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Nuclear Arc Interacts with the Histone Acetyltransferase Tip60 to Modify H4K12 Acetylation

Arc Interacts with Tip60 and H4K12

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Abstract

Arc is an immediate-early gene whose genetic ablation selectively abrogates long-term memory, indicating a critical role in memory consolidation. Although Arc protein localizes to neuronal synapses, it also localizes to the neuronal nucleus, where its function is less understood. Nuclear Arc forms a complex with the β-spectrin isoform βSpIVΣ5 and associates with PML bodies, sites of epigenetic regulation of gene expression. We report here a novel interaction between Arc and Tip60, a histone-acetyltransferase and subunit of a chromatin-remodelling complex, using biochemistry and super-resolution microscopy in primary rat hippocampal neurons. Arc and βSpIVΣ5 are recruited to nuclear Tip60 speckles, and the three proteins form a tight complex that localizes to nuclear perichromatin regions, sites of transcriptional activity. Neuronal activity-induced expression of Arc i) increases endogenous nuclear Tip60 puncta, ii) recruits Tip60 to PML bodies, and iii) increases histone acetylation of Tip60 substrate H4K12, a learning-induced chromatin modification. These mechanisms point to an epigenetic role for Arc in regulating memory consolidation.

Significance Statement

This manuscript reports a novel epigenetic role for the neuronal immediate early gene Arc, a master regulator of synaptic plasticity and critical effector of memory consolidation. Arc protein is localized both to synapses, where its role is well studied, and to the nucleus, where its function is still obscure. We now report that Arc interacts with the histone acetyltransferase Tip60, a subunit of the NuA4 chromatin modifying complex that functions in transcriptional regulation, implicated in Alzheimer's disease. We present data showing that Arc associates with and enhances Tip60's acetylation of its substrate H4K12, an important learning-induced histone mark. This discovery of an epigenetic function of Arc may shed light in the elucidation of mechanisms of learning and memory.

Introduction

Long-term memory formation requires both RNA and protein synthesis (Alberini, 2009; Davis and Squire, 1984). The neuron-specific immediate-early gene Arc (Link et al., 1995; Lyford et al., 1995) plays a critical role in memory consolidation. Arc expression is induced by exposure to novel environments (Chawla et al., 2005; Guzowski et al., 1999), while efficient Arc translation requires concomitant activation of NMDA receptors and second messenger pathways associated with reward and fear (Bloomer et al., 2008). Down-regulation of Arc abrogates both late-phase long term potentiation and memory consolidation (Guzowski et al., 2000; Plath et al., 2006).

While the synthesis, transport and translation of Arc mRNA are well understood, less is known about the functions of Arc protein. One discovered role of Arc is in the regulation of AMPA receptor endocytosis, thus controlling activity-dependent synaptic efficacy (Chowdhury et al., 2006; Rial Verde et al., 2006; Shepherd et al., 2006). Recent results indicate a role for Arc in 'tagging' inactive synapses (Okuno et al., 2012), and eliminating synaptic contacts in cerebellar development (Mikuni et al., 2013).

A significant proportion of Arc protein localizes to the nucleus (Bloomer et al., 2007), where it interacts with a nuclear spectrin isoform (βSpIVΣ5), and associates with PML (Promyelocytic Leukemia) bodies, sites of transcriptional regulation (Torok et al., 2009). Co-expression of Arc and βSpIVΣ5 synergistically increase the number of nuclear PML bodies, suggesting that Arc may regulate PML body function (Bloomer et al., 2007). Increased neuronal activity promotes Arc nuclear localization, an increase in nuclear PML bodies and reduced transcription of the GluA1 AMPA receptor, thereby contributing to homeostatic plasticity (Korb et al., 2013).

The implication of Arc in memory consolidation and transcriptional regulation along with its nuclear localization hints at a role in the epigenetic regulation of gene expression, which has been proposed as a mechanism for long-term memory formation (Zovkic et al., 2013). An important epigenetic modification studied in neurons is the acetylation of histones by acetyltransferases (HATs) (Peixoto and Abel, 2013). Out of the myriad of proteins that reside at nuclear PML bodies, a small number possess HAT activity (Eskiw and Bazett-Jones, 2002). These include the CREB Binding Protein (CBP), p300 and Tip60 (von Mikecz et al., 2000; Wu et al., 2009). CBP and p300 have both been implicated in learning and memory (Alarcon et al., 2004; Barrett et al., 2011; Korzus et al., 2004). Although brain-specific roles for Tip60 has been established in *Drosophila* (Johnson et al., 2013; Pirooznia et al., 2012b), little is known about its function in memory formation.

Here we report the interaction of Arc with Tip60 at nuclear PML bodies. Arc expression in hippocampal neurons induces the formation of endogenous Tip60 speckles, while Arc associates with acetylated H4K12, a known substrate of Tip60 which is critical for age-dependent memory formation (Peleg et al., 2010). Our results suggest that Arc may be recruiting the Tip60 HAT complex to modulate learning-induced H4K12Ac, and we propose a role for this complex in the epigenetic regulation of long-term memory formation.

Materials and Methods

Constructs and cloning

Arc-YFP, Arc-pCDNA3.1, PML-mCherry, PML-CFP, βSpIVΣ5-YFP and βSpIVΣ5-CFP have been previously described in Bloomer et al (2007). To clone βSpIVΣ5-mCherry, the YFP tag of βSpIVΣ5-YFP was excised with EcoRI and BsrGI and replaced with an amplified mCherry sequence containing the respective flanking sites and an in-frame stop codon. To clone βSpIVΣ5-HA, the YFP tag of βSpIVΣ5-YFP was excised with EcoRI and BsrGI and replaced with a double-stranded HA sequence containing the respective flanking sites and an in-frame stop codon. Isoform 1 of Tip60 containing flanking BamH1 and Xho1 restriction sites was amplified off a first strand brain cDNA library and cloned into a pGEMT vector. Xho1-Tip60-BamHI was then cloned into the multiple cloning site of the YFP and CFP vectors to generate Tip60-YFP.

Cell Culture

Hippocampi and cortices from E18 Sprague-Dawley rats of either sex were dissected aseptically, digested using a papain dissociation system (Worthington Biochemical Corporation). Cells were dissociated using a papain dissociation system (Worthington Biochemical Corporation) and plated at a density of 120,000 cells/ml on poly-D-lysine-coated glass-bottom culture dishes (MatTek, Ashland, MA) that had been double-coated with poly-D-lysine. Neurons were cultured in a chemically defined medium (NBactive4, Brainbits) and fed weekly by replacing half of the medium. HEK293 cells were obtained from the Author's University Cell Culture Facility, and were cultured in high glucose DMEM (Gibco) with 10% fetal bovine serum (Invitrogen Corporation, Carlsbad, CA). Like the neurons, these cells were plated on the poly-D-lysine coated glass-bottom dishes for imaging. In addition, HEK293 cells were plated in 10 cm tissue culture plates for western blots.

