

The Nuclear Transcription Factor RAR α Associates with Neuronal RNA Granules and Suppresses Translation*

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All-*trans*-retinoic acid stimulates dendritic growth in hippocampal neurons within minutes by activating mitogen-activated protein kinase and mTOR and increasing dendritic translation of calcium calmodulin-dependent protein kinase II α and the α -amino-3-hydroxyl-5-methyl-4-isoxazole propionate receptor subunit GluR1. Hippocampal neurons express RAR α in dendrites, and knocking down RAR α prevents all-*trans*-retinoic acid effects on dendritic growth. Here we show, by liquid chromatography/mass spectrometry analysis of immunoaffinity isolates of hippocampal neurons, that RAR α partners with many RNA-binding proteins and translation factors conveyed in dendritic RNA transport granules, including the purine-rich element-binding protein, Pur α . The interaction of RAR α with Pur α , an RNA-binding protein required for dendritic RNA transport, and other RNA-binding proteins was confirmed by tandem affinity purification. Confocal microscopy confirmed localization of neuronal RAR α in dendritic RNA granules with Pur α and FMRP (the fragile \times mental retardation protein). Hippocampal RAR α also associates with mRNA, e.g. encoding GluR1 and calcium calmodulin-dependent protein kinase II α . Consistent with a granule function of conveying translationally silenced mRNA, RAR α inhibits translation initiation, independent of 7-methylguanylate cap or poly(A) tail, and prompts mRNA redistribution to silencing ribonucleoprotein particles. These data afford a mechanism for rapid stimulation of dendritic growth by all-*trans*-retinoic acid and reveal that the ligand-dependent transcription factor RAR α also regulates translation.

The developed nervous system relies on atRA² for synaptic plasticity that underlies hippocampus-dependent spatial learning (1, 2). Restricting the dietary atRA precursor retinol (vitamin A) or genetically impairing atRA signaling impairs hippocampus-dependent learning (3–6). Retinoid-compromised rodents err

markedly more frequently in tests related to hippocampal function, such as the radial arm and Morris water mazes, and have diminished long term potentiation and abolished long term depression. Conversely, atRA dosing reduces frequency of relational memory errors in older mice relative to young mice (7).

Synaptic plasticity relies on protein synthesis in dendrites (8, 9). Large ribonucleoparticles or RNA granules convey translationally silenced mRNA from neuronal cell bodies to dendrites. These RNA encode diverse classes of postsynaptic proteins, including ionotropic glutamate receptors and kinases such as CaMKII α (10). Synaptic activity triggers translation of dendritic mRNA to support rapid synaptic structure and efficacy modifications (10, 11). For example, the α -amino-3-hydroxyl-5-methyl-4-isoxazole propionate receptor subunits GluR1 and GluR2 insert into synaptic membranes after local translation to enhance the number, strength, and stability of synapses (12).

atRA stimulates dendritic growth in primary mouse hippocampal neurons; siRNA knockdown and small hairpin RNA knockdown indicate a central function for RAR α in atRA-induced dendritic growth (13). Responses to atRA occur in less than 30 min and include induction of somatodendritic translation of GluR1 and CaMKII α mRNAs. Intense dendritic expression of RAR α in hippocampus *in vivo* and in primary hippocampal neurons suggests a contribution to translation control, in addition to its established function as a transcription factor. Here we provide direct evidence that RAR α clusters in dendritic RNA granules and can repress translation. These data provide a mechanism for the rapid stimulation of synaptic remodeling by atRA and indicate that a nuclear ligand-dependent transcription factor can regulate dendritic translation.

EXPERIMENTAL PROCEDURES

RAR α -associated Proteins—To produce RAR α bait, COS cells (2×10^7) transfected with the Myc-RAR α chimera were lysed in 0.5% Nonidet P-40, 50 mM Tris, pH 7.3, 150 mM NaCl, containing protease inhibitors. Supernatants (12,000 g, 10 min) were incubated 2.5 h at 4 °C with c-Myc beads. The beads were washed with lysis buffer three times.

To identify proteins associated with RAR α , the beads were incubated 5 h at 4 °C with lysates (0.5% Triton X-100, 50 mM Tris, pH 7.3, 150 mM NaCl, 2.5 mM MgCl₂, 1000 units/ml RNaseOUT, protease inhibitors) from primary hippocampal neurons (2×10^7 ; DIC24–30). The beads were washed four times, eluted with 2 \times loading buffer, and resolved by 4–20% gradient SDS-PAGE. The bands were processed in a DigestPro (INTAVIS Bioanalytical Instruments AG), washed, reduced with dithiothreitol, alkylated with iodoacetamide, and trypsin-

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² The abbreviations used are: atRA, all-*trans*-retinoic acid; cds, coding sequences; DIC, days in culture; FL, firefly luciferase; MAP2, microtubule-associated protein 2; MS2BS, binding sites for the bacteriophage protein MS2; PB, somatic processing bodies; RRL, rabbit reticulocyte lysate; TAP, tandem affinity purification; CaMKII, calmodulin kinase II; siRNA, small interfering RNA; EGFP, enhanced green fluorescent protein; AF-1, activation function-1; GST, glutathione S-transferase; LBD, ligand-binding domain.

RAR α Suppresses Translation

TABLE 1

RAR α -interacting proteins

Lysates of DIC14–21 hippocampal neurons were probed with RAR α bound to anti-Myc-conjugated beads. The isolate was subjected to gradient gel electrophoresis, and 22 bands were analyzed with electrospray ionization-quadrupole-time-of-flight mass spectrometry. Bold type designates select interactions confirmed by TAP.

