40**, 347-359 (2003).

34. Job, C. & Eberwine, J. Localization and translation of mRNA in dendrites and axons. *Nat. Rev. Neurosci.* **2**, 889-898 (2001).

35. Glanzer, J., *et al.* RNA splicing capability of live neuronal dendrites. *Proc. Natl. Acad. Sci. USA* **102**, 16859-16864 (2005).

36. Yi, J.J. & Ehlers, M.D. Ubiquitin and protein turnover in synapse function. *Neuron* **47**, 629-632 (2005).

37. Sutton, M.A. & Schuman, E.M. Local translational control in dendrites and its role in long-term synaptic plasticity. *J. Neurobiol.* **64**, 116-131 (2005).

38. Pang, P.T., *et al.* Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. *Science* **306**, 487-491 (2004).

39. Deisseroth, K., Mermelstein, P.G., Xia, H. & Tsien, R.W. Signaling from synapse to nucleus: the logic behind the mechanisms. *Curr. Opin. Neurobiol.* **13**, 354-365 (2003).

40. Steward, O. & Worley, P.F. A cellular mechanism for targeting newly synthesized mRNAs to synaptic sites on dendrites. *Proc. Natl. Acad. Sci. USA* **98**, 7062-7068 (2001).

41. Steward, O. & Worley, P. Local synthesis of proteins at synaptic sites on dendrites: role in synaptic plasticity and memory consolidation? *Neurobiol. Learn. Mem.* **78**, 508-527 (2002).

42. Lyford, G.L., *et al.* Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. *Neuron* **14**, 433-445 (1995).

43. Link, W., *et al.* Somatodendritic expression of an immediate early gene is regulated by synaptic activity. *Proc. Natl. Acad. Sci. USA* **92**, 5734-5738 (1995).

44. Sheng, M. & Greenberg, M.E. The regulation and function of c-fos and other immediate early genes in the nervous system. *Neuron* **4**, 477-485 (1990).

45. Guzowski, J.F. Insights into immediate-early gene function in hippocampal memory consolidation using antisense oligonucleotide and fluorescent imaging approaches. *Hippocampus* **12**, 86-104 (2002).

46. Kobayashi, H., Yamamoto, S., Maruo, T. & Murakami, F. Identification of a cis-acting element required for dendritic targeting of activity-regulated cytoskeleton-associated protein mRNA. *Eur. J. Neurosci.* **22**, 2977-2984 (2005).

47. Wallace, C.S., Lyford, G.L., Worley, P.F. & Steward, O. Differential intracellular sorting of immediate early gene mRNAs depends on signals in the mRNA sequence. *J. Neurosci.* **18**, 26-35 (1998).

48. Steward, O., Wallace, C.S., Lyford, G.L. & Worley, P.F. Synaptic activation causes the mRNA for the IEG Arc to localize selectively near activated postsynaptic sites on dendrites. *Neuron* **21**, 741-751 (1998).

49. Malenka, R.C. & Nicoll, R.A. Long-term potentiation--a decade of progress? *Science* **285**, 1870-1874 (1999).

50. Malinow, R., Mainen, Z.F. & Hayashi, Y. LTP mechanisms: from silence to four-lane traffic. *Curr. Opin. Neurobiol.* **10**, 352-357 (2000).

51. Bortolotto, Z.A., Nistico, R., More, J.C., Jane, D.E. & Collingridge, G.L. Kainate receptors and mossy fiber LTP. *Neurotoxicology* **26**, 769-777 (2005).

52. Steward, O. & Worley, P.F. Selective targeting of newly synthesized Arc mRNA to active synapses requires NMDA receptor activation. *Neuron* **30**, 227-240 (2001).

53. Yin, Y., Edelman, G.M. & Vanderklish, P.W. The brain-derived neurotrophic factor enhances synthesis of Arc in synaptoneurosomes. *Proc. Natl. Acad. Sci. USA* **99**, 2368-2373 (2002).

54. Pinkstaff, J.K., Chappell, S.A., Mauro, V.P., Edelman, G.M. & Krushel, L.A. Internal initiation of translation of five dendritically localized neuronal mRNAs. *Proc. Natl. Acad. Sci. USA* **98**, 2770-2775 (2001).

55. McIntyre, C.K., *et al.* Memory-influencing intra-basolateral amygdala drug infusions modulate expression of Arc protein in the hippocampus. *Proc. Natl. Acad. Sci. USA* (2005).

56. Moga, D.E., *et al.* Activity-regulated cytoskeletal-associated protein is localized to recently activated excitatory synapses. *Neuroscience* **125**, 7-11 (2004).

57. Husi, H., Ward, M.A., Choudhary, J.S., Blackstock, W.P. & Grant, S.G. Proteomic analysis of NMDA receptor-adhesion protein signaling complexes. *Nat. Neurosci.* **3**, 661-669 (2000).

58. Mokin, M., Lindahl, J.S. & Keifer, J. Immediate-early gene-encoded protein Arc is associated with synaptic delivery of GluR4-containing AMPA receptors during in vitro classical conditioning. *J. Neurophysiol.* **95**, 215-224 (2006).

59. Rial Verde, E.M., Worley, P., Malinow, R. & Cline, H. The immediate-early gene Arc regulates AMPA-receptor trafficking. *Soc. Neurosci. Abstr.* **29**, 55.57 (2003).

60. Rial Verde, E.M., Worley, P., Malinow, R. & Cline, H. The role of Arc in synaptic function in rat hippocampus. *Soc. Neurosci. Abstr.* **28**, 839.835 (2002).

61. Shepherd, J.D., Chowdhury, S., Petralia, R., Huganir, R. & Worley, P. Arc modulates AMPA receptor trafficking via its interaction with the endocytic machinery. *Soc. Neurosci. Abstr.* **30**, 971.915 (2004).

62. Wang, H. & Pickel, V.M. Activity-regulated cytoskeleton-associated protein Arc is targeted to dendrites and coexpressed with mu-opioid receptors in postnatal rat caudate-putamen nucleus. *J. Neurosci. Res.* **77**, 323-333 (2004).

63. Liu, D., Bei, D., Parmar, H. & Matus, A. Activity-regulated, cytoskeleton-associated protein (Arc) is essential for visceral endoderm organization during early embryogenesis. *Mech. Dev.* **92**, 207-215 (2000).

64. Guzowski, J.F., *et al.* 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**, 3993-4001 (2000).

65. Rodriguez, J.J., *et al.* Long-term potentiation in the rat dentate gyrus is associated with enhanced Arc/Arg3.1 protein expression in spines, dendrites and glia. *Eur. J. Neurosci.* **21**, 2384-2396 (2005).

66. Montag-Sallaz, M. & Montag, D. Learning-induced arg 3.1/arc mRNA expression in the mouse brain. *Learn. Mem.* **10**, 99-107 (2003).

67. Guzowski, J.F., Setlow, B., Wagner, E.K. & McGaugh, J.L. Experience-dependent gene expression in the rat hippocampus after spatial learning: a comparison of the immediate-early genes Arc, c-fos, and zif268. *J. Neurosci.* **21**, 5089-5098 (2001).

68. Kelly, M.P. & Deadwyler, S.A. Acquisition of a novel behavior induces higher levels of Arc mRNA than does overtrained performance. *Neuroscience* **110**, 617-626 (2002).

69. Ulloor, J. & Datta, S. Spatio-temporal activation of cyclic AMP response element-binding protein, activity-regulated cytoskeletal-associated protein and brain-derived nerve growth factor: a mechanism for pontine-wave generator activation-dependent two-way active-avoidance memory processing in the rat. *J. Neurochem.* **95**, 418-428 (2005).

70. Pinaud, R., Penner, M.R., Robertson, H.A. & Currie, R.W. Upregulation of the immediate early gene arc in the brains of rats exposed to environmental enrichment: implications for molecular plasticity. *Brain Res. Mol. Brain Res.* **91**, 50-56 (2001).

71. Vazdarjanova, A., McNaughton, B.L., Barnes, C.A., Worley, P.F. & Guzowski, J.F. Experience-dependent coincident expression of the effector immediate-early genes arc and Homer 1a in hippocampal and neocortical neuronal networks. *J. Neurosci.* **22**, 10067-10071 (2002).

72. Guzowski, J.F., McNaughton, B.L., Barnes, C.A. & Worley, P.F. Environment-specific expression of the immediate-early gene Arc in hippocampal neuronal ensembles. *Nat. Neurosci.* **2**, 1120-1124 (1999).

73. Ramirez-Amaya, V., *et al.* Spatial exploration-induced arc mRNA and protein expression: evidence for selective, network-specific reactivation. *J. Neurosci.* **25**, 1761-1768 (2005).