Transfections and Stimulations

Neuronal cultures were transfected overnight between DIV 12 and 21 using Lipofectamine 2000 per manufacturer's instructions. HEK293 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 10 cm cell culture dishes, 10 µg of plasmid DNA plus 100 µl serum-free DMEM and 10 µl lipofectamine 2000 plus 100 µl DMEM were used. To

stimulate protein expression of endogenous Arc or Arc constructs, Forskolin at a final concentration of 50 μM or the same volume of vehicle (DMSO) was added to neurons for 4 hours before fixing. Expression of endogenous Arc was induced by stimulating spontaneous network activity in DIV18-23 neurons with 4AP (10 μM) and bicuculline (10 μM) for 4 hours.

Immunofluorescence

For construct co-expression experiments, transfected neurons or HEK cells were fixed with a solution containing 4% paraformaldehyde (PFA), 4% sucrose, and 1× PBS for 15 min at 4 °C. The cells were subsequently incubated with 1 uM DAPI for 10 minutes, and preserved with FluorSave. For immunostaining, the cells were fixed with 100% MeOH at -20°C for 10 min. Neurons/HEK cells were blocked with a solution containing 10% goat serum, 2% bovine serum albumin (BSA), and 1× PBS for 1 hour at room temperature (RT), except when of goat-anti-Tip60 (K-17) (Santa Cruz) goat antibody was used, in which case blocking was done with 3% BSA in DPBS. The primary antibodies were incubated for 1 hour at RT in a dilution buffer containing 1:1 block solution and PBS-Triton X solution at the following dilutions: mouse anti-Arc (C7) 1:300 (Santa Cruz), goat anti-Tip60 (K17) 1:300, rabbit anti-Tip60 (Novus Biologicals), rabbit anti-H4K12ac (Abcam ab61238), rabbit-anti-PML (Abcam), mouse-anti-PML (Sigma). The dishes were washed 2x 10 minutes with PBS-Triton X and incubated with Alexafluor488 or Alexafluor564 conjugated secondary antibodies (Molecular Probes-Invitrogen) 1:1000 in dilution buffer for 1 hour at RT. The dishes were washed as above for 2 x 10 min and stained with 1 uM DAPI for 10 minutes to label DNA, followed by addition of FluorSave.

Imaging and data analysis

Fluorescence images were obtained using a motorized inverted wide-field epifluorescence microscope (Nikon Eclipse Ti-E), using 40x and 60x Plan-Apo oil objectives, with numerical apertures of 1.35 and 1.49, respectively. Motorized excitation and emission filter wheels (Ludl electronics, Hawthorne, NY) fitted with a DAPI/CFP/YFP/DsRed quad filter set (#86010, Chroma, Rockingham, VT) were used together with filter cubes for DAPI, CFP, YFP and TxRed and Cy5 (Chroma) to select specific fluorescence signals. Z-stacks were obtained spanning the entire nucleus and out-of-focus fluorescence was removed using the AutoQuant 3D deconvolution

algorithm (Media Cybernetics, Rockville, MD, USA). Images were digitized using a cooled EM-CCD camera (iXon EM+ 885, Andor, Belfast, Northern Ireland). Image acquisition was performed using NIS Elements AR 3.1 software (Nikon). NIS Elements analysis tools were used to outline the nuclei based on their DAPI images and to measure areas of the various nuclear substructures, as illustrated in Figure 1 for Arc-YFP puncta in the nucleus of a 20 DIV hippocampal neuron.

3D structured illumination microscopy (3D-SIM)

A DeltaVision OMX V4 microscope (Applied Precision-GE) equipped with 405, 488 and 568 nm lasers for excitation and the BGR filter drawer (emission wavelengths 436/31 for DAPI, 528/48 for Alexa488 and 609/37 for Alexa568) was used for acquisition of 3D-SIM images. An Olympus Plan Apochromat 100x/1.4 PSF oil immersion objective lens was used with liquid-cooled Photometrics Evolve EM-CCD cameras for each channel. 15 images per section per channel were acquired (made up of 3 rotations and 5 phase movements of the diffraction grating) at a z-spacing of 0.125 µm, as previously described (Gustafsson, 2008; Schermelleh et al., 2008). Structured illumination reconstruction and alignment was completed using the SoftWorX (Applied Precision-GE) program with figure preparation in Fiji (Schindelin et al., 2012).

Localization microscopy (PALM and STORM)

3D photo-activated localization microscopy (PALM) and direct stochastic optical reconstruction microscopy (dSTORM) experiments were performed on the Elyra-PS.1 platform (Zeiss, Jena, Germany) with a 63x PLAN-APO objective (NA = 1.4). Excitation and dark-state conversion were facilitated by a 150 mW 642 nm diode laser (to excite Alexa-647) and a 200 mW 488 nm diode laser (to excite Atto-488) operating at 100% laser power and further enhanced by ultrahigh power TIRF illumination. PALM of Arc-mEOS2 was performed by alternating excitation with a 405 nm laser operating in Transfer mode while imaging with the 561nm laser, whereas dSTORM used continuous excitation with the 488 nm and 642 nm laser lines and the reflected light was allowed to pass through a multi-band pass filter (set 77HE). 3D PALM/dSTORM was obtained with PRILM (Phase Ramp Imaging Localization Microscopy) by placing a double phase ramp with wedge angle of 1' at the DIC slider position, which was calibrated on the day of the experiment using low density multi-spectral fluorescent beads (500 nm) to generate

a Point Spread Function Localization Precision LUT. Time-lapse images were taken at 16ms exposure times with the gain of the EMCCD camera set at 250 (Andor iXon DU 897). The images were cropped at 256x256 and the resulting images were processed using the PALM module of the ZEN software (Zeiss, Jena, Germany).

Immunoprecipitations and Western blotting

Transfected HEK293 cells growing in 10 cm tissue culture dishes were transfected as described above and allowed to express overnight at 37 °C. The cultures and subsequent lysates were kept on ice or at 4 °C throughout the entire procedure. The cultures were washed once with 1 ml of PBS, and lysed in 500 µl of lysis buffer for 30 min, then scraped into 1.5 ml tubes. Lysis buffer consisted of 5 mM HEPES pH 7.2, 0.5% NP40, 250 mM NaCl, 2 mM EDTA, 10% glycerol, 1:100 dilution of protease inhibitor cocktail (Sigma-Aldrich). The lysates were spun down for 20 min at 16,000×g to pellet cell debris. Lysates from multiple dishes were combined, and 1 ml of the supernatant was then incubated on a rotator with 10 µl mouse-anti GFP (Roche) for 90 min, followed by 100 µl of Protein-A/G Plus-Agarose (Santa Cruz Biotechnology) for another 60 min on a rotator. Each reaction was split into two equal volumes to allow for subsequent loading into 2 wells. The beads were spun down at 1000×g for 5 min and the supernatant was removed. The beads were washed and re-suspended in 1 ml lysis buffer. This was repeated 3 times. The beads and input lysates were resuspended and boiled at 95 °C for 5 min in sample buffer, resolved by SDS-PAGE with Tris-glycine gels (Bio-Rad), transferred to nitrocellulose membranes (Invitrogen), and imunoblotted. The primary antibodies used were anti-GFP (rabbit polyclonal; Invitrogen), anti-Arc (rabbit poly-clonal; Santa Cruz) or anti-HA (mouse monoclonal; Santa Cruz).

