Translational Control of New Protein Synthesis at Activated Synapse

: Role of Synaptic Polyadenylation of Pre-Existing mRNAs

Chan young Shin, Ph.D.
Assistant Professor
Center for Geriatric Neuroscience Research and Department of Pharmacology
School of Medicine, Konkuk University

Cognitive deficit and learning impairment is one of the hallmark features of several forms of neurological diseases such as Alzheimer's disease (AD) and Fragile X mental retardation syndrome (FXS). To gain a better control of the mental retardation and age-related cognitive deficit, it is crucial to understand the mechanisms underlying learning and memory. It is more than obvious that synapses are basic units of neural information processing. Synapses utilize various structural and biochemical strategies to efficiently transmit a signal from an input to a target. Synapses are also equipped with biochemical and morphological methods to adjust their signaling properties according to the previous history of neural stimulation. This use-dependent changes in synaptic efficacy; also called synaptic plasticity is a basis of information processing and storage in the brain. The synaptic plasticity has two different temporal components. One is short-term synaptic plasticity, which occurs within milliseconds to minutes after appropriate synaptic stimulation and lasts only transiently at best up to 1-2 hours. While this type of synaptic plasticity does not require new protein synthesis at the activated synapse, which means the modification of existing protein is sufficient to mediate the required synaptic changes, the other type of synaptic plasticity called long-

term synaptic plasticity is essentially dependent on the new protein synthesis. Long-term synaptic plasticity lasts days to years and maybe during the last of the life of an individual (Lisman and Fallon, 1999) and is generally accepted as a mechanistic basis for the memory storage in the brain.

Regarding the protein synthesis dependency of long-term synaptic plasticity, several immediate questions are arising. For example, what are the proteins newly synthesized after synaptic stimulation and what is the role of those proteins in establishing and maintaining synaptic plasticity? Another obvious question is the mechanism of the regulation of new protein synthesis at the activated synapse. This is very important considering the highly polarized structure of mammalian neurons. There exist approximately ten thousands synapses per average excitatory neurons and in extreme cases one hundred thousands synapses in neurons like cerebellar Purkinje cells. How a neuron can achieve specificity of new protein synthesis only at the activated synapses among tens of thousands of other inactive synapses? Neurons utilize at least three strategies for the activity dependent regulation of protein synthesis and targeting. First, some proteins are translated in the soma from newly transcribed mRNAs. For example, transcription factors such as CREB (cAMP-response-element-binding protein) and C/EBP (CCAAT enhancer binding protein) are activated in response to particular forms of synaptic stimulation (Alberini et al., 1994). The mechanism to regulate the transport of newly synthesized proteins to a remote synaptic site is not fully understood yet but several researches suggest the involvement of the formation of a kind of "tag" at the activated synapse to specifically recruit the newly synthesized proteins (Frey and Morris, 1997). Second, newly transcribed mRNAs may be transported to activated

synapses, where they are translated. This mechanism has been recently described for *Arc*, an immediate-early gene whose transcription is tightly regulated by synaptic activity (Steward et al., 1998). It has been also reported that the transport of alpha-CAMKII mRNA to activated synapses increased after neural stimulation both in mammals and Drosophila (Rook et al., 2000; Ashraf et al., 2006), which is regulated by specific cis-element(s) in its 3'-UTR. It is also acknowledged that the transport of mRNA containing granules to denritic spine is facilitated by neural activity (Krichevski and Kosik, 2001). The supply of mRNAs into the dendritic region is presumably accompanied by concomitant protein synthesis upon their arrival at the appropriate target synaptic region. The third mechanism regulating the synaptic protein synthesis is the regulation of protein translation using mRNAs existing at the synapse before the arrival of synaptic activation. This hypothesis is originated from the fact that almost all the constituents required for the protein translation such as mRNAs, tRNA, ribosomes, initiation and elongation factors and even satellite protein secretory pathways are localized in and around the dendritic spine (Steward and Levy, 1982: Tiedge and Brosius, 1996; Kleiman et al., 1990; Pierce et al., 2001). Several different family members of RNA binding proteins have been also reported to exist at synapses including fragile mental retardation protein (FMRP), staufen and cytoplasmic polyadenylation binding proteins (CPEB), which might be important in the regulation of mRNA transport, stability and translational efficiency. The local regulation of new protein synthesis from existing mRNAs has temporal and spatial advantages compared with global control of new protein synthesis combined with specific targeting. Actually, it has been suggested to be critical in protein synthesis dependent phase of long-term synaptic plasticity (Kelleher et al., 2004; Klann et al., 2004).

