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Synaptotagmin1 synthesis induced by synaptic plasticity in mouse hippocampus through activation of nicotinic acetylcholine receptors

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ABSTRACT

We have reported that systemic application of nicotinic agonists expresses a long-term potentiation (LTP)-like facilitation, a model of synaptic plasticity, *in vivo* in the mouse hippocampus. The present study conducted to clarify the involvement of synaptotagmin1 in synaptic plasticity by investigating the time-dependent change of the mRNA and protein levels of synaptotagmin1 during LTP-like facilitation in the mouse hippocampus. The mRNA expression of synaptotagmin1 increased during 2- to 8-h period by intraperitoneal application of nicotine (3 mg/kg), returning to the basal level in 12-h. Also, the protein level of synaptotagmin1, but not synaptophysin, in a total fraction from hippocampus increased during 4- to 12-h period by the same treatment, returning to the basal level in 24-h. The protein level of synaptotagmin1 in a membrane fraction from hippocampus also increased during 4- to 8-h period by nicotine, returning to the basal level in 12-h. This nicotine-enhanced synaptotagmin1 protein in a membrane fraction was inhibited by pretreatment of mecamylamine (0.3 mg/kg, i.p.), a nonselective nicotinic acetylcholine receptors (nAChRs) antagonist. Furthermore, choline (30 mg/kg, i.p.), a selective α7 nAChR agonist, or ABT-418 (10 mg/kg, i.p.), a selective α4β2 nAChR agonist, enhanced the level of synaptotagmin1 in a membrane fraction. Our findings demonstrate that synaptotagmin1 protein following mRNA which is enhanced without increasing the number of synapse gathers around pre-synaptic membrane during hippocampal LTP-like facilitation through activation of α7 and/or α4β2 nAChRs in the brain. These results suggest that new-synthesized synaptotagmin1 following synaptic plasticity may contribute to long-lasting synaptic plasticity via positive, feedfoward mechanisms.

Synaptic plasticity which is a long-lasting increase in the efficacy of synaptic transmission is assumed to underlie plastic changes associated with learning and memory [2,3]. Long-term potentiation (LTP) in the hippocampus being a center for learning and memory is the most studied form of synaptic plasticity. The hippocampus also has cholinergic innervations and dense nicotinic acetylcholine receptors (nAChRs) expression [25,30,32], which are implicated in the basic processes underlying learning and memory [16,20,21]. nAChRs containing α7 and α4β2 subunits are the most abundant in the brain [7,25]. Both α7 and α4β2 nAChRs have significant Ca^{2+} permeability [23,25,29], indicating that these receptors induce Ca^{2+}-sensitive process such as neurotransmitter release. Indeed, activation of presynaptic α7 nAChRs in the hippocampus increases glutamate release [11]. Nicotine facilitates LTP induction in hippocampal slice preparations [8]. We have shown that LTP is inhibited by a nAChRs inhibitor and activation of nAChRs expresses a LTP-like facilitation *in vivo* in the intact mouse hippocampus [18,19]. The expression of hippocampal LTP is due to enhanced presynaptic neurotransmitter release and/or enhanced postsynaptic sensitivity [13]. Taken together, the enhancement of presynaptic neurotransmitter release might contribute to synaptic plasticity, a LTP-like facilitation through nAChRs activation.

Synaptotagmin1, the vesicle-associated Ca^{2+}-binding protein, consists of a single transmembrane region and two copies of a Ca^{2+} regulatory domain called the C2 domain (C2A and C2B), and is required for the tight temporal coupling between Ca^{2+} influx

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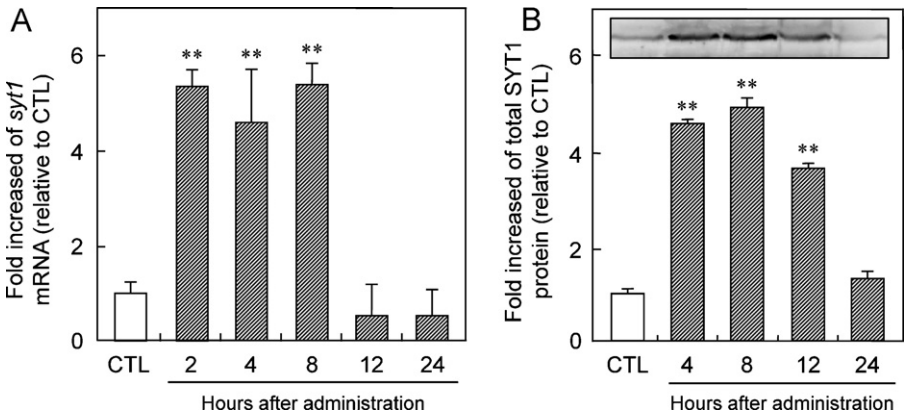

Fig. 1. Increased expression of synaptotagmin1 during synaptic plasticity by activation of nAChRs. (A) The mRNA expression of *synaptotagmin1* (*Syt1*) in mouse hippocampus was analyzed by quantitative RT-PCR. *Gapdh* was used as endogenous control. The indicated time shows the survival term of mouse after nicotine treatment (3 mg/kg, i.p.). Results for *Syt1* mRNA are expressed as a fold change relative to control (CTL). Data are presented as mean ± SEM ($n = 6$). $^{**}P < 0.01$ compared with CTL. (B) Total SYT1 protein from mouse hippocampus increases during synaptic plasticity by activation of nAChRs. Protein in a total fraction from mouse hippocampus was analyzed by western blotting with antibody against SYT1. The indicated time shows the survival term of mouse after nicotine treatment (3 mg/kg, i.p.). As an internal control, GAPDH was monitored with anti-GAPDH antibody (data not shown). Densitometric analysis shows the level of total SYT1 protein as a fold change relative to control (CTL). Data are presented as mean ± SEM ($n = 6$). $^{**}P < 0.01$ compared with CTL.

