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Arc/Arg3.1 Translation Is Controlled by Convergent *N*-Methyl-D-aspartate and G_s-coupled Receptor Signaling Pathways*[S]

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Arc/Arg3.1 is an immediate early gene whose expression is necessary for the late-phase of long-term potentiation (LTP) and memory consolidation. Whereas pathways regulating Arc transcription have been extensively investigated, less is known about the role of post-transcriptional mechanisms in Arc expression. Fluorescence microscopy experiments in cultured hippocampal neurons revealed that Arc protein level was dramatically increased by activation of the cAMP-dependent protein kinase (PKA) pathway, which is implicated in long-term memory. A PKA-dependent increase in Arc protein level was observed after pharmacological or synaptic activation of *N*-methyl-D-aspartate (NMDA) receptors, which play a critical role in both LTP induction and learning. Arc protein was also up-regulated by activation of PKA through G_s-coupled dopamine and β-adrenergic receptors, which regulate the late-phase of LTP and memory. When agonists for the NMDA and G_s-coupled receptors were co-applied, they had an additive effect on Arc protein expression. Interestingly, G_s-coupled receptor stimulation was ineffective in the presence of an NMDA receptor antagonist, suggesting calcium influx through the NMDA receptor plays a gating role in this pathway. Stimulation of the cAMP/PKA pathway did not affect Arc mRNA level or protein stability, identifying translational efficacy as the main determinant of Arc protein expression level. It is concluded that efficient Arc translation requires NMDA receptor activity, whereas a further enhancement can be achieved with activation of G_s-coupled receptors. These experiments have, therefore, revealed remarkable similarities in the signaling pathways that control Arc expression and those that regulate LTP, learning, and memory.

Activity-regulated, cytoskeletal-associated protein Arc, also known as Arg3.1, was identified as an immediate early gene, whose mRNA is induced by patterns of synaptic activity that elicit LTP (1, 2). After transcription Arc mRNA is transported into neuronal dendrites, where it localizes specifically to activated synapses (3). Experiments using antisense or genetic knockdown have shown that Arc expression is necessary for both the late phase of LTP and memory consolidation (4, 5). Thus far, studies of the regulation of Arc expression have focused mainly on mechanisms controlling gene transcription. Arc mRNA levels are increased after treatment with stimulants or growth factors as well as by exposing animals to novel environments (6–13). The signaling pathways controlling Arc transcription include several receptors (for NMDA,[2] dopamine, serotonin, brain-derived neurotrophic factor, insulin, adrenaline, and acetylcholine), second messengers (Ca^{2+} and cAMP), protein kinases (cAMP-dependent protein kinases A and C (PKA and PKC), Src, extracellular signal-regulated kinase), and transcription factors (Egr1 and Egr3) (6, 13–21).

Post-transcriptional regulation of Arc protein expression has not been studied in as much detail. Brain-derived neurotrophic factor and reelin-integrin receptor signaling increase Arc protein synthesis in synaptoneurosome preparations (22, 23). In addition, β-adrenergic receptor activation increases Arc protein expression and enhances memory without an increase in the fluorescence *in situ* hybridization signal of Arc mRNA (15). These results suggest that Arc protein expression can be regulated post-transcriptionally. The level at which this may occur is unknown and could include regulation of mRNA stability, translational efficacy, protein degradation, or a combination of the above.

To further study the post-transcriptional regulation of Arc protein expression, both Arc mRNA and protein levels were determined in cultured hippocampal neurons using quantitative real-time PCR (qRT-PCR) and fluorescence microscopy. Stimulation of the cAMP/PKA pathway by either NMDA receptor or G_s-coupled receptor activation resulted in a strong up-regulation of Arc protein. Activation of the NMDA receptor results in calcium influx, which can activate PKA through calcium-calmodulin (Ca^{2+}-CaM)-dependent adenylyl cyclases (ACs) type I and VIII, whereas D1/D5 dopamine and β-adrenergic receptor receptors activate ACs through the heterotrimeric GTP-binding protein G_s. The effects of activating NMDA and G_s-coupled receptors on Arc protein levels were additive, whereas NMDA receptor function was required for

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[2] The abbreviations used are: NMDA, *N*-methyl-D-aspartate; qRT-PCR, quantitative real-time PCR; FPS, fluorescence/pixel/s; ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; hnRNP, heterogeneous nuclear ribonucleoprotein; miR, microRNA; PKA, cAMP-dependent protein kinase; RT, room temperature; CaM, calmodulin; AC, adenylyl cyclase; UTR, untranslated region; 8-CPT, 8-(4-chlorophenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic-monophosphate; PBS, phosphate-buffered saline; CMV, cytomegalovirus; APV, 2-amino-5-phosphonovaleric acid.

the dopamine and β-adrenergic effects. The PKA-induced increase in Arc protein level occurred without a change in Arc mRNA level or Arc protein stability but was prevented by a translation inhibitor. Together, these results show that NMDA and G_s-coupled receptor signaling pathways converge to regulate Arc translation.

MATERIALS AND METHODS

Cell Culture—Hippocampal rat brain tissue (E18) obtained from Brainbits, Inc. (Springfield, IL) was cultured as previously described (24). Briefly, cells were dissociated and plated at 50,000 cells/cm^2 on poly-D-lysine-coated glass-bottomed dishes (MatTek, Ashland, MA) in a chemically defined medium of B27, glutamate, and glycine in Neurobasal medium (Invitrogen). Neurons were fed once weekly by replacing half the medium. For the Western blot and qRT-PCR experiments neurons were cultured at the same density in 6-well tissue culture plates from BD Biosciences Falcon.

DNA Constructs—The Arc coding region with flanking untranslated regions (UTRs) was amplified by a PCR reaction using CHORI-230 rat BAC Clone DNA as a template (Children's Hospital Oakland-BACPAK Resources, Oakland, Ca) and was subcloned into the pGemT-easy vector (Promega, Madison WI). The region of the gene containing two introns was replaced with DNA amplified by PCR from Marathon rat brain cDNA (Clontech, Cambridge, UK) via restriction digest and ligation. A novel BsrGI restriction site was added directly before the stop codon in the Arc coding region by PCR using the Arc pGemT-easy construct without introns as template. EYFP from the Clontech vector EYFP-N1 was amplified by PCR using primers to incorporate BsrGI sites flanking the coding region. BsrGI-EYFP-BsrGI was subcloned into the Arc-pGemT-easy vector, and the Arc-EYFP DNA was subsequently subcloned into pcDNA3.1+ (Invitrogen). The Arc coding region construct was generated from DNA amplified by PCR from Marathon rat brain cDNA (Clontech) and subcloned into the pGemT-easy vector (Promega). The Arc coding region was then subcloned into EYFP-N1 (Clontech). Plasmids expressing cyan and red Fluorescent proteins (ECFP-C1 and dsRed2-C1, Clontech) were used as controls. The DNA sequence of the coding region of the monomeric form of EosFP was codon-optimized using Oligo Version 4.0 software (Molecular Biology Insights, Cascade, Co) and was synthesized and subcloned into pBluescript-SK by Epoch Biolabs (Sugarland, TX). EYFP was replaced by EosFP in the Arc-EYFPN1 plasmid by restriction digest and ligation. The sequence of the full-length Arc cDNA used in these experiments is identical to the NCBI rat Arc mRNA sequence with GenBank™ accession number NM_019361. In the Arc plasmid containing the coding region only, the Arc 5′-UTR has been replaced with the short optimal Kozak consensus sequence GCCACC, whereas the 3′-UTR was removed.