Even though there are considerable recent advances in this field, the mechanisms mediating local translational control of dormant mRNAs at activated synapses are not clear yet. The most well known molecular mechanism regulating local translation is CPEB dependent, polyadenylation-induced translation of target mRNAs. The regulation of mRNA translation by CPEB is first known in germ cells (Richter, 2001; Wells et al., 2000). In the *Xenopus* oocyte, progesterone stimulation leads to phosphorylation of xCPEB by Aurora-A kinase and translation of CPE containing mRNAs such as cmos, cyclin B1 and cdk2, which lead to the proper cell cycle progression (Stebbins-Boaz et al., 1999). In hippocampal neurons, CPEB1 regulates the synthesis of the subunit of calcium/calmodulin dependent kinase II (CaMKII) following glutamate receptor activation (Huang et al., 2002; Wells et al., 2001; Wu et al., 1998). The CaMKII mRNA translation was induced following the activation of the N-methyl-D-aspartate (NMDA) type glutamate receptor (Wells et al., 2001). In addition to the Aurora-A kinase, CaMKII is capable of phosphorylating CPEB on a site critical to its activation (T^{171}, S^{174}), which leads to CPEB-dependent protein-synthesis (Atkins et al., 2004). The same group of authors also suggested that the balance between CaMKII and protein phosphatase 1 (PP1) activity is important during hippocampal LTP induction (Atkins et al., 2005). This suggests that independent mechanisms can regulate CPEB-mediated protein synthesis in the same cell.

During development, light exposure induces massive synaptic activation in the visual cortex that leads to functional and structural synaptic plasticity. In such paradigms, light stimulation induced rapid, synaptic polyadenylation of mRNA encoding CaMKII followed by new protein synthesis of CaMKII by a translational

mechanism (Wu et al., 1998). This suggested a role for CPEB in synaptic plasticity. Indeed, knockout of CPEB-1 caused a modest deficit in certain forms of LTP and LTD in the mouse hippocampus and hippocampus-dependent learning (Alarcon et al., 2004; Berger-Sweeney et al., 2006).   In Aplysia, a neuron-specific isoform of CPEB regulates the synthesis of   -actin mRNA following synaptic activity (Si et al., 2003). At activated synapses, Aplysia CPEB protein is up regulated locally, which is needed for the stable maintenance of long-term facilitation (Si et al., 2003).

One of the most important questions regarding CPEB dependent local translational control in neuron is the identification of the complement of the mRNAs regulated by CPEB and delineation of its function at activated synapses. The cis-elements where CPEB can bind are composed of CPE (UUUUUAU) and polyadenylation signal (AAUAAA).   The distances between CPE and hexanucleotide sequences confer additional constraint for the CPEB binding.   Those two elements should be within 100 nt from each other (Mendez et al., 2002), even though it's not clear whether physical or spatial proximity is more important.   Bioinformatic searches based on these criteria revealed hundreds of candidate target mRNAs, which are important in synaptic transmission, structural organization, cell to cell interaction, migration, apoptosis and cell cycle regulation (data not shown).   Among these, we are especially interested in the tissue plasminogen activator (tPA) for several additional reasons not to mention the existence of CPE and hexanucleotide sequences in it's 3'-UTR.   First, tPA knock out mice or the treatment of tPA inhibitor prevented late phase long-term potentiation (LTP), which is a widely accepted model of long term synaptic plasticity.   Likewise, the over-expression of tPA increased L-LTP.   Second, tPA is also involved in long term depression (LTD) induction, which is another well-known model

of long-term synaptic plasticity.   Third, a high concentration of tPA is toxic to neuron

and it is rapidly released from neuron after stimulation, which suggests the local

concentration of tPA at activated synapse should be rigorously regulated.   Fourth, an

unidentified protein regulates polyadenylation and translation of tPA mRNA in mouse

oocytes.

To investigate whether tPA mRNA is regulated by CPEB-dependent

polyadenylation at activated synapse, we first tried to determine the subcellular

localization of tPA and tPA mRNAs.   tPA is released from presynaptic terminal upon

neural stimulation.   However, it is not clear yet whether it is expressed and released

from dendritic site, where translational control of dormant mRNA is supposed to occur.

Immunoreactivity of tPA is localized in dendrite.   Some of the immunoreactive

puncta colocalized with synaptic marker protein synaptophysin.   These data suggest

that tPA is localized in dendrite as well as in axon and some of the dendritic tPA is

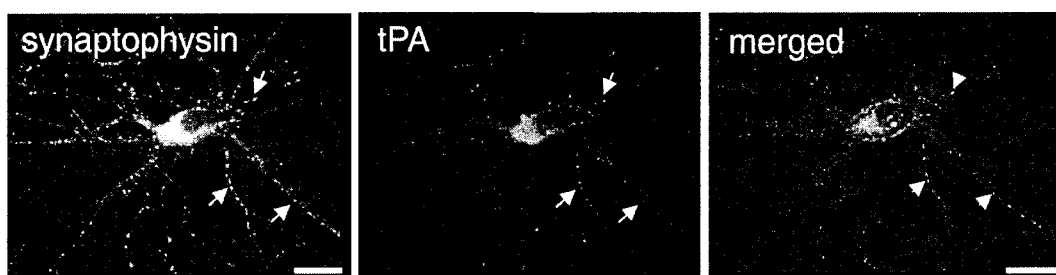localized at the synapse presumably postsynaptically (Fig. 1).

Figure 1. Figure 1. Dendritic localization of tPA.   Cultured rat hippocampal neuron was
stained for a synaptic marker synaptophysin and tPA.   Arrows indicate tcolocalization
of synaptophysin and tPA.

