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Hasan Ali Irier _____

Date

Role of 3' untranslated regions in translation regulation of GluR2 mRNAs

By

Hasan Ali Irier

Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Pharmacology

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M.S., Georgia State University, 2002

B.S., Ankara University, 1997

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An abstract of

A dissertation submitted to the Faculty of the Graduate School of Emory University

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ABSTRACT

Role of 3’ untranslated regions in translation regulation of GluR2 mRNAs

Hasan Ali Irier

GluR2 expression is regulated at the transcription level by cell-specific transcription factors that target the promoter, and GluR2 is also subject to translational control by the GU repeats residing in the long 5' untranslated region (UTR). In this study, the translational regulation of GluR2 mRNAs by alternative 3’UTRs was explored. GluR2 mRNAs exist as two major GluR2 transcripts of 6 kb and 4 kb, differing only in the length of their 3’UTRs (~2750 bp or “long” and ~750 bp or “short”, respectively) in rats and mice. Both short and long GluR2 mRNAs are abundantly expressed in CA1 and CA3 pyramidal neurons, and dentate granule cells (DG). Pilocarpine-induced status epilepticus (SE) significantly reduced GluR2 mRNA levels in CA1 and CA3 but not DG. In *Xenopus* oocytes, the expression profiles of luciferase reporters bearing alternative GluR2 5’ and 3’ UTRs were studied. In the absence of long 5’UTR, which contains translation repressor elements, the long 3’UTR serves as a translational suppressor for GluR2 transcripts. In rat hippocampus**,** the majority of endogenous GluR2 transcripts exhibited strong association with polysomes, which is indicative of active translation, whereas GluR2 transcripts bearing long 3’UTRs were associated with ribosome-free ribonucleoprotein complexes. A de-repression of translation of GluR2 mRNAs bearing long 3’UTRs after prolonged seizures was observed. The mechanism of the long 3’UTR mediated translation repression was studied using the luciferase reporter mRNAs bearing alternative GluR2 UTRs in rabbit reticulocyte lysates treated with translation elongation inhibitors and translation initiation modulators. Translation of the reporter mRNAs bearing the long GluR2 3’UTR was insensitive to low concentrations of the elongation inhibitors cycloheximide and anisomycin, in contrast to a reporter bearing the short 3’UTR, which was inhibited, suggesting that initiation is the site of translation regulation for GluR2 mRNAs bearing the long 3’UTRs. The translation initiation modulator kasugamycin selectively induced the expression of reporter mRNAs bearing either of the long UTRs of GluR2. These findings overall suggest that GluR2 transcripts have distinct translation patterns due to alternative 5’ and 3’UTRs. The mechanisms of UTR-mediated translation regulation present potential targets for therapeutic modulation of GluR2 expression in a transcript-specific manner.

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It's been a wonderful journey!

This dissertation is dedicated to

my wife Lorraine Irier,

and

my parents, Gulbahar & Nuri Irier

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid
ANOVA	analysis of variance
ARE	AU rich element
ARC	activity-regulated cytoskeleton-associated protein
BDNF	brain derived neurotropic factor
bp	base pairs
BSA	bovine serum albumin
CA1	cornu ammonis 1
CA2	cornu ammonis 2
CA3	cornu ammonis 3
CaMKII	calcium/calmodulin-dependent protein kinase II
CNS	central nervous sytem
CPE	cytoplasmic polyadenylation element
CPEB	ctyoplasmic polyadenylation element binding protein
CPSF	cytoplasmic polyadenylation specific factor
DMSO	dimethyl sulfoxide
dNTPs	deoxynuclotide triphophates
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
eIF2	eukaryotic initiation factor 2
eIF4A	eukaryotic initiation factor 4A
eIF4B	eukaryotic initiation factor 4B
eIF4F	eukaryotic initiation factor 4F
eIF4G	eukaryotic initiation factor 4G
EPSC	excitatory postsynaptic currents
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GDP	guanosine diphosphate
GRIP	glutamate receptor interacting protein
GTP	guanosine triphosphate
H3	histone 3
H4	histone 4
i.p.	intraperitoneal
IRE	iron response element
IRES	internal ribosome entry site/segment
KA	kainic acid (or kainate)
mRNA	messenger RNA
NMDA	N-methyl-D-aspartate
NRF1	nuclear respiratory factor 1
NRSE	neuron restrictive silencer element
PABP	poly-(A) binding protein
PAP	poly-(A) polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PICK1	protein interacting with C kinase 1
Q/R editing	glutamine/arginine editing
Q-RT-PCR	quantitative real time PCR
RE1	repressive element 1
REST	RE1-silencing transcription factor
RNase	ribonuclease
RPM	revolution per minute
RT-PCR	reverse transcription polymerase chain reaction
SE	status epilepticus
Sp1	specific protein1

tRNA	transfer RNA
UTR	untranslated region

Chapter I. BACKGROUND & SIGNIFICANCE

A. Glutamate Receptors: Classification & Biological Functions

Almost 60 years ago, the excitatory actions of the amino acid glutamate were first suggested by Hayashi et al. (Hayashi, 1954), who observed convulsions in dogs and monkeys that had been injected with low concentrations of sodium glutamate into their motor cortex. Five years later, Curtis et al. (Curtis et al., 1959) discovered that direct application of glutamate on cat spinal cord evoked repetitive discharges from all types of interneurons. Starting from the early 1970s, the identification of multiple glutamate receptors and the discovery of associated selective antagonists have provided further evidence for glutamate as a neurotransmitter and the biological function of its receptors (Watkins and Jane, 2006).

Based on molecular biological and pharmacological studies, two major glutamate receptor families were recognized: those which cause slower synaptic responses and biochemical changes through second messenger production, called metabotropic glutamate receptors (mGluR) (Schoepp et al., 1999), and those which mediate fast synaptic responses via ion channels causing rapid and large changes in membrane conductance, called ionotropic glutamate receptors (iGluR) (Dingledine et al., 1999). Excessive activation of glutamate receptors is implicated in cell death and/or neurodegeneration by increasing the intracellular Ca2+ levels in neurons, a process that leads to activation of proteases,

phospholipases and endonucleases, and generation of free radicals (Coyle and Puttfarcken, 1993).

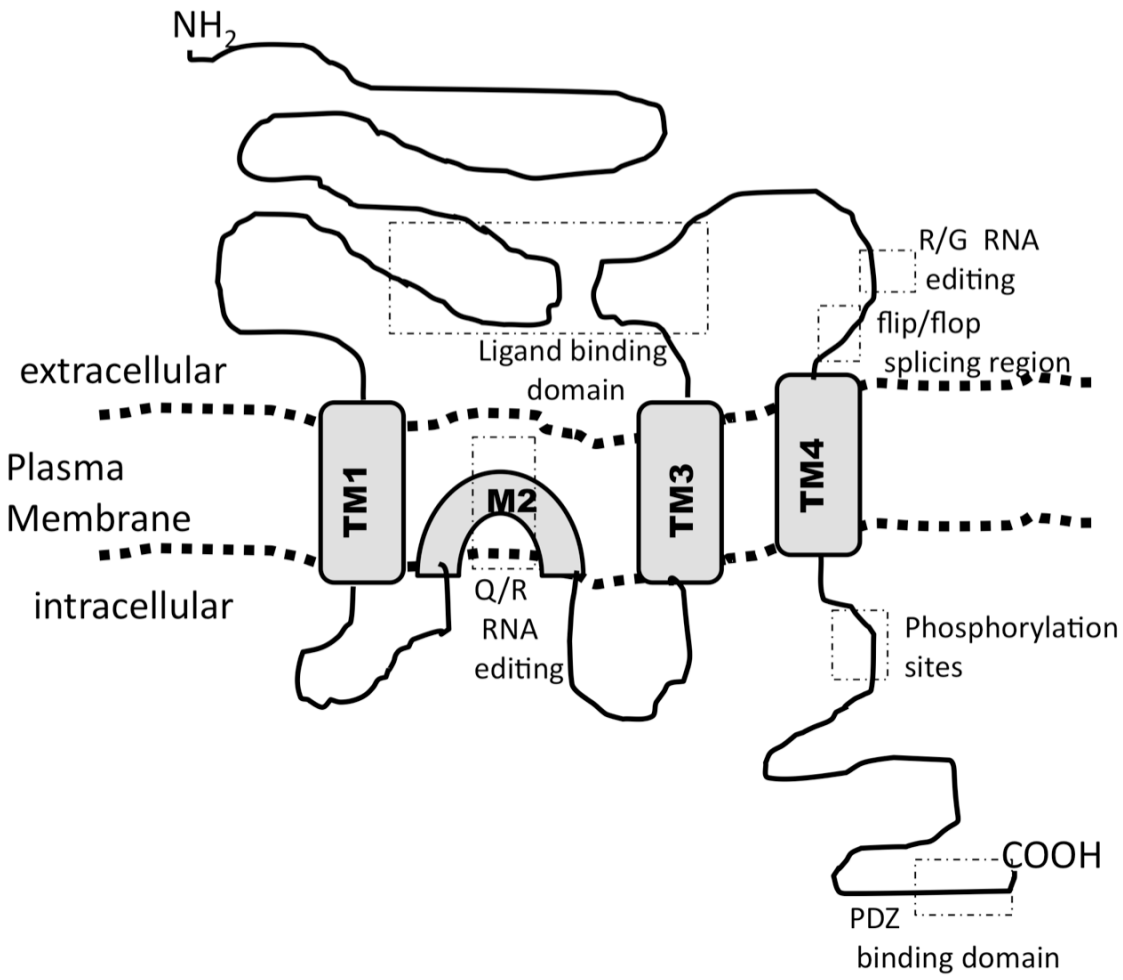
a. Ionotropic Glutamate Receptors.

The ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels that mediate the majority of the fast excitatory synaptic transmission in the central nervous system (CNS). The glutamate released into the synaptic cleft binds to iGluRs and allows Na^+ and Ca^{2+} ions to pass through a channel formed near the center of the receptor. This influx of ions into the neuron results in depolarization of the plasma membrane and generation of an electric current that is transmitted through the membrane and spreads to the processes of the neighboring neurons in the CNS. Over the last 20 years, 17 different genes have been identified that encode protein subunits for functional iGluRs (Gasic and Hollmann, 1992; Myers et al., 1999; Nakanishi and Masu, 1994; Wisden and Seeburg, 1993b). Assembly of these 17 subunits into functional tetrameric receptors constitutes the major families of iGluRs, which were originally named based on their pharmacological responses to selective agonists: (*i*) N-methyl-D-aspartate (NMDA) receptors (NR1, NR2A, NR2B, NR2C, NR2D, NR3A and NR3B subunits; recently nomenclature replaced NR with GluN), (*ii*) α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) receptors (GluR1, GluR2, GluR3, GluR4 subunits), and (*iii*) 2-carboxy-3-carboxymethly-4-isopropeny;pyrorolidine (Kainate) receptors (GluR5, GluR6, GluR7, KA1 and KA2 subunits, recently named as GluK1-5). In addition to these functionally defined receptor classes, GluR delta receptors constitute a fourth subfamily, based on their sequence

homology to the ligand binding domains of iGluRs receptors, though whether the delta receptors function as ion channels remains unresolved (Lomeli et al., 1993; Schmid and Hollmann, 2008). Native receptors of all subfamilies of iGluRs are thought to be assembled from tetramers typically comprising more than one type of subunit. Even though the average overall amino acid identity among the NMDAR, Kainate, and AMPA receptors is about 30 %, all three have common structural features that put them under a single superfamily. Each iGluR subunit has an extracellular amino terminal domain followed by a first transmembrane domain (TM1), and then a pore-forming domain that resides in the membrane as a re-entrant loop to the cytoplasm. The second and third transmembrane domains (TM2 and TM3) are linked by a large extracellular loop, and TM3 is followed by an intracellular carboxy terminus (Dingledine et al., 1999). The agonist binding domain is located in a pocket formed between two extracellular regions (Mayer and Armstrong, 2004) **(Figure I-1).**

Figure I-1. Schematic illustration of an AMPA receptor subunit. Each subunit contains a large extracellular N-terminal domain, four hydrophobic transmembrane domains (TM1-4), and an intracellular C-terminal domain. The ligand binding site is a region between the N-terminus and a loop that links TM3 to TM4. This loop also contains R/G editing and “flip” or “flop” alternative splice regions. An intracellular loop between TM1 and M2 participates in forming a cation pore channel and also the Q/R RNA editing site in the GluR2 subunit. The intracellular C-terminus contains several phosphorylation and protein interaction sites such as PDZ domain-containing proteins.

Fig. I-1


NH2
R/G RNA
editing
flip/flop
splicing region
Ligand binding
domain
extracellular
Plasma
Membrane
intracellular
TM1
M2
TM3
TM4
Q/R
RNA
editing
Phosphorylation
sites
COOH
PDZ
binding domain

b. Ionotropic glutamate receptors in health and disease

Given that glutamate is the most abundant neurotransmitter in the brain, the great density and diversity of the ionotropic glutamate receptors (iGluRs) in the central nervous systems (CNS) are expected. Under physiological conditions, activation of iGluRs by endogenous glutamate produces diverse responses in neuronal cells. These responses form the basis for neurotransmission and synaptic plasticity, which are essential in the normal functioning of the CNS. Thus, it is not surprising that iGluRs are increasingly discovered to be associated with many disorders affecting the CNS, and are implicated in numerous other disease states targeting the nervous system. I will briefly summarize the role of iGluR subfamilies in the healthy brain and then present a few examples of the role played by iGluRs in extensively studied disorders and disease states.

NMDA receptors (NMDARs) mediate the slow component of the glutamatergic excitatory postsynaptic current (EPSC), and are involved in other physiological and pathophysiological events in the CNS (Dingledine et al., 1999). Activation of NMDA receptors requires binding of both glutamate (to the NR2A-D subunits) and the co-agonist glycine (to the NR1 subunit) to receptors for the opening of the ion channel (Anson et al., 1998; Kleckner and Dingledine, 1988; Kuryatov et al., 1994; Laube et al., 1997). Because of their high Ca^{2+}-permeability, NMDARs are essential in triggering Ca^{2+}-dependent signaling events in the cytoplasm (MacDermott et al., 1986). NMDARs also function as mediators of coincidence detection and synaptic efficacy in which a correlated pre- and post-synaptic activity produces long-term changes in the synaptic strength (Magee and

Johnston, 1997; Markram et al., 1997; Stanton and Sejnowski, 1989). Overstimulation of NMDARs in neurons can lead to Ca^{2+}-overload (Frandsen and Schousboe, 1992; Lei et al., 1992) and subsequent cell death, which are implicated in neuronal insults such as stroke (Goldberg and Choi, 1990), or in neurodegenerative conditions a such as Alzheimer's and Parkinson's Disease (Koutsilieri and Riederer, 2007).

AMPARs, often found co-localized with NMDARs, are widely expressed in the CNS and involved in neurotransmission and synaptic plasticity mechanisms (Bekkers and Stevens, 1989). They mediate the majority of the post-synaptic response at most fast excitatory synapses in the CNS (Dingledine et al., 1999). The subunit composition of AMPARs is important in determining the Ca^{2+}-permeability of the ion channel. Heteromeric AMPARs containing edited GluR2 subunits have been shown to have low Ca^{2+} permeability (Geiger et al., 1995; Washburn et al., 1997). Because of their abundance and functional diversity, AMPARs are involved in numerous neurodevelopment disorders such as Fragile-X syndrome (Hu et al., 2008; Li et al., 2002; Pilpel et al., 2009), schizophrenia (Akbarian et al., 1995; Dracheva et al., 2008; Eastwood et al., 1997; Zavitsanou et al., 2008), neurodegenerative conditions such as Alzheimer's disease (Chappell et al., 2007; Jansen et al., 1990), motor neuron diseases such as Amyotrophic Lateral Sclerosis (ALS) (Andries et al., 2007; Couratier et al., 1994; Zhao et al., 2008), stroke (Koistinaho et al., 1999; Soundarapandian et al., 2005), and neuronal damage associated with ischemia (Oguro et al., 1999) and epilepsy (Dingledine et al., 1990; Higuchi et al., 2000).

Kainate receptors (KARs) are also widely expressed in the brain, though less abundant compared to the AMPARs, and mostly appear to be involved in modulating synaptic neurotransmission (Castillo et al., 1997; Vignes and Collingridge, 1997; Wisden and Seeburg, 1993a). Heteromeric or homomeric assemblies of GluR5, GluR6, GluR7, KA1 and KA2 subunits form functional KARs, except that homomeric assemblies of KA1 and KA2 subunits are incapable of producing functional channels (Herb et al., 1992). Potential roles for KARs have been suggested in CNS disorders such as Huntington's Disease and temporal lobe epilepsy (Coyle, 1987; MacDonald et al., 1999), memory loss (Ko et al., 2005), and neuropathic pain (Sutton et al., 1999).

c. Identification and Physiological function of AMPA receptors

Ionotropic glutamate receptors showing greater selectivity to a synthetic glutamate analog α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) (Honore et al., 1982) are pharmacologically classified as AMPA receptors (AMPARs) (Wisden and Seeburg, 1993b) though they are also, to a lesser extent, responsive to kainate and quisqualate (Shinozaki and Ishida, 1981). Cloning of the first AMPAR subunit, GluR1 (Hollmann et al., 1989), greatly intensified the study of recombinantly expressed AMPARs for their distinct properties. To date, four distinct genes, *gria1, gria2, gria3* and *gria4,* have been shown to express four distinct subunits of AMPARs, GluR1, GluR2, GluR3 and GluR4, respectively. Each subunit comprises ~ 900 amino acids and has a molecular weight of ~150kDa (Rogers et al., 1991). There is approximately 70% homology among the genes encoding for AMPAR subunits. Post-transcriptional

modifications of individual subunits add to the diversity of functions. All subunits of AMPARs undergo alternative splicing at the extracellular region prior to the TM4-terminal domain, generating “flip” and “flop” forms (Monyer et al., 1991; Sommer et al., 1990). The functional consequence of this is that AMPARs with flip forms desensitize four times more slowly than those with flop splice variant (Mosbacher et al., 1994). The expression of each form is region- and cell-type-specific, is developmentally regulated, and presents a different sensitivity to allosteric modulators such as 4-[2-(phenylsulfonyamino) ethylthio)]-2, 6-difluoro-phenoxyacetamide, cyclothiazide (Hagino et al., 2004; Sekiguchi et al., 1998), or zinc (Shen et al., 2000). In addition to the flip/flop splice variants, AMPAR subunits GluR1, GluR2 and GluR4 can also be alternatively spliced at the intracellular C-terminus, which generates “long” and “short” isoforms. While the short isoform of GluR2 is the most abundant subunit (90% of total GluR2 protein), GluR4 long isoform is the predominant form in rat hippocampus (Gallo et al., 1992; Kohler et al., 1994). The GluR3 subunit does not have alternative isoforms due to the absence of an appropriate splicing site. The presence of the alternative isoforms of AMPAR subunits greatly contributes to the kinetic properties of the channel as well as intracellular protein-protein interactions.

AMPARs are thought be formed by tetrameric or pentameric assemblies of their subunits, which vary with the stage of development, cell type, and brain region (Rosenmund et al., 1998; Song and Huganir, 2002). AMPARs are widely expressed in the CNS, in which specific neurons and glia show selective expression of AMPARs composed of different subunit combinations (Gallo and

Russell, 1995; Jonas et al., 1994; Martin et al., 1993). In hippocampus, for example, GluR4 is mainly expressed in early stages of development while GluR1, GluR2 and GluR3 expression increases with the development (Zhu et al., 2000). The AMPARs are also found in peripheral sites, such as the heart, pancreas, postganglionic sympathetic neurons, and enteric ganglia (Bertrand et al., 1992; Carlton et al., 1998; Gill et al., 1998; Liu et al., 1997).

Depending on the subunit composition, AMPARs allow influx of Na^+, and Ca^{2+} ions in response to the ligand binding. In the mammalian CNS, AMPAR-mediated Na^+ influx causes the majority of the postsynaptic response at most of the fast excitatory synapses (Dingledine et al., 1999). AMPARs are critical for NMDA receptor activation. At normal membrane potentials, the NMDAR channel is blocked by Mg2+; however, when the repeated activation of AMPARs by glutamate produces a sustained postsynaptic depolarization, the Mg^{2+} block of NMDAR is relieved and the NMDAR-activation allows Ca^{2+} entry into the cell (Mayer et al., 1984; Nowak et al., 1984).

Dynamic cycling of the AMPARs localized precisely at the glutamatergic postsynaptic terminals is determined by neuronal activity (e.g. NMDA-receptor activation) (Craig et al., 1994; Man et al., 2000), structural motifs in the C-terminus domains, and a host of signaling and scaffolding proteins that interact with the C-terminus domain (Kim and Sheng, 2004; Palmer et al., 2005; Shi et al., 2001). For example, AMPARs are clustered in the post synaptic density (PSD) of synapses by the interaction of closely associated intracellular proteins known as TARPs (transmembrane AMPAR regulatory proteins) and PSD-95 (El-

Husseini et al., 2000; Nicoll et al., 2006). Interactions between TARPs and PSD-95 are critical for removal or insertion of the synaptic AMPARs, which in turn contribute to the long-term potentiation (LTP) and depression (LTD) of synaptic strength in excitatory synapses (Bhattacharyya et al., 2009; Harms et al., 2005; Hayashi et al., 2000). Phosphorylation of AMPAR subunits is a major factor in the dynamin-dependent endocytosis of the AMPARs, during which redistribution of the subunits into and out of synapses occurs in an activity-dependent manner (Barria et al., 1997; Luscher et al., 1999).

Overstimulation of the synaptic AMPARs by excessive release of glutamate in acute (e.g. trauma, stroke, and epilepsy) or chronic (e.g. PD and ALS) neurological disorders is thought to contribute to neurodegeneration or cell death. Several AMPA receptor antagonists have been shown to provide neuroprotection under experimental conditions, though the therapeutic applications of these antagonists are limited due to adverse side effects (Catarzi et al., 2007).

d. Significance of GluR2 subunits in functional AMPA receptors.

Each AMPA receptor subunit (GluR1, GluR2, GluR3 and GluR4) has a channel-lining re-entrant hairpin loop (M2) that contains a glutamine/arginine (Q/R) site, named after the amino acid that normally resides in that position (see Figure I-1). The GluR2 subunit plays a critical role in determining AMPA receptor function, because the 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; Sommer et al., 1991). Thus, ion permeation properties of functional

AMPA receptors are defined by the relative abundance of edited GluR2 subunits (Washburn et al., 1997). In particular, three properties of the ion channels are strongly reduced by the presence of edited GluR2 subunit in the functional AMPAR; (i) Ca^{2+}-permeability of the ion channel (Geiger et al., 1995; Hollmann et al., 1991; Verdoorn et al., 1991), (ii) single channel conductance (Burnashev et al., 1992; Swanson et al., 1997), and (iii) voltage dependent channel blocking by endogenous intracellular polyamines and by polyamine toxins (Bowie and Mayer, 1995; Kamboj et al., 1995; Koh et al., 1995; Washburn et al., 1997).

