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Proteasome regulates the mediators of cytoplasmic polyadenylation signaling during late-phase long-term potentiation

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Abstract

The ubiquitin-proteasome pathway is essential for long-term synaptic plasticity, but its exact roles remain unclear. Previously we established that proteasome inhibition increased the early, induction part of late-phase long-term potentiation (L-LTP) but blocks the late, maintenance part. Our prior work also showed that the proteasome modulates components of the mammalian target of rapamycin pathway for translation. In this study, we tested the possible role of the proteasome in regulating the cytoplasmic polyadenylation signaling required for translation during L-LTP. We found that a polyadenylation inhibitor cordycepin diminishes the enhancement of early L-LTP mediated by proteasome inhibition. Furthermore, blocking Aurora-A kinase and calcium-calmodulin-dependent kinase II reduces the increase in early L-LTP brought about by proteasome inhibition. Our results suggest a link between polyadenylation-mediated translational control and protein degradation during induction of long-term synaptic plasticity.

1. Introduction

Long-term synaptic plasticity, which underlies long-term memory, depends on new protein synthesis through gene transcription and translation of newly transcribed genes [1;2]. The induction of long-term synaptic plasticity in the mammalian central nervous system also requires local translation of pre-existing mRNAs in dendrites which is thought to confer synapse-specificity of plasticity [3]. Many studies in the past several years have indicated that protein degradation by the ubiquitin-proteasome pathway (UPP) has a role in long-term synaptic plasticity and long-term memory [4–6]. Previously, using hippocampal late-phase long-term potentiation (L-LTP) in the mouse as a model system, we showed that protein degradation has opposite roles with respect to L-LTP induction and maintenance [7]. We showed that proteasome inhibition enhances the induction part of L-LTP but blocks maintenance. Our results suggested that under normal physiological conditions, the

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proteasome limits L-LTP induction by degrading proteins that are locally translated from pre-existing mRNAs in dendrites but promotes L-LTP maintenance by facilitating transcription in the nucleus. There are two major signaling cascades that regulate local protein synthesis in dendrites. One is the signaling pathway mediated by mammalian target of rapamycin (mTOR) [8]. The second is the cytoplasmic polyadenylation signaling pathway [9]. We recently established that proteasome inhibition stabilizes the proteins whose synthesis is regulated by the mTOR pathway [10]. In this study, we tested the possible role of the cytoplasmic polyadenylation signaling pathway.

The translation of a subset of mRNAs in dendrites depends on extension of the poly-A tail in their 3' untranslated regions, which occurs in the cytoplasm and is thus termed cytoplasmic polyadenylation [11–13]. The lengthening of the poly-A tail depends on a nucleotide sequence (typically UUUUUAU) called the cytoplasmic polyadenylation element (CPE). A protein called CPE-binding protein (CPEB) plays a key role in regulated polyadenylation of a subset of mRNAs and thus affects their translation [14].

CPEB plays a key role in activity-dependent local translation of mRNAs in dendrites [15]. NMDA receptor (NMDA-R)-mediated signaling regulates polyadenylation of mRNAs in dendrites [16]. NMDA-R activates Aurora-A kinase which phosphorylates CPEB. In the non-phosphorylated state, CPEB is bound by neuroguidin (or maskin in non-neuronal cells) which in turn is bound to the translation initiation factor eIF4E. The binding of neuroguidin prevents eIF4E from binding to eIF4G and since the interaction of eIF4E and eIF4G is critical for driving the translation forward, translation remains repressed. For relief of translational repression by neuroguidin, polyadenylation of the mRNAs needs to occur which is a highly regulated process. Regulation of polyadenylation requires two sequence elements in the 3'-UTR of the mRNAs, a hexanucleotide sequence AAUAAA and the CPE. When CPEB bound to the CPE is phosphorylated, it recruits cleavage and polyadenylation specificity factor (CPSF) to the nearby polyadenylation hexanucleotide. CPSF enables poly-A addition to the mRNA by poly-A polymerase. Poly-A binding protein (PABP) binds the elongated poly-A tail and recruits eIF4G. The PABP-eIF4G dimer helps dislodge neuroguidin from eIF4E and allows the interaction of eIF4E with eIF4G which initiates translation [17]. CPEB is also a substrate for Calcium-calmodulin-dependent protein kinase II (CaMKII) [18].

