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Development- and activity-dependent regulation of SNAP-25 phosphorylation in rat brain

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Abstract

Synaptosomal-associated protein of 25 kDa (SNAP-25), a member of the SNARE proteins essential for neurotransmitter release, is phosphorylated at Ser^{187} in PC12 cells and in the rat brain in a protein kinase C-dependent manner. It remains unclear how the phosphorylation of SNAP-25 is regulated during development and by neuronal activity. We studied the mode of SNAP-25 phosphorylation at Ser^{187} in the rat brain using an anti-phosphorylated SNAP-25 antibody. Both the expression and phosphorylation of SNAP-25 increased remarkably during the early postnatal period, but their onsets were quite different. SNAP-25 expression was detected as early as embryonic Day 18, whereas the phosphorylation of SNAP-25 could not be detected until postnatal Day 4. A delay in the onset of phosphorylation was also observed in cultured rat hippocampal neurons. The phosphorylation of SNAP-25 was regulated in a neuronal activity-dependent manner and, in the rat hippocampus, decreased by introducing seizures with kainic acid. These results clearly indicated that the phosphorylation of SNAP-25 at Ser^{187} is regulated in development- and neuronal activity-dependent manners, and is likely to play important roles in higher brain functions.

Keywords: SNAP-25; Phosphorylation; Development; Activity-dependent

Neurotransmitter release is regulated by several different mechanisms, and these regulations are some of the important mechanisms of synaptic plasticity that underlie learning and memory. In the short-term, many protein kinases regulate neurotransmitter release both positively and negatively, for which various protein substrates have been identified [2,11,24,32,40]. However, little is known about the precise mechanisms and functional relevance to brain functions.

Synaptosomal-associated protein of 25 kDa (SNAP-25) is a membrane protein expressed in neurons and endocrine cells [29]. In neurons, SNAP-25 is localized primarily in axons and nerve terminals, with a large part associated with the plasma membrane through palmitoylated cysteine residues. A significant population of SNAP-25 is also localized in cytoplasmic vesicular membranes [43]. SNAP-25 is one of the so-called soluble *N*-ethylmaleimide-sensitive fusion protein receptor (SNARE) proteins and plays an essential role in neurotransmitter release through the formation of a core complex with other SNARE proteins, VAMP-2/synaptobrevin 2 and syntaxin 1 [4,5,17,33,37–39]. In addition to its essential role in neurotransmitter release, the possible involvement of SNAP-25 in neurite extension and sprouting [3,28], regulation of ion channel functions [13,14,20,44,46], and neurotransmitter-receptor incorporation into the plasma membrane [18,19] has been suggested. Thus, SNAP-25 is likely to play multiple roles in synapses, and the elucidation of its regulatory mechanisms will contribute to the understanding of synaptic plasticity.

Previously, we showed that the treatment of clonal rat pheochromocytoma PC12 cells with phorbol-12-myristate-13-acetate (PMA), a potent protein kinase C (PKC) activator,

Abbreviations: BAP, bacterial alkaline phosphatase; HFS, high frequency stimulation; PKC, protein kinase C; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMA, phorbol-12-myristate-13-acetate; SDS, sodium dodecyl sulfate; SNAP-25, synaptosomal-associated protein of 25 kDa; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein receptor

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resulted in the phosphorylation of SNAP-25 at Ser^{187} and concomitant suppression of the association with syntaxin 1 [35]. Acute activation of PKC enhanced the Ca^{2+}-dependent release of catecholamine from PC12 cells and bovine adrenal chromaffin cells by recruiting secretory vesicles to the plasma membrane, for which SNAP-25 phosphorylation was essential [23,36]. SNAP-25 phosphorylation was also regulated by a more sustained mechanism, and the phosphorylation level and preferential localization of SNAP-25 in the plasma membrane was upregulated after prolonged incubation with nerve growth factor (NGF) [15]. All such results suggest the importance of SNAP-25 phosphorylation in neuronal function, but little is known of how and when SNAP-25 is phosphorylated *in vivo*.

All procedures involving animals complied with the guidelines of the National Institutes of Health and were approved by the Animal Care and Use Committee of the Mitsubishi Kagaku Institute of Life Sciences. To obtain seizure-induced animals, adult male Wister rats (7 weeks) were injected with kainic acid (15 mg/kg; Sigma, St Louis, MO, USA) or an equivalent volume of phosphate-buffered saline (PBS). Animal behavior was then monitored for 1 h to detect seizures. The surviving animals that exhibited seizures were sacrificed either 2 or 3 h after intraperitoneal (i.p.) or subcutaneous (s.c.) injections, respectively. After sacrificing the animals, hippocampi were dissected out and frozen immediately in liquid nitrogen, as previously described [21]. The hippocampi from the untreated animals were used as controls. The hippocampal samples were homogenized in sodium dodecyl sulfate (SDS) sample buffer and supplied for Western blotting. For embryonic and postnatal studies, timed pregnancies were established in female Wister rats. Estrus phases were determined by vaginal lavage and mating was confirmed by the presence of a sperm plug, which was recorded as embryonic Day 0 (E0) of gestation. P_2 fractions were prepared from the whole brain of various embryonic rats and from the forebrain without cerebellum of postnatal rats.