Type (number)	Identity
RNA transport (4)	Pur α , Pur β , FXR, Tho complex 4
Protein synthesis (19)	PABP1 , eIF3 α , eIF3 β , ribosomal proteins S3, S4, S5, S6, S12, S13, S16, S17, S19, S25, S26, L23, 40 S ribosomal protein SA (p40), 60 S ribosomal protein, putative ribosomal S4, Hsp70
RNA helicases (3)	DDX1, DDX3, DDX5
hnRNP (4)	hnRNP U , hnRNP A/B, hnRNP D0 (AU-rich element RNA-binding protein), hnRNP Q (SYNCRIP),
Other RNA associated (9)	NonO , PSF, GP137 (RNA granule protein 103), EWS, FUS, cold-inducible RBP, RNA-binding motif protein 3, G3BP, single strand DNA-binding protein 1
Endocytotic (4)	AP2A1, AP2A2, AP2B1, clathrin coat assembly AP50 (AP2 ml)
Cytoskeletal (5)	vimentin, desmin, tubulin α 3, β -actin, tropomodulin
Others (10)	REP1 , 14-3-3 ζ , VCP, 14-3-3 β , 14-3-3 γ , VG potassium channel α -2 subunit, p100-coactivator, hypothetical protein LOC 68045, nucleotide-diphosphate kinase 2, tumor metastatic process associated protein NM23

digested. The digests were loaded onto a C18 cartridge and eluted at 250 nl/min with A (0.5% acetic acid in water) and B (0.5% acetic acid in 80% acetonitrile/20% water) in a 15-min linear gradient from 2% B at 0 min to 80% B, held at 80% B for 5 min, returned to 2% B over 10 min from a reverse phase column (Vydac 238EV5.07515, 75 μ m \times 150 mm) fitted with a coated spray tip (FS360-50-5-CE; New Objective, Inc.). Nano-liquid chromatography/electrospray ionization/tandem mass spectrometry was done with an AB QStar Pulsar, with a Proxeon Biosystems nano-electrospray source, with resolution >10,000. A 5-s tandem mass spectrometry scan followed a 1-s survey scan. Ions between m/z 400 and 1,000, charges between +2 to +5, and intensities >40 counts were fragmented. Liquid chromatography/tandem mass spectrometry data were submitted to Mascot for analyses.

TAP determination of RAR α interacting proteins was done with a kit (Stratagene) (14).

RAR α -associated mRNA—RAR α was immunoprecipitated from lysates of primary hippocampus neurons (DIC14–15) with anti-RAR α (Santa Cruz). mRNA was released by boiling twice (5 min each, 10 mM Tris, pH 7.5, 1 mM EDTA, 1% SDS), purified with MegaClear (Ambion), and reverse transcribed with Superscript II (Invitrogen). Primers used following RAR α immunoprecipitation were (forward and reverse, respectively): GluR1, 5'-AGTCGAAGCGGATGAAGGG-3', 5'-GTTGTG-GTGGTTGGAGGC-3'; GluR2, 5'-TTCTAACAGCATA-CAGATAGG-3', 5'-AAGCATTGGTGACTGCGAAAC-3'; CaMKII α 5'-ATCGCCTATATCCGCATCAC-3', 5'-GGAC-AAAGAGCGGATCTCTG-3'; laminin B1, 5'-CATTGAGAA-CGTGGTCAACCAC-3', 5'-GAACGAGCTCTCACAGTCG-TAG-3'; and RAR α , 5'-TCACAGACCTTCGGAGCATC-3', 5'-CCAGTTCTGTCTGAGAGGAC-3'.

Plasmids and Cell Culture—NonO, 14-3-3 ζ , hnRNP U, PSF, PABP1, Pur α , RAR α , VCP, and REP1 were PCR-amplified

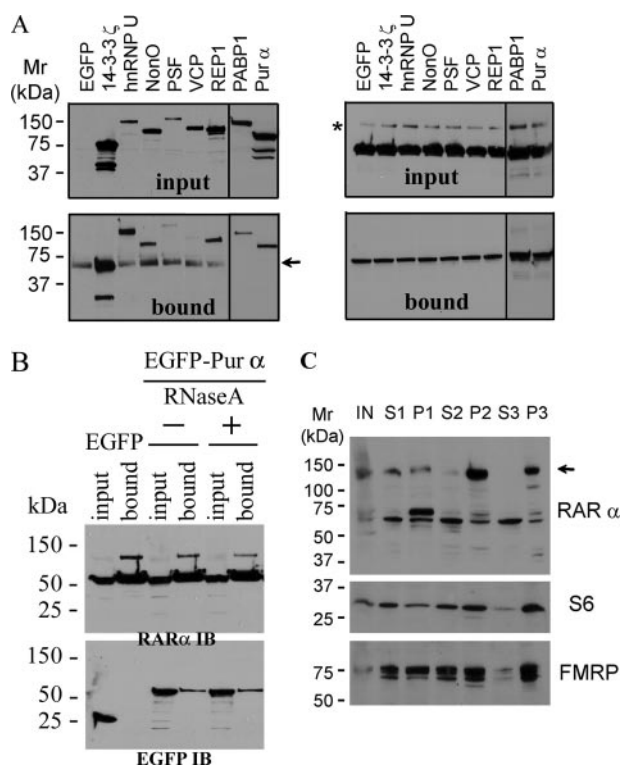


FIGURE 1. RAR α associates with neuronal RNA granule proteins. *A*, RAR α and Myc-tagged neuronal proteins selected from Table 1 were co-transfected into COS cells to confirm their association. RAR α was isolated by TAP. Total (*input*) and RAR α pull-down fractions (*bound*) were immunoblotted for Myc (*left*) or RAR α (*right*, C-terminal antibody). The *arrow* indicates a nonspecific band. The *asterisk* indicates a high molecular weight form of RAR α . *B*, RAR α and either EGFP or EGFP-tagged Pur α were co-transfected into COS cells; RAR α was isolated by TAP. Total (*input*) and RAR α pull-down fractions (*bound*) were immunoblotted for RAR α (*top*, RAR α IB) or EGFP (*bottom*, EGFP IB) with (+) or without (–) RNase A treatment. *C*, subcellular distribution of RAR α in mouse brain homogenates. Equal amounts of protein from each fraction were immunoblotted for RAR α , S6, or FMRP: S1, 1000 \times g supernatant; S2, 10,000 \times g supernatant; P2, 10,000 \times g pellet; S3, 100,000 \times g supernatant; P3, 100,000 \times g pellet. The *arrow* indicates a modified form of RAR α .