GluR2 subunits constitute the majority of heteromeric AMPARs in the mammalian central nervous system (Wenthold et al., 1996). In specific mammalian brain regions such as the hippocampus and cerebral cortex, the GluR2 along with GluR1 is the predominantly expressed subunit compared to GluR3 and GluR4 (Craig et al., 1993; Jia et al., 1996; McBain and Dingledine, 1993; Tsuzuki et al., 2001). A number of scaffolding proteins known to interact with GluR2 subunits are also involved in these processes. The glutamate receptor interacting protein 1 (GRIP1), a PDZ domain containing scaffolding protein found in PSD, interacts with the C-terminus of GluR2 (and GluR3) subunits, and this interaction is required for the dendritic expression of GluR2 in hippocampal neurons (Dong et al., 1997; Guo and Wang, 2007; Hoogenraad et al., 2005). The continuous cycling of GluR2-containing AMPARs between non-synaptic and synaptic sites is regulated by the interactions between N-ethylmaleimide-sensitive factor (NSF) and GluR2 subunits, which in turn determine the surface receptor numbers (Nishimune et al., 1998; Noel et al.,

1999; Shi et al., 2001; Song and Huganir, 2002). PICK, a protein that interacts with protein C kinase 1, binds to the GluR2 subunit (along with a number of membrane proteins) via its PDZ domain, and is required for the internalization of AMPARs from the synaptic plasma membrane in response to NMDA receptor activity (Dixon et al., 2009; Hanley and Henley, 2005; Hanley et al., 2002; Terashima et al., 2008). In addition to the trafficking proteins that interact with the GluR2 subunit, the state of the Q/R editing at the M2 domain of GluR2 subunit also determines the dimerization and ER exit of the GluR2 subunits from the endoplasmic reticulum. For example, the unedited GluR2 subunits can form tetramers and be trafficked to the membrane surface to form Ca^{2+}-permeable AMPA receptors (Greger et al., 2006; Greger et al., 2003; Greger et al., 2002). Overexpression of the edited GluR2 subunits in the CA1 region of the rat hippocampus protects pyramidal neurons in that region from ischemia-induced cell death (Liu et al., 2004). The GluR2 subunit is a crucial target in the activity-dependent AMPAR trafficking (Carroll et al., 2001) and in determinant of abundance and assembly of the functional AMPA receptors, as well as synaptic plasticity (Collingridge et al., 2004; Jia et al., 1996) under physiological conditions. Thus, any changes in the expression level of GluR2 subunits are expected to have significant physiological consequences.

GluR2 expression is regulated at the transcription level by the conserved regulatory elements located at the promoter regions, and at the translation level by the 5' and 3' untranslated regions (UTRs) of mRNAs (Huang et al., 1999; Irier et al., 2009; Myers et al., 1999; Myers et al., 2004). As a prelude to the function

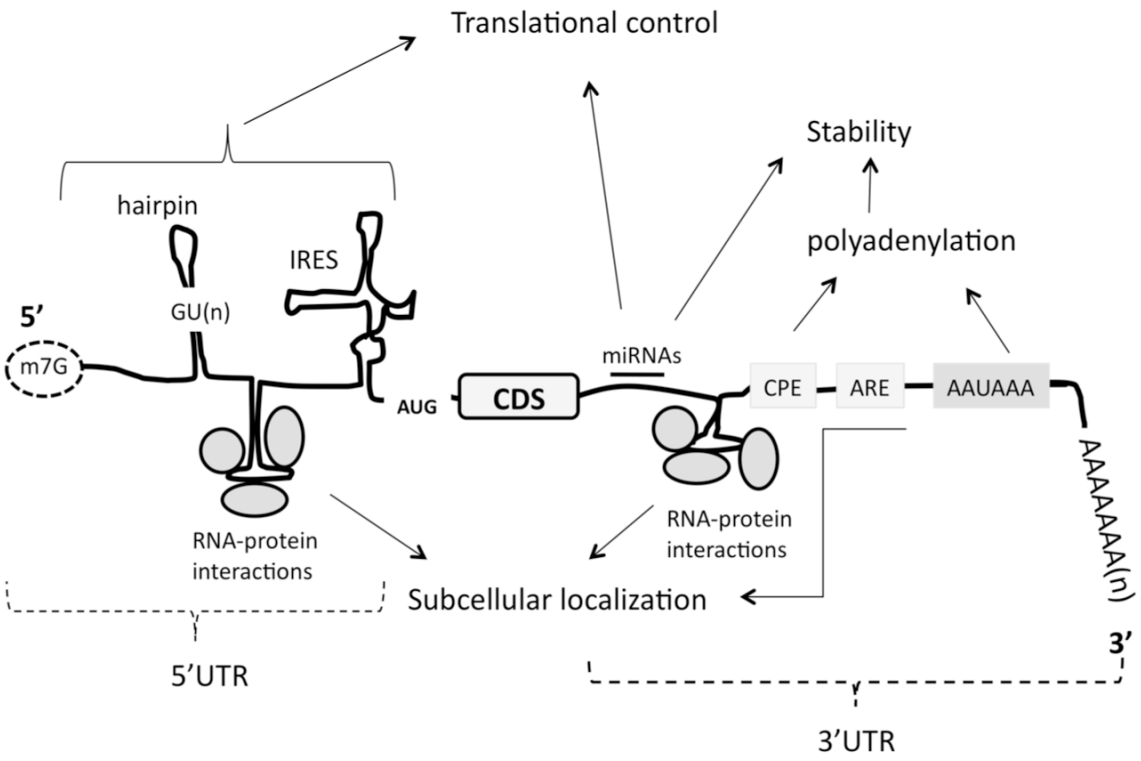
of UTRs in GluR2 mRNA translation regulation, a brief overview of eukaryotic mRNA translation is presented, and well studied examples of UTR-mediated translation regulation of other mRNAs are presented in the following sections B and C.

B. Role of untranslated regions of mRNA in RNA processing.

Although it is a complex and multistep process, regulation of gene expression can be categorized in two major steps: (*i*) transcription control, in which cis-acting DNA elements such as promoters, enhancers, and silencers modulate the transcription process to produce mature mRNAs, and (*ii)* post-transcriptional regulation of mRNA, in which cis-acting RNA elements located in 5'- and 3'- untranslated regions (5'UTRs, and 3'UTRs) and trans-acting factors such as proteins or micro RNAs(miRNAs) mediate the processing, intracellular transport, stability, and translation of mRNAs (Conne et al., 2000; Gao, 1998; Sonenberg, 1994) (Figure I-2).

Figure I-2. Illustration of some of the post-transcriptional regulatory elements and structures on a eukaryotic mRNA untranslated region (UTR). 5’ UTR may include the methylated guanosine cap (m7G), hairpin structures formed in GU-rich regions, internal ribosomal entry sites/segments (IRES), and RNA-interacting proteins. 3’UTRs may include miRNA targets involving multiple interacting proteins, cytoplasmic polyadenylation elements (CPE), AU-rich elements (ARE), alternative AAUAAA polyadenylation signals, and a poly(A) tail. (Adapted from Pesole et al., 2001, with modifications)

Fig.I-2


Translational control
Stability
polyadenylation
hairpin
IRES
GU(n)
5'
m7G
AUG
CDS
miRNAs
CPE
ARE
AAUAAA
AAAAAA(n)
3'
RNA-protein interactions
RNA-protein interactions
Subcellular localization
5'UTR
3'UTR

The genesis of mature mRNAs starts with the recognition of DNA by specific transcription factors and RNA polymerases that target specific regions of DNA which are abundant in regulatory elements such as promoters, silencers, and enhancers. A concerted effort of these transcription factors and RNA polymerases produces pre-mRNA molecules, which then undergo further modifications. The introns are removed, the 5'm7GpppN (where N is any nucleotide) cap structure (Shatkin, 1976) and 100- 250 bases-long adenine residues [poly (A) tail] (Manley, 1983; Manley and Levine, 1985) are added at the 3'end, which itself is formed by endonucleolytic cleavage of pre-mRNA(Nevins and Darnell, 1978). Resulting mature mRNAs then can undergo further modifications, collectively known as "RNA editing," which involve alterations, additions, or deletions of nucleosides (Simpson and Thiemann, 1995). The final product is a mature mRNA with three distinct regions: (i), a 5' untranslated region (UTR) that is a stretch of bases between the cap and start (AUG) codons, (ii) a coding region, and (iii) a 3'UTR that is a stretch of bases between the stop codon and poly(A) tail (Figure I-2).

a. 5' untranslated regions of mRNAs

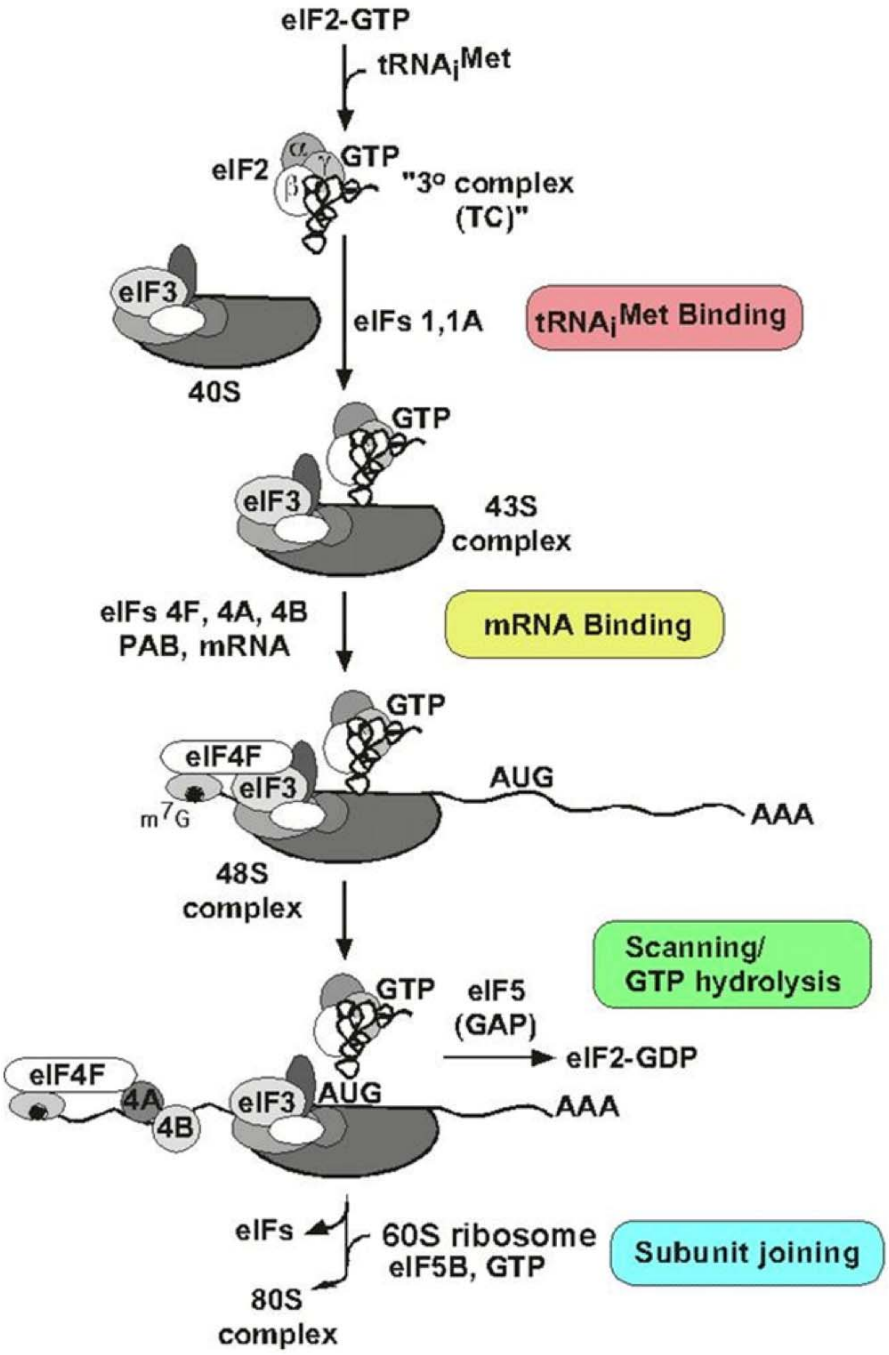
Studies in the early1970s demonstrated that the mRNAs from higher organisms contain modified structures such as 5' terminal m^7Gppp (cap structure) and 3' terminal poly(A), (Adams and Cory, 1975; Brawerman, 1974), and that the size of mRNAs exceeds the size required to code for protein (Proudfoot and Brownlee, 1976). These findings led to further interest in deciphering the structure of mRNAs and their function in protein synthesis, and laid the foundation for

studying the molecular biology of eukaryotic mRNA translation. The “scanning model” of eukaryotic mRNA translation was originally postulated in 1980, and is still accepted as a main paradigm today (Kozak, 1980; Kozak, 1989). Since then, significant advances have been made in deciphering the molecular mechanisms and protein factors involved in the process. Currently the eukaryotic mRNA translation is categorized into four consecutive steps: initiation, elongation, termination, and ribosome recycling. Figure I-3 summarizes the known steps in the translation initiation of a eukaryotic mRNA. The translation of mRNA is initiated once the 43S preinitiation complex is formed by interaction of 40S ribosomal subunits with Met-tRNAi. This preinitiation complex then binds to the 5’ cap structure of the mRNA, by a process facilitated by translation factor complex eIF4; begins to scan through the 5’UTR; and unwinds the secondary structure until it meets the first qualified AUG codon of the mRNA placed on the P (peptidyl) site of the ribosome. Once the start signal is recognized, 60S ribosome (the large subunit) joins 40S ribosome (the small subunit) to form the final 80S initiation complex, which is ready to accept appropriate aminoacyl-tRNA into the A (aminoacyl) site on the ribosome (Cheung et al., 2007; Pestova and Kolupaeva, 2002; Sonenberg and Hinnebusch, 2009). The next step is the elongation of the nascent peptide, during which a sequential and rapid addition of amino acid residues to the COOH-end of the nascent peptide occurs. In addition to the cap-dependent translation initiation, which the majority of the eukaryotic mRNAs undergo, there are also cap-independent translations of mRNAs in which internal ribosomal entry segments (IRES) located in 5’UTRs are utilized for

alternative initiation of translation (van der Velden and Thomas, 1999). However, the proposed molecular mechanisms of the IRES-driven alternative initiations in eukaryotic mRNAs remain unresolved (Baird et al., 2006; Dmitriev et al., 2007; Kozak, 2005b).

Figure I-3. A summary of general eukaryotic translation initiation pathway. An early step in the translation initiation pathway is the binding of Met-tRNA$_i^{Met}$ to the 40S ribosomal subunit in a ternary complex (TC) composed of Met-tRNA$_i^{Met}$ GTP and eIF2. The recruitment of TC to 40S subunits is promoted *in vitro* by eIF1, eIF1A and the eIF3 complex resulting in the 43S pre-initiation complex. Interaction of mRNA with the 43S pre-initiation complex (48S complex) is stimulated by eIF4F (eIF4A-eIF4E-eIF4G), poly(A)-binding protein, and eIF3, and the 48S complex scans the mRNA until the Met-tRNA$_i^{Met}$ base-pairs with an AUG triplet. AUG recognition triggers GTP hydrolysis by eIF2 in a reaction stimulated by eIF5, and the eIF2-GDP and other eIFs are ejected from the ribosome. The eIF1, eIF1A, and eIF4G regulate the scanning process *in vitro* (Pestova and Kolupaeva, 2002). In the final reaction, eIF5B bound to GTP promotes joining of the 60S subunit with the 40S- Met-tRNA$_i^{Met}$-mRNA complex to produce the 80S initiation complex (copied from www.biomed.cas.cz/mbu/lrge/research.html with permission).

Fig. I-3


eIF2-GTP
tRNAiMet
eIF2
GTP
"3° complex
(TC)"
eIF3
eIFs 1,1A
tRNAiMet Binding
40S
GTP
eIF3
43S
complex
eIFs 4F, 4A, 4B
PAB, mRNA
mRNA Binding
GTP
eIF4F
eIF3
AUG
AAA
m7G
48S
complex
Scanning/
GTP hydrolysis
GTP
eIF5
(GAP)
eIF2-GDP
eIF4F
4A
4B
eIF3
AUG
AAA
eIFs
60S ribosome
eIF5B, GTP
Subunit joining
80S
complex

Once the mRNA translation is initiated, the efficiency of translation can be influenced by specific properties of 5'UTRs, such as GC content, the length of the 5'UTR, and the location of upstream initiation codons (uAUG usually precedes the start AUG in the open reading frame). In most cases, mRNAs bearing shorter 5'UTRs without any secondary structures or uAUG are translated more efficiently than mRNAs bearing long and/or structured 5'UTRs (Kochetov et al., 1998). Translation of mRNAs bearing structured 5'UTRs (e.g. β-globin) are thought to be restricted because these mRNAs require ATP and helicase activity for an efficient binding of 43S pre-initiation complex at the cap and the subsequent scanning through the structured 5'UTR (Kozak, 2005a; Pestova and Kolupaeva, 2002; Pisareva et al., 2008). For example, secondary structures and alternative start codons(uAUGs) in the full length 5'UTR of NR2a (a subunit of NMDA receptors) causes significant repression on NR2A mRNAs *in vitro* and *in vivo* (Wood et al., 1996). Similarly, the secondary structures and uAUGs of the 5'UTR of protein kinase C mRNAs are involved in the translation regulation (Morrish and Rumsby, 2001). Moreover, the long 5'UTRs of GluR2 mRNAs that contain GU repeats which are predicted to form a stable secondary structure, cause translation repression (Myers et al., 2004), as will be discussed in detail in section C of this chapter. In some cases the secondary structures in the 5'UTRs of mRNAs can lead to mRNA decay by post-termination ribosomes, thereby determining the stability of the mRNA (Vilela et al., 1999). Proteins that bind to the 5'UTRs can also interrupt the ongoing translation initiation process. A well-studied example of this interruption is the binding of iron response element (IRE)-

binding proteins to the IRE on the 5'UTR of ferritin mRNA, which blocks interaction between 43S pre-initiation complex and the cap-associated translation initiation factors (Muckenthaler et al., 1998). Mutations in 5'UTRs can also result in disruption of translation regulation. For example, 5'UTR of c-myc (a proto-oncogene) contains an internal ribosome entry segment (IRES) that allows cap-independent translation of c-myc mRNA; however, a single mutation in the IRES can result in significant increase in the expression of c-myc (Chappell et al., 2000; Nanbru et al., 1997). More examples of mRNA translation regulation at the initiation level by RNA binding proteins, secondary structures, and positioning of AUG codons in the 5'UTRs are being discovered (Jackson and Wickens, 1997; Sonenberg and Hinnebusch, 2009).

b. 3'untranslated regions of mRNA

A recent computational analysis of a large UTR database suggested that while the average length of 5'UTRs remains consistent in organisms ranging from fungi to higher plants, the average length of the 3'UTRs increases with the complexity of the organism (Mignone et al., 2002; Pesole et al., 2001). Compared to 5'UTRs, the 3'UTRs are intrinsically more diverse in their structural and functional features. In addition to translation initiation, they are also utilized in the mRNAs processing steps, such as polyadenylation, stability, subcellular localization, and miRNA-induced RNA degradation. The 3'UTRs should be essential in post-transcriptional regulation, because they are positioned between a termination codon and poly(A) tail. With the exception of some histone mRNAs, almost all eukaryotic mRNAs contain poly(A) tails added post-transcriptionally at the 3'end

(Brawerman, 1974). Although the exact mechanism of the 3'end formation remains unknown, it has been well established that in most eukaryotic mRNAs, the presence of highly conserved AAUAAA elements in the 3'UTR and of the interacting proteins CPSF (cleavage and polyadenylation specificity factor) and CstF (cleavage stimulating factor) are all required for efficient 3'-end processing, in which pre-mRNA is cleaved and subsequently polyadenylated in the nucleus (Colgan and Manley, 1997; Keller, 1995; Proudfoot and Brownlee, 1976; Wahle and Kuhn, 1997; Zhao et al., 1999). The number and positioning of AAUAAA elements harbored on a 3'UTR are important for alternative polyadenylation of the 3'end, which in turn may result in production of multiple RNA transcripts with subtle differences, such as the length of 3'UTRs, even though they code for the same protein (Lutz, 2008). In the nucleus, polyadenylation is carried out by poly(A) polymerase (PAP), which adds up to ~250 adenine nucleotides to the 3'end of mRNA molecule, though determining the final length of poly(A)-tails requires some additional protein factors such as nuclear poly(A)-binding protein II (PABPN1) (Wahle, 1991; Wahle, 1995). The length of the poly(A) tail, however, may further be regulated by specific protein factors targeting conserved elements embedded in the 3'UTR during or after the transport of mature mRNA to the cytoplasm. In *Xenopus leavis* oocytes, for example, the cytoplasmic polyadenylation machinery, which includes but is not limited to cytoplasmic element (CPE) binding proteins (CPEBs), CPSF, symplekin, and Gld-2, collectively facilitates the activation of translationally dormant mRNAs during oocyte maturation (or after fertilization) by increasing the length of poly(A) tails

(Gorgoni and Gray, 2004; Hake and Richter, 1994; Radford et al., 2008). In the cytoplasm, there is a host of 3'UTR-interacting proteins that play important roles in stability, transport and translation of mature mRNAs. For example, studies on mRNAs regulated by cytoplasmic polyadenylation, such as c-mos, Cdk2, cyclinB1, and Wee1 mRNAs (in *Xenopus*), and αCAMKII and Arc mRNAs (in mice), concluded that the presence of a conserved CPE element (UUUUUAU) and its variations in the 3'UTRs are required for CPEB function in cytoplasm (Charlesworth et al., 2000; Gebauer et al., 1994; Mendez et al., 2000; Sheets et al., 1994; Stebbins-Boaz et al., 1996). Neuronal counterparts of CPEB proteins (CPEB3 and CPEB4) also regulate trafficking and translation of mRNAs via 3'UTR-interactions; however, direct recognition of the CPE elements (or its variants) by neuronal CPEB proteins does not seem to be a requirement for all mRNAs which contain the CPE elements (Du and Richter, 2005; Huang et al., 2003; Huang et al., 2002b; Huang et al., 2006; Theis et al., 2003). In neurons, the 3'UTRs of some mRNAs are used for trafficking of mRNAs from soma to dendrites. For example, while activity dependent trafficking of αCAMKII mRNA to the dendrites requires presence of full length 3'UTR (Rook et al., 2000), a cis-acting dendritic targeting element (DTE) within the initial fragment of Arc mRNAs 3'UTR is sufficient to provide strong trafficking activity (Kobayashi et al., 2005). Instead of having one dominant 3'UTR, some mRNAs species exist as distinct mRNA populations differing only in length of the 3'UTRs. For example, brain-derived neurotrophic factor (BDNF) mRNAs are produced with either a short or long 3'UTR, though both still encode for the same proteins in the rat brain

(Timmusk et al., 1993). Only the BDNF mRNAs bearing a long 3'UTR are targeted to dendrites (An et al., 2008), and most likely serve as a template for local translation upon synaptic activity (Bramham and Wells, 2007). Another interesting feature of 3'UTRs is their involvement in the circularization of mRNA by translation initiation factors. The circularization, or "closed loop" structure, of eukaryotic mRNAs was first proposed after the observation that poly(A)-binding protein (PBAP) in yeast also interacts with a translation initiation factor eIF4G, which in turn interacts with cap-binding translation factor eIF4E, essentially bringing 5' and 3'UTRs into a closed loop structure (Tarun and Sachs, 1996). The proposed circularization of mRNA was also visualized by reconstituting purified translation initiation factors, PBAP and mRNA, *in vitro,* and viewing the closed loop with atomic force microscopy (Wells et al., 1998). It is thought that circularization of mRNAs facilitates translational silencing (Mazumder et al., 2003; Sampath et al., 2003). Apart from translation regulation of mRNAs by 3'UTR-interacting proteins, there are also small (20-25 nucleotide) micro RNAs (miRNAs) (e.g. *let-7* and *lin-4*) that imperfectly bind to the 3'UTRs of their target mRNAs thereby repressing translation or initiating RNA degradation process (Bagga et al., 2005; Maroney et al., 2006). The miRNA-mediated degradation seems to target mRNAs that are already translationally repressed (Wu et al., 2006). Findings that let-7 miRNA controls translation by specifically inhibiting 5'-cap binding process and poly(A) tail function (Humphreys et al., 2005; Mathonnet et al., 2007) suggest that circularized mRNAs are most likely the preferred targets for miRNAs.

