Modulation of cPKCγ on Synapsin-Ia/b–Specific Phosphorylation Sites in the Developing Visual Cortex of Mice

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PURPOSE. To explore the role of synapsin-Ia/b in visual cortical plasticity, the dynamic changes in total protein expression (T-) and conventional protein kinase C (cPKC)γ-modulated phosphorylation (P-) levels of synapsin-Ia/b were observed in the developing visual cortex of mice.

METHODS. The Western blot analysis was used to determine the levels of T- and P-synapsin-Ia/b at site of Ser9, 549, and 603; the cPKCγ gene wild-type (cPKCγ+/+) and knockout (cPKCγ−/−) mice were applied to explore the modulation of cPKCγ on synapsin-Ia/b phosphorylation status in visual cortex of mice at postnatal 7 to 60 days (P7–P60, n = 6 per group).

RESULTS. The results showed that T-synapsin-Ia/b protein levels significantly increased at P14 to P35 and peaked at P42 to 60 (P < 0.001) in visual cortex when compared with that of P7 cPKCγ+/+ mice, and cPKCγ−/− did not affect this pattern of T-synapsin-Ia/b protein expressions. For synapsin-Ia/b phosphorylation status, the levels of P-Ser9 and 603 synapsin-Ia/b significantly elevated at P21 to P28 (P < 0.05 or 0.001), and then went down and maintained at lower levels at P35 to P60 (P < 0.05 or 0.001) compared with P7 cPKCγ+/+ mice. In addition, the cPKCγ gene knockout could significantly (P < 0.001) inhibit both the increase and decrease of P-Ser9 and 603 synapsin-Ia/b levels when compared with cPKCγ+/+ mice at P7 to P60. However, there were no significant changes of P-Ser549 synapsin-Ia/b in the developing visual cortex of both cPKCγ+/+ and cPKCγ−/− mice at P7 to P60.

CONCLUSIONS. These results suggested that both protein expression levels and cPKCγ-modulated phosphorylation status at Ser9 and 603 of synapsin-Ia/b may play important role in developing visual cortex of mice.

Keywords: visual cortex, synapsin-Ia/b, cPKCγ, protein expression, phosphorylation

The visual system has increasingly been recognized to retain a degree of plasticity for rewiring neural networks over the entire life.1,2 During the critical period, substantial changes in expression of pre- and postsynaptic proteins interact with experience to facilitate synaptic development, fine tuning of cortical circuits, and maturation of the cortical connectome.3 The presynaptic proteome has hundreds of proteins, many of which are directly involved with the cycling of synaptic vesicles (SV).4 As the most abundant SV proteins, synapsins have distinct functional roles and were used as specific marker for presynaptic terminals to capture presynaptic development.5 Synapsins are neuron-specific phosphoproteins and highly conserved evolutionarily.6–8 In mammals, synapsins are encoded by three genes and consist of at least 10 isoforms, including synapsin-Ia and b that stored in vesicle of presynaptic nerve terminals.9–12 Synapsin-Ia/b has been reported in synaptogenesis, synapse function, synapse maintenance, and synaptic plasticity.13–14 For example, the deletion of synapsin-I could block the enhancements of learning, presynaptic plasticity, and long-term potentiation (LTP).15

As a variety of protein kinase substrate, synapsin-Ia/b contains multiple phosphorylation sites and their phosphorylation levels affect the presynaptic plasticity.6 The protein kinase A (PKA)/Ca2+-calmodulin dependent kinase (CaMK) IV could phosphate Ser9 of site one that is shared by all the synapsins in the N-terminal domain A.6,16–18 The Ser9 phosphorylation regulates neurite elongation and SV mobilization, which has a prominent role in the expression of posttetanotic potentiation and short-term synaptic enhancement.19 In addition, a nonhydrolysable analog of platelet-activating factor (PAF) enhances LTP and promotes learning and memory through increasing synapsin-I phosphorylation at sites one (PKA and CaMKI/IV) and three (CaMKII) that is associated with decreased binding of synapsin-I to SV or actin and increased neurotransmitter release.18,20 The underlying mechanism involves elevated Ca2+ within presynaptic boutons and PKC activation.21 Synapsin-I also contained multiple consensus sites four to six for extracellular signal-regulated kinase 1/2 (ERK1/2) and cyclin-dependent kinase 1/5 (CDK1/5), and the phosphorylation at these sites contributes to release neurotransmitters.6,16,18

