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Research Report

Activity-regulated cytoskeleton-associated protein Arc/Arg3.1 binds to spectrin and associates with nuclear promyelocytic leukemia (PML) bodies

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ABSTRACT

Activity-regulated cytoskeleton-associated protein (Arc/Arg3.1) is an immediate early gene, whose expression in the central nervous system is induced by specific patterns of synaptic activity. Arc is required for the late-phase of long-term potentiation (LTP) and memory consolidation, and has been implicated in AMPA receptor trafficking. Since Arc's molecular function remains incompletely understood, we have determined its subcellular localization in cultured hippocampal neurons and HEK 293T cells. Fluorescence microscopy experiments revealed that both endogenous and exogenous Arc protein was primarily found in the nucleus, where it concentrated in puncta associated with promyelocytic leukemia (PML) bodies, proposed sites of transcriptional regulation. Arc co-localized and interacted with the βIV spectrin splice variant βSpIVΣ5, a nuclear spectrin isoform associated with PML bodies and the nuclear matrix. A small region of Arc containing the coiled-coil domain is also restricted to β-spectrin-positive puncta, while the isolated spectrin homology domain is diffusely localized. Finally, Arc and βSpIVΣ5 synergistically increased the number of PML bodies. These results suggest that Arc functions as a spectrin-binding protein, forming a complex that may provide a role at sites of transcriptional regulation within the nucleus.

1. Introduction

Arc, also known as Arg3.1, is an immediate early gene (IEG) whose expression is strongly induced by neuronal activity patterns that elicit long-term alterations in synaptic strength (Link et al., 1995; Lyford et al., 1995). Arc is unique among IEGs because its mRNA is rapidly transported to distal dendrites and selectively localizes at activated synapses, where it has the potential to be locally translated (Steward and Worley, 2001; Steward et al., 1998). Arc plays a critical role in the late-phase of long-term potentiation (LTP) and is required for the consolidation of long-term memory. Induction of LTP in the hippocampus increases Arc protein expression, while blocking Arc expression by antisense oligonucleotide infusion or gene knockout causes defects in both late-phase LTP and memory tasks (Guzowski et al., 2000; Rodriguez et al., 2005; Plath et al., 2006). In addition, novel sound or taste stimuli, as well as spatial exploration of novel environments, rapidly induce Arc expression in a subset of neurons in the cortex and hippocampus (Guzowski et al., 1999; Montag-Sallaz et al., 1999; Bock et al., 2005; Chawla et al., 2005; Ramirez-Amaya et al., 2005).

It has been hypothesized that locally translated Arc interacts with existing cytoskeletal proteins, leading to

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specific modifications of synapses that are undergoing activity-dependent remodeling. Recently, Arc was proposed to regulate AMPA receptors trafficking (Chowdhury et al., 2006; Plath et al., 2006; Rial Verde et al., 2006; Shepherd et al., 2006). In addition, a number of findings suggest that Arc associates with the cytoskeleton. Arc interacts with CaMKII, leading to an increase in neurite length mediated by cytoskeletal remodeling (Donai et al., 2003). Arc expression also reduces the immunoreactivity of microtubule-associated protein 2 (MAP2), a protein associated with the actin cytoskeleton (Fujimoto et al., 2004). Finally, Arc co-sediments with actin in crude cell extracts and has been proposed to share homology with α-spectrin (Lyford et al., 1995). However, Arc has not yet been shown to directly interact with a component of the cytoskeleton. In addition to its synaptic localization, Arc protein has been found in the cell body and nucleus (Irie et al., 2000), which may contain a cytoskeletal structure known as the nuclear matrix (Tsutsui et al., 2005). While a role for Arc in the dendritic compartment has recently been defined, Arc's role in the nucleus remains poorly understood.

In order to further study the function of Arc protein, its subcellular localization was determined in cultured hippocampal neurons and HEK 293T cells. Arc was found primarily in the nucleus and concentrated in puncta associated with promyelocytic leukemia (PML) bodies, which are proposed sites of transcriptional regulation (Wang et al., 2004). Arc co-localized and directly interacted with βSpIVΣ5, a spectrin associated with PML bodies and the nuclear matrix (Tse et al., 2001). Finally, co-expression of Arc and βSpIVΣ5 increased the number of PML bodies in HEK 293T cells, suggesting a role for this complex in PML body function.

2. Results

In earlier experiments using rats that received electric stimulation, Arc protein was localized by antibody staining to the cell body, nucleus, and dendrites of neurons in hippocampal slices (Steward and Worley, 2001). Here we have re-examined the subcellular localization of Arc in cultured hippocampal neurons, which provide superior resolution.

2.1. *Localization of Arc protein in hippocampal neurons*

The distribution of Arc protein in hippocampal neurons was first investigated using a construct in which the Arc coding region was fused to enhanced yellow fluorescent protein (EYFP). The Arc-EYFP construct contained full-length 5′ and 3′ untranslated regions (UTRs) of the Arc mRNA, in order not to disturb UTR-dependent translation and trafficking processes. Arc-EYFP was co-expressed with ECFP, which allows the subcellular enrichment of Arc to be accurately evaluated by comparing the yellow and cyan fluorescence signals. While ECFP filled the entire cell, including the dendrites, Arc was primarily found in the cell body, where it accumulated in a region that resembled the nucleus (Fig. 1).

In order to compare the localization of exogenous and endogenous Arc, immunofluorescence experiments were performed using an Arc-specific antibody. Basal levels of Arc expression are low in the hippocampus and are upregulated by activity (Link et al., 1995; Lyford et al., 1995). Therefore, in order to obtain an immunofluorescence signal, the neurons were treated with forskolin, an activator of adenylyl cyclases (ACs), which has previously been shown to upregulate Arc mRNA synthesis (Waltereit et al., 2001). Four hours after forskolin stimulation, the neurons were fixed and stained with the Arc antibody (Fig. 2A). Endogenous Arc protein was localized primarily in the nucleus, as shown by its co-localization with the nuclear Hoechst dye, while significantly lower levels were found in the dendritic compartment. A similar distribution was recently reported by Chowdhury et al. (2006). Quantification of the antibody signal within and outside the nucleus indicated that endogenous Arc protein levels were 7.7-fold higher in the nuclear compartment. Forskolin treatment may change the localization of endogenous Arc protein, although the majority of Arc protein expression requires stimulation (Lyford et al., 1995; Steward and Worley, 2001).

Next, neurons were transfected with a plasmid encoding full length Arc mRNA, in which the coding region was flanked by intact UTRs (Fig. 2B). Expressed Arc protein was detected by immunofluorescence using the Arc-specific antibody. Although the expressed Arc mRNA contains a full length 3′ UTR, which has previously been shown to be important in dendritic

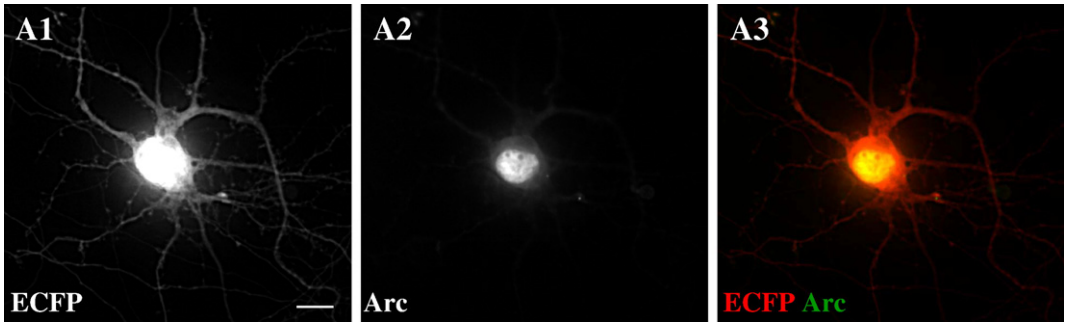

Fig. 1 – Arc is primarily found in the nucleus of hippocampal neurons. Hippocampal neurons were co-transfected with ECFP and Arc tagged with EYFP. Neurons were imaged live 16-h post-transfection. ECFP (A1) localized throughout the neuron, showing the overall morphology of the cell, while Arc (A2) was found primarily in the cell body, with lower levels in the dendrites. A3 shows the merged image of A1 and A2. Scale bar is 10 μm.

