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N-copine: a novel two C2-domain-containing protein with neuronal activity-regulated expression

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Abstract Neuronal activity is often associated with changes in gene expression. By a two-dimensional cDNA-display system, restriction landmark cDNA scanning, we identified a novel gene whose expression in the hippocampus was up-regulated by kainate stimulation. The mRNA expression was detected only in brain and up-regulated by the stimulation evoking CA3-CA1 long-term potentiation. The encoded protein contains two copies of C2-domain, known as the Ca^{2+}-binding domain of PKC-γ, and shows 49% identity with human copine I. We designated this protein N-copine (neuronal-copine). N-copine may have a role in synaptic plasticity.

Key words: N-copine; C2-domain; Gene expression; Kainate; Hippocampus; Plasticity; Long-term potentiation

1. Introduction

Activation of synapse transmission induces changes in biochemical events of neurons including gene expression. These changes lead to physiological changes in the central nervous system, like kindling, degeneration, and long-term potentiation of synaptic response (LTP) [1–3]. LTP at CA3-CA1 in the hippocampus (CA1-LTP) is one of the most studied forms of LTP [4]. For induction of CA1-LTP, the activation of NMDA-type glutamate receptor (NMDA-R) is necessary [5]. Moreover, gene expression is involved when CA1-LTP lasts more than 6 h [6]. The changes in gene expression induced by synapse activity seem to cause biochemical and structural changes in neurons which then lead to long-term changes in synaptic response.

Several groups have isolated the genes induced by synapse activity in the central nervous system [7–9]. However, there must be much more synapse activity-induced genes, as Fazeli et al. estimated the ratio of such genes to be about 1% [10]. To search for them, we have analyzed the changes in gene expression of the hippocampus from kainate-injected mice. Injection of kainate induces an LTP-like change in synaptic response of hippocampal neurons [3]. Using a novel cDNA-display system, restriction landmark cDNA scanning (RLCS) [11], we have identified the genes whose expressions were regulated by kainate in an NMDA-R-dependent manner. In the present study, we isolated cDNA of one of them. The encoded protein was a novel one and showed a high homology with human copine I, which was identified recently [12]. We designated this protein N-copine (neuronal-copine).

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2. Materials and methods

2.1. Animal procedures

All animals were treated ethically according to the rules of Shionogi Animal Use and Care Committee. Kainate (Sigma) was injected i.p. into 6–7 weeks female BALB/c mice (8 mg/kg). NMDA-R antagonist MK801 (RBI) was injected i.p. (5 mg/kg diluted in PBS) 30 min before kainate injection. Mice were killed by cervical dislocation and the brains were dissected.

2.2. RLCS and cloning of N-copine cDNA

RLCS analysis was performed according to the method described previously [11]. The cDNA fragment corresponding to spot 50 was subcloned into a pSPORT2 vector (GIBCO BRL) by the PCR-mediated method [11]. Using this as a probe in plaque hybridization, the full-length cDNA of mouse N-copine was isolated from λgt10 mouse brain cDNA library (CLONTECH). Human N-copine cDNA was isolated by PCR for human brain cDNA library (GIBCO BRL). The primers were designed from the mouse N-copine cDNA and a human EST clone sequence that corresponds to the 3′ non-coding region (GenBank accession number T15557). The sequences of the isolated cDNAs were determined on both strands. In the mouse cDNA, the *Bgl*II and *Hin*fI sites used for RLCS in this study were present at positions near the 3′-terminus that were expected from the RLCS profile. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession numbers AB008893 and AB009288.

2.3. Northern blot analysis

For multi-tissue Northern blot analysis, MTN blot membrane (CLONTECH) was used. For developmental analysis, 2 μg of poly $(A)^+$ RNA from mouse brain was separated on formaldehyde/1% agarose gel and then blotted onto a nylon membrane. Membranes were hybridized with ^{32}P-labeled 1.5 kbp DNA probe corresponding to the nucleotides 509–2019 of N-copine cDNA.

2.4. Electrophysiology

Preparation of transverse hippocampal slices from female BALB/c mouse (8 weeks) and electrophysiological experiments were performed as described previously [13]. Test stimuli (0.1 ms width, 0.033 Hz) were given to the Schaffer collateral/commissural pathway and field excitatory post synaptic potentials were recorded extracellularly in the pyramidal cell layer of the CA1 region. LTP was induced by three trains of tetanus (100 Hz, 1 s) given at 5-min intervals; field excitatory post synaptic potentials were potentiated more than 300% for 6 h.