Induction of Arc expression by stimulation of network activity

Hippocampal and cortical neuronal cultures were grown on glass-bottom dishes (Mattek) to maturity (DIV 18-21) and inspected on a brightfield microscope for formation of neurites and extensive networks. Spontaneous synaptic activity was increased in the culture on the day of the experiment for a predetermined period of time (4 hours) by the addition of 100 µM 4-Aminopyridine (4AP), a presynaptic K^+ channel antagonist that facilitates transmitter release, in conjunction with 50 µM Bicuculline (Bic), a $GABA_A$ receptor antagonist that reduces the inhibitory tone of the network. The 4AP-Bic combination increases synchronous network bursting and results in synaptic NMDA re-

ceptor activation (Hardingham et al., 2002). In order to overcome the translational impediment that limits endogenous Arc protein expression, 50 μM Forskolin (Fors), an adenylyl cyclase activator, was added (Bloomer et al. 2008). This 4AP-Bic-Fors combination induces the expression of Arc in a subset of neurons, which is revealed by immunohistochemistry using the anti-Arc (C7, Santa-Cruz Biotechnology) antibody. At the end of 4 hours of treatment, cultures are fixed in the presence of 4% PFA and processed for immunohistochemistry.

Data Analysis and Statistics

Fluorescence intensity values were determined from segmented nuclei (Fig. 1) and the mean value was calculated for each channel (Cyan, Yellow, Red, Far-red), to represent the nuclear levels of the corresponding protein, which were fluorescently labeled by antibodies (for endogenous proteins) or fusion to a fluorescent protein (CFP, YFP, mCherry) for exogenously expressed proteins. The Mean and Standard Error of the Mean (SEM) were calculated for each data set and they were compared pair-wise using the Student's t-test for the Null hypothesis that the means are identical, assuming unequal variances. Significance was evaluated using the 2-tailed p-value and the difference between two means was considered significant when $p<0.01$. Levels of significance are denoted by asterisks: $p<0.01$ = *, $p<0.001$ = **, $p < 0.0001$ = ***

Results

Arc, βSpIVΣ5 and Tip60 form distinct, highly localized nuclear puncta

Arc-YFP was expressed in cultured hippocampal neurons by transient transfection. Basal expression of Arc protein in cultured neurons is severely retarded due to a translation impediment, which can be rescued by activation of the cAMP-dependent protein kinase A pathway (Bloomer et al., 2008). Neurons transfected with Arc-YFP were allowed to express overnight after which they were treated with forskolin for four hours before fixation. As previously reported (Bloomer et al., 2007), a significant portion of Arc protein localizes to the nucleus, where it is enriched in puncta (Fig. 2A). These nuclear Arc puncta vary in size and number and are found in close proximity to nuclear domains densely labeled by the DNA stain DAPI (Fig 2A, insets). At the light microscopy level, Arc and DAPI do not clearly overlap.

The neuron-specific nuclear beta-spectrin isoform βSpIVΣ5 (Tse et al., 2001) has been shown to form a tight complex with PML (Tse et al., 2001) and with nuclear Arc (Bloomer et al., 2007). Expression of βSpIVΣ5-YFP in cultured hippocampal neurons reveals small puncta both in the cytoplasm (soma and dendrites) and nucleus (Figure 2B). When expressed alone, the βSpIVΣ5 puncta are smaller in size and there are more puncta per nucleus compared to Arc. Like Arc, βSpIVΣ5 is often seen adjacent to DAPI-dense regions (Figure 2B, insets). There is no clear difference in size between nuclear and cytoplasmic βSpIVΣ5 puncta.

When Tip60-YFP was expressed in hippocampal neurons it was found both in the cytoplasm (mostly in the soma) and the nucleus, where it formed rather large speckles that were variable in size (Fig 2C). Tip60 puncta were significantly larger than those for either Arc or βSpIVΣ5 (Fig 2C, inset), filling a large portion of the inter-chromatin space. To investigate possible interactions between these three proteins, they were fused to spectrally separated GFP isoforms and co-expressed in hippocampal neurons.

Tip60 associates with both Arc and βSpIVΣ5

Co-expression of Arc-YFP and Tip60-mCherry revealed a strong overlap of these two proteins in hippocampal nuclei (Fig. 2D), demonstrating co-localization of Arc with a histone modifying enzyme. The structures that are dual-labeled for both Tip60 and Arc resemble the Tip60 puncta (Fig 2C) in size and number, suggesting that the Tip60 speckles have recruited Arc. The result obtained for co-expression of βSpIVΣ5-YFP and Tip60-mCherry was

even more striking. Co-expression with Tip60 causes βSpIVΣ5 to redistribute from the numerous well-defined small puncta to a few much larger speckles (Fig. 2E), again resembling the pattern seen for Tip60 alone. It therefore appears that Tip60 recruits both Arc and βSpIVΣ5 upon co-expression, changing both the size and number of their puncta.

Arc and βSpIVΣ5 have been shown to form a complex and each individually interacts with Tip60. We therefore co-expressed the three proteins after fusing them to spectrally distinct GFP isoforms (CFP, YFP, mCherry) in both neurons and human embryonic kidney (HEK293) cells. As illustrated in Figure 3, upon co-expression, Arc, βSpIVΣ5, and Tip60 form nuclear complexes in both hippocampal neurons and HEK293 cells. The fluorescence signal for the three fluorophores had a fixed ratio, indicated by the fact that almost all pixels were white, following normalization of the red, green and blue channels. This preponderance of white pixels indicates that the stoichiometry of Arc, βSpIVΣ5 and Tip60 is well-defined in the complex.

A statistical analysis was performed measuring the number of puncta and their average size (area) for each nucleus (see Material and Methods). This analysis was performed for Arc, βSpIVΣ5, and Tip60 expressed alone or in combination. Figure 3I shows a scatterplot graphing, for each nucleus, the average area of the different puncta versus their number. When expressed alone, each protein occupies a distinct region of the scatterplot (Fig. 3I). βSpIVΣ5 has the smallest puncta and is most numerous, consistent with its role as a nuclear matrix protein, while Arc is intermediate in size and number, and Tip60 puncta are larger than both and are the most sparse. Co-expression of Tip60+Arc, Tip60+βSpIVΣ5 or Tip60+Arc+βSpIVΣ5 results in co-localization of the components to puncta which have similar properties to those formed by Tip60 alone, indicating recruitment of all three proteins into the same complex.

