Translational regulation of GluR2 mRNAs in rat hippocampus by alternative 3′ untranslated regions

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Abstract

The GluR2 subunit determines many of the functional properties of the AMPA subtype of glutamate receptor. The roles of untranslated regions (UTRs) in mRNA stability, transport or translation are increasingly recognized. The 3′end of the GluR2 transcripts are alternatively processed to form a short and long 3′UTR, giving rise to two pools of GluR2 mRNA of 4 and 6 kb in length respectively in the mammalian brain. However, the role of these alternative 3′UTRs in GluR2 expression has not been reported. We demonstrate that in the cytoplasm of rat hippocampus, native GluR2 mRNAs bearing the long 3′UTR are mostly retained in translationally dormant complexes of ribosome-free messenger ribonucleoprotein (mRNP), whereas GluR2 transcripts bearing the short 3′UTR are predominantly associated with actively translating ribosomes. One day after pilocarpine-induced status epilepticus (SE), the levels of both long and short GluR2 transcripts were markedly decreased in rat hippocampus. However, after SE GluR2 mRNAs bearing long 3′UTRs were shifted from untranslating mRNP complexes to ribosome-containing complexes, pointing to a selective translational derepression of GluR2 mRNA mediated by the long 3′UTR. In *Xenopus* oocytes, expression of firefly luciferase reporters bearing alternative GluR2 3′UTRs confirmed that the long 3′UTR is sufficient to suppress translation. The stability of reporter mRNAs in oocytes was not significantly influenced by alternative 5′ or 3′UTRs of GluR2 over the time period examined. Overall our findings that the long 3′UTR of GluR2 mRNA alone is sufficient to suppress translation, and the evidence for seizure-induced derepression of translation of GluR2 via the long 3′UTR, strongly suggests that a regulatory signaling mechanism exists that differentially targets GluR2 transcripts with alternative 3′UTRs.

Introduction

The AMPA subtype of ionotropic glutamate receptors, which exist as either homomeric or heteromeric assemblies of GluR1-4 subunits that form functionally distinct receptors (Swanson et al., 1997), mediates the majority of the postsynaptic response at most fast excitatory synapses in the CNS (Dingledine et al., 1999). The subunit composition of AMPA receptors varies with the stage of development, cell type, and brain region (Song and Huganir, 2002). AMPA receptors are precisely localized opposite to glutamatergic presynaptic terminals (Craig et al., 1994), and dynamic cycling of the receptors occurs in an activity-dependent manner (Man et al., 2000). Each AMPA receptor subunit has a channel-lining re-entrant hairpin loop that contains a glutamine/arginine (Q/R) site. However, RNA editing at the Q/R site of almost all GluR2 subunits replaces glutamine with arginine, making the site more positively charged (Dingledine et al., 1999). Thus, ion permeation properties of functional AMPA receptors are defined by the relative abundance of edited GluR2 subunits (Hollmann et al., 1991; Koh et al., 1995; Swanson et al., 1997). Moderate changes in GluR2 expression are expected to have

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significant physiological consequences by affecting synaptic AMPA receptor phenotype. The expression of GluR2 transcript levels relative to other AMPA receptor subunits is changed following seizures (Ekonomou et al., 2001; Grooms et al., 2000; Huang et al., 2002), after ischemia (Colbourne et al., 2003; Gorter et al., 1997; Pellegrini-Giampietro et al., 1992), following administration of drugs of abuse (Fitzgerald et al., 1996; Mameli et al., 2007) or antipsychotics (Martinez-Turrillas et al., 2002). GluR2 expression is known to be influenced strongly at the transcriptional level by positive and negative regulatory elements found in the 5′ proximal region of the promoter (Fig.1A) (Huang et al., 1999; Myers et al., 1998). Earlier findings that some hippocampal interneurons contain GluR2 mRNA but little or no GluR2 protein (Washburn et al., 1997), and that *de novo* synthesis of GluR2 protein is required for LTD in dopaminergic neurons of the ventral tegmental area (Mameli et al., 2007), suggest that neuron-specific or activity-specific regulatory mechanisms for GluR2 translation are also in place. Thus it is worthwhile to better understand molecular mechanisms regulating translation of GluR2 transcripts and their effects on physiological properties of AMPA receptors.

GluR2 mRNAs are translationally suppressed by involvement of an imperfect, polymorphic GU-repeat in the longer 5′ untranslated region (UTR) (Myers et al., 2004). In many other mRNAs including those encoding the NR1 subunit of NMDA receptors, 3′ UTRs play a major role in regulating RNA stability, cellular localization and translation through interaction with RNA binding proteins (Awobuluyi et al., 2003a; Conne et al., 2000; de Moor et al., 2005; Kuersten and Goodwin, 2003; Mazumder et al., 2003). For example in PC12 cells, translation of reporter mRNA bearing 3′UTRs of NMDA receptor subunit NR1 is significantly inhibited (Awobuluyi et al., 2003b). In neurons, cytoplasmic polyadenylation elements (CPEs) located within the 3′UTRs of NMDA receptor transcripts have been shown to interact with binding proteins (CPEBs), and this interaction appears to regulate translation in an activity-dependent manner (de Moor and Richter, 1999; Wells et al., 2001). GluR2 mRNAs in mice contain alternative 3′UTRs differing in their length (~750 and ~2750 bases) (Kohler et al., 1994). However, the physiological roles of alternative GluR2 3′UTRs are unknown. Although GluR2 3′UTRs contain many predicted conserved sites for RNA binding proteins (Huang et al., 2006), none has been confirmed to be functional. Here, we provide evidence that the long 3′ UTR but not the short 3′UTR of GluR2 represses translation in the presence of either 5′UTR, and that this effect is partially relieved following status epilepticus.

Material and Methods

GluR2 3′UTR constructs

Four firefly luciferase reporter constructs were designed to evaluate the role of alternative GluR2 5′ and 3′UTRs on translation regulation in Xenopus oocytes. The luciferase protein coding region was flanked by all four combinations of naturally existing alternative GluR2 5′ and 3′UTRs. First, a KpnI - HindIII fragment of pGL2-nrfl1-luc, which contains a T3 RNA polymerase initiation site, a CMV promoter, and the rat GluR2 long 5′UTR (-429 bases from initial AUG start codon) was cloned into the multiple cloning site of the pSP-luc+ cloning vector (Promega, Madison, WI) using existing KpnI and Hind III sites. Next, we removed the CMV promoter from the construct using SacI and SacII so that T3 promoter directly preceded the long 5′UTR of GluR2 followed by the firefly luciferase coding regions. The resulting plasmid was digested with KpnI and XbaI to remove the desired fragment, which contains (5′→3′) a T3 promoter, the long 5′UTR of GluR2, and the firefly luciferase coding region. This fragment was then cloned into the pSP73 vector (Promega) using existing KpnI and XbaI sites, allowing us to further utilize remaining the restriction sites in MCS for cloning alternative GluR2 3′UTRs. This construct was named p75UL. Rat GluR2 short and long 3′UTRs (750 and 2750 bases relative to the stop codon, respectively) were amplified from cDNA generated from adult male Sprague Dawley rat brain tissue (Charles River Laboratories, Wilmington, MA)

using 3′UTR-specific primers designed to contain 5′-PstI and 3′-BstE II restriction sites. The amplified short and long GluR2 3′UTR fragments flanked by Pst I and BstE II sites were cloned into the pCRII-TA cloning vector (Promega), then were excised using Pst I and BstEII restriction enzymes and cloned downstream of the firefly luciferase coding region using the same sites in p75UL. The resulting constructs contained (5′→3′) a T3 RNA polymerase initiation site followed by GluR2 long 5′UTR, firefly luciferase coding region and short or long GluR2 3′UTR. To generate constructs bearing the short 5′UTR, we removed 65 bases from the long 5′UTR (between bases -429 to -365, which contains the GU repeat region) by taking advantage of the existing unique restriction sites BssH II and NheI. All constructs were sequenced to confirm integrity.