74. Chawla, M.K., *et al.* Sparse, environmentally selective expression of Arc RNA in the upper blade of the rodent fascia dentata by brief spatial experience.

Hippocampus (2005).

75. Vazdarjanova, A. & Guzowski, J.F. Differences in hippocampal neuronal population responses to modifications of an environmental context: evidence for distinct, yet complementary, functions of CA3 and CA1 ensembles. *J. Neurosci.* **24**, 6489-6496 (2004).

76. Ons, S., Marti, O. & Armario, A. Stress-induced activation of the immediate early gene Arc (activity-regulated cytoskeleton-associated protein) is restricted to telencephalic areas in the rat brain: relationship to c-fos mRNA. *J. Neurochem.* **89**, 1111-1118 (2004).

77. Kelly, M.P. & Deadwyler, S.A. Experience-dependent regulation of the immediate-early gene arc differs across brain regions. *J. Neurosci.* **23**, 6443-6451 (2003).

78. Huff, N.C., *et al.* Amygdala regulation of immediate-early gene expression in the hippocampus induced by contextual fear conditioning. *J. Neurosci.* **26**, 1616-1623 (2006).

79. Guzowski, J.F., *et al.* Mapping behaviorally relevant neural circuits with immediate-early gene expression. *Curr. Opin. Neurobiol.* **15**, 599-606 (2005).

80. Gusev, P.A., Cui, C., Alkon, D.L. & Gubin, A.N. Topography of Arc/Arg3.1 mRNA expression in the dorsal and ventral hippocampus induced by recent and remote spatial memory recall: dissociation of CA3 and CA1 activation. *J. Neurosci.* **25**, 9384-9397 (2005).

81. Temple, M.D., Worley, P.F. & Steward, O. Visualizing changes in circuit activity resulting from denervation and reinnervation using immediate early gene expression. *J. Neurosci.* **23**, 2779-2788 (2003).

82. Wang, D.C., Chen, S.S., Lee, Y.C. & Chen, T.J. Amyloid-beta at sublethal level impairs BDNF-induced arc expression in cortical neurons. *Neurosci. Lett.* (2006).

83. Palop, J.J., *et al.* Vulnerability of dentate granule cells to disruption of arc expression in human amyloid precursor protein transgenic mice. *J. Neurosci.* **25**, 9686-9693 (2005).

84. Dickey, C.A., *et al.* Amyloid suppresses induction of genes critical for memory consolidation in APP + PS1 transgenic mice. *J. Neurochem.* **88**, 434-442 (2004).

85. Rosi, S., *et al.* Neuroinflammation alters the hippocampal pattern of behaviorally induced Arc expression. *J. Neurosci.* **25**, 723-731 (2005).

86. Kunizuka, H., *et al.* Activation of Arc gene, a dendritic immediate early gene, by middle cerebral artery occlusion in rat brain. *Neuroreport* **10**, 1717-1722 (1999).

87. Guthrie, K., Rayhanabad, J., Kuhl, D. & Gall, C. Odors regulate Arc expression in neuronal ensembles engaged in odor processing. *Neuroreport* **11**, 1809-1813 (2000).

88. Zou, Z. & Buck, L.B. Combinatorial effects of odorant mixes in olfactory cortex. *Science* **311**, 1477-1481 (2006).

89. Matsuoka, M., *et al.* Rapid induction of Arc is observed in the granule cell dendrites in the accessory olfactory bulb after mating. *Brain Res.* **975**, 189-195

(2003).

90. Bock, J., Thode, C., Hannemann, O., Braun, K. & Darlison, M.G. Early socio-emotional experience induces expression of the immediate-early gene Arc/arg3.1 (activity-regulated cytoskeleton-associated protein/activity-regulated gene) in learning-relevant brain regions of the newborn chick. *Neuroscience* **133**, 625-633 (2005).

91. Velho, T.A., Pinaud, R., Rodrigues, P.V. & Mello, C.V. Co-induction of activity-dependent genes in songbirds. *Eur. J. Neurosci.* **22**, 1667-1678 (2005).

92. Leitner, S., Voigt, C., Metzdorf, R. & Catchpole, C.K. Immediate early gene (ZENK, Arc) expression in the auditory forebrain of female canaries varies in response to male song quality. *J. Neurobiol.* **64**, 275-284 (2005).

93. Nishimura, M., Yamagata, K., Sugiura, H. & Okamura, H. The activity-regulated cytoskeleton-associated (arc) gene is a new light-inducible early gene in the mouse suprachiasmatic nucleus. *Neuroscience* **116**, 1141-1147 (2003).

94. Maier, B., Medrano, S., Sleight, S.B., Visconti, P.E. & Scrable, H. Developmental association of the synaptic activity-regulated protein arc with the mouse acrosomal organelle and the sperm tail. *Biol. Reprod.* **68**, 67-76 (2003).

95. Castro, E., Tordera, R.M., Hughes, Z.A., Pei, Q. & Sharp, T. Use of Arc expression as a molecular marker of increased postsynaptic 5-HT function after SSRI/5-HT1A receptor antagonist co-administration. *J. Neurochem.* **85**, 1480-1487 (2003).

96. Pei, Q., Zetterstrom, T.S., Sprakes, M., Tordera, R. & Sharp, T. Antidepressant drug treatment induces Arc gene expression in the rat brain. *Neuroscience* **121**, 975-982 (2003).

97. Tordera, R., *et al.* Effect of different 5-HT1A receptor antagonists in combination with paroxetine on expression of the immediate-early gene Arc in rat brain. *Neuropharmacology* **44**, 893-902 (2003).

98. Larsen, M.H., *et al.* Regulation of activity-regulated cytoskeleton protein (Arc) mRNA after acute and chronic electroconvulsive stimulation in the rat. *Brain Res.* **1064**, 161-165 (2005).

99. Ziolkowska, B., Urbanski, M.J., Wawrzczak-Bargiela, A., Bilecki, W. & Przewlocki, R. Morphine activates Arc expression in the mouse striatum and in mouse neuroblastoma Neuro2A MOR1A cells expressing mu-opioid receptors. *J. Neurosci. Res.* (2005).

100. Fosnaugh, J.S., Bhat, R.V., Yamagata, K., Worley, P.F. & Baraban, J.M. Activation of arc, a putative "effector" immediate early gene, by cocaine in rat brain. *J. Neurochem.* **64**, 2377-2380 (1995).

101. Tan, A., Moratalla, R., Lyford, G.L., Worley, P. & Graybiel, A.M. The activity-regulated cytoskeletal-associated protein arc is expressed in different striosome-matrix patterns following exposure to amphetamine and cocaine. *J. Neurochem.* **74**, 2074-2078 (2000).

102. Fujiyama, K., Kajii, Y., Hiraoka, S. & Nishikawa, T. Differential regulation by

stimulants of neocortical expression of mrt1, arc, and homer1a mRNA in the rats treated with repeated methamphetamine. *Synapse* **49**, 143-149 (2003).

103. Schiltz, C.A., Kelley, A.E. & Landry, C.F. Contextual cues associated with nicotine administration increase arc mRNA expression in corticolimbic areas of the rat brain. *Eur. J. Neurosci.* **21**, 1703-1711 (2005).

104. Mahlke, C. & Wallhausser-Franke, E. Evidence for tinnitus-related plasticity in the auditory and limbic system, demonstrated by arg3.1 and c-fos immunocytochemistry. *Hear. Res.* **195**, 17-34 (2004).

105. Kim, K.A., Chakraborti, T., Goldstein, G., Johnston, M. & Bressler, J. Exposure to lead elevates induction of zif268 and Arc mRNA in rats after electroconvulsive shock: the involvement of protein kinase C. *J. Neurosci. Res.* **69**, 268-277 (2002).

106. Guzowski, J.F., *et al.* Recent behavioral history modifies coupling between cell activity and Arc gene transcription in hippocampal CA1 neurons. *Proc. Natl. Acad. Sci. USA* **103**, 1077-1082 (2006).

107. Valverde, A.M., Sinnett-Smith, J., Van Lint, J. & Rozengurt, E. Molecular cloning and characterization of protein kinase D: a target for diacylglycerol and phorbol esters with a distinctive catalytic domain. *Proc. Natl. Acad. Sci. USA* **91**, 8572-8576 (1994).

108. Johannes, F.J., Prestle, J., Eis, S., Oberhagemann, P. & Pfizenmaier, K. PKCu is a novel, atypical member of the protein kinase C family. *J. Biol. Chem.* **269**, 6140-6148 (1994).

109. Rozengurt, E., Rey, O. & Waldron, R.T. Protein kinase D signaling. *J. Biol. Chem.* **280**, 13205-13208 (2005).

110. Sturany, S., *et al.* Molecular cloning and characterization of the human protein kinase D2. A novel member of the protein kinase D family of serine threonine kinases. *J. Biol. Chem.* **276**, 3310-3318 (2001).