2.5. In situ hybridization

In situ hybridization was performed as described [14]. The tissues with and without stimulations of kainate or LTP were embedded in a cryostat block, freshly frozen and cut into 8-μm-thick sections. The sections were subjected to hybridization with ^{35}S-labeled or digoxigenin-labeled riboprobe. For the template of riboprobe synthesis, 1.5 kbp fragment (nucleotide residues 509–2019) of the N-copine cDNA inserted into the pSPORT2 vector (GIBCO BRL) was used. As negative control, the sections were incubated with solution-containing sense probes; no signal was obtained.

2.6. Western blot analysis

GST protein fused with amino acid residues 4–161 of mouse N-copine was produced (Pharmacia). Antisera against the GST-N-copine fusion protein were raised in New Zealand White rabbits. The antibody specific for N-copine was purified by affinity chromatogra-

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phy with the N-copine fusion protein-conjugated Sepharose. Mouse brain homogenate was subjected to SDS-PAGE [15] and transferred to polyvinylidene difluoride membrane. After blocking with 10% skim milk in PBS, the membrane was incubated with the anti-N-copine antibody (70 ng/ml) and then with HRP-conjugated anti-rabbit IgG antiserum (1:2000 dilution, CAPPEL). Peroxidase-coupled detection was performed with ECL system (Amersham).

3. Results

3.1. Regulation of N-copine mRNA expression in hippocampus by neuronal activity

To find genes whose expression in hippocampus was up-regulated by kainate injection, we used the RLCS system, a cDNA-display system on a two-dimensional gel matrix. In RLCS, each cDNA species appears as a spot at a certain position on a two-dimensional gel; the changes in the intensity of each spot reflect the changes in the expression of the corresponding gene [11]. We compared the RLCS profiles of the hippocampus from the kainate-injected mice with that of the hippocampus from the mice not injected, and found out spots whose intensities were enhanced by kainate injection (Fig. 1A). N-copine cDNA was derived from one of them, spot 50. The intensity of spot 50 was enhanced 6 h but not 2 and 24 h after kainate injection. Administration of the NMDA-R antagonist MK801 before kainate injection inhibited the enhancement (Fig. 1B). This result shows that the expression of N-copine mRNA is up-regulated in an NMDA-R-dependent manner.