Arc localizes the Arc-βSpIVΣ5-Tip60 complex to the perichromatin region

As shown in the insets of Figure 2A and 2B, Arc and βSpIVΣ5 puncta localize to interchromatin domains, but are often found adjacent to DAPI-dense structures. Figure 4 illustrates the relationship of the Arc-βSpIVΣ5-Tip60 complex with DNA labeled by DAPI staining (4A), and compares this with the localization of Tip60 (4F) and Arc (4K) alone, following expression of GFP fusion constructs in HEK293 cells. Arc-βSpIVΣ5-Tip60c complexes appear as white puncta due to the tight co-localization of the three components. Many of these complexes localize to the interface between the interchromatin space, which is devoid of DNA, and the compact chromatin domains,

which is stained heavily by DAPI. This interface is sometimes referred to as the 'perichromatin' region, and it has important functions, including transcription and RNA processing (Fakan and van Driel, 2007; Niedojadlo et al., 2011). In several instances the Arc-βSpIVΣ5-Tip60 complexes appear to be engulfed by a small amount of DAPI, indicative of loosely packed DNA characteristic of perichromatin regions. In Figure 4 the interchromatin domains are delineated by a line created by segmenting the DAPI structure, to show various locations of the tripartite complex puncta: in the center of an interchromatin domain (4B), adjacent to or embedded in the perichromatin region (4CD), and completely surround by chromatin (4E). The Arc-containing complexes are seen only rarely at locations B and E, and predominantly localize to the perichromatin regions CD.

In order to investigate if this interesting sub-nuclear localization could be attributed to an individual component of the complex, we studied their relationship with chromatin. In HEK293 cells, the tripartite complexes (4D-E) are smaller than the Tip60 speckles (4G-J), which filled large parts of the interchromatin space. In contrast, Arc-YFP expressed by itself in HEK293 cells (4K) forms small puncta that localized predominantly to the perichromatin region (4L-O). Expression of Arc-YFP in hippocampal neurons (Fig. 4P) resulted in a comparable pattern where the puncta showed a strong preference for the perichromatin regions (Fig 4Q-T). When expressed by itself in neurons, βSpIVΣ5 forms numerous small nuclear puncta which localize both to the inter-chromatin and perichromatin compartments, while a clear association with dense DAPI structure can be seen (Fig 2B).

Tip60 binds to Arc and βSpIVΣ5

The co-localization seen in the imaging results discussed above suggests that Tip60 can bind to both Arc and βSpIVΣ5. To test this idea more directly, we performed co-immunoprecipitation (co-IP) experiments (Fig. 5). Arc was co-expressed in HEK293 cells with either Tip60-YFP, or YFP as a negative control. Tip60-YFP was immunoprecipitated with a mouse anti-GFP antibody; the precipitate was resolved by SDS-PAGE, and transferred to a nitrocellulose filter, where Arc was detected with a rabbit anti-Arc antibody. These experiments showed that Arc co-immunoprecipitated with Tip60 (Fig 5A). Tip60 was also found to bind to βSpIVΣ5. HEK293 cells were transfected with either βSpIVΣ5-HA and YFP (negative control) or βSpIVΣ5-HA and Tip60-YFP. Tip60-YFP was immunoprecipitated with a mouse anti-GFP antibody, and ßSpIVΣ5-HA was detected with a mouse anti-HA antibody (Fig 5B). Finally, Tip60 was seen to physically interact with the complex formed by Arc and βSpIVΣ5:

HEK293 cells were transfected with Arc, ßSpIVΣ5-HA, and either YFP or Tip60-YFP. Tip60-YFP was immunoprecipitated with a mouse anti-GFP antibody, and Arc or βSpIVΣ5-HA was detected with a rabbit anti-Arc or mouse anti-HA antibody respectively (Fig 5C), suggesting that complex formation between Arc and βSpIVΣ5 does not interfere with Tip60 binding.

Endogenous Arc interacts with Tip60 in a rich variety of dynamic nuclear structures

The finding that nuclear Arc co-localizes with and physically binds to Tip60 was novel and therefore prompted a more careful look into the nature of the endogenous protein-protein interactions. To this end we employed two differing yet complementary super-resolution microscopy approaches, 3D-Stimulated Emission Depletion Microscopy (3D-STED) and 3D-Stochastic Optical Reconstruction Microscopy (3D-STORM), which push the boundaries of the resolution limit of light microscopy. Upon stimulation of network activity by a combination of 4AP, Bicuculline and Forskolin (4AP-Bic-Fors, see Methods), a subset of neurons expressed endogenous Arc which we immunostained using an antibody recognizing Arc protein. At resolutions narrowly eclipsing 200 nm, 3D-STED revealed that in these activated neuronal nuclei, the distribution of Tip60 protein highly paralleled that of Arc (Fig. 6, top panel). The two proteins abutted each other in many distinct conformations (Fig. 6, bottom panel). In order to definitively delineate this interaction, we proceeded to use 3D-STORM which has the capability of imaging single molecules, and observed that in support of the results obtained through widefield and STED microscopy, molecules of Arc and Tip60 do indeed interact in the activated neuronal nucleus with unforeseen clarity (Fig. 7).

Arc increases nuclear Tip60 puncta

To investigate the effect of Arc expression on endogenous Tip60 protein, hippocampal neurons were transiently transfected with Arc-YFP, fixed and stained with an anti-Tip60 antibody. Tip60 antibody staining in neuronal nuclei was mostly homogenous (Fig 8A), with at most one or two detectable hotspots. However, Arc expression strongly induced the formation of bright endogenous Tip60 puncta in the nucleus (Fig 8B, C). Figure 8 illustrates that Arc-positive nuclei contained more endogenous Tip60 puncta (~10 per nucleus) than untransfected controls (0-2 per nucleus). Although some of the Tip60 puncta were seen to associate with Arc (insets, Fig 8B, C), the two proteins did not overlap as closely as when they were both over-expressed (Fig. 2D). Co-expression of Arc-YFP and

βSpIVΣ5-CFP in hippocampal neurons also induced formation of extra Tip60 hotspots and the Arc- βSpIVΣ5 complex still associated with Tip60 speckles (Fig 8D). Our findings that Arc expression modulates the concentration of endogenous Tip60 protein in the nucleus suggest that Arc may be modulating the function of Tip60.

Arc recruits Tip60 to PML bodies

Over-expression of Arc and βSpIVΣ5 in HEK293 cells, cooperatively increases the number of PML bodies (Bloomer et al, 2007), while regulation of transcription of the GluA1 AMPA receptor by Arc depends on PML function (Korb et al., 2013). We therefore investigated the interaction between Arc, Tip60 and PML in hippocampal neurons and HEK293 cells. When Tip60 and PML were expressed together they both localized to the interchromatin domains, where they occupied mostly non-overlapping regions (Fig 9ACE). However, when Tip60 and PML were co-expressed together with Arc, the three proteins formed a well-defined complex (Fig 9BDF). This recruitment of Tip60 to PML nuclear bodies was elucidated through the use of Structured Illumination Microscopy, using HEK293 cells, which revealed that Tip60 puncta permeated and enveloped PML bodies more strongly when Arc is present (Figure 9E).

