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Expression of c-Jun N-Terminal Kinase (JNK)-Interacting Protein (JIP) in Cultured Rat Hippocampal Neurons

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c-Jun N-terminal kinase (JNK)-interacting protein 1 (JIP1), also known as Islet-brain 1 (IB1), is a scaffold protein that is highly expressed in neurons and pancreatic β-cells. In this study subcellular localization of JIP was investigated in cultured rat hippocampal neurons using an antibody that recognize all variants of JIP1, JIP-2 and JIP-3. The overall expression profile of JIP is punctate throughout soma and dendrites. Statistic analysis showed that 54.8 ± 4.0% and 94.1 ± 4.5% of total JIP immunopuncta overlapped with those of excitatory postsynaptic markers SD-95 and αCamik, respectively. In contrast, only 8.6 ± 0.5% and 7.3 ± 0.5% of JIP clusters overlapped with those of inhibitory postsynaptic markers glycine receptor (GlyR) and gephyrin, respectively. JIP clusters overlapped or juxtaposed with SV2 but not GAD, markers for general and inhibitory nerve terminals, respectively. A substantial fraction (29.3 ± 1.0%) of flotillin immunopuncta, a marker for lipid rafts, clusters overlapped with those of JIP. In addition, JIP was highly expressed in some select ends of dendrites but minimal in axons. These data suggest important roles of JIP in excitatory postsynaptic sites, lipid rafts and dendritic ends.

Key words : Cytoskeleton, excitatory, hippocampal culture, inhibitory, JIP1, lipid raft

Introduction

c-Jun N-terminal kinase (JNK)-interacting protein 1 (JIP1), also known as Islet-brain 1 (IB1), is a scaffold protein that participates in the organization of the JNK signaling pathway. JIP1 has been implicated in cell survival [4], in regulating expression of the glucose transporter type 2 (GLUT2) [3] and in glucose-induced insulin secretion [25]. JIP1 is a highly conserved protein [3,17,21] composed of 707, 711, and 715 amino acids in mice, humans, and rats, respectively. It has a molecular weight of 120 kDa containing several protein-protein interaction domains such as a JNK-binding domain, an Src homology 3 domain (SH3), and a phosphotyrosine interaction domain. JIP1 serves as an organizing center for signal transduction. JIP1 is also related to the microtubule motor protein kinesin [24]. It may influence vesicles and mitochondria in axons [12], and is essential for the transport of phosphorylated amyloid-β precursor protein [19].

JIP1 is highly expressed in neurons and in pancreatic β-cells [13,21,26]. In the brain, JIP1 expression was localized in the synaptic regions of the olfactory bulb, retina, cerebral and cerebellar cortex and hippocampus in the adult mouse brain [21]. JIP1 was also detected in a restricted number of axons, such as the mossy fibers from dentate gyrus and axons of cerebellar Purkinje cells [21]. Prominent localization of JIP1 in synapses suggests that the protein may be critical for cell signalling in mature nerve terminals [21]. Recently, it is reported that the JIP1/2 group of scaffold proteins is critically required for normal N-methyl-D-aspartic acid (NMDA) receptor function. Kennedy *et al.* [13] examined the phenotype of compound mutant mice that lack expression of both JIP1 and JIP2. These mice were found to exhibit severe defects in N-methyl-D-aspartic acid (NMDA) receptor function, including decreased NMDA-evoked current amplitude, cytoplasmic Ca^{2+}, and gene expression. Alteration in NMDA receptor signaling in this mutant mice suggests localization of JIP at excitatory synapses. Indeed, synaptic localization of JIP1 was recently reported. At the electron microscopic level using DAB staining, Borsello *et al.* [5] showed that JIP1 was found in axonal and synaptic elements and in dendritic elements. The staining was rather local and discontinuous. The cytoplasmic staining was associated with cytoskeletal elements and some membranes. However, it was difficult to attribute the staining to microtubules, microfilaments, or neurofilaments.

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Our knowledge on the subcellular distribution of JIP1 in neurons is far from satisfactory. In this study I took advantage of cultured hippocampal neurons and double-staining and show that JIP proteins are localized to excitatory postsynaptic compartments and flotillin-positive lipid rafts.

