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Developmental Expression of Eukaryotic Initiation Factor 4E (eIF4E) and eIF4E-binding Protein 1 (eIF4EBP1) in Rat Hippocampal Neurons

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Local protein synthesis at subsynaptic sites plays a key role in the regulation of the protein composition in local domains. In this study, we carried out immunocytochemistry of cultured rat hippocampal neurons in various developmental stages to investigate the expression of eIF4E and its binding protein, eIF4EBP1. Both proteins were distributed in dendrites. In addition, eIF4EBP1 was highly expressed in the nucleus throughout the development, whereas eIF4E was not expressed in the nucleus. Punctate expression of eIF4E and eIF4EBP1 was evident in DIV 3. The colocalization rates of eIF4E or eIF4EBP1 puncta with PSD95 were higher in the dendrogenic than in the mature stages. In contrast, the colocalization rates of eIF4E and eIF4EBP1 puncta were higher in the mature than in the dendrogenic stages. As eIF4E is inactive when it is bound to eIF4EBP1, these data indicate that most dendritic eIF4E's are active during development but that they are mostly under inhibition in mature neurons.

Key words : Development, eukaryotic translation initiation factor 4E (eIF4E), eIF4E-binding protein 1 (eIF4EBP1), hippocampal neuron, immunocytochemistry

Introduction

Functions of the brain are based on the neural networks which are dynamically remodeled by changes in the strength of synaptic connections. The long-lasting changes in synaptic strength, such as long-term potentiation (LTP), require new protein synthesis [4, 8, 13]. The local protein synthesis at subsynaptic sites plays a key advantageous mechanism that would regulate the protein composition in local domains on a moment-by-moment basis. In the translation process of protein synthesis, initiation is the rate limiting step where most of translational control occurs [14]. During translation initiation, eukaryotic translation initiation factor 4E (eIF4E) binds the 5'-cap structure of mRNA and the ribosome-associated scaffold protein eIF4G [17] in the cytoplasm. The eIF4E-binding protein 1 (eIF4EBP1) acts as a negative regulator of this process by preventing interaction of eIF4E with eIF4G in a competitive manner [7].

In addition to the organelles such as rough endoplasmic reticulum and Golgi outpost that are necessary for protein synthesis, many translation factors were found located in the dendrite. Presence of eIF4E in postsynaptic region was revealed by electron microscopic study [1]. Immunocytochemistry (ICC) and western blotting also showed the postsynaptic localization of eIF4E, eIF4EBP1, eIF4EBP2 [16]. High resolution confocal microscopy and detergent extraction experiments showed that the eIF4E, eIF4G, eIF5, eIF5A and eIF6 were localized to the postsynaptic sites in association with the postsynaptic density (PSD) [3]. Furthermore, eIF4E and eIF4EBP1 mRNAs are localized, and upregulated by neuronal stimulation, in dendrites of cultured rat hippocampal neurons [9, 11].

Neurons undergo complex morphological development. Dendrites will initially outgrow, followed by extensive branching and lengthening during dendrogenic stage. As a dendrite matures it produces small protrusion that would eventually develop into spines. We still have little information on spatial distribution of eIF4E and eIF4EBP1 during neuronal development. In this study, we carried out ICC of rat hippocampal cultures to investigate developmental expression of eIF4E and eIF4EBP1.

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Materials and Methods

Primary culture of rat hippocampal neurons

Hippocampi from Sprague-Dawley rat pups at embryonic day 18 (E18) were dissected, dissociated by trypsin treatment and mechanical trituration, and plated onto 12 mm-diameter polylysine/laminin-coated glass coverslips at a density of ~ 150 neurons/mm^2 as described [2]. Cells were plated initially in Neurobasal medium supplemented with B27 (Invitrogen, Carlsbad, CA, USA), 25 μM glutamate, and 500 μM glutamine, and fed 5 days after plating and weekly thereafter with the same media (without added glutamate) containing 1/3 (v/v) 'conditioned' Neurobasal media by incubating for 24 h on astrocyte cultures [6] For developmental study, cultured neurons were fixed on DIV (days in vitro) 0.5, 3, 7, 10 and DIV 20. Culture coverslips were rinsed briefly in Dulbecco's phosphate-buffered saline (D-PBS, Invitrogen) and cells were fixed by a sequential paraformaldehyde/methanol fixation procedure [incubation in 4% paraformaldehyde in PBS (20 mM sodium phosphate buffer, pH 7.4, 0.9% NaCl) at RT for 10 min followed by incubation in methanol at -20°C for 20 min] [10].