2. Materials and Methods

2.1. Hippocampal slice electrophysiology

We used C57/Bl6 mice obtained from Harlan Laboratories (Frederick, MD, USA) for experimental protocols approved by the Institutional Animal Care and Use Committee of Wake Forest University Health Sciences. For L-LTP experiments we prepared transverse hippocampal sections (400 μ m) from 6 to 12-week-old male mice using a tissue chopper in an oxygenated and chilled artificial cerebrospinal fluid (ACSF) as described previously [7]. All recordings were obtained from slices maintained at 32°C. We used theta-burst stimulation (TBS) protocol to induce L-LTP. The stimulation protocol consisted of 4 pulses at 100 Hz, with bursts repeated at 5 Hz and each tetanus including 3 ten-burst trains separated by 30 seconds [7]. The same stimulus was repeated after 5 min. For L-LTP

experiments on isolated dendrites, we separated the dendrites by means of a surgical cut to the slice which has been shown to be effective [7;19–21]. We placed the hippocampal slices in ice-cold ACSF containing sucrose instead of NaCl and MgCl₂ instead of CaCl₂ (replacement chemicals were of same molarity), and made a cut below the cell body layer of CA1 region. The slices were then placed in ACSF and allowed to recover for 2 h before the experiment. L-LTP was induced as described previously [7;22].

2.2. Pharmacological reagents

Cordycepin was purchased from R & D Systems (Minneapolis, MN, USA). MLN8237 was from Selleck Chemicals (Houston, TX, USA). KN-93 and KN-92 were obtained from EMD Millipore (Billerica, MA, USA). Stock solutions of all these reagents were prepared in DMSO and were diluted to the final concentrations in ACSF. For these reagents, the same concentration of DMSO alone was used for controls. A membrane-permeant proteasome inhibitor β -lactone (25 μ M final; Cayman Chemical, Ann Arbor, MI, USA) was applied to the hippocampal slices after treatment with the individual inhibitors mentioned above.

2.3 Statistical analysis

The data were analyzed by using Student's t-test. The values are presented as mean $\pm$ standard error. The sample size (n) in each dataset corresponds to the number of animals (not slices) used to collect the data.

3. Results

3.1. Effect of cordycepin, an inhibitor of polyadenylation, on enhancement of early part of L-LTP mediated by proteasome inhibition

L-LTP induction requires local translation of mRNAs in dendrites [3]. Our previous work, using a series of experiments, showed that proteasome inhibition enhances the early part of L-LTP (Ep-L-LTP), which encompasses the induction phase, by stabilizing locally translated proteins [7]. Subsequently, we showed that the mTOR pathway plays a critical role in proteasome inhibition-mediated augmentation of Ep-L-LTP [10]. Since cytoplasmic polyadenylation signaling pathway is also known to play a role in local translation and has been shown to be necessary during L-LTP [23], we hypothesized that proteasome inhibition-mediated augmentation of Ep-L-LTP requires this pathway. If our hypothesis is correct, proteasome inhibition by β -lactone should enhance Ep-L-LTP, and blockade of polyadenylation by an inhibitor (cordycepin) [23–25] should diminish the β -lactone-mediated enhancement in Ep-L-LTP.

To test our hypothesis, we pre-incubated the slices for 1 h with cordycepin (200 μ g/ml), before applying β -lactone (25 μ M) for 30 min. We then induced L-LTP with TBS. We found that cordycepin significantly reduced the effect of β -lactone on Ep-L-LTP (at 30 min: β -lactone, 154% $\pm$ 4.5%, cordycepin + β -lactone, 129 $\pm$ 2.9, n=9, P < 0.01, t-test) (Fig. 1A). We found that cordycepin does not affect basal synaptic transmission (Fig. 1B). Also, the pattern of TBS remains unaltered in the presence of cordycepin (Fig. 1C).

Since polyadenylation signaling is expected to regulate translation locally in dendrites and our previous data showed that the Ep-L-LTP-augmenting effect of proteasome inhibition occurs in dendrites [7], it was important to test whether proteasome inhibition affected the cytoplasmic polyadenylation signaling pathway locally in dendrites. To test this, we used a hippocampal slice preparation in which we made a surgical cut to separate the cell bodies of pyramidal neurons from their dendrites in the stratum radiatum (Fig. 1D). We then induced L-LTP after treatment of hippocampal slices with β -lactone alone or with β -lactone treatment after pre-incubation with cordycepin. We observed that pre-treatment with cordycepin in these 'cut slice' preparations significantly decreased the augmentation in Ep-L-LTP brought about by β -lactone (at 30 min: β -lactone, $180\% \pm 5.3\%$, cordycepin + β -lactone, $150.9\% \pm 3.3$, $n=9$, $P < 0.001$, t-test) (Fig. 1E).