The conventional Ca2+-activated, phospholipid-dependent protein kinase C (cPKC) belongs to serine/threonine protein kinase family.22,23 The cPKCγ, one of the PKC family
members, is neuron-specific and only expressed in neurons of cerebral cortex and spinal cord. The inactive form of cPKCγ was present in the cytoplasm, and then redistributed to the membrane compartment in response to the stimulation. In addition, our previous works demonstrated that synapsin-la/b is a cPKCγ-interacting protein and may participate in cerebral ischemic/hypoxic preconditioning formation and development by using functional proteomics technology. In this study, we further explored the dynamic changes of synapsin-Ia/b protein expressions and its site-specific phosphorylation status modulated by cPKCγ in the developing visual cortex of mice.

**METHODS**

**Experimental Animals**

The C57BL/6j wild type (+/+) and cPKCγ heterozygote (+/−) mice were purchased from The Jackson Laboratories (Animal qualification: 002466; Bar Harbor, ME, USA) and bred in the Animal Department of Capital Medical University that complies with the requirement of medical experimental animal environmental facilities. Eighty-four clean neonatal cPKCγ+/+ and cPKCγ knockout (+/−) mice were randomized into seven groups (n = 6 per group), respectively. The mice were killed at postnatal 7, 14, 21, 28, 35, 42, and 60 days (P7–P60). The primary visual cortex was obtained based on the Mouse Brain postnatal 7, 14, 21, 28, 35, 42, and 60 days (P7–P60). The reagents were purchased from Sigma-Aldrich Company (St. Louis, MO, USA), such as proteinase inhibitors and phosphatase inhibitors.

**Sample Preparation**

The frozen samples taken out from the liquid nitrogen were thawed first and then completely dissolved by being homogenized and sonicated in Buffer C containing 2% SDS in Buffer A (50 mM Tris–Cl, pH 7.5, containing 1 mM EGTA, 2 mM EDTA, 5 mg/mL each of leupeptin, aprotinin, pepstatin A, and chymostatin, 50 mM okadacid, 50 mM potassium fluoride, and 5 mM sodium pyrophosphate). Finally, the BCA kit (Pierce Company, Rockford, IL, USA) was used to determine the protein concentration of the samples, which were then preserved at −80°C for later electrophoresis.

**Western Blot Analysis**

To explore the effect of cPKCγ on synapsin-I phosphorylation status, the Western blot analysis was performed as our previous report. First, the SDS-PAGE (10% SDS gel) was prepared, and the total protein of 30 μg per lane was loaded for electrophoresis. Following the electrophoresis, the nitrocellulose (NC) membrane (Schleicher and Schell, Berlin, Germany) was used as a carrier, and then the transferred NC membrane was rinsed with TTBS (20 mM Tris, pH7.5, containing 0.05% Tween-20, and 0.15 M NaCl). After that the NC membrane was blocked with 10% nonfat milk for 1 hour, and then washed by using TTBS for three times (10 minutes for each time). The blocked NC membrane was then incubated with primary rabbit polyclonal antibodies against phospho-synapsin I at the sites one (Ser9, PP084; R&D Systems, Minneapolis, MN, USA), three (Ser549, ab119370, Abcam, Cambridge, UK), or six (Ser603, PP056; R&D) at 1:1000 for 1 hour, against T-synapsin ( #6710, 1:1000; Cell Signaling Technology, Boston, MA, USA) for 3 hours, and mouse monoclonal antibody against β-actin (1:10000, #60008-I-lg; Proteintech Corp., Chicago, IL, USA) for 1 hour to verify equal loading. The secondary antibodies included horsedarsh peroxidase-conjugated goat anti-rabbit (Stressgen Biotechnologies Corporation, Victoria, BC, Canada) or anti-mouse IgG (The Jackson Laboratories) at 1:4,000 dilutions for 1 hour. The ECL-plus Kit (Perkin-Elmer Life Science Inc., Waltham, MA, USA) was used to detect the immunoblotting signals.