Primary cultures of rat hippocampal neurons from 18-day embryos were performed, as previously described [27]. To obtain the protein samples, culture dishes were washed with PBS and cells were disrupted in SDS sample buffer.

Anti-phosphorylated SNAP-25 polyclonal antibody (pAb SN25Pi) was raised to a peptide with the sequence (C)MEKADS(Pi)NKTRI, corresponding to SNAP-25 residues 182-192, which includes a phospho-serine at position 187 [12]. A monoclonal anti-SNAP-25 antibody, BR05 [25] and anti-SNAP-25-Ct antibody raised against a peptide corresponding to SNAP-25 residues 195-207 [12] were used for the detection of total SNAP-25. An anti-synaptotagmin 1 antibody (1D12 [47]) was used for the normalization of immunoblots.

Protein samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE), followed by immunoblotting according to the standard protocol [15]. Immunoreactive bands were quantified by a luminescent image (LAS-1000, Fuji Photo Film, Tokyo, Japan).

Previously, we demonstrated that SNAP-25 was phosphorylated at Ser^{187} in clonal rat pheochromocutoma PC12 cells by stimulation with PMA [9,12,36]. Initially, we examined whether SNAP-25 could be phosphorylated at Ser^{187} in rat brain neurons by using a specific anti-phosphorylated SNAP-25 antibody, SN25Pi. In cultured rat hippocampal neurons, a faint immunoreactive band was detected with SN25Pi in the absence of PMA, indicating that a small but significant fraction of SNAP-25 was phosphorylated under normal culture conditions. The intensity of the immunoreactive band to SN25Pi increased remarkably after treatment of the culture with PMA (Fig. 1A), indicating that the Ser^{187} of SNAP-25 could be phosphorylated by the activation of endogenous PKC in cultured neurons. An immunoreactive band to SN25Pi was also visible in the P_2 membrane fraction of rat brain homogenate (Fig. 1B). Immunoreactivity decreased remarkably by treatment with bacterial alkaline phosphatase (BAP). In contrast, immunoreactivity to a conventional anti-SNAP-25 antibody (BR05) was not affected by BAP treatment. These results indicate that SNAP-25 is phosphorylated at Ser^{187} in rat brain neurons.

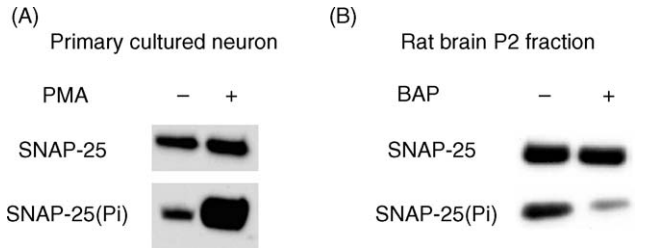

Fig. 1. SNAP-25 phosphorylation in rat brain. (A) Effect of PKC activation on SNAP-25 phosphorylation in rat hippocampal primary culture. The 10-day culture was incubated in the presence (+) or absence (−) of PMA (1 μmol/L) for 30 min. Then the same amount of cellular proteins samples (1 μg for SNAP-25 blot and 10 μg for SNAP-25(Pi) blot) was supplied for immunoblotting with either anti-SNAP-25 (BR05; upper) or anti-phosphorylated SNAP-25 (SN25Pi; lower) antibodies. (B) SNAP-25 phosphorylation in rat brain. The same amount of solubilized P_2 fraction from postnatal Day 20 rat was incubated in the presence (+) or absence (−) of BAP (10 units) for 30 min. The samples were then separated by SDS-PAGE and analyzed by immunoblotting as in Panel A.

To determine whether SNAP-25 phosphorylation is regulated in a development-dependent manner, immunoblotting analysis was performed using rat brain P_2 fractions of various developmental stages (Fig. 2A). As previously described [30], SNAP-25 expression was detected as early as 20 embryonic days, then increased almost linearly thereafter, and reached a plateau around 20 days after birth. In contrast, SNAP-25 phosphorylation could not be detected clearly until postnatal Day 4 and increased significantly up to 60 days after birth. A quantitative analysis revealed that the ratio of SNAP-25 protein level and that of SNAP-25 phosphorylation level at P8 to P14 was 0.67 ± 0.08 (S.D., $n = 3$) and 0.28 ± 0.02 (S.D., $n = 3$), respectively (Fig. 2B). These results suggest that SNAP-25 phosphorylation is regulated by development-dependent mechanisms with a mode distinct from that of SNAP-25 protein expression.

Since embryonic neurons continue to maturate *in vivo*, it would be interesting to see whether SNAP-25 phosphorylation is regulated in cultured neurons in a maturation-dependent manner. Fig. 2C shows that the amount of SNAP-25 increased almost linearly and reached a plateau around 21 days in culture. In contrast, SNAP-25 phosphorylation could not be detected at 6 days in culture, but dramatically increased between 14 and 21 days,