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Regulation of Protein Kinase C Isozymes During Early Post-Natal Hippocampal Development

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Abstract

During neonatal hippocampal development, serotonin 1A receptor-mediated signaling initially employs PKC ϵ to boost neuronal proliferation and then uses PKC α to promote synaptogenesis. Such stage-specific involvement of a PKC isozyme could be determined by its relative expression level. In mouse hippocampi, we detected relatively low levels of α , β , γ , and δ isozymes at post-natal days 2–6 (P2–6), which was followed by a large increase in their expression. In contrast, the PKC isozymes ϵ and θ were relatively abundant at P6, following which they underwent a further increase by P15. Comparison with purified proteins confirmed that the PKC ϵ levels at P6 and P15 were respectively 1.75 and 7.36 ng per 60 μ g of protein, whereas PKC α levels at P6 and P15 were respectively 160 pg

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and 1.186 ng per 60 µg of protein. Therefore, at P6, PKCε was about 11-fold more abundant than PKCα. Consequently, signaling cascades could use the relatively abundant PKCε (and possibly PKCθ) molecules for early events at P2-6 (e.g. neurogenesis), following which PKCα (and the β, γ, or δ isoforms) could guide maturation or apoptosis. Notably, at P6 but not P15, PKCε, was localized to the nuclei of neuroblasts, probably directing mitosis. In contrast, at P15 but not P6, PKCα was highly expressed in the processes of the differentiated hippocampal neurons. In summary, PKC isoforms follow differential profiles of expression in neonatal hippocampus and the relative abundance of each may determine its mode and stage of involvement in hippocampal development.

Keywords

PKC Isozymes; Neonatal; 5-HT_{1A}receptor; Neurogenesis; Hippocampus

1. Introduction

Brain development is regulated by a number of signaling molecules. Transient expression of different developmental factors at the cellular level at definite time points in ontogeny and growth is critical for normal development of the nervous system. A number of transducer molecules regulate signal transduction pathways by phosphorylating various intracellular components. The serine/threonine kinases termed as protein kinase C (PKC) are often involved in such signal transduction cascades. They influence numerous cellular functions, including cell differentiation, tumor promotion, membrane protein function [28], cell proliferation [1], fertilization [5,8–10], and embryonic compaction [42]. The signaling pathway has also been implicated in modulation of motor behavior, learning and memory [3].

The PKC family consists of 11 different serine/threonine kinases that are subdivided into three groups based on sequence homology, as well as activator and cofactor requirements. These groups include the conventional (cPKC α, β and γ), the novel (nPKC δ, ε, θ, μ, and η), and the atypical (aPKC λ and ζ) isoforms [25,40]. The conventional PKC isotypes are activated by phosphatidylserine (PS), Ca²⁺, and diacylglycerol. The novel PKCs are not Ca²⁺-dependent, but are still activated by diacylglycerol and PS. The atypical PKCs are lipid-dependent, but they are neither Ca²⁺-sensitive, nor do they respond to diacylglycerol [25,27,39]. Ca²⁺-dependent isotypes have different substrate specificity and phospholipid dependency compared to the Ca²⁺-independent isoforms. The Ca²⁺-independent PKCs are suggested to be involved in different cellular functions than the Ca²⁺-dependent isoforms [18].

Despite having closely related structures, specific PKC isoforms appear to be localized differentially in a cell and their roles are specific to individual species, tissues, and possibly the stage of development [21]. For example, differentiation of the embryonic teratocarcinoma cell line F9 is associated with an increase in PKCα mRNA and protein and a decrease in mRNAs for both PKCβ and γ [17]. Specific signaling cascades, which are stimulated at various stages of brain development, cause activation of the different isotypes of PKC. Multipotent PCC7Mz1 embryonic carcinoma cells showed upregulation of PKC βII, δ and ε three days after retinoic acid treatment when cells mature morphologically [30]. Oehrlein and coworkers also observed that PKCδ was expressed in all differentiated cell types while PKCα, λ and ζ, were expressed in stem cells and newly arising cell types. In contrast, PKCβII was abundant only in the somata of only 2.5% of all neurons. They also found PKCε to be localized in the somata and the axon. Sposi and coworkers studied gene expression for PKCα, β and γ during ontogenic development of human and murine CNSs [37], and Pauken and Capco analyzed both mRNA and protein expression for the PKC isoforms in mouse preimplantation development [31]. The latter group found PKCα and PKCδ proteins are most likely synthesized during oogenesis for use during

embryogenesis whereas PKC β was not detected at all before or during embryonic development. PKC γ and PKC ζ transcription is probably initiated during or immediately after fertilization.