To examine the localization of tPA mRNA in dendrite, indirect fluorescent in

situ hybridization (FISH) was performed using probes specific for tPA mRNA.

Specific tPA mRNA signals were found in dendrite.   The signal density was usually

Figure 2. tPA-FISH
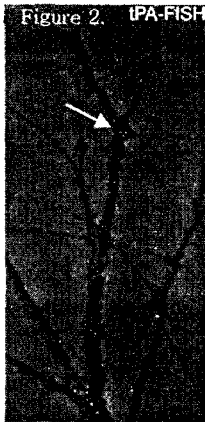
high in dendritic branch point, which might suggest that the transport of tPA mRNA along the dendrite is a regulated process (Fig. 2. look an arrow indicating tPA FISH signal).

Next, we checked the release of tPA from the dendritic side. Cultured primary rat hippocampal neurons were transfected with green fluorescent protein labeled tPA (GFP-tPA). Consistent with the immunocytochemistry results, transfected tPA was localized in dendrite as a punctate pattern, which might suggest that tPA is localized within transport and/or secretory granules. Stimulation of the neuron with glutamate rapidly decreased dendritic tPA signal, which suggests the loss of tPA from dendritic side by mechanisms like exocytosis (Fig. 3).
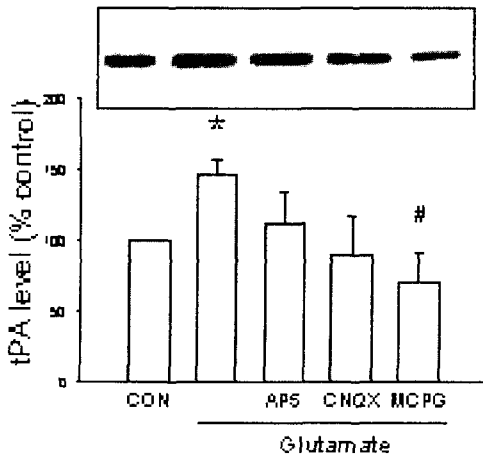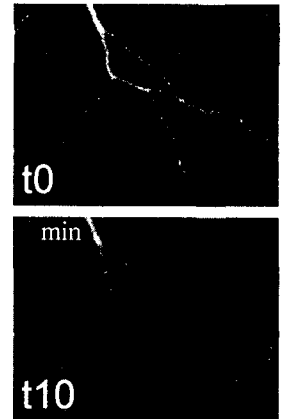

Figure 3. Glutamate Induced tPA release.

Figure 4. An mGluR antagonist MCPG inhibits glutamate induced tPA increase from synaptoneurosome.

Stimulation of the cultured neuron and isolated synaptoneurosome fraction rapidly increased tPA level, which is dependent on translation but not transcription. Interestingly, polyadenylation inhibitor cordycepin inhibited the increase of tPA level. To identify glutamate receptor subtypes involved in glutamate induced tPA increase, various antagonists were used to block the effect of glutamate. MCPG, a specific antagonist against metabotropic glutamate receptor (mGluR) blocked glutamate

induced tPA increase. In addition, a specific group I mGluR agonist DHPG alone increased tPA level both in cultured neuron and isolated synaptoneurosome (Fig. 4). These data suggest that neuronal stimulation increases tPA synthesis at local synapse by mGluR dependent translational control.

To determine the length of poly A tail of tPA mRNA, RT-PCR based polyadenylation test (PAT assay) was employed. Glutamate stimulation increased poly A tail length within 2 minutes of stimulation and it was mGluR activation dependent (Fig. 5).

tPA

glutamate	—	+	+	+
CRDCP	—	—	+	—
MCPG	—	—	—	+

Figure 5. Glutamate induced polyadenylation of tPA is inhibited by cordycepin and MCPG. Smearing represents polyadenylation

Finally, the association of tPA mRNA with CPEB was investigated. Recombinant glutathione S-transferase (GST) fused CPEB was mixed with brain lysate and full down with glutathione-agarose. TPA mRNA but not negative control mRNA coding glial fibrillary acidic protein was recovered from precipitate. Immunoprecipitation using an antibody specific against CPEB also gave similar recovery of tPA mRNA but not GFAP mRNA.

Taken together, CPEB regulates tPA mRNA translation at activated synapses by regulating the extent of polyadenylation, which is mediated by mGluR activation. Identifying mRNA targets for local synaptic translational control and understanding the mechanism of their translational regulation will contribute not only to the acquisition of better information for the mechanisms regulating synaptic plasticity but also to the development of methods to modulate the learning and memory impairments both in pathological and normal aging situations
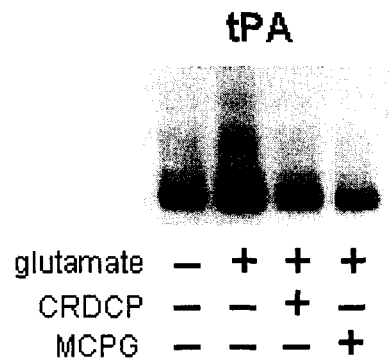
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