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Ultrastructural features of hippocampal CA1 synapses with respect to synaptic enhancement following repeated PKA activation

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Abstract

We reported previously that repeated activations, but not a single activation, of cyclic AMP-dependent protein kinase (PKA), led to a slowly developing (requiring ∼1 week to develop) long-lasting (lasting ≥3 weeks) enhancement of synaptic transmission efficiency in the organotypic slice culture of the rat hippocampus. It was accompanied by an increase in the number of synapses identified immunohistochemically. To answer the question of whether the "perforated synapse", which is known to occur transiently after the induction of long-term potentiation (LTP) in combination with the enlargement of postsynaptic density (PSD), is involved also in this slow/persistent synaptic enhancement, we examined the ultrastructural changes after the repeated activations of PKA. The answer was partially yes (occurrence of perforated synapses was increased) but partially no (the increase in the number of perforated synapses was not transient but persistent; mean apparent size of PSD did not increase). These results suggest that the mechanism of the slow/persistent synaptogenesis shares limited features with the mechanism of the quick/transient morphogenesis after LTP.

Keywords: Electron microscopy; Hippocampal slice culture; PKA activation; Synaptic plasticity; Synaptogenesis; Long-term memory

Synaptic plasticity is assumed to be the neural basis of memory. Although the cellular and molecular mechanisms for its relatively short-lasting phase (<24 h) have been intensively studied, those for its long-lasting phase (>24 h) have been analyzed less well. We reported previously that repeated activations, but not a single activation, of cyclic AMP-dependent protein kinase (PKA), led to a slowly developing (requiring ∼1 week to develop) long-lasting (lasting ≥3 weeks) enhancement of synaptic transmission efficiency in the CA1 area of the stable organotypic culture of the rat hippocampus [17]. This long-lasting enhancement was accompanied by an increase in the number of pre- and postsynaptic structures identified immunohistochemically. We proposed that this phenomenon should serve as a good *in vitro* model system for the analysis of long-lasting synaptic plasticity.

As to the morphological changes coupled with synaptic enhancement, those following the induction of long-term potentiation (LTP) after tetanic stimulation have been well documented. Synaptic input at high frequency provokes an enlargement of postsynaptic spines [5,9] and/or perforation (*i.e.* being horseshoe-shaped or doughnut-shaped) of synapses [12,19]. Then the question arises of whether the slow/persistent synaptic enhancement after the repeated PKA activations which we reported previously would also be accompanied by morphological changes similar to those after LTP, although the time courses of development are quite different between the two phenomena. To answer this question we examined here the ultrastructural features of the CA1 region of cultured slices after the repeated activations of PKA and performed a quantitative analysis of those features.

As described previously [17], organotypic slice cultures of the hippocampus were prepared from rat pups of the Wistar strain (Nihon SLC) at 8–9 days of age and maintained for 10–11 days before stimulation, during which period the cultures underwent maturation [10,17]. Animals were treated properly following our institutional guidelines for animal welfare.

Abbreviations: ACSF, artificial cerebrospinal fluid; BSS, basal salt solution; ddFK, dideoxyforskolin; DIV, day(s) *in vitro*; EPSP, excitatory postsynaptic potential; EM, electron microscopy (or microscopic); FK, forskolin; LTP, long-term potentiation; PKA, protein kinase A or cyclic AMP-dependent protein kinase; PSD, postsynaptic density

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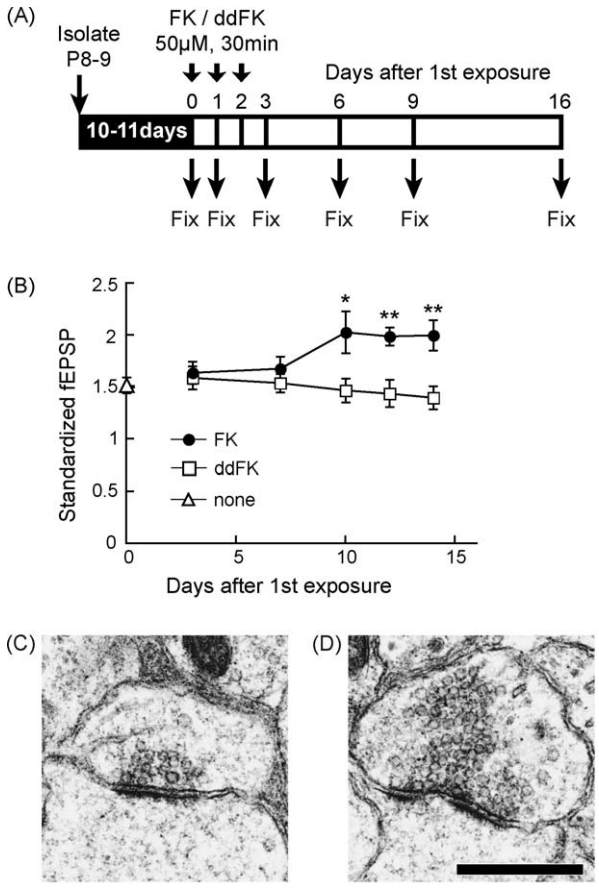