from DIC14 hippocampal neuron cDNA and cloned in frame into EGFP-N1 (Clontech). An N-terminal Myc tag was included in PCR primers. DsRed-Pur α and NTAP-RAR α were generated by releasing Pur α or RAR α cds from EGFP expression vectors and inserting them into DsRed-N1 (Clontech) or NTAP-B (Stratagene) in frame with DsRed and TAP tag, respectively. RAR α deletion mutants with N-terminal hemagglutinin were PCR-amplified from EGFP-RAR α and inserted into EGFP-N1. An hemagglutinin tag was included in the primer. A dominant negative RAR α was made by ligating cds residues 1–403 lacking the AF-2 core in frame with EGFP in EGFP-N1 (15). The FL cds was PCR-amplified from pGL3 (Promega) and inserted into pcDNA3.1(+) (Invitrogen) to generate pcDNA-FL. pSL-MS2 12X and Pol II-MS2-GFP were gifts from Robert H. Singer (Albert Einstein College of Medicine). MS2 12X was subcloned into pcDNA-FL. MS2 cds was subcloned into EGFP-N1 to generate EGFP-MS2. MS2-RAR α -EGFP and MS2-RAR α /domains-EGFP were generated by PCR amplification of MS2 cds from Pol II-MS2-GFP and cloned into EGFP expression vectors. Nontargeting siRNA and GW182 siRNA were obtained from Dharmacon. COS cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine

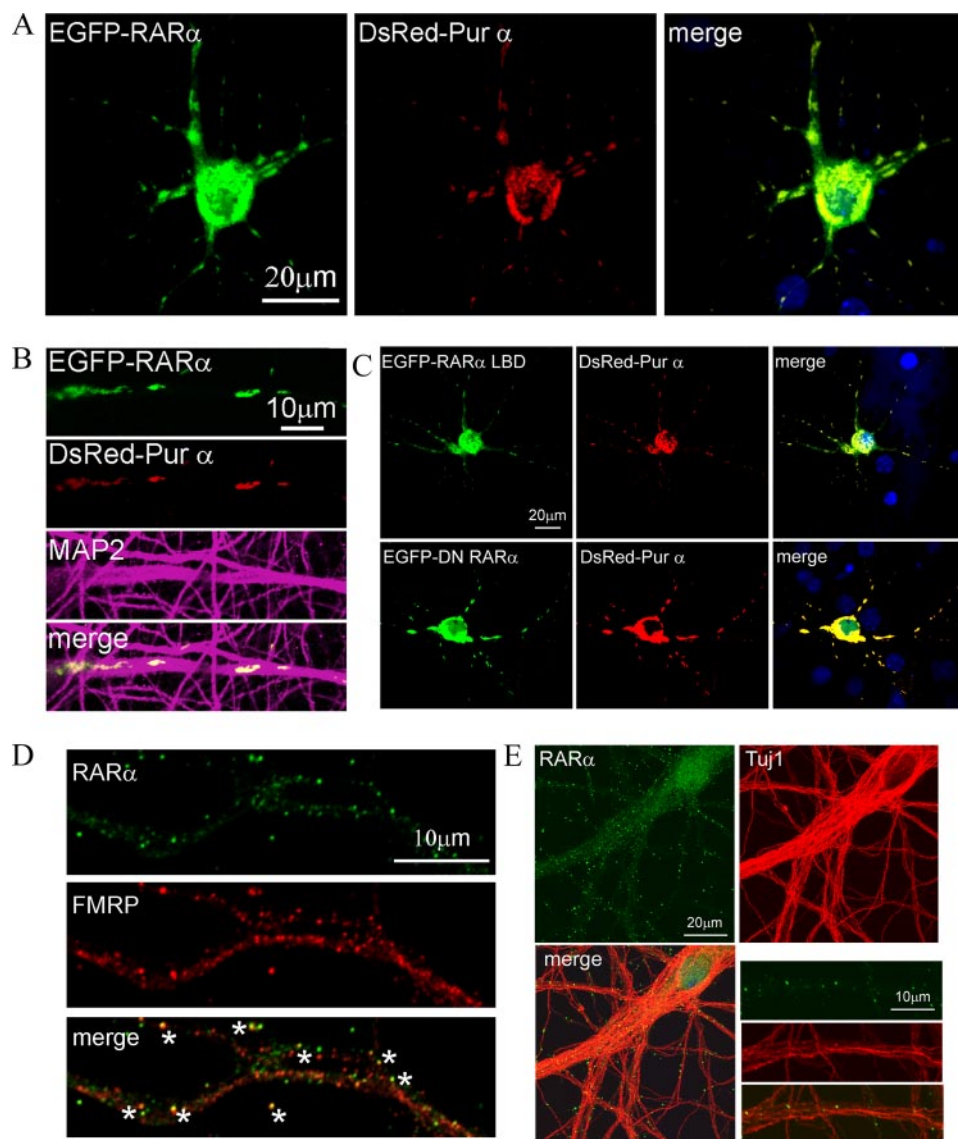


FIGURE 2. Localization of RAR α in neuronal granules. *A*, co-localization in DIC12 hippocampal neurons of RAR α (green) with Pur α (red). *B*, enlargement of dendrites from *A*, and co-localization of RAR α with MAP2 (purple). *C*, localization of the RAR α LBD (EGFP-RAR α -LBD) and a dominant negative RAR α mutant (EGFP-DN-RAR α) in DIC12 hippocampal neurons with DsRed-Pur α . *D*, a representative dendrite showing partial co-localization of endogenous RAR α (green) and FMRP (red) in DIC12 hippocampal neurons. Asterisks denote co-localizing puncta. *E*, RAR α puncta intersperse among α -tubulin III-labeled (*Tuj1*) neuronal microtubules. The larger panels show a cell, whereas the smaller panels show a magnified dendritic region.

serum (Invitrogen). Primary cultures of hippocampal neurons were prepared from e17 mouse embryos. Neurons and COS cells were transfected with Lipofectamine 2000 (Invitrogen) according to instructions, except Lipofectamine was 0.5 μ l/well in 24-well plates for neurons, and the medium was unchanged.