111. Hayashi, A., Seki, N., Hattori, A., Kozuma, S. & Saito, T. PKCnu, a new member of the protein kinase C family, composes a fourth subfamily with PKCmu. *Biochim. Biophys. Acta* **1450**, 99-106 (1999).

112. Van Lint, J., *et al.* Protein kinase D: an intracellular traffic regulator on the move. *Trends Cell Biol.* **12**, 193-200 (2002).

113. Mellor, H. & Parker, P.J. The extended protein kinase C superfamily. *Biochem. J.* **332**, 281-292 (1998).

114. Scheid, M.P. & Woodgett, J.R. PKB/AKT: functional insights from genetic models. *Nat. Rev. Mol. Cell. Biol.* **2**, 760-768 (2001).

115. Toker, A. Signaling through protein kinase C. *Front. Biosci.* **3**, D1134-1147 (1998).

116. Manning, G., Whyte, D.B., Martinez, R., Hunter, T. & Sudarsanam, S. The protein kinase complement of the human genome. *Science* **298**, 1912-1934 (2002).

117. Gschwendt, M., *et al.* Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase c isoenzymes. *FEBS Lett.* **392**, 77-80 (1996).

118. Nishikawa, K., Toker, A., Johannes, F.J., Songyang, Z. & Cantley, L.C. Determination of the specific substrate sequence motifs of protein kinase C isozymes. *J. Biol. Chem.* **272**, 952-960 (1997).

119. Iglesias, T. & Rozengurt, E. Protein kinase D activation by mutations within its pleckstrin homology domain. *J. Biol. Chem.* **273**, 410-416 (1998).

120. Iglesias, T. & Rozengurt, E. Protein kinase D activation by deletion of its cysteine-rich motifs. *FEBS Lett.* **454**, 53-56 (1999).

121. Iglesias, T., Waldron, R.T. & Rozengurt, E. Identification of in vivo phosphorylation sites required for protein kinase D activation. *J. Biol. Chem.* **273**, 27662-27667 (1998).

122. Waldron, R.T., *et al.* Activation loop Ser744 and Ser748 in protein kinase D are transphosphorylated in vivo. *J. Biol. Chem.* **276**, 32606-32615 (2001).

123. Van Lint, J.V., Sinnett-Smith, J. & Rozengurt, E. Expression and characterization of PKD, a phorbol ester and diacylglycerol-stimulated serine protein kinase. *J. Biol. Chem.* **270**, 1455-1461 (1995).

124. Zugaza, J.L., Waldron, R.T., Sinnett-Smith, J. & Rozengurt, E. Bombesin, vasopressin, endothelin, bradykinin, and platelet-derived growth factor rapidly activate protein kinase D through a protein kinase C-dependent signal transduction pathway. *J. Biol. Chem.* **272**, 23952-23960 (1997).

125. Rey, O., Young, S.H., Cantrell, D. & Rozengurt, E. Rapid protein kinase D translocation in response to G protein-coupled receptor activation. Dependence on

protein kinase C. *J. Biol. Chem.* **276**, 32616-32626 (2001).

126. Matthews, S.A., Pettit, G.R. & Rozengurt, E. Bryostatin 1 induces biphasic activation of protein kinase D in intact cells. *J. Biol. Chem.* **272**, 20245-20250 (1997).

127. Storz, P., Doppler, H. & Toker, A. Protein kinase D mediates mitochondrion-to-nucleus signaling and detoxification from mitochondrial reactive oxygen species. *Mol. Cell. Biol.* **25**, 8520-8530 (2005).

128. Storz, P. & Toker, A. Protein kinase D mediates a stress-induced NF-kappaB activation and survival pathway. *EMBO J.* **22**, 109-120 (2003).

129. Storz, P., Doppler, H. & Toker, A. Activation loop phosphorylation controls protein kinase D-dependent activation of nuclear factor kappaB. *Mol. Pharmacol.* **66**, 870-879 (2004).

130. Song, J., Li, J., Lulla, A., Evers, B.M. & Chung, D.H. Protein Kinase D Protects Against Oxidative Stress-Induced Intestinal Epithelial Cell Injury via Rho/ROK/PKC-{delta} Pathway Activation. *Am. J. Physiol. Cell. Physiol.* (2006).

131. Matthews, S.A., Iglesias, T., Rozengurt, E. & Cantrell, D. Spatial and temporal regulation of protein kinase D (PKD). *EMBO J.* **19**, 2935-2945 (2000).

132. Sidorenko, S.P., *et al.* Protein kinase C mu (PKC mu) associates with the B cell antigen receptor complex and regulates lymphocyte signaling. *Immunity* **5**, 353-363 (1996).

133. Dequiedt, F., *et al.* Phosphorylation of histone deacetylase 7 by protein kinase D

mediates T cell receptor-induced Nur77 expression and apoptosis. *J. Exp. Med.* **201**, 793-804 (2005).

134. Parra, M., Kasler, H., McKinsey, T.A., Olson, E.N. & Verdin, E. Protein kinase D1 phosphorylates HDAC7 and induces its nuclear export after T-cell receptor activation. *J. Biol. Chem.* **280**, 13762-13770 (2005).

135. Matthews, S.A., Rozengurt, E. & Cantrell, D. Protein kinase D. A selective target for antigen receptors and a downstream target for protein kinase C in lymphocytes. *J. Exp. Med.* **191**, 2075-2082 (2000).

136. Jamora, C., *et al.* Gbetagamma-mediated regulation of Golgi organization is through the direct activation of protein kinase D. *Cell* **98**, 59-68 (1999).

137. Vantus, T., *et al.* Doxorubicin-induced activation of protein kinase D1 through caspase-mediated proteolytic cleavage: identification of two cleavage sites by microsequencing. *Cell Signal.* **16**, 703-709 (2004).

138. Zhang, W., Zheng, S., Storz, P. & Min, W. Protein kinase D specifically mediates apoptosis signal-regulating kinase 1-JNK signaling induced by H2O2 but not tumor necrosis factor. *J. Biol. Chem.* **280**, 19036-19044 (2005).

139. Matthews, S., Iglesias, T., Cantrell, D. & Rozengurt, E. Dynamic re-distribution of protein kinase D (PKD) as revealed by a GFP-PKD fusion protein: dissociation from PKD activation. *FEBS Lett.* **457**, 515-521 (1999).

140. Rey, O., Sinnett-Smith, J., Zhukova, E. & Rozengurt, E. Regulated nucleocytoplasmic transport of protein kinase D in response to G protein-coupled

receptor activation. *J. Biol. Chem.* **276**, 49228-49235 (2001).

141. Hausser, A., *et al.* Protein kinase C mu selectively activates the mitogen-activated protein kinase (MAPK) p42 pathway. *FEBS Lett.* **492**, 39-44 (2001).

142. Wang, Y., *et al.* The RAS effector RIN1 directly competes with RAF and is regulated by 14-3-3 proteins. *Mol. Cell. Biol.* **22**, 916-926 (2002).

143. Brandlin, I., *et al.* Protein kinase C (PKC)eta-mediated PKC mu activation modulates ERK and JNK signal pathways. *J. Biol. Chem.* **277**, 6490-6496 (2002).

144. Diaz Anel, A.M. & Malhotra, V. PKCeta is required for beta1gamma2/beta3gamma2- and PKD-mediated transport to the cell surface and the organization of the Golgi apparatus. *J. Cell Biol.* **169**, 83-91 (2005).

145. Ghanekar, Y. & Lowe, M. Protein kinase D: activation for Golgi carrier formation. *Trends Cell Biol.* **15**, 511-514 (2005).

146. Hausser, A., *et al.* Protein kinase D regulates vesicular transport by phosphorylating and activating phosphatidylinositol-4 kinase IIIbeta at the Golgi complex. *Nat. Cell Biol.* **7**, 880-886 (2005).

147. Liljedahl, M., *et al.* Protein kinase D regulates the fission of cell surface destined transport carriers from the trans-Golgi network. *Cell* **104**, 409-420 (2001).

148. Bowden, E.T., Barth, M., Thomas, D., Glazer, R.I. & Mueller, S.C. An invasion-related complex of cortactin, paxillin and PKCmu associates with invadopodia at sites of extracellular matrix degradation. *Oncogene* **18**, 4440-4449 (1999).

149. Marklund, U., Lightfoot, K. & Cantrell, D. Intracellular location and cell context-

dependent function of protein kinase D. *Immunity* **19**, 491-501 (2003).

150. Celil, A.B. & Campbell, P.G. BMP-2 and insulin-like growth factor-I mediate Osterix (Osx) expression in human mesenchymal stem cells via the MAPK and protein kinase D signaling pathways. *J. Biol. Chem.* **280**, 31353-31359 (2005).