Fig. 1. Experimental design. (A) The timeline of the experiment. The hippocampal slices were isolated from rat pups at postnatal age 8–9 days, cultured for 10–11 days until maturation, exposed 3× to 50 μM FK or ddFK (30 min each time) and fixed for EM examination 1, 3, 6, 9 and 16 days after the first exposure (and immediately before the first exposure). (B) Development of slow/persistent enhancement of synaptic strength after three exposures to FK. This panel was adapted from our previous report [17] with the permission of the publisher for the readers' convenience for comparison with morphological changes observed in the present study. The ordinate indicates the electrical index of synaptic strength, which is the maximal field excitatory postsynaptic potential amplitude standardized by the maximal field spike amplitude. (C and D) Macular and perforated asymmetrical synapses, respectively, in the CA1 apical dendritic region of organotypic slice culture of the hippocampus. The bar indicating 0.5 μm in (D) applies also to (C).

The timeline of stimulation and examination is diagrammatically shown in Fig. 1A. For stimulation, the culture medium was replaced with fresh medium containing 50 μM forskolin (FK; Sigma), an adenylyl cyclase activator, or 50 μM dideoxyforskolin (ddFK; Sigma), a structurally related inactive analog of FK, which was replaced with medium free of FK/ddFK 30 min later. For the repetitive exposure, the same procedures were repeated after an interval of 24 h. All treatments were carried out at 34 °C. The number of days after the FK/ddFK treatment was reckoned from the day of the first exposure throughout this study.

Conventional electron microscopy was carried out on the cultures [20]. Briefly, on the predetermined day, selected from the time-course of the enhancement of synaptic transmission (Fig. 1B), the cultures were pre- and post-fixed with a mixture of 2% glutaraldehyde/2% paraformaldehyde and 2% osmium tetraoxide, respectively. The fixed culture was dehydrated and embedded in Quetol-812 resin (Nisshin EM). After trimming out the culture's CA1 area, sections of 70–80-nm thickness were cut tangentially to the surface of the culture at the middle of its thickness (correspondingly to the electrical measurement [17]).

In the pursuit of morphological changes, it is desired to make serial sections and to reconstruct the three-dimensional configuration of the region of interest. However, in this study where comparison among many specimen groups were necessary, we adopted a simplified methodology. Typically, two photographs were taken from one culture, one for proximal apical dendritic layer ($\leq$100 μm from cell soma) and the other for distal apical dendritic layer ($\geq$150 μm from cell soma) without intentional selection. This was done because the functional features of synapses were reported to be different between proximal and distal layers of the CA1 region in the developing hippocampus [13]. On those representative photographs, boxes having 10 μm × 10 μm dimension were set on and magnified digitally to 90,000×. Then the number of asymmetrical synapses (Fig. 1C) was counted over each of the boxes, adopting following criteria: the presence of clusters of clear spherical (not ellipsoidal) vesicles ~50 nm in diameter in the presynaptic cytoplasm; the presence of electron-dense submembraneous structure of $\geq$20 nm in thickness in the postsynaptic cytoplasm (postsynaptic density [PSD]); the presence of clear gap between pre- and postsynaptic compartments (*i.e.* synapses sectioned nearly in parallel to the synaptic cleft were excluded). At the same time, the apparent length of PSD was measured on each asymmetrical synapse. This was done for two purposes: to know whether the size of synapse would change during the course of synaptic enhancement; to obtain a factor for correction, since the density of synapses should be corrected taking their size into account (see below).

We enumerated the apparently "perforated" synapses on the same EM photographs (Fig. 1D). In this study, the criterion for the perforated synapse was an interrupted postsynaptic density apposed to a single presynaptic terminal (whether the clusters of synaptic vesicles were split or not). As explained above, we did not make three-dimensional reconstruction every time. Thus, the number of perforated synapses presented here might be an underestimation considering the possibility that the tangential section of an actually perforated synapse should give an image of macular synapse in appearance. However, since this situation is common to all the samples, comparisons should be valid.

All enumerations and measurements were performed on a blind-test basis, where tens of photographs were shuffled and the origin of each photograph was concealed during the examination.

As depicted in Fig. 2A and B, the number of asymmetrical synapses (most probably of excitatory synapses) appearing in a photograph of the CA1 proximal dendritic layer of the culture 9 days after FK-exposures was greater than that of the culture 9 days after ddFK-exposures. This was also the case in the CA1 distal dendritic layer of the culture 9 days after FK-exposures (Fig. 2C and D).