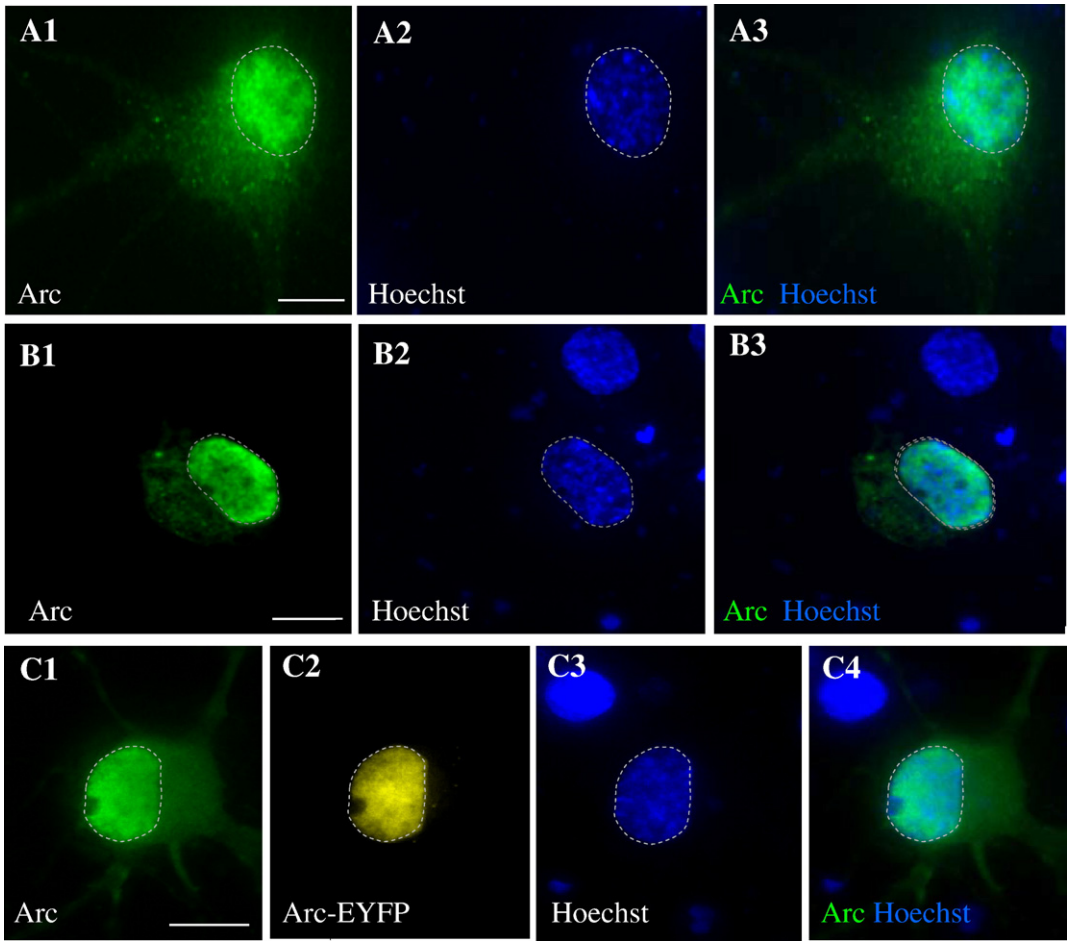

Fig. 2 – Both endogenous and exogenous Arc localize primarily to the nucleus. (A) Endogenous Arc expression was induced in cultured hippocampal neurons with 50 μM forskolin for 4 h. The cells were fixed, permeabilized, and Arc protein was detected with an Arc-specific primary antibody (C7) and an AlexaFluor488-conjugated secondary antibody (A1). Nuclei were stained with the Hoechst 33258 dye (A2) and are outlined by a dashed gray line in all panels. The merged image (A3) shows that endogenous Arc protein is found primarily in the nucleus. The Arc antibody signal is 7.7-fold stronger in the nucleus than outside (*N*=15, SEM=0.77). (B) Hippocampal neurons were transfected with untagged Arc. After 16 h, the neurons were fixed, permeabilized, and stained with the Arc and Alexafluor488-conjugated antibodies (B1). The nucleus was stained with Hoechst dye (B2). The merged image (B3) shows that exogenous Arc is found primarily in the nucleus. Arc antibody signal is 7.9-fold stronger in the nucleus than outside (*N*=15, SEM=0.37). (C) EYFP-tagged Arc was expressed for 16 h, after which the neurons were fixed, permeabilized, and stained with the Arc and Alexafluor488-conjugated antibodies (C1). Arc-EYFP signal (C2). Nucleus stained with the Hoechst dye (C3). A composite of the antibody signal and Hoechst image (C4) shows that Arc is localized primarily in the nucleus. Arc antibody signal is 7.6-fold higher in the nucleus than outside (*N*=15, SEM=0.64). Scale bars are 10 μm.

targeting (Kobayashi et al., 2005), the majority of exogenous Arc protein was localized to the nucleus. Similar to what was found for endogenous Arc, exogenous Arc concentrated 7.9-fold in the nucleus. In order to compare the antibody staining with the localization of exogenously expressed Arc protein, the EYFP-tagged version of Arc was expressed in hippocampal neurons. Arc-EYFP displayed the same subcellular distribution as the untagged Arc protein labeled by antibody (Fig. 2C). Arc-EYFP was enriched in the nucleus 7.6-fold. These results show that the subcellular localization of endogenous and exogenous Arc is comparable, with both proteins being predominantly found in the nucleus. In addition, it appears that the EYFP tag does not disrupt Arc's localization.

2.2. *Nuclear Arc is localized to puncta*

Arc-EYFP and ECFP were also co-expressed in HEK 293T cells (Fig. 3A), which have been used extensively in studies of subnuclear domains. The subcellular distribution of Arc protein in HEK 293T cells was similar to that seen in hippocampal neurons; the majority of Arc was found in the nucleus. Interestingly, Arc concentrated in several discrete nuclear puncta. This finding raised the question whether Arc could be associated with similar puncta in hippocampal neurons. It has previously been shown that stimulation is needed not only for the induction of Arc gene expression, but also for proper localization of both its mRNA and protein (Steward and Worley, 2001). Hippocampal neurons co-

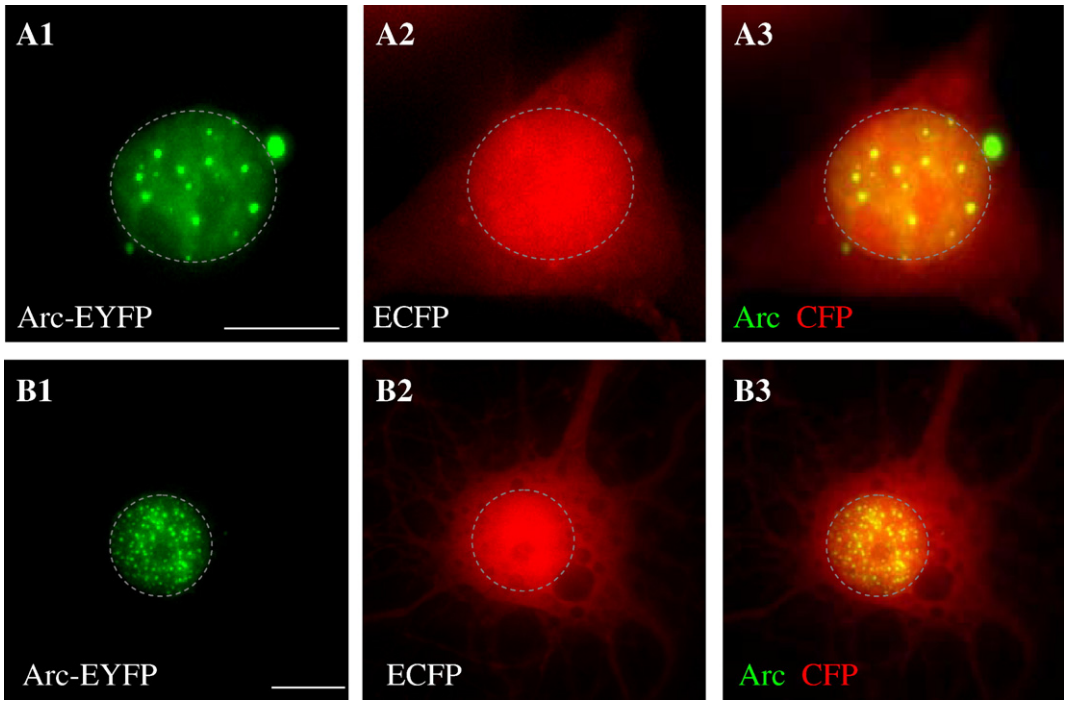

Fig. 3 – Arc concentrates in discrete nuclear puncta. (A) Arc-EYFP (A1) and ECFP (A2) were co-expressed (A3) in HEK 293T cells overnight for 16 h. Arc was found primarily in the nucleus (outlined by the gray dashed circle) and concentrated in discrete puncta. Scale bar is 5 μm. (B) Arc-EYFP (B1) and ECFP (B2) were co-expressed (B3) in cultured hippocampal neurons overnight for 16 h. The following day, neurons were stimulated with 50 mM forskolin for 4 h. Arc was found primarily in the nucleus, where it concentrated in discrete puncta. Scale bar is 10 μm.

expressing Arc-EYFP and ECFP were therefore stimulated by treatment with forskolin. In these experiments, Arc-EYFP displayed a punctate subnuclear distribution similar to that seen in HEK 293T cells (Fig. 3B).

The size and number of Arc puncta suggested they corresponded to either promyelocytic leukemia (PML) bodies or nuclear speckles (Spector, 2001). Cells generally contain between 10 and 30 PML bodies and between 25 and 50 speckles. PML bodies have been implicated in many nuclear functions, including transcriptional control, and contain numerous transcription factors, while speckles are involved in pre-mRNA splicing and contain many splicing factors, including the spliceosome assembly factor SC35. We next set out to determine if Arc associates with one of these two subnuclear domains.

2.3. *Arc is associated with PML bodies*

In order to investigate the molecular nature of the nuclear Arc puncta, Arc-EYFP was expressed in HEK 293T cells, and its localization was compared with that of PML and SC35. PML and SC35, visualized using specific antibodies, were found in distinct nuclear domains (Figs. 4A, B). The Arc puncta partially co-localized with PML bodies, while they were only found in proximity to SC35 speckles. Co-localization was quantified using the Pearson's correlation coefficient (*r*), which indicated that Arc was co-localized with PML ($r=0.77$), but not with SC35 ($r=0.25$). An *r* value of 1.0 depicts 100% co-localization. Additional experiments in which Arc-EYFP and cherry-PML were co-expressed in HEK 293T cells resulted in puncta that were closely associated ($r=0.78$, Fig. 4C). In both conditions, all Arc puncta were associated with PML puncta, and vice versa. The Pearson's coefficients, which are pixel-based, therefore indicate that these two structures only partially overlap.