Reporter expression and RNA stability in Xenopus oocytes

Reporter cDNA constructs bearing alternative GluR2 5′ and 3′ UTRs [see Fig. 4A, designated SS for Luciferase protein coding region(ff) flanked by Short 5′UTR and Short 3′UTR of GluR2; SL, ff flanked by Short 5′UTR and Long 3′UTR of GluR2; LS, ff flanked by Long 5′UTR and Short 3′UTR of GluR2; and LL, ff flanked by Long 5′UTR and Long 3′UTR of GluR2)] were linearized at the 3′-end using the *BstE* II restriction site. In vitro synthesis of 5′-capped mRNAs from the linearized reporter constructs was performed using T3 RNA polymerase following the instructions provided in the T3 mMESSAGEmMACHINE (Ambion, Austin, TX). The resulting mRNAs were quantified and quality checked in the RNA Nano Chips apparatus using a 2100 Agilent Bioanalyzer (Agilent, Waldbronn, Germany). Oocytes (stage V-VII) were harvested from *Xenopus laevis* and injected with mRNAs as described by Dingledine et al. (1992). Each of the firefly reporters representing alternative GluR2 UTRs was separately microinjected into oocytes (0.05 - 5 fmol/oocyte). Firefly luciferase activities were measured from individual oocytes 0, 4, 16, 24 and 40 hours after injection. Oocytes were individually homogenized by trituration in a 96-well plate containing 100 μl of Passive Lysis Buffer (Promega, Madison WI). To measure luciferase activity, homogenate from an individual oocyte was mixed by pipetting and 20ul was transferred into Microlite1 96-well white flat bottom plates (Thomas Scientific, Swedesboro, NJ). The firefly luciferase activity, defined as relative light units (RLU) measured at 570 nm from individual wells, was measured using the luciferase assay substrate from Dual-Luciferase Reporter Assay System (Promega, Madison, WI). The RLU from the homogenates of uninjected Xenopus oocytes (0.2-0.7 RLU) constituted background, which did not differ significantly among different batches of oocytes. The lowest experimental sample RLUs were at least 200 fold higher than that of the background. In parallel, to monitor RNA stability, five microinjected oocytes from the same batch were pooled for each time point between 4 and 40 hours. To recover a sufficient amount of reporter mRNAs from the oocytes, we pooled five microinjected oocytes into 1ml Trizol, which was stored at -80 ° C. After collection of oocytes for all time points, the pooled oocytes in Trizol were homogenized by pipetting up and down (30-40 strokes), and the RNAs were extracted from the Trizol homogenates by the standard phenol-chloroform method. The isolated RNA pools were dissolved in 20 μl *in vitro* reverse transcription reaction containing Thermoscript Reverse Transcriptase (Invitrogen) and the reaction run for 50 min at 55 °C. The resulting cDNAs were then quantified by Q-RT-PCR analysis using a single primer set that amplified GluR2 mRNA bearing both short and long 3′UTRs along with standard dilutions of the luciferase reporter cDNAs.

Sucrose gradient analysis of endogenous GluR2 transcripts

Linear sucrose gradient fractionation of cytoplasmic extract derived from rat hippocampal homogenates was based on the procedure described by Feng et al. (1997), with modifications. Briefly, rats (n=6-8) were anesthetized deeply with isoflurane (NOVAPLUS, Lake Forest, IL), decapitated, and the hippocampus quickly dissected out in ice-cold phosphate buffered saline (pH 7.4). The hippocampal tissue was minced and homogenized in 1 ml of either polysome-

preserving or polysome-disrupting buffer using a Dounce type-B tight-fitting pestle (40 strokes/hippocampus/1 ml). Both buffers contained the following components: 20 mM Tris-HCl, pH 7, 100 mM KCl, 100 μg/ml cycloheximide, 5 μl/ml RNAse inhibitor (Applied Biosystems). Polysome-preserving buffer, in addition, contained 5 mM $MgCl_2$, whereas polysome-disrupting buffer contained 10 mM EDTA. In both cases Triton-X100 (Sigma, St. Louis, MO) was added immediately after the homogenization to a final concentration of 0.5% and the homogenate was incubated on ice for 10 min. Cellular debris was pelleted in sterile microfuge tubes initially at $3,000 \times g$ for 10 min, then at $13,000 \times g$ for 30 min. The resulting supernatant (~700 μl) was loaded onto the top of a 15-45% linear sucrose gradient for subsequent fractionation. The gradients were centrifuged at $39,000 \times g$ for 60 min at 4 °C. Ten 1.2 ml fractions from each gradient tube were collected into RNase-free microfuge tubes using Isco gradient fractionators (Isco, Lincoln,NE). Total RNA from each fraction was extracted using a standard phenol-chloroform method. The resulting RNA pellets were reconstituted in 40 μl RNase-free water and stored at -80 °C until reverse transcription to cDNA and subsequent Q-RT-PCR analysis.

Quantitative Real Time PCR analysis of reporter and native GluR2 mRNAs

Reporter mRNAs isolated from Xenopus oocytes, the endogenous mRNAs recovered from the linear sucrose gradient fractions, and from the subcellular fractions of the rat hippocampus were all reverse transcribed into template cDNAs using random hexamers (50 ng/μl) and Thermoscript reverse transcriptase from ThermoScript RT–PCR Assay System (Invitrogen, Carlsbad, CA). Primers specific to short and long GluR2 3′UTRs, and GAPDH coding region were identified using Primer Quest (Integrated DNA Technologies, IA) and were synthesized by IDT. All primer sets resulted in a single product as assessed by 2% agarose gel electrophoresis and had similar primer efficiencies as determined by melt-curve analysis. The first strand cDNAs were used as template in 25 μl PCR reactions with 400 nM primers and SYBR GREEN PCR Master Mix (Applied Biosystems, Warrington, WA1 4SR,UK). The final PCR mixes were transferred into a 96-well thin-wall PCR plate, which was covered with a piece of optically clear sealing film. PCR conditions were 3 min at 95 °C, followed by 45 cycles of 15 sec at 95°C and 30 sec at 63°C. PCR was performed using an iCycler iQ Detection system and software (Biorad, CA). The threshold cycles (C_t) of the samples run in triplicate were averaged and quantified relative to a standard curve (diluted cDNA from total rat hippocampal RNA, or known quantities of reporter plasmid DNAs). The cycle threshold number was log linear with input cDNA up to a CT of 34. Control reactions with primer sets but without added template consistently did not produce products.

