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The EJC Factor eIF4AIII Modulates Synaptic Strength and Neuronal Protein Expression

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SUMMARY

Proper neuronal function and several forms of synaptic plasticity are highly dependent on precise control of mRNA translation, particularly in dendrites. We find that eIF4AIII, a core exon junction complex (EJC) component loaded onto mRNAs by pre-mRNA splicing, is associated with neuronal mRNA granules and dendritic mRNAs. eIF4AIII knockdown markedly increases both synaptic strength and GLUR1 AMPA receptor abundance at synapses. eIF4AIII depletion also increases ARC, a protein required for maintenance of long-term potentiation; *arc* mRNA, one of the most abundant in dendrites, is a natural target for nonsense-mediated decay (NMD). Numerous new NMD candidates, some with potential to affect synaptic activity, were also identified computationally. Two models are presented for how translation-dependent decay pathways such as NMD might advantageously function as critical brakes for protein synthesis in cells such as neurons that are highly dependent on spatially and temporally restricted protein expression.

INTRODUCTION

In eukaryotes, nearly every aspect of cytoplasmic mRNA metabolism, including subcellular localization, translational efficiency, and mRNA half-life, is highly regulated (Moore, 2005). Cytoplasmic control of gene expression is particularly important for elaboration of such key asymmetric functions as cell division, oogenesis, body patterning, cell motility, and synaptic plasticity (Du et al., 2007; Steward and Schuman, 2003). In the case of neurons, dendritically localized mRNAs encode membrane receptors, neurotrophins, cytoskeletal proteins, and key regulatory kinases that participate in synaptic activity. Local protein synthesis from dendritic mRNAs is important for the maintenance of synaptic function as well as several forms of synaptic plasticity (Amrani et al., 2004; Sutton and Schuman, 2005; Ule and Darnell, 2006).

Cytoplasmic mRNA metabolism is mediated by a host of *trans*-acting factors that, along with the mRNA, form a messenger ribonucleoprotein particle (mRNP; Kindler et al., 2005). Many cytoplasmic mRNP constituents are initially acquired in the nucleus; thus the nuclear history of an mRNA is of significant consequence for its cytoplasmic fate (Czaplinski and Singer, 2006; Farina and Singer, 2002; Giorgi and Moore, 2007). One clear example is *oskar* mRNA, whose localization at the posterior pole of *Drosophila* oocytes requires the transcript to have undergone pre-mRNA splicing (Hachet and Ephrussi, 2004). *Trans*-acting factors required for this process include eIF4AIII, BARENTSZ, TSUNAGI, and MAGO NASHI, the *Drosophila* homologs of mammalian eIF4AIII, MLN51, Y14, and MAGOH, respectively (Palacios et al., 2004; and references therein). These four proteins constitute the core of a larger assemblage known as the exon junction complex (EJC; Ballut et al., 2005; Tange et al., 2005).

EJCs are deposited on mRNAs upstream of exon–exon junctions in a manner independent of sequence and as a consequence of pre-mRNA splicing (Le Hir et al., 2000; Tange et al., 2004). Structurally, the EJC consists of a stably-bound tetrameric core that serves as a binding platform for other more transiently associated factors. Within this core, the DEAD-box protein eIF4AIII collaborates with its binding partner MLN51 to serve as the main RNA binding constituent (Ballut et al., 2005; Shibuya et al., 2004). EJCs travel with spliced mRNAs to the cytoplasm, where those located within open reading frames (ORFs) can be positive effectors of ribosome recruitment (Nott et al., 2004) prior to their displacement by the first or "pioneer" round of translation (Dostie and Dreyfuss,

2002; Ishigaki et al., 2001). Conversely, EJCs located downstream of an ORF can trigger nonsense-mediated mRNA decay (NMD; Conti and Izaurralde, 2005). In mammals, NMD substrates are presumably targeted for decay coincident with the pioneer round (Ishigaki et al., 2001). This targeting involves interaction between the key NMD factor UPF1 and a ribosome stalled at a termination codon. UPF1 recruitment occurs by way of its interaction with UPF2 and UPF3, which directly associate with any downstream EJCs. This pathway not only serves to downregulate aberrant mRNAs (e.g., those produced from mutant alleles or by improper pre-mRNA processing), it also modulates protein expression from natural NMD targets. Such natural targets include mRNAs containing upstream open reading frames (uORFs) and those produced from transcripts harboring introns in the 3′-UTR (Hillman et al., 2004; Mendell et al., 2004; Wittmann et al., 2006).

During *Drosophila* oocyte maturation, all four EJC core factors accumulate at the posterior pole along with *oskar* mRNA. Also exhibiting this same localization pattern is STAUFEN, a double-stranded RNA-binding protein required for both transport and regulated translation of *oskar* (Micklem et al., 2000; St Johnston, 2005). In addition to its role in oogenesis, STAUFEN has been implicated as an mRNA transport factor in both *Drosophila* and mammalian neurons (Kiebler et al., 1999; Li et al., 1997). In *Drosophila* neurons, dendritic STAUFEN granules contain UPF1 and other factors involved in mRNA translational regulation and decay (Barbee et al., 2006). In mammalian neurons, both ubiquitous STAUFEN1 (STAU1) and brain-specific STAUFEN2 (STAU2) exhibit somatodendritic localization, and STAU1 has been shown to function in the dendritic delivery of some mRNAs (Kanai et al., 2004; Tang et al., 2001). Also exhibiting dendritic localization in mammalian neurons are the EJC core factors MLN51, Y14, and MAGOH (Glanzer et al., 2005; Macchi et al., 2003; Monshausen et al., 2004), although no functional consequence of this localization has yet been reported.

Here we investigated participation of the key EJC factor eIF4AIII (referred to hereafter as 4AIII) in mRNA expression in mammalian neurons. Functionally, 4AIII knockdown in neurons leads to increased miniature excitatory postsynaptic currents (mEPSCs) and increased abundance of the AMPA receptor GLUR1 at synapses. Structurally, 4AIII is associated with neuronal mRNA transport granules and dendritic mRNAs. One such mRNA, *arc* (encoding the Activity-regulated cytoskeletal-associated protein; Arg3.1), is a natural target for NMD by virtue of two EJCs situated on its 3′-UTR. Bioinformatic analysis indicates that several other brain-expressed mRNAs, as well as numerous mRNAs in other tissues, could be regulated in an analogous fashion. Finally, two models are presented for how translation-dependent mRNA decay pathways such as NMD might advantageously function as critical brakes on protein synthesis in cells such as neurons that are highly dependent on precise control of protein expression in the cytoplasm.

RESULTS

4AIII Displays Somatodendritic Localization in Rat Hippocampal and Cortical Neurons

In HeLa cells, 4AIII exhibits primarily nuclear localization (Figure 1A; Palacios et al., 2004; Shibuya et al., 2004). To examine its distribution in neurons, we used an antiserum against human 4AIII that also recognizes the rat protein (Figure S1A). The hippocampus is particularly well suited for examining subcellular compartmentalization of neuronal proteins because cell bodies and neurites reside in separate and easily distinguishable layers (Figure 1B, left panel). Immunofluorescence of coronal brain sections revealed endogenous 4AIII in the dendritic layer as well as the nuclear and cytoplasmic (somatic) compartments of neurons within the CA1 field (Figure 1B, right panels). Other hippocampal fields (e.g., dentate gyrus; Figure S3) displayed similar 4AIII distributions. We also examined 4AIII localization in cultured rat cortical neurons. Whereas 4AIII was primarily nuclear in the glial feeder cells (similar to HeLa; Figure S1B, top panels), cortical pyramidal neurons showed both somatic and dendritic staining similar to the hippocampal neurons (Figure 1C). An analogous distribution pattern was observed using a different α-4AIII serum (Figure S1B, bottom panels). Thus, in both brain sections and cultured neurons, a significant fraction of 4AIII resides outside the nucleus.