151. Ernest Dodd, M., Ristich, V.L., Ray, S., Lober, R.M. & Bollag, W.B. Regulation of protein kinase D during differentiation and proliferation of primary mouse keratinocytes. *J. Invest. Dermatol.* **125**, 294-306 (2005).

152. Ristich, V.L., Bowman, P.H., Dodd, M.E. & Bollag, W.B. Protein kinase D distribution in normal human epidermis, basal cell carcinoma and psoriasis. *Br. J. Dermatol.* **154**, 586-593 (2006).

153. Prestle, J., Pfizenmaier, K., Brenner, J. & Johannes, F.J. Protein kinase C mu is located at the Golgi compartment. *J. Cell Biol.* **134**, 1401-1410 (1996).

154. Vega, R.B., *et al.* Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. *Mol. Cell. Biol.* **24**, 8374-8385 (2004).

155. Matthews, S.A., *et al.* Essential role for protein kinase D family kinases in the regulation of class II histone deacetylases in B lymphocytes. *Mol. Cell. Biol.* **26**, 1569-1577 (2006).

156. Jenuwein, T. & Allis, C.D. Translating the histone code. *Science* **293**, 1074-1080 (2001).

157. Cheung, P., Allis, C.D. & Sassone-Corsi, P. Signaling to chromatin through

histone modifications. *Cell* **103**, 263-271 (2000).

158. Strahl, B.D. & Allis, C.D. The language of covalent histone modifications. *Nature* **403**, 41-45 (2000).

159. Verdin, E., Dequiedt, F. & Kasler, H.G. Class II histone deacetylases: versatile regulators. *Trends Genet.* **19**, 286-293 (2003).

160. Rey, O., Zhukova, E., Sinnett-Smith, J. & Rozengurt, E. Vasopressin-induced intracellular redistribution of protein kinase D in intestinal epithelial cells. *J. Cell. Physiol.* **196**, 483-492 (2003).

161. Woods, A.J., White, D.P., Caswell, P.T. & Norman, J.C. PKD1/PKCmu promotes alphavbeta3 integrin recycling and delivery to nascent focal adhesions. *EMBO J.* **23**, 2531-2543 (2004).

162. Iwata, M., *et al.* PKCepsilon-PKD1 signaling complex at Z-discs plays a pivotal role in the cardiac hypertrophy induced by G-protein coupling receptor agonists. *Biochem. Biophys. Res. Commun.* **327**, 1105-1113 (2005).

163. Lemonnier, J., Ghayor, C., Guicheux, J. & Caverzasio, J. Protein kinase C-independent activation of protein kinase D is involved in BMP-2-induced activation of stress mitogen-activated protein kinases JNK and p38 and osteoblastic cell differentiation. *J. Biol. Chem.* **279**, 259-264 (2004).

164. Wood, C.D., Marklund, U. & Cantrell, D.A. Dual phospholipase C/diacylglycerol requirement for protein kinase D1 activation in lymphocytes. *J. Biol. Chem.* **280**, 6245-6251 (2005).

165. Stafford, M.J., Watson, S.P. & Pears, C.J. PKD: a new protein kinase C-dependent pathway in platelets. *Blood* **101**, 1392-1399 (2003).

166. Jaggi, M., *et al.* E-cadherin phosphorylation by protein kinase D1/protein kinase C{mu} is associated with altered cellular aggregation and motility in prostate cancer. *Cancer Res.* **65**, 483-492 (2005).

167. Cabrera-Poch, N., Sanchez-Ruiloba, L., Rodriguez-Martinez, M. & Iglesias, T. Lipid raft disruption triggers protein kinase C and Src-dependent protein kinase D activation and Kidins220 phosphorylation in neuronal cells. *J. Biol. Chem.* **279**, 28592-28602 (2004).

168. Wang, Y., *et al.* Interaction between protein kinase Cmu and the vanilloid receptor type 1. *J. Biol. Chem.* **279**, 53674-53682 (2004).

169. Iglesias, T., *et al.* Identification and cloning of Kidins220, a novel neuronal substrate of protein kinase D. *J. Biol. Chem.* **275**, 40048-40056 (2000).

170. Chawla, S., Vanhoutte, P., Arnold, F.J., Huang, C.L. & Bading, H. Neuronal activity-dependent nucleocytoplasmic shuttling of HDAC4 and HDAC5. *J. Neurochem.* **85**, 151-159 (2003).

171. Guan, Z., *et al.* Integration of long-term-memory-related synaptic plasticity involves bidirectional regulation of gene expression and chromatin structure. *Cell* **111**, 483-493 (2002).

172. Lin, J.W., *et al.* Yotiao, a novel protein of neuromuscular junction and brain that interacts with specific splice variants of NMDA receptor subunit NR1. *J.*

Neurosci. **18**, 2017-2027 (1998).

173. Dell'acqua, M.L., *et al.* Regulation of neuronal PKA signaling through AKAP targeting dynamics. *Eur. J. Cell Biol.* (2006).

174. Carnegie, G.K., Smith, F.D., McConnachie, G., Langeberg, L.K. & Scott, J.D. AKAP-Lbc Nucleates a Protein Kinase D Activation Scaffold. *Mol. Cell* **15**, 889-899 (2004).

175. Sieburth, D., *et al.* Systematic analysis of genes required for synapse structure and function. *Nature* **436**, 510-517 (2005).

176. Doppler, H., Storz, P., Li, J., Comb, M.J. & Toker, A. A phosphorylation state-specific antibody recognizes Hsp27, a novel substrate of protein kinase D. *J. Biol. Chem.* **280**, 15013-15019 (2005).

177. Pei, Q., Tordera, R., Sprakes, M. & Sharp, T. Glutamate receptor activation is involved in 5-HT2 agonist-induced Arc gene expression in the rat cortex. *Neuropharmacology* **46**, 331-339 (2004).

178. Kremerskothen, J., Wendholt, D., Teber, I. & Barnekow, A. Insulin-induced expression of the activity-regulated cytoskeleton-associated gene (ARC) in human neuroblastoma cells requires p21(ras), mitogen-activated protein kinase/extracellular regulated kinase and src tyrosine kinases but is protein kinase C-independent. *Neurosci. Lett.* **321**, 153-156 (2002).

179. Dong, E., *et al.* A reelin-integrin receptor interaction regulates Arc mRNA translation in synaptoneurosomes. *Proc. Natl. Acad. Sci. USA* **100**, 5479-5484

(2003).

180. Ichikawa, H., Fujimoto, T., Taira, E. & Miki, N. The Accumulation of Arc (an Immediate Early Gene) mRNA by the Inhibition of Protein Synthesis. *J. Pharmacol. Sci.* **91**, 247-254 (2003).

181. Brackmann, M., Zhao, C., Kuhl, D., Manahan-Vaughan, D. & Braunewell, K.H. MGluRs regulate the expression of neuronal calcium sensor proteins NCS-1 and VILIP-1 and the immediate early gene arg3.1/arc in the hippocampus in vivo. *Biochem. Biophys. Res. Commun.* **322**, 1073-1079 (2004).

182. Teber, I., Kohling, R., Speckmann, E.J., Barnekow, A. & Kremerskothen, J. Muscarinic acetylcholine receptor stimulation induces expression of the activity-regulated cytoskeleton-associated gene (ARC). *Brain Res. Mol. Brain Res.* **121**, 131-136 (2004).

183. Messaoudi, E., Ying, S.W., Kanhema, T., Croll, S.D. & Bramham, C.R. Brain-derived neurotrophic factor triggers transcription-dependent, late phase long-term potentiation in vivo. *J. Neurosci.* **22**, 7453-7461 (2002).

184. Ying, S.W., *et al.* Brain-derived neurotrophic factor induces long-term potentiation in intact adult hippocampus: requirement for ERK activation coupled to CREB and upregulation of Arc synthesis. *J. Neurosci.* **22**, 1532-1540 (2002).

185. Krug, M., Lossner, B. & Ott, T. Anisomycin blocks the late phase of long-term potentiation in the dentate gyrus of freely moving rats. *Brain Res. Bull.* **13**, 39-42 (1984).

186. Rutherford, L.C., DeWan, A., Lauer, H.M. & Turrigiano, G.G. Brain-derived neurotrophic factor mediates the activity-dependent regulation of inhibition in neocortical cultures. *J. Neurosci.* **17**, 4527-4535 (1997).

187. Ramakers, G.J., Corner, M.A. & Habets, A.M. Development in the absence of spontaneous bioelectric activity results in increased stereotyped burst firing in cultures of dissociated cerebral cortex. *Exp. Brain Res.* **79**, 157-166 (1990).