Specific mutations in 3'UTRs are linked to diseases such as Myotonic dystrophy (DM), which occurs as a result of an expanded number of trinucleotide (CTG) repeats in the 3'UTRs of a cAMP-dependant protein kinase gene (DMPK)(Timchenko, 1999). Deletions of AU-rich elements(AREs) from the 3'UTRs of some genes as a result of nonrandom chromosomal alterations results in shortening of the half-life of wild-type mRNAs such as CCND1 (a member of Cyclin G1 gene family)(Rimokh et al., 1994).

The structure and features of 5'and 3' UTRs in an mRNA provide a myriad of possibilities for translational control in response to cellular signals that can be tailored to specific transcripts. Thus 5' and 3'UTRs play a role in translational regulation that is indispensable to cell viability, growth, and response to environmental signals. The GluR2 mRNAs bear alternative combinations of 5' and 3'UTR, differing in the length of UTRs. The role of these alternative UTRs in translation regulation and mRNA stability is discussed in the following sections.

C. Molecular Control of GluR2 subunit expression

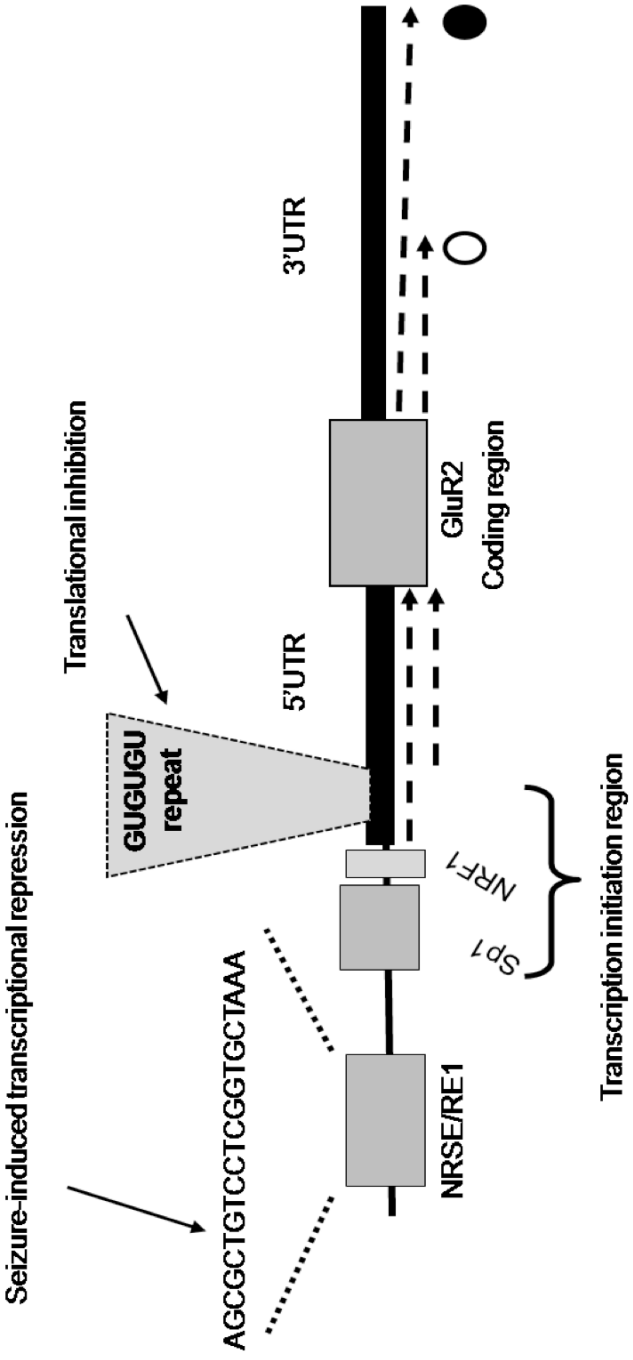
a. Transcriptional regulation of GluR2 expression

The organization of mouse GluR2 gene (*Gria2*) structure was first described by Kohler et al, (Kohler et al., 1994), and the functional evaluation of its promoter region was studied by Myers et al. (Myers et al., 1998). These studies on the GluR2 promoter revealed several features that are largely shared among by other ionotropic glutamate receptors (Myers et al., 1999), including high GC content, the presence of multiple transcriptional initiation sites, positive

and negative transcriptional regulatory elements such as Sp1 and NRF1, and neuron-restricted silencer RE1/NRSE elements (Figure III-4). Transcription of GluR2 has been shown to start at the TATA-less promoter region, where the strongest initiation site is located between -429 and -431bps from the 5' end and to the translation initiation codon. GluR2 promoter activity is regulated by histone deacetylases in neuronal cultures, and negatively influenced by the transcription factor REST/NRSF, which targets RE1/NRSE elements located at the GluR2 promoter in non-neuronal cells (Huang et al., 1999; Myers et al., 1998). Moreover, organizational and structural features of GluR2 promoter may determine the efficacy of the transcriptional regulation of GluR2 gene expression, as indicated by findings that the downregulation of GluR2 transcription after seizure in rats is mediated by rapid deacetylation of H3 and H4 histones that are associated with the GluR2 promoter (Huang et al., 2002a).

Figure I-4. Schematic of known transcriptional and translational regulation on GluR2expression. NRF, nuclear respiratory factor; NRSE, neuron specific silencer element

Fig. I-4


Seizure-induced transcriptional repression
AGCGCTGTCCTCGGTGCTAAA
NRSE/RE1
SP1
NRF1
Transcription initiation region
5'UTR
GUGUGU repeat
Translational inhibition
GluR2 Coding region
3'UTR

b. Translation regulation of GluR2 expression

The populations of GluR2 transcripts *in vivo* (in rat and mouse brains) have been shown to contain multiple 5'UTRs with different lengths, the longest being 481 bases from the translation initiation codon (Kohler et al., 1994; Myers et al., 1998). Significant discoveries have been made on the translation regulation of GluR2 mRNAs in our laboratories within the last decade. Myers et al. showed that the majority of the GluR2 transcripts contain alternative 5'UTRs ranging from 310 to 429 bases in length, and that translation of GluR2 mRNAs bearing long 5'UTRs (-429 bases from the translation initation codon) was significantly repressed due to the presence of a polymorphic repeat (GU) found only in the long 5'UTRs of GluR2 **(Figure I-4)** (Myers et al., 2004)**.** Using reticulocyte lysates *in vitro* and Xenopus oocytes and cultured neurons *in vivo*, Myers et al. demonstrated that the GluR2 transcripts exhibit different translation efficiencies resulting from the presence or absence of a short polymorphic sequence (GU repeat) in the long 5'UTR of GluR2 transcripts (Myers et al., 2004). These findings were further supported by demonstrating that the GluR2 mRNAs bearing the short, but not long, 5'UTRs were associated with polyribosomes in the rat brain homogenates, indicating that the translation of GluR2 mRNAs bearing a long 5'UTR is repressed. The mechanisms of this repression remain unclear. However, modeling of the long 5'UTR structure by an RNA-fold algorithm (FoldRNA) predicted a structured hairpin loop. Based on the energy requirements of the proposed Kozak model of 5'capped-translation initiation (Kozak, 1989), the predicted secondary structure in the long 5'UTR of GluR2

mRNAs appears to be energetically stable enough to stop ribosome scanning during translation initiation. Myers et al. showed for the first time that the long 5'UTRs of GluR2 mRNAs are targets for a strong translation repression mechanism tailored to distinct GluR2 mRNAs (Myers et al., 2004). However, the role of alternative 3'UTRs in the translation regulation has not been addressed in these studies because the reporter mRNAs used in these studies were designed to contain only a single short (~700bases) 3'UTR.

D. The goals for my thesis research

The GluR2 subunit determines several properties of functional AMPA receptors including the Ca^{2+}-permeability, channel conductance, and cell surface expression, all of which will be affected by the changes in the expression of GluR2. In addition to transcription control and post-translational modifications, translation regulation is used as an alternative mechanism to regulate GluR2 expression in neurons. Previous studies investigating the regulations of GluR2 expression revealed that distinct GluR2 mRNA populations bearing alternative combinations of 5' and 3'UTRs are generated in the brain and that the length and structural features of 5'UTRs determine the disposition of translation regulation. However, the mechanisms underlying the translation regulation by long 5'UTRs, and the role of 3'UTRs in such regulations, remain unknown. In this thesis research, the alternative 3'UTRs of GluR2 mRNAs were investigated for their role in the translation regulation, cellular distribution, and mRNA stability under normal physiological conditions using *in vivo* and *in vitro* translation assays, and during or after seizures in rat hippocampus.

CHAPTER II. Materials and Methods

A. Materials

Adult male Sprague Dawley rats 40-50 days of age were purchased from Charles River Labs (Wilmington MA, USA). Female frogs (*Xenopus laevis*) were purchased from Xenopus Express (Brooksville, FL, USA). Rabbit reticulocyte lysate were purchased from Applied Biosystems (Foster City CA, USA). Pilocarpine HCl, (-) scopolamine methyl nitrate, terbutaline hemisulfate, Trizol, EDTA, DMSO, Triton-X, ETOH, Cycloheximide (Cat #s 01811) and Anisomycin (A-9789) were purchased from Sigma (Sigma Aldrich, St Louis, MO, USA). Kasugamycin was purchased from Biomol International (Plymouth Meeting, PA, USA). DMDA-PatA was kindly made available to us by Dr. Daniel Romo at Department of Chemistry, Texas A&M University. Formamide and Isopropyl alcohol were purchased from Fluka Biochemika (Buchs, Switzerland).

Restriction enzymes were purchased from New England Biolabs (Beverly, MA). T4 DNA ligase, T4 DNA and Klenow polymerases, the pSP-Luc+ cloning vector, Passive Lysis Buffer, Luciferase substrate LARII were purchased from Promega (Madison, WI, USA). Microlite 96-well white flat bottom plates were purchase from Thomas Scientific (Swedesboro, NJ, USA).

B. Methods.

a. Preparation of GluR2 3'UTR constructs. Four firefly luciferase reporter constructs were designed to evaluate the role of alternative GluR2 5' and 3'UTRs in 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 the remaining 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 (a region 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.

b. Reporter expression and RNA stability in Xenopus oocytes. Reporter cDNA constructs bearing alternative GluR2 5' and 3' UTRs were linearized at the 3'-end using the *BstE* II restriction site [see Fig. III-6, designated SS for Luciferase protein coding region (ff) if flanked by Short 5'UTR and Short 3'UTR of GluR2; SL, if flanked by Short 5'UTR and Long 3'UTR of GluR2; LS if flanked by Long 5'UTR and Short 3'UTR of GluR2; and LL, if flanked by Long 5'UTR and Long 3'UTR of GluR2)]. *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 was 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.

c. In vitro translation and RNA stability of reporters in rabbit reticulocyte lysates (RRL).

In vitro synthesis of 5'-capped mRNAs from the linearized reporter constructs was performed as previously described above. Briefly, 5' capped mRNAs were synthesized from the linearized reporter constructs using T3 RNA polymerase following the instructions provided in the T3 mMESSAGEmMACHINE (Ambion, Austin, TX, USA), and the *in vitro* synthesis of reporter mRNAs without a 5'cap structure was performed using T3 RNA polymerase following the instructions provided in the T3 MEGAScript (Ambion, Austin, TX, USA). The resulting mRNAs were dissolved in RNAse-free water, quantified and quality checked in the RNA Nano Chip apparatus using 2100 Agilent Bioanalyzer (Agilent, Waldbronn, Germany). Small aliquots of 100uM reporter mRNA stocks were stored in a -80^{0}C freezer.

For *in vitro* translation of reporter mRNAs, commercially available rabbit reticulocyte lysates were used following the instructions provided with Retic Lysate IVT^{TM} (Ambion, Austin, TX, USA). A reticulocyte lysate translation assay mix was assembled in a 0.5 µl thin-walled PCR reaction tube on ice, and the components were added in the following order; drug (or vehicle), 34 µl rabbit reticulocyte lysate, 20X Translation Mix(-met), 1mM methionine, 0.8Unit RNase inhibitor, reporter mRNA, and RNase-free water to a final 50ul translation

reaction mix. The following antibiotics/translation inhibitors were mixed with reticulocyte lysates (34ul) and incubated on ice for 5-10min prior to adding the remaining components of the final translation mix. Cycloheximide (CHX) was dissolved in RNase-free water and used at final concentrations of 70, 7 and 0.7nM. Anisomycin (ANS) was dissolved in DMSO, and used at concentrations of 750, 75 and 7.5nM. Kasugamycin was dissolved in RNAse-free water, and used at final concentrations of 460, 4.6 and 0.46nM. DMDA-PateamineA was dissolved in DMSO, and used at concentrations of 10 to 0.001uM. The final translation mix was incubated at 30^0C over 40 min using a digitally monitored heat block. A 5ul sample was removed by pipetting from the final translation, mixed at 10 min intervals, and flash frozen in a 96-well plate (Microlite white flat bottom plates, Thomas Scientific, Swedesboro, NJ, USA) that was placed on dry-ice. The luciferase activity, defined as relative light units (RLU) measured at 570nm, from the individual wells was measured using the luciferase assay substrate (Dual-Luciferase Reporter Assay System, Promega). The RLU from the 5 µl reticulocyte samples without a reporter mRNAs (0.05 to 0.5 RLU) constituted background, which was consistent for each 96-well plate used. The lowest experimental sample RLUs at 20 min were at least 100-fold higher than that of the background.

To determine reporter mRNAs stability, each firefly reporter mRNA was extracted from the rabbit reticulocyte lysates at time 0 and 40 min using a standard phenol-chlorophorm method. The extracted mRNA was dissolved in 20ul *in vitro* reverse transcription reaction that contained Thermoscript Reverse Transcriptase

(Invitrogen, Carlsbad, CA,USA), and the reaction was run for 50min at 50^0C. The resulting cDNAs were quantified by quantitative real time PCR analysis (SyberGreen Supermix, Applied Biosystems) using primer sets that amplified UTR-specific regions along with standard dilutions of the luciferase reporter cDNA.

d. 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 X *g* for 10 min, then at 13,000 X *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 X *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.

e. Quantitative Real Time PCR analysis of reporter and native GluR2 mRNAs. Reporter mRNAs isolated from Xenopus oocytes and Rabbit reticulocyte lysates, 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 templates 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 95^0C and 30 sec at 63^0C. 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.

f. 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 X g, which was followed by a second spin at 13,000 X g for 30 min at 4^0C. 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.

g. 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).

h. 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 ^{0}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^{0}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 ^{0}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 ^{0}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.

i. 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., 2002a). 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.

CHAPTER III: Translational regulation of GluR2 mRNAs in rat hippocampus by alternative 3' untranslated regions.*

(This chapter was previously published on Feb 07 2008 as a research article in Journal of Neurochemistry)*

A. 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 is 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.

B. 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 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., 2002a), 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 **(see Fig.I-4 in Chapter I)** (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 (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., 2003). 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.

C. Results

a.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 **(Figure III-1).** 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 **(Figure III-2).** 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. 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).

Figure III-1. 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).

Fig. III-1

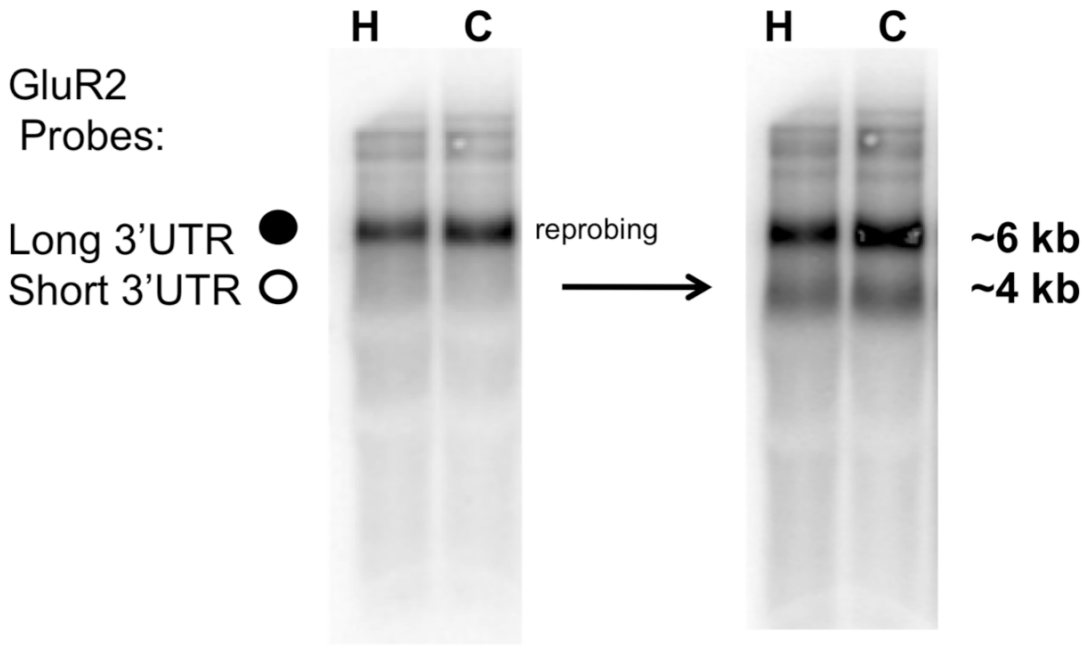
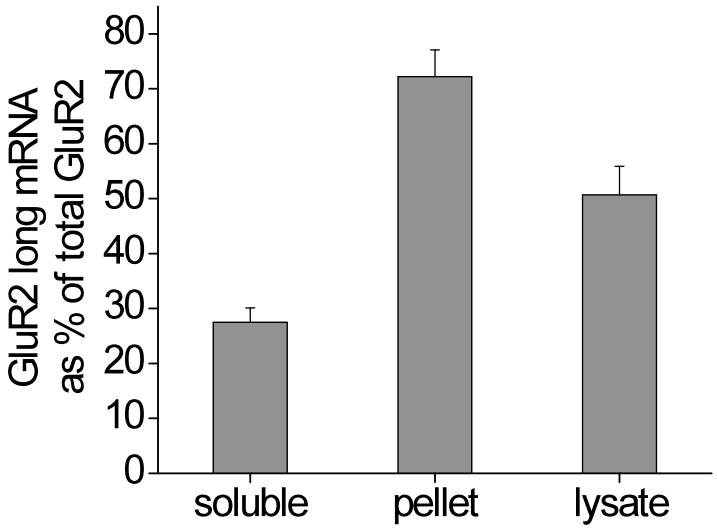


Figure III-2. Subcellular distribution of native GluR2 transcripts in rat hippocampus; supernate and pellet fractions of detergent-treated hippocampal homogenates were analyzed by Q-RT-PCR for their native GluR2 mRNA content and compared to that of untreated total lysate from the hippocampus of same animals.

Fig. III-2

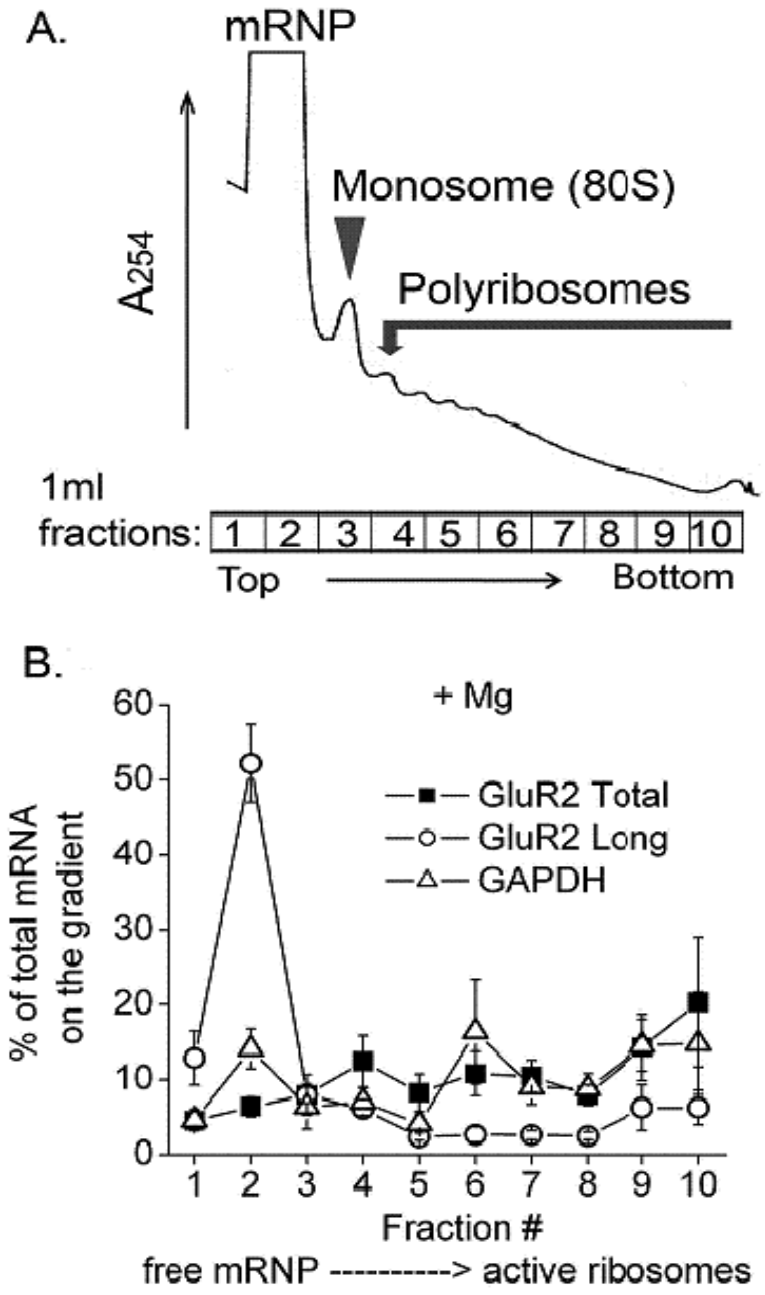

GluR2 long mRNA
as % of total GluR2
80
70
60
50
40
30
20
10
0
soluble
pellet
lysate

b. 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 paradigm, ribosomal particles were 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) determined the density of translating polysomes. 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 **(Figure III-3**). 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 **(Figure III-3).**

Figure III-3. Association of native GluR2 transcripts with ribosomes on a sucrose gradient. Fractionation of cytoplasmic extracts of rat hippocampus on a linear sucrose gradient (15-45 %) revealed ribosome-free mRNPs (fractions 1 and 2), 80S monosome peak (fraction 3) and translating polyribosomes (fractions 4 to 10). 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).