Materials and Methods

Antibodies

The following antibodies were used. JIP1 (1:250; rabbit polyclonal M-3000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). αCaMKII (1:500; mouse monoclonal 6G9, Upstate Biotechnology Inc., Lake Placid, NY, USA). glutamic acid decarboxylase (GAD-6) (1:100; mouse monoclonal, Chemicon International Inc., Temecula, CA, USA), gephyrin (1:500; mouse monoclonal mAb7a) and glycine Receptor (1:500; mouse monoclonal mAb4a) (Synaptic Systems, Göttingen, Germany). Neurofilament 200 (NF200; 1:250, mouse monoclonal NE14) and neurofilament 160 (NF160; 1:250, moue monoclonal) are from Sigma (Saint Louis, MO, USA), synaptic vesicle 2 (SV2) (1:500; mouse monoclonal SV2) and α-tubulin (1:2,000; mouse monoclonal 12G10) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA), flotillin (1:500; mouse monoclonal clone 18 (BD Biosciences, Franklin Lakes, NJ, USA). PSD95 (1:1,000; chicken polyclonal UCT-C1, a kind gift from Dr. Randall Walikonis, University of Connecticut, CT, USA).

Dissociate hippocampal cell culture

Culture of embryonic rat hippocampal cells was previously described [14-16]. Briefly, hippocampi from time-pregnant rats (Sprague-Dawley) at embryonic day 18 (E18) were dissected, dissociated by trypsin treatment and mechanical trituration, and plated onto polylysine/laminin-coated glass coverslips (12 mm in diameter) at a density of ~150 neurons/mm^2 in 24-well culture plates as described [6]. 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 glutamate) containing 1/3 (v/v) Neurobasal media conditioned by incubating for 24 h on astrocyte cultures [10].

Immunocytochemistry (IC)

After 21 days *in vivo* (DIV), cells were fixed through the PFA/MeOH fixation method [16]. Coverslips were rinsed briefly in phosphate buffered saline (PBS) and with 4% paraformaldehyde (PFA) in PBS at room temperature (RT) for 10 min. Coverslips were rinsed in PBS and then incubated in -20°C methanol at -20°C for 20 min. The cells were then rinsed once with PBS and blocked overnight at 4°C in preblocking buffer [5% normal goat serum, 0.05% Triton X-100 in H-PBS (450 mM NaCl and 20 mM phosphate buffer, pH 7.4)]. Primary antibodies were diluted in preblocking buffer (250 ul per well of 24-well culture plate), replaced with preblocking buffer, and incubated overnight at 4°C. Coverslips were rinsed (15 min x 3) in preblocking buffer, and incubated with secondary antibodies [Alexa Fluor 488-conjugated Streptavidin, Alexa Fluor 568 conjugated-goat anti-rabbit and Alexa Fluor 647-conjugated goat ant-chicken IgG (each diluted 1:1,000 in blocking buffer; Invitrogen)] at RT for 1-2 hr. Coverslips were rinsed once in preblocking buffer for 15 min, twice in PBS, and mounted on slides with 4% n-propylgallate in 90% glycerol and 10% sodium carbonate buffer (pH~8.7).

Fluorescence light microscopy

A Leica Research Microscope DM IRE2 (Leica Microsystems AG, Wetzlar, Germany) equipped with filter systems I3 S, N2.1 S and Y5. Digital images were acquired with a HCX PL FL 100X oil-immersion lens and a high-resolution CoolSNAP^{TM} CCD camera (Photometrics Inc., Germany) under the control of a computer equipped with FW4000 (Leica) software. Images (1388 x 1039 pixels) were processed with the use of Photoshop 5.0 (Adobe Systems).

Analysis

IC clusters per 20 μm of proximal dendrites of typical pyramidal neurons were counted, and expressed in % of total (mean±SD).