4AIII Colocalizes and Physically Associates with Known Dendritic mRNP Proteins

In both dendrites and somata of cultured neurons, endogenous 4AIII exhibited extensive colocalization with two well-known dendritic mRNP proteins, STAU1 (Figure 1D) and the FRAGILE X protein (FMRP; Figure 1E). FMRP is a translational repressor of dendritic mRNAs and associates with neuronal mRNA transport granules (Kanai et al., 2004; Vanderklish and Edelman, 2005). Immunoprecipitation (IP) assays confirmed a RNA-dependent biochemical association between 4AIII and FMRP in brain lysates (Figure S1C). Taken together, these data suggest that 4AIII is associated with FMRP and STAU1 in dendritic mRNPs.

Also colocalizing with FMRP was the essential NMD factor UPF1, which like 4AIII, distributes throughout somata and dendrites (Figure 1F). This observation nicely parallels recent data showing that UPF1 and other factors involved in mRNA decay and microRNA function colocalize with STAUFEN- and FMRP-containing granules in *Drosophila* neurons (Barbee et al., 2006).

4AIII Associates with Dendritic mRNAs

To test directly the idea that 4AIII is physically associated with dendritic mRNAs, cytosolic extracts of rat cortex were incubated with α-4AIII or nonimmunized (rIgG) rabbit serum, and the immunoprecipitated complexes were analyzed by semiquantitative RT-PCR (Figure 2A). For dendritic mRNAs, we chose *arc*, *dendrin*, *map2*, *camkIIα*, *gluR1*, and *grin1*, all of which have been

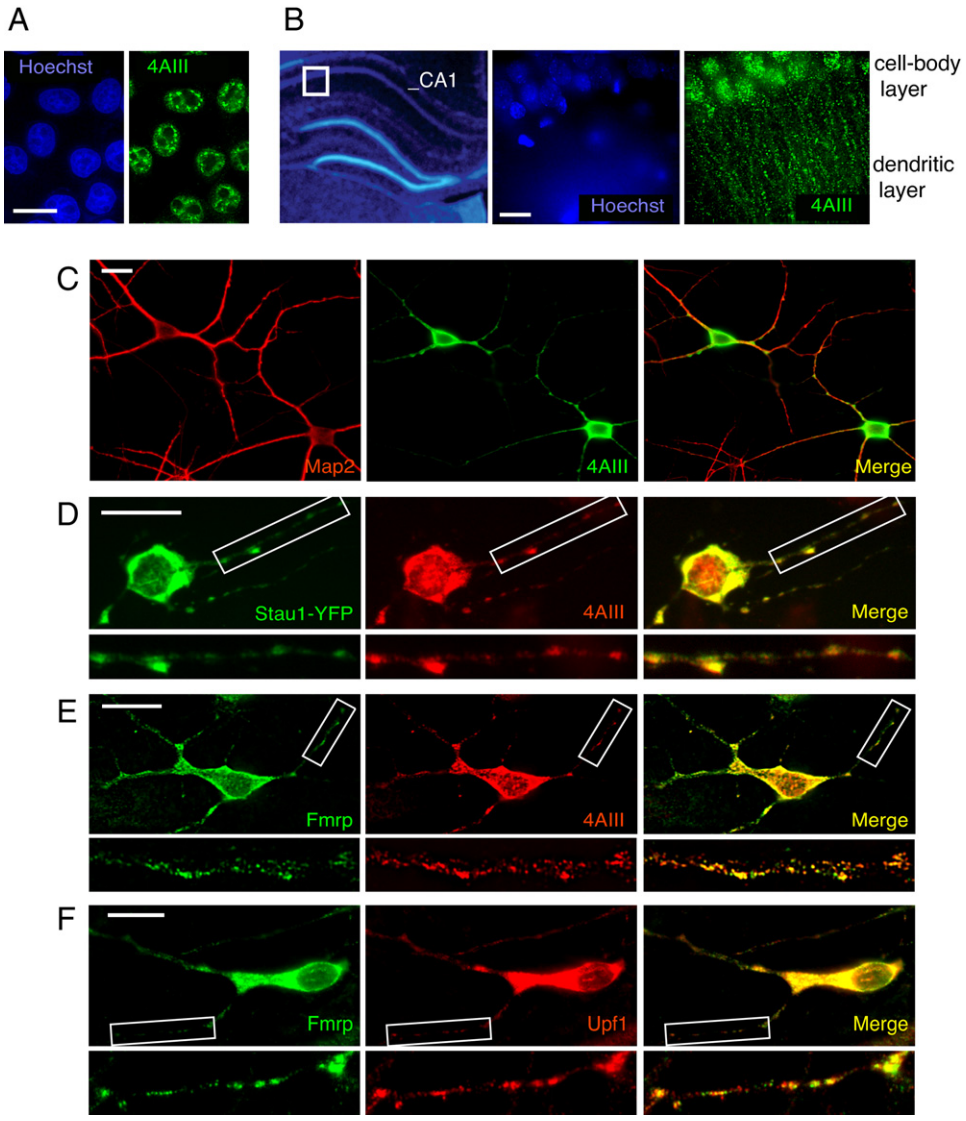

Figure 1. 4AIII Exhibits Somatodendritic Localization in Neurons and Colocalizes with STAU1 and FMRP

(A) HeLa cells stained for DNA (blue) and endogenous 4AIII (green; scale bar: 30 μm).

(B) Left: Coronal slice (p25 rat; 25 days old) showing hippocampus stained for DNA (blue) and the CA1 field (white box) enlarged in middle and right panels, which show DNA (blue) and endogenous 4AIII (green; scale bar: 30 μm).

(C) Endogenous 4AIII (green) and MAP2 (red) in DIV7 cultured cortical neurons. MAP2 allows for visualization of dendrites.

(D–F) Colocalization of endogenous eIF4AIII or UPF1 (central panels; red) with exogenous STAU1-YFP or endogenous FMRP (left panels; green) in cultured cortical neurons. Right panels: merged signals. Lower panels: enlargements of indicated (white box) dendritic regions.

(In [C]–[F] scale bar is 10 μm).

documented to exhibit dendritic localization (Ju et al., 2004; Steward and Schuman, 2003). For comparative purposes, we also analyzed three housekeeping mRNAs that encode essential components of the glycolytic pathway: *gapdh*, *phgdh*, and *pgam1* (Table S1). For all three housekeeping mRNAs, very little precipitation was observed with either α-4AIII or nonimmune serum. In contrast, all six dendritic mRNAs were significantly enriched in α-4AIII precipitates. This preferential association could be quantified by normalizing all IP efficiencies to that of *gapdh* (Figure 2B). Whereas all three housekeeping mRNAs exhibited very low coIP efficiencies, the dendritic mRNAs were between 5- and 30-fold enriched in the α-4AIII pellet compared to *gapdh*. Thus 4AIII exhibits preferential association with dendritic mRNAs.