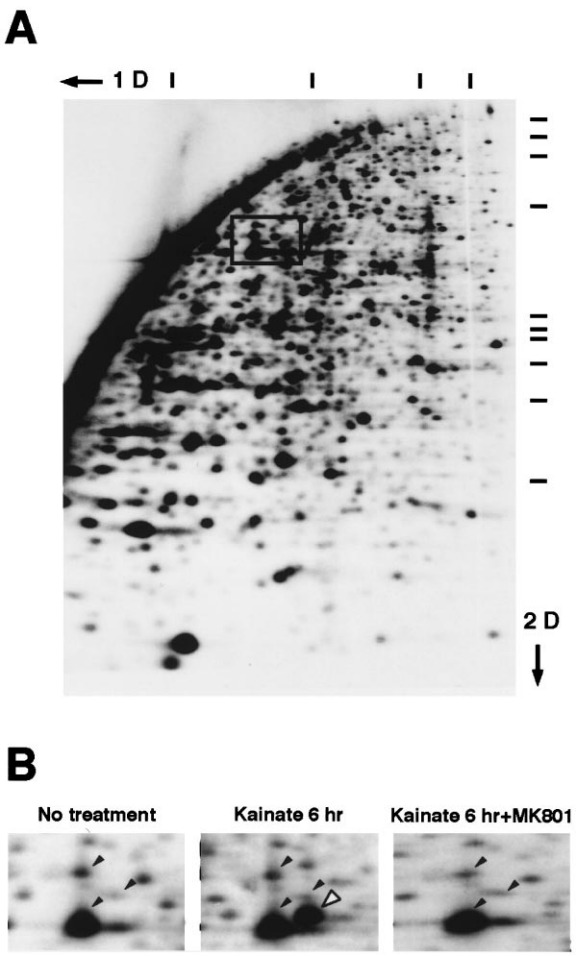

Fig. 1. RLCS analysis of hippocampus cDNA from kainate-injected mice. A: Whole profile of non-treated mice. Arrows show the directions of first- and second-dimensional electrophoresis (1D and 2D, respectively). The scales are 1.88, 1.49, 0.93, and 0.42 kbp (from right to left) in 1D, and 1357, 1078, 872, 603, 310, 281/271, 234, 194, 118 and 72 bp (from top to bottom) in 2D. The region around spot 50 (surrounded by a square) is zoomed up in B. B: Profiles around spot 50 from mice not treated, 6 h after kainate injection and 6 h after kainate injection with MK801 pretreatment. Open arrowhead shows spot 50. Closed arrowheads show the spots whose intensities are not changed.

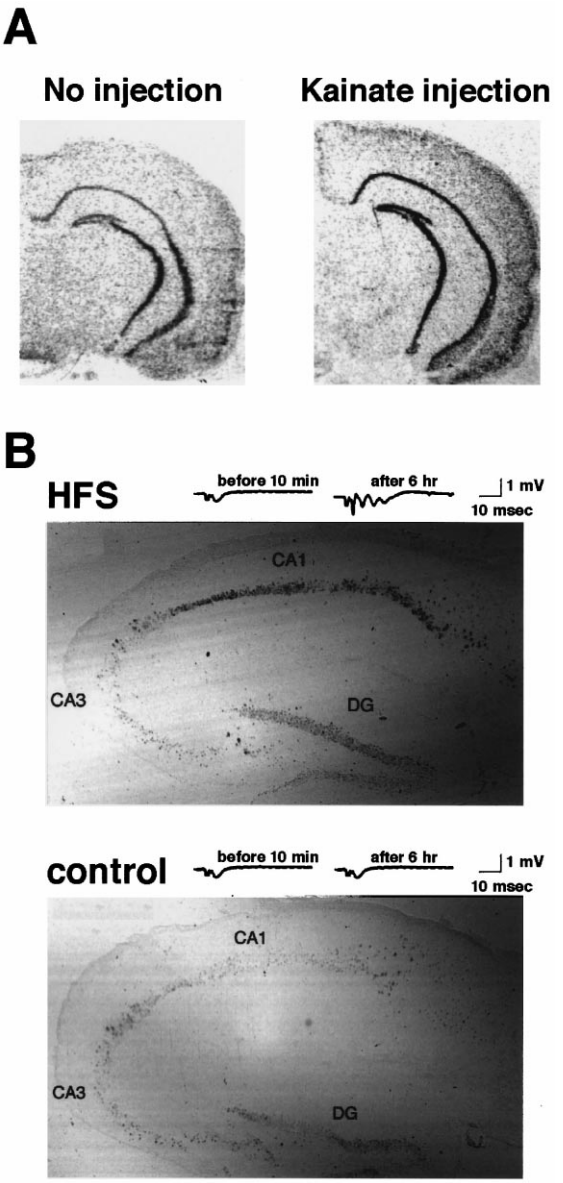

Fig. 2. Up-regulation of N-copine mRNA expression in hippocampus by neuronal activity. A: In situ hybridization of brain frontal sections from mice 6 h after kainate injection or not injected. ^{35}S-labeled riboprobe specific for N-copine mRNA was used. B: In situ hybridization of hippocampal slices 6 h after high-frequency stimulation (HFS) evoking CA1-LTP or no stimulation (control). Digoxigenin-labeled riboprobe specific for N-copine mRNA was used. fEPSPs of 10 min before and 6 h after the stimulation are also shown.