Arc increases H4K12 acetylation

Following experimental DNA damage induction, Tip60 is recruited to PML bodies, resulting in its activation (Cheng et al., 2008; Legube et al., 2004; Wu et al., 2009). Since Arc induces a tight interaction between Tip60 and PML bodies, we investigated whether Arc expression could affect acetylation of known Tip60 histone substrates. We selected lysine 12 of histone H4, because it's the only Tip60 substrate whose acetylation is induced by learning (Peleg et al., 2010). Interestingly, learning-induced acetylation of H4K12 selectively declines with aging, and Ruvbl1, a subunit of the Tip60 complex, is shown to decrease with aging (Blalock et al., 2003).

Arc-YFP was expressed in cortical neurons for one day, after which the cells were fixed and stained using an antibody specific for acetylated lysine 12 of histone H4 (H4K12Ac). We have performed additional experiments comparing the effect of Arc-YFP and YFP over-expression on H4K12 acetylation, following treatment with Forskolin, which rescues Arc translation (Bloomer et al., 2008), and the pharmacological combination of 4AP-Bicuculine-Forskolin, which induces endogenous Arc expression. Over-expression of YFP did not affect

H4K12 acetylation, whereas Arc-YFP significantly increased H4K12 acetylation (Fig. 10B). Interestingly, the increase was much higher after network stimulation than with Forskolin treatment only. In addition, these experiments revealed that endogenous Arc expression levels correlated strongly with H4K12Ac levels. The relationship between endogenous Arc expression and H4K12Ac levels was investigated by analyzing the 95% of the neurons that were not transfected. Segmented nuclei were ranked according to endogenous Arc expression level and analyzed for H4K12 acetylation (Fig. 10C). The increasing trend displayed by the Arc-sorted H4K12Ac levels indicates a positive correlation between endogenous Arc levels and H4K12 acetylation status.

Tip60 regulates H4K12 acetylation in hippocampal neurons

Several enzymes including p300, CBP, and PCAF have been correlated to the increase of the H4K12Ac mark in the hippocampus of learning animals (Bousiges et al., 2013; Bousiges et al., 2010), but a direct demonstration of enzyme-substrate interaction has not been reported. In order to investigate whether Tip60 may be playing a role in the acetylation of this memory-related histone mark, we expressed an enzymatically inactive mutant of Tip60Q377E/G380E (or Tip60dm for double mutant), where two critical residues in the acetyltransferase domain of the protein are mutated (Ikura et al., 2000), in hippocampal neurons. Aside from these two amino acid mutations, the chromodomain, zinc-finger domain, and the majority of the C-terminal part of Tip60dm remain intact, resulting in an enzymatically inactive yet fully structured protein (Squatrito et al., 2006). Tip60dm therefore can be expected to act as a dominant-negative, by associating with endogenous complexes, displacing endoTip60 and removing their histone acetyltransferase activity (Sun et al., 2005). Upon overexpression of Tip60dm, we found that the intensity of H4K12Ac staining per nucleus was decreased, resulting in two clearly distinguishable distributions for Tip60dm–positive and –negative neurons (Fig. 11). This finding suggests that Tip60 may be one of the HATs responsible for the acetylation of H4K12 in hippocampal neurons.

Arc associates with the learning-induced histone mark H4K12Ac

The learning-induced mark H4K12Ac is enriched in transcribed regions of gene bodies and therefore stained most of the nucleus (Fig. 12A), which made it likely to co-localize to the discrete Arc puncta (Fig. 12B) by chance alone. However, an interesting pattern emerged when the analysis was limited to H4K12Ac 'hotspots', those areas in the

nucleus where the H4K12Ac signal was greater than 50% of the maximum signal (Fig. 12D-E). Arc puncta were seen to either overlap with or be adjacent to regions with the most concentrated H4K12Ac signal. Since H4K12Ac is known to be important for transcriptional elongation and each of these hotspots may correspond to several intragenic transcribed regions, we opted to analyze this association better using Structured Illumination Microscopy (SIM). Data from SIM imaging confirmed the widefield data results. The H4K12Ac staining which appears fairly homogenous in Figure 12A, actually consists of a large number of very small structures, with a diameter less than 200 nm, which cannot be resolved by conventional light microscopy. The over-expressed Arc puncta maintain their solid appearance in the SIM images (Fig. 12F-G), suggesting they form large aggregates. The H4K12Ac structures were seen to cluster at several regions of the nucleus, and some of these concentrations were observed in close proximity of Arc puncta (Fig. 12G, top row). This sporadic association suggests that either Arc puncta can be recruited to pre-existing H4K12Ac dense regions or that this histone mark may be relocated to Arc puncta.

Discussion

In this study, we report physical and functional interactions between Arc protein and the histone acetyltransferase (HAT) Tip60. Arc not only interacts with Tip60, but also induces the formation of local concentration of endogenous Tip60. While these endogenous puncta do not interact as closely with Arc as over-expressed Tip60, they are located in close proximity. Tip60 was original discovered as a HIV-Tat interacting protein (Kamine et al., 1996; Yamamoto and Horikoshi, 1997) and is a component of a multimeric nuclear complex that is involved in the acetylation of six lysines on histones, which include H2A-K5, H3-K14, and K5, K8, K12, and K16 of histone H4 (Cai et al., 2003; Doyon et al., 2004; Kimura and Horikoshi, 1998). Tip60 is a subunit of a chromatin remodeling complex that plays roles in transcription regulation and DNA repair. In addition to HAT activity, the Tip60 complex possesses ATPase, DNA helicase and structural DNA binding capabilities (Ikura et al., 2000).

Several previous findings support a unique role for Tip60 in the central nervous system. Tip60 controls sleep in the fruit fly *Drosophila* by epigenetically regulating axonal growth of pacemaker cells (Pirooznia et al., 2012a), while increasing Tip60 levels rescues an axonal transport defect in an Alzheimer's disease (AD) model (Johnson et al., 2013; Pirooznia et al., 2012b). Tip60 binds to Fe65 and AICD, the cytoplasmic C-terminal domain of amyloid

precursor protein (APP), and the complex is targeted to the nucleus where it regulates transcription (Cao and Sudhof, 2001; Muller et al., 2013). Like Tip60, Arc has been implicated in Alzheimer's disease: patients with AD can express anomalously high levels of Arc, while transgenic mouse models of AD have shown that genetic deletion of Arc reduces Aβ load (Wu et al., 2011), as well as disruption of experience-drive Arc responses (Rudinskiy et al., 2012). There is evidence for deregulation of epigenetic processes in AD, and histone modification is being considered for therapy (Stilling and Fischer, 2011).