188. Corner, M.A. & Ramakers, G.J. Spontaneous firing as an epigenetic factor in brain development--physiological consequences of chronic tetrodotoxin and picrotoxin exposure on cultured rat neocortex neurons. *Brain Res. Dev. Brain Res.* **65**, 57-64 (1992).

189. Hardingham, G.E., Arnold, F.J. & Bading, H. Nuclear calcium signaling controls CREB-mediated gene expression triggered by synaptic activity. *Nat. Neurosci.* **4**, 261-267 (2001).

190. Robinson, H.P., *et al.* Periodic synchronized bursting and intracellular calcium transients elicited by low magnesium in cultured cortical neurons. *J. Neurophysiol.* **70**, 1606-1616 (1993).

191. Brewer, G.J., Torricelli, J.R., Evege, E.K. & Price, P.J. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J. Neurosci. Res.* **35**, 567-576 (1993).

192. Poo, M.M. Neurotrophins as synaptic modulators. *Nat. Rev. Neurosci.* **2**, 24-32 (2001).

193. Li, Y.X., Zhang, Y., Lester, H.A., Schuman, E.M. & Davidson, N. Enhancement of neurotransmitter release induced by brain-derived neurotrophic factor in cultured hippocampal neurons. *J. Neurosci.* **18**, 10231-10240 (1998).

194. Hartmann, M., Heumann, R. & Lessmann, V. Synaptic secretion of BDNF after high-frequency stimulation of glutamatergic synapses. *EMBO J.* **20**, 5887-5897 (2001).

195. Shelton, D.L., *et al.* Human trks: molecular cloning, tissue distribution, and expression of extracellular domain immunoadhesins. *J. Neurosci.* **15**, 477-491 (1995).

196. Rutherford, L.C., Nelson, S.B. & Turrigiano, G.G. BDNF has opposite effects on the quantal amplitude of pyramidal neuron and interneuron excitatory synapses. *Neuron* **21**, 521-530 (1998).

197. Yu, W. & Miller, R.F. NBQX, an improved non-NMDA antagonist studied in retinal ganglion cells. *Brain Res.* **692**, 190-194 (1995).

198. Donevan, S.D. & Rogawski, M.A. GYKI 52466, a 2,3-benzodiazepine, is a highly selective, noncompetitive antagonist of AMPA/kainate receptor responses. *Neuron* **10**, 51-59 (1993).

199. Lomeli, H., *et al.* Control of kinetic properties of AMPA receptor channels by nuclear RNA editing. *Science* **266**, 1709-1713 (1994).

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

201. Stoppini, L., Buchs, P.A. & Muller, D. A simple method for organotypic cultures of nervous tissue. *J. Neurosci. Methods* **37**, 173-182 (1991).

202. West, A.E., *et al.* Calcium regulation of neuronal gene expression. *Proc. Natl. Acad. Sci. USA* **98**, 11024-11031 (2001).

203. Murphy, T.H., Worley, P.F. & Baraban, J.M. L-type voltage-sensitive calcium channels mediate synaptic activation of immediate early genes. *Neuron* **7**, 625-635 (1991).

204. Dolmetsch, R.E., Pajvani, U., Fife, K., Spotts, J.M. & Greenberg, M.E. Signaling to the nucleus by an L-type calcium channel-calmodulin complex through the MAP kinase pathway. *Science* **294**, 333-339 (2001).

205. Weick, J.P., Groth, R.D., Isaksen, A.L. & Mermelstein, P.G. Interactions with PDZ proteins are required for L-type calcium channels to activate cAMP response element-binding protein-dependent gene expression. *J. Neurosci.* **23**, 3446-3456 (2003).

206. Thiagarajan, T.C., Lindskog, M. & Tsien, R.W. Adaptation to synaptic inactivity in hippocampal neurons. *Neuron* **47**, 725-737 (2005).

207. Helton, T.D., Xu, W. & Lipscombe, D. Neuronal L-type calcium channels open quickly and are inhibited slowly. *J. Neurosci.* **25**, 10247-10251 (2005).

208. Perkinton, M.S., Sihra, T.S. & Williams, R.J. Ca(2+)-permeable AMPA receptors induce phosphorylation of cAMP response element-binding protein through a phosphatidylinositol 3-kinase-dependent stimulation of the mitogen-activated

protein kinase signaling cascade in neurons. *J. Neurosci.* **19**, 5861-5874 (1999).

209. Turetsky, D.M., *et al.* Cortical neurones exhibiting kainate-activated Co2+ uptake are selectively vulnerable to AMPA/kainate receptor-mediated toxicity. *Neurobiol. Dis.* **1**, 101-110 (1994).

210. Jensen, J.B., Schousboe, A. & Pickering, D.S. Development of calcium-permeable alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors in cultured neocortical neurons visualized by cobalt staining. *J. Neurosci. Res.* **54**, 273-281 (1998).

211. Liu, S.Q. & Cull-Candy, S.G. Synaptic activity at calcium-permeable AMPA receptors induces a switch in receptor subtype. *Nature* **405**, 454-458 (2000).

212. Blaschke, M., *et al.* A single amino acid determines the subunit-specific spider toxin block of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate/kainate receptor channels. *Proc. Natl. Acad. Sci. USA* **90**, 6528-6532 (1993).

213. Wheeler, D.G. & Cooper, E. Weak synaptic activity induces ongoing signaling to the nucleus that is enhanced by BDNF and suppressed by low-levels of nicotine. *Mol. Cell. Neurosci.* **26**, 50-62 (2004).

214. Zhu, J.J., Esteban, J.A., Hayashi, Y. & Malinow, R. Postnatal synaptic potentiation: delivery of GluR4-containing AMPA receptors by spontaneous activity. *Nat. Neurosci.* **3**, 1098-1106 (2000).

215. Bradley, J., Carter, S.R., Rao, V.R., Wang, J. & Finkbeiner, S. Splice variants of the NR1 subunit differentially induce NMDA receptor-dependent gene

expression. *J. Neurosci.* **26**, 1065-1076 (2006).

216. Hardingham, G.E., Fukunaga, Y. & Bading, H. Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat. Neurosci.* **5**, 405-414 (2002).

217. Bredt, D.S. & Nicoll, R.A. AMPA receptor trafficking at excitatory synapses. *Neuron* **40**, 361-379 (2003).

218. Schenk, U., *et al.* A novel pathway for presynaptic mitogen-activated kinase activation via AMPA receptors. *J. Neurosci.* **25**, 1654-1663 (2005).

219. Hayashi, T., Umemori, H., Mishina, M. & Yamamoto, T. The AMPA receptor interacts with and signals through the protein tyrosine kinase Lyn. *Nature* **397**, 72-76 (1999).

220. Passafaro, M., Nakagawa, T., Sala, C. & Sheng, M. Induction of dendritic spines by an extracellular domain of AMPA receptor subunit GluR2. *Nature* **424**, 677-681 (2003).

221. Huettner, J.E. Spine-tingling excitement from glutamate receptors. *Sci. STKE* **2003**, pe53 (2003).

222. Lopez-Bayghen, E., Aguirre, A. & Ortega, A. Transcriptional regulation through glutamate receptors: Involvement of tyrosine kinases. *J. Neurosci. Res.* **74**, 717-725 (2003).

223. Wang, Y., Small, D.L., Stanimirovic, D.B., Morley, P. & Durkin, J.P. AMPA receptor-mediated regulation of a Gi-protein in cortical neurons. *Nature* **389**, 502-

504 (1997).

224. Kawai, F. & Sterling, P. AMPA receptor activates a G-protein that suppresses a cGMP-gated current. *J. Neurosci.* **19**, 2954-2959 (1999).

225. Huang, E.J. & Reichardt, L.F. TRK Receptors: Roles in neuronal signal transduction. *Annu. Rev. Biochem.* (2003).

226. Aoki, C., *et al.* Localization of brain-derived neurotrophic factor and TrkB receptors to postsynaptic densities of adult rat cerebral cortex. *J. Neurosci. Res.* **59**, 454-463 (2000).

227. Du, J., Feng, L., Yang, F. & Lu, B. Activity- and Ca(2+)-dependent modulation of surface expression of brain-derived neurotrophic factor receptors in hippocampal neurons. *J. Cell. Biol.* **150**, 1423-1434 (2000).

228. Du, J., *et al.* Regulation of TrkB receptor tyrosine kinase and its internalization by neuronal activity and Ca2+ influx. *J. Cell. Biol.* **163**, 385-395 (2003).

229. Meyer-Franke, A., *et al.* Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons. *Neuron* **21**, 681-693 (1998).

230. Lee, F.S. & Chao, M.V. Activation of Trk neurotrophin receptors in the absence of neurotrophins. *Proc. Natl. Acad. Sci. USA* **98**, 3555-3560 (2001).