By in situ hybridization, we confirmed that N-copine expression was up-regulated in the whole hippocampus, particularly in the CA1 region, at 6 h after kainate injection but not

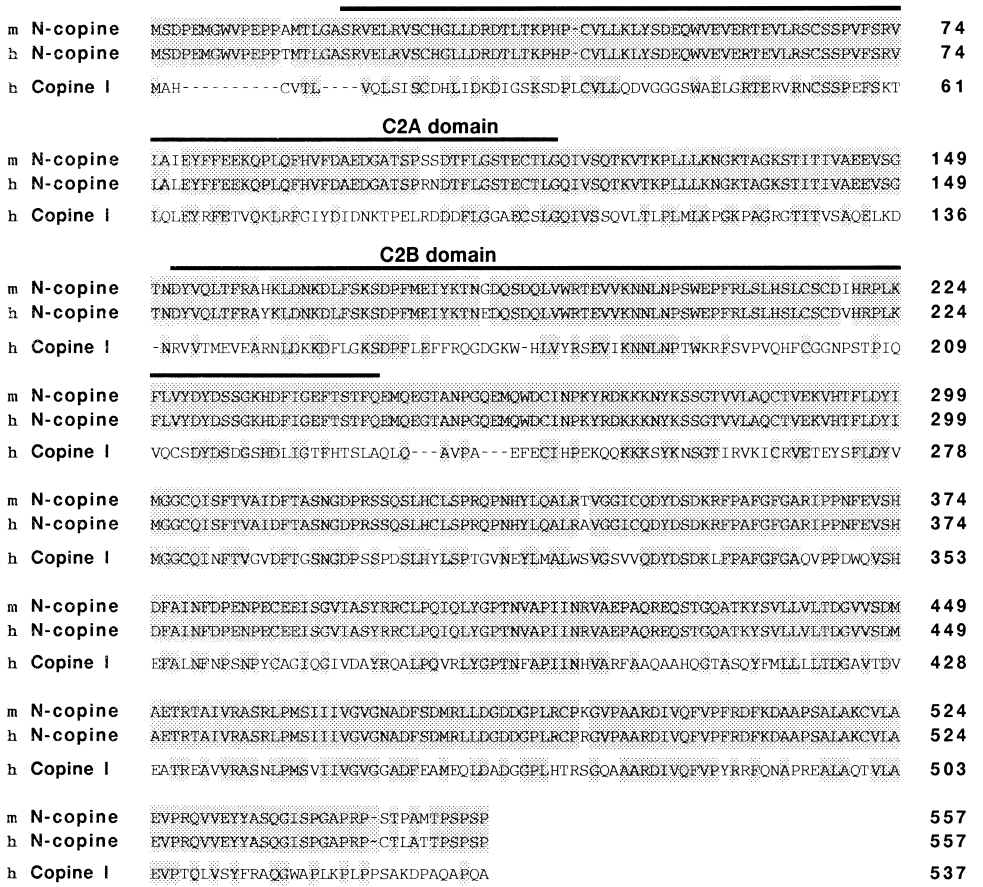

Fig. 3. Alignment of amino acid sequences of mouse and human N-copine (m, h N-copine), and of human copine I (h copine I). Identical amino acid residues are shaded. Hyphens show gaps. Solid lines show C2-domains.

at 2 h after kainate injection (Fig. 2A). Because we did not detect any signals in glia by more precise investigation (data not shown), we conclude that N-copine expression is neuron-specific. Then we evoked CA1-LTP in the mouse-hippocampal slices that lasted for more than 6 h with the high-frequency stimulation and performed in situ hybridization (Fig. 2B). The expression of N-copine mRNA was significantly up-regulated in pyramidal cells in CA1, but not in neurons in other regions. In the adjacent section of the same slice, we confirmed the up-regulation of proenkephalin mRNA, whose expression was reported to be enhanced by the stimulation evoking LTP [7]. These results show that the expression of N-copine mRNA in CA1 pyramidal cells was associated with the CA1-LTP.

3.2. Cloning and structure of N-copine cDNA

Using cDNA fragment extracted from spot 50 as a probe, we isolated the full-length cDNA of N-copine from the mouse brain cDNA library. Its size was 2.1 kbp, which corresponds well to the size of the transcript estimated by Northern blot analysis (2.2 kbp, Fig. 5A). We also isolated the cDNA of the human counterpart from the human brain cDNA library. In both human and mouse cDNAs, there was only one large ORF of 1674 bases followed by a poly(A) signal. The sequence around the initiating Met is in accordance with the Kozak rule [16]. The ORF of N-copine cDNA encodes a protein which consists of 557 amino acid residues; it is hydrophilic and does not seem to have a signal sequence or transmembrane region (Fig. 3). The identity of the amino acid sequences between human and mouse was 98%. From the GenBank database, we identified human copine I [12] as the protein most homologous to N-copine. Identity between human N-copine and human copine I is 49%.