When similar experiments were performed in hippocampal neurons, it was found that both PML and SC35 antibodies showed a ubiquitous staining pattern; neither of the antibodies specifically labeled the nucleus (data not shown). A similar staining pattern has been shown previously for SC35 in cultured hippocampal neurons (Glanzer et al., 2005), while PML bodies have not been recognized immunohistochemically in rat neurons (Lam et al., 1995; Hayashi et al., 2001). Although this discrepancy may suggest that neurons do not contain PML bodies, these structures have been shown to exist in human neurons (Yamada et al., 2001). It therefore appears that PML bodies do exist in neurons, but that available antibodies, which have been raised to human epitopes, do not recognize rat PML in neurons. Given the antibody recognition problems and the insoluble nature of Arc, which is most likely due to its association with the cytoskeleton, co-immunoprecipitation (co-IP) of endogenous Arc with endogenous PML would be very difficult. Therefore, the subcellular localization of exogenous PML tagged with the red fluorescent protein (cherry) was determined in neurons. When Arc-EYFP and cherry-PML were co-expressed in cultured hippocampal neurons (Fig. 5A), both proteins concentrated in nuclear puncta, which partially co-localized ($r=0.80$). Finally, we determined whether endogenous Arc could co-localize with

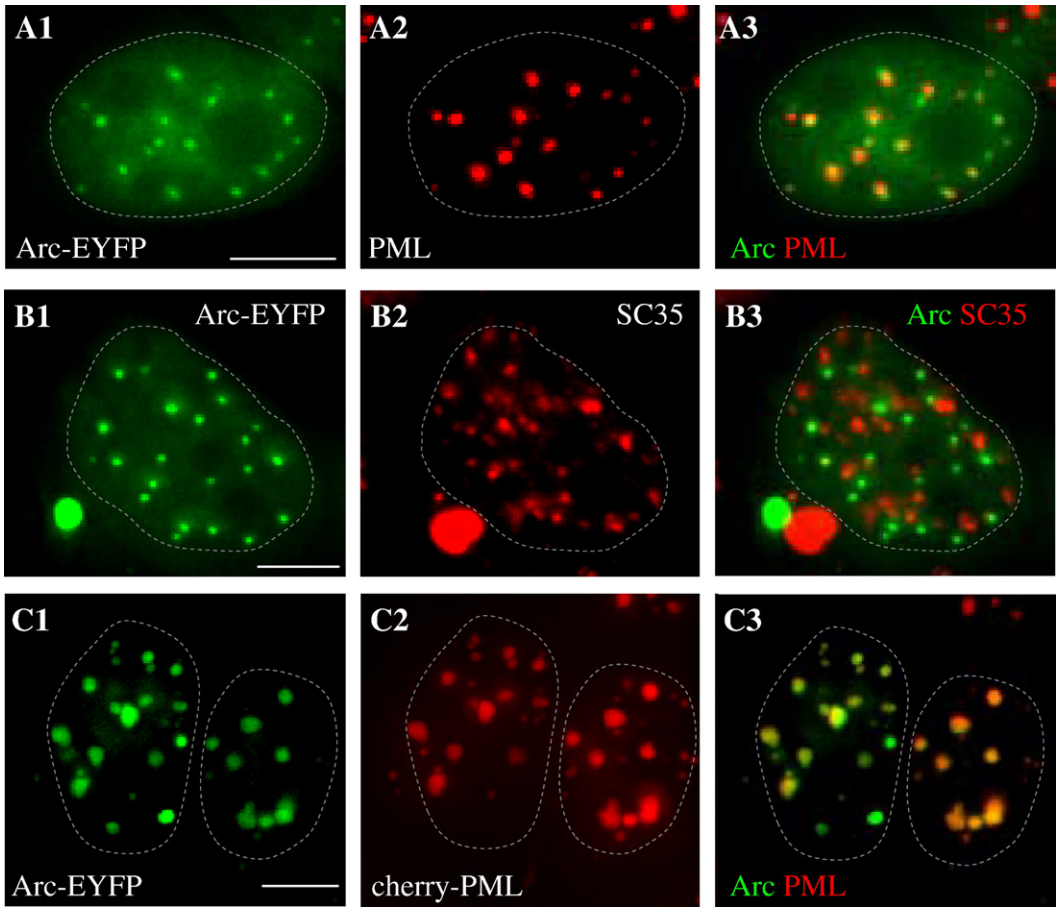

Fig. 4 – Arc associates with PML bodies. (A) Arc-EYFP was expressed in HEK 293T cells (A1) and endogenous PML bodies were labeled with a PML primary antibody (PG-M3) and AlexaFluor568-conjugated secondary antibody (A2). The merge image shows association of the Arc puncta with PML bodies (A3). The Pearson's correlation coefficient for Arc and PML is 0.77, where 1.0 implies 100% co-localization (N=25, SEM=0.01). (B) Arc-EYFP was expressed in HEK 293T cells (B1) and endogenous nuclear speckles were labeled with a SC35 primary antibody and AlexaFluor568-conjugated secondary antibody (B2). The merge image shows Arc puncta and SC35 speckles are not associated (B3). The Pearson's correlation coefficient for Arc and SC35 is 0.25 (N=17, SEM=0.018). (C) Arc-EYFP (C1) and cherry-PML (C2) were co-expressed in HEK 293T cells. The merge image shows their partial co-localization in the nucleus (C3). The Pearson's correlation coefficient for Arc and PML is 0.78 (N=47, SEM=0.016). Scale bars are 5 μm.

exogenous PML in neurons. Neurons expressing cherry-PML were treated with forskolin to induce Arc expression, fixed and labeled with the Arc antibody (Fig. 5B). Whereas endogenous Arc is normally present throughout the nucleus (Fig. 2A), expression of exogenous PML resulted in puncta that recruited Arc (r=0.78). In both cases Arc protein concentrated near and partially co-localized with all the exogenous PML puncta, therefore the Pearson's coefficient depicts partial co-localization or association of the two proteins. These results show that endogenous and exogenous Arc is enriched near PML-containing bodies in the nuclei of both HEK 293T cells and hippocampal neurons. Next we wanted to determine whether Arc directly interacts with a protein that associates with PML bodies.

2.4. *Arc interacts with βSpIVΣ5*

A portion of Arc has been previously proposed to share homology with α-spectrin (Lyford et al., 1995). α-spectrins form complexes with β-spectrins that can bind actin, calmodulin, ankyrin, and numerous other proteins (Bennett and Gilligan, 1993). We therefore investigated whether Arc, acting as an α-spectrin, could bind to a β-spectrin. An interesting candidate was the Σ5 splice variant of βIV spectrin (βSpIVΣ5), which has been shown to associate with nuclear PML bodies and the nuclear cytoskeleton, also known as the nuclear matrix (Tse et al., 2001). We therefore expressed Arc-EYFP and βSpIVΣ5-ECFP, either alone or together, in hippocampal cultures and HEK 293T cells (data not shown) in order to evaluate their association. The localization of βSpIVΣ5 protein in neurons has not been previously shown, even though the transcript was found predominantly in the brain of developing mouse embryos (Tse et al., 2001). Our results show that Arc alone was primarily nuclear, while βSpIVΣ5 was found in the nucleus and the dendrites in a punctate manner (Figs. 6A, B). When both proteins were co-expressed, they tightly co-localized in nuclear puncta (Fig. 6C).

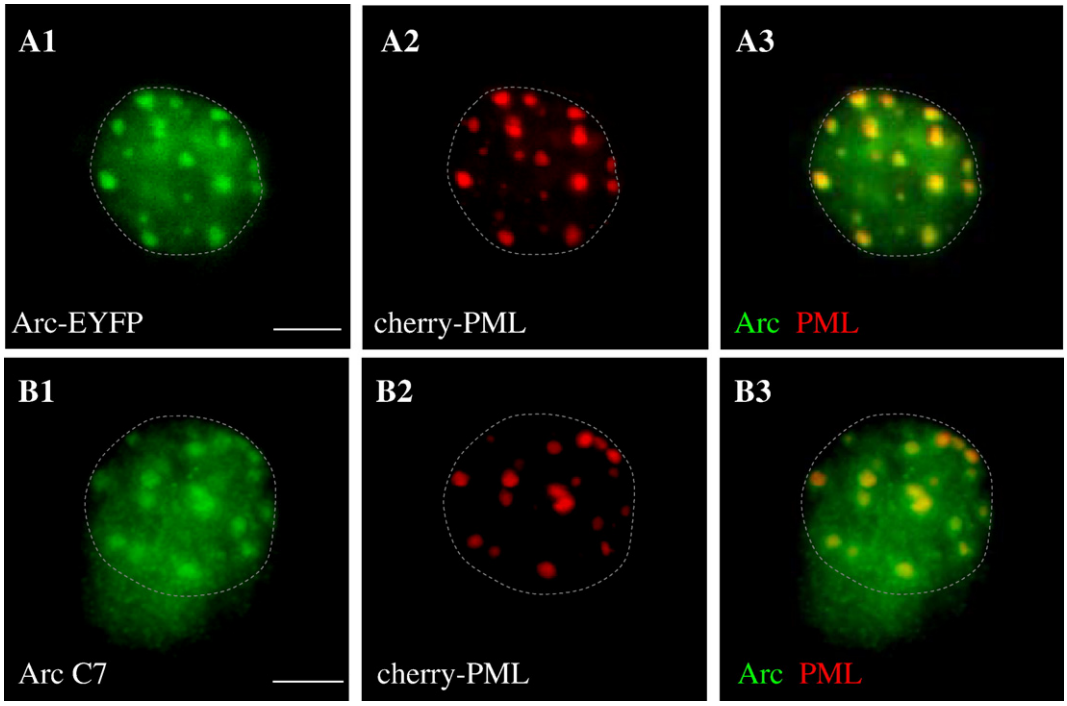

Fig. 5 – Arc associates with PML bodies in hippocampal neurons. (A) Arc-EYFP (A1) and cherry-PML (A2) were co-expressed in cultured hippocampal neurons. The merged image (A3) shows their partial co-localization in the nucleus. The Pearson's correlation coefficient for Arc and PML is 0.80 (*N*=14, SEM=0.016). (B) Cherry-PML was expressed in cultured neurons overnight for 16 h (B2). The next day endogenous Arc expression was induced with 50 mM forskolin for 4 h, and the protein was detected with the Arc specific antibody and AlexaFluor488-conjugated secondary antibody (B1). The merged image (B3) shows their partial co-localization in the nucleus, with Arc being enriched near PML-positive structures. The Pearson's correlation for endogenous Arc and over-expressed PML is 0.78 (*N*=19, SEM=0.013). Scale bars are 5 mm.