**Statistical Analysis**

The quantitative analysis for immunoblotting was conducted by using Gel Doc 2000 imaging system with Quantitative-One software (Bio-Rad Company, Hercules, CA, USA). The T-synapsin-la/b protein levels were expressed as the ratio that band density of T-synapsin-la/b or b/the corresponding β-actin band density, as well as the band density of P-synapsin-la/b or b/T-synapsin-la/b density for synapsin-la/b phosphorylation levels.

The data appeared in the form of mean ± standard error. The dynamic changes of T and P-synapsin la/b were analyzed by using one-way ANOVA for either gene type or two-way ANOVA for comparing two gene types, and then the followed Bonferroni test was applied for all pair wise multiple comparison. The significance was regarded as at least P < 0.05.

**RESULTS**

To study the modulation of cPKCγ on synapsin-la/b phosphorylation, the levels of T-synapsin-la/b expression and P-synapsin-la/b at sites one, three, and six were analyzed by using Western blot analysis in the visual cortex of developing cPKCγ+/+ and cPKCγ+/− mice.

**Effect of cPKCγ on the Levels of T-Synapsin-Ia/b Expression in Visual Cortex of Mice During Postnatal Development**

At postnatal day 7 (P7), the expression of T-synapsin-la/b in visual cortex of cPKCγ+/+ and cPKCγ+/− mice was approximately 0.14 ± 0.05 and 0.16 ± 0.06, respectively, and then reached 0.52 ± 0.09 and 0.35 ± 0.10 at P14, and approximately 0.64 ± 0.15 and 0.64 ± 0.14 at P55 (Fig. 1, cPKCγ+/+: F(6,35) = 48.95, P < 0.001, cPKCγ+/−: F(6,35) = 57.59, P < 0.001). What is more, the relative protein levels of T-synapsin-la/b in cPKCγ+/+ and cPKCγ+/− mice increased with the age enlarged showing consistent with that of T-synapsin-la/b, approximately 0.28 ± 0.08 and 0.32 ± 0.08, respectively, at P7, 0.56 ± 0.12 and 0.56 ± 0.08 at P14, and then 0.90 ± 0.15 and 0.91 ± 0.10 at P55 (Fig. 1, cPKCγ+/+: F(6,35) = 67.77, P < 0.001; cPKCγ+/−: F(6,35) = 92.48, P < 0.001). In addition, there were no significant difference in T-synapsin-la/b expressions between cPKCγ+/+ and cPKCγ+/− mice (Fig. 1, F(6,70) = 0.52(ia) / 0.68(ib), P > 0.05). The results indicated that cPKCγ had no obvious influence on the developmental expressions of T-synapsin la/b.

**Effect of cPKCγ on the Levels of P-Synapsin-Ia/b at Site One (Ser 9) in Visual Cortex of Mice During Postnatal Development**

As shown in Figure 2, the postnatal development of P-synapsin-la/b (ser 9) was different between cPKCγ+/+ mice and