Fig. III-3


A.
mRNP
Monosome (80S)
Polyribosomes
A254
1ml
fractions: 1 2 3 4 5 6 7 8 9 10
Top
Bottom
B.
+ Mg
GluR2 Total
GluR2 Long
GAPDH
% of total mRNA on the gradient
0
10
20
30
40
50
60
1 2 3 4 5 6 7 8 9 10
Fraction #
free mRNP ----------> active ribosomes

As expected, when translating polysomes in the cytoplasmic lysate were disrupted by EDTA treatment **(Figure III-4**), pan GluR2 mRNAs as well as glyceraldehyde-3-phosphate deydrogenase (GAPDH) mRNAs was largely recovered in the ribosome-free mRNPs on the linear sucrose gradient **(Figure III-4).** 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 (**Figure III-5A).** The distribution pattern of GluR2 mRNAs bearing long 3'UTRs in these regions was similar to that of pan GluR2 (**Figure III-5B).** The sense probes hybridized to the tissues from the same animals showed only background signal **(Figure III-5C and 5D).**

c. 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 **(Figure III-6).** In vitro synthesized 5'-capped mRNAs from the reporter constructs were quality checked and

Figure III-4. Treatment of the lysate with EDTA, an 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.

Fig. III-4

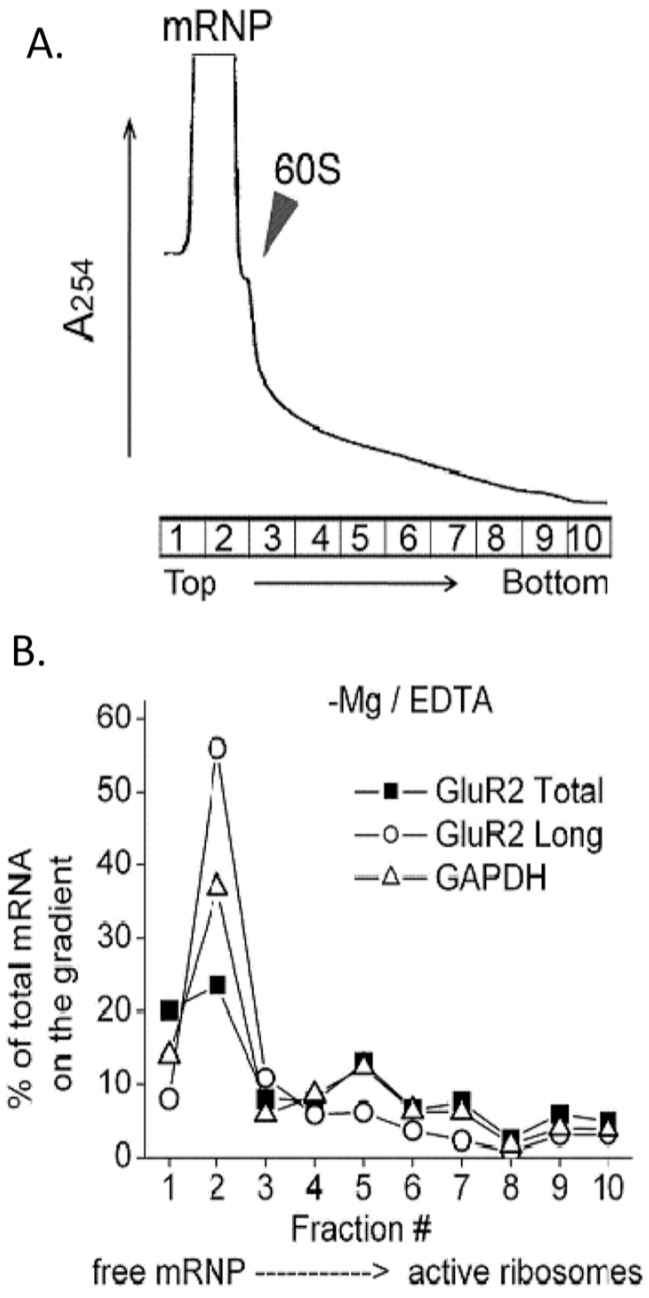

A.
mRNP
60S
A254
1 2 3 4 5 6 7 8 9 10
Top
Bottom
B.
-Mg / EDTA
GluR2 Total
GluR2 Long
GAPDH
% of total mRNA on the gradient
Fraction #
free mRNP ----------> active ribosomes

Figure III-5. 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.

Fig. III-5

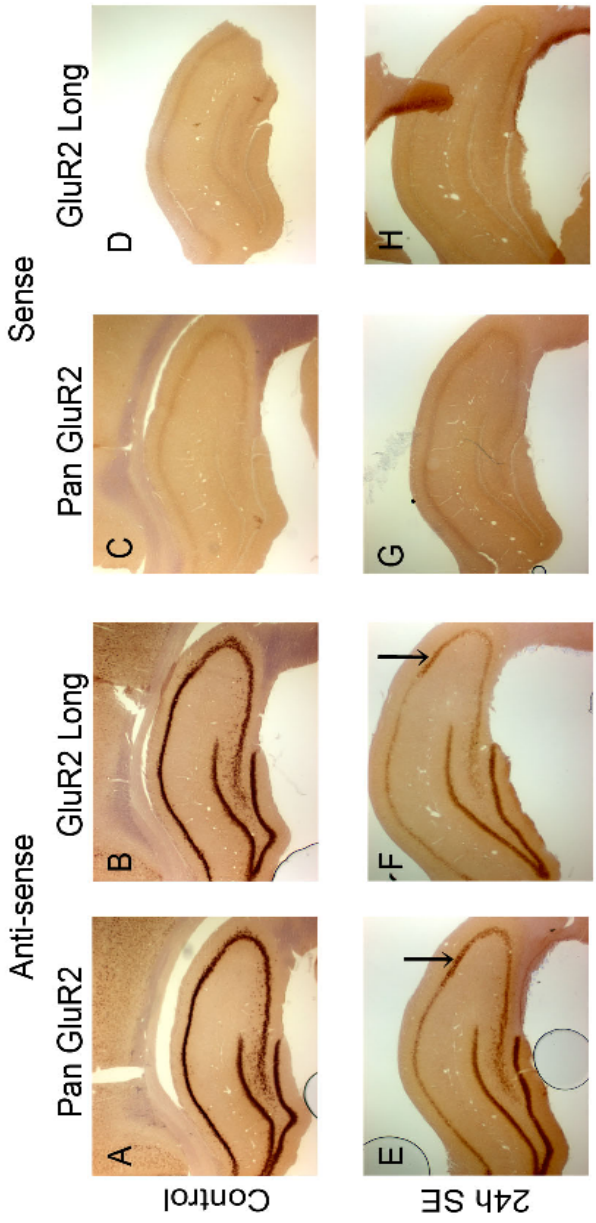
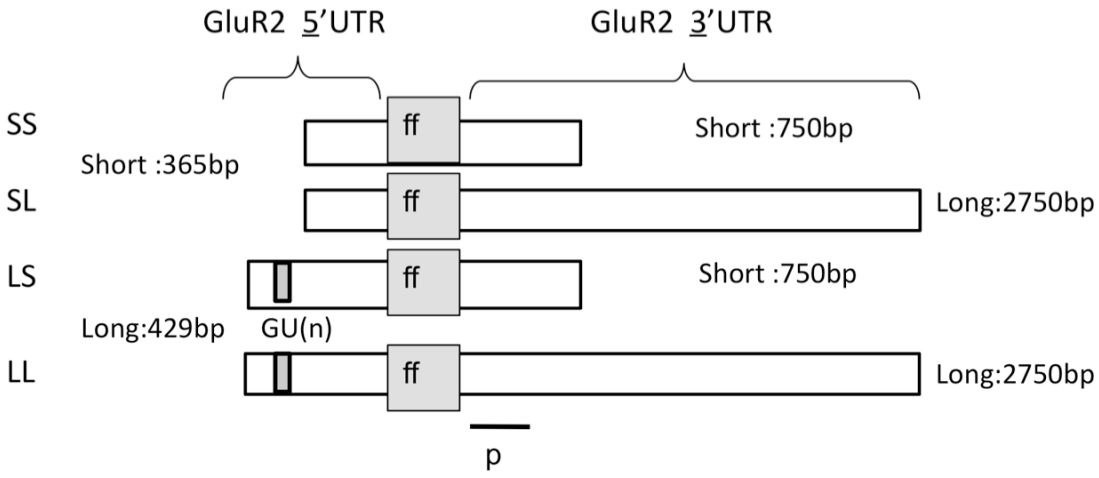

Anti-sense
Sense
Pan GluR2
GluR2 Long
Pan GluR2
GluR2 Long
Control
24h SE
A
B
C
D
E
F
G
H

Figure III-6 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.

Fig. III-6


GluR2 5'UTR
GluR2 3'UTR
SS
Short :365bp
ff
Short :750bp
SL
ff
Long:2750bp
LS
ff
Short :750bp
Long:429bp
GU(n)
LL
ff
Long:2750bp
p

the RNA amount measured with the Agilent mRNA Nano bioanalyzer **(Figure III-7)**. 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 III-8 and -9). Figure III-10** shows that the long 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 **(Figure III-11),** 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 III-11)**. 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 **(Figure III-12).** This was particularly evident with constructs bearing the short 5'UTR (cf SS with SL in **Figure III-12**). 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.

d. 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.

Figure III-7 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.

Fig. III-7

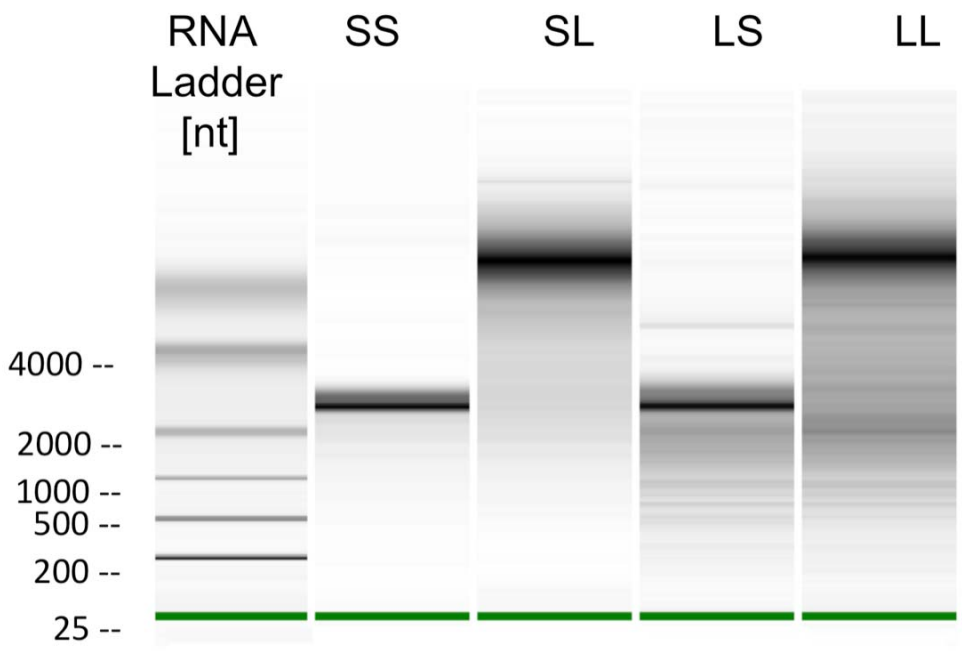


Figure III-8 Activity of Luciferase expressed from the reporter mRNAs bearing short 5’ and short 3’UTRs of GluR2 was proportional to both mRNA amount and time after injection into Xenopus oocytes. for the SS and SL populations of mRNA.

Figure III-8

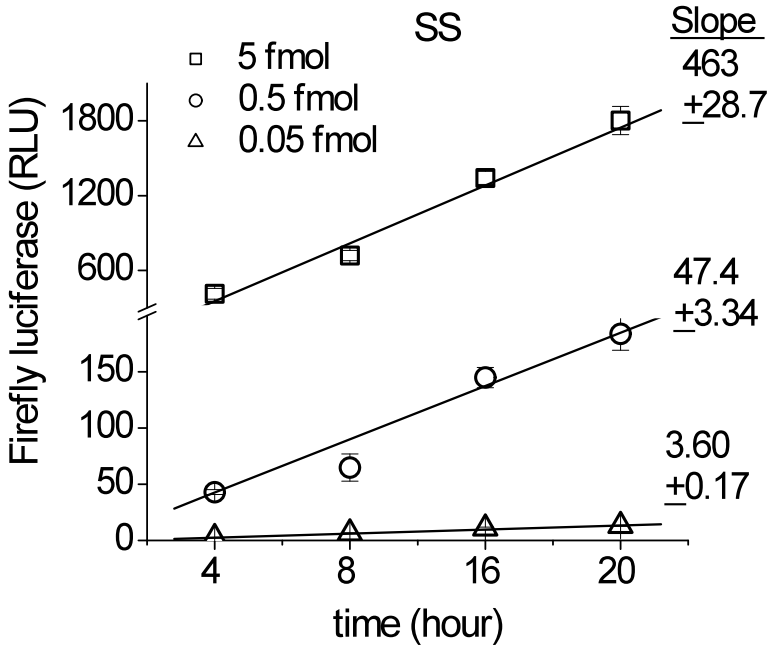

SS
Slope
5 fmol
0.5 fmol
0.05 fmol
463
±28.7
47.4
±3.34
3.60
±0.17
Firefly luciferase (RLU)
1800
1200
600
150
100
50
0
4
8
16
20
time (hour)

Figure III-9 Activity of Luciferase expressed from the reporter mRNAs bearing the short 5' and **long** 3'UTRs of GluR2 was proportional to both mRNA amount and time after injection into *Xenopus oocytes*.

Fig. III-9

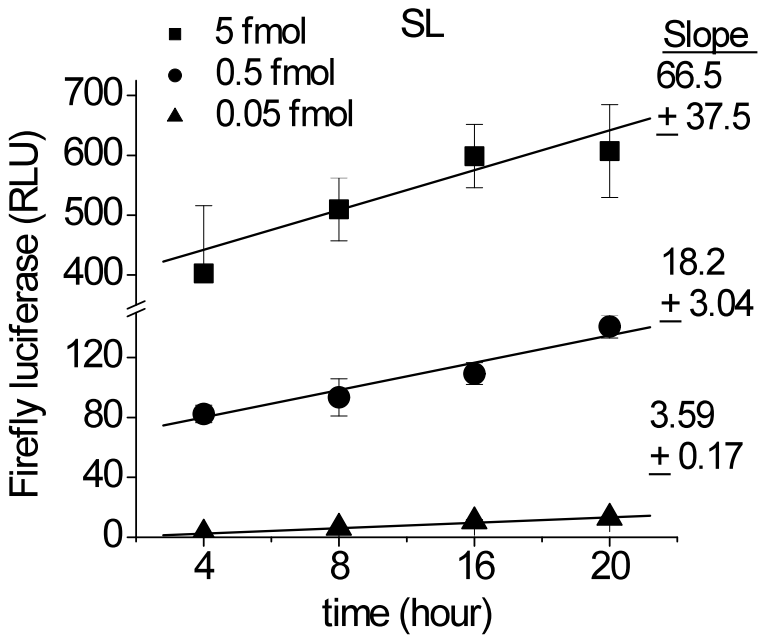

SL
5 fmol
0.5 fmol
0.05 fmol
Slope
66.5
± 37.5
18.2
± 3.04
3.59
± 0.17
Firefly luciferase (RLU)
700
600
500
400
120
80
40
0
4
8
16
20
time (hour)

Figure III-10. Expression profile of firefly reporter mRNAs bearing alternative GluR2 UTRs in *Xenopus* oocytes. Individual Xenopus oocytes were microinjected with reporter mRNAs (5 fmol/oocyte) and incubated at 17^0C. 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.

Fig. III-10

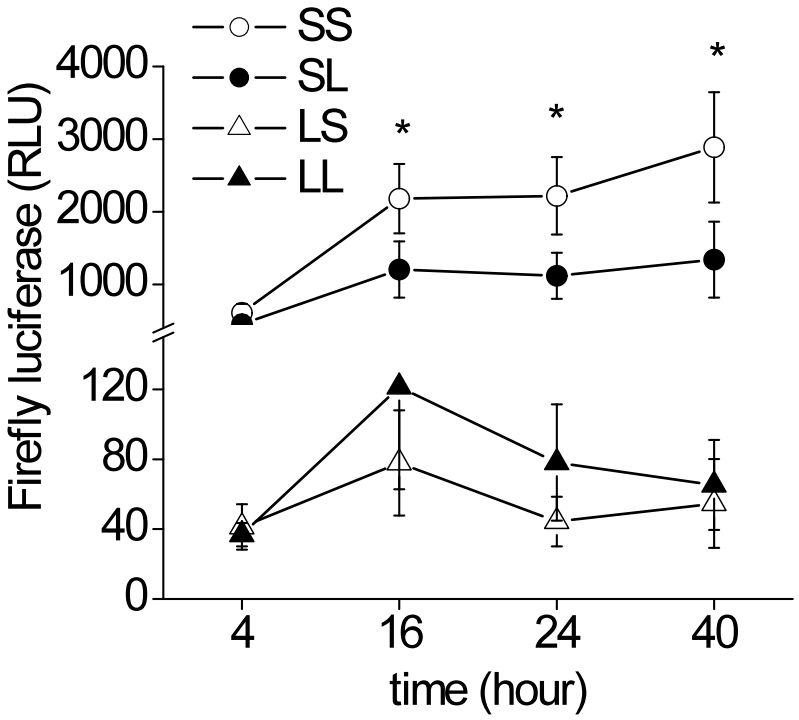

SS
SL
LS
LL
Firefly luciferase (RLU)
4000
3000
2000
1000
120
80
40
0
*
*
*
4
16
24
40
time (hour)

Figure III-11. Stability of the reporter mRNAs in *Xenopus oocytes*. 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).

Fig. III-11

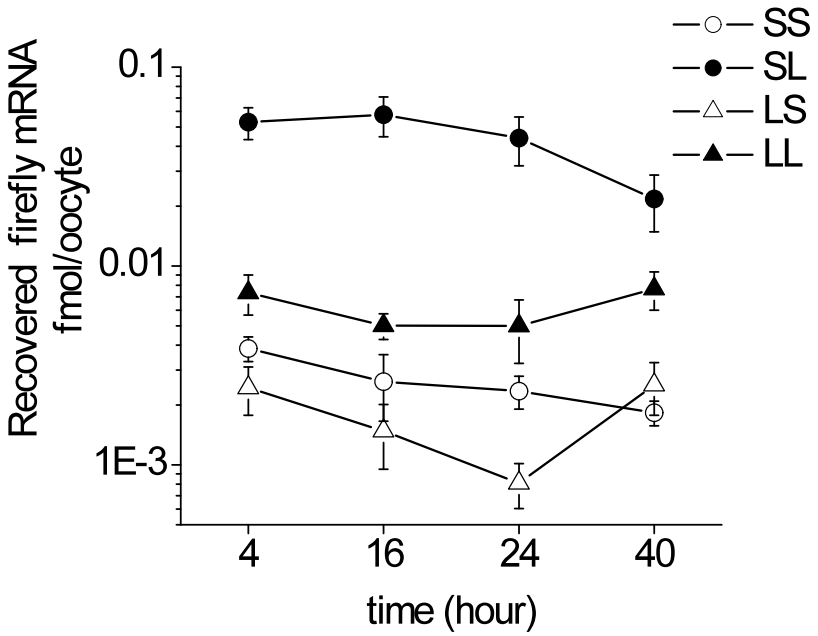
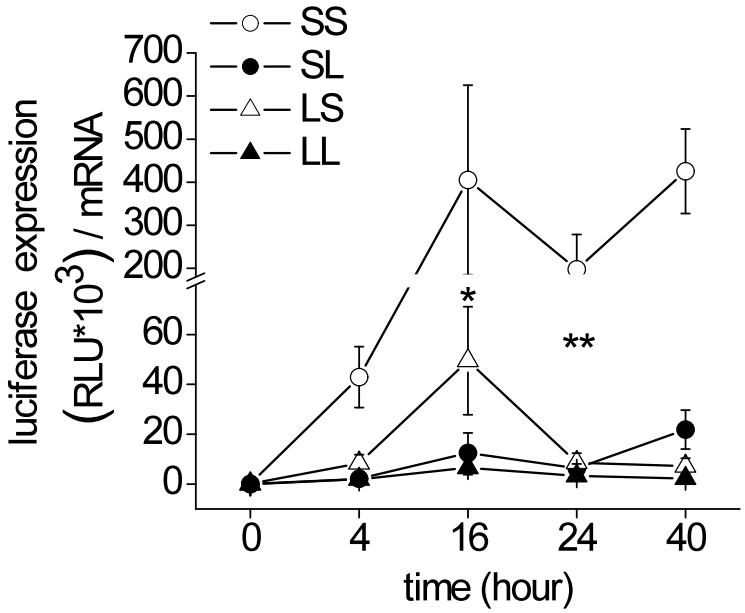

SS
SL
LS
LL
Recovered firefly mRNA
fmol/oocyte
0.1
0.01
1E-3
4
16
24
40
time (hour)

Figure III-12, 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.

Fig. III-12


SS
SL
LS
LL
700
600
500
400
300
200
60
40
20
0
*
**
luciferase expression
(RLU*10^3) / mRNA
0
4
16
24
40
time (hour)

hippocampus within 16 to 24 hours after the onset of SE (Grooms et al., 2000; Huang et al., 2002a). 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 **(Figure III-13).** 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 **(Figure III-14 and III-16),** 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. Figure III-15 and III-16)** 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 **(Figure III-15).**

Figure III-13 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.

Fig. III-13

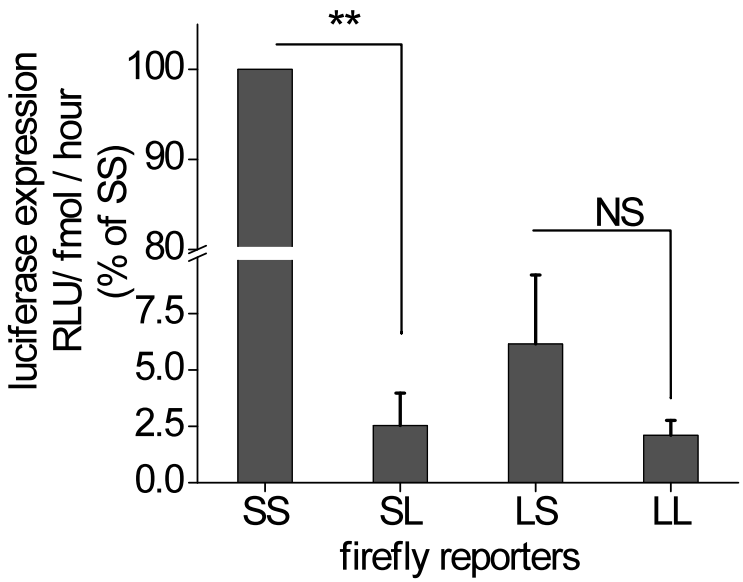

**
NS
luciferase expression
RLU/ fmol / hour
(% of SS)
100
90
80
7.5
5.0
2.5
0.0
SS
SL
LS
LL
firefly reporters

Figure III-14. Effects of pilocarpine-induced status epilepticus (SE) on native GluR2 transcripts. 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).