The most interesting characteristic of the amino acid sequence of N-copine is the two C2-domains, which were first characterized as a Ca^{2+}-binding domain of protein kinase C [17]. The C2-domains of N-copine conserve the amino acid residues typical of other C2-domains (Fig. 4A). Especially, the second C2-domain conserves the five Asp residues that are considered essential to Ca^{2+} binding [18]. As a family of proteins that contain two C2-domains, Südhof et al. propose a 'double C2-domain protein family', including synaptotagmin, rabphilin-3A, and Doc2 [19]. In spite of the existence of two C2-domains, N-copine and copine I are rather different from the members of the conventional 'double C2-domain protein family'. First, the phylogenetic tree shows that two C2-domains of N-copine and copine I are more similar to those of PLC-γ than to those of synaptotagmin and Doc2 (Fig. 4B). Second, N-copine and copine I have two C2-domains in the N-terminal regions, whereas those of the proteins of 'double C2-domain protein family' have C2-domains in their C-terminal regions.

3.3. Brain-specific expression of N-copine

Multi-tissue Northern blot analysis showed that the N-copine-specific 2.2 kbp transcript was detected only in the brain

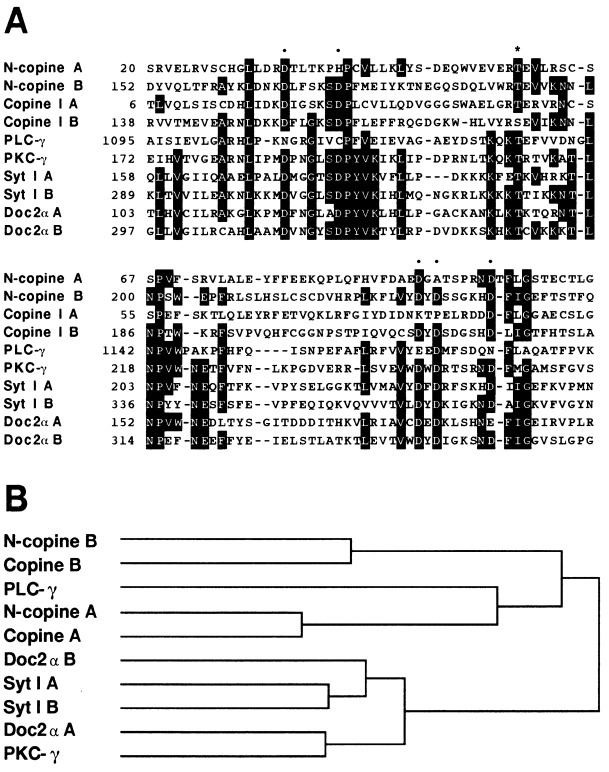

Fig. 4. Domain structure of N-copine. A: Sequence alignment of two C2-domains (classified as A, B) present in N-copine, copine I, synaptotagmin I, Doc2α. The C2-domains in phospholipase C-γ (PLC-γ) and protein kinase C-γ (PKC-γ) are also aligned. Amino acid residues conserved in more than five kinds of C2-domains are shown on black backgrounds. Dots show the five conserved aspartate residues that are considered to be important for Ca^{2+} binding. The asterisk shows the conserved threonine residue, which serves as a putative phosphorylation site. B: Phylogenetic tree. Evolutionary distances between the C2-domains from various proteins were estimated with the Gene Works (IntelliGenetics).

but not in other tissues (Fig. 5A). During the development of the brain, expression of N-copine mRNA was strikingly increased after birth (Fig. 5B). In Western blot analysis with anti-N-copine antibody, we detected N-copine in the mouse brain homogenate; its size was 62 kDa (Fig. 5C), which corresponds well with the molecular weight calculated from the amino acid sequence (61.7 kDa).

4. Discussion

In this study, we identified a cDNA encoding a novel protein, N-copine. N-copine shows a high homology with human copine I, which was isolated as a Ca^{2+}-dependent phospholipid-binding protein. Creutz et al. suggest that human copine I composes a protein family by searching the other copine-related EST clones [12]. We conclude that N-copine is a brain-specific member of the copine family.