Subcellular distribution of endogenous GluR2 transcripts

Hippocampi from 6 adult rats were homogenized individually in 1.2 ml polysome-preserving lysis buffer. Three hundred μl of the 1.2 ml homogenate was combined with 1 ml Trizol for total RNA extraction. The remaining 900 μl of the homogenate was treated with Triton-X to a final concentration of 0.5% on ice for 10 min. Cell debris was pelleted at $3,000 \times g$, which was followed by a second spin at $13,000 \times g$ for 30 min at 4°C. Total RNA was extracted from the supernatant, which constitutes the fraction containing cytoplasm and ribosomes (Feng et al., 1997), from the pellet (collected after each spin, and combined as total pellet) enriched in large organelles and nuclei, and from the 300 μl total homogenate that constitutes the whole cell content.

Northern blot analysis of native GluR2 transcripts

Rat whole hippocampus (~50 mg/rat) and an equivalent amount of cortex tissue were individually homogenized in 2 ml Trizol for 40 sec using a PT 2100 Polytron Homogenizer (KINAMATICA, Littau, Luzern). Total RNA (20 μg/lane) was resolved on a 2% agarose gel

containing 8% formaldehyde, then transferred onto Zeta-Probe GT membrane (BioRad, Hercules, CA) by capillary action. The membrane was initially probed with a ^{32}P-labeled cDNA fragment specific to GluR2 long 3′UTR (rat cDNA position 4958-5335), then stripped and re-probed for pan GluR2 (rat cDNA position 2769 -3119).

Non-isotopic in situ hybridization of native GluR2 mRNAs

Rats under isoflurane anesthesia were transcardially perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 20 min at room temperature. Their brains were removed and placed in 4% PFA / 20% sucrose at 4 °C for 1-3 days prior to sectioning. Coronal sections of whole brain (35-40 micron) were prepared using a standard sliding microtome Model 860 (American Optical Corp, Buffalo, NY, USA), and the sections were post-fixed in 4% PFA at 4°C for 3-5 days. Digoxigenin-labeled GluR2 pan (2661-2922) and long (4858-5330) 3′UTR RNA probes (both sense and anti-sense strands) were prepared from cDNA inserts cloned into the pCRII-TOPO plasmid. After linearization of the plasmid, digoxigenin-labeled riboprobes were synthesized with the SP6-T7 DIG-RNA labeling kit (Roche, Mannheim, Germany), according to manufacturer's instructions. In situ hybridization was performed as previously described (Tongiorgi et al., 1998) with the following modifications. Briefly, the post-fixed free-floating coronal sections at the level of dorsal hippocampus were washed twice in 0.5X SSC and PBS, treated with proteinase K (2 mg/ml for 40 min at room temperature), and then washed again in PBS. Prehybridization was carried out at 42 °C for 2 hours in plastic 24-well plates (Costar,Corning, NY) containing 50% formamide (Fluka Biochemica, Steinheim, Switzerland) 1X SSC (Invitrogen), 1X Denhardt's solution, 0.5 mg/ml yeast tRNA, 0.5 mg/ml salmon sperm DNA, 0.5 mg/ml heparin in DEPC-treated water. Using glass hooks, slices were transferred into hybridization solution, composed of prehybridization solution to which was added 10% dextransulfate and 500 ng/ml digoxigenin labeled riboprobes. In situ hybridization was carried out overnight (≥18 h) in sealed multiwall plates at 55 °C without agitation. RNAse-free pipette tips and glassware were used throughout. Post hybridization and immunodetection of the digoxigenin labeled riboprobes were carried out as described by Tongiorgi et al. (1998). Using water-based AquaPolymont (Polysciences, Inc., Warrington, PA), sections were mounted on glass slides, covered with a glass cover slip, and sealed with clear nail protector (Wet'n' Wild, North Arlington, NJ). Images of hippocampus from the sections were taken with a digital camera under a light microscope.

Pilocarpine-induced status epilepticus

Adult male Sprague Dawley rats (Charles River Labs, Wilmington, MA), 40-50 days of age and 200-250 gram body weight, were used in all experiments. Status epilepticus (SE) was induced in these rats as previously described (Huang et al., 2002). In brief, rats were injected with a mixture of methylscopolamine and terbutaline (2.5 mg/kg i.p.). After 20 min, rats were injected with pilocarpine HCl (380-400 mg/kg s.c.) or an equivalent volume of saline. In rats, pilocarpine-induced seizures consist of distinct motor behaviors including forelimb clonus, tail extension, rearing and falling. Animals presenting these behaviors with increased seizure intensity, duration and frequency 20- 40 min after the injection of pilocarpine were declared to be in SE, which is characterized by periodic rearing and falling accompanied by clonus. To increase survival of animals in SE, hyperthermia was minimized by periodic cooling of the animal with chilled air. Twenty four hours after SE onset, treated rats along with the controls were processed for in situ hybridization or sucrose gradient analysis as described above.

Results

GluR2 mRNA bearing long 3′UTR is the majority GluR2 species in rat hippocampus but is underrepresented in the cytoplasm

Two distinct populations of GluR2 transcripts (long ~6kb, and short ~4kb) were detected by Northern blot analyses of total RMNA isolated from rat hippocampus and cortex (Fig. 1B). To determine whether the abundance of GluR2 transcripts in cytoplasm is 3′UTR-specific, whole hippocampi from SD male rats were homogenized in 1.2ml lysis buffer. Total homogenate was fractionated into a supernatant enriched in cytoplasmic RNAs and ribosomes, and a pellet that consists largely of nuclear extract and cellular debris. Total RNA was extracted from the supernatant, from the pellet, and from an unfractionated total hippocampal homogenate. In vitro synthesized cDNA from the total RNA preparation was quantified by Q-RT-PCR using primers specific to the GluR2 long 3′UTR. The level of GluR2 transcripts with the long 3′UTR was normalized to the total GluR2 mRNA level in same fractions. Although approximately half of the total GluR2 mRNA in the lysate had the long 3′UTR, more than 70% of the GluR2 transcripts in the pellet fraction contained the long 3′UTR, and less than 30% of the GluR2 mRNAs recovered from the ribosome-rich supernatant had the long 3′UTR (Fig. 1C). This suggests that a minority of the endogenous GluR2 transcripts in the cytoplasm bears the long 3′UTR, and that GluR2 mRNAs bearing the short 3′UTR are the dominant GluR2 transcript in the cytoplasm.