231. Wasylyk, B., Hagman, J. & Gutierrez-Hartmann, A. Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. *Trends Biochem. Sci.* **23**, 213-216 (1998).

232. Ramanan, N., *et al.* SRF mediates activity-induced gene expression and synaptic

plasticity but not neuronal viability. *Nat. Neurosci.* **8**, 759-767 (2005).

233. Waltereit, R., *et al.* Arg3.1/Arc mRNA induction by Ca2+ and cAMP requires protein kinase A and mitogen-activated protein kinase/extracellular regulated kinase activation. *J. Neurosci.* **21**, 5484-5493 (2001).

234. Kelleher, R.J., 3rd, Govindarajan, A. & Tonegawa, S. Translational Regulatory Mechanisms in Persistent Forms of Synaptic Plasticity. *Neuron* **44**, 59-73 (2004).

235. Kelleher, R.J., 3rd, Govindarajan, A., Jung, H.Y., Kang, H. & Tonegawa, S. Translational control by MAPK signaling in long-term synaptic plasticity and memory. *Cell* **116**, 467-479 (2004).

236. Si, K., *et al.* A neuronal isoform of CPEB regulates local protein synthesis and stabilizes synapse-specific long-term facilitation in aplysia. *Cell* **115**, 893-904 (2003).

237. Schratt, G.M., Nigh, E.A., Chen, W.G., Hu, L. & Greenberg, M.E. BDNF regulates the translation of a select group of mRNAs by a mammalian target of rapamycin-phosphatidylinositol 3-kinase-dependent pathway during neuronal development. *J. Neurosci.* **24**, 9366-9377 (2004).

238. Wilkie, G.S., Dickson, K.S. & Gray, N.K. Regulation of mRNA translation by 5'- and 3'-UTR-binding factors. *Trends Biochem. Sci.* **28**, 182-188 (2003).

239. Atkins, C.M., Davare, M.A., Oh, M.C., Derkach, V. & Soderling, T.R. Bidirectional regulation of cytoplasmic polyadenylation element-binding protein phosphorylation by Ca2+/calmodulin-dependent protein kinase II and protein

phosphatase 1 during hippocampal long-term potentiation. *J. Neurosci.* **25**, 5604-5610 (2005).

240. Aakalu, G., Smith, W.B., Nguyen, N., Jiang, C. & Schuman, E.M. Dynamic visualization of local protein synthesis in hippocampal neurons. *Neuron* **30**, 489-502 (2001).

241. Wells, D.G., *et al.* A Role for the Cytoplasmic Polyadenylation Element in NMDA Receptor-Regulated mRNA Translation in Neurons. *J. Neurosci.* **21**, 9541-9548 (2001).

242. Atkins, C.M., Nozaki, N., Shigeri, Y. & Soderling, T.R. Cytoplasmic polyadenylation element binding protein-dependent protein synthesis is regulated by calcium/calmodulin-dependent protein kinase II. *J. Neurosci.* **24**, 5193-5201 (2004).

243. Takei, N., Kawamura, M., Hara, K., Yonezawa, K. & Nawa, H. Brain-derived neurotrophic factor enhances neuronal translation by activating multiple initiation processes. Comparison with the effects of insulin. *J. Biol. Chem.* **276**, 42818-42825 (2001).

244. Takei, N., *et al.* Brain-derived neurotrophic factor induces mammalian target of rapamycin-dependent local activation of translation machinery and protein synthesis in neuronal dendrites. *J. Neurosci.* **24**, 9760-9769 (2004).

245. Sutton, M.A., Wall, N.R., Aakalu, G.N. & Schuman, E.M. Regulation of dendritic protein synthesis by miniature synaptic events. *Science* **304**, 1979-1983 (2004).

246. Sherman, M.Y. & Goldberg, A.L. Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. *Neuron* **29**, 15-32 (2001).

247. Ehlers, M.D. Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system. *Nat. Neurosci.* **6**, 231-242 (2003).

248. Patrick, G.N., Bingol, B., Weld, H.A. & Schuman, E.M. Ubiquitin-mediated proteasome activity is required for agonist-induced endocytosis of GluRs. *Curr. Biol.* **13**, 2073-2081 (2003).

249. Bingol, B. & Schuman, E.M. A proteasome-sensitive connection between PSD-95 and GluR1 endocytosis. *Neuropharmacology* **47**, 755-763 (2004).

250. Bence, N.F., Sampat, R.M. & Kopito, R.R. Impairment of the ubiquitin-proteasome system by protein aggregation. *Science* **292**, 1552-1555 (2001).

251. Shyu, A.B., Greenberg, M.E. & Belasco, J.G. The c-fos transcript is targeted for rapid decay by two distinct mRNA degradation pathways. *Genes Dev.* **3**, 60-72 (1989).

252. Fukuchi, M., Tabuchi, A. & Tsuda, M. Activity-dependent transcriptional activation and mrna stabilization for cumulative expression of pituitary adenylate cyclase-activating polypeptide mRNA controlled by calcium and cAMP signals in neurons. *J. Biol. Chem.* **279**, 47856-47865 (2004).

253. Wolke, U., Weidinger, G., Koprunner, M. & Raz, E. Multiple levels of posttranscriptional control lead to germ line-specific gene expression in the zebrafish. *Curr. Biol.* **12**, 289-294 (2002).

254. Finkbeiner, S. New roles for introns: sites of combinatorial regulation of Ca2+- and cyclic AMP-dependent gene transcription. *Sci. STKE* **2001**, PE1 (2001).

255. Herman, J.P., Schafer, M.K., Watson, S.J. & Sherman, T.G. In situ hybridization analysis of arginine vasopressin gene transcription using intron-specific probes. *Mol. Endocrinol.* **5**, 1447-1456 (1991).

256. Wierenga, C.J., Ibata, K. & Turrigiano, G.G. Postsynaptic expression of homeostatic plasticity at neocortical synapses. *J. Neurosci.* **25**, 2895-2905 (2005).

257. O'Brien, R.J., *et al.* Activity-dependent modulation of synaptic AMPA receptor accumulation. *Neuron* **21**, 1067-1078 (1998).

258. Ju, W., *et al.* Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors. *Nat. Neurosci.* **7**, 244-253 (2004).

259. Turrigiano, G.G., Leslie, K.R., Desai, N.S., Rutherford, L.C. & Nelson, S.B. Activity-dependent scaling of quantal amplitude in neocortical neurons. *Nature* **391**, 892-896 (1998).

260. Watt, A.J., van Rossum, M.C., MacLeod, K.M., Nelson, S.B. & Turrigiano, G.G. Activity coregulates quantal AMPA and NMDA currents at neocortical synapses. *Neuron* **26**, 659-670 (2000).

261. Watt, A.J., Sjostrom, P.J., Hausser, M., Nelson, S.B. & Turrigiano, G.G. A proportional but slower NMDA potentiation follows AMPA potentiation in LTP. *Nat. Neurosci.* **7**, 518-524 (2004).

262. Turrigiano, G.G. & Nelson, S.B. Homeostatic plasticity in the developing nervous

system. *Nat. Rev. Neurosci.* **5**, 97-107 (2004).

263. Lester, R.A., Quarum, M.L., Parker, J.D., Weber, E. & Jahr, C.E. Interaction of 6-cyano-7-nitroquinoxaline-2,3-dione with the N-methyl-D-aspartate receptor-associated glycine binding site. *Mol. Pharmacol.* **35**, 565-570 (1989).

264. Wang, Y. & Durkin, J.P. alpha-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, but not N-methyl-D-aspartate, activates mitogen-activated protein kinase through G-protein beta gamma subunits in rat cortical neurons. *J. Biol. Chem.* **270**, 22783-22787 (1995).

265. Paul, S., Nairn, A.C., Wang, P. & Lombroso, P.J. NMDA-mediated activation of the tyrosine phosphatase STEP regulates the duration of ERK signaling. *Nat. Neurosci.* **6**, 34-42 (2003).

266. Bennett, M.K., Erondu, N.E. & Kennedy, M.B. Purification and characterization of a calmodulin-dependent protein kinase that is highly concentrated in brain. *J. Biol. Chem.* **258**, 12735-12744 (1983).

267. Miller, S.G. & Kennedy, M.B. Distinct forebrain and cerebellar isozymes of type II Ca2+/calmodulin-dependent protein kinase associate differently with the postsynaptic density fraction. *J. Biol. Chem.* **260**, 9039-9046 (1985).

268. Thiagarajan, T.C., Piedras-Renteria, E.S. & Tsien, R.W. alpha- and betaCaMKII. Inverse regulation by neuronal activity and opposing effects on synaptic strength. *Neuron* **36**, 1103-1114 (2002).