Under basal neuronal culture conditions Arc protein expression is very low or undetectable (Bloomer et al., 2008), despite the fact that these cultures are spontaneously active (Habets et al., 1987). As both Arc and Tip60 have clear neuronal functions, we performed experiments where we induced Arc expression in hippocampal neurons by stimulating network bursting using a combination of 4AP, Bicuculline and Forskolin (see Methods), which causes a subset of neurons (typically 30%) in the network to express endogenous Arc protein. This 'success rate' is similar to what has been seen in the dentate gyrus of animals exposed to novel environments (Chawla et al., 2005). We observed that endogenous Tip60 puncta associated with endogenous Arc protein in the neuronal nucleus. The data shown in our study therefore link Tip60 to Arc, a neuron-specific immediate early gene implicated in memory consolidation, supporting a distinctive neuronal function for Arc in regulating memory consolidation by modulating the learning-induced histone mark H4K12Ac through its association with the acetyltransferase Tip60.

Dynamic nuclear interactions between Arc, βSpIVΣ5, Tip60 and PML bodies

We demonstrate here that both Arc and βSpIVΣ5 can directly interact with Tip60, both separately and as a complex. Since Tip60 itself exists as a multimeric complex, it is possible that other proteins within the complex may also play a role in the Tip60-Arc interaction. For example, γ-actin and BAF53, an actin-related protein, are components of the Tip60 nuclear complex (Ikura et al., 2000), and may possibly bind to the spectrin homology domain of Arc or to spectrin βSpIVΣ5 itself, which may bind to nuclear actin (Young and Kothary, 2005).

We also found that the interactions between Arc, βSpIVΣ5 and Tip60 are dynamic, directional and center around nuclear PML bodies. In our over-expression studies, we saw that Tip60 could cause a redistribution of Arc and βSpIVΣ5, suggesting tight binding affinities of Arc and βSpIVΣ5 for Tip60. Interestingly, Tip60 has been shown to cause the redistribution of various other Tip60-binding proteins to nuclear speckles, sometimes even from the cytoplasm (Legube et al., 2004; Logan et al., 2004; von Rotz et al., 2004). In contrast, expressing both Arc and

βSpIVΣ5 together could exert a morphological change on Tip60 nuclear structure, where Tip60 no longer exists as large speckles but as round puncta that completely co-localize with Arc and βSpIVΣ5, which localize to the perichromatin regions, sites of transcription and RNA processing (Fakan and van Driel, 2007; Niedojadlo et al., 2011). Images taken of activated neuronal nuclei show that endogenous Arc and endogenous Tip60 strongly associate with each other and co-localize in the perichromatin region where only a select few proteins including PML are known to reside in (Cmarko et al., 2003). Furthermore, while Tip60 and PML rarely overlap, Arc expression forms a complex with Tip60 that co-localize tightly with PML bodies, suggesting a possible role of Arc in recruiting Tip60 to these known sites of transcription. Indeed, super-resolution imaging data presented here indicate a possible dose-dependent recruitment of Tip60 to PML bodies by Arc protein (Figure 9).

The interaction between Tip60 and Arc is reciprocal: over-expression of Tip60 recruits co-expressed Arc to the inter-chromatin domain, thereby increasing its size and reducing the number of puncta (Figure 3I). Over-expression of Arc also alters the distribution of endogenous Tip60 by causing it to form local 'hotspots' (Fig. 8BC). This effect of Arc on Tip60 was not seen when both were over-expressed. Overall, our results suggest that that the Arc- complex may be able to recruit and redistribute Tip60 at a sub-nuclear level.

Over-expressed Arc puncta (Fig. 2A and Fig. 4P) were significantly larger than endogenous Arc structures (Fig. 6). The sub-nuclear localization of endogenous Arc proteins probably depends on its binding partners, only few of which are known. It is possible that over-expression of Arc exhausts the pool of available binding partners, resulting in mislocalization. In this respect, our data obtained using STED microscopy of endogenous Arc and Tip60 confirms their association in a biologically relevant context.

A possible epigenetic role for Arc via Tip60

Studies of Arc's functional roles have focused on the synapse, where it regulates AMPA receptor endocytosis. Only a handful of papers so far have implicated Arc as a nuclear protein. Arc has been demonstrated to interact with Amida, a nuclear apoptosis-inducing protein, and may negatively regulate Amida-induced cell death (Irie et al., 2000). However, since Arc knockout mice do not have detectable neuron damage (Plath et al., 2006), protection against cell death is unlikely to be a major function of nuclear Arc. In 2007, Arc was localized to nuclear PML bodies (Bloomer et al., 2007), which play a crucial role in transcriptional regulation. Arc and βSpIVΣ5 synergis-

tically increase the number of endogenous PML bodies in HEK293 cells, suggesting a role for Arc in regulating transcription. Consistent with this earlier finding, a recent study has demonstrated that nuclear Arc regulates GluA1 transcription and homeostatic plasticity through a PML-dependent mechanism (Korb et al., 2013).

In this paper we have identified the HAT Tip60 as a novel Arc interacting protein, which appears to be regulated both directly and indirectly by Arc and βSpIVΣ5 nuclear expression. This result is interesting especially in light of recent findings implicating histone acetylation in memory consolidation, all of which involve known Tip60 substrates (Fischer et al., 2007; Levenson et al., 2004; Levenson and Sweatt, 2005; Levenson and Sweatt, 2006; Peleg et al., 2010). One such study demonstrated that histone acetylation increases after contextual fear conditioning, and is triggered by the same signal transduction mechanisms that are implicated in LTP and memory consolidation (Levenson et al., 2004). Furthermore, treatment with drugs that promote acetylation helped to enhance long-term memory formation (Vecsey et al., 2007). Recent experiments have shown that acetylation levels increase in young mice with learning, for Lysines 9 and 14 on histone H3, and Lysines 5, 8, 12 and 16 on histone H4. In older mice, only acetylation of H4K12 failed to increase, suggesting that the loss of histone H4K12 acetylation is associated with age-related memory decline (Peleg et al., 2010).

Using a pharmacological network stimulation paradigm that specifically activates synaptic NMDA receptors (Hardingham et al., 2002), we show that expression levels of endogenous Arc protein are highly correlated with acetylation of H4K12, a modification marking regions containing gene bodies and transcriptional start sites (Park et al., 2013). While causality cannot be inferred from such a correlation, we noted that over-expression of exogenous Arc is able to increase H4K12 acetylation. The extent of this effect depends on the network activation status: under basal conditions a small but statistically significant increment is seen, while following pharmacological network activation a much more robust increase in H4K12 acetylation was evident. Therefore, activity-dependent Arc expression may make a critical contribution to the modulation of this learning-induced chromatin modification. Further evidence linking Arc with H4K12 acetylation comes from our microscopy data demonstrating the close association of individual molecules of Arc with Tip60, an enzyme responsible for this epigenetic modification, as well as with regions of the chromatin densely acetylated at this histone mark. By targeting an epigenetic regulator, such as Tip60, Arc would be able to more effectively control gene transcription. It will be interesting to see if H4K12 acetylation (or other substrates of Tip60) is impaired in Arc knockout animals. Taken together, our find-

452 ings point toward a function of nuclear Arc in memory consolidation, which involves modulation of the learn-
453 ing-induced H4K12Ac by direct association with the histone acetyltransferase Tip60. Further exploration of the
454 interaction between Arc and Tip60 or other chromatin-modifying enzymes in the nucleus would likely shed light on
455 the epigenetic mechanisms of memory consolidation.
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Legends

Figure 1. Segmentation of nuclear substructures.