Brain development begins very early in prenatal life, but hippocampal development and most of the pruning and stabilization of synapses in various neural circuits are completed during the early post-natal stages. This is extremely important for the blueprinting of behavioral patterns that characterize each human being, and any aberrance in this process is believed to cause various emotional disorders. Many intricate and interdependent molecular and morphological changes orchestrate such events, wherein the various members of the PKC family selectively participate to regulate the course of brain development promoted by neurotransmitter receptors.

The serotonin 1A receptor (5-HT_{1A}-R) is one such neurotransmitter receptor, which is essential for early post-natal development of the front brain [11]. The hippocampus, which is a major 5-HT_{1A}-R-expressing signaling hub, matures mainly during the early post-natal stages of brain development. In our earlier studies, we focused on this structure to investigate if 5-HT_{1A}-R-mediated signaling played a stage-specific role in hippocampal maturation in mice [23]. We observed that at postnatal day 6 (P6), hippocampal 5-HT_{1A}-R-mediated signaling through the mitogen activated protein kinase (MAPK) pathway required a PKC isozyme (possibly PKC ϵ) to cause increased division of pre-neuronal cells. In contrast, later, in the P15 hippocampus, the 5-HT_{1A}-R $\Rightarrow$ MAPK signaling pathway becomes PKC-independent, but stimulates PKC α , which in turn bolsters synaptic strength at the CA1 dendrites, evidenced by an increase in excitatory postsynaptic potential (EPSP) in the Schaffer collateral pathway [23]. Such temporal participation of different PKC isozymes could be stoichiometrically controlled by stage-specific expression of PKC isozymes. Thus, a clear idea of the expression profiles of various PKC isozymes during the early post-natal stages is seriously needed for the advancement of research on hippocampal development.

The only report so far on postnatal levels of PKC isozymes in the hippocampus addressed this question in the rat [16]. This study found PKC ζ , PKM ζ and PKC δ to be present at birth, following which their expression did not increase postnatally. In contrast, the expression of the other isoforms (α , β , γ , ϵ , and η) gradually increased in the first four postnatal weeks. Despite many similarities, many significant developmental dissimilarities exist between rat and mouse brains [32,33]. Since the mouse model has been used for most functional studies involving gene deletion, it is imperative to have a clear idea about the expression profiles of the PKC isozymes in mice. To the best of our knowledge, this important piece of information is not available in the literature. In the present study, we have investigated the expression profiles of six PKC isozymes at mRNA and protein levels during the critical period of mouse brain development P2 to P20. Our results show that post-natal regulation of these PKC isozymes, which have diverse mechanisms of activation, is quite distinct in mice, wherein PKC ϵ is possibly an early signal in neonatal hippocampal development. Furthermore, cell type-specific expression patterns for PKC ϵ and PKC α in the hippocampus were different from one another and dissimilar at P6 and P15, thus suggesting that these isozymes play differential functional roles at these two developmental stages.

2. Results

The major reason for our interest in two specific post-natal stages was that P6 and P15 were two crucial points in hippocampal development when GABAergic signaling switched from excitatory to inhibitory, and synaptogenesis attained its peak, respectively [20,35]. The PKC isozymes PKC ϵ and PKC α appeared to be differentially involved during early post-natal development of the hippocampus [23]. These results also indicated that 5-HT_{1A} receptor-promoted hippocampal development involved PKC α at P15, but most probably PKC ϵ at P6. As mentioned earlier, to know if this was due to a stoichiometric control, we sought to compare

the levels of PKC α and PKC ϵ at P6 and P15. Since no information was available on the neonatal expression profiles for PKC α and PKC ϵ and several other isozymes in mouse brain, this study was undertaken and we report protein and mRNA expression profiles for six PKC isozymes and also determine if there was a significant change in expression of each isozyme between P6 and P15.