Fig. III-14

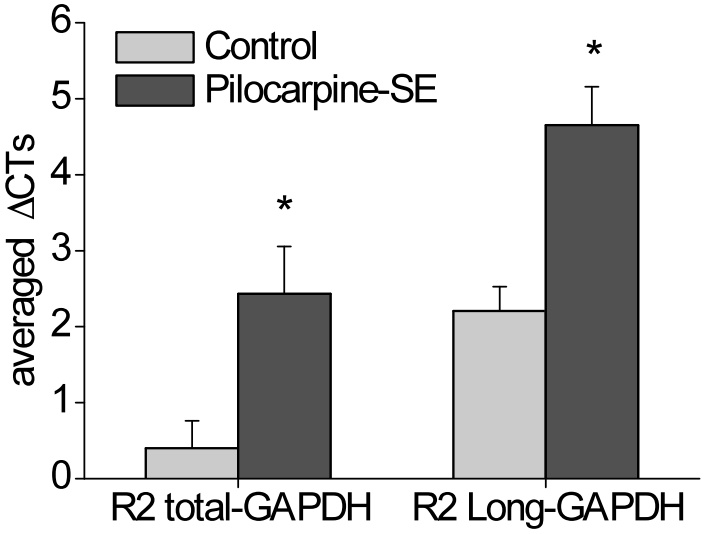
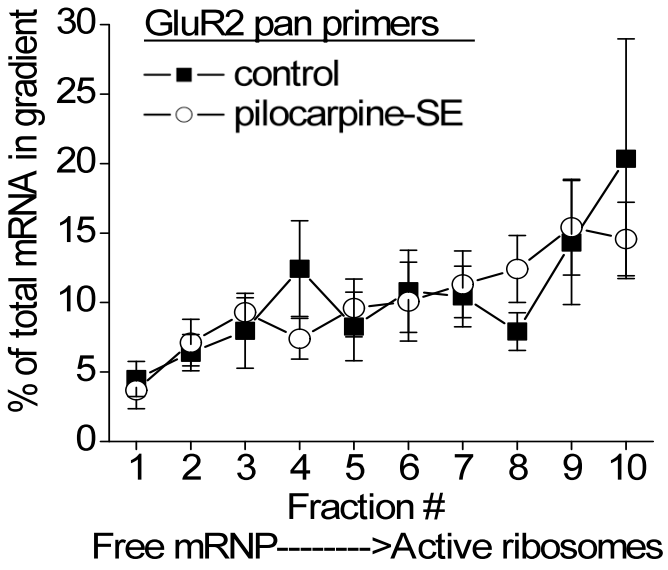

6
5
4
3
2
1
0
averaged ΔCTs
Control
Pilocarpine-SE
*
*
R2 total-GAPDH
R2 Long-GAPDH

Figure III-15. To determine the effects of pilocarpine-induced SE on the association of GluR2 mRNAs with ribosomes in rat hippocampus, polyribosome association of pan GluR2 (top panel) and GluR2 bearing long 3'UTRs (bottom panel) was examined over a sucrose gradient assay.

Fig. III-15


GluR2 pan primers
control
pilocarpine-SE
% of total mRNA in gradient
30
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10
Fraction #
Free mRNP-------->Active ribosomes

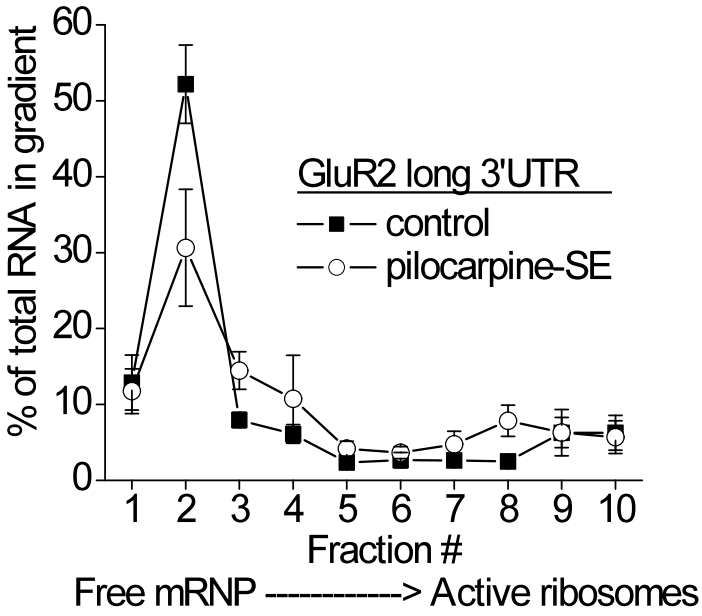
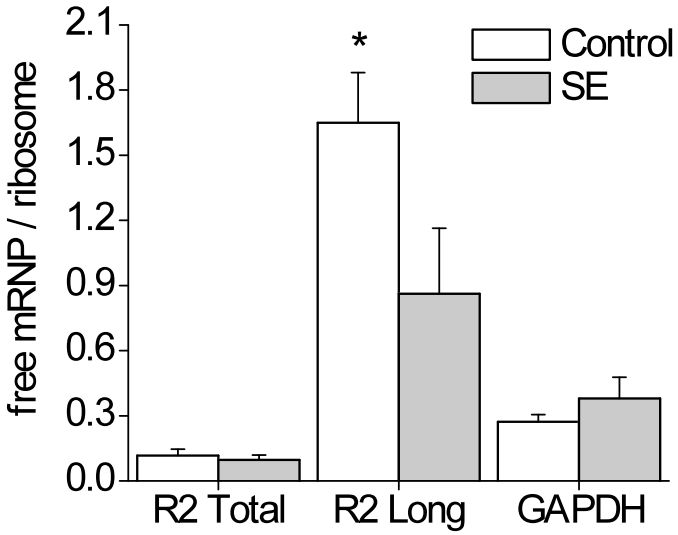

GluR2 long 3'UTR
control
pilocarpine-SE
% of total RNA in gradient
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10
Fraction #
Free mRNP ---------> Active ribosomes

Figure III-16 Native GluR2 transcripts recovered from free mRNP (fraction 1 and 2 see Fig.III-15) and active ribosomes (fraction 3 to 10, see Fig.III-15) 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).

Fig. III-16


free mRNP / ribosome
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0.0
*
Control
SE
R2 Total
R2 Long
GAPDH

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 (Figure III-5E 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 Figure III-5E and F).

D. 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; FigureIII-1), 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.

CHAPTER IV. Control of GluR2 translational initiation by its alternative 3'UTRs.*

A. ABSTRACT

Four major GluR2 transcripts differing in size (~4 and ~6 kb) due to alternative 3'UTRs, and also containing alternative 5'UTRs, exist in the brain. Both the long 5'UTR and long 3'UTR repress translation of GluR2 mRNA; repression by the 3'UTR is relieved following seizures. To understand the mechanism of repression, we used rabbit reticulocyte lysates as an *in vitro* translation system to examine the expression profiles of firefly reporter mRNAs bearing alternative combinations of GluR2 5'UTR and 3'UTR in the presence of inhibitors of either translational elongation or initiation. Translation of reporter mRNAs bearing the long GluR2 3'UTR was insensitive to low concentrations of the translation elongation inhibitors cycloheximide (0.7-70 nM) and anisomycin (7.5-750 nM), in contrast to a reporter bearing the short 3'UTR, which was inhibited. These data suggest that the rate-limiting step for translation of GluR2 mRNA bearing the long 3'UTR is not elongation. Regardless of the GluR2 UTR length, translation of all reporter mRNAs was equally sensitive to DMDA-Pateamine A (0.2-200 nM), an initiation inhibitor. Kasugamycin, which can facilitate recognition of certain mRNAs by ribosomes leading to alternative initiation, had no effect on translation of a capped reporter bearing both short 5'UTR and short 3'UTR, but increased the translation rate of reporters bearing either the long GluR2 5'UTR or long

3’UTR. Our findings suggest that both the long 5’UTR and long 3’UTR of GluR2 mRNA repress translation at the initiation step, but by different mechanisms.

B. INTRODUCTION

AMPA receptors, a subfamily of ionotropic glutamate receptors mediating the majority of fast excitatory neurotransmission in the central nervous system (Dingledine et al., 1999), are ligand-gated ion channels formed by heteromeric or homomeric combinations of GluR1, GluR2, GluR3 and GluR4 subunits (Kohler et al., 1994; Rosenmund et al., 1998). Although most well-known examples of gene regulation involve transcription, the efficiency of mRNA *translation* can be regulated in a transcript-specific manner by structural motifs residing in the 5' or 3'UTR, alternate or additional 5'-UTR AUG codons (or their cognate short open reading frames), RNA binding proteins, or the nucleotide context of the initiator AUG (Gray and Wickens, 1998). Control of translation and trafficking of GluR2 subunits is involved in long term synaptic potentiation (Gainey et al., 2009; Isaac et al., 2007). Multiple GluR2 transcripts exist in hippocampus that has alternative 5’- and 3’-untranslated regions (UTRs) (Irier et al., 2009; Kohler et al., 1994; Myers et al., 1998). GluR2 mRNAs with at least two different 3'UTRs, long (~2750 bases) and short (~750 bases), exist in the brain (Kohler et al., 1994) but encode the same protein. GluR2 transcription is regulated by positive and negative regulatory elements in the promoter region(Huang et al., 1999; Myers et al., 1998) .Translation is repressed by GU repeats located in the long 5’- UTR, and also by unknown elements in the long 3’UTR (Irier et al., 2009; Myers et al., 2004). Seizures reduce overall GluR2 mRNAs level but de-repress the

translation of GluR2 mRNAs bearing the long 3'UTRs in rat hippocampus (Irier et al., 2009). These findings suggest that GluR2 mRNAs with alternative combinations of GluR2 5' and 3'UTRs are subject to transcript-specific regulation of translation, but the regulated step of translation is unknown.

In general, eukaryotic mRNA translation occurs in four consecutive phases: initiation, elongation, termination and ribosome recycling. In the initiation phase, a 43S pre-initiation complex is formed by interaction of 40S ribosomal subunits with Met-tRNAi. This pre-initiation ribosomal complex then binds to mRNA at the 5' cap structure (methylated guanosine triphosphate m7GpppN) in a process facilitated by translation factor complex eIF4, and begins to scan through 5'UTR, unwinding the secondary structure until it encounters the first eligible AUG codon, which is then oriented onto the P (peptidyl) site of the scanning ribosome. Once this start signal is recognized, the larger 60S subunit joins the 40S to form an 80S initiation complex that is ready to accept appropriate aminoacyl-tRNA into the A (aminoacyl) site on the ribosome, thus starting the elongation phase of translation (Kozak, 1989; Pestova et al., 2001; Sonenberg and Hinnebusch, 2009) .

One approach to determine whether initiation or elongation is the rate-limiting step in protein expression is to treat cells with low concentrations of modulators of these processes (Chen and Sarnow, 1995; Lodish and Jacobson, 1972; Walden et al., 1981) . If elongation is the rate limiting step, then translation rate should be sensitive to a low concentration of elongation inhibitors. On the contrary, if translation of an mRNA is insensitive to low concentrations of

translation elongation inhibitors or is sensitive to translation initiation modulators, then the rate limiting step for such mRNAs is likely initiation. Taking this approach with rabbit reticulocyte lysates as an *in vitro* translation system, we report that translational repression caused by both the long 5'UTR and long 3'UTR is mediated at the initiation step.

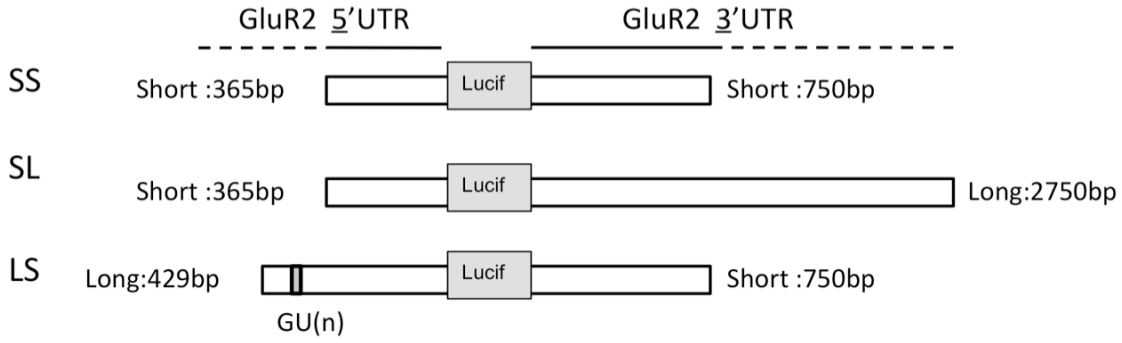
C. RESULTS

a. Effects of elongation inhibitors on translation of GluR2 reporter mRNAs.

To study the mechanisms of transcript-specific regulation of GluR2 translation, we employed a set of reporter constructs previously designed in our laboratory (Irier et al., 2009) **(Figure IV-1)** . Briefly, these constructs contain a firefly luciferase coding region

Figure IV-1. Schematic illustration of firefly luciferase reporter mRNAs bearing alternative combinations of GluR2, 5'and 3'UTRs [designated SS for firefly luciferase coding region if flanked by Short 5'UTR and Short 3'UTR of GluR2; SL if flanked by Short 5' and Long 3'UTR of GluR2, and LS if flanked by Long 5'UTR and Short 3'UTR of GluR2]. The luciferase coding region is common to all reporters. The dark gray box indicates the position of GU repeats on the long 5'UTR (Myers et al., 2004).

Fig. IV-1


GluR2 5'UTR
GluR2 3'UTR
SS
Short :365bp
Lucif
Short :750bp
SL
Short :365bp
Lucif
Long:2750bp
LS
Long:429bp
Lucif
Short :750bp
GU(n)

flanked by alternative combination of GluR2 5'- and 3'-UTRs [designated in Fig. IV-1 **SS** for Luciferase protein coding region if flanked by Short 5'UTR and Short 3'UTR of GluR2; **SL**, if flanked by Short 5'UTR and Long 3'UTR of GluR2; **LS**, if flanked by Long 5'UTR and Short 3'UTR of GluR2]. *In vitro* synthesis of mRNAs from linearized reporter constructs yielded good quality mRNAs as determined by the Agilent 2100 bioanalyzer **(Figure IV-2).** The rate of firefly luciferase expression by rabbit reticulocyte lysate was proportional to the amount of reporter mRNA added (**Figure IV-3, 4 and 5)** Over half of the reporter mRNAs added was recovered after 40min incubation at 30^0C (**Figure IV-6**).

Using rabbit reticulocyte lysates as *in vitro* translation system, we examined the expression of firefly reporter mRNAs bearing alternative combinations of GluR2 5'- and 3'-UTRs (SS, SL and LS) in the presence or absence of translation elongation inhibitors cycloheximide (0.7-70nM) or anisomysin D (7.5-75nM). At low concentrations, cycloheximide has been shown to inhibit the elongation of nascent peptide by blocking the translocation of peptidyl-tRNA on the translating (80S) ribosomes (Baliga et al., 1968; Lodish, 1971; Munro et al., 1968) . In rabbit reticulocyte lysates, the translation of reporter mRNAs bearing a short 5'- and a short 3'-UTR (SS) was inhibited by low concentrations of cycloheximide (0.7-7nM), whereas translation of the reporter bearing GluR2 short 5' and long 3'UTR (SL) was insensitive (**Figure IV-7**). Moreover, translation of reporters bearing a long 5' and a short 3' UTR (LS) was also sensitive to low concentrations of cycloheximide. At higher concentrations of cycloheximide (≥70nM), expression of all three reporter mRNAs was equally inhibited (data not shown). Likewise,

Figure IV-2 The quality and amount of each in vivo transcribed firefly reporter were evaluated with an Agilent 2100 Bioanalyzer, which produces a gel-like image. The expected sizes of the transcripts is SS: 2760; SL: 4710; LS: 3140 bp.

Fig. IV-2

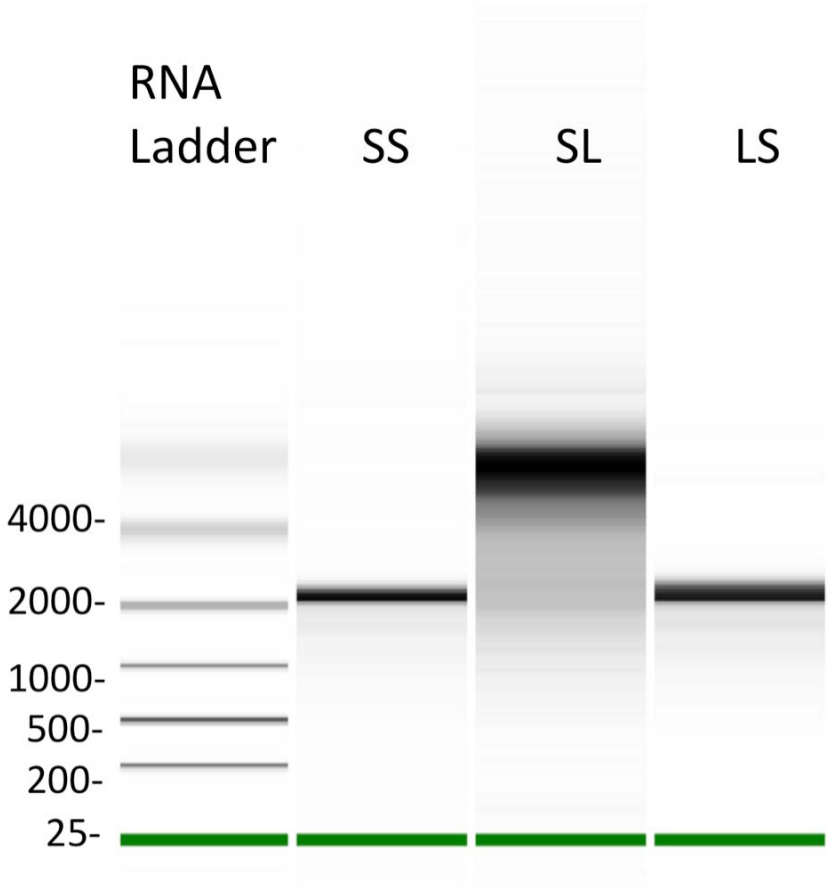


Figure IV-3 The activity of luciferase expressed from the firefly reporter mRNAs bearing the short 5'- and short 3'-UTRs of GluR2 (**SS**) was proportional to both mRNA amount and time in rabbit reticulocyte lysate translation mix.

Fig. IV-3

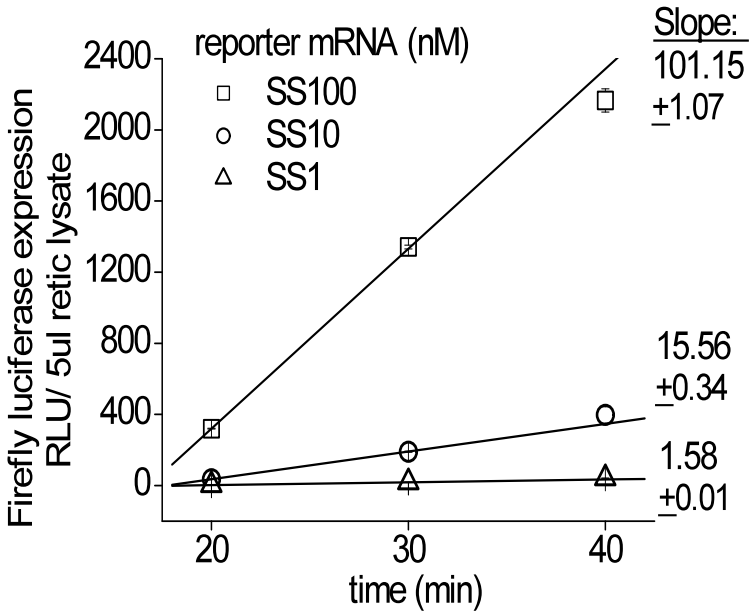

reporter mRNA (nM)
SS100
SS10
SS1
Slope:
101.15
±1.07
15.56
±0.34
1.58
±0.01
Firefly luciferase expression
RLU/ 5ul retic lysate
2400
2000
1600
1200
800
400
0
20
30
40
time (min)

Figure IV-4 The activity of luciferase expressed from the firefly reporter mRNAs bearing the short 5'- and long 3'-UTRs of GluR2 (**SL**) was proportional to both mRNA amount and incubation time in rabbit reticulocyte lysate translation mix.

Fig. IV-4

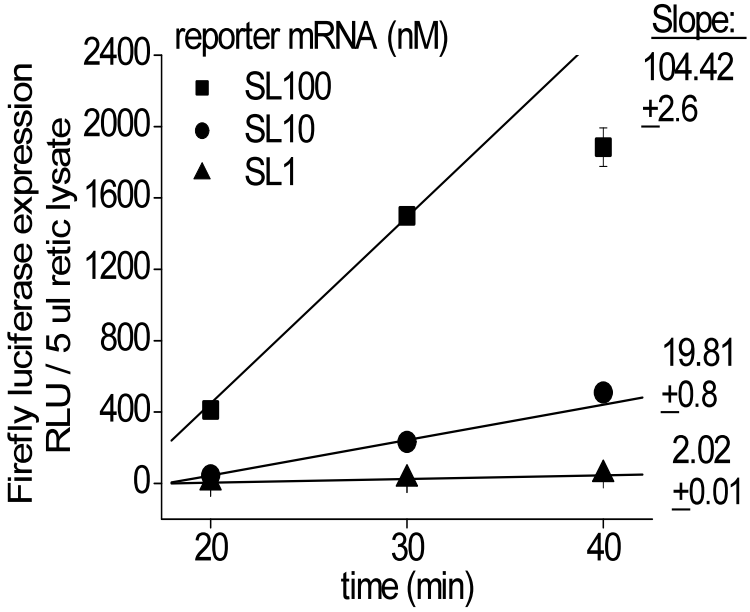

reporter mRNA (nM)
SL100
SL10
SL1
Slope:
104.42
±2.6
19.81
±0.8
2.02
±0.01
Firefly luciferase expression
RLU / 5 ul retic lysate
2400
2000
1600
1200
800
400
0
20
30
40
time (min)

Figure IV-5 The activity of luciferase expressed from the firefly reporter mRNAs bearing the long 5'UTR and short 3'UTR of GluR2 (**LS**) was proportional to both mRNA amount and time in rabbit reticulocyte lysate translation mix.