A region of interest containing Arc-YFP puncta in a single neuronal nucleus is segmented to outline the Arc structures using Elements AR. The area of each structure is calculated and the mean and SEM are reported for each nucleus, together with the number of puncta (N).

Figure 2. Arc, β-Spectrin IV and Tip60 co-localize in nuclei of hippocampal neurons.

Fluorescently tagged versions of each protein were (co-)expressed in cultured hippocampal neurons. See text for details. DNA was labeled using DAPI (blue). Wide-field z-stacks were deconvolved using AutoQuant 3D deconvolution and a representative optical section through the center of the nucleus is shown. Scale bars are 2 μm. Four insets on the right show the structures at higher resolution. Inset scale bars are 0.5 μm.

Figure 3. Arc, β-Spectrin IV and Tip60 form a tight complex.

Fluorescently-tagged versions of Arc, β-Spectrin IV and Tip60 were co-expressed in hippocampal neurons (A-D) or HEK293 cells (E-H) and imaged as described in Fig. 2. Scale bar is 2 μm. I. Arc, βSpIVΣ5 and Tip60 fused to spectrally distinct GFP isoforms were expressed in hippocampal neurons, either individually, as pairs and all three together. Neuronal nuclei were outlined using DAPI staining and sub-nuclear structures ('puncta') were segmented as described in the Methods section. For each nucleus, the mean area and number of puncta was calculated. The scatter plot graphs 'number of puncta' versus 'mean area' for the different experiments. Arc, βSpIVΣ5 and Tip60, when expressed alone, each occupy a distinct region of the plot. Paired and tripartite complexes have properties similar to Tip60 alone.

Figure 4. Arc localizes the Arc-βSpectrin-Tip60 complex to the perichromatin region.

A. Fluorescently-tagged versions of Arc (green), β-Spectrin (red) and Tip60 (blue) were co-expressed in HEK293 cells and imaged as described in Fig. 3. Because of their strict overlap, Arc- β-Spectrin-Tip60 complexes appear as white puncta. The DAPI signal is shown in magenta. The insets (**B-E**) show the relationship between individual

complexes and chromatin with higher magnification. Segmentation of the DAPI structures is indicated with a magenta line. B shows a complex in the center of an interchromatin domain. Panels C and D show complexes localized to the perichromatin region, while E illustrates a complex surrounded by dense DAPI staining. Analysis of five nuclei indicated that the majority of puncta were localized to the perichromatin region (84%, n=151), while the puncta were less frequently observed in the center of the interchromatin region (9%, n=16) or in densely packed chromatin (7%, n=12).

F. Tip60-YFP was expressed in HEK293 cells, which were fixed as stained for DNA by DAPI. Tip60 occupies large parts of the inter-chromatin (IC) domains. Insets (**G-J**) show individual Tip60 speckles in higher magnification. Not the larger size of Tip60 compared to the tripartite complexes shown in B-E.

K. Arc-YFP was expressed in HEK293 cells, which were fixed and stained with DAPI. Many small Arc puncta are seen, which associate with the interface of the IC domains and the dense chromatin strongly labeled with DAPI, indicated with a red line that segments the DAPI structure. Insets (**L-M**) show the relationship of Arc puncta with chromatin at higher magnification.

P. Arc-YFP was expressed in hippocampal neurons (18 DIV), which were fixed and labeled with DAPI. White arrows indicate Arc puncta that localize to the IC-chromatin interface indicated with the red line. Insets (Q-T) show Arc localization in higher magnification.

Figure 5. Arc and βSpIVΣ5 interact with Tip60.

A. Tip60 interacts with Arc. HEK293T cells were transfected with either Arc in pCDNA3.1 and YFP vector, or Arc-pCDNA3.1 and Tip60-YFP. Tip60-YFP was immunoprecipitated with a mouse anti-GFP antibody, and Arc was detected with a rabbit anti-Arc antibody.

B. Tip60 interacts with ßSpIV∑5. HEK293T cells were transfected with either ßSpIV∑5-HA and YFP vector or ßSpIV∑5-HA and Tip60-YFP. Tip60-YFP was immunoprecipitated with a mouse anti-GFP antibody, and ßSpIV∑5-HA was detected with a mouse anti-HA antibody. **C**. Tip60 interacts with both Arc and ßSpIV∑5. HEK293T cells were transfected with Arc, ßSpIV∑5-HA, and either YFP or Tip60-YFP. Tip60-YFP was immunoprecipitated with a mouse anti-GFP antibody, and Arc or ßSpIV∑5-HA was detected with a rabbit anti-Arc or mouse anti-HA antibody respectively.

Figure 6. 3D Stimulated Emission Depletion Microscopy shows association of endogenous Arc and Tip60. The left panel shows the distribution of endogenous Arc protein (red) and endogenous Tip60 protein (green) in a representative z-plane of a hippocampal neuronal nucleus following network activation by a 4 hour treatment with 4AP-Bicuculline-Forskolin (see Methods). In the right panel, Tip60 structures have been segmented and are shown with a green outline to highlight their relationship with Arc. Scale bar is 1 μm. The bottom panel shows examples of the rich variety of association patterns formed by Arc and Tip60 puncta, with many unique conformations. Scale bar represents 300 nm.

Figure 7. Dual-color super-resolution microscopy of Arc-mEOS2 and endogenous Tip60. A representative z-plane of a hippocampal neuronal nucleus after 4 hours of network activation by 4AP-Bicuculline-Forskolin treatment (see Methods), showing the single-molecule distribution Arc-mEOS2 (green) imaged using three-dimensional 3D PALM and endogenous Tip60 (red) imaged using direct Stochastic Optical Reconstruction Microscopy (dSTORM). Cyan-colored squares outline distinct areas of association between Arc and Tip60 proteins, which were consistently found across reconstructed z-sections. Scale bar is 2 um. The bottom panel shows detailed images at higher magnification. Scale bar is 200 nm.

Figure 8. Arc increases nuclear Tip60 puncta.

A-D. DIV18 hippocampal neurons were transfected with Arc-YFP or YFP as a control, and imaged the next day. Arc-YFP expression was stimulated for 4hrs with 50μM Forskolin or DMSO as a control, fixed and stained for endogenous Tip60 (red). Comparing Arc-negative (**A**) with Arc-positive (**B**, **C**) neurons, it was found that over-expression of Arc-YFP induced the formation of endogenous Tip60 nuclear puncta, which associate with Arc-YFP puncta (B, C insets). 58 ± 4% (n=24) of endogenous Tip60 puncta were associated with exogenous Arc-YFP spots. Over-expression of YFP alone did not induce the formation of Tip60 hotspots (**D**).