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**Figure 1.** Effect of cPKCγ on the levels of T-synapsin-Ia/b expression in visual cortex of mice during postnatal development. (A) The typical result of Western blot showed the changes of total (T)-synapsin Ia and Ib protein levels in visual cortex of both cPKCγ<sup>+/+</sup> and cPKCγ<sup>−/−</sup> mice during postnatal development. The quantitative analysis results demonstrated a significant increase of T-synapsin Ia (B) and Ib (C) protein levels in visual cortex of both cPKCγ<sup>−/−</sup> and cPKCγ<sup>−/−</sup> mice from P7 to P60 days. ***P < 0.001 versus their corresponding P7, n = 6 per group.
FIGURE 2. Effect of cPKCγ on the levels of P-synapsin-Iα/b at site one (ser 9) in visual cortex of mice during postnatal development. (A) The typical result of Western blot showed increasingly strong signals of P-synapsin Iα/b at P7 to P28, and then quickly decreased to a low level at P35 to P60 in visual cortex of cPKCγ+/+ mice. The P-synapsin Iα/b signals gradually increased in visual cortex of cPKCγ−/− mice from P7 to P60 days. (B, C) The results of quantitative analysis demonstrated that the relative level of P-synapsin-Iα/b increased to the highest level at P21, but decreased rapidly to a low level at P35 to P60 in cPKCγ+/+ mice. The relative levels of P-synapsin-Iα/b in the visual cortex of cPKCγ+/+ mice were significantly higher than cPKCγ−/− mice at P35 to P60. *P < 0.05, **P < 0.01, and ***P < 0.001 versus their corresponding P7; #P < 0.05, ##P < 0.01, and ###P < 0.001 versus cPKCγ+/+ mice, n = 6 per group.
cPKCγ−/− mice. In cPKCγ+/+ mice, there were increasingly strong signals of P-synapsin Ia/b at P7 to P28, and then quickly decreased at P35 to P60 (Fig. 2A). The relative level of P-synapsin Ia/b at P7 was 0.69 ± 0.07 / 0.70 ± 0.07, and then increased with development to the peak at P21 0.95 ± 0.70 / 1.00 ± 0.04, but decreased quickly to a low level at P35 to P60 (Fig. 2, F(6,35) = 19.84(1a) / 56.67(1b), P < 0.001). In cPKCγ−/− mice, there were slowly increasing signals of P-synapsin Ia/b in visual cortex. At P7, the relative level of P-synapsin Ia/b was 0.23 ± 0.04 / 0.24 ± 0.11, then increased with development at P21 0.42 ± 0.07 / 0.55 ± 0.04, and then increased to a high level at P35 to P60 (Fig. 2, F(6,35) = 38.00(1a) / 38.91(1b), P < 0.001).

The relative expressions of P-Synapsin-Ia/b (ser 9) in the visual cortex of cPKCγ+/+ mice were significantly higher than that of cPKCγ−/− mice at approximately P7 to P28 and lower than cPKCγ+/+ mice at P35 to P60 (Fig. 3A). The relative level of P-synapsin Ia/b at P7 was 0.74 ± 0.04 / 0.74 ± 0.04, and then increased with development to the highest level at P21 1.00 ± 0.03 / 0.97 ± 0.09, but decreased quickly to a low level at P35 to P60 (Fig. 3, F = 125.31(1a) / 70.98(1b), P < 0.001). In cPKCγ−/− mice, there were slowly increasing signals of P-synapsin Ia/b in visual cortex. At P7, the relative level of P-synapsin Ia/b was 0.22 ± 0.02 / 0.24 ± 0.02, then increased with development at P21 0.39 ± 0.04 / 0.47 ± 0.05, and then increased rapidly to a high level at P35 to P60 (Fig. 3, F(6,35) = 109.54(1a) / 86.85(1b), P < 0.001).

The relative expressions of P-Synapsin-Ia/b (ser 603) in the visual cortex of cPKCγ+/+ mice were significantly higher than cPKCγ−/− mice at approximately P7 to P28 and lower than cPKCγ+/+ mice at P35 to P60 (Fig. 3A). The relative level of P-synapsin Ia/b at P7 was 0.11(Ia) / 0.27(Ib), P > 0.05, which suggested that cPKCγ may not modulate the specific phosphorylation site six of synapsin-Ia/b in visual cortex.

Effect of cPKCγ on the Levels of P-Synapsin-Ia/b at Site Three (Ser 603) in Visual Cortex of Mice During Postnatal Development