and synaptic vesicle fusion as the Ca^{2+} sensor [4,14]. Mice carrying a mutation in the *synaptotagmin1* (*Syt1*) gene revealed defects in neurotransmitter release in hippocampal neurons [10]. The impairment of Ca^{2+} binding in the C2B domain causes a dramatic decrease in neurotransmitter release [17]. Thus, this particular property of the Ca^{2+} sensor is observed as a cooperative increase in neurotransmitter release with increasing concentration of Ca^{2+}.

LTP occurs in two temporally distinct phases: early LTP (E-LTP) depends on modification of preexisting proteins and lasts only 1- to 2-h, whereas late LTP (L-LTP) requires transcription and synthesis of new proteins and persists for many hours [6]. Taken together, synaptotagmin1 is an attractive candidate for the critical proteins synthesized during L-LTP. However, there are no direct reports of synaptotagmin1 linking with synaptic plasticity under the influence of nAChRs. To elucidate the involvement of synaptotagmin1 (SYT1) protein in synaptic plasticity, we investigated the time-dependent change of hippocampal SYT1 protein of mice treated by nAChRs agonists.

Male C57BL/6 mice (25–30 g) were housed under standard illumination parameters (12-h light/dark cycle), and were given free access to food and water. Animal care and handling were done strictly in accordance with the "Guidelines for Animal Experimentation at Himeji Dokkyo University."

Nicotine (3 mg/kg, RBI, Natick, MA), choline (30 mg/kg, Wako Pure Chemicals, Osaka, Japan), mecamylamine (0.5 mg/kg, RBI), ABT-418 (10 mg/kg, Sigma-RBI, St. Louis, MO) was dissolved in sterile saline. These drugs or vehicle (sterile saline) at a volume of 0.1 ml were injected intraperitoneally at the indicated time.

C57BL/6 mice treated with each drugs were sacrificed at the indicated time under anesthesia administered by intraperitoneal injection of pentobarbital sodium salt. Mouse hippocampus was isolated, and used for the quantitative analysis for mRNA and protein of synaptotagmin1.

Total RNA was prepared from the isolated mouse hippocampus by using SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer's instructions. For quantitative RT-PCR analysis, first-strand cDNAs were synthesized from 5 μg of total RNA by using Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics, Indianapolis, IN) at 55 °C for 30 min after denaturation at 65 °C for 10 min. The cDNA was amplified by use of a Fast Real-Time PCR system, Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), and gene specific primers as follows: *Syt1*, forward primer 5′-gctttgaagttccgttcgag-3′ and reverse primer 5′-agcatgtctgaccagtgtcg-3′; *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase), forward primer 5′-cgtgttcctacccccaatgt-3′ and reverse primer 5′-tgtcatcatacttggcaggtttct-3′. All oligonucleotide primers were synthesized by Invitrogen (Tokyo, Japan). Duplicate PCR products were evaluated by melting curve analysis following the manufacturer's instructions and checked after agarose gel electrophoresis. Data analysis was performed using a comparative CT method [31]. The endogenous control *Gapdh* was used to normalize quantification of mRNA target.

Mouse hippocampal homogenate was prepared by sonication in a lysis buffer (20 mM Tris–HCl, pH 7.2, 2 mM EDTA, 1% TritonX-100 and 6 M urea) containing protease inhibitor cocktail (Roche Diagnostics). After centrifugation of its homogenate at 15,000 rpm for 10 min at 4 °C, the supernatant was used as a total fraction. Membrane fraction from mouse hippocampus was isolated using ProteoExtract Native Membrane Protein Extraction Kit (Calbiochem, San Diego, CA) according to the manufacturer's instructions. SYT1 or synaptophysin (SYP) were separated in 7.5% SDS-polyacrylamide gel, and blotted onto an Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking and washing, SYT1 or SYP was visualized using antibodies against SYT1 (1/1000, Synaptic Systems, Goettingen, Germany) or SYP (1/1000, Synaptic Systems) and ECL Western blotting detection kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions.

Statistical significance of the differences between groups was determined by Student's *t*-test or one-way ANOVA followed by Dunnett's multiple-comparisons tests.

To investigate whether the expression of *synaptotagmin1* (*Syt1*) mRNA changes during synaptic plasticity, we performed quantitative RT-PCR (qRT-PCR) analysis using mouse hippocampus which expresses a LTP-like facilitation by systemic application of nicotine (3 mg/kg, i.p.) [18]. qRT-PCR analysis showed that the mRNA expression of *Syt1* significantly increased about 5.3-, 4.6- and 5.4-fold 2-, 4- and 8-h after nicotine administration (3 mg/kg, i.p.) as compared with control, respectively, and returned to the basal level in 12-h (Fig. 1A). *Gapdh* gene, which was used as an internal control, induced no change 2- to 24-h after nicotine treatment (data not shown).

Next, we examined the level of SYT1 protein by western blot analysis with antibody against SYT1 in a total fraction from hippocampus. SYT1 protein in a total fraction significantly increased about 4.6-, 4.9- and 3.7-fold 4-, 8- and 12-h after nicotine adminis-