3.2. Effect of Aurora-A kinase inhibition on β -lactone-mediated enhancement of Ep-L-LTP

We next tested the role of Aurora-A kinase that has been previously shown to regulate cytoplasmic polyadenylation in hippocampal neurons [16]. Previous work by others showed that NMDA-R-mediated signaling causes activation of Aurora-A kinase, which phosphorylates CPEB. Phosphorylation of CPEB results in polyadenylation of mRNAs that are known to be localized to dendrites such as CaMKII α mRNA [16]. We made use of a small molecule inhibitor of Aurora-A kinase called MLN8237 [26;27]. Thus far the effect of MLN8237 on L-LTP has not been studied. We pre-incubated the hippocampal slices with MLN8237 (1.0 μ M) for 1 h and induced L-LTP. We found that MLN8237 significantly inhibited L-LTP (at 30 min: control, 30min: $170\% \pm 11\%$; MLN8237, $130\% \pm 8\%$; $n=9$; $P < 0.0001$, t-test) (Fig. 2A). We found that MLN8237 does not affect basal synaptic transmission (Fig. 2B) and does not cause any changes in the pattern of TBS (Fig. 2C).

Next we tested the effect of MLN8237 on β -lactone-mediated enhancement in Ep-L-LTP. We found that incubation of hippocampal slices with MLN8237 prior to application of β -lactone greatly reduced the enhancement in Ep-L-LTP (at 30 min: β -lactone, $193\% \pm 10\%$; MLN8237 + β -lactone, $128\% \pm 11\%$; $n=7$; $P < 0.0001$, t-test) (Fig. 2D).

3.3. Effect of calcium-calmodulin-dependent kinase II (CaMKII) inhibition on β -lactone-mediated increase in Ep-L-LTP

The role of CaMKII in synaptic plasticity in general and LTP in particular has been well established [28;29]. Previous work by others has implicated CaMKII in activation of CPEB and thus a potential role in controlling translation. It has been found that CaMKII activation by NMDA receptors causes phosphorylation of mouse CPEB at its regulatory site (Ser-171) and that inhibition of CaMKII by KN-93 blocks CPEB phosphorylation and the expression of a reporter gene containing CPE [18]. A second study showed that CaMKII regulates CPEB phosphorylation during L-LTP [30]. Thus, in addition to the Aurora-A kinase, CaMKII appears to regulate cytoplasmic polyadenylation in hippocampal neurons. Since the previous work implicating the role of CaMKII in CPEB phosphorylation and L-LTP used a different protocol (4 X 100 Hz), we first tested the effect of KN-93 (5 μ M) on L-LTP induced by TBS. Pre-incubation of hippocampal slices with KN-93 for 1 h significantly inhibited L-LTP. As control, we used an inactive analog KN-92 (at 30min: KN-92, $156\% \pm 9\%$; KN-93, $133\% \pm 10\%$; $n=8$, $P < 0.0001$, t-test) (Fig. 3A). In addition, we tested the effect

of KN-93 on basal synaptic transmission and TBS and found that neither of these is affected by application of KN-93 to the slices (Fig. 3B & Fig. 3C)

Next, we applied KN-93 (or KN-92) prior to incubating the hippocampal slices with β -lactone. We found that KN-93 (but not KN-92) significantly reduced the increase in Ep-L-LTP brought about by β -lactone (at 30 min: KN-92, $182\% \pm 12\%$; KN-93, $136\% \pm 9\%$; $n=8$, $P < 0.0001$, t-test) (Fig. 3D).