Results and Discussion

JIP is present in excitatory postsynaptic compartment

The anti-JIP1 antibody used in this work recognizes all variants of JIP1, JIP-2 and JIP-3. The overall expression profile of JIP1 is punctate throughout soma and dendrites. Previous reports indicated that JIP proteins are associated with excitatory spines [13]. Therefore, we first investigated

if JIP colocalizes with PSD-95 and αCaMKII, excitatory synaptic markers. Many JIP immunopuncta overlapped with those of PSD-95 and αCaMKII (Fig. 1A, 1B). In general, JIP immunopuncta in proximal dendrites overlapped better than those in distal dendrites, where JIP-only immunopuncta were frequent. Statistic analysis (Fig. 6) showed that 54.8 ± 4.0% of total JIP immunopuncta overlapped with those of PSD95. For PSD95, 69.7 ± 4.0% of the immunopuncta overlapped with those of JIP. JIP-only and PSD95-only immunopuncta were 45.2 ± 3.7% and 23.8 ± 2.2%, respectively. This result indicates that, although majority of PSD95-positive clusters include JIP, there are many JIP proteins that are not associated with PSD95 clusters. For αCaMKII clusters (Fig. 6), 94.1 ± 4.5% of total JIP immunopuncta overlapped with those of αCaMKII. There were, however, many (22 ± 2.4%) αCaMKII immunopuncta that did not overlap with those of JIP. These results indicate that most of JIP-positive clusters contain αCaMKII but also that there are many αCaMKII clusters that do not include JIP proteins. Since PSD95 and αCaMKII are markers for excitatory synapses, these results indicate that most excitatory spines have JIP protein in the postsynaptic compartment. Also is indicated that there are clusters that are not excitatory postsynaptic compartments. One point to note is that highest immunostain signals were associated with some, not all, dendritic ends (Fig. 1, arrows). Although the meaning of this result is not elucidated, this is reproducible.

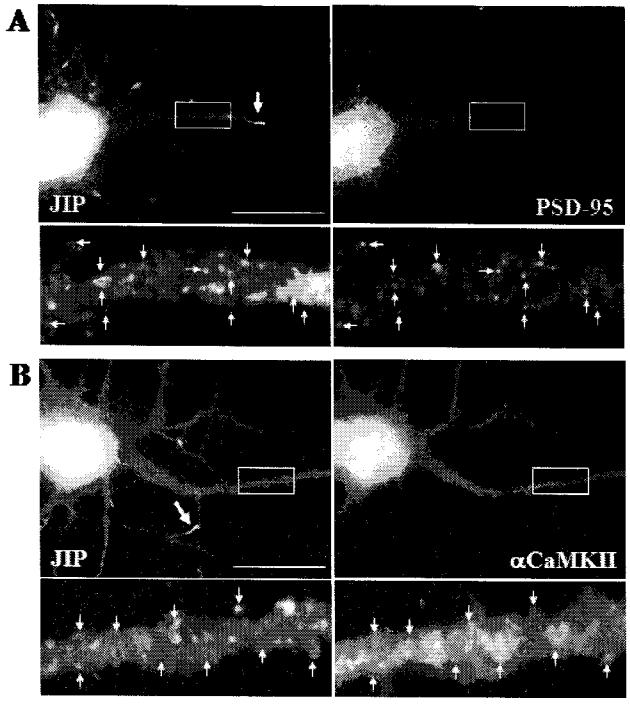

Fig. 1. JIP is expressed at excitatory postsynaptic compartments. E18 rat hippocampal neurons were grown for 21 days *in vitro* (DIV21), fixed and double-stained with antibodies against JIP and PSD-95 (A), and JIP and αCaMKII (B). Strongly stained dendritic ends are marked by arrows. Boxed areas are shown enlarged (insets). Some colocalizing clusters were marked with small arrows in insets. Scale bar; 30 μm.