Transfections and Stimulation—Neuronal cultures were transfected between day 17 and 25 as previously described (24). Briefly, cells were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen). For each transfection, 2 μg of DNA (1 μg of each plasmid) was added to 50 μl of Neurobasal medium, and 3 μl of Lipofectamine 2000 was added to a separate 50 μl of Neurobasal medium. After a 5-min room temperature (RT) incubation, the samples were combined and allowed to incubate for an additional 20 min at RT. Culture media was removed from the neurons, and the DNA/Lipofectamine 2000 solution was added dropwise to the cultures. After a 10-min incubation at 37 °C, the medium was replaced, and the dishes were returned to the incubator for 16 h. When stated, hippocampal neurons were stimulated with 50 μM forskolin, 1 mM 8-bromo-cAMP, 10 μM NMDA, 100 μM SKF-38393, 10 μM isoproterenol, 20 μM H89, 20 μM anisomycin, 10 μM MK-801, 10 μM APV, 10 μM 8-(4-chlorophenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic-monophosphate (8-CPT), all from Sigma-Aldrich, or 10 μM MG-132 (Calbiochem) in the defined media. For the APV withdrawal experiments, neurons were cultured with conditioned media containing APV starting on day 5 in culture (day 5). Neurons were transfected on D23, and APV free media was added on D24.

Immunofluorescence and Imaging—For immunofluorescence experiments we used the monoclonal C-7 Arc antibody (Santa Cruz Biotechnology, Santa Cruz, CA). C-7 is highly specific for Arc (Fig. 1*A*) and has been used previously for immunofluorescence (25, 26). Transfected neuron dishes were rinsed once with Dulbecco's phosphate-buffered saline with glucose and pyruvate (PBS) and fixed with a solution containing 4% paraformaldehyde, 4% sucrose, and 1× PBS for 15 min at 4 °C. Cells were then washed and imaged in PBS. Cells used for immunofluorescence were fixed with paraformaldehyde as above and permeabilized with ice-cold methanol for 20 min at −20 °C. After permeabilization, the cells were blocked with a solution containing 10% goat serum, 2% bovine serum albumin, and 1× PBS for 1 h at RT. Primary C-7 antibody diluted 1:100 in block was incubated overnight at 4 °C. The dishes were washed 3 times with block for 10 min each and incubated with the secondary antibody (Alexa Fluor 488-goat anti-mouse IgG, Molecular Probes-Invitrogen) diluted 1:500 in block solution for 1–2 h at RT. The dishes were washed as above with block then once with PBS for 10 min and imaged in PBS. Neurons were imaged as previously described (24). Briefly, cells were imaged with a Nikon (Tokyo, Japan) Diaphot inverted fluorescence microscope with a 40× oil immersion objective. A Hamamatsu (Shizuka, Japan) cooled digital camera and Openlab Version 3.1.6 software (Improvision, Lexington, MA) were used to acquire the images. For the EosFP experiments neurons were treated with UV light for 30 s using a blue filter (Chroma Technology, Rockingham, VT) before imaging.

Western Blot Analysis—Neuronal cultures were dissolved in lysis buffer (radioimmune precipitation assay buffer; 50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1:100 dilution of protease inhibitor mixture (Sigma-Aldrich)), scraped into 1.5 ml tubes, and sonicated (24). To assure equal protein loading, the concentrations of the lysates were determined with the DC Protein Assay kit according to the microplate protocol (Bio-Rad). Samples consisting of 25 μg of lysate protein were separated using SDS-PAGE on 10% Tris-glycine gels (Invitrogen). Proteins were transferred to polyvinylidene difluoride membranes (Invitrogen) and immunoblotted as follows. The polyvinylidene difluoride membranes were blocked with 5% nonfat milk in PBS for

1 h at RT. The blots were incubated with the Arc antibody diluted 1:100 in the blocking solution overnight at 4 °C and then were washed 3 times for 10 min each with PBS plus 0.1% Tween 20 (Bio-Rad) at RT. The membranes were incubated with goat anti-mouse horseradish peroxidase in block (1:1000; Santa Cruz Biotechnology) for 1 h at RT. The membranes were then washed 8 times for 15 min each with PBS plus 0.1% Tween 20, exposed with SuperSignal West Pico chemiluminescent horseradish peroxidase) substrate (Pierce), and developed on x-ray film according to the instructions.

qRT-PCR—Neurons in 6-well tissue culture plates were transfected with 1 μg each of Arc-EYFP and dsRed2C1 plasmids (Clontech). 16 h post-transfection cells were treated with 50 μM forskolin or vehicle for 4 h. RNA was isolated with the Qiashredder kit according to instructions (Qiagen, Valencia, CA). RNA was harvested from a 6-well plate via a Qiagen column, subjected to a DNase treatment, and re-purified with a second Qiagen column. 1 μg of RNA was reverse-transcribed with the Bio-Rad I-script kit according to the manufacturer's instructions. A standard curve was generated by serial dilution of all the cDNAs. Reactions were set up in 96-well PCR plates (USA Scientific, Inc. Ocala, CA) with iQ SYBR Green Supermix according to the instructions (Bio-Rad) and run in the iCycler Thermal Cycler (Bio-Rad).

Data Analysis—Grayscale images (12-bit resolution) of fluorescent neurons were acquired separately for the cyan (ECFP) and yellow (EYFP) channels and analyzed using ImagePro Plus Version 4.5 (Media Cybernetics, Silver Spring, MD). Outlines of fluorescent neurons in the images were detected by the ImagePro "automatic bright objects" tool, and both the area (A) and total fluorescence intensity (F) within the outline were measured. For each neuron, the average fluorescence (F/A) was divided by the exposure time (T) used to acquire the image to obtain fluorescence/pixel/s (FPS = $F/A/T$), which was used as a relative measure of protein expression. FPS was measured for n neurons (n = at least 20 per condition) to allow calculation of a mean and S.E. for each condition. Cyan (control) and yellow (Arc) FPS values were obtained for each neuron, and their ratio was calculated. FPS was calculated for the red EosFP signals in a similar manner. qRT-PCR results were analyzed with iCycler iQ optical system software Version 3.0a (Bio-Rad).

RESULTS

Post-transcriptional regulation of Arc expression was investigated in cultured hippocampal neurons. First, treatments were identified that have been reported to induce endogenous Arc expression. Previous work has shown that Arc mRNA level is increased after treatment with NMDA, dopamine receptor agonist, or forskolin (13, 14, 20). Forskolin directly activates ACs, resulting in cAMP production and activation of both PKA and the exchange protein directly activated by cyclic-AMP (Epac). Activation of NMDA and dopamine receptors can also result in AC activation and cAMP production, mediated by Ca^{2+}-CaM and the stimulatory G-protein G_s, respectively. Therefore, the effect of these agonists on Arc protein level was investigated in hippocampal neurons. Because forskolin can have nonspecific effects independent of its action on ACs, neurons were also stimulated with 8-Br-cAMP, a membrane-permeable analogue of cAMP.

Endogenous Arc Protein Expression—The effect of the agonists on Arc protein level was first evaluated by Western analysis using a monoclonal Arc-specific antibody. Fig. 1*A* illustrates that this antibody recognizes Arc protein, seen as a single band at ~50 kDa. Arc protein levels increased after a 4-h treatment with each of the following agonists: forskolin, 8-Br-cAMP, NMDA, and the D1/D5 dopamine receptor agonist SKF-38393. Pharmacological activation of PKA by either forskolin or 8-Br-cAMP resulted in a stronger induction of Arc than activation of NMDA receptors or dopamine receptors. Western analysis reflects a combined effect of the compounds on Arc protein expression for all cell types present in the hippocampal culture, including neurons and glial cells, both of which express Arc protein (27).

To determine Arc expression solely in neurons, hippocampal cultures were treated with agonists, fixed, and analyzed by immunofluorescence microscopy using the Arc antibody and Alexa Fluor 488-conjugated secondary antibody. Only neurons were selected for data analysis. Arc protein displayed a punctate distribution and was found primarily in the soma, with lower levels in the distal dendrites (Fig. 1*B*). A time course of Arc expression was determined by stimulating cultured neurons with forskolin for different time periods over a 24-h period. Arc expression detected with the Arc antibody peaked at 4 h, reaching a 12-fold increase over unstimulated neurons (Fig. 1*C*). How Arc expression level was affected by the previously used agonists was also tested (Fig. 1*D*). Arc protein level increased ~12-fold for forskolin, 11-fold for 8-Br-cAMP, 7-fold for NMDA, and 4-fold with SKF-38393. NMDA and dopamine signaling are both expected to activate PKA through Ca^{2+}/CaM and G_s, respectively. Therefore, the co-application of NMDA and SKF-38393 was investigated. This resulted in an 11-fold increase in Arc protein expression (Fig. 1*D*), indicating the effects of NMDA and the dopamine agonist are additive.