4. Discussion

Our data suggest a role for proteasome-mediated degradation in controlling the translation through the cytoplasmic polyadenylation signaling pathway during the early part of L-LTP. Previously we showed that the proteasome has opposite roles in different parts of the neuron during induction and maintenance of L-LTP [7]. Our results indicated that in dendrites, the proteasome normally limits induction of L-LTP by degrading proteins that are locally translated from pre-existing mRNAs. We showed that in the nucleus, however, the proteasome has a positive role in L-LTP maintenance because it facilitates transcription [7]. Our present study indicates that proteolysis might control cytoplasmic polyadenylation-mediated translation required for L-LTP, especially during the induction phase of L-LTP although direct proof of this concept must await future molecular studies. It is likely that proteasome inhibition initially stabilizes positive regulators of L-LTP giving rise to an enhancement of Ep-L-LTP. This notion is supported by the fact that pre-incubation of hippocampal slices with the protein synthesis inhibitor anisomycin prior to β -lactone treatment significantly diminishes the increase in Ep-L-LTP brought about by β -lactone [7]. Our results presented here and published previously show that the increase in Ep-L-LTP is not sustained even in isolated dendrites. Therefore, it is highly likely that proteasome also stabilizes negative regulators of plasticity that turn over slowly. In the cytoplasmic signaling pathway, the phosphatase calcineurin, which is a substrate for ubiquitin-proteasome-mediated degradation [31], is known to negatively regulate polyadenylation-mediated translation by dephosphorylating maskin and enhance its binding to eIF4E thus blocking translation initiation [32].

How might proteolysis by the UPP regulate cytoplasmic polyadenylation signaling? Some of the key players in the cytoplasmic polyadenylation signaling pathway have been shown to be substrates for ubiquitin-proteasome-mediated degradation. For example, CPEB is a substrate for the ubiquitin-proteasome pathway [33] and CPEB dimers are degraded through the action of a multi-subunit E3 ligase containing the substrate-binding protein β -TrCP [34;35]. Aurora-A kinase is a substrate for proteolysis by the UPP as well. In *Xenopus* Aurora-A kinase is a target for the E3 ubiquitin ligase anaphase-promoting complex consisting of a regulatory subunit called Cdh1/Fizzy-related [36]. It is possible that the amount of Aurora-A is limiting for CPEB-mediated translation required for induction of L-LTP.

Based on our results, during Ep-L-LTP, the following scenario can be envisaged for the role of ubiquitin-mediated proteolysis. The proteins that positively impact L-LTP induction are synthesized and degraded rapidly. Synthesis of a subset of these rapidly-turning over

proteins are likely to be regulated by the cytoplasmic polyadenylation signaling pathway. Under normal physiological circumstances, ubiquitin-proteasome-mediated degradation of components of the cytoplasmic polyadenylation signaling pathway as well as proteolytic removal of the proteins synthesized through this pathway limits induction of L-LTP in the absence of adequate stimuli. With sufficiently strong stimulation, however, these proteins would somehow be stabilized, perhaps through post-translational modifications such as phosphorylation, and thus L-LTP is induced (shown schematically in Fig. 3E). Therefore, proteolysis might play a role in determining the threshold for L-LTP induction. Tight control of translation likely prevents synaptic strength from being saturated thus allowing room for future synaptic plasticity. Also, unfettered translation could have adverse effect on synaptic function. Recent studies show that overexpression of eIF4E or deletion of the translational repressor eukaryotic initiation factor 4E-binding protein 2 (4E-BP2) causes synaptic abnormalities and autism-like behavior. Translational control is also an important factor in aging and thus for age-related cognitive impairments [37;38]. Perturbations in proteolysis are observed in many neurodegenerative and other diseases of the nervous system [39–41]. Regulation of both protein synthesis and proteolysis is critical for other forms of plasticity such as those mediating nociception [42–45]. Thus regulation of translation by ubiquitin-mediated proteolysis is likely to be important for normal physiological function and thus disruption of this regulation might be a critical factor in diseases and disorders of the brain.

5. Conclusion

Taken together the data indicate that the proteasome is likely to regulate elements of cytoplasmic polyadenylation signaling that controls translation of mRNAs during L-LTP.

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Highlights

We tested the link between the proteasome and translation during L-LTP.

We examined components of cytoplasmic polyadenylation signaling.

A polyadenylation inhibitor blocks the increase in L-LTP by proteasome inhibition.

Blocking Aurora-A kinase reduces the L-LTP increase by proteasome inhibition.

CaMKII inhibition diminishes the L-LTP increase by proteasome inhibition.

Figure 1. Cordycepin treatment significantly decreases β -lactone-mediated enhancement of Ep-L-LTP in intact slices and isolated dendrites

(A) Incubation of intact hippocampal slices with cordycepin prior to β -lactone significantly ($P < 0.01$) reduces the extent of Ep-L-LTP. Inset: Representative traces taken at 30 min for treatment with β -lactone alone and Cordycepin + β -lactone. TBS: theta-burst stimulation.