RRL Translation—FL RNA was transcribed with T7 message machine (Ambion) using linearized pcDNA-FL. For the uncapped transcript, the nonfunctional cap analog ApppG was used in place of GpppG (NEB). Fifty–100 base poly(A) tails were added with *Escherichia coli* poly(A) polymerase (Ambion). Translation was done with a nuclease-treated RRL following instructions (Promega), except reactions used 90 ng of RNA, 0.6 mM MgCl₂, and 125 mM KCl to promote cap-poly(A) synergy (16, 17). FL and *Renilla* luciferase activities were measured with luciferase systems (Promega). Northern blotting was done with Northern-

Max (Ambion) using psoralen-biotin-labeled FL or β -actin probes generated by *in vitro* transcription. The signals were developed with a Bright-Star BioDetect kit (Ambion) and quantified with Image J.

Subcellular Fractionation—Brains from 1-month-old mice were homogenized in a lysis buffer of 10 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, and a protease inhibitor mixture (Roche Applied Science). The lysates were centrifuged 1000 \times *g* for 10 min, 10,000 \times *g* for 10 min, and 100,000 \times *g* for 1 h to obtain S1, S2 and P2, and S3 and P3, respectively. Equal amounts of protein from each fraction were loaded onto SDS-PAGE for Western analysis with anti-RAR α (Santa Cruz, 1:750), anti-FMRP (Chemicon, 1:1000) and anti-S6 (Cell signaling, 1:1000). For solubilization, COS cells (3 \times 10⁶) transfected 24 h with pcDNA-FL-MS2BS and MS2-EGFP or MS2-RAR α or domains-EGFP were lysed in 200 μ l of buffer C (0.25 M sucrose, 10 mM Tris, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 2 mM dithiothreitol, 30 units/ml RNaseOUT) with digitonin (50 mg/ml; Sigma) on ice for 15 min. The lysates were centrifuged 1000 \times *g* for 5 min. Supernatants were spun 14,000 \times *g* for 5 min to obtain RNA for purification with TRIzol reagent (Invitrogen). The pellets were washed with buffer C twice and resuspended in TRIzol. RNA was isolated according to the TRIzol protocol (Invitrogen). Cytoplasmic and nuclear RNA were isolated with a cytoplasmic and nuclear RNA isolation kit (Norgen). In a

separate experiment, brains from 23-day-old mice (littermates) were homogenized in lysis buffer and divided into two groups. One group was supplemented with 80 units/ml RNaseOUT. The other group was not and was used for RNase treatment. The S2 fraction was divided, and one portion was treated with 33 μ g/ml RNase A at 37 $^{\circ}$ C for 30 min and then separated into S3 and P3 fractions.

Sucrose Density Gradients—Polysome profiling was done with 15–45% sucrose gradients (18). Nine hundred- μ l fractions were collected from tube bottoms. RNA was isolated by two-round precipitation with 8 M guanidine HCl and ethanol. Protein was precipitated with 2 volumes of acetone.

Immunostaining—DIC12 hippocampal neurons were fixed in 4% paraformaldehyde with 4% sucrose, permeabilized with 0.2% Triton X-100 5 min, and blocked with 5% goat serum and

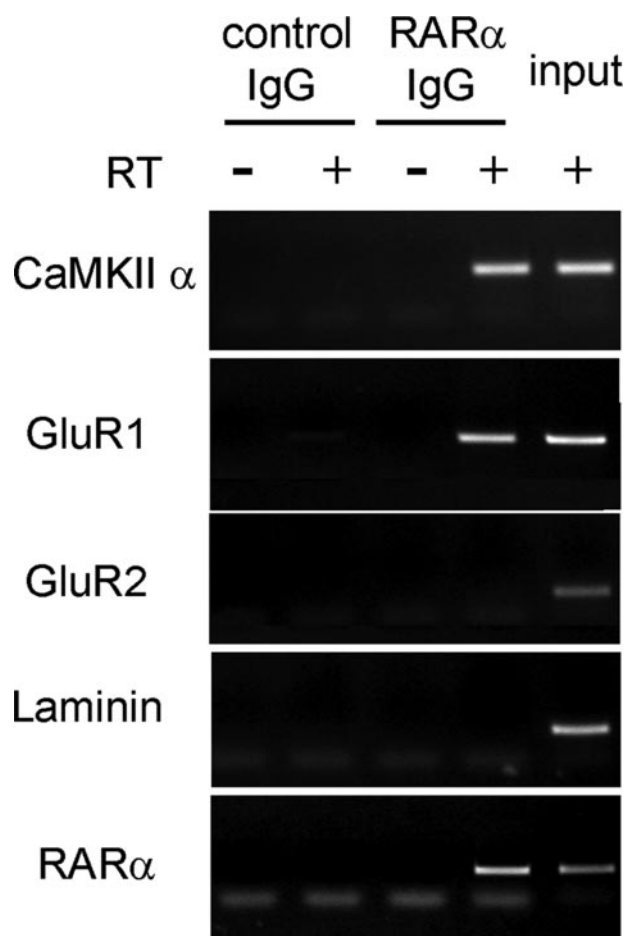


FIGURE 3. RAR α associates specifically with CaMKII α and GluR1 mRNA in neurons. Lysates of DIC14–15 hippocampal neurons were immunoprecipitated with IgG or anti-RAR α and analyzed by reverse transcription-PCR without (–RT) or with (+RT) reverse transcriptase with primers for mRNA indicated at the left.