Fig. IV-5

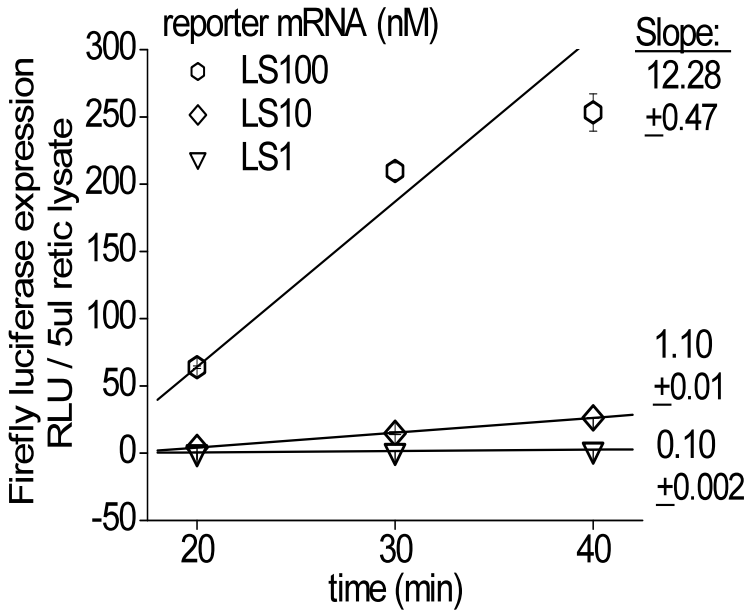

reporter mRNA (nM)
LS100
LS10
LS1
Slope:
12.28
±0.47
1.10
±0.01
0.10
±0.002
Firefly luciferase expression
RLU / 5ul retic lysate
300
250
200
150
100
50
0
-50
20
30
40
time (min)

Figure IV-6 Recovered reporter mRNAs from RRL translation mix were quantified by quantitative real time PCR using primers specific to short 3'UTR of GluR2, and known quantities of cDNA standard. Amounts of reporter mRNAs recovered from the lysate at 40 min were normalized to the reporter mRNAs added at time 0min.

Fig. IV-6

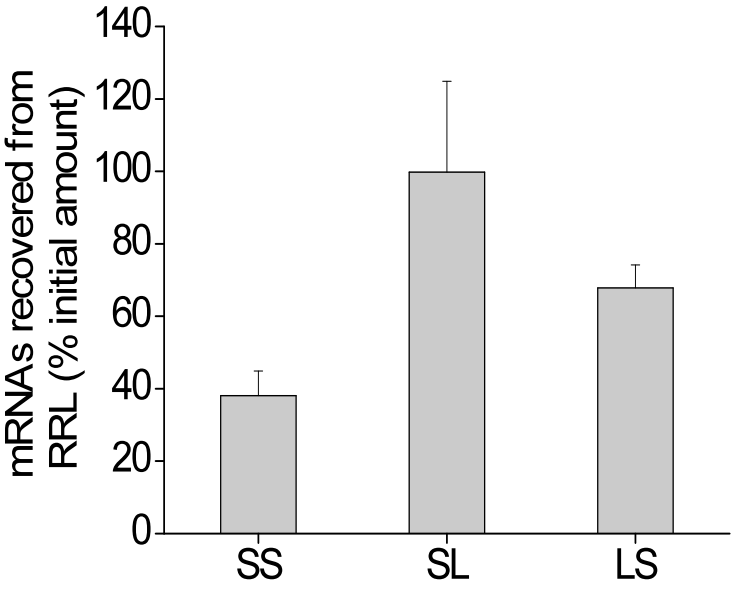
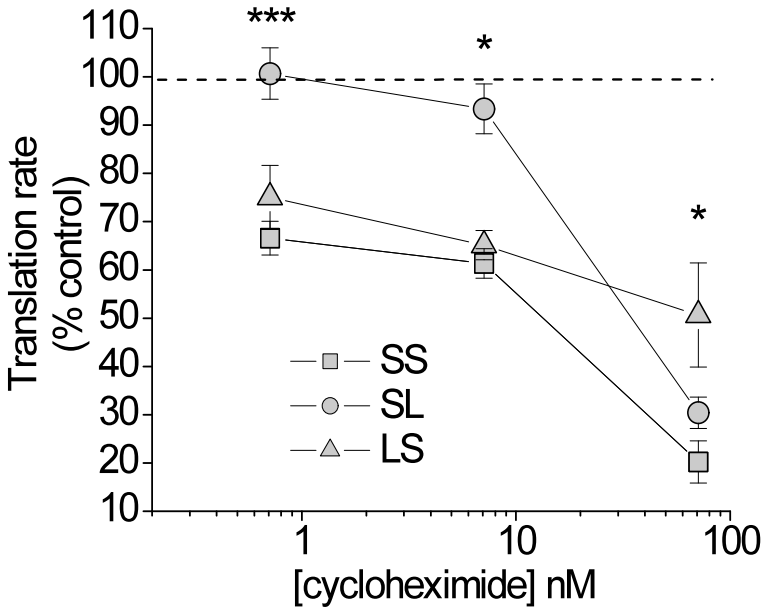

mRNAs recovered from RRL (% initial amount)
140
120
100
80
60
40
20
0
SS
SL
LS

Figure IV-7. At indicated concentrations, effects of **cycloheximide,** a translation elongation inhibitor, on the expression profiles of the 5’ capped firefly reporter mRNAs bearing alternative combinations of GluR2 5’ and 3’UTRs in rabbit reticulocyte lysate. The translation rates of individual firefly reporters were determined by measuring firefly luciferase activities as recorded luminescence units (RLU) from a 5 µl RRL sample collected at indicated time points. Slopes of RLU from the 5 µl samples collected between 20 to 40min were calculated for the drug-treated samples and normalized to that of vehicle -treated (RNase-free water) samples and presented as % control. Translation rates of the controls were set to 100% (dotted line) (* $P < 0.01$ for SS vs. SL at 7nM CHX, ***$P<0.001$ for SS vs. SL at 0.7nM CHX, by ANOVA, post hoc Bonferroni). N=5-6 per reporters per drug treatment, error bars indicate standard errors.

Fig. IV-7


***
*
*
110
100
90
80
70
60
50
40
30
20
10
Translation rate
(% control)
SS
SL
LS
1
10
100
[cycloheximide] nM

anisomycin also inhibits the elongation phase of translation by blocking a peptidyl transferase, a protein that catalyzes translocation of peptidyl-tRNA on translating ribosomes (Grollman, 1967). In the translation mix treated with a relatively high concentration of anisomycin (750nM), the translation rate of luciferase reporters bearing all three combinations of GluR2 UTRs were equally inhibited; however the lower concentrations of anisomycin (7.5-75nM) significantly repressed the expression of SS, but not SL, reporter **(Figure IV-8)** providing further evidence that translation elongation is not the rate limiting step for the expression of the SL reporter mRNA bearing a long 3'UTR of GluR2. The LS reporter exhibited intermediate sensitivity to anisomycin (**Figure IV-8**).

b. Effects of initiation modulators on translation of GluR2 reporters.

We first tested the effects of kasugamycin, an antifungal aminoglycoside antibiotic that inhibits translation initiation in prokaryotes by binding to a specific region between the 30S ribosome and 16S rRNA within the 70S ribosome thereby impeding the binding of initiator tRNA (fMet-tRNA) to the ribosome (Schluenzen et al., 2006; Schuwirth et al., 2006). In rabbit reticulocyte lysates, none of the luciferase reporter mRNAs bearing alternative combinations of GluR2 5' and 3'UTR was inhibited by kasugamycin at the concentrations tested; on the contrary, and to our surprise, the expression of the SL and LS reporters were selectively potentiated by kasugamycin (**Figure IV-9**), with no effect on the SS reporter. Kasugamycin has been shown to accelerate the translation of some prokaryotic mRNAs by a mechanism involving creation of a novel ribosomal

Figure IV-8 At indicated concentrations, effects of **anisomycin D,** a translation elongation inhibitors, on the expression profiles of the 5’ capped firefly reporter mRNAs bearing alternative combinations of GluR2 5’ and 3’UTRs in rabbit reticulocyte lysate, and translation initiation modulators. The translation rates of individual firefly reporters were determined by measuring firefly luciferase activities as recorded luminescence units (RLU) from a 5 µl RRL sample collected at indicated time points. Slopes of RLU from the 5 µl samples collected between 20 to 40min were calculated for the drug-treated samples and normalized to that of vehicle -treated (dimethyl sulfoxide) samples and presented as % control. Translation rates of the controls were set to 100% (dotted line) (* P < 0.01 for SS vs. SL 7.5 and 75nM ANS, for SS vs. LS at 75nM ANS, for SS vs. LS at 7.5nM ANS, ***P<0.001 for SS vs. LS at 7.5nM ANS, ANOVA, post hoc Bonferroni). N=5-6 per reporters per drug treatment, error bars indicate standard errors.

Fig. IV-8

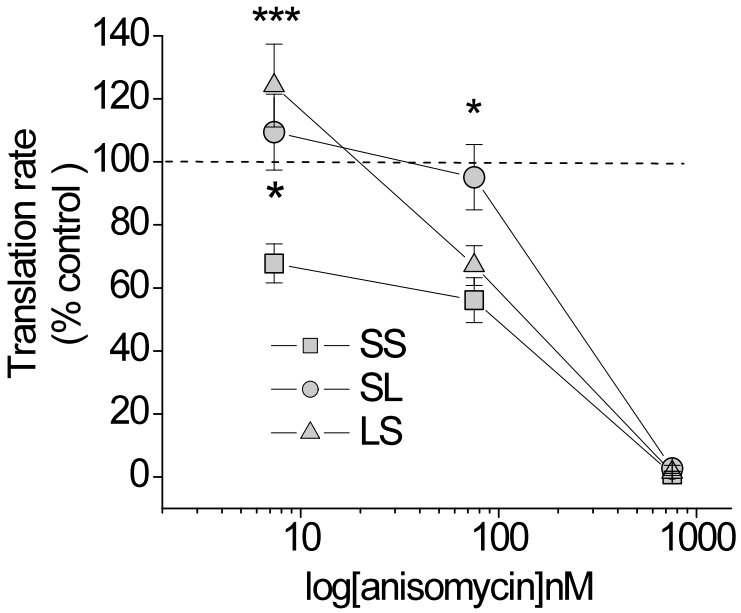
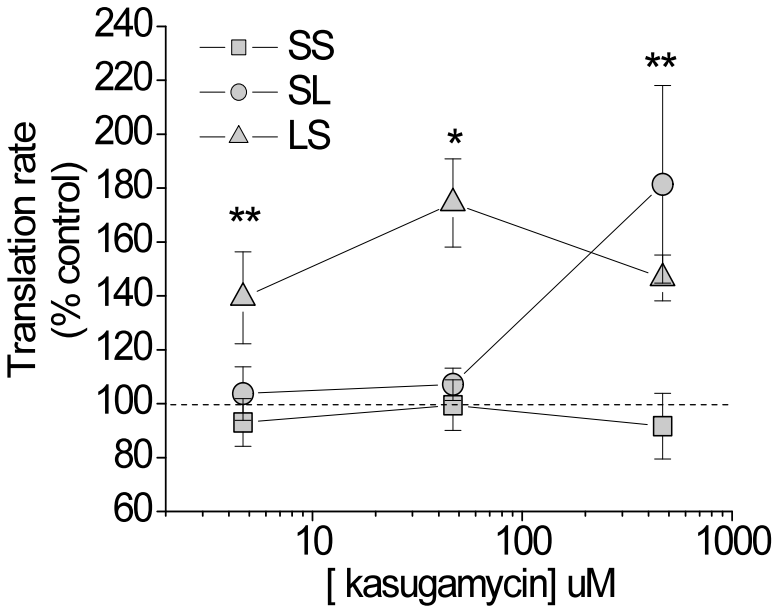

***
*
*
140
120
100
80
60
40
20
0
Translation rate
(% control)
SS
SL
LS
10
100
1000
log[anisomycin]nM

Figure IV-9 At indicated concentrations, effects of **Kasugamycin,** a translation initiation modulator, on the expression profiles of the 5’ capped firefly reporter mRNAs bearing alternative combinations of GluR2 5’ and 3’UTRs in rabbit reticulocyte lysate, and translation initiation modulators. The translation rates of individual firefly reporters were determined by measuring firefly luciferase activities as recorded luminescence units (RLU) from a 5 µl RRL sample collected at indicated time points. Slopes of RLU from the 5 µl samples collected between 20 to 40min were calculated for the drug-treated samples and normalized to that of vehicle -treated (RNAse-free water) samples and presented as % control. Translation rates of the controls were set to 100% (* $P < 0.01$ for SS vs. LS at 46µM KSG; ** $P < 0.05$ for SS vs. SL at 460µM KSG, for SS vs. LS at 4.6-460µM KSG by ANOVA, post hoc Bonferroni). N=5-6 per reporters per drug treatment, error bars indicate standard errors.

Fig. IV-9


240
220
200
180
160
140
120
100
80
60
Translation rate
(% control)
SS
SL
LS
**
*
**
10
100
1000
[kasugamycin] uM

initiation complex (Kaberdina et al., 2009); a similar mechanism could operate in eukaryotes.

We then studied the effects of desmethyl, desamino Pateamine A (DMDA-Pat A), a simplified structural analog of marine natural product Pateamine A (Romo et al., 2004) . DMDA-Pat A is thought to inhibit translation initiation of 5'capped mRNAs by disrupting

the interaction of the eIF4F protein complex with eIF4A (Bordeleau et al., 2006; Low et al., 2005) , both of which are essential protein factors during ribosome scanning at the 5'UTR of capped mRNAs leading to a final 80S ribosome assembly at the start codon (Kapp and Lorsch, 2004; Pestova et al., 2001). In rabbit reticulocyte lysates, the translation of reporters bearing all three combinations of GluR2 UTRs was approximately equally sensitive to inhibition by DMDA-Pateamine A (**Figure IV-10**), suggesting that initiation influences translation rate of these luciferase reporters bearing alternative combinations of GluR2 5'- and 3'-UTRs.

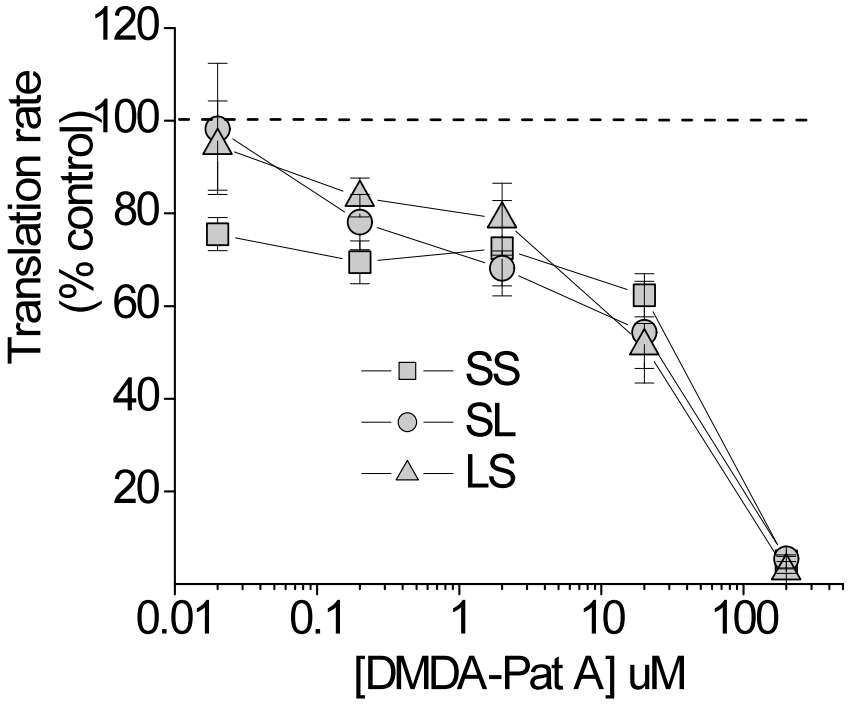
D. DISCUSSION

The major finding of this study is that alternative GluR2 3'UTRs determine the sensitivity to inhibition of translation by two well-characterized elongation inhibitors, cycloheximide and anisomycin D. The long 3'UTR of GluR2 imparts relative insensitivity to these elongation inhibitors compared to the short 3'UTR, which strongly suggests that elongation is not the rate-limiting step for translation of GluR2 bearing the long 3'UTR. Further, kasugamycin, which can promote

formation of an alternative 61S ribosomal complex that improves initiation of translationally-restricted mRNAs (Kaberdina et al., 2009), increased translation of reporters bearing the long but not the short 3'UTR.These results taken together suggest that initiation is a rate limiting step for translation of GluR2 mRNAs bearing the long 3'UTR. Thus, the site of action for the translation repression of the GluR2 transcripts bearing the long 3-UTR (Irier et al., 2009) in vivo, and its derepression following seizures, is likely to involve the initiation phase of translation.

Figure IV-10 At indicated concentrations, effects of **DMDA-Pateamine A,** a translation initiation modulator, on the expression profiles of the 5’ capped firefly reporter mRNAs bearing alternative combinations of GluR2 5’ and 3’UTRs in rabbit reticulocyte lysate. The translation rates of individual firefly reporters were determined by measuring firefly luciferase activities as recorded luminescence units (RLU) from a 5 µl RRL sample collected at indicated time points. Slopes of RLU from the 5 µl samples collected between 20 to 40min were calculated for the drug-treated samples and normalized to that of vehicle -treated (dimethyl sulfoxide) samples and presented as % control. Translation rates of the controls were set to 100% (dotted line) (NS, not statistically significant by ANOVA, post hoc Bonferroni). N=5-6 per reporters per drug treatment.

Fig. IV-10


Translation rate
(% control)
120
100
80
60
40
20
SS
SL
LS
0.01
0.1
1
10
100
[DMDA-Pat A] uM

a. Effects of DMDA-Pateamine A on the GluR2 mRNA translation initiation. The finding that the initiation inhibitor, DMDA-pateamine A, reduces the translation rate of reporters bearing the short 3'UTR of GluR2 suggests that the rates of elongation and initiation of this transcript are likely to be approximately the same. DMDA-Pateamine A disrupts eIF4F complex by impairing the interaction between eIF4A and eIF4G, which is required in the process of 5'UTR scanning by the pre-initiation 48S complex (Kaberdina et al., 2009). Moreover, regardless of the 5'UTR length or structure, some mRNAs are susceptible to inhibition by eIF4A mutants (Svitkin et al., 2001). The observation that DMDA-Pateamine A inhibits indiscriminately all the reporters bearing GluR2 UTRs may be due to a direct impairment of the 5'UTR scanning process, which is common among all GluR2 transcripts during translation initiation. Alternatively, the rates of elongation and initiation of the transcript bearing short 5'UTR and short 3'UTR may be approximately the same such that this transcript would be sensitive to inhibitors of both elongation and initiation.

b. Transcript-specific alternative initiation of GluR2 mRNA translation by Kasugamycin. A recent report suggests that kasugamycin can facilitate translation of leaderless mRNAs (mRNAs starting with a 5'-AUG codon only) by forming 61S ribosomal particles that function as an alternative initiation complex in prokaryotes (Kaberdina et al., 2009). To our knowledge, the inducing effects of kasugamycin have not been reported for eukaryotic mRNA translation. Our observation that kasugamycin selectively induces the expression of reporter mRNAs bearing either of the long UTRs of GluR2 raises the possibility that it

favors mRNAs bearing structured or long UTRs. The mechanism of induction in eukaryotes is currently unknown, however the subunits comprising 61S ribosomal particles are evolutionary conserved among prokaryotes and eukaryotes (Kaberdina et al., 2009; Wilson and Nierhaus, 2005). Whether 61S-like ribosomal particles are formed in eukaryotes in the presence of this aminoglycoside and constitute alternative translational machinery would be interesting to determine. Nevertheless, the effects of kasugamycin present further evidence that GluR2 mRNAs can have distinct translation patterns due to alternative 5' and 3'UTRs.

In eukaryotes ribosomes bind most transcripts at the 5'cap end and scan along the 5'UTR until an appropriate AUG codon is encountered (Kozak, 1989). However, alternative modes of translational initiation have been described (Chen and Sarnow, 1995; Jackson, 2005; Sonenberg and Hinnebusch, 2009) . For example, initiation of fibroblast growth factor (FGF)-2 mRNA occurs at an internal ribosomal entry site (IRES) of 5'UTR in neurons of specific brain regions (Audigier et al., 2008; Vagner et al., 1995). Secondary structures within the IRES and IRES-trans activating proteins are thought to be involved in initiation (Sonenberg and Hinnebusch, 2009). GluR2 long 5'UTRs (≥429 bases from the start codon) exhibit a high GC-content, and an imperfect GU repeat region that mediate translational repression (Myers et al., 2004). The GU repeats in the GluR2 long 5'UTRs are predicted to form stable secondary structures that stall the ribosome scanning process (Myers et al., 1998). Understanding the mechanisms underlying the selective effects of kasugamycin on translationally

restricted GluR2 mRNAs may present a potential target for therapeutic interventions.

Previous studies demonstrated that GluR2 mRNAs are transported from soma to dendrites in an activity-dependent manner (Ju et al., 2004). Approximately half of the GluR2 mRNAs in rat hippocampus bear a long 3'UTR (Irier et al., 2009), and about 75% bear the short 5'UTR (unpublished), suggesting that a considerable fraction of native GluR2 transcripts are translationally regulated by the long 3'UTR alone.

Owing to its essential role in AMPA receptor function, the GluR2 subunit has been of considerable interest. Our results present additional evidence that in addition to transcriptional regulation, expression of the GluR2 subunit is highly regulated at the level of mRNA translation by the presence of alternative UTRs. Understanding how the physiological properties of glutamate receptor channels are regulated at the translational level should be relevant to the late phase of LTP, learning, and the response to seizures, as well as other situations in which AMPA receptor phenotype is remodeled.

CHAPTER V. Summary and Future Directions

A. Summary of major findings

During the course of this thesis research, two major GluR2 RNA species were identified of ~6 kb and ~4 kb in the Northern blots of the rat hippocampus and

cortex, as described by others in mice. I showed that *in situ* hybridization of digoxigenin-labeled RNA probes specific to GluR2 short or long 3'UTRs in the principal hippocampal regions indicated that both short and long GluR2 mRNAs are abundantly expressed in CA1, CA3 and DG. Moreover, 24 hours after pilocarpine-induced status epilepticus (SE), the GluR2 mRNA levels in CA1 and CA3, but not DG, were significantly reduced, as previously shown by others in a kainate-induced seizure model using male rats.

Endogenous mRNAs that are undergoing active translation are typically associated with polyribosomes. The association of endogenous GluR2 mRNAs from a rat hippocampus with ribosome-free ribonucleoproteins and polyribosomes was examined using a sucrose gradient assay. Briefly, after centrifugation of the hippocampal lysate layered over a sucrose gradient, the bottom fractions contain denser polyribosomes. Therefore, mRNAs detected in these fractions are considered to be actively translated, whereas RNA found in the top fractions, which contain ribosome-free ribonucleoproteins, are typically considered to be poorly translated or stalled. While the majority of the overall endogenous GluR2 transcripts was detected in the polyribosome (bottom) fractions, the large majority of GluR2 transcripts bearing the long 3'UTRs was detected in the ribosome-free ribonucleoprotein (top) fractions.