The ability of the agonists to induce Arc in the presence of the PKA antagonist H89 was also investigated (Fig. 1*D*). Pretreatment with H89 abolished the effect for all the agonists tested, indicating that in each case the induction of Arc protein expression depended on PKA activation. The effects of the agonists on endogenous Arc protein level may have resulted from transcriptional and/or post-transcriptional regulation. This issue was investigated next.

Post-transcriptional Regulation of Arc Protein Expression—To determine whether PKA activation could result in a post-transcriptional effect on Arc protein level, a yellow fluorescent Arc fusion protein (Arc-EYFP) was expressed in cultured hippocampal neurons through transient transfections (Fig. 2). As a control, enhanced cyan fluorescent protein (ECFP) was co-transfected with Arc-EYFP, resulting in co-expression of both cDNAs in the same subset of neurons. In these experiments, a full-length Arc cDNA construct was used that contained complete 5′- and 3′-UTRs, whereas transcription was driven by the strong viral CMV promoter. Because of its high constitutive activity, the CMV promoter should be relatively insensitive to regulation by cellular signaling pathways, allowing us to evaluate post-transcriptional mechanisms that regu-

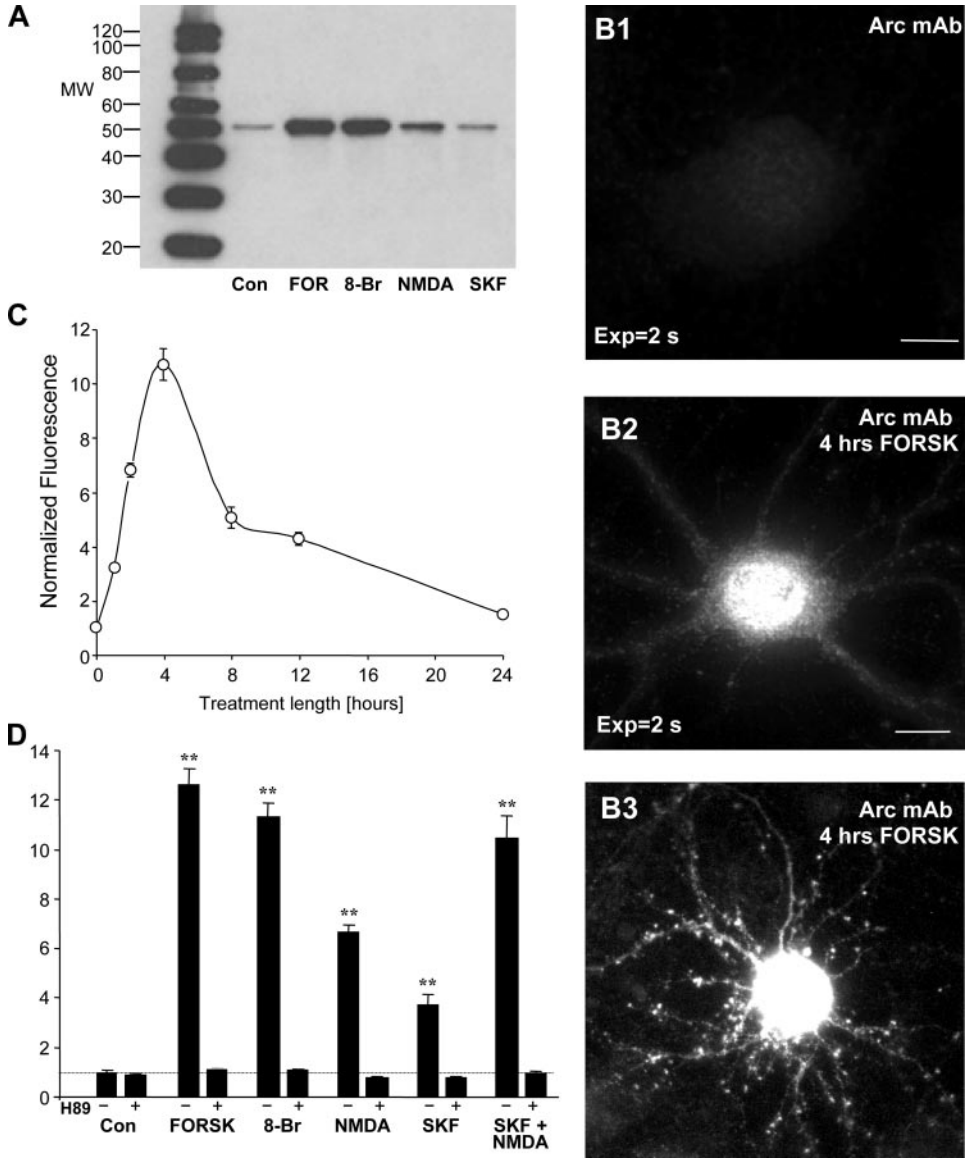

FIGURE 1. **Endogenous Arc protein expression is induced by PKA agonists.** *A*, Arc immunoblot of hippocampal cultures treated 4 h with vehicle, 50 μM forskolin (*FOR*), 1 mM 8-Br-cAMP, 10 μM NMDA, or 100 μM SKF-38393 (*SKF*). All agonists induced Arc protein expression with forskolin > 8-Br-cAMP > NMDA plus SKF-38396 > NMDA > SKF-38393. *Con*, control. *B*, Arc immunohistochemistry signal of cultured neurons stimulated for 4 h with vehicle (*B1*) or 50 μM forskolin (*B2* and *B3*). Exposure times were the same (2 s) for *B1* and *B2*, allowing direct comparison of Arc protein levels. Arc immunoreactivity has a punctate appearance and localizes primarily to the soma with lower amounts in the dendrites. For the neuron shown in *B3*, the brightness was increased 10-fold to visualize the distribution of endogenous Arc in the dendritic compartment. Arc puncta are mostly localized to dendritic spines. *Scale bars* are 10 μm. *mAb*, monoclonal antibody. *C*, quantification of Arc immunofluorescence signal in cultured neurons treated with 50 μM forskolin for different time periods. Cultures were treated so that all cells were fixed at the same time. Based on morphology, only neurons were imaged. Arc expression peaks at 4 h. *D*, quantification of Arc immunofluorescence signal in cultured neurons treated with PKA agonists. Cells were pretreated with vehicle or 20 μM H89 for 1 h and then were treated with the PKA agonists for 4 h. All agonists induced Arc protein expression with forskolin > 8-Br-cAMP > NMDA plus SKF-38396 > NMDA > SKF-38393. H89 pretreatment blocked the effect of all the PKA agonists. n = at least 20 cells per condition for this figure and all other figures unless otherwise stated. The *asterisks* (* and **) in all figures denote a difference from control fluorescence where $p < 0.05$, or $p < 0.01$, respectively.

late Arc expression. Similar to what was seen for endogenous protein, exogenous Arc protein was found primarily in the soma, with lower levels in the dendrites (Fig. 2*A*). Forskolin treatment caused Arc to be sharply up-regulated (notice the difference in exposure times), whereas its distribution became more punctate both in the soma and dendrites (Fig. 2*A*). Expression of both Arc-EYFP and ECFP is driven by the same viral CMV promoter; therefore, they are expected to be subject to the same transcriptional regulation, and a difference in fluorescence signal should reflect post-transcriptional events. To correct for differences in general protein expression between neurons, the Arc-EYFP signal was normalized to ECFP in the same neuron. This normalization also corrects for any effect that an agonist has on general translation within a single cell. Again, the time course of the forskolin effect on Arc protein expression was determined. Arc-EYFP- and ECFP-transfected cultures were stimulated with forskolin for different time periods over 24 h. Arc expression peaked at 4 h with a 9.4-fold increase over unstimulated neurons (Fig. 2*B*). Next, the effect of the agonists on Arc expression level was tested. All agonists increased Arc-EYFP expression, while having a marginal effect on ECFP. Agonist treatment did not have a negative effect on neuronal health (supplemental Fig. 1). Arc-EYFP/ECFP ratios increased 9.1-fold for forskolin, 10.4-fold for 8-Br-cAMP, 4.4-fold for NMDA, and 3.6-fold with SKF-38393 stimulation (Fig. 2*C*). NMDA and SKF-38393 co-application increased Arc expression additively by 8.8-fold. In addition, H89 blocked all of the agonist effects (Fig. 2*D*).