3% bovine serum albumin in phosphate-buffered saline 1 h. The cells were incubated with anti-RAR α (1:100; Santa Cruz Biotechnology sc-551, polyclonal to C terminus epitope) and anti-FMRP (1:100; Chemicon) or anti-Tuj1 (1:100, Chemicon) overnight at 4 °C, followed by Alexa 488- and Alexa 555-conjugated-secondary antibodies (1:100) (Invitrogen) incubation at room temperature for 1 h. MAP2 staining was done using anti-MAP2 (1:100, Chemicon) and Alexa 647-conjugated-secondary antibody (1:20) (Invitrogen) on DIC12 neurons transfected with EGFP-RAR α and DsRed-Pur α for 24 h. The neurons were washed with phosphate-buffered saline three times and mounted for microscopy. Confocal microscope images were obtained with a LSM510 confocal microscope in the College of Natural Resources BioImaging facility.

RESULTS AND DISCUSSION

RAR α Associates with RNA-regulating Proteins in Neuronal Granules—We used a Myc-RAR α chimera bound to anti-Myc-conjugated beads as bait to isolate RAR α protein partners from mouse hippocampal neurons and resolved the isolate by gel electrophoresis. We analyzed 22 gel bands by electrospray ionization-quadrupole-time-of-flight mass spectrometry, which identified 58 proteins (Table 1). RAR α -associated proteins

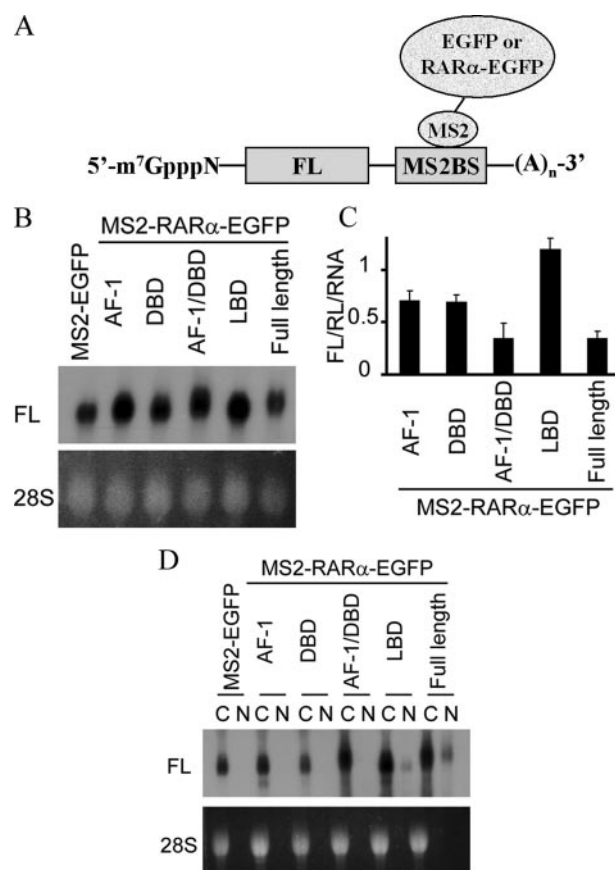


FIGURE 4. RAR α suppresses translation in intact cells. *A*, a tethering assay for evaluating translation suppression relies on co-expressing FL mRNA, harboring tandem repeats of MS2BS in its 3'-untranslated region, with a chimera of MS2 and a query protein. *B*, Northern blotting of FL-MS2BS mRNA expressed during tethering assays. RNA was isolated from COS cells 24 h after transfection with FL-MS2BS and MS2-EGFP or MS2-(RAR α or domains)-EGFP. The 28 S rRNA was ethidium bromide-stained. *C*, FL/*Renilla* luciferase (RL) activities in the presence of MS2-RAR α or domains-EGFP relative to control (FL/*Renilla* luciferase of MS2-EGFP, set as 1), normalized to the mRNA signals of FL-MS2BS mRNA shown in *B*; the data are the means \pm S.E., $n = 9$ (three replicates in each of three experiments). *Renilla* luciferase was co-transfected to normalize for variations in FL activity caused by variations in FL mRNA. *D*, Northern blotting of FL-MS2BS mRNA expressed with MS2-EGFP or MS2-RAR α or domains-EGFP indicating nuclear export. Lanes C, cytoplasm; lanes N, nuclei.

included many RNA-binding proteins and translation factors associated with dendritic RNA transport granules (19, 20). We selected eight proteins to confirm as RAR α -interacting partners by streptavidin/calmodulin tandem affinity purification. This second immunoaffinity technique verified seven of the eight as RAR α partners: Pur α , PABP1, REP1, PSF, NonO, hnRNP U, and 14-3-3 ζ ; the exception was VCP (Fig. 1A). TAP analysis was repeated with EGFP-Pur α and RAR α with RNase A treatment to determine whether this interaction required mRNA (Fig. 1B). RNase A treatment did not abolish the interaction between Pur α and RAR α , indicating RNA independence.