Arc mRNA Is a Natural Target for NMD

Within the dendritic set, *arc* mRNA exhibited the greatest fold enrichment upon α-4AIII IP (Figure 2B). Examination of the *arc* gene revealed it to have a conserved and highly

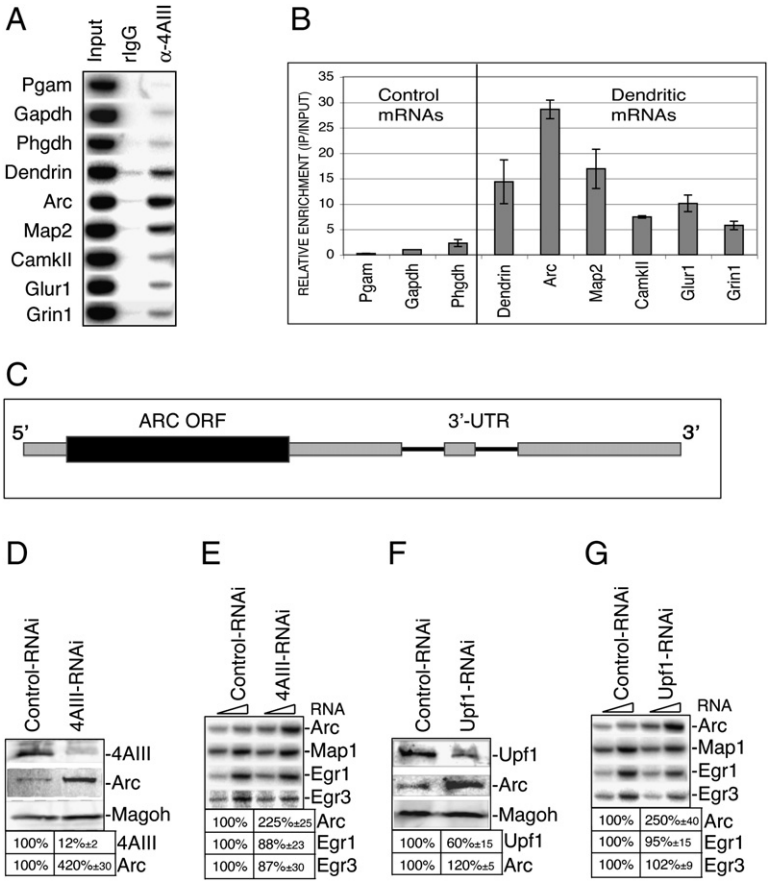

Figure 2. 4AIII Associates with Dendritic mRNAs and Modulates Arc mRNA, a Natural NMD Substrate

(A) Semiquantitative RT-PCR of RNA samples immunoprecipitated from cytosolic cortex extracts with either α-4AIII or control (rabbit IgG; rIgG) serum.

(B) Relative mRNA enrichment upon α-4AIII IP determined by subtracting control serum IP values from 4AIII-IP values, dividing the result by input (total) for that mRNA, and then normalizing all values to that obtained for Gapdh mRNA. Bars are averages of four data points from two separate experiments ± standard deviation (SD).

(C) Schematic representation of mammalian *arc* gene: 5′- and 3′-UTRs (gray boxes), ORF (black box), and introns (lines).

(D–G) RNAi knockdown of 4AIII (D–E) or UPF1 (F–G) in PC12 cells. Western blot signals for 4AIII (D), UPF1 (F), and ARC were normalized to those for MAGOH. (E and G) Semiquantitative RT-PCR signals for *arc*, *egr1*, and *egr3* mRNAs were normalized to those of *map1* mRNA; reported values are averages of three independent experiments ± SD.

unusual structure. In the three mammalian genomes for which *arc* pre-mRNA splicing patterns could be confirmed with extensive EST data (human, mouse, and rat), only two introns are apparent, and both are located in the 3′-UTR (Figures 2C and S2). This contrasts with the vast majority of mammalian genes, where introns are generally excluded from this region because EJC deposition downstream of a termination codon can induce NMD of the product mRNA (see Introduction). Thus mammalian *arc* mRNA would appear to be a natural NMD target.

To examine the possibility that EJC deposition plays a role in modulating *arc* expression, we performed RNAi knockdown of 4AIII in rat PC-12 cells. This neuroendocrine cell line was previously utilized to monitor *arc* mRNA abundance in response to depolarization and/or translational inhibitors (Ichikawa et al., 2003; Waltereit et al., 2001). Whereas 88% knockdown of endogenous 4AIII resulted in a 2-fold increase in *arc* mRNA (Figures 2D and 2E), another dendritic mRNA, *map1*, was unaffected. Also unaffected were two other immediate early mRNAs, *egr1* and *egr3*, indicating that the increase in *arc* expression upon 4AIII knockdown was not due to transcriptional activation. Consistent with elevated *arc* mRNA, ARC protein levels increased 4-fold upon 4AIII depletion (Figure 2D). Coexpression of an exogenous, RNAi-immune human 4AIII construct restored ARC protein levels to their endogenous state, validating the specificity of the 4AIII knockdown (Figure S2C).

To confirm that the increases in *arc* mRNA and protein levels upon 4AIII knockdown were due to disruption of NMD, we repeated the RNAi analysis, targeting instead the essential NMD factor UPF1 (Figure 2F). As observed for knockdown of 4AIII, Arc mRNA levels increased upon Upf1 depletion, whereas *map1*, *egr1*, and *egr3* mRNA levels were unaffected (Figure 2G). ARC protein levels also increased in UPF1 knockdown cells (Figure 2F). We conclude that *arc* mRNA is a natural NMD target.

4AIII Remains Associated with *arc* mRNA upon Induction

Arc is an immediate early gene whose mRNA traffics to dendrites and accumulates at activated synapses. *Arc* expression is modulated by neuronal activity and is required for both maintenance of long-term potentiation (LTP) and long-term memory consolidation (Plath et al., 2006; Steward and Worley, 2002; Tzingounis and Nicoll, 2006). Activity-dependent upregulation of *arc* mRNA and protein can be strongly induced in vivo by electroconvulsive or kainic acid-induced seizures, with particular accumulation in the dentate gyrus (Wallace et al., 1998; Waltereit et al., 2001). This effect can be recapitulated in vitro by stimulation of cultured neurons with, for

example, brain-derived neurotrophic factor (BDNF; Rao et al., 2006).

Having observed a strong association between 4AIII and *arc* mRNA in brain extracts under unstimulated conditions (Figures 2A and 2B), we next assessed whether any change in this interaction occurred upon *arc* mRNA induction. To do so, we examined the association of *arc* mRNA with 4AIII in the dentate gyrus or in cultured cortical neurons pre- and post-kainic-acid-induced seizure or BDNF treatment, respectively. Both protocols significantly increased overall *arc* mRNA and protein levels (Figures 3A, S3, and S4A), and these increases were apparent in both somata and dendrites (Figures 3B and S3). IP of cytoplasmic extracts from dissected dentate gyrus or cultured neurons revealed that 4AIII was equally associated with *arc* mRNA pre- and postinduction (Figures 3C and S4B). Thus, even when *arc* mRNA is strongly induced, its association with 4AIII is unperturbed. This suggests that *arc* mRNA is subject to NMD even when it accumulates in response to strong stimuli.

To rule out the possibility that 4AIII association with induced *arc* mRNA is limited to the somatic compartment, we also analyzed their colocalization in dendrites (Figure 3D). Of 360 dendritic granules analyzed from six neurons following BDNF treatment, 59% of *arc* mRNA granules colocalized with 4AIII. On the other hand, colocalization of 4AIII with *arc* mRNA accounted for only 29% of 4AIII puncta, most likely reflecting association of 4AIII with other dendritic mRNPs. Indeed, our data indicate that only upon induction does the level of Arc mRNA approach those of other dendritic mRNAs (note that in both Figures 2A and 3C, RT-PCR reactions for Arc contained ten times more total RNA than those for other dendritic mRNAs). Further, we observed no significant changes in either the overall levels or subcellular distribution of 4AIII upon *arc* induction in either the dentate gyrus or cultured neurons (Figures S3 and S4C). This is the expected result if *arc* represents just a fraction of dendritic mRNA.