In these experiments transcription of Arc-EYFP and ECFP was driven by the constitutively active strong CMV promoter in order to minimize transcriptional effects of the treatments. Effects of the pharmacological treatments on general transcription were corrected for by normalizing for ECFP expression. The results obtained with the ECFP construct show that the CMV promoter is almost completely insensitive to the various agonist treatments. Consequently, the strong increase in Arc-EYFP levels likely resulted from a post-transcriptional mechanism.

Regulation of Arc Protein Expression Does Not Require the Arc-untranslated Regions—Several post-transcriptional mechanisms control protein levels through an interaction with the 5′- and 3′-UTRs of a target mRNA. Therefore, it was investi-

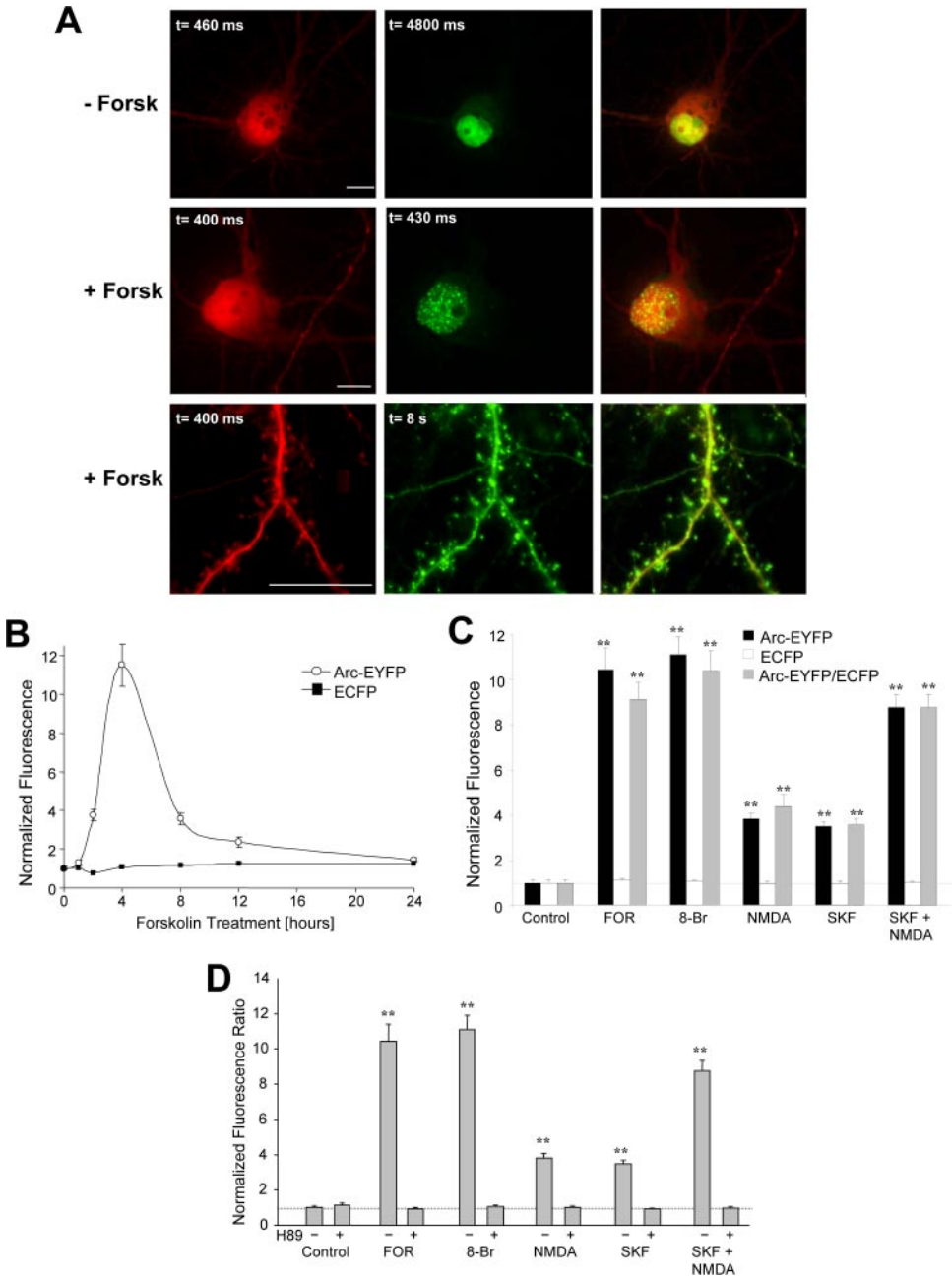

FIGURE 2. **Exogenous Arc protein expression is induced by PKA agonists.** *A*, representative images of neurons expressing Arc-EYFP and ECFP, which were treated with either vehicle or forskolin (*Forsk*) for 4 h. Exposure times for the Arc-EYFP signals were 4800 ms, 430 ms, and 8 s respectively, whereas they were the 460 ms, 400 ms, and 400 ms for the ECFP signals. The merged images show that Arc is enriched in the soma with lower levels in the dendrites similarly to what was seen for endogenous Arc (Fig. 1*B*). Forskolin treatment increased the expression of Arc protein (indicated by the shorter exposure time) and enhanced its punctate appearance in the soma and in the dendrites. *Scale bars* are 10, 10, and 5 μm. *B*, quantification of yellow and cyan fluorescence signals in neurons co-expressing Arc-EYFP and ECFP treated with forskolin for different time periods. Cultures were treated so that all cells were fixed at the same time. Arc-EYFP expression peaked at the same time point (4 h) as endogenous Arc (Fig. 1*C*). Forskolin had a minimal effect on the expression level of the control protein ECFP. *C*, quantification of yellow and cyan fluorescence signals in neurons co-expressing Arc-EYFP and ECFP treated with agonists for 4 h. All agonists increased Arc expression while only minimally effecting ECFP, with the following profile: forskolin (*FOR*) > 8-Br-cAMP > NMDA plus SKF-38393 > NMDA > SKF-38393 (*SKF*). The Arc-EYFP/ECFP ratio measures the Arc-specific post-transcriptional effect on Arc protein expression. *D*, quantification of the Arc-EYFP/ECFP ratio in neurons that were pretreated with vehicle or 20 μM H89 for 1 h followed by a 4-h treatment with agonists. H89 completely blocked the effect of all agonists on Arc expression level.

gated whether the agonist-induced increases in Arc protein levels reported above require the presence of UTRs in the Arc mRNA. Experiments were performed with an Arc construct that lacked both the 5′- and 3′-UTRs. The 3′-UTR of Arc has previously been shown to be important in dendritic targeting, although its role in Arc protein translation is unknown (28). A plasmid encoding Arc-EYFP without the UTRs was co-expressed with ECFP in cultured neurons (Fig. 3). Again, forskolin treatment caused Arc to be up-regulated (notice the difference in exposure times), whereas its distribution became more punctuate in the soma and dendrites (Fig. 3*A*).

Transfected cultures were stimulated with forskolin for different time periods over 24 h. Arc expression ratios peaked at 4 h, with a 7.6-fold increase over unstimulated neurons (Fig. 3*B*). Transfected cultures were also stimulated with the PKA agonists (Fig. 3*C*). All the previously used agonists increased Arc-EYFP expression, while having a marginal effect on ECFP. Arc expression ratios increased ~8-fold for forskolin and 8-Br-cAMP, 4-fold for NMDA, 3.2-fold for SKF-38393, and additively 7.6-fold for NMDA plus SKF-38393 stimulation. NMDA stimulation did not enhance the forskolin effect, possibly because forskolin has already saturated the signaling pathway. Again, the PKA antagonist H89 blocked the effect of all agonists tested (Fig. 3*D*). The two main effectors of cAMP are PKA and Epac, an activator of the small G-protein Rap1. To investigate a possible involvement of Epac in the regulation of Arc expression, we used 8-CPT, a cAMP analogue that specifically activates Epac. 8-CPT was completely ineffective, further verifying that cAMP acted specifically through PKA (Fig. 3*C*). These data show that Arc expression is regulated through the cAMP/PKA pathway in hippocampal neurons by a mechanism that does not require the UTRs of Arc.