Next, Arc-EYFP, βSpIVΣ5-ECFP, and cherry-PML were all three co-expressed in neurons (Fig. 7A). Arc and βSpIVΣ5 tightly co-localized to the same puncta, while PML bodies were found adjacent to the Arc/βSpIVΣ5 complexes (Fig. 7B). The extent of co-localization was quantitated using the Pearson's correlation coefficient, which resulted in values of 0.96 for Arc and βSpIVΣ5, 0.74 for Arc and PML, and 0.75 for βSpIVΣ5 and PML. Arc and βSpIVΣ5 were always found in the same puncta, while PML puncta were always partially associated with the Arc-βSpIVΣ5 puncta. Similar results were obtained with HEK 293T cells (data not shown). The tight co-localization seen between Arc and βSpIVΣ5 in both HEK 293T cells and hippocampal neurons suggested that these two proteins might bind to each other. This hypothesis was investigated using GST-pulldown and BRET^2 assays.

2.5. *Arc directly associates with βSpIVΣ5*

Native Arc protein is very poorly soluble; therefore, it was difficult to test a physical interaction between Arc and βSpIVΣ5 using co-IP experiments. To circumvent this problem an alternative approach was used, in which Arc was expressed as a fusion protein with glutathione-S-transferase (GST). GST-tagged Arc bound to glutathione-agarose was incubated with HEK 293T cell lysates expressing βSpIVΣ5, tagged either N- or C-terminally with green fluorescent protein (GFP). Bound proteins were separated by SDS-PAGE and βSpIVΣ5 was detected with a GFP antibody. Both βSpIVΣ5 GFP fusions bound to GST-Arc, while they did not bind to GST alone (Fig. 8A). These GST-pulldown experiments indicated that Arc and βSpIVΣ5 are in the same complex, although not necessarily through a direct interaction.

To investigate whether Arc binds directly to βSpIVΣ5, a bioluminescence resonance energy transfer (BRET^2) assay was performed. A BRET^2 signal requires that proteins are within 10–100 Å of each other. This assay can therefore be used to verify a direct interaction of two proteins in living cells, without the problems inherent to co-IPs. This assay also circumvents the main problem associated with an Arc co-IP, namely the challenge of solubilizing Arc without disturbing its interactions with other proteins. Plasmids encoding Arc fused to Renilla luciferase (Rluc) and βSpIVΣ5 fused to GFP^2 were co-expressed in HEK 293T cells. Rluc-Arc and GFP^2-βSpIVΣ5 exhibited a strong BRET^2 signal that increased with expression of GFP^2-βSpIVΣ5, while negative controls did not (Fig. 8B). These results show that Arc directly interacts with βSpIVΣ5 in live HEK 293T cells. Since βSpIVΣ5 is known to associate with the nuclear matrix, this is the first cytoskeletal interaction identified for Arc.

2.6. *The Arc–spectrin interaction does not require Arc's spectrin homology domain*

In order to determine the region of Arc protein that is responsible for the βSpIVΣ5 interaction, Arc deletion constructs

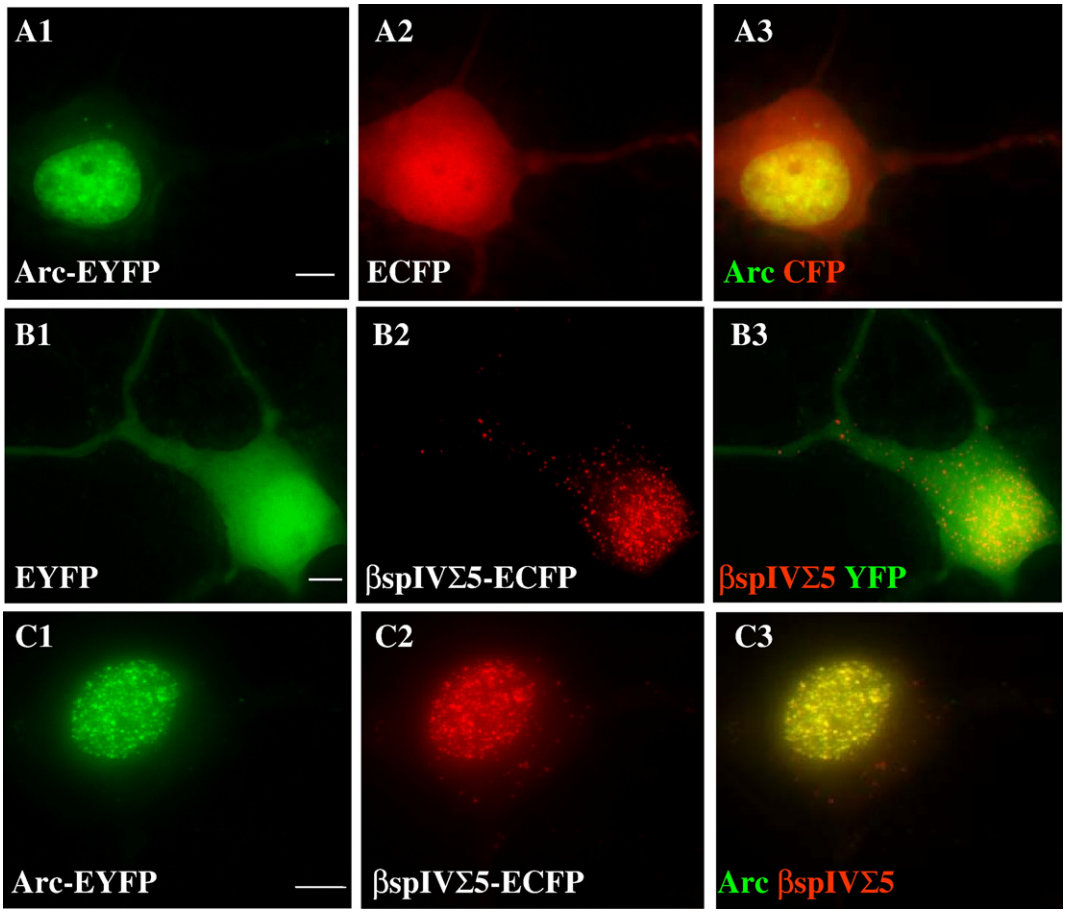

Fig. 6 – Arc and βSpIVΣ5 co-localize in cultured hippocampal neurons. (A) Arc-EYFP (A1) and ECFP (A2) were co-expressed in cultured hippocampal neurons. The merge image (A3) shows the primarily nuclear localization of Arc. (B) βSpIVΣ5-ECFP (B1) and EYFP (B2) were co-expressed in cultured hippocampal neurons. The merge image (B3) shows the punctate nuclear and dendritic localization of βSpIVΣ5. (C) Arc-EYFP (C1) and βSpIVΣ5-ECFP (C2) were co-expressed in cultured neurons. The merge image (C3) shows their tight co-localization. The Pearson's correlation coefficient for Arc and βSpIVΣ5 is 0.96 (*N*=34, SEM=0.003). Scale bars are 5 μm.

tagged with EYFP were expressed in cultured neurons along with βSpIVΣ5-ECFP. The Arc coding region (CR) consists of 396 amino acids (aa). Arc NT includes the N-terminus of the protein, aa 1–25, which shows no homology with α-spectrin. Arc CC includes aa 26–154, and contains a putative coiled-coil domain. Using the COILS software, Arc was predicted to have a coiled-coil domain from amino acid 49 to 79 (score=0.96) (Lupas et al., 1991). Arc CT (aa 155–396) contains the C-terminal portion of Arc previously suggested to share homology with α-spectrin (aa 228–380) (Lyford et al., 1995; Bock et al., 2005). It was hypothesized that Arc constructs containing the spectrin homology domain would co-localize with βSpIVΣ5. However, when expressed in cultured neurons, both full length Arc (CR) and the coiled-coil construct (CC) specifically and strictly co-localized with βSpIVΣ5 to the same puncta, while both the N-terminus and C-terminal spectrin homology domain were diffusely localized (Fig. 9). The *r* values were 0.96 (CR), 0.12 (NT), 0.97 (CC), and 0.17 (CT). These data are consistent with the Arc coiled-coil domain forming the primary spectrin binding region.

2.7. *Arc and βSpIVΣ5 affect PML body number*

Arc-EYFP and βSpIVΣ5-ECFP were expressed in HEK 293T cells and the number of endogenous PML bodies was determined by antibody staining (Fig. 10). In our experiments, Arc-EYFP or βSpIVΣ5-ECFP alone did not significantly increase the number of endogenous PML bodies. Interestingly, when both proteins were co-expressed, they synergistically increased the number of PML bodies by 85%. These results show that Arc and βSpIVΣ5 together regulate PML body number and may therefore have an effect on PML body function, which includes transcriptional regulation. PML bodies can be modulated by a number of factors including viral infection and the interferon response, cell cycle progression, DNA damage, and the over-expression of a subset of PML body-containing proteins (Chang et al., 1995; Regad and Chelbi-Alix, 2001; Boisvert et al., 2001; Dellaire and Bazett-Jones, 2004). In our experiments, a viral CMV promoter was used to drive protein expression. However, the control EYFP and ECFP expression did not affect PML body number. Over-expression of some proteins found in PML bodies, including PML itself and Sp100, can increase PML

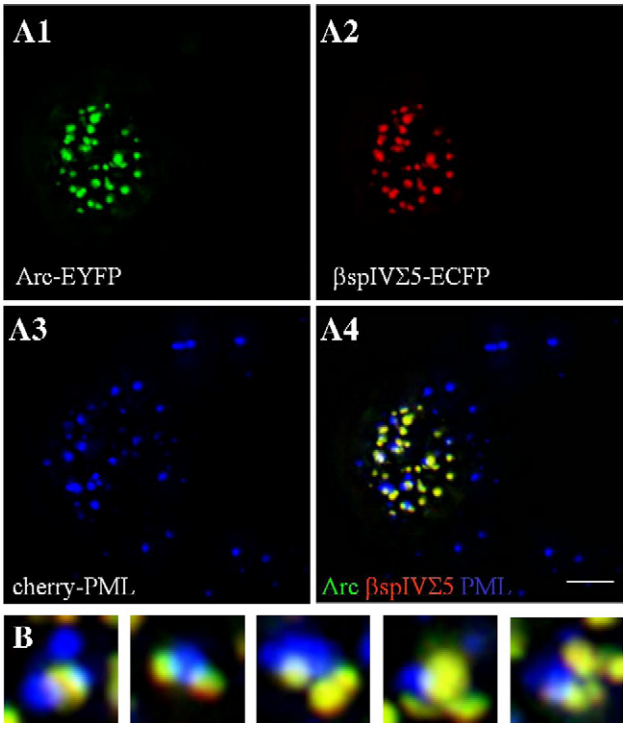

Fig. 7 – Arc and βSpIVΣ5 co-localize and associate with PML bodies in hippocampal neurons. (A) Arc-EYFP (A1), βSpIVΣ5-ECFP (A2), and cherry-PML (A3) were expressed in hippocampal neurons. The merged image (A4) shows their co-localization. (B) Enlarged areas of A4 show that Arc and the spectrin isoform strictly overlap, while the Arc/spectrin complex associates with PML-positive structures. Pearson's coefficients are: 0.96 for Arc-βSpIVΣ5, 0.74 for Arc-PML, and 0.75 for βSpIVΣ5-PML (*N*=10, SEM=0.020, 0.025, and 0.035 respectively). Scale bar is 10 μm.

body number. However, Arc or βSpIVΣ5 alone does not significantly affect PML body number. In addition, it appears that Arc and βSpIVΣ5 are not actual constituents of PML bodies (because they only partially co-localize with PML), but rather associate with these structures, possibly mediating their association with the nuclear matrix.