In *Xenopus* oocytes, the expression profile of firefly reporters bearing alternative GluR2 5' and 3' UTRs was examined. In the absence of long 5'UTR, which contain translation repressor elements, the presence of a long 3'UTR served as a translational suppressor for GluR2 transcripts. The mRNA stability of these

reporter mRNAs bearing alternative GluR2 3'UTRs were similar in *Xenopus* oocytes, as determined by Q-RT-PCR.

Using a sucrose gradient assay, the effects of pilocarpine-induced SE on the association with polysomes of native GluR2 transcripts bearing alternative 3'UTRs were also examined. 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 using 3'UTR-specific primers. While distribution of the pan GluR2 mRNA over the gradient fractions was not affected, a portion of GluR2 mRNAs bearing long 3'UTRs shifted from ribosome-free ribonucleoprotein (top) to polysome (bottom) fractions 24 hrs after pilocarpine-induced SE, suggesting an activity-dependent derepression of translation of GluR2 transcripts bearing long 3'UTRs. The distribution of GAPDH, an internal control, over the gradients was slightly shifted towards ribosome-free ribonucleoproteins, as expected if prolonged seizures reduce general translation. These findings overall indicate that under normal physiological conditions translation of GluR2 transcripts bearing alternative combinations of 5' and 3'UTRs are discriminately processed and that this UTR-specific translation is responsive to strong extracellular signaling.

To determine the mechanism of 3'-UTR mediated translation repression, the translation profile of the luciferase reporter mRNAs bearing GluR2 UTRs were studied in rabbit reticulocyte lysates treated with translation elongation inhibitors and modulators of translation initiation. Translation of the reporter mRNAs

bearing the long GluR2 3'UTR was insensitive to low concentrations of the translation elongation inhibitors cycloheximide and anisomycin, in contrast to a reporter bearing the short 3'UTR, which was inhibited, suggesting that the translation initiation is the site of translation regulation for GluR2 mRNAs bearing the long 3'UTR. Translation initiation modulator kasugamycin selectively induced the expression of reporter mRNAs bearing either of the long UTRs of GluR2 indicting that it favors mRNAs bearing structured or long UTRs. These findings overall suggest that GluR2 transcripts can have distinct translation patterns due to alternative 5' and 3'UTRs. The mechanisms underlying the differences in the translational regulation of GluR2 mRNAs present potential targets for therapeutic interventions.

B. Future Directions

a. Role of 3'UTR-interacting proteins on expression profile of luciferase reporter mRNAs bearing alternative GluR2 UTRs in *Xenopus* oocytes. In many other mRNAs, 3' UTRs play a major role in regulating RNA stability, cellular localization and translation through interaction with a number of RNA binding proteins (Colegrove-Otero et al., 2005; Derry et al., 2006; Wilson and Brewer, 1999). Although GluR2 3'UTRs contain many predicted conserved sites for RNA binding proteins, none have been tested or confirmed to be functional (Huang et al., 2006). During the course of this thesis work, a set of sometimes extensive preliminary experiments have been performed to determine some of the potential

3'UTR-binding proteins using the reporter mRNAs bearing alternative GluR2 UTRs (SS and SL) in *Xenopus* oocytes. The sequential analysis of GluR2 mRNAs shows that the long 3'UTR of GluR2 mRNA contains ten potential CUGBP2 binding sites (AUUUA), and six CPE elements, whereas the GluR2 short 3'UTR contain two CUGBP2 binding sites and one CPE element **(Figure V.1).** CUGBP2 (also known as ETR-3, BRUNOL3, and NAPOR2) is a CUG-binding protein (~50kD) expressed predominantly in muscle tissue and the brain. It is an RNA binding protein that binds to AU-rich sequences (Anant et al., 2001), and in other mRNAs is involved in nuclear and cytoplasmic RNA processing such as splicing, editing and stability (Ladd and Cooper, 2004). CUGBP2 has been shown to bind to AUUUA sequences on the 3'UTR of cyclooxygenase-2 (COX-2), thereby stabilizing the mRNA but inhibiting COX-2 mRNA translation (Mukhopadhyay et al., 2003). In this thesis work, over-expression of CUGBP2 protein was achieved by microinjection of *in vitro* synthesized CUGBP2 encoding mRNAs into the *Xenopus* oocytes **(FigureV-2 and V-3).** These oocytes were then injected with the luciferase reporters bearing alternative combinations of 5'- and 3'-UTRs of GluR2. The over-expression of CUGBP2 in the oocytes caused a transient reduction in the translation of reporters bearing GluR2 long 3'UTRs (SL), but not in those bearing short 3'UTR (SS) **(FigureV-4, V-5 and V-6)**. In a recent study, a significant decrease in CUGBP2 protein was observed in the pyramidal cells of the hippocampal CA1 and CA3 regions of mice hippocampus after transient global ischemia (Otsuka et al., 2009). Both GluR2 mRNA and protein levels are reduced after ischemia in mice and rats (Gorter et al., 1997;

Pellegrini-Giampietro et al., 1992). Thus, the preliminary findings from this thesis work and the previous studies overall raise the possibility that translational repression mediated by the long 3'UTR could be mediated in part by association of CUGBP2 with the long 3'UTRs of GluR2 mRNAs. Does CUGBP2 bind to GluR2 3'UTRs considering that 9 predicted CUGBP2 binding site " AUUUA" exist in the long 3'UTR? One common approach to reveal such protein-3'UTR interactions is reversible cross-linking combined with immunoprecipitation, which takes advantage of the highly reactive, reversible crosslinker, formaldehyde, combined with high-stringency immunoprecipitation to identify specific RNAs associated with a given protein(Niranjanakumari et al., 2002).

Figure V-1. Putative CUGBP2- and CPEB-biding element in GluR2 UTRs (a), Diagram of GluR2 mRNAs with long and short 3'UTR (b) ,the positions of the 11 conserved CUGBP2 –binding element sequences (a-k, dark rectangular), and (c) the 6 conserved CPE sequences (1-6, triangles) are shown.

Fig. V-1

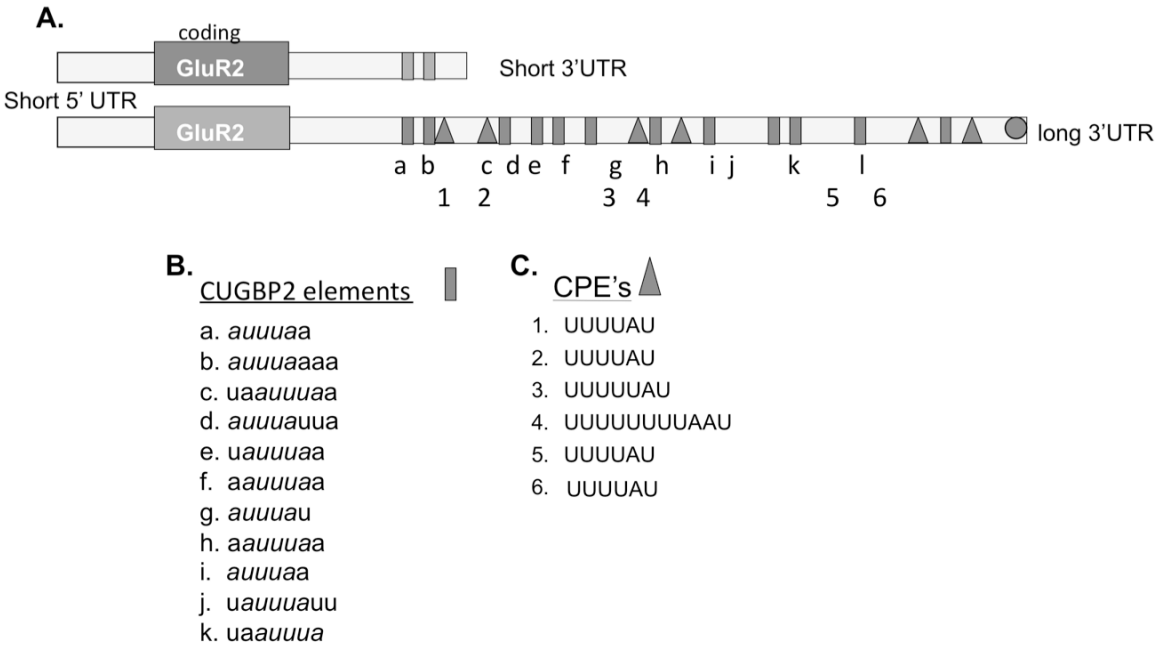
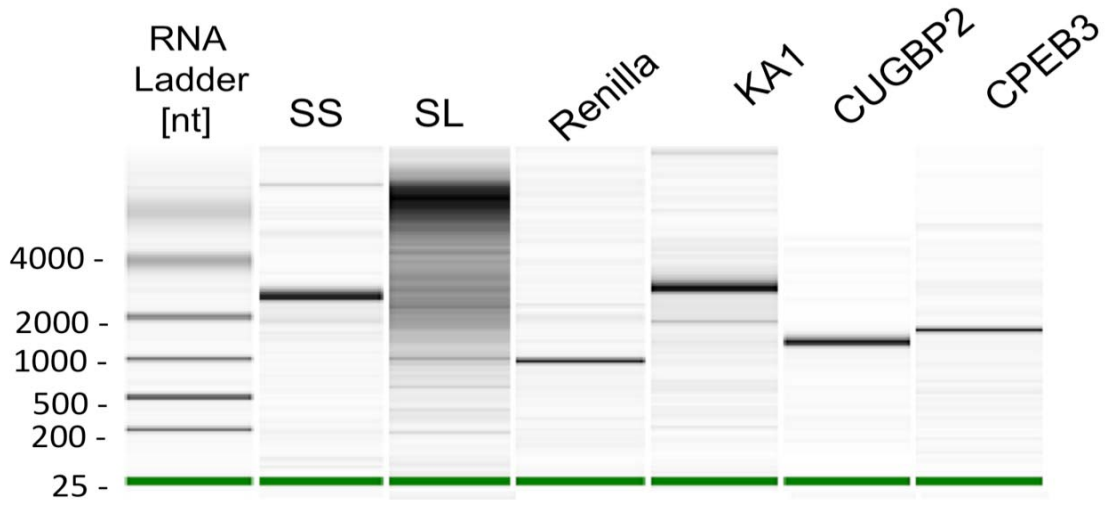

A.
coding
GluR2
Short 3'UTR
Short 5' UTR
GluR2
long 3'UTR
a b c d e f g h i j k l
1 2 3 4 5 6
B.
CUGBP2 elements
a. auuuaa
b. auuuaaaa
c. uaauuuaa
d. auuuauua
e. uauuuaa
f. aauuuaa
g. auuuau
h. aauuuaa
i. auuuaa
j. uauuuauu
k. uaauuua
C.
CPE's
1. UUUUAU
2. UUUUAU
3. UUUUUAU
4. UUUUUUUUAAU
5. UUUUAU
6. UUUUAU

Figure V-2 In vitro synthesized firefly reporter mRNAs bearing combinations of alternative GluR2 5'- and 3'UTRs mRNAs, and mRNAs encoding CUGBP2, CPEB, KA1 proteins were analyzed with an Agilent Bioanalyzer to determine the quality and the quantity of the RNAs.

Fig. V-2.


RNA
Ladder
[nt]
SS
SL
Renilla
KA1
CUGBP2
CPEB3
4000 -
2000 -
1000 -
500 -
200 -
25 -

FigureV-3 Xenopus oocytes were microinjected with either CUGBP2, CPEB3, KA1 (as an non-3'UTR interacting control) mRNAs (25 fmol/oocyte) or equivalent amount of vehicle (water). 20-40 hours later oocytes were injected with the firefly reporters. The injected oocytes were then homogenized by sonication and sedimented at 13,000xg. CUGBP2 and CPEB3 proteins were detected in pellet (P) and supernate (S) fractions, respectively, by western blot.

Fig. V-3

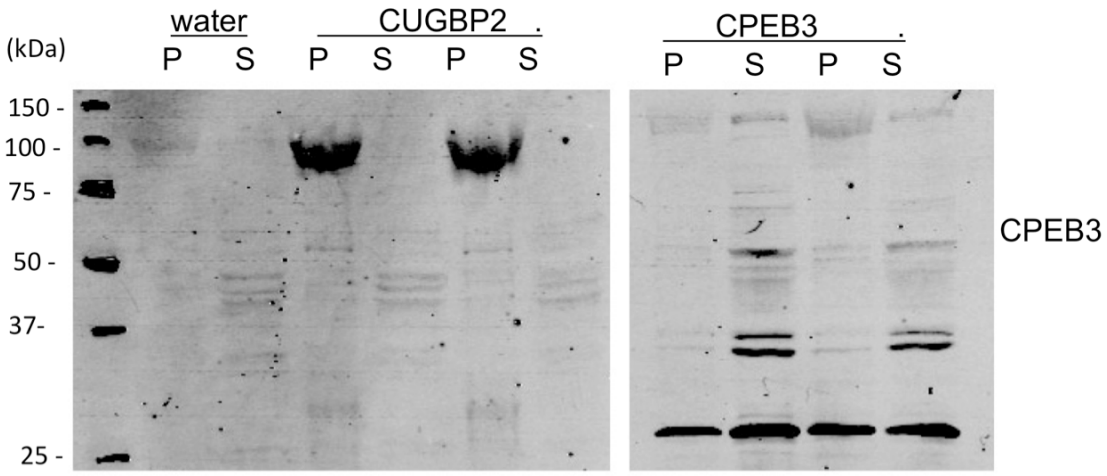


Figure V-4. Xenopus oocytes were injected with mRNA encoding CUGBP2, CPEB or KA1 (25 fmol/oocyte), and then 18-20 hrs later they received a second injection of the reporter mRNAs bearing short 5'- and short 3'-UTR (**SS**) of GluR2 (5 fmol/oocyte). At indicated time points, oocytes were individually homogenized and firefly reporter expression was measured as recorded luminescence unit (RLU).

Fig. V-4

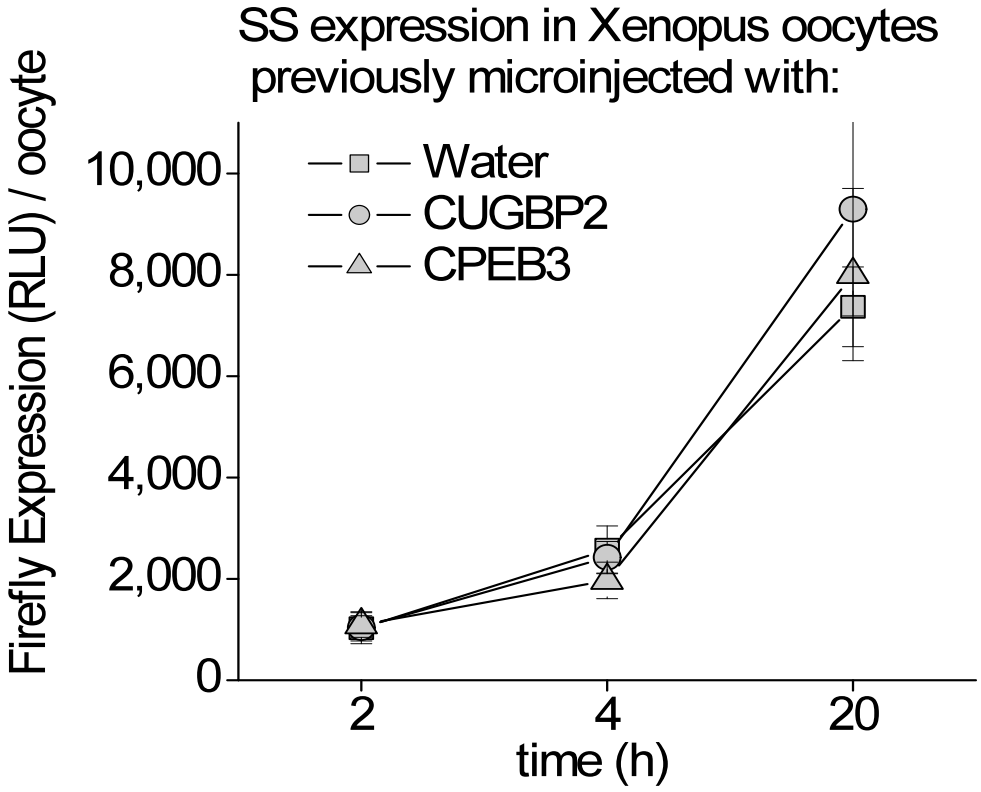

SS expression in Xenopus oocytes
previously microinjected with:
Water
CUGBP2
CPEB3
Firefly Expression (RLU) / oocyte
10,000
8,000
6,000
4,000
2,000
0
2
4
20
time (h)

Figure V-5 Xenopus oocytes were injected with mRNA encoding CUGBP2, CPEB or KA1 (25 fmol/oocyte), and then 18-20 hrs later they received a second injection of the reporter mRNAs bearing short 5'- and long 3'-UTR (**SL**) of GluR2 (5 fmol/oocyte). At indicated time points, oocytes were individually homogenized and firefly reporter expression was measured as recorded luminescence unit (RLU).

Fig. V-5

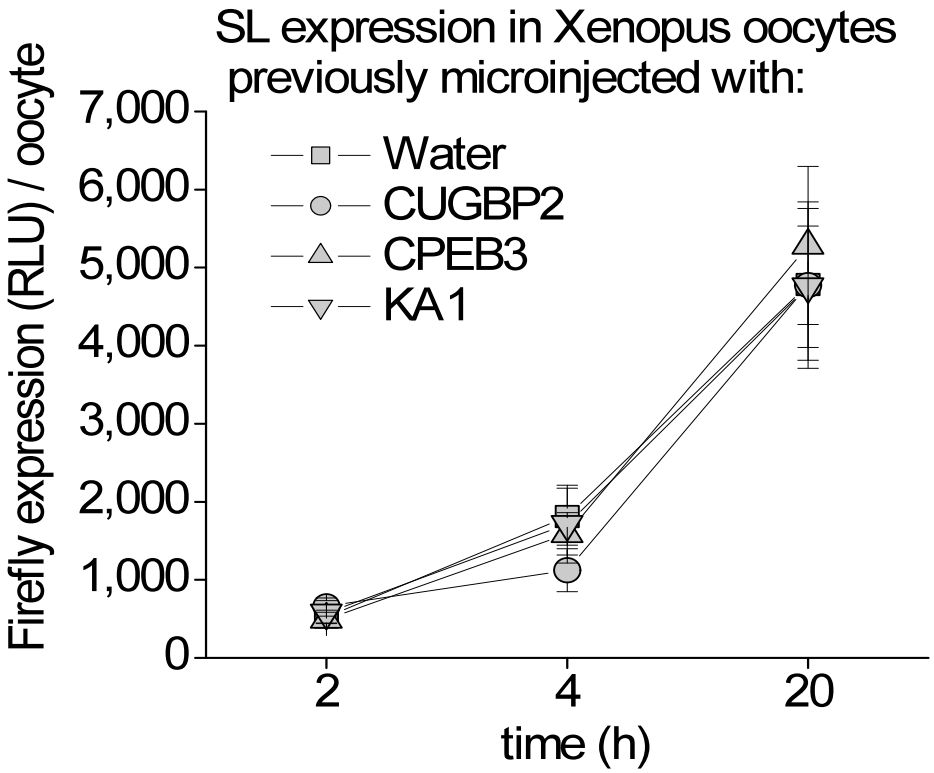
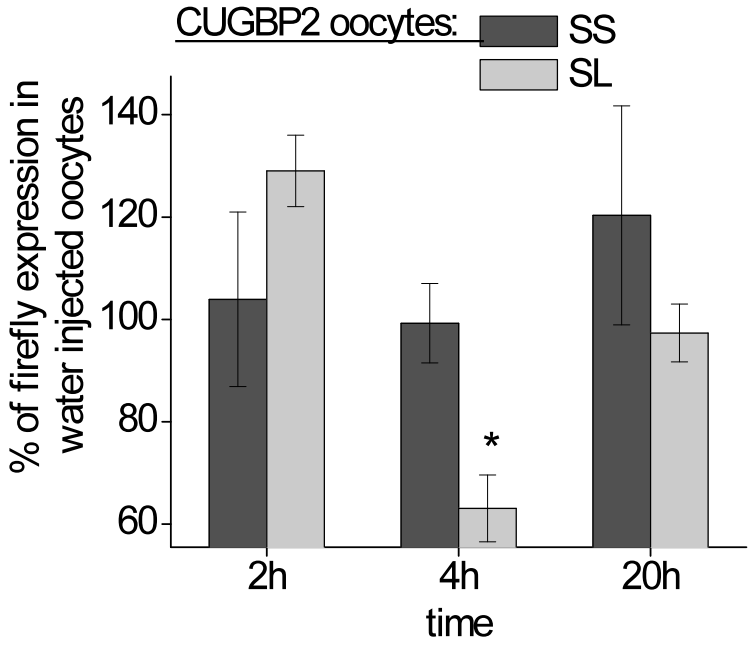

SL expression in Xenopus oocytes
previously microinjected with:
Water
CUGBP2
CPEB3
KA1
Firefly expression (RLU) / oocyte
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
2
4
20
time (h)

Figure V-6 Translation of the firefly reporters SS and SL were evaluated in the presence of CUGBP2. For individual firefly reporters, the expression of the firefly luciferase protein (RLU) was represented as a percent of the firefly reporters in water injected oocytes. (Means and standard error of the mean (sem) of n=5-9 independent experiments, * $p<0.05$ ANOVA, post hoc Bonferroni)

Fig. V-6

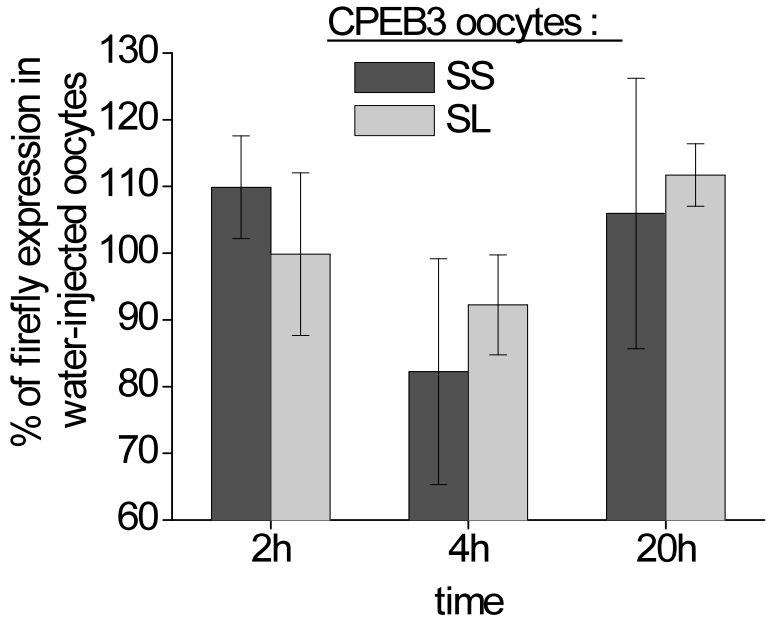

CUGBP2 oocytes:
SS
SL
% of firefly expression in water injected oocytes
140
120
100
80
60
*
2h
4h
20h
time

RNA-protein interactions in vivo (Niranjanakumari et al., 2002) could be a potential experiment to answer this question.