4AIII Knockdown Increases *arc* mRNA and Protein Abundance in Neurons

Having shown that the association of 4AIII with *arc* mRNA is unperturbed upon induction, we next analyzed the effects of 4AIII knockdown on *arc* mRNA and protein levels after BDNF treatment. Coexpression of shRNAs and eGFP from a single plasmid allowed for ready identification of transfected neurons; downregulation of endogenous 4AIII was confirmed by immunofluorescence (Figure S5A). Upon 4AIII knockdown *arc* mRNA and protein levels increased in both somata and dendrites (Figures 4A, 4B and S5B). These mRNA and protein increases were confirmed quantitatively by cumulative frequency analysis (Figure 4, right panels). Significantly, upon 4AIII knockdown, ARC protein levels increased not only in somata and dendrites, but also at synapses (Figure 4B).

4AIII Knockdown Increases Excitatory Synaptic Strength

Having shown that 4AIII knockdown changed the levels of at least one protein at synapses, we next sought to investigate the effects of 4AIII knockdown on postsynaptic strength. We recorded excitatory miniature postsynaptic currents (mEPSCs) mediated by AMPA receptors (Turrigiano et al., 1998; Watt et al., 2000) in cortical neurons. Individual mEPSCs (Figure 5A) reflect the postsynaptic response to release of individual neurotransmitter vesicles; mEPSC amplitude is generally thought to reflect the number of AMPA receptors present in the postsynaptic membrane, whereas mEPSC frequency is dependent on the number of functional synaptic contacts and the probability of spontaneous vesicle fusion.

We recorded from untransfected neurons and from neurons transfected with either the 4AIII-RNAi or a control-RNAi plasmid (DIV7). By expressing the shRNA constructs at very low efficiency (10–20 neurons/culture dish) we could test the effects of 4AIII knockdown in postsynaptic neurons without affecting 4AIII expression presynaptically and without perturbing network activity. Knockdown of 4AIII approximately doubled mEPSC amplitude (Figures 5A, 5B, and 5C) without affecting mEPSC frequency (Figure 5C) or passive cellular properties such as input resistance and resting potential. 4AIII knockdown decreased the rise time constants of the mEPSCs slightly (Figures 5B and 5C; $p < 0.01$), and there was a nonsignificant trend toward reduction in the decay time constant; these small changes in kinetics could reflect changes in receptor properties.

To determine whether the observed changes in mEPSC amplitude caused by 4AIII knockdown were associated with changes in AMPA receptor abundance at synapses, we compared the amounts of synaptic GLUR1 in neurons transfected with either a 4AIII- or control-RNAi plasmid. Coimmunofluorescence of the presynaptic marker synapsin and surface GLUR1 revealed a marked increase of this AMPA receptor at putative synaptic sites upon 4AIII knockdown (Figure 5D).

Overall, we find that 4AIII knockdown leads to an increase in mEPSC amplitude and a corresponding increase of GLUR1 receptor levels at synapses. These data indicate that 4AIII normally acts as a brake on synaptic strength so that reducing levels of 4AIII leads to a dramatic increase in quantal synaptic transmission.

Bioinformatics Reveals a Set of Genes Containing Conserved 3′-UTR Introns

In light of recent evidence that ARC protein overexpression leads to decreased GLUR1 levels at synapses (see Discussion), our observation that 4AIII knockdown increased synaptic GLUR1 was unexpected. A simple interpretation is that *arc* is not the only dendritic mRNA affected by 4AIII depletion. We therefore sought to identify other potential targets for regulation by 4AIII via its ability to induce NMD when bound downstream of a natural stop codon. Previous studies that aimed to identify such

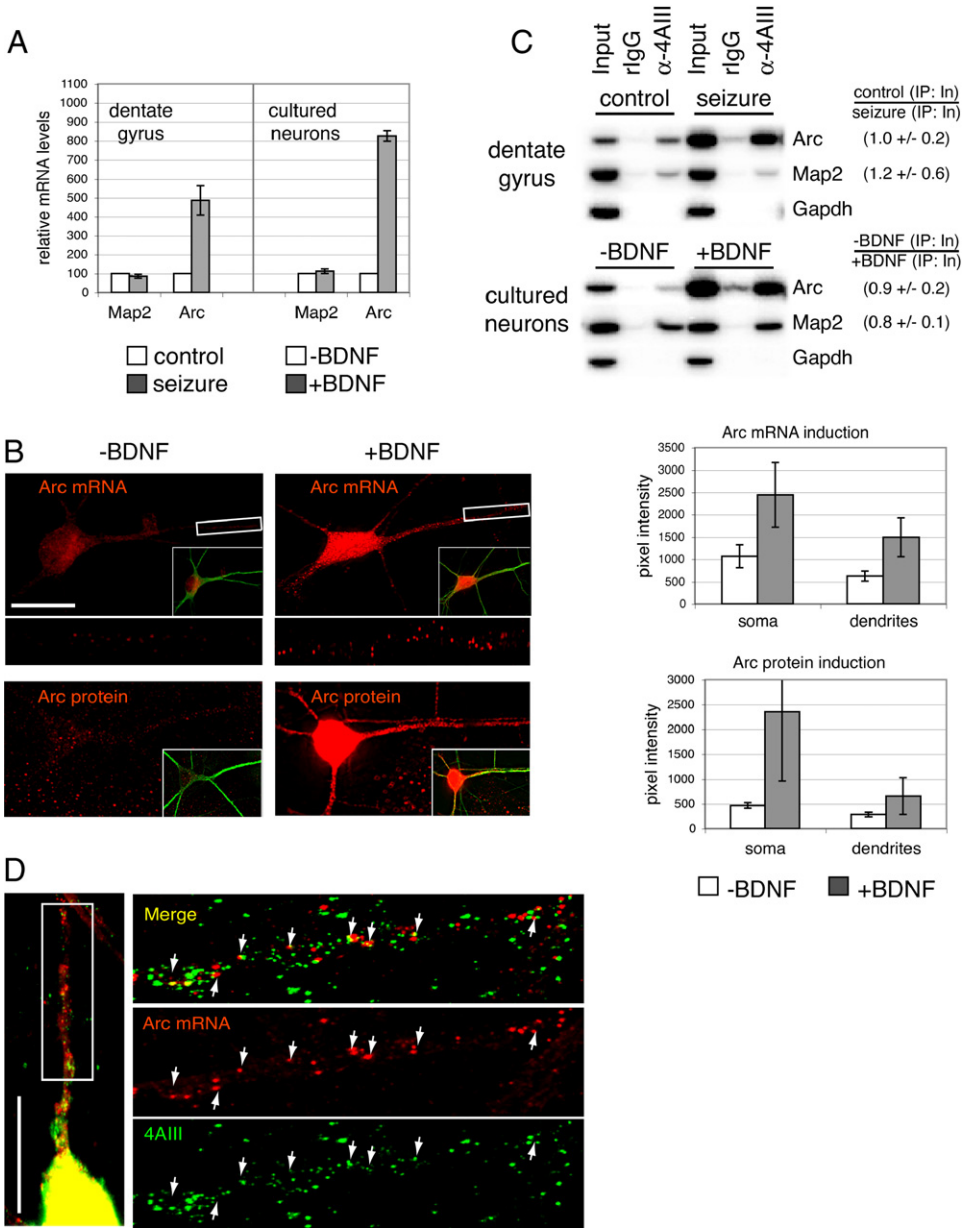

Figure 3. No Change in 4AIII:Arc mRNA Association upon Arc Induction

(A) *Arc* and *map2* mRNAs levels pre- and postinduction (calculated comparing RT-PCR input levels in [C]. Seizure: kainic acid-induced seizure of adult rats; +BDNF: treatment of cultured neurons with BDNF.