β-Adrenergic Receptor Signaling Increases Arc Protein Expression—It has previously been shown that NMDA receptors physically interact with dopamine receptors (29–31). To determine whether PKA activation by other G_s-coupled receptors display additivity with NMDA receptor acti-

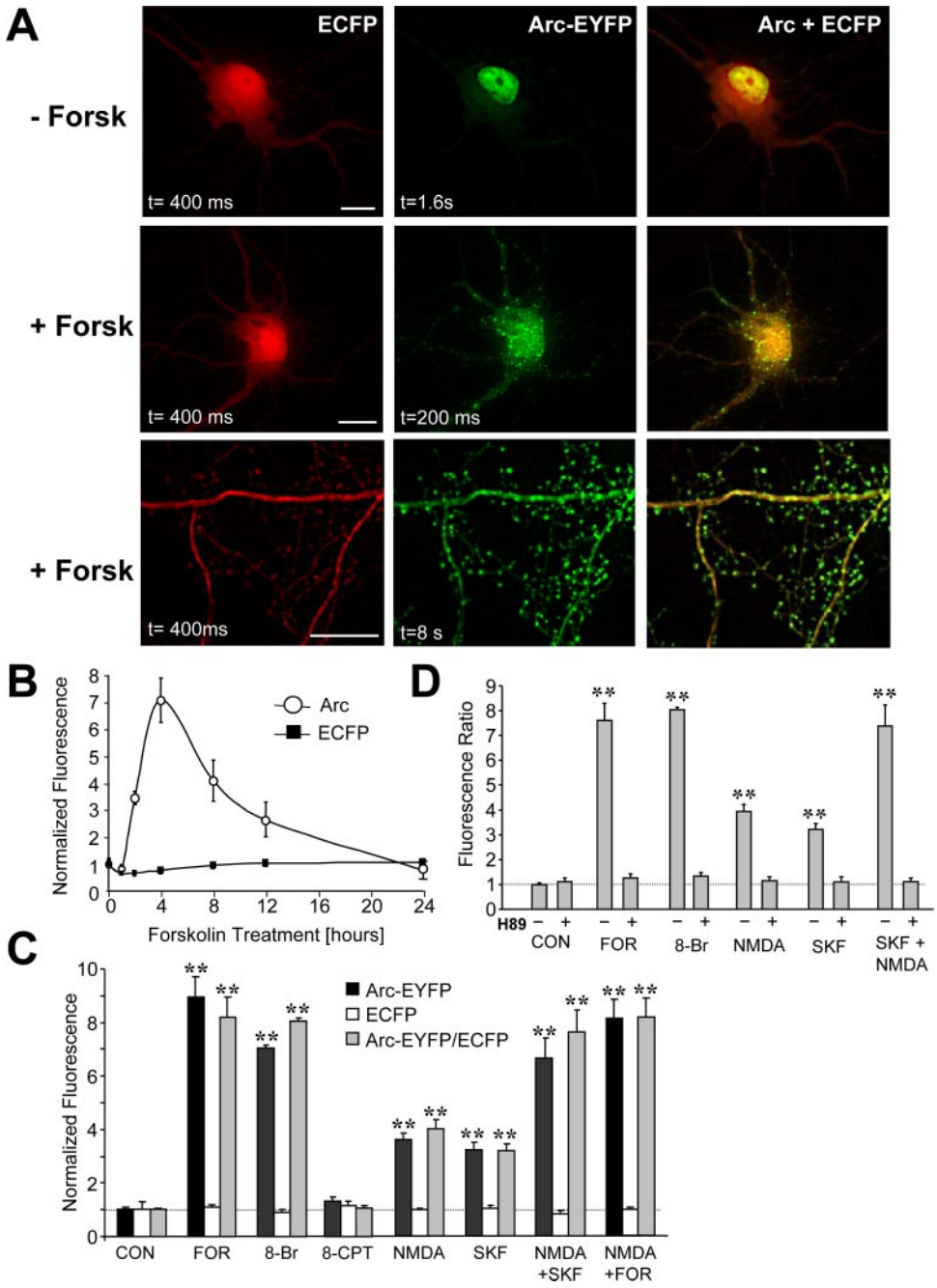

FIGURE 3. **Arc UTRs are not required for Arc induction.** *A*, fluorescence signals of neurons expressing Arc-EYFP (without UTRs) and ECFP treated with vehicle or forskolin (*Forsk*) for 4 h. Exposure times for the Arc-EYFP signals were 1600 ms, 200 ms, and 8 s, respectively, whereas they were 400 ms for ECFP. Arc is enriched in the soma with lower levels in the dendrites. Forskolin treatment increased the expression of Arc protein both in the soma and dendrites in a punctate manner. *Scale bars* are 10, 10, and 5 μm. *B*, quantification of yellow and cyan fluorescence signal from neurons expressing Arc-EYFP (without UTRs) and ECFP. Neurons were treated with forskolin for different time periods. Cultures were treated so that all cells were fixed at the same time. Arc expression peaks at 4 h. *C*, effect of agonists on yellow and cyan fluorescence signals in neurons co-expressing Arc-EYFP (lacking UTRs) and ECFP. All agonists (except 8-CPT) increased Arc expression while having only a minimal effect on ECFP. The profile was as follows: forskolin = 8-Br-cAMP = NMDA plus forskolin > NMDA plus SKF-38393 > NMDA > SKF-38393. *D*, quantification of Arc-EYFP/ECFP ratio in cells pretreated with vehicle or 20 μM H89 for 1 h followed by a 4-h treatment with agonists. H89 blocked the effect of all the agonists on Arc expression level.

vation in respect to Arc protein expression, the β-adrenergic receptor agonist isoproterenol was used to stimulate the cultures (Fig. 4*A*). Isoproterenol had the same effect as the dopamine agonist on endogenous and exogenous Arc protein levels. These results show that Arc expression can be induced by activation of two distinct G_s-coupled receptors and that a physical association between NMDA and G_s-coupled receptors may not be necessary for the effect. Fig. 4*B* illustrates that the PKA antagonist H89 blocked the effect of isoproterenol both alone and in combination with NMDA, indicating that these agonist-induced increases of Arc protein were also dependent on PKA activity.

Endogenous NMDA Receptor Activity Increases Arc Expression—It was next tested whether Arc protein level could be increased by synaptic activation of endogenous NMDA receptors resulting from spontaneous network activity. This was accomplished using an APV-withdrawal paradigm. The competitive reversible NMDA antagonist APV was added to hippocampal cultures at day 5, which resulted in a homeostatic gradual increase of NMDA receptor density (32). APV was removed at day 24, 1 day after transfection, resulting in network activation of the up-regulated NMDA receptors. Removing APV from the medium restores the function of endogenous NMDA receptors, which are now available for activation by spontaneous network activity present in these cultures (33, 34). The APV withdrawal paradigm has been extensively characterized and has been shown to result in stimulation of NMDA receptors by glutamate released from presynaptic terminals activated by spontaneous network activity (35–39).

Four hours after APV withdrawal the neurons were fixed, and Arc protein expression was evaluated by fluorescence microscopy. APV withdrawal strongly increased both endogenous and exogenous Arc protein expression (Fig. 5*A*). Interestingly, the network activity-induced NMDA receptor activation after APV withdrawal resulted in a much stronger up-regulation of Arc protein than direct activation of NMDA receptors by bath-applied NMDA (see Figs. 1, 2, and 4). One explanation for this difference is that the NMDA receptor density would be much higher in these experiments due to the prolonged APV antagonism.