Many RAR α -associated proteins populate granules that house RNA-binding proteins. Therefore, we used differential centrifugation to determine subcellular loci of RAR α in mouse brain. A high molecular weight form of RAR α localized in the RNA granule-containing P3 and the membrane containing P2 fractions with two other granule proteins, FMRP and ribosomal

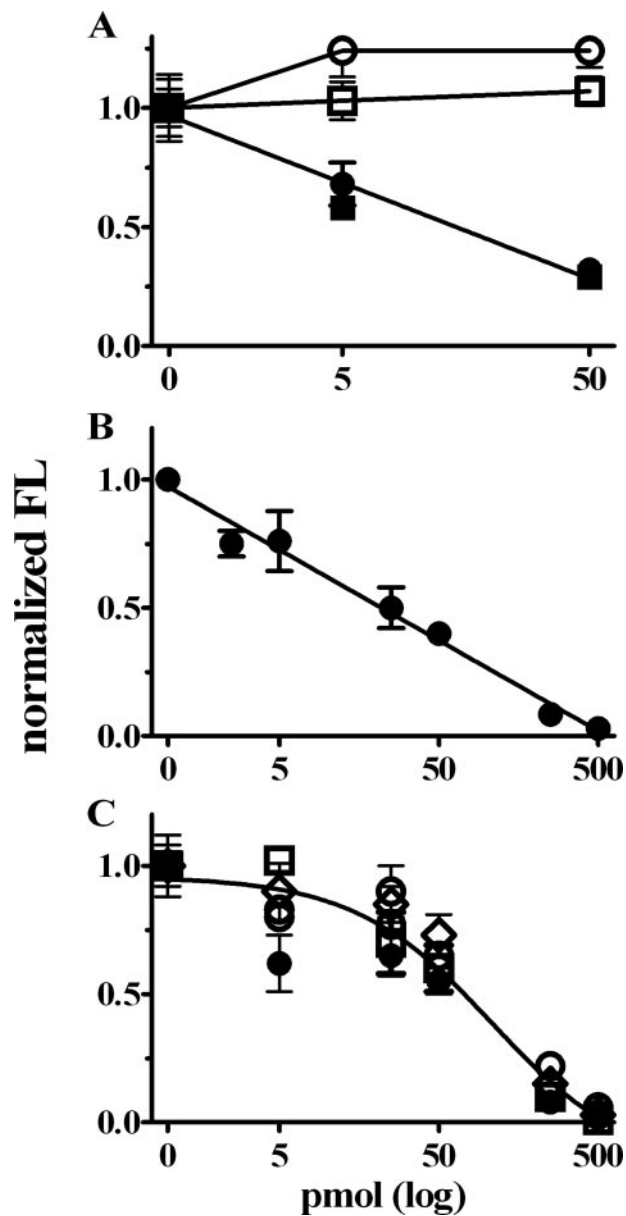


FIGURE 5. **RAR α suppresses translation in RRL.** *A*, sequence independence of RAR α translation suppression. Effect of GST (open symbols) or GST-RAR α (filled symbols) on FL mRNA translation by a RRL programmed with FL (squares) or GluR1-FL mRNA (circles). *B*, dose-dependent inhibition of FL mRNA translation by RAR α . *C*, Cap- and poly(A)-independent inhibition of FL mRNA translation by RAR α . Open circles, uncapped and untailed; open triangles, capped and untailed; open squares, uncapped and tailed; filled circles, capped and tailed. The data in A–C were normalized to FL activity in the absence of GST and RAR α and represent the means \pm S.D., $n = 3$. FL measurements were made in lysates after 60 min of incubation at 37°C.

protein S6 (Fig. 1*B*). This experiment was repeated, except the S2 fraction was treated with RNase A. RAR α again isolated with the P3 fraction, indicating that mRNA is not obligatory to its localization in the RNA granule fraction. This RAR α migrated predominantly as an SDS-resistant band at \sim 140 kDa, suggesting covalent modification and/or a multiprotein complex. We are in the process of analyzing the precise composition of this complex but have confirmed that its major constituent is RAR α by redoing the Western blot with a monoclonal antibody raised against the N terminus of RAR α (Chemicon MAB5346)[em]the first antibody used was a polyclonal raised against the C termi-

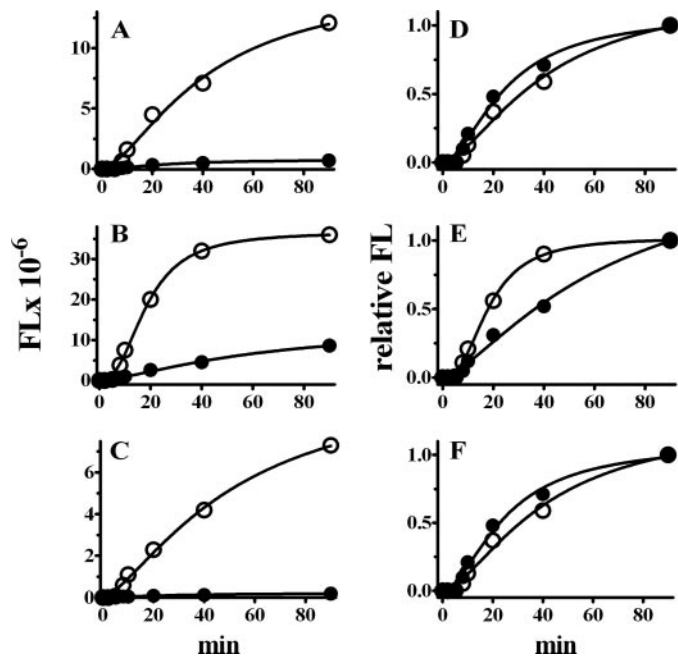


FIGURE 6. **Time courses of RAR α effects on FL mRNA translation.** RAR α (filled circles) and GST (open circles) were compared for effects on FL mRNA translation. *A*, capped and untailed; *B*, capped and tailed; *C*, uncapped and tailed. *D–F*, each data set in A–C, respectively, was normalized to its own highest signal to assess mRNA functional half-life. The data are the means \pm S.D., $n = 3$. FL measurements were made in lysates as described in the legend to Fig. 5 with 500 pmol of either GST or RAR α -GST.

nus[em]and proteomics analysis with liquid chromatography/mass spectrometry (data not shown).