Translation profile in rat hippocampus of native GluR2 transcripts bearing alternative 3′UTRs

Endogenous mRNAs that are undergoing active translation are typically associated with polyribosomes, whereas RNAs associated with ribosome-free ribonucleoproteins (RNP) or monosomes are not being actively translated (Spirin, 1969; Warner et al., 1963). We examined the polyribosome-association profiles of endogenous GluR2 mRNAs from rat hippocampus using linear sucrose gradient fractionation. In such a fractionation paradigm, ribosomal particles are separated according to particle size through sucrose gradient centrifugation in which the number of ribosomes occupying the nucleotides of mRNAs (30-40 nucleotides per ribosome) determines the density of translating polysomes (Hershey et al., 1996). The fractions near the bottom of the gradient contain denser polyribosomes, whereas fractions 1 and 2 contain few or no ribosomes. Thus, mRNAs detected in the bottom fractions are considered to be actively translated whereas RNA found in the top fractions, which contain ribosome-free mRNPs, are not being translated (Feng et al., 1997). We layered cytoplasmic extracts from rat hippocampi on top of a linear sucrose gradient (15-45%) to separate translating polyribosomes from non-translating components including ribosome-free mRNPs, ribosome subunits and monosomes. A clear separation of the aforementioned components on the gradient was observed by monitoring UV absorption values at 254 nm (Fig.2A). Our results showed that whereas the majority of the overall endogenous GluR2 transcripts were detected in the ribosome-containing (bottom) fractions (3 to 10), the large majority of GluR2 transcripts bearing the long 3′UTR were detected in the mRNP fractions 1 and 2 (Fig. 2B). As expected, when translating polysomes in the cytoplasmic lysate were disrupted by EDTA treatment (Fig. 2C), pan GluR2 mRNAs as well as glyceraldehyde-3-phosphate deydrogenase (GAPDH) mRNAs were largely recovered in the ribosome-free mRNPs on the linear sucrose gradient (Fig. 2D). These results demonstrate that, in rat hippocampus, GluR2 transcripts bearing long 3′UTRs are mostly associated with mRNPs and thus in a translationally dormant state. These results also suggest that ~80% of the GluR2 mRNAs that are associated with ribosomes contain short 3′UTRs.

We also sought to determine whether the distribution of GluR2 transcripts in distinct hippocampal regions such as CA1, CA3 and dentate gyrus (DG) is 3′UTR-specific. In order to compare the cellular distribution of GluR2 transcripts in intact tissue, digoxygenin-labelled

GluR2-specific riboprobes (sense or antisense) were hybridized *in situ* to native GluR2 mRNAs on 35-40 micron coronal sections of rat hippocampus. As expected, overall GluR2 mRNA was abundant in the DG, CA1 and CA3 (Fig. 3A). The distribution pattern of GluR2 mRNAs bearing long 3′UTRs in these regions was similar to that of pan GluR2 (Fig. 3B). The sense probes hybridized to the tissues from the same animals showed only background signal (Fig. 3C and 3D).

Translational regulation by alternative GluR2 3′UTRs

Cytoplasmic injection of in vitro synthesized mRNAs into *Xenopus laevis* oocytes is widely employed to study the function of heterologously expressed genes, as well as the role of 3′ UTRs in translation (reviewed in Pique et al., 2006). To determine whether the alternative UTRs would influence translation of a heterologous transcript, we designed firefly reporters bearing all four combinations of GluR2 5′- and 3′- UTRs (Fig. 4A). In vitro synthesized 5′-capped mRNAs from the reporter constructs were quality checked and the RNA amount measured with the Agilent mRNA Nano bioanalyzer (Fig.4B). Firefly reporter mRNAs were microinjected into stage V-VI Xenopus oocytes, and their translation efficiencies were compared by measuring luciferase activity from individually homogenized oocytes. Beginning a few hours after injection, luciferase activity was proportional to both time and the amount of reporter mRNAs microinjected into *Xenopus* oocytes (e.g. Fig 4C and D). Figure 5A shows that the 5′UTR exerted a substantial translational inhibition as previously reported (Myers et al., 2004). All firefly reporter mRNAs had similar stability in Xenopus oocytes between 4 and 16 hours after injection, although the level of the four reporter RNAs recovered from the oocytes varied (Fig. 5B), probably as a result of different rates of rapid degradation (data not shown). When luciferase activity was normalized to the level of reporter mRNAs recovered from the microinjected oocytes, translational inhibition mediated by the long 3′UTR was obvious regardless whether the long or short 5′UTR was present (Figure 5C). The translation rates (luciferase activity per fmol mRNA per hour), measured between 4 and 16 hours after injection, were significantly lower for reporters with long 3′UTRs than for reporters bearing short 3′UTRs (Fig. 5D). This was particularly evident with constructs bearing the short 5′UTR (cf SS with SL in Fig 5D). These data indicate that translational repression of GluR2 mRNAs is mediated by either a long 5′UTR or a long 3′UTR. Moreover, the degree of translational repression by the long 3′UTR or long 5′UTR is approximately the same.

Effect of pilocarpine-induced status epilepticus (SE) on translation and regional distribution of GluR2 transcripts with alternative 3′UTRs

SE is known to reduce the levels of total GluR2 mRNA and protein in the CA1 and CA3 regions of rat hippocampus within 16 to 24 hours after the onset of SE (Grooms et al., 2000; Huang et al., 2002). We homogenized hippocampi from rats treated with pilocarpine or saline 22-24 hr previously, and isolated total RNA from the cytoplasmic/ribosomal fraction. The levels of total GluR2 mRNA and GluR2 mRNA bearing the long 3′UTR were quantified by Q-RT-PCR. The measured change of the average cycle threshold (ΔCT) between GluR2 and GAPDH mRNA was significantly larger in the pilocarpine-treated animals compared to that of saline-treated animals, confirming a substantial decrease of the overall levels of GluR2 mRNAs 24 hours after SE (Fig. 6A). The level of transcripts bearing the long 3′UTR was reduced by approximately the same extent (~75%) as that of total GluR2, in agreement with the in situ hybridization results (see below). Using linear sucrose gradient fractionation, we examined the effects of pilocarpine-induced SE on polyribosome-association of native GluR2 transcripts bearing alternative 3′UTRs. Rats were treated with pilocarpine or saline, and one day later the cytoplasmic/ribosomal fractions of hippocampal homogenates were run on a sucrose gradient. RNAs were isolated from each gradient fraction and were quantified with Q-RT-PCR. Whereas the distribution of the pan GluR2 mRNA over the gradient fractions was not affected (Fig 6B and C), a portion of GluR2 mRNAs bearing long 3′UTRs was shifted from ribosome free mRNP

complexes (top 2 fractions) to actively translating ribosomes 24 hrs after pilocarpine-induced SE (Fig. 6D and C), pointing to an activity-dependent derepression of translation of GluR2 transcripts bearing long 3′UTRs. The shift from mRNPs to polyribosomes was a selective effect of status epilepticus on transcripts bearing the long 3′UTR because it was not seen with pan-GluR2 or with GAPDH transcripts (Fig 6C).

The effect of pilocarpine-induced SE on the cellular distribution of GluR2 transcripts was examined in 40 micron coronal sections of hippocampus one day after rats had been treated with pilocarpine or saline. We observed a visually obvious reduction of total GluR2 mRNA as well as GluR2 transcripts bearing long 3′UTRs in the CA1 and CA3a regions of the rat hippocampus (Fig. 2E and F). There was relative preservation of the levels of pan-GluR2 and GluR2 transcripts bearing the long 3′UTR in both dentate granule cells and CA2 (arrows in Fig 2E, F).