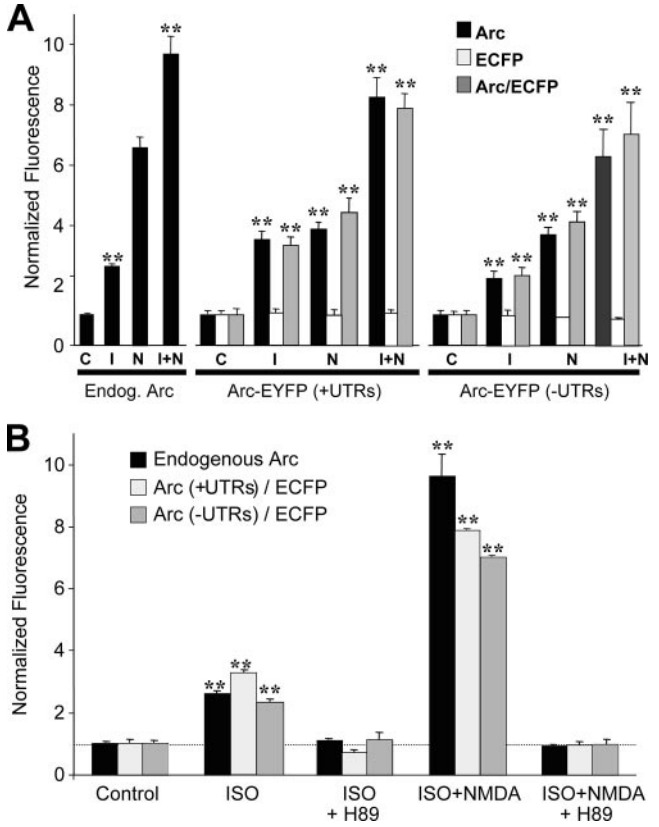

FIGURE 4. **β-Adrenergic signaling increases Arc protein levels.** *A*, the effect of the β-adrenergic receptor agonist isoproterenol on Arc expression was evaluated both in untransfected neurons (using the Arc antibody) or after co-expression of Arc-EYFP (with or without UTRs) and ECFP. Neurons were treated with vehicle (*C*), 10 μM isoproterenol (*I*), 10 μM NMDA (*N*), or 10 μM isoproterenol plus 10 μM NMDA (*I*+*N*) for 4 h. Isoproterenol alone and with NMDA increased Arc protein levels to the same extent as the D1/D5 dopamine agonist. *B*, pretreatment for 1 h with H89 prevented the subsequent effect of a 4-h treatment with isoproterenol (*ISO*, + or −NMDA) on Arc expression level (with or without its UTRs).

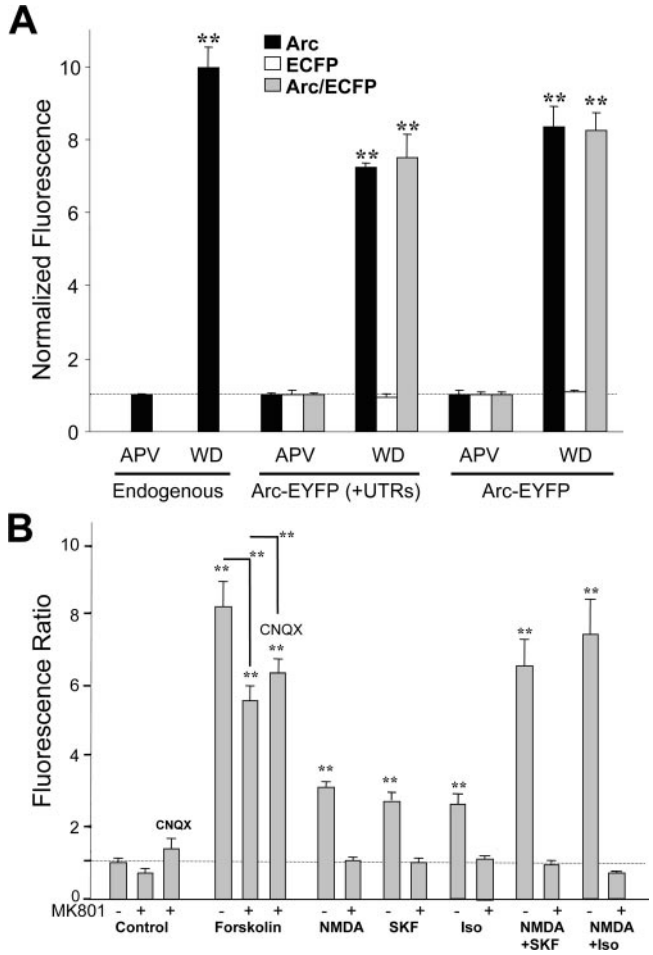

FIGURE 5. **Synaptic NMDA activation increases Arc protein.** *A*, neurons were cultured in the presence of 10 μM APV from day 5 and were either not transfected (for endogenous Arc analysis) or transfected with Arc-EYFP (with or without UTRs) and ECFP on day 23 *in vitro*. The following day the APV-containing medium was replaced with conditioned medium without APV, and the neurons were fixed and imaged 4 h later. Quantification of fluorescence signals showed that APV withdrawal (WD) strongly increased both endogenous and exogenous Arc protein levels. *B*, quantification of the Arc-EYFP/ECFP ratio in cells pretreated with vehicle or 10 μM MK-801 for 1 h followed by a 4-h treatment with the agonists. MK-801 treatment partially blocked the forskolin induction and completely blocked Arc induction by NMDA, SKF-38393, isoproterenol, NMDA plus SKF-38393, and NMDA plus isoproterenol. Pretreatment with 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (*CNQX*) in addition to MK-801 had no further effect.

NMDA Receptor Function Is Necessary for Agonist-induced Arc Up-regulation—It was also determined whether NMDA receptor activation was necessary for the agonist-induced Arc protein increases. MK-801, a non-competitive NMDA receptor antagonist, blocked the effects of NMDA, SKF-38393, isoproterenol, NMDA plus SKF-38393, and NMDA plus isoproterenol (Fig. 5*B*). Surprisingly, induction of Arc protein by dopamine or β-adrenergic receptor activation required NMDA receptor signaling. This shows that basal NMDA receptor activity is necessary for the G_s-mediated effects and suggests a gating role for intracellular calcium ions. In addition, MK-801 partially blocked the ability of forskolin to enhance Arc expression (Fig. 5*B*). Forskolin directly activates all AC isoforms, except type IX. However, for the Ca^{2+}/CaM-dependent AC1 subtype, the degree to which forskolin is able to activate this enzyme is modulated by calcium concentration (40). By blocking the basal NMDA receptor activation produced by spontaneous network activity, MK-801 is expected to lower intracellular calcium concentration and reduce the effectiveness of forskolin to activate the Ca^{2+}/CaM-dependent ACs. Blocking AMPA receptor activity by 6-cyano-7-nitroquinoxaline-2,3-dione (*CNQX*) in the presence of MK-801 had no additional effect (Fig. 5*B*), showing specificity for the NMDA receptor.