269. Scheetz, A.J., Nairn, A.C. & Constantine-Paton, M. NMDA receptor-mediated

control of protein synthesis at developing synapses. *Nat. Neurosci.* **3**, 211-216 (2000).

270. Huang, Y.S., Jung, M.Y., Sarkissian, M. & Richter, J.D. N-methyl-D-aspartate receptor signaling results in Aurora kinase-catalyzed CPEB phosphorylation and alphaCaMKII mRNA polyadenylation at synapses. *EMBO J.* **21**, 2139-2148 (2002).

271. Brocke, L., Chiang, L.W., Wagner, P.D. & Schulman, H. Functional implications of the subunit composition of neuronal CaM kinase II. *J. Biol. Chem.* **274**, 22713-22722 (1999).

272. Donai, H., *et al.* Interaction of Arc with CaM kinase II and stimulation of neurite extension by Arc in neuroblastoma cells expressing CaM kinase II. *Neurosci. Res.* **47**, 399-408 (2003).

273. Vazdarjanova, A., *et al.* Behavior induces expression of the plasticity-related immediate-early gene Arc in excitatory and inhibitory CaMKII-positive neurons. *Soc. Neurosci. Abstr.* **30**, 329.314 (2004).

274. Rechsteiner, M. & Rogers, S.W. PEST sequences and regulation by proteolysis. *Trends Biochem. Sci.* **21**, 267-271 (1996).

275. Campbell, R.E., *et al.* A monomeric red fluorescent protein. *Proc. Natl. Acad. Sci. USA* **99**, 7877-7882 (2002).

276. Xia, Z., Dudek, H., Miranti, C.K. & Greenberg, M.E. Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP

kinase/ERK-dependent mechanism. *J. Neurosci.* **16**, 5425-5436 (1996).

277. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254 (1976).

278. Power, J.M. & Sah, P. Nuclear calcium signaling evoked by cholinergic stimulation in hippocampal CA1 pyramidal neurons. *J. Neurosci.* **22**, 3454-3462 (2002).

279. Berridge, M.J. Neuronal calcium signaling. *Neuron* **21**, 13-26 (1998).

280. Thompson, K.R., *et al.* Synapse to nucleus signaling during long-term synaptic plasticity; a role for the classical active nuclear import pathway. *Neuron* **44**, 997-1009 (2004).

281. Meffert, M.K., Chang, J.M., Wiltgen, B.J., Fanselow, M.S. & Baltimore, D. NF-kappa B functions in synaptic signaling and behavior. *Nat. Neurosci.* **6**, 1072-1078 (2003).

282. Martin, K.C., *et al.* MAP kinase translocates into the nucleus of the presynaptic cell and is required for long-term facilitation in Aplysia. *Neuron* **18**, 899-912 (1997).

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

284. Lint, J.V., Rykx, A., Vantus, T. & Vandenheede, J.R. Getting to know protein

kinase D. *Int. J. Biochem. Cell Biol.* **34**, 577-581 (2002).

285. Zugaza, J.L., Sinnett-Smith, J., Van Lint, J. & Rozengurt, E. Protein kinase D (PKD) activation in intact cells through a protein kinase C-dependent signal transduction pathway. *EMBO J.* **15**, 6220-6230 (1996).

286. Rozengurt, E., Sinnett-Smith, J., Van Lint, J. & Valverde, A.M. Protein kinase D (PKD): a novel target for diacylglycerol and phorbol esters. *Mutat. Res.* **333**, 153-160 (1995).

287. Rey, O. & Rozengurt, E. Protein kinase D interacts with Golgi via its cysteine-rich domain. *Biochem. Biophys. Res. Commun.* **287**, 21-26 (2001).

288. Storz, P., *et al.* Protein kinase C μ is regulated by the multifunctional chaperon protein p32. *J. Biol. Chem.* **275**, 24601-24607 (2000).

289. Ulfhake, B. & Kellerth, J.O. A quantitative light microscopic study of the dendrites of cat spinal alpha-motoneurons after intracellular staining with horseradish peroxidase. *J. Comp. Neurol.* **202**, 571-583 (1981).

290. Nagai, T., *et al.* A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat. Biotechnol.* **20**, 87-90 (2002).

291. Shaner, N.C., *et al.* Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. *Nat. Biotechnol.* **22**, 1567-1572 (2004).

292. Wong, C. & Jin, Z.G. Protein kinase C-dependent protein kinase D activation modulates ERK signal pathway and endothelial cell proliferation by vascular

endothelial growth factor. *J. Biol. Chem.* **280**, 33262-33269 (2005).

293. Li, J., *et al.* The role of protein kinase D in neurotensin secretion mediated by protein kinase C-alpha/-delta and Rho/Rho kinase. *J. Biol. Chem.* **279**, 28466-28474 (2004).

294. Nakazawa, K., McHugh, T.J., Wilson, M.A. & Tonegawa, S. NMDA receptors, place cells and hippocampal spatial memory. *Nat. Rev. Neurosci.* **5**, 361-372 (2004).

295. Shen, K. & Meyer, T. Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation. *Science* **284**, 162-166 (1999).

296. Lipscombe, D., Helton, T.D. & Xu, W. L-type calcium channels: the low down. *J. Neurophysiol.* **92**, 2633-2641 (2004).

297. Bading, H., Ginty, D.D. & Greenberg, M.E. Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. *Science* **260**, 181-186 (1993).

298. McKinsey, T.A., Zhang, C.L., Lu, J. & Olson, E.N. Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. *Nature* **408**, 106-111 (2000).

299. McKinsey, T.A., Zhang, C.L. & Olson, E.N. MEF2: a calcium-dependent regulator of cell division, differentiation and death. *Trends Biochem. Sci.* **27**, 40-47 (2002).

300. Linseman, D.A., *et al.* Inactivation of the myocyte enhancer factor-2 repressor histone deacetylase-5 by endogenous Ca(2+) //calmodulin-dependent kinase II promotes depolarization-mediated cerebellar granule neuron survival. *J. Biol. Chem.* **278**, 41472-41481 (2003).

301. Ptacek, J., *et al.* Global analysis of protein phosphorylation in yeast. *Nature* **438**, 679-684 (2005).

302. Merkel, J.S., Michaud, G.A., Salcius, M., Schweitzer, B. & Predki, P.F. Functional protein microarrays: just how functional are they? *Curr. Opin. Biotechnol.* **16**, 447-452 (2005).

303. Serra-Pages, C., Medley, Q.G., Tang, M., Hart, A. & Streuli, M. Liprins, a family of LAR transmembrane protein-tyrosine phosphatase-interacting proteins. *J. Biol. Chem.* **273**, 15611-15620 (1998).

304. Kaufmann, N., DeProto, J., Ranjan, R., Wan, H. & Van Vactor, D. Drosophila liprin-alpha and the receptor phosphatase Dlar control synapse morphogenesis. *Neuron* **34**, 27-38 (2002).

305. Zhen, M. & Jin, Y. The liprin protein SYD-2 regulates the differentiation of presynaptic termini in C. elegans. *Nature* **401**, 371-375 (1999).

306. Olsen, O., Moore, K.A., Nicoll, R.A. & Bredt, D.S. Synaptic transmission regulated by a presynaptic MALS/Liprin-alpha protein complex. *Curr. Opin. Cell Biol.* **18**, 223-227 (2006).

307. Wyszynski, M., *et al.* Interaction between GRIP and liprin-alpha/SYD2 is

required for AMPA receptor targeting. *Neuron* **34**, 39-52 (2002).

308. Steindler, C., *et al.* Jamip1 (marlin-1) defines a family of proteins interacting with janus kinases and microtubules. *J. Biol. Chem.* **279**, 43168-43177 (2004).

309. Couve, A., *et al.* Marlin-1, a novel RNA-binding protein associates with GABA receptors. *J. Biol. Chem.* **279**, 13934-13943 (2004).

310. Govek, E.E., Newey, S.E. & Van Aelst, L. The role of the Rho GTPases in neuronal development. *Genes Dev.* **19**, 1-49 (2005).

311. Seoh, M.L., Ng, C.H., Yong, J., Lim, L. & Leung, T. ArhGAP15, a novel human RacGAP protein with GTPase binding property. *FEBS Lett.* **539**, 131-137 (2003).

312. Brondyk, W.H., *et al.* Interaction cloning of Rabin3, a novel protein that associates with the Ras-like GTPase Rab3A. *Mol. Cell. Biol.* **15**, 1137-1143 (1995).

313. Sudhof, T.C. The synaptic vesicle cycle: a cascade of protein-protein interactions. *Nature* **375**, 645-653 (1995).

314. Tomashevski, A., Husseman, J., Jin, L.W., Nochlin, D. & Vincent, I. Constitutive Wee1 activity in adult brain neurons with M phase-type alterations in Alzheimer neurodegeneration. *J. Alzheimers Dis.* **3**, 195-207 (2001).