Discussion

The main conclusions from our studies are i) that GluR2 transcripts possess varying translation efficiencies based on their alternative combinations of 5′- and 3′UTRs, and ii) that translational control by the 3′UTR is itself regulated by mechanisms brought into play following seizures. The principal findings supporting these conclusions are the following: (1) GluR2 transcripts bearing long 3′UTRs are less abundant than those containing short 3′UTRs in the cytoplasmic extract of hippocampus; (2) hippocampal GluR2 mRNAs bearing long 3′UTRs appear to be in a translationally dormant state as they co-sediment mostly with ribosome free mRNP complexes on linear sucrose gradient; (3) For constructs bearing the GluR2 short 5′UTR, the translation rate in *Xenopus* oocytes of firefly reporters with GluR2 long 3′UTR is reduced about 40-fold compared to the reporters bearing the short 3′UTR; (4) 24 hours after pilocarpine-induced seizures, the association of long GluR2 transcripts with mRNPs in whole hippocampus is selectively reduced. These findings taken together indicate that under normal physiological conditions, GluR2 transcripts bearing alternative combinations of 5′ and 3′UTRs are differentially processed and that this UTR-specific translation regulation is responsive to substantial changes in neuronal activity associated with pilocarpine-induced status epilepticus.

It has long been appreciated that GluR2 mRNA and protein levels decline severely after prolonged seizures (Groomes et al. 2000; Huang et al., 2002). We show here that this decline in gross GluR2 mRNA level appears to be partially offset by increased translation of one species of GluR2 mRNA. Thus, 24 hr after pilocarpine-induced status epilepticus, a portion of GluR2 mRNAs bearing long 3′UTRs shifts from ribosome-free mRNPs to polysome fractions in rat hippocampus, although the polysome profile of pan-GluR2 mRNA remains unaltered. These findings suggest that a translational repression mechanism specifically targeting GluR2 mRNAs with long 3′UTRs exists in the cytoplasm of hippocampal neurons. This finding strongly suggests that GluR2 transcripts bearing the long-3′UTR are subject to an activity-induced de-repression of translation that is prominent in the hippocampus after SE. The mechanism of derepression is unclear, but may for example involve miRNA targeted to the long GluR2 3′UTR or binding of a cytoplasmic polyadenylation element binding protein (CPEB), which regulates translation initiation (Richter and Sonenberg, 2005) and facilitates the targeting of mRNAs to dendrites (Huang et al., 2003). Among the CPEB proteins, CPEB3 is expressed specifically in neurons (Theis et al., 2003) and appears to bind to GluR2 long 3′ UTRs. RNAi knock down of CPEB3 mRNA induces GluR2 protein expression in cultured hippocampal neurons (Huang et al., 2006). It is yet to be determined, however, whether the expression of CPEB3 is altered in the hippocampus of animals 24 hours after pilocarpine-induced status epilepticus.

Our finding that GluR2 mRNAs of rat hippocampus bearing the long 3′UTR associate poorly with ribosomes at rest suggests that their translation is less efficient than that of GluR2 mRNAs bearing the short 3′UTR. GluR2 is the first mRNA, to our knowledge, shown to be repressed translationally by both long 5′- and long 3′-UTR. The presence of either long UTR of GluR2 in the firefly reporter mRNAs is sufficient to confer substantial translational repression. These findings raise the possibility that both constitutive and regulated translation of endogenous GluR2 transcripts occurs in vivo. The findings that all principle neurons of CA1, CA3 and DG regions in rat hippocampus express GluR2 mRNAs with long 3′UTR, and that the majority of these GluR2 transcripts associate poorly with ribosomes, further support the hypothesis that cytoplasm of hippocampal neurons contains a pool of translationally regulated GluR2 mRNAs. The effectiveness of translational regulation should be dependent upon the relative abundance of the native GluR2 mRNAs bearing alternative combinations of the 5′- and 3′-UTRs and the protein factors that coordinate potential interactions between alternative 5′ and 3′ UTRs.

In Xenopus oocytes, the observed high translatability of firefly reporters bearing short GluR2 untranslated regions suggests that a subpopulation of native GluR2 mRNAs would be constitutively translated provided that it contains neither a long 5′- nor a long 3′UTR. More than half of the GluR2 mRNAs contain the long 3′UTR (Kohler et al., 1994; Fig 1B), and the majority of GluR2 transcripts bear short 5′UTRs (Myers et al 1998). These findings support the conclusion that GluR2 mRNAs bearing long 3′UTRs constitute a significant portion of the regulated pool of the total GluR2 transcripts.

In cultured neurons, GluR2 mRNAs can be transported from soma to dendrites and translated in physically isolated dendrites upon group mGluR I activation (Ju et al., 2004). Moreover, the abundance of GluR2 protein in dendrites was decreased by NMDA receptor activation (Grooms et al., 2006). Whereas CPEB proteins have been implicated in translational control of the NR1 subunit of NMDA receptors (de Moor and Richter, 1999; Wells et al., 2001), proteins involved in the mechanisms of activation, repression or de-repression of GluR2 translation are unknown, and it is not known whether specific GluR2 mRNA subpopulations are targeted to synapses. It is, however, likely that translation of GluR2 mRNAs bearing a long 3′UTR is regulated because the majority of the GluR2 long transcripts appear to be poorly translated under normal physiological conditions, and their association with ribosomes is increased after seizure activity associated with pilocarpine-induced SE. Taken together, our findings suggest that activity-dependent translational regulation of GluR2 mRNAs bearing a short 5′UTR and long 3′UTR should have physiological significance in forming functional AMPA receptors.