(B) Left: FISH of *arc* mRNA (top panels) and immunofluorescence of ARC protein (bottom panels) in cultured neurons pre- and post-BDNF treatment. MAP2 (green inset) allows for visualization of dendrites. Middle panels: Enlargements of boxed dendritic regions. Right: Relative intensities of *arc* mRNA and protein before and after induction in soma and dendrites. Bars are averages of two separate experiments ± standard deviation (SD).

(C) Semiquantitative RT-PCR of RNA samples immunoprecipitated from cytosolic extracts of dissected dentate gyrus (top) or cultured cortical neurons (bottom) with either α-4AIII or control (rabbit IgG; rIgG) serum. Relative α-4AIII IP efficiencies ± SD are reported at right.

(D) FISH of *arc* mRNA (red) and immunofluorescence of 4AIII (green) after BDNF treatment. Right panels: enlargement of indicated dendritic region. Arrows indicate examples of colocalization. (In [B] and [D] scale bar is 10 μm).

natural mammalian NMD targets employed microarray analysis of UPF1- or UPF2-depleted cells (Mendell et al., 2004; Wittmann et al., 2006). However, *arc* mRNA was not identified in either study, likely because it is poorly expressed in the cell type (HeLa) examined (data not shown). Therefore other potential targets may have been missed as well.

An alternate approach for the prediction of NMD targets is to search for conserved 3′-UTR introns computationally. In order to generate a biologically important set of

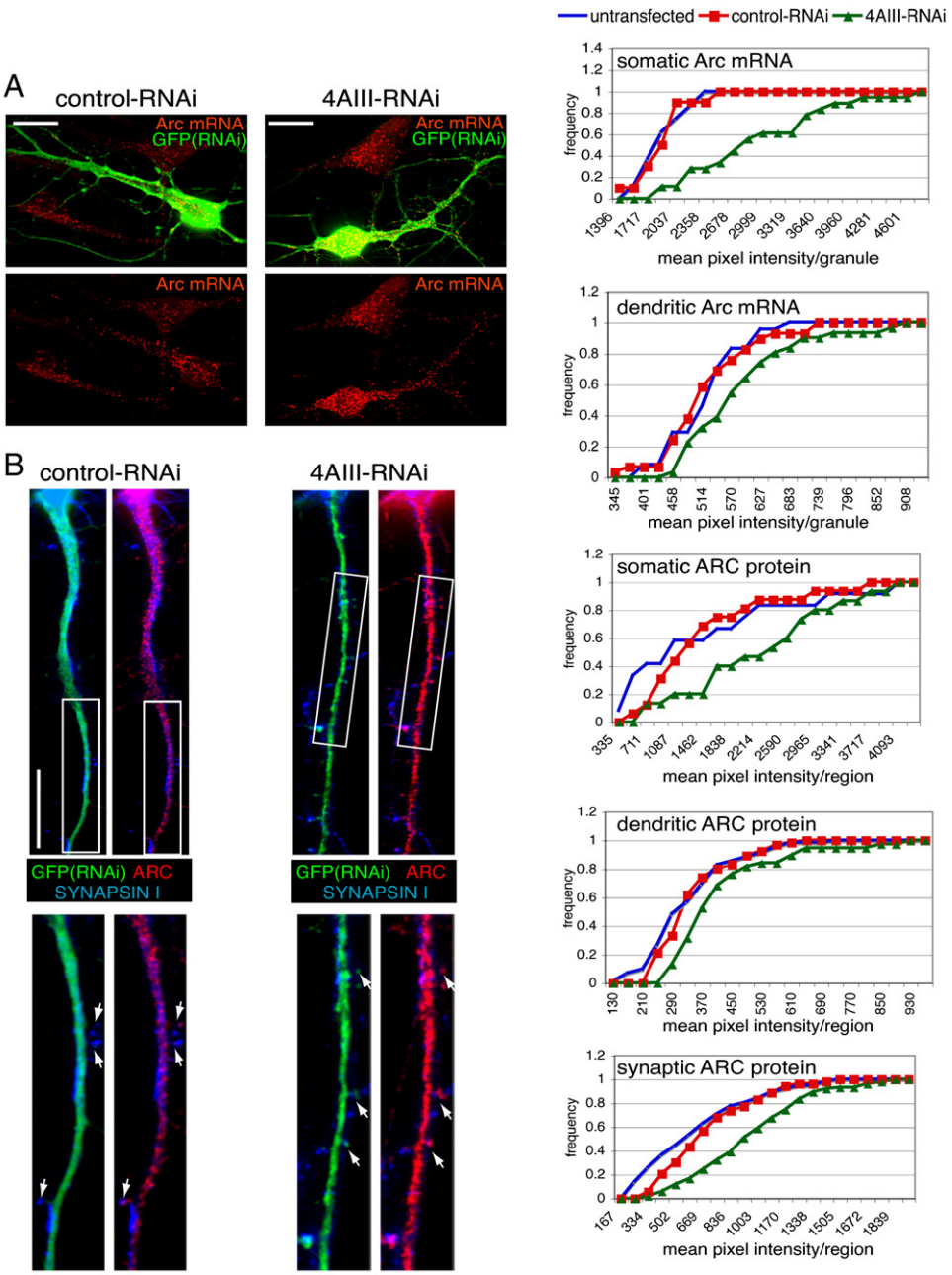

Figure 4. 4AIII Knockdown Increases *arc* mRNA and Protein Levels in Neurons

(A and B) GFP-positive cells (green): neurons transfected with control-RNAi (left panels) or 4AIII-RNAi (right panels) plasmid. Following BDNF treatment, neurons were subjected to immunofluorescence of GFP and either (A) FISH of endogenous *arc* mRNA (red) or (B) immunofluorescence of ARC protein (red) and the presynaptic marker SYNAPSIN1 (blue). Right panels: Cumulative histograms quantifying the distribution of *arc* mRNA and ARC protein in soma (A and B), dendrites (A and B) and synapsin-positive spines (B) of neurons that were either untransfected, transfected with control-RNAi plasmid, or transfected with 4AIII-RNAi plasmid. Distributions are representative of at least two separate experiments. Arrows indicate examples of SYNAPSIN-positive spines. Relative distribution of ARC protein in transfected versus untransfected neurons is shown in Figure S5.

candidates, we focused on identifying human genes where any annotated 3′-UTR intron contains canonical splice signals (GT-AG) that are evolutionarily conserved across multiple mammalian species. As schematized in Figure 6, we implemented an algorithm to identify genes where: (1) alignment of known transcripts to the human genome indicates the presence of at least one 3′-UTR intron; (2) the 5′ and 3′ splice sites are conserved across human, rat, and mouse; (3) the 3′-UTR intron is at least 30 bases long to avoid mistakes in aligning transcripts to