E. DIV18 hippocampal neurons were co-transfected with Arc-YFP (green) and βSpIVΣ5-CFP (blue), treated for 4 hours with Forskolin, fixed and stained for endogenous Tip60 (red). Over-expression of both Arc and βSpIVΣ5

686 similarly induced the formation of endogenous Tip60 nuclear puncta, which associated with the Arc- βSpIVΣ5
687 complex. Scale bars are 2 μm; inset scale bars are 0.5 μm.
688
689 **Figure 9. Arc recruits Tip60 to PML bodies.**
690 **A.** Tip60-YPF (green) and PML-mCherry (red) were co-expressed in 18 DIV hippocampal neurons, which were
691 fixed and stained for DNA by DAPI (blue). Although Tip60 speckles are seen on close proximity of PML bodies in
692 the interchromatin domains, they do not overlap.
693 **B.** When Arc-YFP (green), Tip60-mCherry (blue) and PML-CFP (red) where co-expressed, they were found to
694 tightly overlap, indicated by a puncta containing a preponderance of white pixels in the merged image (far-right
695 panel).
696 **C and D.** When the same experiment was performed in HEK293 cells, Tip60 and PML occupied non-overlapping
697 regions of the interchromatin space (C), while inclusion of Arc resulted in puncta in which all three proteins tightly
698 co-localized (D).
699 **E.** A nucleus of a representative HEK293 expressing Arc (blue), Tip60 (green), and PML (red), showing that in
700 structures containing moderate to high Arc (white arrows 2-4) Tip60 is recruited to PML bodies, which does not
701 occur when Arc is low (white arrow 1). The bottom insets are zoom-in views of PML bodies 1 – 4 as viewed
702 through Structured Illumination Microscopy, showing that Tip60 puncta heavily populate and permeate the porous
703 PML bodies more efficiently when Arc is present. Scale bars are 2 um, except for bottom panel which scale bar is
704 500 nm.
705
706 **Figure 10. Arc expression increases H4K12Ac levels.**
707 Cortical neurons (21 DIV) were transfected with Arc-YFP or YFP as a control. The next day the cultures were
708 treated with either Forskolin or the combination of 4AP-Bicuculline-Forskolin (see Methods section for details).
709 After 4 hours of treatment, the neurons were fixed and stained for both H4K12Ac and endogenous Arc, using a red
710 and far-red secondary antibody, respectively. DNA was labeled using DAPI. Scale bars represent 10 μm.
711 **A.** Representative images for the four experimental conditions. Each field contains a transfected neuron (YFP or
712 Arc-YFP) surrounded by untransfected controls. The top row shows the YFP or Arc-YFP signal, while the bottom

713 row representing the H4K12Ac signal. The transfected neuron is indicated with a white arrow. Nuclear outlines are
714 shown as thin blue lines.
715 **B.** H4K12Ac levels (mean fluorescence intensity per nucleus) were determined for both transfected neurons (YFP
716 or Arc-YFP) and untransfected controls (Con) for both treatment conditions for 30 fields of view containing at least
717 1-4 transfected neurons and 30-90 untransfected controls. The bar graph shows the average H4K12Ac levels,
718 normalized using the mean of the untransfected neurons, with error bars indicating SEMs. YFP over-expression did
719 not significantly alter H4K12Ac levels in either condition: p-values were 0.07 and 0.12 for Forskolin and
720 4AP-Bic-Fors, respectively. Arc-YFP over-expression increased H4K12 acetylation levels for both treatment
721 scenarios, with high statistical significance ($p=3*10^{-5}$ for Forskolin, $p=5*10^{-11}$ for 4AP-Bic-Fors), although the
722 increase was much larger following network activation by 4AP-Bic-Fors (89%) than with Forskolin treatment only
723 (10%).
724 **C.** The relationship between endogenous Arc expression and H4K12Ac levels was investigated by analyzing the
725 neurons that were not transfected. The inset shows DAPI, endogenous Arc and H4K12Ac levels for 15
726 untransfected neuronal nuclei. Five neurons (solid white arrows) strongly expressed endogenous Arc and the same
727 five neurons also displayed high levels of H4K12 acetylation. Eleven neurons (thin gray arrows) had barely de-
728 tectable Arc levels, and H4K12Ac staining was faint as well. Nuclei are outlined by a thin blue line. The graph was
729 generated by sorting 816 nuclei by their endogenous Arc levels and plotting H4K12Ac levels versus the sortation
730 index, from low to high Arc levels. The solid black line is a moving average of 50 H4K12Ac values. All scale bars
731 represent 10 μm.
732
733 **Figure 11. A Tip60 mutant lacking acetyltransferase activity decreases H4K12 acetylation.**
734 Hippocampal neurons (21 DIV) were transfected with a catalytically-inactive double mutant of Tip60
735 (Q377E/G380E, abbreviated as Tip60dm).
736 **A-B**. Representative pair of neuronal nuclei positive (A) and negative (B) for Tip60dm, showing that upon
737 Tip60dm overexpression, the overall staining of H4K12Ac per nucleus is decreased.
738 **C.** Distributions of nuclear H4K12Ac staining in neurons expressing Tip60dm versus negative control neurons.
739 Each horizontal line represents the average H4K12Ac intensity of a nucleus. All averages were normalized to the

mean of the population of Tip60dm-negative neurons. Circles to the left of the distributions indicate the mean and SEM of each population: mean ± SEM (N) is 1.00 ± 0.02 (n=408) for Tip60dm-negative and 0.62 ± 0.06 (n=27) for Tip60dm-positive neurons. P-value = $4*10^{-9}$. Scale bars are 2 µm.

Figure 12. Arc associates with H4K12Ac.

A-E. A representative hippocampal neuronal nucleus showing H4K12Ac staining (**A**), and Arc-YFP puncta (**B**). The same image with increasing the brightness threshold to show only the brightest 50^{th} percentile H4K12ac shows clear H4K12Ac "hotspots" (**C**), which is overlaid with localized Arc-YFP puncta (**D**; scale bar = 1um). Insets below show association of Arc puncta with H4K12ac hotspots (E; scale bar = 200nm). Hippocampal cultures were treated with 4AP, Bicuculline and Forskolin for 4 hours to induce Arc expression. **F**. A representative image of a hippocampal neuron that has been transfected with Arc-YFP, showing Arc-YFP puncta (green), imaged with Structured Illumination Microscopy to observe the localization of H4K12Ac (red) in higher resolution. The increased resolution afforded by SIM did not substantially change the Arc puncta. However, the H4K12Ac staining which appeared fairly homogenous in panel A, can now be seen to consist of many tiny structures, the size of which is just below the resolution of the conventional light microscope. They are distributed fairly uniformly over the nucleus, although local concentrations can be observed, which would correspond to the 'hotspots' in panel C. Some of these clusters of H4K12Ac seemed to associate with Arc puncta. The scale bar is 1.7 µm. Panel **G** shows six Arc puncta at higher magnification. The top row shows an Arc puncta associating with dense H4K12Ac clusters, while the bottom row shows a few bright H4K12Ac structures surrounding Arc puncta. The scale bar is 500 nm.