Arc Expression Is Regulated at the Level of Translation—PKA activation could exert its positive effect on Arc protein level through one or more post-transcriptional mechanisms, including regulating mRNA stability, translational efficacy, or protein degradation. Experiments were performed to determine which of these mechanisms is involved. qRT-PCR was used to determine the Arc mRNA level in cultured neurons expressing both Arc-EYFP and DsRed2. In this experiment Arc-EYFP and wild type dsRed2 were expressed in cultured neurons. The cells were treated with forskolin or vehicle, and the RNA was quantified with specific primers. EYFP primers were used instead of Arc primers to prevent the possibility of genomic contamination. DsRed2, measured with DsRed2 primers, was used instead of ECFP because the EYFP primers would also amplify ECFP (supplemental Fig. 2*A*). Both the EYFP and DsRed2 primers were shown to be specific, amplifying only one product (supplemen-

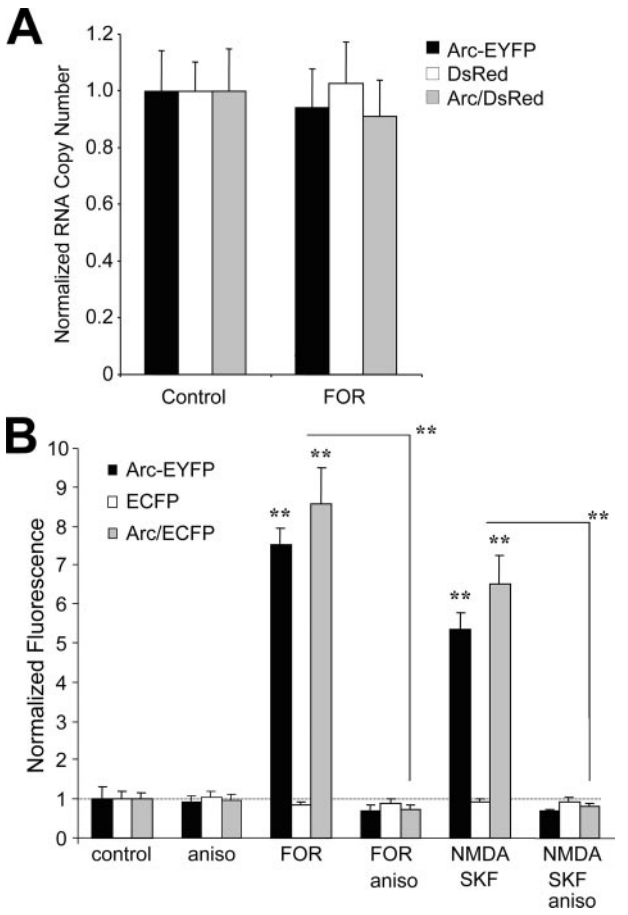

FIGURE 6. **Arc expression is regulated at the level of translation.** *A*, normalized amounts of RNA extrapolated from the qRT-PCR standard curve (supplemental Fig. 2*D*). The amounts of Arc-EYFP and DsRed2 RNA treated with forskolin (*FOR*) did not significantly differ from the control values. $n = 8$ for each condition. *B*, quantification of Arc-EYFP-, ECFP-, and Arc-EYFP/ECFP expressing neurons pretreated with vehicle or 40 μM anisomycin for 1 h followed by a 4-h treatment with agonists. Anisomycin blocked all the agonist-induced increases in Arc protein fluorescence.

tal Fig. 2*B*). Forskolin stimulation did not significantly affect the level of Arc-EYFP or DsRed2 mRNA (supplemental Fig. 2, *C* and *D*, and Fig. 6*A*). Therefore, the forskolin induction of Arc-EYFP is not due to a change in Arc mRNA level.

To investigate whether the effects of forskolin and NMDA plus SKF-38393 on Arc protein level required protein synthesis, neurons were pretreated with the translational inhibitor anisomycin. This resulted in a complete loss of Arc protein induction for the agonists (Fig. 6*B*). Therefore, the effect of the agonists on Arc protein level requires *de novo* protein synthesis.

To determine whether the degradation of Arc protein plays a role, Arc was tagged with EosFP, a photo-convertible fluorescent protein whose emission changes from green to red after UV light treatment (41). At time 0, a portion of wild type EosFP or Arc-EosFP expressed in cultured neurons was converted from green to red. Because any newly synthesized protein is green fluorescent, protein stability can be directly measured by observing the red signal. The levels of red wild type EosFP or red Arc-EosFP were undetectable before UV induction (Fig. 7*A*). After photo-conversion, the failure of the red signal to decline indicated that both proteins were stable over 3 h (Fig. 7*B*). This confirms that Arc protein stability was not a factor in the previous experiments. PKA activation, therefore, primarily increases Arc protein levels post-transcriptionally by increasing translation in our system.

DISCUSSION

The experiments described above have addressed the potential mechanisms of the Arc post-transcriptional regulation in neurons. The results indicate that Arc protein level is relatively low under basal conditions in cultured hippocampal neurons, which are characterized by a low level of spontaneous neuronal network activity. However, activation of the cAMP/PKA pathway either directly or through various receptors resulted in a dramatic and selective increase in Arc protein. The up-regulation of Arc protein occurred without changes in its mRNA level or protein stability, implicating a mechanism by which translational efficacy of Arc is enhanced by PKA activation. The increase in endogenous Arc protein levels that we observed likely resulted from a combination of transcriptional and post-transcriptional mechanisms. Stimulation of PKA has been previously shown to result in a 2.5-fold increase in Arc transcription (20). The increase in endogenous Arc protein levels shown here was much more robust (13-fold), indicating the presence of an additional, post-transcriptional level of control. The experiments with exogenous Arc-EYFP and ECFP allowed us to show unambiguously that Arc expression is regulated at the level of mRNA translation. Taken together, these results indicate that Arc expression is controlled by both transcriptional and translational mechanisms, with the latter being substantially more prominent.

Arc Translation Is Regulated by PKA Activity—It was found that Arc expression is post-transcriptionally regulated at the level of translation by many of the same stimuli that induce its transcription, including NMDA, dopamine, and β-adrenergic receptor agonists. Arc protein levels were significantly increased in hippocampal neurons by forskolin, 8-Br-cAMP, NMDA, SKF-38393, and isoproterenol treatment, which specifically activate ACs, cAMP-dependent enzymes, NMDA receptors, D1/D5 dopamine receptors, and β-adrenergic receptors, respectively. In addition, the co-application of NMDA with either SKF-38393 or isoproterenol increased Arc protein levels more than either agonist alone. In fact, the effects were additive in both cases, suggesting that Arc protein expression acts as an integrator for signaling through NMDA and G_s-coupled receptors.

D1/D5 dopamine and β1/2-adrenergic receptors are G-protein-coupled receptors that couple positively to G_s-dependent ACs, which include all isoforms except type VIII. Calcium entry through NMDA receptors can activate Ca^{2+}/CaM-dependent AC type I and VIII. AC1 is unique in that it is brain-specific and has the ability to integrate Ca^{2+}/CaM and G_s signals (42). The additive effect of NMDA and G-protein-coupled receptor stimulation could, therefore, be mediated by the integrative property of AC1. Further support for an involvement of AC1 comes from the finding that MK-801 ablated both the dopamine and β-adrenergic receptor effects on Arc expression levels. Because activation of AC1 by G_s requires calcium (43), blockade of the basal NMDA receptor activity may reduce the internal calcium concentration to a level that precludes activation of AC1 by G_s.