Acknowledgments

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Abbreviations

UTR

untranslated regions

AMPA

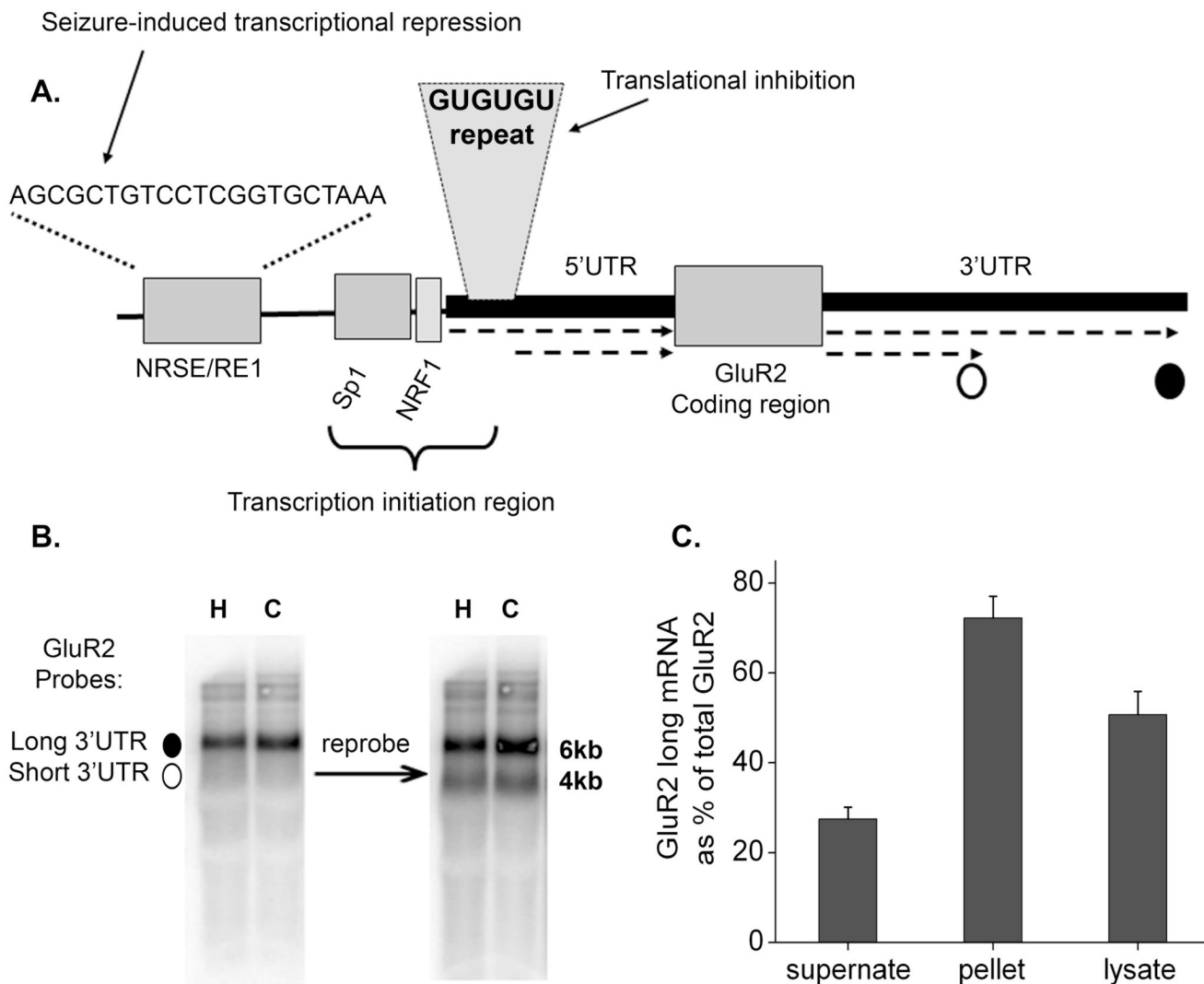α-Amino-3-hydroxy-5-methylisoxazole-4-propionate