3. Discussion

The subcellular localization of Arc protein was investigated in detail using HEK 293T cells and hippocampal neurons. In both cell types, Arc was found to accumulate in the nucleus, where it formed a complex with a β-spectrin isoform. Furthermore, we found that Arc and β-spectrin associated with PML bodies, both individually and as a complex.

3.1. *PML bodies and spectrin*

Exogenous Arc associated with both endogenous and exogenous PML bodies in the nucleus of HEK 293T cells. In hippocampal neurons, endogenous and exogenous Arc formed nuclear puncta that associated with exogenous PML. Exogenous PML protein expressed in neurons, both alone and with Arc, formed structures that resembled PML bodies. Whereas endogenous Arc is normally found throughout the nucleus, expression of exogenous PML caused a redistribution of nuclear Arc into puncta that were associated with the PML bodies. These consequences of PML expression strongly suggest that PML bodies can form in rat neurons. The notion that Arc associates with PML bodies is further supported by Arc's interaction with βSpIVΣ5, a protein known to be associated with PML bodies and the nuclear matrix.

It was found that Arc and βSpIVΣ5 formed complexes in the nucleus of both neurons and HEK 293T cells. The Arc–βSpIVΣ5 interaction was direct and required a portion of the protein containing a coiled-coil domain, but not the spectrin homology domain. This result was surprising, since the interaction between α and β subunits is facilitated by their spectrin repeats (Speicher et al., 1992). It is possible that Arc's spectrin homology domain interacts with other spectrin repeat-containing proteins. In addition, it is also possible that the coiled-coil domain of Arc has multiple binding partners, including other spectrin proteins. For example, αSpII and βSpII localize to the nucleus, while βSpIΣ2, βSpIIΣ1, and βSpIII are found in spines (Tse et al., 2001; Baines et al., 2001; Young and Kothary, 2005), two Arc-containing subcellular domains. In addition, other splice variants of βIV spectrin may interact with Arc (Berghs et al., 2000; Komada and Soriano, 2002). An absolute minimal domain for the Arc–spectrin interaction needs to be identified. Arc was recently found to interact with endophilin and dynamin, two proteins involved in clathrin-mediated receptor endocytosis (Chowdhury et al., 2006). The region in Arc necessary for dynamin binding includes aa 195–214, while endophilin binding requires aa 89–100, which is found in the coiled-coil (CC) Arc construct. It will be interesting to determine whether βSpIVΣ5 binds to the endophilin-binding site. If not, Arc may be able to bind both proteins simultaneously.

3.2. *A nuclear function of Arc and βSpIVΣ5*

Arc and βSpIVΣ5 synergistically increased the number of PML bodies in HEK 293T cells. This result suggests that βSpIVΣ5 and Arc function together in a complex that regulates points of contact between the nuclear matrix and PML bodies. Arc and βSpIVΣ5 may determine the location and number of PML bodies in the nucleus, which in turn could alter their function. More than 60 proteins with many different functions are known to localize in PML bodies (Negorev and Maul, 2001; Eskiw and Bazett-Jones, 2002). PML bodies have been implicated in transcription, DNA replication and repair, apoptosis, tumor suppression, proteolysis, and the antiviral response (Lallemand-Breitenbach et al., 2001; Salomoni and Pandolfi, 2002; Takahashi et al., 2003; Dellaire and Bazett-Jones, 2004; Regad and Chelbi-Alix, 2001; Zhong et al., 2000). Multiple transcription factors are found within these structures where they are regulated by post-translational modifications. PML bodies may play a role in transcriptional regulation by affecting the availability and activation state of transcription factors. These factors include CBP and p300, which have histone acetyltransferase

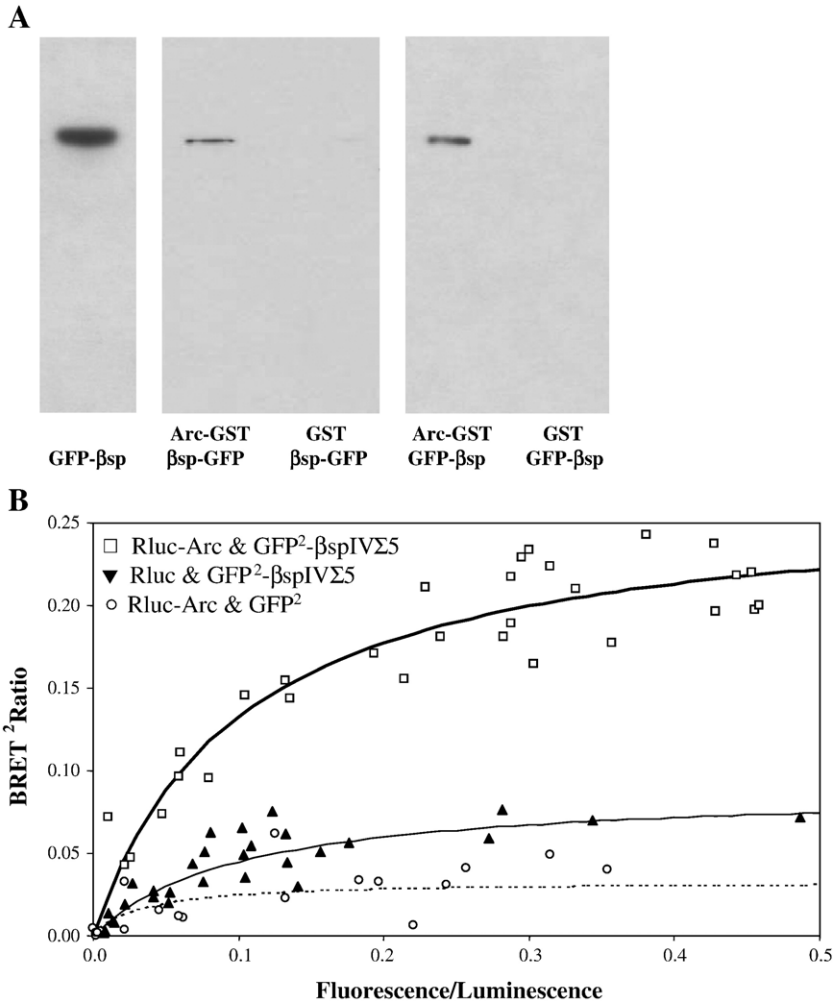

Fig. 8 – Arc directly interacts with βSpIVΣ5. (A) GST-Arc and GST, purified from bacterial lysate using glutathione-agarose beads, were incubated with HEK 293T cell lysates expressing βSpIVΣ5-GFP or GFP-βSpIVΣ5. The bound proteins were separated by SDS-PAGE and blotted with a GFP antibody. The first lane is HEK 293T cell lysate expressing GFP-βSpIVΣ5. Arc-GST (lanes 2 and 4) but not GST alone (lanes 3 and 5) interacts with both versions of GFP tagged βSpIVΣ5. (B) HEK 293T cells were co-transfected with Rluc-Arc and GFP²-βSpIVΣ5, Rluc and GFP²-βSpIVΣ5, or Rluc-Arc and GFP². The BRET² ratio, GFP fluorescence, and Rluc luminescence were measured 48 h after transfection. BRET² levels are plotted as a function of the fluorescence/luminescence ratio. The data shown represent pooled individual readings from three independent experiments. The curves were fitted using the following Langmuir equation: $BR = BR_{max}/(1 + FL_{50}/FL)$, in which BR = Bret ratio, BR_{max} = maximum value of BR, FL = Fluorescence/Luminescence ratio, FL_{50} = half maximum FL.

(HAT) activity, necessary for unwinding DNA prior to transcription. The Arc-βSpIVΣ5 complex localizes to the periphery of PML bodies, where transcription has been proposed to occur (Boisvert et al., 2000). It will be interesting to determine if Arc-βSpIVΣ5 complexes have a role in histone acetylation or transcription.

Although Arc is important in LTP stabilization and memory consolidation, its specific molecular role in these processes is unknown. The late-phase of LTP requires both transcription and translation. A portion of new protein synthesis occurs locally at activated synapses, allowing alteration of the molecular composition of specific dendritic spines (Flexner et al., 1963; Davis and Squire, 1984). Arc may play multiple roles in LTP and memory, both in the nucleus, through transcriptional regulation, and at activated synapses, where it could act as a scaffold to concentrate proteins at the plasma membrane. Since the vast majority of the protein is localized to the nucleus, it is likely that Arc has a substantial function there. It remains to be determined whether such a nuclear function is critical for Arc's role in learning and memory. An intriguing possibility is suggested by the recent proposal that synaptic plasticity and memory formation involve long-term alterations of chromatin structure, and subsequent epigenetic changes in gene expression patterns (Hsieh and Gage, 2005; Levenson and Sweatt, 2006). By altering the acetylation status of histones, HATs localized to PML bodies may play a critical role in this process (Alarcon et al., 2004; Korzus et al., 2004).