As shown in Figure 3, the postnatal development of P-synapsin-Ia/b (ser 603) was similar with P-synapsin-Ia/b (ser 9) in cPKCγ+/+ and cPKCγ−/− mice. In cPKCγ+/+ mice, there were increasingly strong signals of P-synapsin Ia/b (ser 603) at P7 to P28, and then quickly decreased to a low level at P35 to P60 (Fig. 3A). The relative level of P-synapsin Ia/b at P7 was 0.74 ± 0.04 / 0.74 ± 0.04, and then increased with development to the highest level at P21 1.00 ± 0.03 / 0.97 ± 0.09, but decreased quickly to a low level at P35 to P60 (Fig. 3, F = 125.31(1a) / 70.98(1b), P < 0.001). In cPKCγ−/− mice, there were slowly increasing signals of P-synapsin Ia/b in visual cortex. At P7, the relative level of P-synapsin Ia/b was 0.22 ± 0.02 / 0.24 ± 0.02, then increased with development at P21 0.39 ± 0.04 / 0.47 ± 0.05, and then increased rapidly to a high level at P35 to P60 (Fig. 3, F(6,35) = 109.54(1a) / 86.85(1b), P < 0.001).

The relative expressions of P-Synapsin-Ia/b (ser 603) in the visual cortex of cPKCγ+/+ mice were significantly higher than cPKCγ−/− mice at approximately P7 to P28 and lower than cPKCγ+/+ mice at P35 to P60 (Fig. 2, F(6,35) = 19.84(1a) / 56.67(1b), P < 0.001). The results indicated that cPKCγ modulated the specific phosphorylation site three of synapsin-Ia/b in visual cortex.

Effect of cPKCγ on the Levels of P-Synapsin-Ia/b at Site Six (Ser 549) in Visual Cortex of Mice During Postnatal Development

As shown in Figure 4, the postnatal development of P-synapsin-Ia/b (ser 549) was not significantly different between cPKCγ+/+ and cPKCγ−/− mice. No matter in cPKCγ+/+ or in cPKCγ−/− mice, the relative level of P-synapsin Ia/b (ser 549) did not change with the age enlarged (Fig. 4, cPKCγ+/+: F(6,35) = 0.19(1a) / 0.95(1b), P > 0.05; cPKCγ−/−: F(6,35) = 0.28(1a) / 0.51(1b), P > 0.05).

The relative levels of P-synapsin-Ia/b (ser 549) in the visual cortex between cPKCγ+/+ and cPKCγ−/− mice had no significant difference (Fig. 2, F(6,70) = 0.11(1a) / 0.27(1b), P > 0.05), which suggested that cPKCγ may not modulate the specific phosphorylation site six of synapsin-Ia/b in visual cortex.

DISCUSSION

In this study, we first reported that the T-synapsin-Ia/b protein level rapidly increased in visual cortex of wild-type mice (cPKCγ+/+) with postnatal age, reaching the peak at P42 and maintaining high level at adulthood, and cPKCγ gene knockout (cPKCγ−/−) did not affect T-synapsin-Ia/b protein levels and its protein expression patterns. This finding was similar with previous report. The developmental profile showed a steep increase of synapsin-Ia/b protein levels from birth to P20 that was the major period of synaptogenesis,58 and the protein expression pattern in visual cortex of mice at P30 to P46 manifested that T-Synapsin-Ia/b were upregulated with age.57

The phosphorylation (P-) status of synapsin-Ia/b at sites one (Ser9), three (Ser603), and six (Ser549) is important for synaptic function. It has been reported that synapsin-Ia/b dissociating from SV and dispersing from boutons freeing vesicles for release rely on protein kinases-mediated reversible phosphorylation during extensive stimulation.8,12,20,30 In our study, we found that the levels of P-Ser9 and 603 synapsin-Ia/b significantly elevated at P21 to P28, and then went down and maintained at lower levels at P35 to P60 in visual cortex of mice. These results are similar with our previous report that P-synapsin-Ia/b levels at site one increased to the highest level at P21, and then decreased rapidly to a low level at P35 to P60 in visual cortex of mice.31 Then which one of the protein kinases is responsible for sites one and three phosphorylations?