JIP is not present in inhibitory postsynaptic compartment

To investigate if JIP is also present in inhibitory postsynaptic compartments, sister hippocampal cultures were stained with antibodies against inhibitory postsynaptic markers such as gephyrin and glycine receptors (GlyRs). Clusters of glycine receptors are widely expressed in the hippocampus [7]. Gephyrin is a glycine receptor (GlyR)-tubulin-bridging molecule, initially purified in association with the GlyR subunit, and knock-out experiments [8] have shown that GlyR accumulation at synapses relies on gephyrin, the core scaffolding protein of inhibitory post-

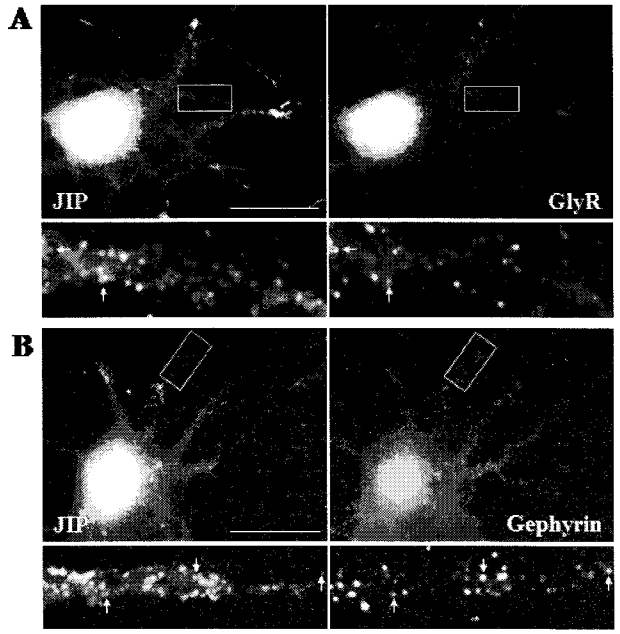

Fig. 2. JIP is not expressed at inhibitory postsynaptic compartments. DIV21 rat hippocampal neurons were fixed and double-stained with antibodies against JIP and GlyR (A), and JIP and gephyrin (B). Boxed areas are shown enlarged (insets). A few colocalizing clusters were marked with small arrows in insets. Scale bar; 30 μm.

synaptic differentiations [18]. Most of the GlyR clusters did not overlapped with those of JIP (Fig. 2A). Statistic analysis (Fig. 6) showed that only small fraction (8.6 ± 0.5%) of JIP clusters overlapped with those of GlyR. Majority (84.8 ± 2.5%) of GlyR clusters did not overlap with those of JIP. For gephyrin (Fig. 6), the overlap with only 7.3 ± 0.5% of JIP clusters overlapped with those of gephyrin (Fig. 2B). Most (86.0 ± 2.6%) of gephyrin clusters did not overlap with those of JIP. These results indicate JIP is not, if any, present in inhibitory postsynaptic compartments.

Absence of JIP in inhibitory postsynaptic compartments were further evidenced by double-staining with JIP and GAD, a marker for inhibitory axon terminal, antibodies (Fig. 3, Fig. 6). Only a small fraction (8.1 ± 0.5%) of GAD clusters overlapped or neighbored with those of JIP. Majority (91.9 ± 4.4%) of GAD clusters were alone. In the contrary, double-staining of the sister cultures with antibodies against JIP and SV2, a general marker for presynaptic terminal, showed that majory (74.5 ± 4.6%) of SV2-positive clusters overlapped with those of JIP. These results strongly support that JIP is present in the positive, but not in inhibitory, postsynaptic compartments.