Immunocytochemistry

Fixed cells were blocked overnight at 4°C in preblocking buffer (5% normal goat serum, 0.05% Triton X-100, and 450 mM NaCl in 20 mM sodium phosphate buffer, pH 7.4). Primary antibodies [mouse anti-eIF4E (1:500, BD Biosciences, Palo Alto, CA, USA; rabbit anti-eIF4EBP1 (1:100, Santa Cruz, CA, USA), chicken anti-PSD95 [12] (1:1,000, a gift from Dr. Randal W. Walikonis, University of Connecticut, CT, USA) were added to the coverslips and incubated overnight at 4°C. Coverslips were rinsed (3×15 min in preblocking buffer) and incubated with secondary antibodies [Alexa Fluor 488-conjugated goat anti-mouse and Alexa Fluor 568-conjugated goat anti-rabbit IgG (each diluted 1:1,000 in blocking buffer; Invitrogen)] at RT for 2 hr. Coverslips were rinsed once in preblocking buffer for 15 min, twice in Dulbecco's phosphate buffered saline (D-PBS), and mounted on slides with 4% n-propylgallate in 90% glycerol and 10% sodium carbonate buffer (pH 8.7).

Image acquisition and processing

Confocal images (1,024×1,024 pixels) were acquired using 100× oil-immersion lens on the Leica TCS SP2 Confocal System with laser lines at 488, 543, and 633 nm and live digital images (1,024×1,024 pixels) were acquired using a fluorescence microscope (Leica DM IRE, Wetzlar, Germany). All of obtained images processed with the use of Adobe Systems Photoshop 7.0 software.

Result and Discussion

To study developmental expression of eIF4E and eIF4EBP1 in neurons, we cultured E18 rat embryonic hippocampal neurons on PDL/laminin-coated coverslips in Neurobasal medium supplemented with B27. In our hands, the cultured hippcampal neurons routinely produce small processes overnight (days in vitro; DIV 0.5). During the following a couple of days (DIV 3), the neurons form distinct axon and dendrites. At this stage, initial dendritic branching takes place. However, dendritic protrusions are very limited. Therefore, we defined this initial development as early stage. Thereafter, dendrites undergo extensive branching and lengthening, and produce many protrusions such as filopodia. We define this period (DIV 7, 10) as dendrogenic stage. Toward the end of the third week of culture, neurons are generally mature with highly branched dendrites and spines. Therefore, we fixed the cultured neurons on DIV 0.5, 3, 7, 10, and 20. Fixed cells were subjected to ICC and localization at synaptic sites was determined by colocalization with PSD95, a postsynaptic marker in DIV 7, 10, 20 neurons.

Expression of eIF4E and eIF4EBP1 in the early neuronal development (DIV 0.5 and 3)

In all developmental stages, confocal microscopic images revealed immunoreactivity of eIF4E and eIF4EBP1 in the cytoplasm and dendrites. However, there was a differential expression of eIF4EBP1 in the nucleus from the very early stage of development (DIV 0.5). While eIF4E was distributed in the perikaryon and initial processes (Fig. 1A-a, arrowhead), the eIF4EBP1 was highly expressed in the nucleus (Fig. 1A-b, asterisk) as well as the cytoplasm and processes. On DIV 3, high expression of eIF4E was associated with perikaryon and dendrites (Fig. 1B-a), while higher expression of eIF4EBP1 was associated with nucleus (Fig. 1B-b, asterisk). Punctate expression of eIF4E and eIF4EBP1 was evident on DIV 3 (Fig. 1B, arrows in *insets*).

Expression of eIF4E and eIF4EBP1 in the dendrogenic stage

Higher nuclear expression of eIF4EBP1 was evident on

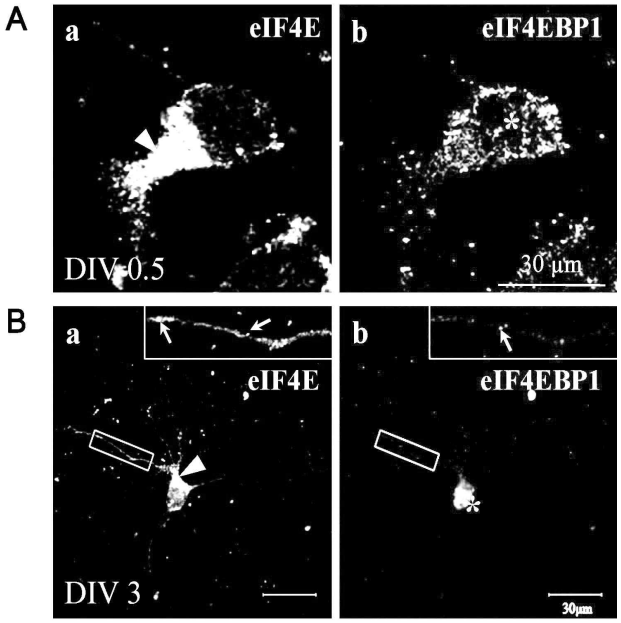

Fig. 1. Expression of eIF4E and eIF4EBP1 in the cultured rat hippocampal neurons at early developmental stages. Embryonic day 18 (E18) rat hippocampal neurons were grown as described in Materials and Methods. They were fixed on DIV 0.5 (A) or DIV 3 (B) and double-stained with antibodies against eIF4E and eIF4EBP1. Strong eIF4E immunoreactivity in the perikaryon is marked by arrowheads, and nuclear expression of eIF4EBP1 by asterisks. The boxed areas were shown enlarged in *insets* in B. Punctate expression of the two proteins are shown in the *insets* of B. Scale bar; 30 μm.