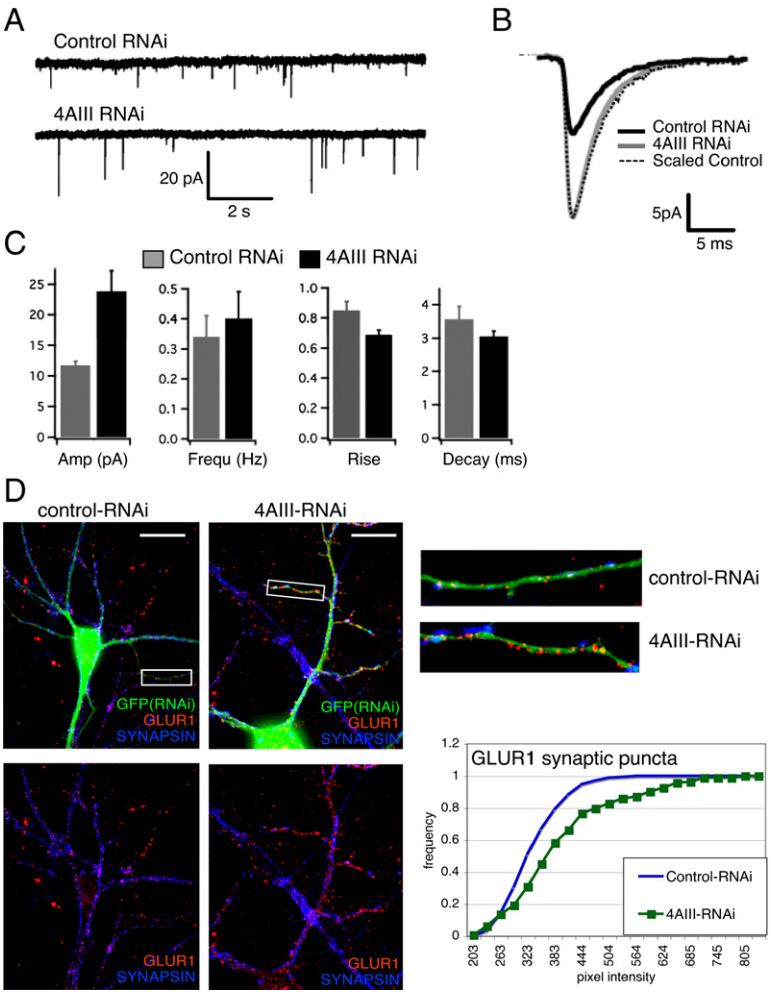

Figure 5. 4AIII Knockdown Increases Excitatory Synaptic Transmission and GLUR1 Levels at Synapses

(A) Example of mEPSC recordings from neurons transfected with either control or 4AIII-RNAi plasmid.

(B) Average mEPSCs from control (black trace, n = 10) or 4AIII-RNAi (gray trace, n = 11) neurons, constructed by averaging all mEPSCs from each neuron and then generating a meta-average for each condition. Scaling the control average mEPSC to the same amplitude as the 4AIII-RNAi average reveals small changes in mEPSC kinetics (dotted line).

(C) Average mEPSC amplitude ($p < 0.001$), frequency ($p = 0.41$), rise times ($p < 0.02$), and decay times ($p = 0.22$).

(D) DIV7 neurons transfected with either control-RNAi (left panels) or 4AIII-RNAi (right panels) plasmid (green) were stained for SYNAPSIN (blue) and surface GLUR1 (red; scale bar is 10 μm). Top right panels: enlargement of indicated dendritic regions. Bottom right panel: cumulative histogram representing the mean intensity distribution of SYNAPSIN-positive GLUR1 puncta. For each condition, 12 neurons and a total of ~200 synapses from termini of apical dendrites were analyzed. The distribution is representative of three separate experiments.

the genome; (4) the intron is at least 50 nucleotides downstream of the conserved stop codon so that the deposited EJC could serve as a trigger for NMD; and (5) there is no alternative splice form in which the splice junction falls within an ORF. The latter requirement ensured that intron conservation was independent of any coding capacity of the flanking exons. This protocol led us to the identification of 152 candidate transcripts from 149 genes in which the conservation of an intron in the 3′-UTR is likely due to some functional role of splicing in that region (Spreadsheet S1). Included in this list are numerous proteins that have been implicated in synapse formation or function, including two cadherins, a neurexin, a neuregulin, a synaptotagmin, and several ion channels.

Using available microarray data from (Su et al., 2004), we also generated a scaled expression profile map for 135 probe-sets that were found to represent 120 of the 152 candidate NMD transcripts (http://expression.gnf.org; Figure S6). Examination of this map reveals several interesting clusters including 5–7 mRNAs expressed primarily in the brain, 6 mRNAs highly enriched in testis, and 40 mRNAs highly expressed across multiple tissues. The largest cluster comprises 44 mRNAs primarily expressed in peripheral blood and bone marrow. Thus it would appear that some feature of hematopoietic cells may particularly favor evolution of genes containing 3′-UTR introns.

DISCUSSION

4AIII Is a Component of Neuronal Transport Granules and Regulates *arc* Expression

This study reveals a novel role for 4AIII and NMD in regulating the expression of *arc*, one of the best-studied dendritic mRNAs. ARC affects neuronal plasticity processes and is essential for the consolidation of long-term memory (Guzowski et al., 2000; Plath et al., 2006; Steward and Worley, 2002). Upon intense synaptic activation, the *arc* gene is induced transcriptionally. A portion of this newly made mRNA is delivered to dendrites, where it specifically localizes to activated synapses in a NMDA receptor-dependent manner and is thought to be locally translated (Steward et al., 1998; Steward and Worley, 2001). Mechanistically, ARC protein was recently

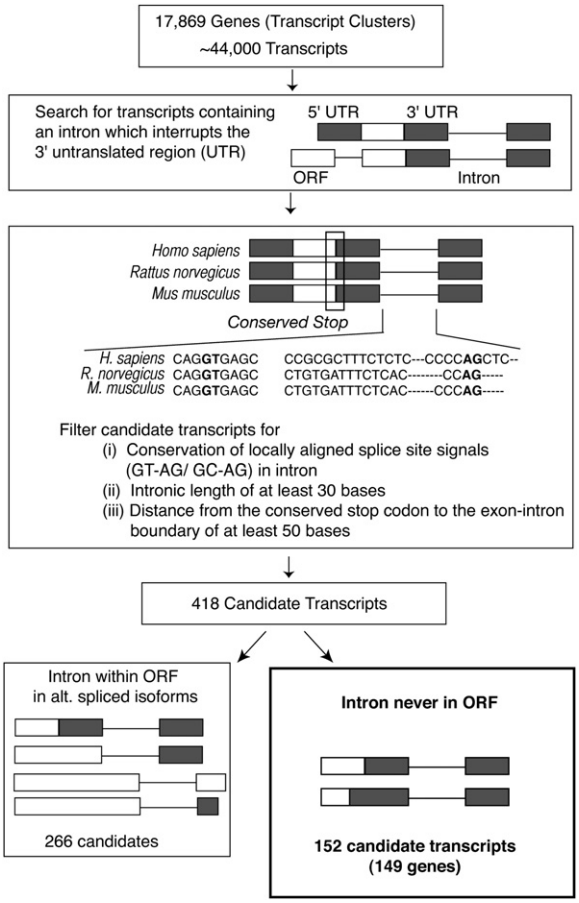

Figure 6. Bioinformatic Strategy to Screen for Potential NMD Target Genes

Strategy utilized to identify mammalian transcripts naturally susceptible to NMD by virtue of a conserved intron in the 3′UTR >50 nucleotides downstream of the stop codon.

demonstrated to regulate synaptic scaling and trafficking of AMPA receptors (Chowdhury et al., 2006; Rial Verde et al., 2006; Shepherd et al., 2006).