Pur α , a granule RNA-binding protein required for dendritic RNA transport, was confirmed as an RAR α -interacting partner in both immunoprecipitation analyses. We therefore applied fluorescence microscopy to determine whether RAR α localized with Pur α in neurons. Soma and dendrites of neurons co-expressed RAR α and Pur α (Fig. 2, *A* and *B*). Dendritic expression of both was punctated. RAR α also co-localized with the dendritic marker MAP2. Extranuclear localization of RAR α required only the LBD (residues 200–390) and not the transcription activation domain or transcription activity, as shown by co-localization of both the RAR α LBD and a dominant negative RAR α mutant with Pur α (Fig. 2*C*). Immunofluorescence also confirmed that \sim 50% of endogenous RAR α co-localized with FMRP (Fig. 2*D*). RAR α puncta interspersed among neuronal microtubules with neuron-specific α -tubulin III (Fig. 2*E*).

RAR α Represses Translation—Synaptic modification relies on dendritic protein synthesis supported by translation of CaMKII α and the α -amino-3-hydroxyl-5-methyl-4-isoxazole propionate receptor subunits GluR1 and GluR2 (21, 22). atRA induces dendritic translation of CaMKII α and GluR1 mRNA but not of GluR2 mRNA (13). Therefore, we immunoprecipitated RAR α from mouse hippocampus and analyzed associated mRNA (Fig. 3). The precipitate included mRNA encoding CaMKII α , GluR1, and RAR α but not GluR2 or the nondendritic protein laminin B1, consistent with RAR α modifying translation of CaMKII α and GluR1, but not of GluR2.

To determine whether RAR α affect translation directly, we applied a tethering assay that has established translation regulation by exon junction complex proteins, DEAD box heli-

RAR α Suppresses Translation

cases, etc. (23–25). Translation was evaluated from an FL mRNA harboring tandem repeats of the MS2BS in its 3'-untranslated region (Fig. 4A). This construct was co-expressed with constructs expressing chimera of the bacteriophage protein MS2 with EGFP or MS2-RAR α -EGFP. *Renilla* luciferase also was co-transfected to normalize for variations in FL mRNA expression. The RAR α construct reduced FL translation (FL/*Renilla* luciferase signal) $86 \pm 2\%$ (mean \pm S.E., $n = 9$, 3 replicates in each of three experiments) relative to MS2-EGFP. A second experiment compared RAR α to each of its domains. In this case, to exclude mRNA expression differences as underlying differences in FL activity, the ratios FL/*Renilla* luciferase activity were normalized to amounts of FL mRNA (Fig. 4B). The degree of suppression by full-length RAR α remained the same (Fig. 4C). The combined ligand-independent activation function-1 (AF-1) and DNA-binding domains of RAR α (residues 1–199) reduced translation as well as full-length RAR α , but the individual AF-1 (residues 1–87) or DNA-binding domain (res-

idues 87–199) were less potent. The LBD did not repress translation. MS2-RAR α -EGFP did not reduce translation from FL mRNA lacking MS2BS (not shown). Insertion of MS2BS into FL mRNA did not hinder its nuclear export (Fig. 4D). These data indicate that RAR α suppresses FL activity through changes in translation effected by proximity of the first 199 RAR α residues to FL mRNA.

RAR α Functions as a Sequence-independent Translation Inhibitor—To determine whether RAR α represses translation directly, we added purified RAR α to a RRL programmed with FL mRNA or with an FL mRNA that included the entire 5'- and 3'-untranslated regions of GluR1. RAR α suppressed translation of both FL constructs in a dose-dependent manner, with 50 pmol causing $\sim 70\%$ inhibition. Control GST did not suppress translation (Fig. 5A). To exclude mRNA degradation, quantitative PCR was done with the FL mRNA in each of the three replicates for the six groups exposed to the three amounts of GST or GST-RAR α . The quantities of FL mRNA did not vary, excluding mRNA degradation as the cause of decreased translation (data not shown). Extended dose-dependent analysis showed ~ 80 – 90% repression by 250 pmol RAR α (Fig. 5B). To examine whether suppression requires mRNA capping and/or tailing, we tested the effect of RAR α on FL mRNA capped with a functional m⁷GpppN or a nonfunctional analog, ApppG, in the presence or absence of a poly(A) tail. Low levels of mRNA were used in a RRL assay modified so that translation was susceptible to 5'-cap and 3'-poly(A) synergy (16, 17). Similar concentrations of RAR α inhibited translation regardless of capping or tailing (Fig. 5C). RAR α suppressed FL expression from capped and untailing, capped and tailed, and uncapped and tailed from the beginnings of the assays. The functional half-lives of FL RNA did not change with GST *versus* RAR α treatment, indicating that the stabilities of the mRNAs had not changed during the assays of the various groups (Fig. 6). These data demonstrate that RAR α functions independently of mRNA sequence and does not affect mRNA turnover.

RAR α Inhibits Initiation of Translation—We used polysomal profiling to provide insight into the mechanism of translation suppression (18, 29). FL-mRNA shifted toward the top of the gradient, away from polysomes, only when tethered with RAR α (Fig. 7). Neither MS2-RAR α -EGFP nor MS2-EGFP affected β -actin mRNA. This indicates that RAR α inhibits polysome loading of associated mRNA, impeding initiation.