(B) Normal basal synaptic transmission in cordycepin -treated hippocampal slices ($n=6$) compared to vehicle (DMSO)-treated control ($n=6$) slices. The graph shows input-output curves of fEPSP slope (mV/ms) versus stimulus at the Schaffer collateral pathway upon treatment with the vehicle (Control) or cordycepin.

(C) Cordycepin does not affect the pattern of theta-burst stimulation (TBS). Representative traces collected after the first burst and the tenth burst under vehicle (DMSO)-treated control hippocampal slices and cordycepin-treated slices. Calibration: 2 mV, 20 ms.

(D) Schematic illustration of isolation of dendrites. A cut (black bar) is made just below the pyramidal cell body layer in the CA1 region. Recording electrode (Rec) is placed in the dendritic layer of pyramidal cells. Position of the stimulation electrode (Stim) and the layers stratum radiatum and stratum oriens are also indicated. DG: dentate gyrus.

(E) Treatment of cut slices with cordycepin prior to β -lactone significantly ($P < 0.001$) decreases the magnitude of Ep-L-LTP in isolated dendrites. Inset: Representative traces taken at 30 min for β -lactone alone and Cordycepin + β -lactone.

Figure 2. Aurora-A kinase inhibition decreases L-LTP and β -lactone-mediated enhancement of Ep-L-LTP

(A) Pre-treatment with Aurora-A kinase inhibitor MLN8237 significantly ($P < 0.0001$) reduces L-LTP. Inset: Representative traces taken at different time points (1 = 30 min; 2 = 180 min) for vehicle (DMSO) treatment (Control) and treatment with MLN8237.

(B) Normal basal synaptic transmission in MLN8237-treated hippocampal slices ($n=7$) compared to vehicle (DMSO)-treated control ($n=7$) slices. The graph shows input-output curves of fEPSP slope (mV/ms) versus stimulus at the Schaffer collateral pathway upon treatment with the vehicle (Control) or MLN8237.

(C) MLN8237 does not affect the pattern of theta-burst stimulation (TBS). Representative traces collected after the first burst and the tenth burst under vehicle (DMSO)-treated control hippocampal slices and in MLN8237-treated slices. Calibration: 2 mV, 20 ms.

(D) Incubation with MLN8237 prior to β -lactone application significantly ($P < 0.0001$) decreases the enhancement in Ep-L-LTP. Inset: Representative traces taken at different time points (1 = 30 min; 2 = 180 min) for β -lactone alone and MLN8237 + β -lactone.

Figure 3. CaMKII inhibition decreases L-LTP and β -lactone-mediated enhancement of Ep-L-LTP

(A) Pre-treatment with CaMKII inhibitor KN-93 significantly ($P < 0.0001$) reduces L-LTP. Inset: Representative traces taken at 30 min for treatment with an inactive analog (KN-92) and treatment with KN-93.

(B) Normal basal synaptic transmission in KN-93 -treated hippocampal slices ($n=6$) compared to vehicle (DMSO)-treated control ($n=6$) slices. The graph shows input-output curves of fEPSP slope (mV/ms) versus stimulus at the Schaffer collateral pathway upon treatment with the vehicle (Control) or KN-93.

(C) KN-93 does not affect the pattern of theta-burst stimulation (TBS). Representative traces collected after the first burst and the tenth burst under vehicle (DMSO)-treated control hippocampal slices and in KN-93 -treated slices. Calibration: 2 mV, 20 ms.

(D) Incubation with KN-93 prior to β -lactone application significantly ($P < 0.0001$) decreases the enhancement in Ep-L-LTP relative to pre-treatment with the inactive analog KN-92. Inset: Representative traces taken at 30 min for treatment with β -lactone alone and KN-93 + β -lactone.

(E) Schematic skeletal diagram of the cytoplasmic signaling pathway connecting NMDA receptors to L-LTP induction. This pathway leads to elevation of the poly-A tail of mRNAs and results in increased protein synthesis which in turn facilitates L-LTP induction. Proteasome activity negatively regulates Aurora-A and CaMKII signaling, prevents accumulation of newly synthesized proteins through degradation, and thus keeps L-LTP

induction in check. Therefore, proteasome inhibition by β -lactone increases L-LTP induction.