DIV 7 (Fig. 2A-a, arrow) and DIV 10 (Fig. 2B-a, arrow). The expression of eIF4E and eIF4EBP1 was very high forming dense puncta in dendrites (Fig. 2, eIF4E, eIF4EBP1). The punctuate expression of postsynaptic protein PSD95 were also evident throughout dendrites (Fig. 2, PSD95). The merge images showed that many eIF4E or eIF4EBP1 puncta were colocalized with those of PSD95 (arrowheads in Fig. 2A-b, and 2B-b). Statistical analysis (Fig. 4) showed that the colocalization rate of eIF4E or eIF4EBP1 with PSD95 on DIV 7 were 39.1±9.6% and 70.5±5.2% (n=4), respectively (numbers of counted puncta were 313, 117, 223, respectively, for PSD95, eIF4E, eIF4EBP1). On DIV 10, the colocalization rate of eIF4E or eIF4EBP1 with PSD95 were 57.7±8.2% and 36.0±3.1% (numbers of counted puncta were 272, 160, 99, respectively, for PSD95, eIF4E, eIF4EBP1). The colocalization of eIF4E and its inhibitor eIF4EBP1 on DIV 7 and DIV 10 were shown in Fig. 2A-c and 2B-c. As shown in the merge images (eIF4E/eIF4EPB1 panels), they were not well colocalized. Statistical analysis (Fig. 4) showed that the colocalization rates were 18.5±2.6% (DIV 7) and 11.1±3.9% (DIV 10). The low colocalization of eIF4E and eIF4EBP1 indicates that most of the eIF4E are active being engaged in protein synthesis. The DIV 7 and 10 are the highly dendrogenic period producing branches, lengthening branches, and producing dendritic protrusions such as filopodia and spines. Protein synthesis machinery are thought to be highly active in this period of neuronal development. The high colocalization rates of eIF4E and eIF4EBP1 with PSD95 could be due to high concentration of these proteins. Since dendritic spines are not formed intensively at this developmental stage, the colocalization of these translation proteins with PSD95 is interpreted being occurring in the dendritic cytoplasm, not in the spine.

Expression of eIF4E and eIF4EBP1 in the mature neuron

In mature neurons (DIV 20), the nuclear expression of eIF4E and eIF4EBP1 was most contrasting. While eIF4EBP1 was very strongly expressed in the nucleus (Fig. 3A, asterisk in the *inset*), the nuclear expression of eIF4E was very weak (Fig. 3A, *insets*). This result was in accordance with the previous report. DIV 16 hippocampal neurons expressed eIF4E throughout dendrites but not in the nucleus, while eIF4EBP1 was distributed throughout the neuron with highest expression in the nucleus [9]. There were many eIF4E, eIF4EBP1 and PSD95 puncta in the dendrites of DIV 20 neurons (Fig. 3B). The colocalization rates of eIF4E or eIF4EBP1 with PSD95 were 29.9±2.9% and 40.2±11.7%, respectively (numbers of counted puncta were 267, 80, 106, respectively, for PSD95, eIF4E, eIF4EBP1). Since most of PSD95 puncta are associated with postsynaptic sites, these results indicate that large portions of eIF4E and eIF4EBP1 are also associated with postsynaptic sites. The colocalization of eIF4E with eIF4EBP1 is shown in Fig. 3C. Compared to those of earlier developmental stages, colocalization of eIF4E and eIF4EBP1 were less frequent. Statistical analysis showed the colocalization rate was 38.6±5.6%, indicating that a large portion of eIF4E is under inhibition.

Changes in the colocalization rates were graphically shown Fig. 4. Although the high rates of eIF4E or eIF4EBP1 colocalization with PSD95 during early developmental stages (DIV 7 and 10) may be due to higher expression of these proteins, still large portion of eIF4E and eIF4EPB1 were colocalized with PSD95. These results indicate postsynaptic localization of these translation factors and their roles in syn-

Fig. 2. Expression of eIF4E and eIF4EBP1 in the cultured rat hippocampal neurons at highly dendrogenic stages. Hippocampal neurons were triple-labeled with antibodies against eIF4E, eIF4EBP1 and PSD95 on DIV 7 (A) or DIV 10 (B). Panel a's show merge images of three channels. The boxed areas were shown enlarged in b and c in single or various merges. Colocalized puncta were marked by arrowhead (b) and arrow (c). Note that eIF4E and eIF4EBP1 are well colocalized with PSD95 but they do not colocalize well each other at this developmental stages. Scale bar; 30 μm.