Previous studies had shown that inhibitors of translation led to marked increases in *arc* mRNA abundance, suggesting that *arc* mRNA is somehow destabilized upon translation (Ichikawa et al., 2003; Steward et al., 1998; Steward and Worley, 2001; Wallace et al., 1998). Our data readily explain this by demonstrating that *arc* mRNA is a natural NMD target. Mammalian *arc* genes share the unusual and conserved feature of harboring two 3′-UTR introns. We showed that both *arc* mRNA and ARC protein levels increase in PC-12 cells upon shRNA-mediated depletion of either 4AIII or UPF1. Analogous increases in *arc* mRNA and protein levels were also observed upon 4AIII depletion in cortical neurons, yet the subcellular *arc* mRNA and protein distributions were unaffected. These latter results indicate that 4AIII and, by inference, the EJC are not required for the dendritic localization of *arc* mRNA. Rather, by targeting *arc* mRNA for destruction upon translation, 4AIII serves as a general brake on ARC protein expression.

Putting the Brakes on Protein Synthesis via Translation-Dependent mRNA Decay

Using a bioinformatic approach, we identified 152 transcripts from 149 different genes harboring 3′-UTR introns conserved across multiple mammalian species. Whereas a few of these transcripts had previously been shown to be affected by UPF1 knockdown in HeLa cells (Table S2), most had not. The advantage of our bioinformatic approach was that it relied on splice site conservation across species rather than chance mRNA expression in a particular cell type. By limiting our list to 3′-UTR introns conserved across multiple species, our pool should be highly biased toward genes whose proper expression is dependent on this unusual structural feature. It has been estimated that ~35% of all alternatively spliced mRNA isoforms are targets of NMD and that by eliminating such transcripts, NMD serves to mute genomic noise (Mendell et al., 2004). However, since we also limited our final candidate pool to those genes in which the 3′-UTR intron is never coding, our set likely represents a separate class of transcripts for which the presence of a 3′-UTR intron confers some specific selective advantage.

In all organisms tested to date, including mammals, NMD is not strictly EJC dependent—3′-UTR length and UPF1-interacting proteins are also key determinants (Amrani et al., 2006; Buhler et al., 2006; Weil and Beemon, 2006). For example, when bound to the 3′-UTR, STAU1 can elicit translation-dependent mRNA decay through its direct interaction with Upf1 (STAUFEN-mediated mRNA decay [SMD]; Kim et al., 2005). Given that STAU1 is known to associate with dendritic mRNA transport granules, numerous neuronal mRNAs may also be natural SMD targets.

NMD and SMD are two specific examples of a broader phenomenon we will term collectively "translation-dependent mRNA decay" (TDD). What might be the benefits of intentionally targeting mRNAs to TDD? In the case of ARC, our data indicate that TDD serves as a natural brake on ARC protein expression throughout the cell. Upon BDNF induction of cultured neurons, *arc* mRNA and protein levels increased in both somata and dendrites. In both compartments, *arc* mRNA and protein levels were further enhanced by 4AIII knockdown. A simple interpretation is that *arc* mRNA is translated and subject to NMD in both somata and dendrites. Additionally, 4AIII knockdown increased the level of ARC protein at synapses, and there were no overall changes in the association of 4AIII with *arc* mRNA under two different induction regimens. Thus it would appear that the targeting of *arc* to NMD is neither modulated at synapses nor by synaptic activation. Rather, NMD simply limits the number of protein molecules synthesized per Arc mRNA, wherever that mRNA molecule is translated in the cell (Figure 7, upper panel).

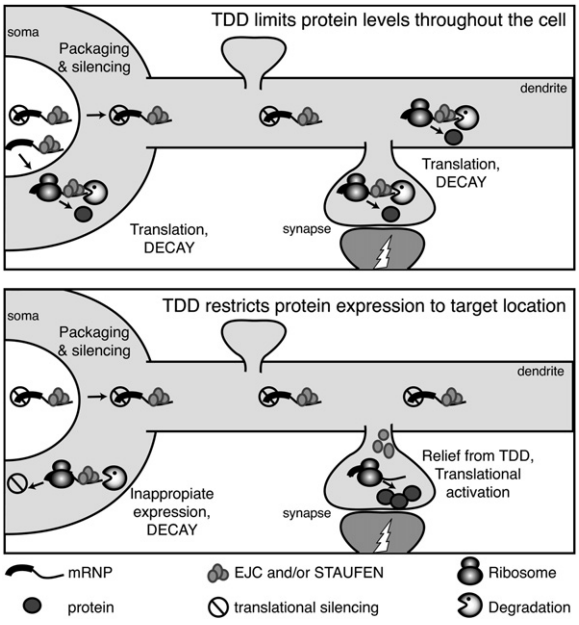

Figure 7. Models by which Translation-Dependent mRNA Decay Could Modulate Neuronal Protein Expression
TDD indicates "translation-dependent mRNA decay."

For mRNAs subject to tighter spatial and/or temporal translational regulation, one can imagine two further advantages of TTD. First, TDD could act as a brake to create a strictly limited burst of protein synthesis upon translational activation at the target location. Because TDD substrates are stable as long as they remain translationally silent, such mRNAs could accumulate at high levels in distal reaches of the cytoplasm (such as neuronal dendrites), where they would be poised for rapid response to an external signal. Upon translational activation, TDD would quickly curtail protein synthesis, producing a tightly time-limited, quantity-limited, and mRNA-specific translational response. This mechanism could certainly apply to those *arc* mRNA molecules localized to dendrites. If TDD operates at synapses in this way, it would parallel emerging data that synaptic protein abundance is also limited by activity-dependent activation of the proteasome (Bingol and Schuman, 2005, 2006).

An alternate possibility is that, for some mRNAs, TDD could serve to prevent improper protein synthesis at undesired times or locations. For example, if a translationally silenced mRNA lost a key inhibitory factor during transport and became inappropriately activated, TDD would quickly eliminate that mRNA. In this scenario, translational activation at the target location might be accompanied by release from TDD (e.g., by removal of the TDD triggering factors), allowing both the mRNA and protein to accumulate there (Figure 7, lower panel). Such a model parallels the activity-dependent relief of dendritic mRNAs from microRNA-mediated repression recently observed in rat and *Drosophila* neurons (Ashraf et al., 2006; Schratt et al., 2006).

A Role of 4AIII in Regulating Synaptic Strength

Electrophysiological recordings demonstrated that 4AIII knockdown in cultured cortical neurons doubled mEPSC amplitude (Figure 5). Functionally this is a dramatic change in synaptic transmission, equivalent in magnitude to changes produced by several forms of long-lasting synaptic plasticity such as long term potentiation (Malenka and Bear, 2004) and synaptic scaling (Turrigiano and Nelson, 2004). Changes in mEPSC amplitude could come about through changes in packaging of glutamate into presynaptic vesicles or through changes in the number or composition of postsynaptic AMPA receptors (Malenka and Bear, 2004; Sutton et al., 2006). Because 4AIII was depleted only in the postsynaptic neuron, an effect on presynaptic glutamate packaging is unlikely, pointing toward a role for 4AIII in regulation of postsynaptic AMPA receptors. Indeed, we found that 4AIII depletion resulted in a significant increase in surface GLUR1 at putative postsynaptic sites.