mRNA

messenger RNA

Figure 1.
Characterization of GluR2 3′UTRs. A. Schematic of known transcriptional and translational regulation domains of GluR2. B, Northern blots of rat hippocampus (H) and cortex (C) mRNAs hybridized to 3′UTR-specific probes that recognize total GluR2 mRNA (open circle ~4kb) and GluR2 mRNA with long 3′UTR (dark filled circle ~6kb). C, Subcellular distribution of native GluR2 transcripts in rat hippocampus; supernate and pellet fractions of detergent-treated hippocampal homogenates were analyzed by Q-RT-PCR for their native GluR2 mRNA content and compared to that of untreated total lysate from the hippocampus of same animals.
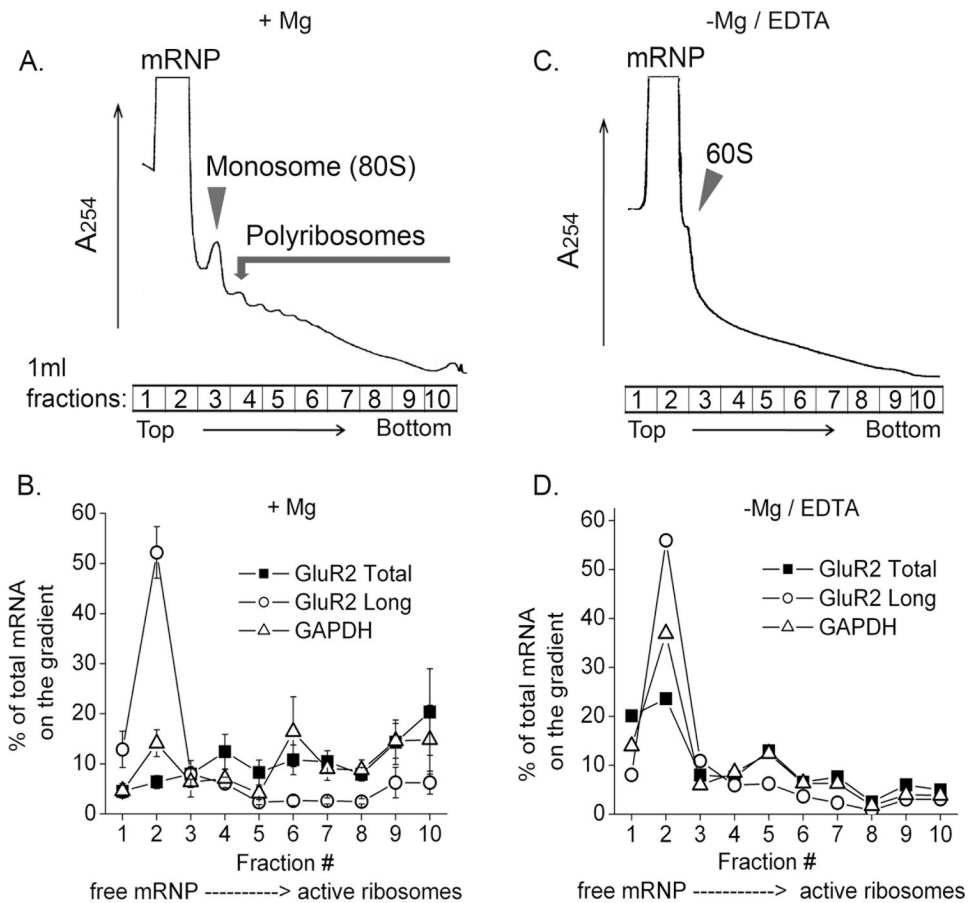
Figure 2.
Differential association of native GluR2 transcripts with ribosomes. A, Fractionation of cytoplasmic extracts of rat hippocampus on a linear sucrose gradient revealed ribosome-free mRNPs (fractions 1 and 2), 80S monosome peak (fraction 3) and translating polyribosomes (fractions 4 to 10). B, The majority of total GluR2 transcripts are detected in ribosomal fractions (both monosomes and polysomes) (fractions 3 to 10) whereas the majority of GluR2 transcripts bearing the longer 3′UTRs are associated mainly with free mRNPs (fractions 1 and 2). C, Treatment of the lysate with EDTA, a Mg^{2+} chelator that disrupts polysome formation, dissociated translating polyribosomes into 40S and 60S ribosomal subunits in the gradient, as expected. D, A major shift of mRNAs (including GAPDH as internal control) from ribosomal to mRNP fractions was observed in EDTA-treated lysates.
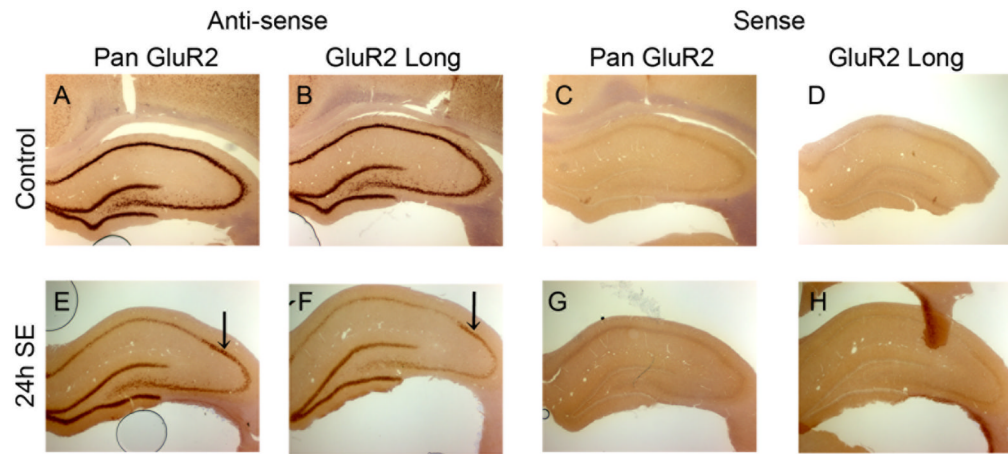
Figure 3.
Cellular distribution of native GluR2 mRNAs in rat hippocampus. *In situ* hybridization of digoxigenin-labeled RNA probes specific to GluR2 coding region (pan GluR2) or the long 3′ UTR reveal the tissue distribution of GluR2 transcripts in CA1, CA3 and DG regions of hippocampus in control rats (A and B), and pilocarpine-treated animals that experienced status epilepticus 24 hr before (E and F). The arrows in panels E and F indicate increased signal associated with the long 3′UTR of GluR2 in the CA2 region after pilocarpine. The sense probes hybridized to the tissues from the control (C and D) and SE animals (G and H) show only background staining.
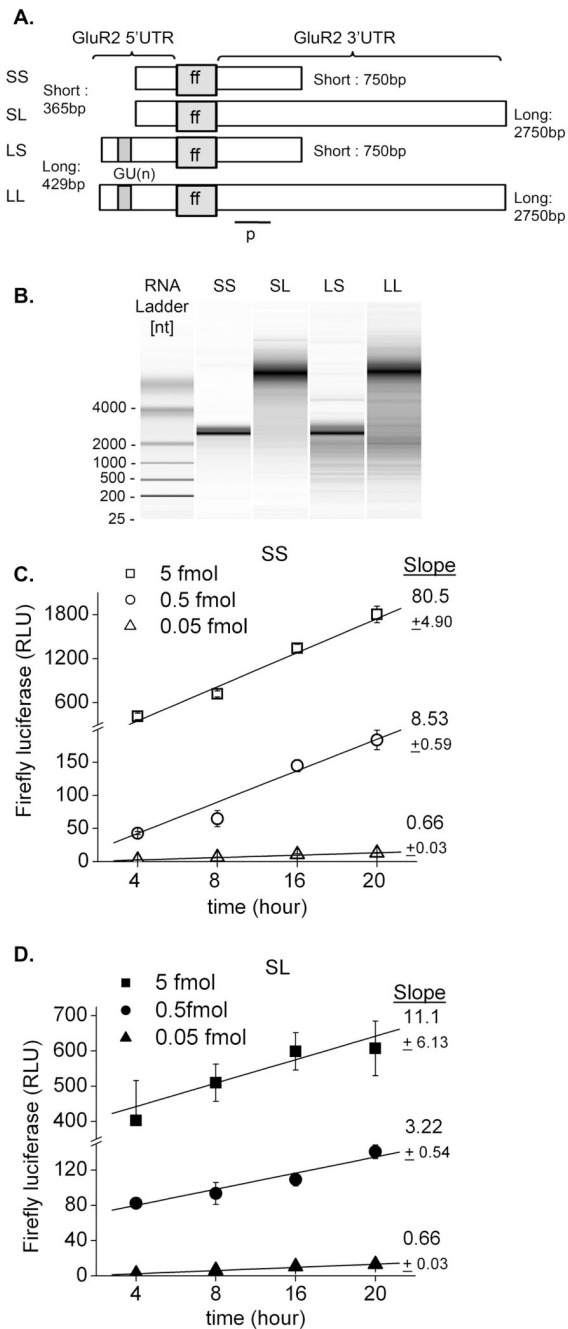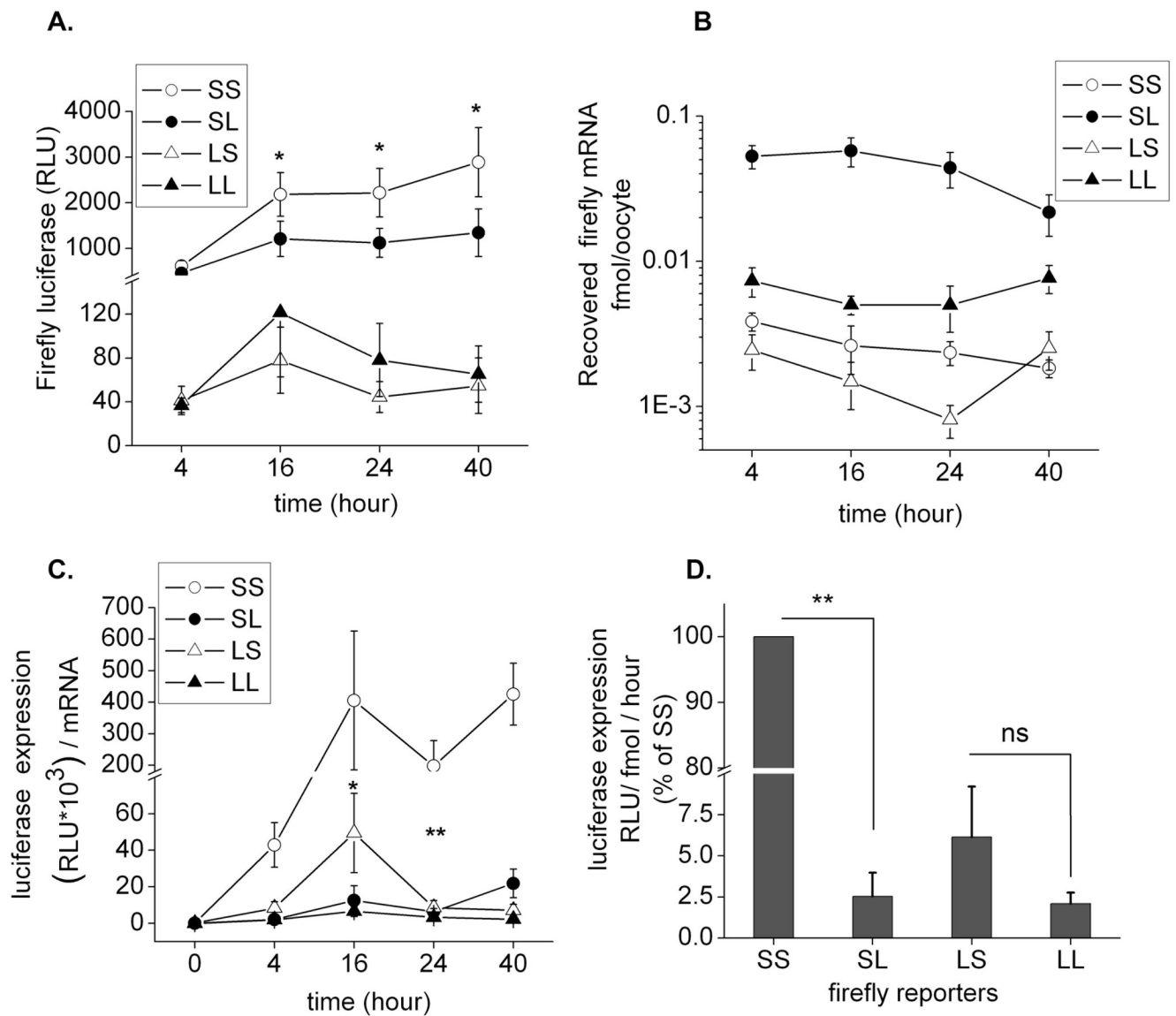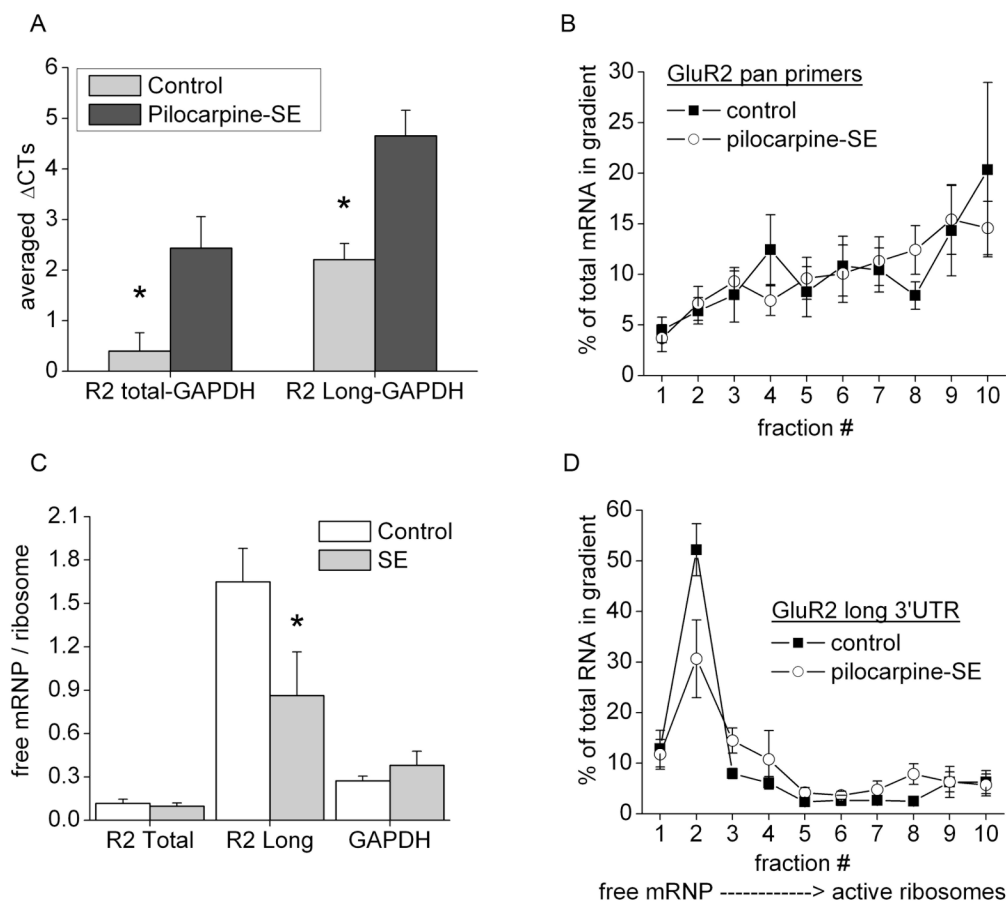
Figure 4.
Translation of reporter mRNAs bearing alternative GluR2 UTRs. A. Schematic of firefly luciferase reporters bearing alternative combinations of GluR2 5′ and 3′ UTRs. Gray boxes indicate the position of GU repeats on the long 5′UTRs (Myers et al., 2004), and the firefly coding region (ff) common to all constructs. The primers (p) used in Q-RT-PCR recognize all four species of reporter mRNA. B. The quality and amount of each in vitro transcribed reporter mRNA were evaluated with an Agilent bioanalyzer. The expected size of the transcripts is SS: 2760; SL: 4710; LS: 3140; and LL: 5110 bp. C and D, Luciferase activity was proportional to both mRNA amount and time after injection into Xenopus oocytes, for the SS and SL populations of mRNA.