In this thesis study, the role of CPEB3 in regulation of GluR2 mRNA translation was also examined. CPEB3 is a (Cytoplasmic polyadenylation element binding) protein highly expressed in CNS. Similarly, kainate induced seizures strongly induce CPEB3 expression in mice hippocampus (within 1 hour in DG, CA1 and CA3, by 2 hrs only in CA1 and CA3 but not DG) (Theis et al., 2003). In *Xenopus* oocytes, over-expression of CPEB3 proteins **(Figure V-2 and V-3)** did not have a detectible effect on the translation of the luciferase reporters bearing alternative combinations of GluR2 **(Figure V-4, V-5 and V-7**). Previously, it has been shown that CPEB3 binds to long GluR2 in vitro (RNA-gel shift assay), and represses translation in cultured hippocampal neurons (Huang et al., 2006). It must be noted that the same study also showed that CPEB3 does not bind to CPE elements located in the GluR2 long 3'UTRs. The repressive effects of CPEB3 on the GluR2 mRNAs were not reproducible in the *Xenopus* oocytes (Figure V-7). One possible explanation for this result is that because CPEB3 is a neuron specific protein, it may require additional protein factors or signaling pathways specific to neuronal cells. It would be interesting to determine which GluR2 mRNA subpopulation is targeted by CPEB3-mediated translation repression in cultured hippocampal neurons in vivo. The preliminary findings gathered on some of the 3'UTR binding proteins are promising although further experiments are required.

Figure V-7 Translation of the firefly reporters SS and SL were evaluated in the presence of CPEB3. For individual firefly reporters, the expression of the firefly luciferase protein (RLU) was represented as a percent of the firefly reporters in water injected oocytes.

Fig. V-7


CPEB3 oocytes :
SS
SL
% of firefly expression in water-injected oocytes
130
120
110
100
90
80
70
60
2h
4h
20h
time

b. Determining the GluR2 mRNAs bearing predominant combinations of 5'- and 3'UTRs in rat hippocampus.

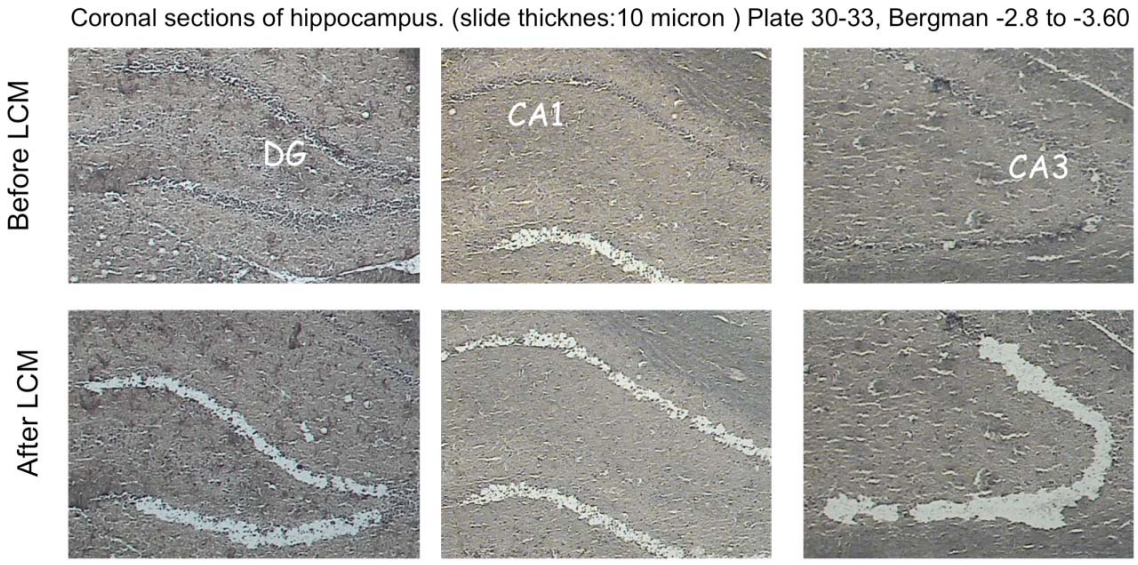
Translation of GluR2 transcripts are differentially mediated by the alternative UTRs. From the *in situ* hybridization studies, it appears that GluR2 mRNAs bearing long 3'UTRs, which are translationally restricted, are detected in all three specific regions (CA1, CA3 and DG) of the hippocampus. What is the abundance of translationally restricted GluR2 transcripts relative to the unrestricted GluR2 mRNAs, which bear shorter UTRs, in the specific regions of the rat hippocampus? During the course of this thesis research, two different approaches have been taken to address this question. As a first approach, the Laser-Capture Microdissection (LCM) technique was used to determine region-specific distribution of GluR2 mRNAs in CA1, CA3 and DG regions. The second approach involved separation of rat total hippocampal RNAs by their size using agarose-gel electrophoresis followed by gel extraction of RNA and quantification by Q-RT-OCR.

For LCM analysis, whole hippocampi were dissected from the adult rats and flash-frozen in an embedding media on dry-ice and then stored at -80^{0}C freezer. The 10micron coronal sections of the whole hippocampus (stereotaxic coordinates;Bergman -2.80 to -3.6, and plates 30-33 on a rodent map) were cut using a cryostat (LEICA LM 1850) at -20^{0}C and mounted on sterile microscope slides. Neuronal cell bodies from the specific hippocampal regions were micro-dissected via the LCM **(Figure V-8)** onto a cap device from which the total RNAs were extracted using extraction buffers provided with PicoPure RNA Isolation

Assay kit (Arcturus KIT0202). The RNAs were reverse transcribed to cDNAs using Thermoscript Reverse transcriptase (Invitrogen).

Figure V-8 For Laser Captured Microscopy (LCM) analysis, the whole brain was dissected from the adult male rats and flash-frozen in an embedding media on dry-ice and then stored at -80^{0}C freezer. The 10micron thick coronal sections of the whole hippocampus (stereotaxic coordinates; Bergman -2.80 to -3.6, and plates 30-33 on a rodent map) were cut using a cryostat (LEICA LM 1850) at -20^{0}C and mounted on sterile microscope slides. Images were taken before and after capturing the neuronal cell bodies from CA1 (A vs. B), CA3 (C vs. D) and DG (E vs. F) onto a cap device.

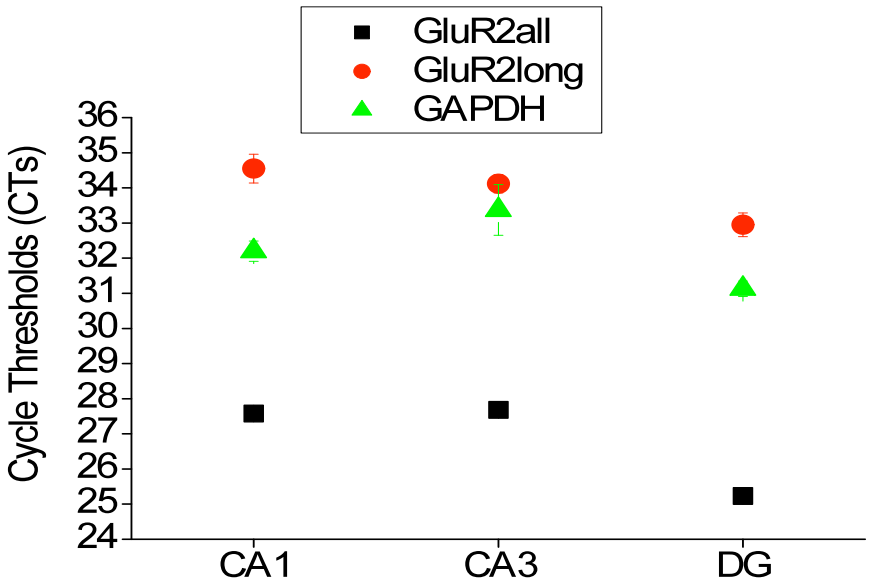
Fig. V-8


Coronal sections of hippocampus. (slide thicknes:10 micron) Plate 30-33, Bergman -2.8 to -3.60
Before LCM
After LCM
DG
CA1
CA3

The resulting cDNAs were quantified by Q-RT-PCR using 3'UTR-specific primers. The GluR2 mRNAs bearing long 3'UTRs were detected in all three hippocampal regions **(Figure V-9).** However, it was not possible to determine the abundance of GluR2 mRNA bearing long 3'UTRs relative to the pan GluR2 transcripts in these regions due to low RNA yield from the captured cells, and incompatibility between the efficiencies of the long 3'UTR- and short 3'UTR-specific Q-RT-PCR primers. Previously The LCM technique has been successfully used in determining changes in gene expression patterns in various brain regions (Borges et al., 2007; Haqqani et al., 2005; Mojsilovic-Petrovic et al., 2004). Once properly optimized for the experimental conditions, the LCM could still be a feasible approach for determining the relative abundance of the GluR2 mRNAs bearing long 3'UTR in cell bodies (soma) vs. dendrites. Use of single primer pairs specific to the GluR2 long 3'UTR, careful identification of the regions on the hippocampal sections that are enriched in dendrites, and proper selection of internal controls (e.g β-actin, GAPDH) may increase the efficiency of this approach in determining the relative abundance of GluR2 subunits bearing alternative UTRs. The second approach to determine the relative abundance of GluR2 mRNAs with alternative 5' and 3'UTRs involved separation of the total RNA under denaturing conditions by size using an agarose-gel electrophoresis. Once denatured in 2.2M formaldehyde at 65^0C for approximately 10 min, the large denatured RNA molecules travel slower than the smaller RNAs through the porous gel matrix (e.g. 2% low-melt agarose gel) when an electric current applied to the matrix under buffered conditions (Maniatis and Efstratiadis, 1980). Such

Figure V-9. Quantification of mRNAs extracted from neuronal cell bodies captured by laser-capture microdissection from the specific hippocampal regions. Total RNAs from the indicated regions were reverse transcribed to cDNAs by Thermoscript reverse transcriptase. The resulting cDNAs were amplified using primers specific to GluR2 all, GluR2 long and GAPDH mRNAs. Cycle thresholds (CTs) are inversely correlated to the starting amount of cDNA prior to amplification.

Fig. V-9.


GluR2all
GluR2long
GAPDH
Cycle Thresholds (CTs)
36
35
34
33
32
31
30
29
28
27
26
25
24
CA1
CA3
DG

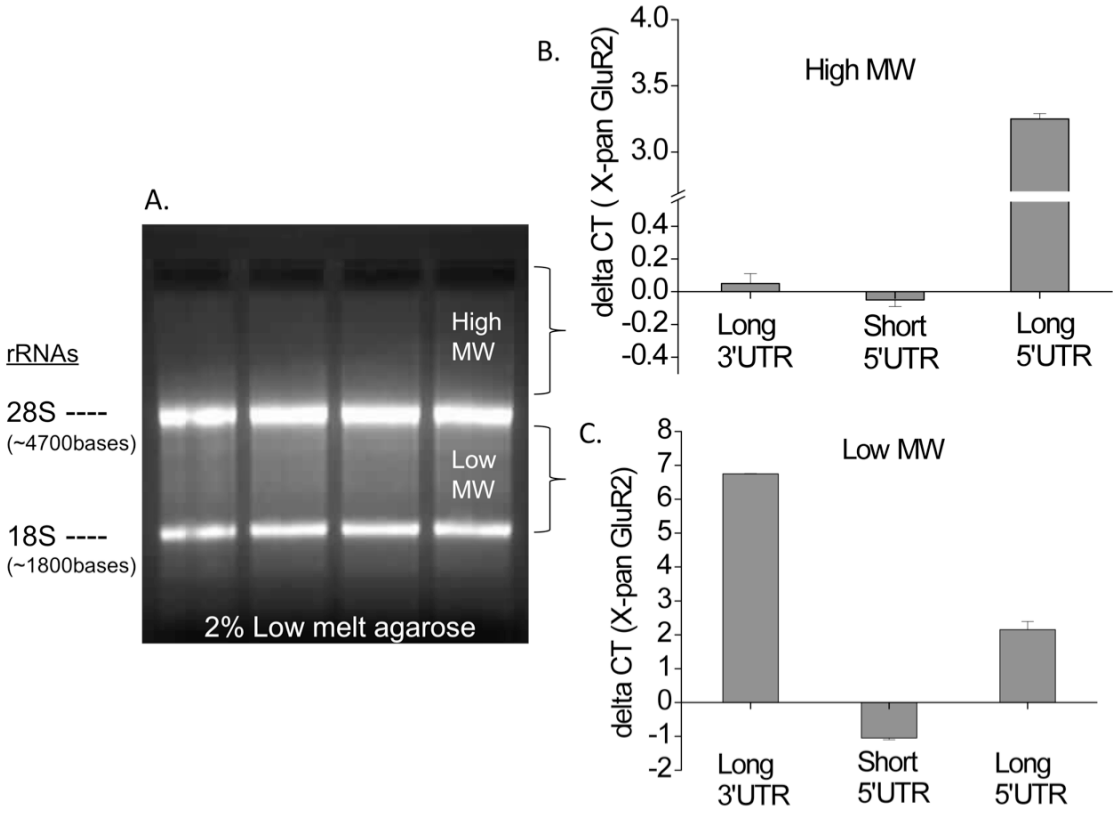
separations of the denatured RNAs (1-10µg/well) in the gel matrix for 1 to 2 hours at a constant electric potential of 100V results in clearly visible bands of 28S and 18S ribosomal RNAs (rRNA) (~4700 and ~1800 bases, respectively) under the short wave (254nm) ultraviolet light exposure. During the course of this thesis research, preliminary experiments were performed to extract RNA from the agarose gel. RNAs from total rat hippocampal homogenates were purified via a standard phenol-chloroform extraction. On-column DNAse treatment of total RNA was carried out using an RNA-purification column (PureLink RNA mini, Invitrogen, cat# 12183-018A) to eliminate potential genomic DNA contamination. Purified and DNAse-treated total RNAs were denatured as described above, and then applied onto a 2% low-melt agarose gel for separation.

A clearly visible separation of 28S and 18S ribosomal RNA bands (~4700 bases and ~1800 bases, respectively) was observed on the gel (Figure V-10). The total RNAs above the 28S rRNA band (high molecular weight region), which are most likely to contain GluR2 mRNAs bearing long 3'UTRs (~6kb), and the total RNAs between the 28S and 18S rRNAs (lower molecular weigth region), which are likely to contain GluR2 mRNAs bearing shorther 3'UTRs (~4kb), were excised from the gel with a surgical scalpel. The total RNA was extracted from the excised sections using a gel extraction buffer and RNA purification columns. The gel- purified RNAs were then reverse transcribed to cDNAs by Thermoscript Reverse transcriptase. The resulting cDNAs were amplified using 5' and 3'UTR-specific primers by Q-RT-PCR. Quantification of specific GluR2 transcripts in the

high and low MW areas of the agarose gel was calculated as cycle threshold (CT) values and normalized to the CT (ΔCTs) values of pan GluR2 from the same regions. GluR2 mRNAs bearing short 5' and long 3'UTRs constitute the majority of the pan GluR2 mRNAs detected in the high MW region (**Figure V-10B**). The low MW area of the agarose gel contains GluR2 mRNAs bearing long 5' and short 3'UTRs with little or no GluR2 mRNAs bearing long 3'UTRs. This approach would provide valuable information about the composition of endogenous GluR2 mRNAs bearing alternative UTRs in the rat hippocampus. However, the main caveat is that very low amounts of long 3'UTRs are still detected in the low MW area of the gel complicating the interpretation of the results. This could possibly be due to partial degradations of RNAs or the inefficiencies in *in vitro* reverse transcription of full length GluR2 mRNAs, which would result in amplification of fragmented long 3'UTRs in the low MW area.

Figure V-10. Determining predominant GluR2 mRNAs bearing alternative 5' and 3'UTRs. **(a),** Total RNAs isolated from rat hippocampal homogenates were separated on 2% low-melt agarose gel by electrophoresis. The ribosomal RNAs (28S and 18S) were visualized under short wave UV light exposure to determine relative location of the GluR2 trancripts. Area above the 28S (high MW) constitute mRNAs longer than 4700bases, while the area between 28S and 18S (low MW) constitute mRNAs smaller than 4700 bases. The mRNAs from **(b)** the high MW area or **(c)** the low MW area of the agarose gel were extracted and were reverse transcribed to cDNAs by Thermoscript reverse transcriptase. Resulting cDNAs were amplified in a Q-RT-PCR reaction using primers specific to GluR2 UTRs. Cycle threshold values are inversely correlated with the amount of GluR2 cDNA. Delta Cycle threshold (ΔCT) values indicate mRNA quantities relative to pan GluR2.

Fig. V-10.


A.
rRNAs
28S ----
(~4700bases)
18S ----
(~1800bases)
High
MW
Low
MW
2% Low melt agarose
B.
High MW
delta CT (X-pan GluR2)
4.0
3.5
3.0
0.4
0.2
0.0
-0.2
-0.4
Long
3'UTR
Short
5'UTR
Long
5'UTR
C.
Low MW
delta CT (X-pan GluR2)
8
7
6
5
4
3
2
1
0
-1
-2
Long
3'UTR
Short
5'UTR
Long
5'UTR

c. What is the mechanism underlying seizure-induced translational de-repression of GluR2 mRNAs that are translationally restricted due to presence of long 5' or long 3'UTRs?

Our observation that seizures induce a translation de-repression of GluR2 mRNAs bearing long 3'UTRs in rat hippocampus (Irier et al., 2009) suggested that an activity dependent cell signaling mechanism which specifically targets the GluR2mRNAs with long 3'UTRs exists in the brain. Moreover, the observation that translational repression of GluR2 transcripts bearing long and structured UTRs could also be reversed pharmacologically *in vitro* provides further evidence that the transcript-specific regulation of GluR2 mRNA is mediated by the presence of alternative combinations of 5' and 3'UTRs. Activity-dependent transport of GluR2 mRNAs from soma to denrites and their translation in mechanically isolated dendrites have been reported (Ju et al., 2004). These observations collectively raise the following questions; (i) Are the activity-dependent transport and dendritic translation of GluR2 mRNAs in neurons UTR-specific?; (ii) Do GluR2 mRNAs bearing long 3'UTRs exist in dendrites?; (iii) Does the translational de-repression of GluR2 mRNAs bearing long and structured UTRs occur in dendrites upon neuronal activity?

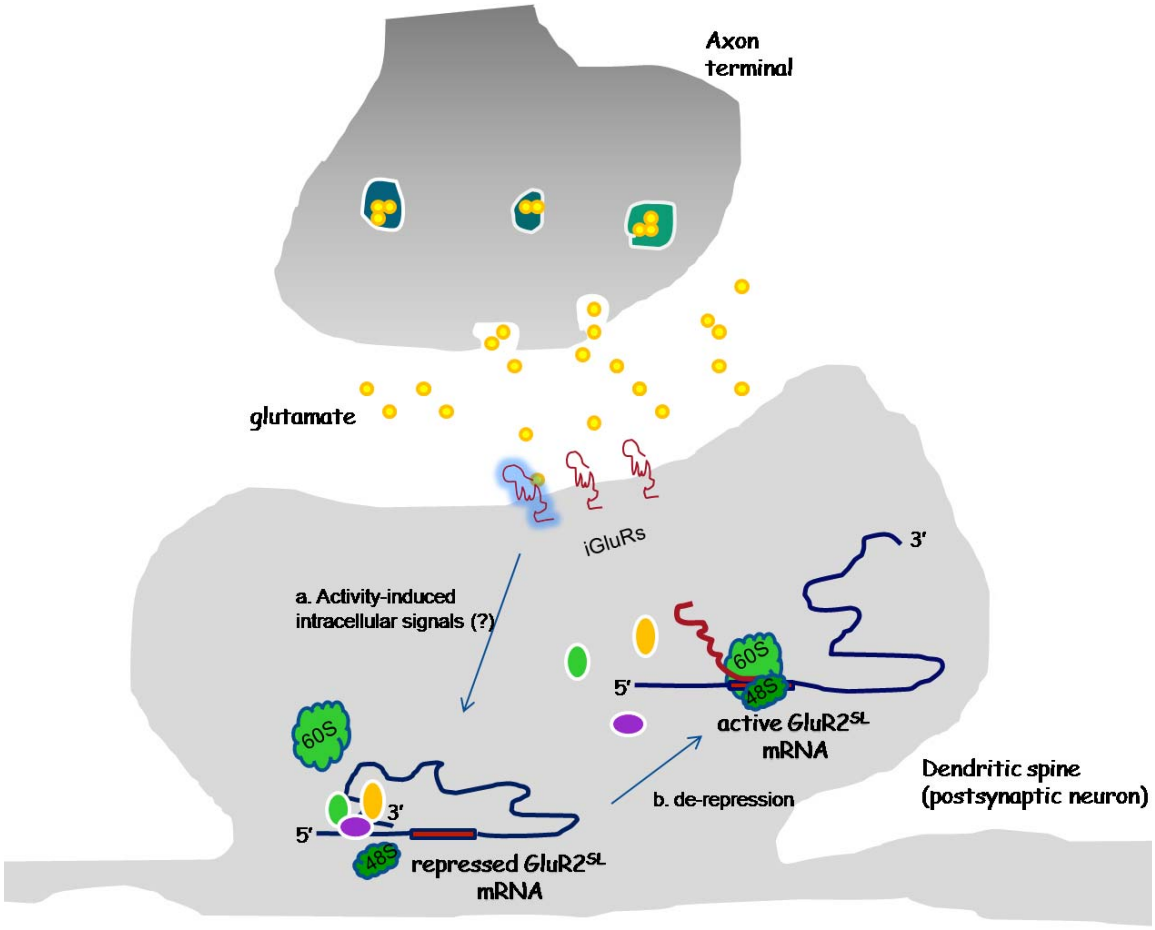
C. Implications

In this study, I have demonstrated that the translation of GluR2 mRNAs bearing long 3'UTRs are repressed, that the translational de-repression of the GluR2 mRNAs with long 3'UTRs occurs 24 hours after pilocarpine-induced status

epilepticus in rat hippocampus, and that de-repression of the translationally restricted GluR2 mRNAs bearing structured and long UTRs could also be pharmacologically induced **(Figure V-11**). An overall implication of these findings is that understanding the mechanisms of translation regulation of GluR2 expression could have a real impact in drug discovery process for neurological disorders (e.g epilepsy, memory and learning disabilities) where alterations of GluR2 expression result in significant physiological consequences.

Figure V-11. A schematic of the translational de-repression of translationally restricted GluR2 mRNAs in a dendrite of a postsynaptic neuron. A, Pre-synaptically released excitatory neurotransmitter glutamate activates the glutamate receptors on the post-synaptic membrane. Intracellular signals generated as a result of the glutamate receptor activation may potentially target translation of GluR2 mRNAs bearing long 3'UTRs at the translation initiation. B, Intracellular signals specifically targeting translationally restricted GluR2 mRNAs de-repress the translation of these transcripts, which can also be achieved pharmacologically.

Fig. V-11


Axon terminal
glutamate
iGluRs
a. Activity-induced intracellular signals (?)
60S
48S
5′
3′
repressed GluR2SL mRNA
b. de-repression
active GluR2SL mRNA
Dendritic spine (postsynaptic neuron)

Chapter VI: References

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