The recent construction of an Arc knockout mouse implicates Arc in AMPA receptor trafficking. The Arc knockout

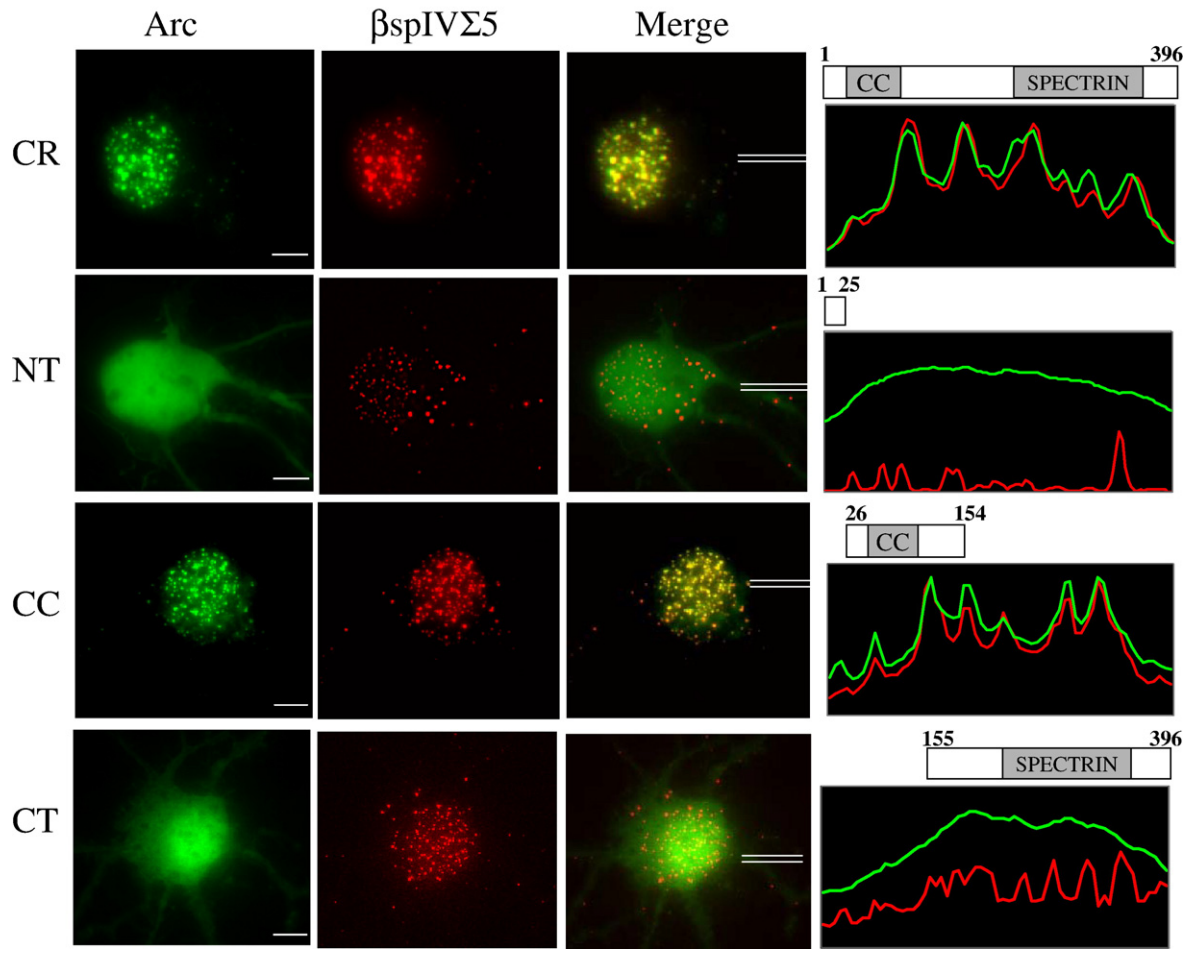

Fig. 9 – Arc's spectrin homology domain is not necessary for the Arc–βSpIVΣ5 interaction. Arc deletion constructs tagged C-terminally with EYFP were co-expressed with βSpIVΣ5-ECFP in cultured neurons. The full-length coding region (CR) of Arc is 396 amino acids. The N-terminal domain of Arc (NT) consisted of the first 25 amino acids (aa). The portion of Arc containing a coiled-coil region (CC) is contained within the aa 26–154 construct. The C-terminal (CT) region of Arc that has previously been proposed to have homology with spectrin is contained in the aa 155–396 construct. Only Arc containing the coiled-coil region co-localized with βSpIVΣ5. Double white bars on the merged picture indicate the *y*-position and width of the line profile graphs of Arc-EYFP (green) and βSpIVΣ5-ECFP (red). For constructs that contain the coiled-coil region, the Arc and βSpIVΣ5 fluorescence signals (*y*-axis) show maxima at the same position (*x*-axis). The Pearson's coefficients for βSpIVΣ5 and the Arc truncations were: 0.96 (CT), 0.12 (NT), 0.97 (CC), and 0.17 (CT), with SEMs of 0.003, 0.020, 0.007, and 0.013, respectively). Scale bars are 5 μm.

mouse has enhanced early-phase LTP, which is explained by the mouse's increase in synaptic AMPA receptors (Plath et al., 2006). In addition, the knockout animals are deficient in late-phase LTP, which led the authors to conclude that "the finding that Arc is required for the maintenance of plasticity and memory is not easily explained by altered AMPA receptor insertion but is likely to entail additional mechanisms" (Plath et al., 2006). Interestingly, the concurrent study that identified the Arc–endophilin and dynamin interactions proposed a mechanism in which endosomes, containing endocytosed AMPA receptors and Arc, traffic back to the nucleus to deliver a signal (Chowdhury et al., 2006). This retrograde endosomal trafficking could explain Arc's enrichment in the nucleus. The delivery of Arc to the nucleus by such a mechanism may result in a change in the transcriptional properties of the neuron, mediated by an Arc-βSpIVΣ5 interaction with PML bodies, and the stabilization of long-term memories.

4. Experimental procedure

4.1. *Cell culture*

Hippocampal rat brain tissue (E18) was obtained from Brainbits, Inc. (Springfield, IL) and was cultured as previously described (Van de Ven et al., 2005). HEK 293T cells were obtained from the Duke University Cell Culture Facility, and were cultured in high glucose DMEM (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (FBS) (Invitrogen Corporation, Carlsbad, CA). Like the neurons, these cells were plated on the poly-D-lysine coated glass-bottom dishes for imaging

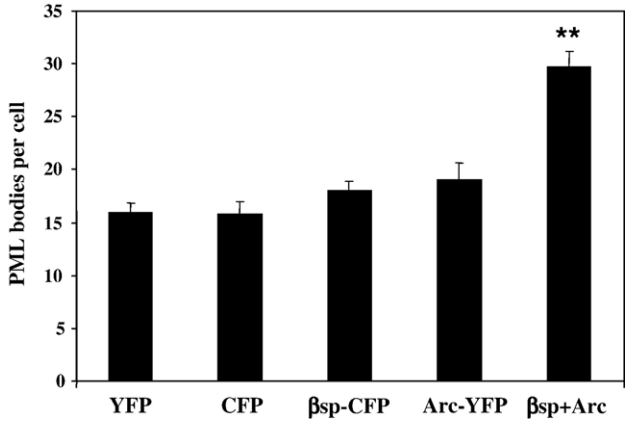

Fig. 10 – Arc and βSpIVΣ5 synergistically increase the number of PML bodies. EYFP, ECFP, Arc-EYFP, βSpIVΣ5-ECFP, and Arc-EYFP plus βSpIVΣ5-ECFP were expressed in HEK 293T cells. The cells were fixed and endogenous PML bodies were labeled with a primary PML antibody and an AlexaFluor568-conjugated secondary antibody. PML bodies per cell were quantified with the Count/Size function of ImagePro v.4.5 software (n=20). ** denotes P-value <0.01.

experiments. In addition, HEK 293T cells were plated in 24 well tissue culture plates (BD Falcon, Franklin Lakes, NJ) and 100×20 mm tissue culture dishes (BD Biosciences, Bedford, MA) for the BRET^2 and GST pulldown experiments.

4.2. *DNA constructs*

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 pGem T-easy vector (Promega, Madison WI). The region of the gene containing two introns (located in the 3′ UTR) was replaced by DNA amplified from Marathon rat brain cDNA via restriction digest and ligation (Clontech, Cambridge, UK), and the complete Arc sequence was subsequently subcloned into pcDNA3.1+ (Invitrogen). A novel *Bsr*GI 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 *Bsr*GI sites flanking the gene. *Bsr*GI-EYFP-*Bsr*GI was subcloned into the Arc-pGemT-easy vector, and the Arc-EYFP DNA was subsequently subcloned into pcDNA3.1+ (Invitrogen). The coding region of Arc was PCR amplified from the previous construct and subcloned into pGEX5x-1 (Amersham Biosciences, Piscataway, NJ) for the GST pulldown assays. βSpIVΣ5-EGFP was a generous gift from Dr. William Tse (Children's Memorial Research Center, Northwestern University, Chicago, IL). For the imaging experiments EGFP was replaced with ECFP (Clontech) by restriction digest and ligation. EGFP-PML was a generous gift from Dr. Tim Ley (Washington University School of Medicine, Department of Medicine, St. Louis, MO) and mCherry was a generous gift from Dr. Roger Tsien (University of California, Department of Pharmacology, La Jolla, CA). For the imaging experiments, Cherry was amplified by PCR, incorporating novel restriction sites and was subcloned into the PML vector replacing the EGFP. The BRET^2 assay vectors were a kind gift from Dr. Donald McDonnell (Duke University, Department of Pharmacology, Durham, NC). For the BRET^2 assay, the Arc coding region and βSpIVΣ5 were subcloned into a modified version of pENTR-2B (Invitrogen), pENTR-T7, and were subsequently recombined into pRlucGB and pGFP^{2}GB respectively using the Gateway LR clonase enzyme mix (Invitrogen).