Figure 5.
Expression profile of firefly reporter mRNAs bearing alternative GluR2 UTRs in *Xenopus* oocytes. A, Individual Xenopus oocytes were microinjected with reporter mRNAs (5 fmol/oocyte) and incubated at 17°C. At indicated time points after injection, the oocytes were individually homogenized and firefly reporter expression was detected as recorded luminescence units (RLU). *p<0.05 ANOVA, post-hoc Bonferroni, comparing SS with SL at 16h, 24h and 40h. B, Recovered reporter mRNAs from microinjected-oocytes were quantified by Q-RT-PCR using UTR-specific primers and known quantities of cDNA standards. The apparent increase in LS mRNA levels between 24 and 40 hours was not statistically significant (ANOVA, post-hoc Bonferroni). C, Expression of firefly luciferase protein presented as luciferase activity per fmol mRNA recovered from oocytes [n=10-15 oocytes for each time point from each of five different animals, *p< 0.05, **p< 0.001 comparing SS with SL, ANOVA, pot-hoc Bonferroni. D, Rate of luciferase expression from 4 to 16 hours after injection presented as percent of SS expression, **p< 0.001 ANOVA, post-hoc Bonferroni, comparing SS vs SL, ns (no significant difference) compared for LS vs LL.

Figure 6.
Effects of pilocarpine-induced status epilepticus (SE) on native GluR2 transcripts. A. The levels of mRNAs are inversely proportional to the average number of PCR cycles needed to reach detection threshold (CT). The difference between the CT values (ΔCT) of GluR2 transcripts and that of the GAPDH mRNA in both control and SE animals indicates that the levels of both pan-GluR2 mRNA, and GluR2 bearing the long 5′UTR, are reduced by pilocarpine-induced SE (N=8, *P<0.01, ANOVA, Post hoc Bonferroni). B-D. To determine the effects of pilocarpine-induced SE on the association of GluR2 mRNAs with ribosomes in rat hippocampus, polyribosome association of pan GluR2 (B) and GluR2 bearing long 3′UTRs (D) was examined over a sucrose gradient assay. (C) native GluR2 transcripts recovered from free mRNP (fraction 1 and 2) and active ribosomes (fraction 3 to 10) are quantified. The ratio of RNA levels in ribosome-free mRNP and ribosome-containing fractions is shown (n=6, * p< 0.01, ANOVA, post-hoc Bonferroni).