315. Li, D. & Roberts, R. WD-repeat proteins: structure characteristics, biological function, and their involvement in human diseases. *Cell. Mol. Life Sci.* **58**, 2085-2097 (2001).

316. Van der Zee, E.A. & Douma, B.R. Historical review of research on protein kinase

C in learning and memory. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **21**, 379-406 (1997).

317. Angenstein, F. & Staak, S. Receptor-mediated activation of protein kinase C in hippocampal long-term potentiation: facts, problems and implications. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **21**, 427-454 (1997).

318. Wu, J., Huang, K.P. & Huang, F.L. Participation of NMDA-mediated phosphorylation and oxidation of neurogranin in the regulation of Ca2+- and Ca2+/calmodulin-dependent neuronal signaling in the hippocampus. *J. Neurochem.* **86**, 1524-1533 (2003).

319. Hori, K., *et al.* NMDA receptor-dependent synaptic translocation of insulin receptor substrate p53 via protein kinase C signaling. *J. Neurosci.* **25**, 2670-2681 (2005).

320. Dolmetsch, R. Excitation-transcription coupling: signaling by ion channels to the nucleus. *Sci. STKE* **2003**, PE4 (2003).

321. Westphal, R.S., *et al.* Regulation of NMDA receptors by an associated phosphatase-kinase signaling complex. *Science* **285**, 93-96 (1999).

322. Grozinger, C.M., Hassig, C.A. & Schreiber, S.L. Three proteins define a class of human histone deacetylases related to yeast Hda1p. *Proc. Natl. Acad. Sci. USA* **96**, 4868-4873 (1999).

323. Cajal, S.R.y. *Histology of the Nervous System* (Oxford University Press, New York, 1995).

324. Legutko, B., Li, X. & Skolnick, P. Regulation of BDNF expression in primary neuron culture by LY392098, a novel AMPA receptor potentiator. *Neuropharmacology* **40**, 1019-1027 (2001).

325. Lauterborn, J.C., Lynch, G., Vanderklish, P., Arai, A. & Gall, C.M. Positive modulation of AMPA receptors increases neurotrophin expression by hippocampal and cortical neurons. *J. Neurosci.* **20**, 8-21 (2000).

326. Zafra, F., Lindholm, D., Castren, E., Hartikka, J. & Thoenen, H. Regulation of brain-derived neurotrophic factor and nerve growth factor mRNA in primary cultures of hippocampal neurons and astrocytes. *J. Neurosci.* **12**, 4793-4799 (1992).

327. Wu, X., *et al.* AMPA protects cultured neurons against glutamate excitotoxicity through a phosphatidylinositol 3-kinase-dependent activation in extracellular signal-regulated kinase to upregulate BDNF gene expression. *J. Neurochem.* **90**, 807-818 (2004).

328. Zafra, F., Hengerer, B., Leibrock, J., Thoenen, H. & Lindholm, D. Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. *EMBO J.* **9**, 3545-3550 (1990).

329. Rodriguez-Moreno, A. & Lerma, J. Kainate receptor modulation of GABA release involves a metabotropic function. *Neuron* **20**, 1211-1218 (1998).

330. Rozas, J.L., Paternain, A.V. & Lerma, J. Noncanonical signaling by ionotropic kainate receptors. *Neuron* **39**, 543-553 (2003).

331. Jones, M.W., *et al.* A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. *Nat. Neurosci.* **4**, 289-296 (2001).

332. Pintchovski, S.A., Rao, V.R., Verdin, E. & Finkbeiner, S. Identification of putative Arc regulatory sites with a genomic screen. *Soc. Neurosci. Abstr.* **31**, 498.496 (2005).

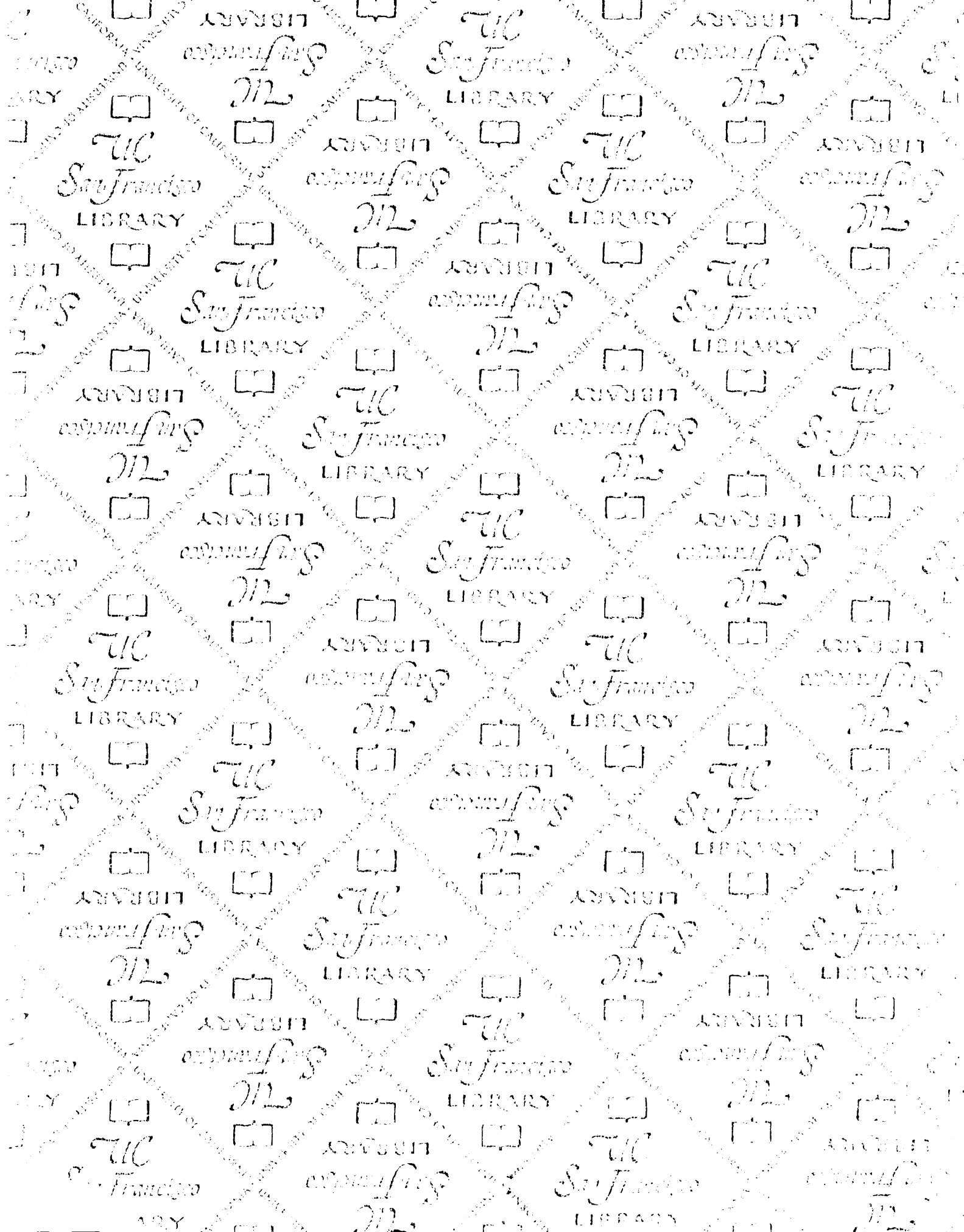
333. Li, L., Carter, J., Gao, X., Whitehead, J. & Tourtellotte, W.G. The neuroplasticity-associated arc gene is a direct transcriptional target of early growth response (Egr) transcription factors. *Mol. Cell. Biol.* **25**, 10286-10300 (2005).

334. Flavell, S.W., *et al.* Activity-dependent regulation of MEF2 transcription factors suppresses excitatory synapse number. *Science* **311**, 1008-1012 (2006).

335. Meffert, M.K. & Baltimore, D. Physiological functions for brain NF-kappaB. *Trends Neurosci.* **28**, 37-43 (2005).

336. Irie, Y., *et al.* Molecular cloning and characterization of Amida, a novel protein which interacts with a neuron-specific immediate early gene product arc, contains novel nuclear localization signals, and causes cell death in cultured cells. *J. Biol. Chem.* **275**, 2647-2653 (2000).

337. Fujimoto, T., Tanaka, H., Kumamaru, E., Okamura, K. & Miki, N. Arc interacts with microtubules/microtubule-associated protein 2 and attenuates microtubule-associated protein 2 immunoreactivity in the dendrites. *J. Neurosci. Res.* **76**, 51-63 (2004).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