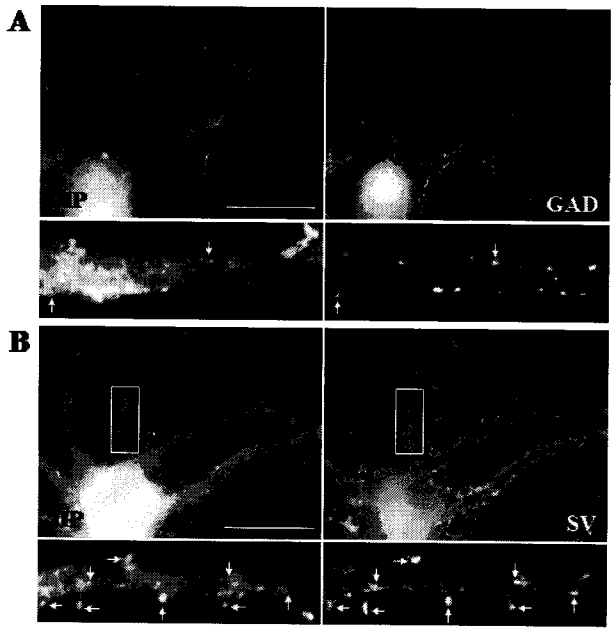

Fig. 3. JIP clusters overlap or juxtapose with SV2 but not with GAD. DIV21 rat hippocampal neurons were fixed and double-stained with antibodies against JIP and SV2, a general marker for nerve terminals (A), and JIP and GAD, a mkarker for inhibitory nerve terminals (B). Boxed areas are shown enlarged (insets). Colocalizing clusters were marked with small arrows in insets. Scale bar; 30 μm.

JIP is present in some lipid rafts

The results so far indicate that there are JIP clusters that do not overlap with synaptic marker. These nonsynaptic JIP clusters may include lipd rafts, specialized structures on the plasma membrane that have an altered lipid composition as well as links to the cytoskeleton [1,9]. To identify some of these nonsynaptic JIP clusters, hippocampal cultures were double-stained with antibodies against JIP and flotillin-1, an established lipid raft marker [2]. There were many flotillin-positive clusters throughout soma and dendrites (Fig. 4). Statistic analysis (Fig. 6) showed that a substantial fraction (29.3 ± 1.0%) of flotillin clusters overlapped with those of JIP, indicating that JIP is present in flotillin-positive lipid rafts. However, majority (70.1 ± 8.9%) of flotillin clusters were alone. It is reported that lipid rafts are present in synapses and dendritic spines [11]. Together with the present findings, a substantial fraction of these flotillin-positive clusters may represent excitatory spines. The statistics were summarized by a graph in Fig. 6.

JIP expression in axons is minimal

Finally, it is tested if JIP is present in axons. Cultured hippocampal neurons were first double-stained with antibodies against JIP and β-tubulin. As shown in Fig. 5A, immunostaining with anti-α-tubulin antibody revealed the overall structure of the neuron including an axon (an array of arrows in Fig. 5A). Staining intensity of the axon with JIP antibody was very weak, indicating absence of JIP in

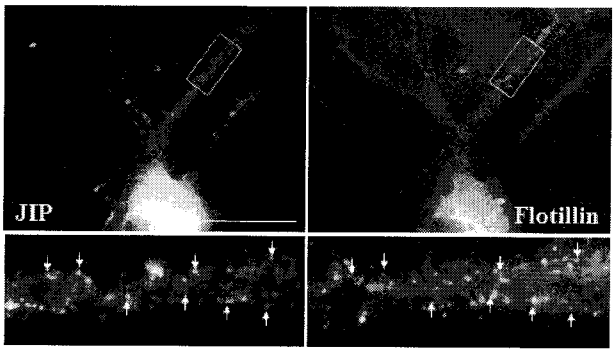

Fig. 4. JIP is expressed at some lipid rafts. DIV21 rat hippocampal neurons were fixed and double-stained with antibodies against JIP and flotillin, a marker for lipid rafts. Boxed areas are shown enlarged (insets). Some olocalizing clusters were marked with small arrows in insets. Scale bar; 30 μm.

axons. However, strong immunostainings were associated with soma and dendrites. To see if JIP clusters were associated with dendritic microtubules, a portion of the double-stain was enlarged. As shown in the inset of Fig. 5A, there is no similarity between the distribution profiles of JIP and microtubule. Next, sister cultures were immunostained with antibodies against neurofilament 200 (NF200) and neurofilament 160 (NF160). As shown in Fig. 5B and 5C, these antibodies most strongly stained axons, where JIP immunostainings were very weak or absent. Instead, strongest immunostains were associated with some dendritic ends (Fig. 5B, inset). These results confirm that JIP is present in somatodendritic domains.