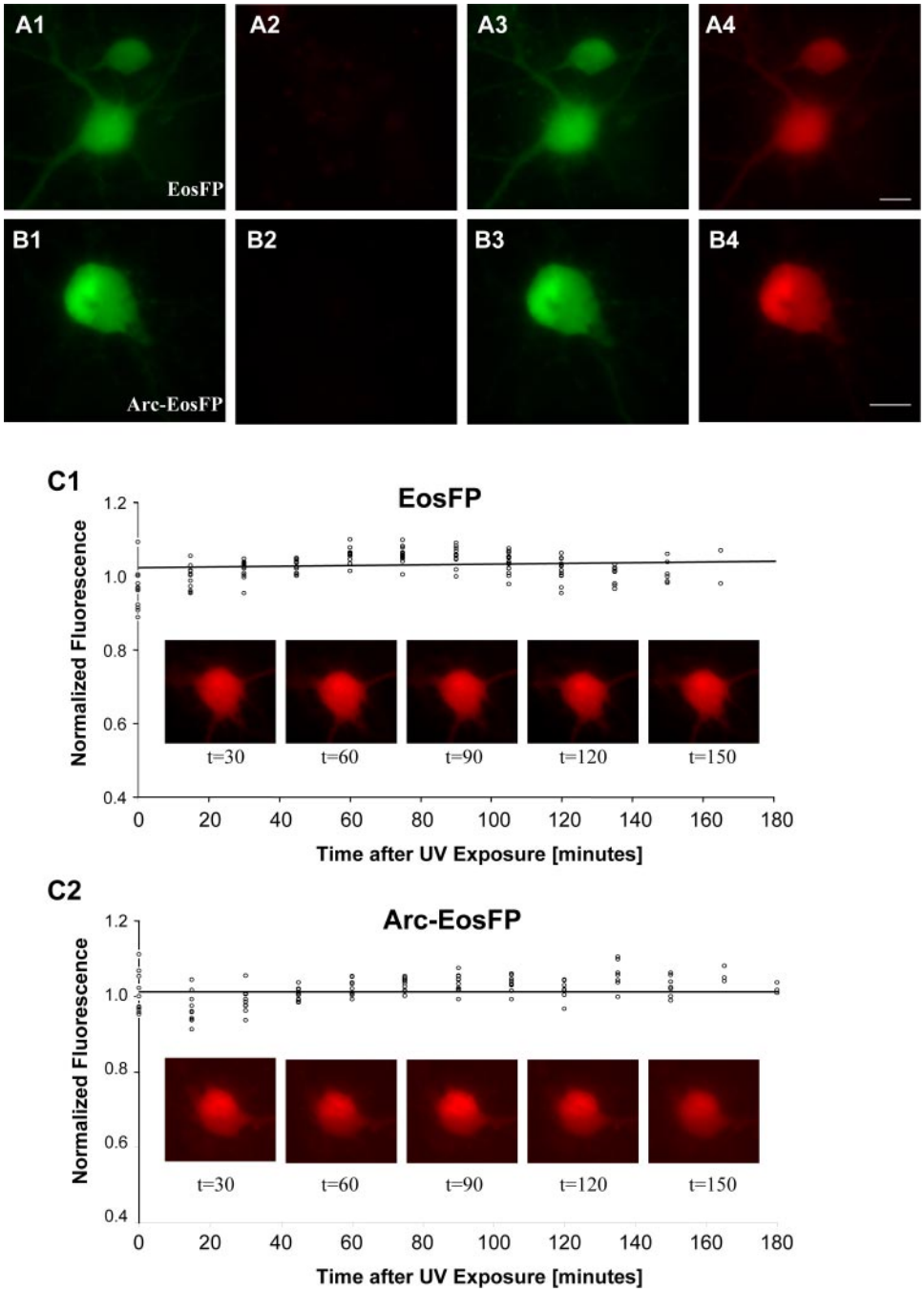

FIGURE 7. **EosFP and Arc-EosFP are stable proteins.** *A* and *B*, EosFP (*A1–4*) and Arc-EosFP (*B1–4*) were expressed in cultured hippocampal neurons. Neurons were imaged with green (*A1*, *B1*, *A3*, *B3*) and red filters (*A2*, *B2*, *A4*, *B4*) before (*A1*, *A2*, *B1*, *B2*) and after (*A3*, *A4*, *B3*, *B4*) UV treatment. The same exposure times were used for all cells. *Scales bars* are 10 μm. *C*, quantification of the red EosFP (*C1*) and red Arc-EosFP (*C2*) signals over 3 h. Images were acquired every 15 min to follow protein degradation. At least 10 cells were quantified per condition. *Inset*, pictures show an example cell for each condition at t = 30, 60, 90, 120, and 150 min after UV exposure. Red wild type EosFP and red Arc-EosFP signal do not diminish over 3 h, indicating that Arc protein does not undergo appreciable degradation over the time period used in the previously discussed experiments.

A PKA inhibitor blocked all of the agonist effects. In addition, a specific activator of Epac, the other major cAMP effector, failed to enhance Arc protein expression. Together these findings firmly implicate PKA activation as a necessary step in the receptor mediated up-regulation of Arc protein.

Translational Regulation of Arc—The effect of forskolin on Arc expression was inhibited by the translational inhibitor anisomycin, whereas the Arc mRNA levels remained the same in our system. In addition, Arc tagged with EosFP was stable over the time period tested. Taken together, these results show that Arc protein levels are regulated post-transcriptionally primarily at the level of translation in our system.

Translational regulation could occur at many different levels, including mRNA sequestration and polyadenylation as well as initiation, elongation, and termination. The up-regulation of translation during LTP is mediated primarily by regulation of initiation, whereas controlled elongation is involved to a lesser extent (44). A role for translation termination or cap-independent internal ribosome entry site-mediated initiation has not been shown to contribute to synaptic plasticity. The best-characterized example of synaptic activity-induced dendritic translation is the regulated polyadenylation of α-calmodulin kinase II, mediated by two cytoplasmic polyadenylation elements in its 3′-UTR (45). Arc mRNA does not contain any cytoplasmic polyadenylation element sequences and is, therefore, not likely to be regulated by this mechanism. PKA phosphorylation of the elongation factor eEF2 under conditions in which α-calmodulin kinase II is polyadenylated leads to a general decrease in protein synthesis (46). It has been proposed that phosphorylation of eEF2 may slow down elongation, making it the rate-limiting step of translation. This would favor the translation of abundant mRNAs that are poorly initiated, such as α-calmodulin kinase II or Arc (46, 47). However, in several of our experiments, Arc expression was driven by a short, optimal 5′-UTR for which initiation is expected to be efficient. Slower

Finally, MK-801 partially blocked the ability of forskolin to enhance Arc expression. This further implicates AC1, because the degree to which forskolin is able to activate this enzyme is modulated by calcium concentration (40). The involvement of AC1 in Arc expression may be particularly relevant since the Ca^{2+}/CaM-dependent ACs have been implicated in both the late-phase of LTP and memory (42).

elongation mediated by phosphorylated eEF2 can, therefore, not explain the increase in Arc translation, suggesting PKA regulates another factor involved in Arc protein synthesis.

AKAPS are scaffolding proteins that localize PKA to certain areas of the cell, including the nucleus and dendrites, and orient enzyme activity toward select substrates (48). PKA has also been implicated as playing a role in synaptic tagging. Pharmacological and genetic inhibition or activation of the cAMP/PKA pathway are sufficient to impair or produce a synaptic tag that leads to long-term LTP expression (49). Specific activation of PKA at synapses could contribute to local Arc translation. How PKA activation contributes to Arc translational regulation is unknown but may include direct phosphorylation of inhibitory proteins bound to its mRNA. RNA-binding proteins, including heterogeneous nuclear ribonucleoproteins (hnRNPs) and microRNAs (miRs) have been found in transport granules that also contain Arc mRNA (50).

In general, mRNAs within granules are translationally repressed during transit. After depolarization, some mRNAs, including α-calmodulin kinase II, are released from granules and associate with translationally active polyribosomes (51). However, the amount of Arc mRNA associated with polyribosomes does not change under these conditions, supporting the hypothesis that Arc is already associated with polyribosomes, but its translation is inhibited by another factor bound to its mRNA (52). Arc interacts with BC1, a non-coding miR associated with the hnRNP family member fragile X mental retardation protein (50). The Arc coding region also contains a putative binding domain for hnRNP A2 (53). Both fragile X mental retardation protein and hnRNP A2 are found in granules and are involved in RNA trafficking and translational repression (50, 54, 55). In addition, recent studies have shown a role for miRs in the translation of proteins involved in activity-dependent synaptic plasticity, learning, and memory (50, 56). The role of hnRNPs and miRs in Arc translation needs to be further explored.

Arc, LTP, Learning, and Memory—Arc plays a critical role both in the stabilization of LTP and in the consolidation of memory (4, 5, 27). The data shown here indicate that Arc expression is regulated by signaling pathways implicated in both of these processes. Whereas calcium influx through the NMDA receptor induces LTP, activation of the cAMP/PKA pathway is critical for the conversion of early-phase LTP (lasting 1–3 h) to late-phase LTP (lasting days or longer). Interestingly, Arc expression peaks during the beginning of late-phase LTP (at 4 h). In addition to the ability of β-adrenergic and D1/D5 dopaminergic receptor activation to lengthen the duration of LTP, activation of these receptors is implicated in mechanisms by which emotions affect memory (57). Dopamine signaling is associated with reward or pleasure, whereas β-adrenergic receptor signaling is associated with anxiety and stress. Similar to their effects on LTP stability, enhancing, or inhibiting these pathways leads to the equivalent alterations in memory (57). Our results have, therefore, revealed that the convergence and timing of signaling pathways regulating Arc expression display remarkable similarities with the processes that govern LTP and modulate learning and memory.

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