4.3. *Transfections and stimulation*

Neuronal cultures were transfected between days 17 and 25 as previously described (Van de Ven et al., 2005). When specified, hippocampal neurons were stimulated with forskolin in DMSO for 4 h at a final concentration of 50 μM or with the same volume of vehicle (DMSO) as a control. HEK 293T cells were transfected similarly, except that DMEM with high glucose media was used and the Lipofectamine 2000/DNA mixture was added directly to existing media. For the Bret^2 assays a total of 1 μg of DNA was transfected per well of HEK 293T cells. For the GST-pulldown experiments 100×20 mm dishes of HEK 293T dishes were transfected similarly, except 10 μg of plasmid DNA plus 750 μl high glucose DMEM and 15 μl lipofectamine 2000 plus 750 μl high glucose DMEM were used.

4.4. *Immunofluorescence and imaging*

Transfected neuron dishes were rinsed once with Dulbecco's phosphate buffered saline with glucose and pyruvate (PBS) and then fixed with a solution containing 4% paraformaldehyde (PFA), 4% sucrose, and 1× PBS for 15 min at 4 °C. Cells were then washed with PBS, permeabilized with ice-cold methanol for 20 min at −20 °C, rinsed with PBS, and blocked with a solution containing 10% goat serum, 2% bovine serum albumin (BSA), and 1× PBS for 1 h at room temperature (RT). The primary antibodies were incubated overnight at 4 °C in the block solution at the following dilutions: anti-Arc C-7 1:100 to 1:2000, anti-PML PG-M3, H-238, and N-19 1:100 (all from Santa Cruz Biotechnology, Santa Cruz, CA) and anti-SC35 1:1000 (Sigma-Aldridge). The dishes were washed 3 times with block and incubated with Alexafluor488 or Alexafluor568-conjugated goat anti-mouse IgG (Molecular Probes-Invitrogen) 1:500 in block solution for 1–2 h at RT. The dishes were washed as above and once with PBS for 10 min. Hoechst dye 33258 (Sigma-Aldridge) was used to stain the nuclei. The fixed neurons were incubated with a 120 ng/ml solution of Hoechst dye in PBS for 15 min at RT. The neurons were then washed 5 times with PBS and imaged in PBS. HEK 293T cells were fixed with ice-cold methanol for 20 min at −20 °C. The cells were then rinsed with PBS and incubated with the primary antibodies (anti-PML 1:100, anti-SC35 1:1000) in PBS for 30 min RT, washed once with PBS, and incubated with the secondary antibodies (Alexafluor488 and Alexafluor568-conjugated goat anti-mouse IgG) in PBS for 30 min at RT. The cells were washed once with PBS and were either imaged in PBS or Hoechst dye applied as above.

4.5. *Imaging and data analysis*

Cells were imaged live unless otherwise specified, as in the case of immunofluorescence experiments. Both live and fixed cells were imaged in PBS and analyzed as previously described (Van de Ven et al., 2005). Openlab v3.1.6 (Improvision, Lexington, MA) was used to acquire the images, which were taken as z-stacks, including slices from 0.1 to 0.3 μm apart. Out-of-focus fluorescence was removed by de-convolution (Openlab). In addition, Pearson's coefficient values (*r*) and PML body number were determined using the co-localization and count/size functions of Image Pro Plus v4.5 (Media Cybernetics, Silver Springs, MD), where *N* is the number of cells used for analysis, and SEM is the standard error of the mean.

4.6. *GST pulldowns*

GST-tagged Arc protein was generated as follows. Arc-pGEX5x-1 and empty pGEX5x-1 were transformed into Top10F' competent cells and plated on Luria Bertini (LB) plates (Invitrogen) with ampicillin (Sigma-Aldrich). The plates were incubated overnight for 15 h at 37 °C. A single colony was chosen from each plate and was grown in 100 ml LB + ampicillin overnight for 16 h at 37 °C and 250 rotations per minute (rpm). 50 ml of overnight cultures was added to 450 ml of LB without antibiotic and was grown for 1 h at 37 °C and 250 rpm. IPTG (Isopropyl β-D-1-thiogalactopyranoside) (Invitrogen), final concentration 0.1 mM, was added to the culture, and the cells were allowed to grow for 5 more hours. The cultures were spun down, resuspended in ice-cold PBS plus protease inhibitors (PIs) (Sigma-Aldrich), sonicated on ice, and Triton X-100 (Sigma-Aldrich) was added with a final concentration of 1%. The cells were centrifuged and the supernatant was added to a 500 μl slurry of glutathione-agarose beads (Sigma-Aldrich). The beads and lysates were mixed by rocking for 5 min at RT and were washed with ice-cold PBS. 1 ml of ice-cold PBS + PIs was added to the beads.

HEK 293T cells were transfected with βSpIVΣ5-EGFP or GFP^2-βSpIVΣ5 in 100×20 mm tissue culture dishes as described above and were allowed to express overnight at 37 °C. The cells were washed with PBS, 500 μl of lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% Deoxycholate, 1:100 dilution of protease inhibitor cocktail (Sigma-Aldrich)) was added, and the cells were scraped into 1.5 ml tubes. The lysates were sonicated and spun down at 4 °C for 30 min at 16,000×*g* to pellet cell debris. 350 μl of supernatant was added to 40 μl of plain GST beads or Arc bound beads, and 500 μl of binding buffer was added to each sample (50 mM Tris pH 7.5, 100 mM NaCl, 10 μM $MgCl_2$, 0.5 mg/ml BSA, 0.5 mM DTT, 10 μM NaF). The samples were mixed by rocking for 1 h at 4 °C, spun down by centrifugation at 4 °C for 5 min at 2000×*g*, and the supernatant was removed. The beads were washed twice with 1.5 ml binding buffer and centrifuged at 4 °C for 5 min at 2000×*g* after each wash. The beads and GFP^2-βSpIVΣ5 input lysate were re-suspended in sample buffer, separated using SDS-PAGE with 10% Tris-glycine gels (Invitrogen), transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen), and immunoblotted as previously described (Van de Ven et al., 2005).

4.7. *$BRET^2$ assay*

Assays were performed using HEK 293T cells plated in 24 well tissue culture plates. Each well was transfected with a total of 1 μg of plasmid DNA encoding Renilla luciferase (Rluc), GFP^2 conjugated proteins, and β-galactosidase to normalize for transfection efficiency. Two days after transfection the cells were trypsinized to release them from the wells and neutralized with white DMEM + 10% charcoal stripped FBS (Invitrogen). The cells were transferred to a 96 well PCR plate (USA scientific Inc, Ocala, CA), spun down, and resuspended in PBS. Cells were counted with a Perkin Elmer Fusion α-FP HT machine. The cells were then spun down again and resuspended with PBS to give 5000 cells/μl. For the control GFP reading, 187.5 μl PBS and 12.5 μl of the 5000 cell/μl suspension were added to a black 96 well plate and read with the Fusion machine. For the $BRET^2$ reading (GFP/Renilla luciferase), 20 μl of the 5000 cell/μl suspension, 20 μl of PBS, and 10 μl of 1 mM DeepBlueC (BioSignal Packard, Montreal, Canada) in ethanol were added to a white 96 well plate and read 12 wells at a time with the Fusion machine. For the control luciferase readings, 30 μl of PBS and 12.5 μl of the 5000 cell/μl suspension were added to a 96 well white plate. 12.5 μl of 1 mM coelenterazine H (Sigma-Aldrich) in ethanol was added to each well and incubated at RT for 18 min covered with aluminum foil. The whole plate was read with the Fusion machine. $BRET^2$ ratios (GFP^2/Rluc emission in the presence of DeepBlueC) were graphed versus the control fluorescence divided by the control luminescence.

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REFERENCES

Alarcon, J.M., Malleret, G., Touzani, K., Vronskaya, S., Ishii, S., Kandel, E.R., Barco, A., 2004. Chromatin acetylation, memory, and LTP are impaired in $CBP^{+/-}$ mice: a model for the cognitive deficit in Rubinstain–Taybi sundrome and its amelioration. Neuron 42, 947–959.

Baines, A.J., Keating, L., Phillips, G.W., Scott, C., 2001. The postsynaptic spectrin/4.1 membrane protein "accumulation machine". Cell. Mol. Biol. Lett. 6, 691–702.

Bennett, V., Gilligan, D.M., 1993. The spectrin-based membrane skeleton and micron-scale organization of the plasma membrane. Ann. Rev. Cell Biol. 9, 27–66.

Berghs, S., Aggujaro, D., Dirkx, R., Maksimova, E., Stabach, P., Hermel, J.M., Zhang, J.P., Philbrick, W., Slepnev, V., Ort, T.,