N-copine has two C2-domains. Various functions have been reported for C2-domains of other proteins: Ca^{2+}-dependent and Ca^{2+}-independent binding to phospholipid, inositol polyphosphate binding, Ca^{2+} binding, interaction with other proteins [19]. The C2-domains of N-copine are expected to have some of these functions. The five Asp residues considered to be important for Ca^{2+} binding of a C2-domain [18] are conserved in the second C2-domain of N-copine. N-copine may be able to bind to Ca^{2+}. In spite of the existence of two C2-domains, N-copine and copine I are not members of the 'double C2-domain protein family' from their structures. Members of the 'double C2-domain proteins' in neuron are involved in the transport of synaptic vesicles during neurotransmitter release [20,21]. N-copine may have different functions from those of 'double C2-domain proteins'.

Interestingly, comparison of the sequence of human N-copine with that of human copine I reveals another characteristic of proteins of this family. Although the identity of the amino acid sequence of the C2-domains between N-copine and copine I is 40%, that of the C-terminal half region is about 60%. Therefore the C-terminal half region is expected to have some unidentified functions. Analyses of such functions would be helpful to reveal a role of N-copine and other family proteins.

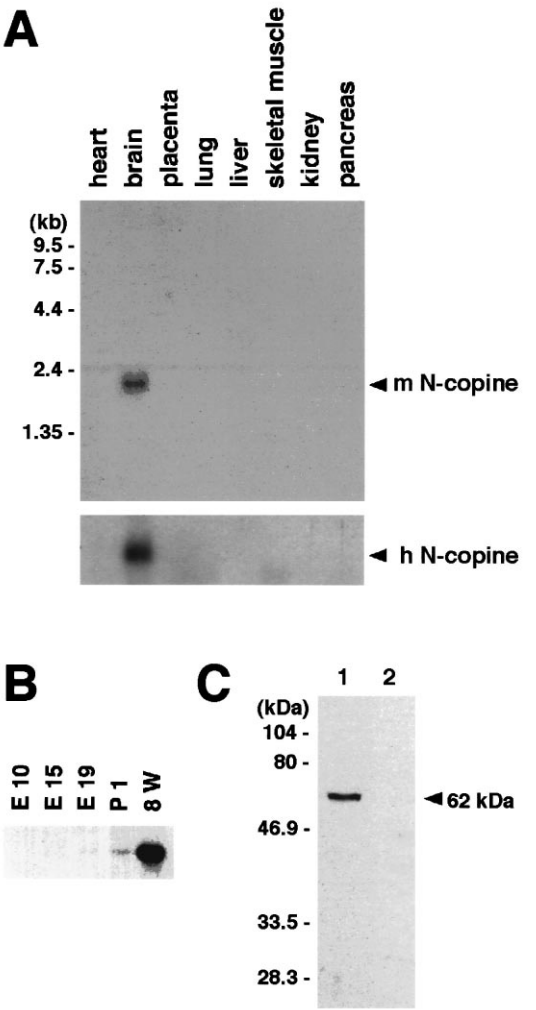

Fig. 5. Brain-specific expression of N-copine. A: Multi-tissue Northern blot analyses of N-copine mRNA expression in adult mouse (upper panel) and human (lower panel). B: Developmental expression of N-copine mRNA in mouse brain. Poly(A)$^+$ RNA derived from mouse brain of embryonic day 10, 15, and 19 (E10, E15, and E19, respectively), and postnatal 1 day and 8 weeks (P1 and 8W, respectively) was used for the analysis. C: Detection of N-copine in mouse brain homogenate with anti-N-copine antibody (lane 1). As a control, the antibody was absorbed with the excess amount of the GST-N-copine fusion protein (lane 2). Position of N-copine (62 kDa) is indicated.

The expression of N-copine mRNA in the hippocampus was up-regulated by kainate injection in an NMDA-R-dependent manner. Kainate injection in vivo has been reported to induce LTP-like potentiation in the hippocampus [3]. Also, Ca^{2+}-influx via NMDA-R is essential to CA1-LTP [5]. In fact, we found that the expression of N-copine mRNA was up-regulated by the stimulation that induced CA1-LTP. Therefore, N-copine is likely to be involved in LTP. Analyses of N-copine functions would reveal a new aspect on the mechanism of synaptic plasticity.

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