Recently, genetic knockout of *arc* was shown to increase both synaptic strength and the number and intensity of GLUR1 puncta. Conversely, *ARC* overexpression in cultured hippocampal neurons led to a defect in TTX-induced synaptic scaling and a marked reduction of surface GLUR1 (Chowdhury et al., 2006; Shepherd et al., 2006). Given that depletion of 4AIII in cultured cortical neurons leads to increases in ARC throughout the cell (Figure 4), the above results are in apparent conflict with the opposite effect on synaptic strength and GLUR1 abundance we observed in 4AIII-depleted neurons (Figure 6). However, numerous kinases and scaffolding proteins have been shown to regulate the trafficking and/or properties of synaptic AMPA receptors. There are thus many potential routes through which 4AIII, by influencing the local composition of synaptic proteins, could influence synaptic strength. Our bioinformatic analysis, for example, revealed a number of other mRNAs expressed in the brain in addition to *arc* that are potential targets of 4AIII regulation via NMD. Regardless, our data clearly indicate that 4AIII does play a critical role in maintaining synaptic strength and suggest that local synthesis of proteins subject to regulation by 4AIII could contribute to synaptic plasticity.

Dendritic mRNAs and the Pioneer Round of Translation

Given that most EJCs are displaced by the first or "pioneer" round of translation, the association of EJC proteins with cytoplasmic mRNAs reports on their translational history. Previous studies had shown that the EJC core factor 4AIII, a known nucleocytoplasmic shuttling protein, exhibits almost exclusive nuclear localization in nongermline cells. Presumably in the cell types examined (e.g., HeLa), the majority of mRNAs are translated soon after nucleocytoplasmic export, allowing rapid recycling of 4AIII to the nucleus. In neurons, however, a distinct subset of mRNAs is translated in a temporally and spatially restricted manner (reviewed in Kindler et al., 2005; Klann and Dever, 2004). Our finding that 4AIII is associated

with both dendritic mRNAs and neuronal transport granules supports the idea that at least a portion of these localized mRNAs are translationally silent. It further indicates that these silent mRNAs experienced no pioneer round of translation in the soma prior to their dendritic localization. This is also true of *arc* mRNA; even though both its EJCs are located in the 3′-UTR and would not be subject removal by a pioneer round, this same feature destabilizes *arc* mRNA upon translation. In mammalian cells, all available evidence indicates that NMD occurs coincident with the pioneer round (Maquat, 2004). So in the case of *arc* mRNA, its mere presence in the dendrite can be considered strong evidence that those mRNA molecules have not yet undergone a pioneer round.

A growing number of studies in diverse cell types suggest that much of cytoplasmic mRNP assembly occurs within the nuclear compartment (Czaplinski and Singer, 2006; Giorgi and Moore, 2007). In addition to the EJC, other examples of nuclear-acquired mRNP components that modulate cytoplasmic mRNA expression include the zipcode-binding (ZBP) and hnRNPA/B families of proteins. STAUFEN and FMRP have also been reported to shuttle between the nucleus and the cytoplasm, possibly assembling with their mRNA targets in the nucleus (Kiebler et al., 2005; Lai et al., 2006). If many such factors necessary for mRNA localization and regulated expression in the cytoplasm are acquired in the nucleus, then retention of these key proteins likely necessitates special precautions to avoid a pioneer round of translation immediately upon nucleocytoplasmic export.

EXPERIMENTAL PROCEDURES

Plasmids, buffers, antibodies, primer sequences, and sources are described in Supplemental Data.

Seizures, Brain Extracts, and Coronal Slices

All experimental procedures involving animals conformed to NIH guidelines and were carried out with approval of the Brandeis Animal Care and Use Committee. Seizures were induced by intraperitoneal kainic acid injection (12 mg/kg); control animals received comparable injections of vehicle. Animals were anesthetized and sacrificed 1 hr after seizure onset. For cytosolic extract preparations, visual cortex (p27 rats) or dentate gyrus (adult rats) was isolated in ACSF and homogenized manually in Lysis Buffer 150 with ~20 strokes in a 2 ml Wheaton dounce homogenizer. Following two rounds centrifugation (10 min each at 1K × g, and 10K × g), supernatant (cytosolic fraction; ~20 μg protein/ul) was utilized for subsequent IP or western blot analysis. For immunofluorescence analysis of coronal hippocampal slices, anesthetized p24–27 rats were perfused with PBS intracardiacally followed by fixing solution. Fixed brains were cut in 50 μm thick slices.

Cell Cultures, Transfections, Extract Preparation, and BDNF Treatment

Visual cortical cultures were derived from p3–5 Long-Evans rat pups as previously described (Turrigiano et al., 1998; Watt et al., 2000), except neurons were plated onto astrocyte beds and supplemented with B27. DIV 7 neurons were transfected with Lipofectamine 2000 and 1–4 μg DNA per 6 cm culture dish. For cytosolic extracts, cortical neurons were lysed in Lysis Buffer 150 and fractionated as above. That these extracts were not significantly contaminated with nuclear material was confirmed by western blotting (Figure S4B). BDNF treatment consisted of a 6 hr incubation of DIV7-9 neurons with 100 ng/ml BDNF in growth medium. HeLa and PC-12 cells were cultured according to ATCC guidelines. PC-12 cells (3×10^6) were transfected by electroporation (Amaxa) with 10 μg of indicated plasmids. Protein samples were prepared by forcing cells in 1% SDS through a 25 gauge needle followed by 5 min at 95°C. Except for neurons in Figures 1D, 5A, 5B, and 5C, which were analyzed at 24 hr, all transfection experiments were analyzed 48 hr posttransfection.

Immunofluorescence and FISH of Cultured Neurons

Paraformaldehyde-fixed DIV 7 neurons were permeabilized, incubated with either primary antibodies or digoxigenin-labeled RNA probes, and then incubated with secondary antibodies followed by extensive washing. Following staining for DNA (Hoechst dye), samples were mounted in DABCO media. Details on these procedures, as well as immunofluorescence of fixed hippocampal slices and detection of surface GluR1, are described in Supplemental Data.

Semiquantitative RT-PCR

RT-PCR analyses employed the Superscript III One-Step RT-PCR kit. Different transcripts required different amounts of input cellular RNA to be in the linear range after 24 rounds of PCR: 0.1 ng/ul for *gapdh*; 1 ng/ul for *map1* and other dendritic mRNAs; and 10 ng/ul for *arc* mRNA. That reactions were quantitative was verified using two different dilutions of starting RNA. All RT-PCR experiments were performed at least two times on independently prepared samples, and results were averaged. All RNA samples were extracted using Trireagent and treated with RQ DNase I prior to RT-PCR analyses.

Immunoprecipitations

Cytosolic extracts (~4–6 mg total protein per sample) were precleared by two incubations with protein G agarose beads, incubated with either α-4AIII or control (preimmune) serum for one hour at 4°C, and then with Protein G agarose beads (40 μl 50% slurry) overnight at 4°C. Following five 30 min washes in Lysis Buffer 250, samples were treated for either RNA or protein extraction (see Supplemental Data). To ensure that 4AIII-precipitated transcripts were chromatin free, the final wash included DNase I. Input samples represented one-fortieth of the starting material for IP.

Electrophysiology

MEPSCs from pyramidal neurons were recorded and quantified as previously described (Turrigiano et al., 1998; Watt et al., 2000). Two-tailed t tests (corrected for multiple comparisons as appropriate) were used to determine statistical significance. EGFP-expressing neurons were identified via fluorescence, and recordings were then obtained after switching to DIC. Recordings were discarded if resting potentials were >−55 mV, input resistances were <200 MΩ, or either parameter changed more than 10% during the course of recording. Only neurons from which >25 mEPSCs were obtained were included. Recordings from untransfected neurons and from neurons transfected with the control-RNAi plasmid were indistinguishable in terms of cellular and mEPSC properties and were combined.

Supplemental Data

Supplemental Data include Experimental Procedures, six figures, one table, and one spreadsheet and can be found with this article online at http://www.cell.com/cgi/content/full/130/1/179/DC1/.

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