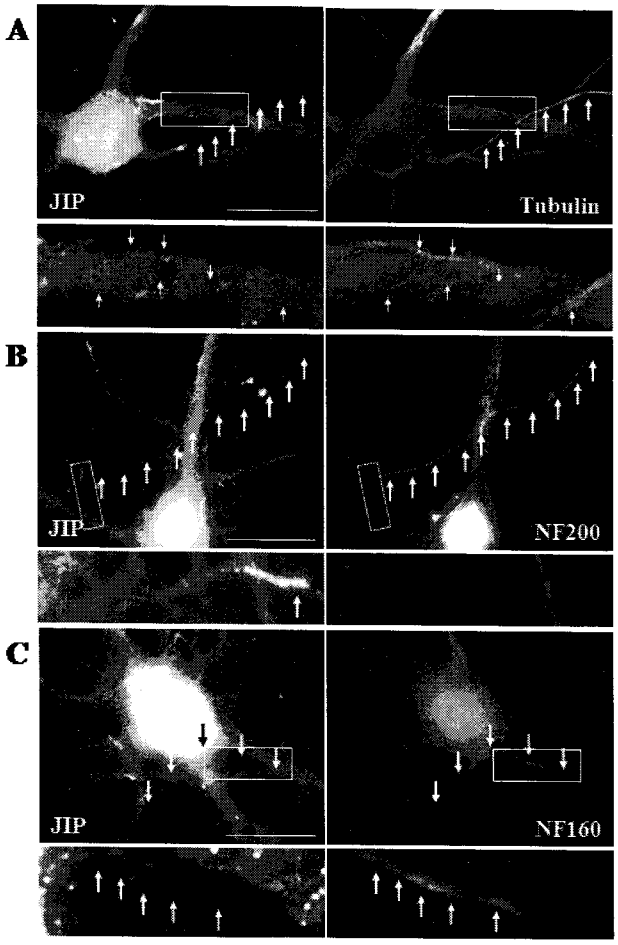

Fig. 5. JIP is minimally expressed in axons. DIV21 rat hippocampal neurons were fixed and double-stained with antibodies against JIP and α-tubulin (A), JIP and NF200 (B), and JIP and NF160. Axons are delineated by a series of arrows. Boxed areas are shown enlarged (insets). Several small arrows were put for positional orientation in inset of panel A. An example of dendritic ends where JIP is highly expressed is marked by a large arrow in the inset of panel B. Scale bar; 30 μm.

In this study it is shown that JIP proteins form clusters in somatodendritic domains. The JIP clusters included excitatory postsynaptic markers such as PSD-95 and α CaMKII but excluded inhibitory postsynaptic markers such as GlyR and gephyrin. Furthermore, JIP colocalized with SV2, a general marker for nerve terminals, but not with GAD, a marker for inhibitory nerve terminals. These results indicate that JIP is expressed in excitatory postsynaptic compartment. In addition, this study also showed that JIP is included in flotillin-positive lipid rafts, and that its expression in axon is minimal.

The main finding of the present study is the presence of JIP in excitatory postsynaptic compartments that is evidence by colocalization of JIP and PSD-95 or αCaMKII. Localization of JIP1 at synaptic sites is not unprecedented. Borsello *et al.* [5] have identified JIP1 in presynaptic terminal and postsynaptic elements. However, they did not identify whether they are excitatory or inhibitory synapses. A more direct hint for localization of JIP1 in excitatory spines has come from a physiological study. Kennedy *et al.* [13] examined the phenotype of compound mutant mice that lack expression of both JIP1 and JIP2. These mice exhibited severe defects in N-methyl-D-aspartic acid (NMDA) receptor function, including decreased NMDA-evoked current amplitude, cytoplasmic Ca^{2+}, and gene expression. The reduced NMDA receptor function observed in JIP1/2-deficient neurons was not caused by decreased expression of NMDA receptors but by marked decrease in Tyr phosphorylation of NR2B, an NMDA receptor substrate that is