RAR α Localizes in Neuronal Ribonucleoprotein Particles—Neuronal ribonucleoprotein particles are structurally and functionally related to PB, which store and/or degrade translationally repressed mRNAs in conjunction with stress granules (26, 27). To determine whether these silencing structures include RAR α -suppressed transcripts, we co-transfected COS cells with FL-MS2BS and MS2-RAR α -EGFP and probed for the PB marker

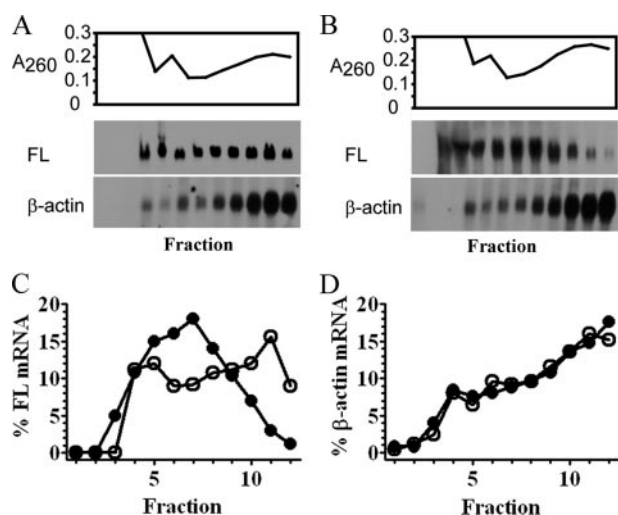


FIGURE 7. **RAR α inhibits translation initiation.** A and B, polysome distribution of FL-MS2BS mRNA *versus* endogenous β -actin in COS cells co-transfected with MS2-EGFP (A) or MS2-RAR α -EGFP (B). RNA was monitored at A_{260} (top). mRNA precipitated from each fraction was analyzed by Northern blot with FL or β -actin probes (bottom). C and D, distribution of FL (C) or β -actin mRNA (D) tethered with MS2-EGFP (open circles) or MS2-RAR α -EGFP (filled circles) in each fraction, expressed as a percentage of the sum of Northern blot signals in all fractions.

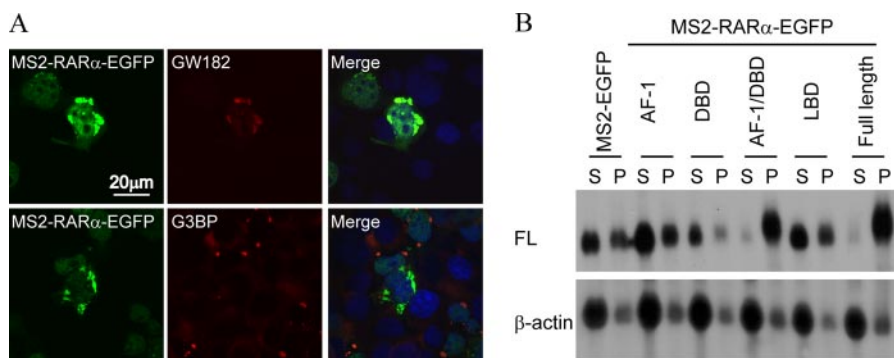


FIGURE 8. **RAR α redistributes mRNA to PB.** A, immunocytochemistry with anti-GW182 or G3BP antibodies (red) of COS cells co-transfected with FL-MS2BS and MS2-RAR α -EGFP (green). The nuclei were counterstained with 4',6'-diamino-2-phenylindole (blue). B, Northern blots of supernatants (lanes S) and pellets (lanes P) from digitonin-solubilized COS cells transfected with FL-MS2BS and MS2-RAR α or domains-EGFP. DBD, DNA-binding domain.

GW182 or the stress granule marker G3BP (Fig. 8A). The majority of RAR α isolated in large aggregates with GW182. In contrast, MS2-RAR α -EGFP did not co-localize with G3BP. To confirm association of repressed transcripts with PB, COS cells were co-transfected with FL-MS2BS and MS2-RAR α or domains-EGFP or MS2-EGFP and were solubilized with digitonin, which preserves PB (18). FL mRNA tethered with EGFP, or the RAR α AF-1, DNA-binding domain or LBD partitioned primarily to soluble fractions, whereas FL mRNA tethered with the combined AF-1/DNA-binding domain or with full-length RAR α partitioned primarily to PB (Fig. 8B). We next dissolved PB by siRNA knockdown of GW182 (28). This did not prevent translation suppression by RAR α (not shown). These data indicate that RAR α silences mRNA independently of PB but sorts silenced mRNA to PB.

Conclusions—Previously, we showed that atRA stimulates dendritic growth within minutes, by activating mitogen-activated protein kinase and mTOR regulation of neuronal translation and increasing dendritic translation of CaMKII α and GluR1 mRNA, without affecting GluR2 (13). We also showed that the atRA effects on dendritic growth were prevented by knocking down RAR α with siRNA or small hairpin RNA and hippocampal neurons expressed RAR α in dendrites, suggesting a function in translation, in addition to transcription.

Here we have shown by immunoaffinity and proteomic analyses that RAR α forms partnerships in neurons with multiple RNA-binding proteins and translation factors and confirmed select RAR α partnerships by TAP. Consistent with a function in translation, mouse brain RAR α localized in the RNA granule-containing fraction. RAR α partnered with the granule RNA-binding protein Pur α and RAR α co-localizes in neuronal somatic PB with Pur α and FMRP. Neuronal RAR α associated specifically with mRNA encoding CaMKII α and GluR1. Both a tethering assay and a RRL translation assay showed that RAR α can repress translation, and polysome profiling showed that RAR α moves mRNA away from polysomes, consistent with prevention of translation initiation. These data broaden insight into the mechanisms of plasticity, translation regulation, retinoid action, and the action of RAR α , well recognized as a ligand-activated transcription factor but not known as a regulator of translation (30–32).

We propose that RAR α silences translation and facilitates delivery of specific mRNA to neuronal dendrites. atRA would stimulate transcription and translation in neurons, providing for rapid insertion of receptors into synapses and generating mRNA in preparation for a new cycle. This mechanism could also modulate homeostatic scaling, a form of metaplasticity that engages local synthesis of GluR1 and other synaptic proteins (8, 12).

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