Fig. 3. Expression of eIF4E and eIF4EBP1 in the cultured rat hippocampal neurons at the mature stage. Neurons were triple-labeled with antibodies against eIF4E, eIF4EBP1 and PSD95 on DIV 20. (A) Merge images of three channels. The boxed area was shown enlarged in single or various merges (B). The expression of eIF4E and eIF4EBP1 in the boxed area of B are shown in C. Colocalized puncta were marked by arrowheads (B) and arrows (C). Note that eIF4E and eIF4EBP1 are well colocalized with PSD95, and that a significant fraction of eIF4E and eIF4EBP1 puncta colocalizes each other. Scale bar; 30 μm.

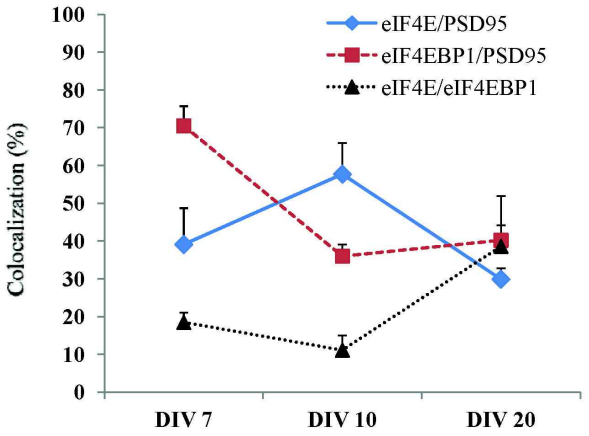

Fig. 4. Statistics. The data show colocalization rates (% ± SD) on different days *in vitro* (DIV)

aptic dynamics. A more intriguing finding is the changes in the colocalization rate of eIF4E and its inhibiting protein eIF4EBP1. The colocalization rates were low (10~20%) in the earlier stages (DIV 7 and DIV 10). However, in the mature neuron, the colocalization rate increased significantly (~40%). These results imply that eIF4E would be constantly and highly engaged in protein synthesis during early neuronal morphological development. The high rate of protein synthesis would require uncoupling of eIF4E and eIF4EBP1. However, in mature neurons, which have finished morphological development, dendritic protein synthesis would be less active in general. Instead, local protein synthesis in specific loci of dendrites would be resumed where it is necessary for synaptic plasticity and dynamics of dendrites. This localized protein synthesis would require most eIF4E be kept under inhibition.

In summary, local dendritic protein synthesis is the most important factor for strengthening of synaptic connections. Generally, eIF4E is a limiting factor in translation initiation and is an important effector of cell proliferation, survival, and malignant transformation [5, 15]. We have shown in this study that eIF4E and its inhibitor eIF4EBP1 are highly expressed in neuronal dendrites. We further showed that the dendritic eIF4E were not under inhibition in the early developmental stages, but they were under inhibition in the mature neuron. Our results imply that the protein synthesis in dendrites would occur in very restricted local areas in mature neurons.

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초록 : 발생단계별 해마신경세포에서 eIF4E 및 eIF4EBP1의 표현

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신경세포의 가지돌기 내 단백질합성은 필요한 단백질을 실시간으로 제공할 수 있는 이점을 제공한다. 본 연구에서는 단백질합성인자 eIF4E와 그 억제 단백질인 eIF4EBP1의 발생단계별 표현을 배양한 해마신경세포를 면역염색하여 조사하였다. eIF4E는 가지돌기에 점박이 모양으로 표현되었으며, 핵에는 표현되지 않았다. 그러나 eIF4EBP1는 가지돌기 뿐 아니라 발생초기(DIV 0.5)부터 핵에서 표현되었으며 성숙한 세포에서 핵에 더욱 뚜렷이 표현되었다. eIF4E 혹은 eIF4EBP1의 PSD95과의 colocalization은 39.1±9.6% 및 70.5±5.2% (DIV 7), 57.7±8.2% 및 36.0±3.1% (DIV 10), 29.9±2.9% 및 40.2±11.7% (DIV 20)이었다. eIF4E와 eIF4EBP1의 colocalizatin은 18.5±2.6% (DIV 7), 11.1±3.9% (DIV 10), 38.6±5.6% (DIV 20)이었다. 이 결과는 eIF4E 및 eIF4EBP1의 많은 부분이 연접후에 위치하며, 발생초기에는 eIF4E가 활동적인 형태로 존재하지만, 성숙 신경세포에서는 eIF4EBP1과 결합하여 비활성적인 형태로 존재함을 의미한다.