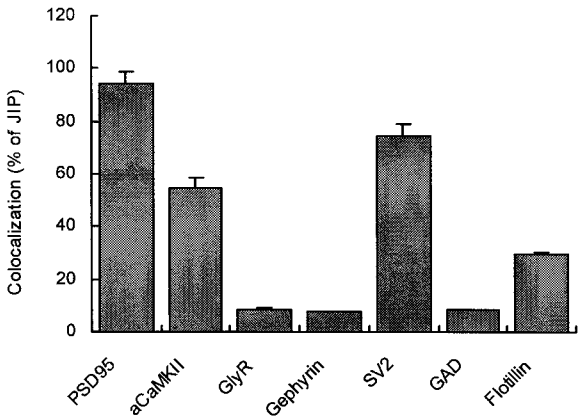

Fig. 6. Statistics. Each 10 segments of 20 μm of proximal dendrites from several neurons were used for counting clusters. Colocalization rates were express in percentage of total JIP clusters.

established to be phosphorylated by the c-Fyn tyrosine kinase *in vivo* [20]. Loss of tyrosine phosphorylation of NR2B is established to cause reduced NMDA receptor signaling [22] and may therefore contribute to the decreased NMDA receptor function observed in JIP1/2-deficient neurons. These data indicate that JIP1/2 is localized to the excitatory postsynaptic compartment playing import role in NMDA receptor signaling, and the present study confirms the localization of JIP at excitotory postsynaptic sites.

The present data showed that JIP is present in lipid rafts, special lipid microdomains in the lipid membrane that play various roles in cellular functions. Neurons are polarized cells whose function depends on the segregation of proteins to specific microdomains of the membrane. A multiprotein-signaling complex is assembled at the postsynaptic membrane of dendritic spines. The assembly of multiprotein-signaling complex could depend on cholesterol-rich lipid rafts. Indeed, Hering *et al.* [11] presented evidence that lipid rafts exist in the dendrites of neurons, where they are associated with a set of postsynaptic proteins. Flotillin-1 is a marker for lipid rafts. Its distribution in the spines of the rat brain at the electron microscopic level suggested that lipid rafts are localized, at least partly, to the postsynaptic cytoplasm, immediately below the PSD in the dendritic spines, and play a role in membrane trafficking (endocytosis and exocytosis) in the region near synaptic active sites [23]. The present data showed that about 30% of flotillin clusters includes JIP. Some of these JIP-containing lipid rafts may represent those in postsynaptic compartments.

In relevance to cytoskeletal elements, JIP did not colocalize with any of NF160, NF200 or microtubule. The expression in axons is very low or absent. Interestingly, JIP was highly expressed some select ends of dendrites. The role of JIP at this region needs to be elucidated in the future.

Acknowledgment

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초록 : 배양한 흰쥐 해마신경세포에서 c-Jun N-terminal kinase (JNK)-interacting protein (JIP)의 표현

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c-Jun N-terminal kinase (JNK)-interacting protein 1(JIP1)은 비계단백질(scaffold protein)로서 신경세포와 췌장β세포에서 많이 발현된다. 본 연구에서는 배양한 흰쥐 해마신경세포에서 JIP1, JIP-2 및 JIP-3을 모두 인식하는 항체를 이용하여 이들의 세포내 표현을 조사하였다. 전반적으로 JIP은 세포체와 가지돌기에 반점 모양으로 표현되었다. 이 JIP 반점들을 통계적으로 분석한 결과 흥분성 연접후표지인 PSD95 및 αCaMKII 반점과 각각 54.8 ± 4.0% 및 94.1 ± 4.5%가 겹쳐졌다. 반면에 억제성 연접후표지인 그리신수용체 및 gephyrin 반점과는 각각 단지 8.6 ± 0.5% 및 7.3 ± 0.5%만 겹쳐졌다. 한편 lipid raft의 표지인 flotillin 반점의 상당부분(29.3 ± 1.0%)이 JIP 반점과 겹쳐졌다. 또한, JIP은 일부 가지돌기의 끝부분에 매우 강하게 발현되었으며 축삭에는 표현이 미미하였다. 이 결과들은 JIP 단백질이 흥분성 연접후구역, 일부 lipid raft, 그리고 일부 가지돌기 끝부분에 주로 위치함을 의미한다.