It has been reported that PAF increased synapsin-Ia/b phosphorylation at sites one and three in a time-dependent manner, and PKC activation could increase dispersion of synapsin-Ia/b from the presynaptic compartment of hippocampal neuron during stimulation in vitro.52 As a neuron-specific member of PKC, cPKCγ expresses exclusively in central nervous system neurons and modulates synaptic efficacy.59 Our previous work has identified synapsin-Ia/b as one of cPKCγ-interacting proteins in hippocampus and cortex.60 In present study, we further found that cPKCγ gene knockout could significantly inhibit both the increase and decrease of p-Ser9 and 603 synapsin-Ia/b levels, but no significant changes of p-Ser549 synapsin-Ia/b were observed in the developing visual cortex of both cPKCγ+/+ and cPKCγ−/− mice at P7 to P60. In addition, the cPKCγ expression levels in cerebral cortex were found peaking at P21 days and decreasing to a lower level in adult rats.61 In this study, we observed that the cPKCγ gene knockout significantly block the increase of P-synapsin-Ia/b levels at sites one and three in the visual cortex of mice at P7 to P28 days. These results suggested that cPKCγ plays the key role in modulating synapsin-Ia/b phosphorylation at sites one and three, and this cPKCγ-synapsin-Ia/b pathway might be involved in the visual plasticity as the peak of the critical period of visual plasticity in mice is also approximately 4 weeks after birth.55

In addition, the phosphorylation status of synapsin-Ia/b at sites one and three in visual cortex of cPKCγ−/− mice at P7 to P60 days also suggested the involvement of other isoforms of PKC or kinases as compensatory substance. It has been reported that the p-synapsin-Ia/b was mediated by PKA/CaMK and PKC in hippocampus and cerebral cortex of adult rats.62 The well-confirmed upstream kinases of synapsin-Ia/b include PKA/CaMKI mediating the phosphorylation at site one,10,57 CaMKII at site three,10, and mitogen-activated protein kinase (MAPK)/ERK/CDK at site six.59–61 The phosphorylation status of synapsin-Ia/b at sites one, three, or six could mediate its binding ability to SV and actin cytoskeleton, respectively. Specifically, PKC is implicated in regulating the size of readily releasable pool and the probability of release after high-frequency stimulation.43,44
FIGURE 3. Effect of cPKCγ on the levels of P-synapsin-Ia/b at site three (ser 603) in visual cortex of mice during postnatal development. (A) The typical result of Western blot showed that P-synapsin Ia/b signals of cPKCγ+/+ mice were significantly higher than cPKCγ−/− mice at P7 to P28 days and lower than cPKCγ−/− mice at P35 to P60. (B, C) The results of quantitative analysis demonstrated that the relative level of P-synapsin-Ia/b increased to the highest level at P21, but decreased rapidly to a low level at P35 to P60 days in cPKCγ+/+ mice. The relative level of P-synapsin Ia/b increased with development to the highest level at P42 to P60 days in cPKCγ−/− mice. The relative levels of P-synapsin-Ia/b in the visual cortex of cPKCγ+/+ mice were significantly higher than cPKCγ−/− mice at P7 to P28 and lower than cPKCγ−/− mice at P35 to P60. *P < 0.05, **P < 0.01 and ***P < 0.001 versus their corresponding P7; #P < 0.05, ##P < 0.01, and ###P < 0.001 versus cPKCγ+/+ mice, n = 6 per group.
FIGURE 4. Effect of cPKCγ on the levels of P-synapsin Ia/b at site six (ser 549) in visual cortex of mice during postnatal development. (A) The typical result of Western blot showed neither increasing nor decreasing signals of P-synapsin Ia/b in visual cortex of cPKCγ+/+ and cPKCγ−/− mice. (B, C) The results of quantitative analysis demonstrated that the levels of P-synapsin Ia/b did not change with development at P7-60 in cPKCγ+/+ and cPKCγ−/− mice, and the P-synapsin-Ia/b levels between cPKCγ+/+ and cPKCγ−/− mice had no significant difference (n = 6 per group).
In conclusion, this was the first report to identify cPKCγ-synapsin pathway by observing P-synapsin-Ia/b levels at specific phosphorylation sites in developing visual cortex of cPKCγ+/− and cPKCγ−/− mice. Our findings supported that cPKCγ might play a key role in modulating the P-synapsin-Ia/b levels on sites one and three in visual cortex of mice especially at P7 to P28 days.

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