Solimena, M., 2000. βIV spectrin, a new spectrin localized at axon initial segments and nodes of Ranvier in the central and peripheral nervous system. J. Cell Biol. 151, 985–1002.
Bock, J., Thode, C., Hannemann, O., Braun, K., Darlison, M.G., 2005. 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.
Boisvert, F.M., Hendzel, M.J., Bazett-Jones, D.P., 2000. Promyelocytic Leukemia (PML) nuclear bodies are protein structures that do not accumulate RNA. J. Cell Biol. 148, 283–292.
Boisvert, F.M., Kruhlak, M.J., Box, A.K., Hendzel, M.J., Bazett-Jones, D.P., 2001. The transcription coactivator CBP is a dynamic component of the promyelocytic leukemia nuclear body. J. Cell Biol. 152, 1099–1106.
Chang, K.S., Fan, Y.H., Andreeff, M., Liu, J., Mu, Z.M., 1995. The PML gene encodes a phosphoprotein associated with the nuclear matrix. Blood 85, 3646–3653.
Chawla, M.K., Guzowski, J.F., Ramirez-Amaya, V., Lipa, P., Hoffman, K.L., Marriott, L.K., Worley, P.F., McNaughton, B.L., Barnes, C.A., 2005. Sparse, environmentally selective expression of Arc RNA in the upper blade of the rodent fascia dentata by brief spatial experience. Hippocampus 15, 579–586.
Chowdhury, S., Shepherd, J.D., Okuno, H., Lyford, G., Petralia, R.S., Plath, N., Kuhl, D., Huganir, R.L., Worley, P.F., 2006. Arc/Arg3.1 interacts with the endocytic machinery to regulate AMPA receptor trafficking. Neuron 52, 445–459.
Davis, H.P., Squire, L.R., 1984. Protein synthesis and memory: a review. Psychol. Bull. 96, 518–559.
Dellaire, G., Bazett-Jones, D.P., 2004. PML nuclear bodies: dynamic sensors of DNA damage and cellular stress. Bioessays 26, 963–977.
Donai, H., Sugiura, H., Ara, D., Yoshimura, Y., Yamagata, K., Yamauchi, T., 2003. 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.
Eskiw, C.H., Bazett-Jones, D.P., 2002. The promyelocytic leukemia nuclear body: sites of activity? Biochem. Cell Biol. 80, 301–310.
Flexner, J., Flexner, L., Stellar, E., 1963. Memory in mice as affected by intracerebral puromycin. Science 141, 57–59.
Fujimoto, T., Tanaka, H., Kumamaru, E., Okamura, K., Miki, N., 2004. Arc interacts with microtubules/microtubule-associated protein 2 and attenuates microtubule-associated protein 2 immunoreactivity in the dendrites. J. Neurosci. Res. 76, 51–63.
Glanzer, J., Miyashiro, K.Y., Sul, J.Y., Barrett, L., Belt, B., Haydon, P., Eberwine, J., 2005. RNA splicing capability of live neuronal dendrites. Proc. Natl. Acad. Sci. U. S. A. 102, 16859–16864.
Guzowski, J.F., McNaughton, B.L., Barnes, C.A., Worley, P.F., 1999. Environment-specific expression of the immediate-early gene Arc in hippocampal neuronal ensembles. Nat. Neurosci. 2, 1120–1124.
Guzowski, J.F., Lyford, G.L., Stevenson, G.D., Houston, F.P., McGaugh, J.L., Worley, P.F., Barnes, C.A., 2000. Inhibition of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory. J. Neurosci. 20, 3993–4001.
Hayashi, T., Sasaki, C., Iwai, M., Sato, K., Zhang, W.R., Warita, H., Abe, K., 2001. Induction of PML immunoreactivity in rat brain neurons after transient middle cerebral artery occlusion. Neurol. Res. 23, 772–776.
Hsieh, J., Gage, F.H., 2005. Chromatin remodeling in neural development and plasticity. Curr. Opin. Cell Biol. 17, 664–671.
Irie, Y., Yamagata, K., Gan, Y., Miyamoto, K., Do, E., Kuo, C.H., Taira, E., Miki, N., 2000. 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.
Kobayashi, H., Yamamoto, S., Maruo, T., Murakami, F., 2005. Identification of a *cis*-acting element required for dendritic targeting of activity-regulated cytoskeleton-associated protein mRNA. Eur. J. Neurosci. 22, 2977–2984.
Komada, M., Soriano, P., 2002. βIV-spectrin regulates sodium channel clustering through ankyrin-G at axon initial segments and nodes of Ranvier. J. Cell Biol. 156, 337–348.
Korzus, E., Rosenfeld, M.G., Mayford, M., 2004. CBP histone acetyltransferase activity is a critical component of memory consolidation. Neuron 42, 961–972.
Lallemand-Breitenbach, V., Zhu, J., Puvion, F., Koken, M., Honore, N., Doubeikovsky, A., Duprez, E., Pandolfi, P.P., Puvion, E., Freemont, P., de, T.H., 2001. Role of promyelocytic leukemia (PML) simulation in nuclear body formation, 11S proteasome recruitment, and As2O3-induced PML or PML/retinoic acid receptor alpha degradation. J. Exp. Med. 193, 1361–1371.
Lam, Y.W., Ammerlaan, W., O., W.S., Kroese, F., Opstelten, D., 1995. Cell type- and differentiation stage-dependent expression of PML domains in rat, detected by monoclonal antibody HIS55. Exp. Cell Res. 221, 344–356.
Levenson, J.M., Sweatt, J.D., 2006. Epigenetic mechanisms: a common theme in vertebrate memory formation. Cell. Mol. Life Sci. 63, 1009–1016.
Link, W., Konietzko, U., Kauselmann, G., Krug, M., Schwanke, B., Frey, U., Kuhl, D., 1995. Somatodendritic expression of an immediate early gene is regulated by synaptic activity. Proc. Natl. Acad. Sci. U. S. A. 92, 5734–5738.
Lupas, A., Van, D.M., Stock, J., 1991. Predicting coiled coils from protein sequences. Science 252, 1162–1164.
Lyford, G.L., Yamagata, K., Kaufmann, W.E., Barnes, C.A., Sanders, L.K., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Lanahan, A.A., Worley, P.F., 1995. Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. Neuron 14, 433–445.
Montag-Sallaz, M., Welzl, H., Kuhl, D., Montag, D., Schachner, M., 1999. Novelty-induced increased expression of immediate-early genes c-*fos* and Arg3.1 in the mouse brain. J. Neurobiol. 38, 234–246.
Negorev, D., Maul, G.G., 2001. Cellular proteins localized at and interacting within ND10/PML nuclear bodies/PODs suggest functions of a nuclear depot. Oncogene 20, 7234–7242.
Plath, N., Ohana, O., Dammermann, B., Errington, M.L., Schmitz, D., Gross, C., Mao, X., Engelsberg, A., Mahlke, C., Welzl, H., 2006. Arc/Arg3.1 is essential for the consolidation of synaptic plasticity and memories. Neuron 52, 437–444.
Ramirez-Amaya, V., Vazdarjanova, A., Mikhael, D., Rosi, S., Worley, P.F., Barnes, C.A., 2005. Spatial exploration-induced Arc mRNA and protein expression: evidence for selective, network-specific reactivation. J. Neurosci. 25, 1761–1768.
Regad, T., Chelbi-Alix, M.K., 2001. Role and fate of PML nuclear bodies in response to interferon and viral infections. Oncogene 20, 7274–7286.
Rial Verde, E.M., Lee-Osbourne, J., Worley, P.P.s., Malinow, R., Cline, H.T., 2006. Increased expression of the immediate-early gene Arc/Arg3.1 reduces AMPA receptor-mediated synaptic transmission. Neuron 52, 461–474.
Rodriguez, J.J., Davies, H.A., Silva, A.T., De, S.I., Peddie, C.J., Colyer, F.M., Lancashire, C.L., Fine, A., Errington, M.L., Bliss, T.V., Stewart, M.G., 2005. 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.
Salomoni, P., Pandolfi, P.P., 2002. The role of PML in tumor suppression. Cell 108, 165–170.
Shepherd, J.D., Rumbaugh, G., Wu, J., Chowdhury, S., Plath, N., Kuhl, D., Huganir, R.L., Worley, P.F., 2006. Arc/Arg3.1 mediates

homeostatic synaptic scaling of AMPA receptors. Neuron 52, 475–484.
Spector, D.L., 2001. Nuclear domains. J. Cell Sci. 114, 2891–2893.
Speicher, D.W., Weglarz, L., DeSilva, T.M., 1992. Properties of human red cell spectrin heterodimer (side-to-side) assembly and identification of an essential nucleation site. J. Biol. Chem. 267, 14775–14782.
Steward, O., Worley, P.F., 2001. A cellular mechanism for targeting newly synthesized mRNAs to synaptic sites on dendrites. Proc. Natl. Acad. Sci. U. S. A. 98, 7062–7068.
Steward, O., Wallace, C.S., Lyford, G.L., Worley, P.F., 1998. Synaptic activation causes the mRNA for the IEG Arc to localize selectively near activated postsynaptic sites on dendrites. Neuron 21, 741–751.
Takahashi, J., Fujigasaki, H., Iwabuchi, K., Bruni, A.C., Uchihara, T., El Hachimi, K.H., Stevanin, G., Durr, A., Lebre, A.S., Trottier, Y., 2003. PML nuclear bodies and neuronal intranuclear inclusion in polyglutamine diseases. Neurobiol. Dis. 13, 230–237.
Tse, W.T., Tang, J., Jin, O., Korsgren, C., John, K.M., Kung, A.L., Gwynn, B., Peters, L.L., Lux, S.E., 2001. A new spectrin, beta IV, has a major truncated isoform that associates with promyelocytic leukemia protein nuclear bodies and the nuclear matrix. J. Biol. Chem. 276, 23974–23985.
Tsutsui, K.M., Sano, K., Tsutsui, K., 2005. Dynamic view of the nuclear matrix. Acta Med. Okayama 59 (4), 113–120.
Van de Ven, T.J., VanDongen, H.M.A., VanDongen, A.M.J., 2005. The nonkinase phorbol ester receptor alpha1-chimerin binds the NMDA receptor NR2A subunit and regulates dendritic spine density. J. Neurosci. 25, 9488–9496.
Waltereit, R., Dammermann, B., Wulff, P., Scafidi, J., Staubli, U., Kauselmann, G., Bundman, M., Kuhl, D., 2001. Arg3.1/Arc mRNA induction by Ca^{2+} and cAMP requires protein kinase A and mitogen-activated protein kinase/extracellular regulated kinase activation. J. Neurosci. 21, 5484–5493.
Wang, J., Shiels, C., Sasieni, P., Wu, P.J., Islam, S.A., Freemont, P.S., Sheer, D., 2004. Promyelocytic leukemia nuclear bodies associate with transcriptionally active genomic regions. J. Cell Biol. 164, 515–526.
Yamada, M., Sato, T., Shimohata, T., Hayashi, S., Igarashi, S., Tsuji, S., Takahashi, H., 2001. Interaction between neuronal intranuclear inclusions and promyelocytic leukemia protein nuclear and coiled bodies in CAG repeat diseases. Am. J. Pathol. 159, 1785–1795.
Young, K.G., Kothary, R., 2005. Spectrin repeat proteins in the nucleus. Bioessays 27, 144–152.
Zhong, S., Salomoni, P., Pandolfi, P.P., 2000. The transcriptional role of PML and the nuclear body. Nat. Cell Biol. 2, E85–E90.