PKC α protein level increases sharply during neonatal hippocampal development

The protein level of PKC α is relatively low at P2, and from P8 it undergoes a gradual and a prominent increase to 3.5-fold of the PKC α level at P2 (Fig. 1a). The increase in the β -actin normalized band intensity between P6 and P15 was statistically significant ($P = 0.003$; $n = 3$). Reverse transcriptase-PCR analysis of PKC α revealed a trend of increase in its mRNA and the induction between P6 and P15 was significant ($P = 0.001$; $n = 5$) (Fig. 1b). This suggested that the observed developmental profile of the PKC α protein could be due to transcriptional regulation of the *PKC α* gene.

PKC β protein as well as mRNA levels increase during neonatal hippocampal development

Like PKC α , the PKC β protein also underwent a gradual increase in expression, peaking at P20 when its normalized band intensity was about 6.5-fold of that at P2 (Fig. 2a). The increase in protein level between P6 and P15 was statistically significant ($P < 0.0001$; $n = 3$). Overall, a 3.5-fold increase in the PKC β mRNA was observed and the induction between P6 and P15 was significant ($P = 0.034$; $n = 6$). Thus, it is likely that PKC β expression is transcriptionally regulated.

PKC γ protein and mRNA increase during neonatal hippocampal development

The third conventional PKC isozyme PKC γ increased in a more dramatic manner from P8, reaching 70-fold of the level at P2 on P20 (Fig. 3a). Although the increase in the mRNA level for PKC γ was not as dramatic, at P20, it reached a level that was three-fold of that at P2 (Fig. 3b). The increase in normalized protein band intensity between P6 and P15 was statistically significant ($P < 0.001$; $n = 3$). Also, the increase in mRNA between P6 and P15 was significant ($P = 0.009$; $n = 4$). Therefore, transcriptional control could be the mechanism for the observed increase in expression of PKC γ protein.

PKC δ expression gradually increases during neonatal hippocampal development

Redirecting our attention from the conventional PKC isozymes to the novel PKC isozyme PKC δ , we observed a profile that was somewhat similar to that for PKC α (Fig. 4a). Thus the normalized PKC δ band intensity gradually increased from P2 to reach 550% of P2 at P20. The increase in PKC δ protein level between P6 and P15 was highly significant ($P < 0.0001$; $n = 3$). The mRNA levels for the PKC δ mRNA showed a trend of increase between P6 and P15, but it was not significant ($P = 0.074$; $n = 5$). Thus the developmental increase in PKC δ expression could be only partially controlled by transcriptional regulation (Fig. 4b).

PKC ϵ is already high at an early stage and it undergoes only a small increase during neonatal development

Though belonging to the same family of novel PKC isozymes, the developmental profiles of PKC δ and PKC ϵ were quite different. The expression of PKC ϵ appeared high at P2, following which its normalized band intensity increased by 40% during the later stages (P15 and P20) and this increase was significant between P6 and P15 ($P = 0.00047$; $n = 3$) (Fig. 5a). The mRNA levels for PKC ϵ appeared to increase at P15-P20 and this change in mRNA levels was also significant between P6 and P15 ($P < 0.0001$, $n = 6$) (Fig. 5b).

PKC θ is moderately high at an early stage and it undergoes a two-fold increase during neonatal development

The temporal profile of PKC θ was similar to that of PKC ϵ . The expression of PKC θ was reasonably high at P2, following which its normalized band intensity increased by 50–100% during the later stages (P10–P20) of neonatal hippocampal development (Fig. 6a). The increase in band intensity from P6 to P15 was significant ($P = 0.006$; $n = 3$). Although the mRNA levels showed a trend of increase at P15 and P20, the change between P6 and P15 was not significant ($P = 0.129$; $n = 3$). Thus PKC θ expression could be only partially regulated at the transcriptional level (Fig. 6b).

Determination of the actual levels of PKC ϵ and PKC α at P6 and P15

The normalized band intensities for the PKC isozymes displayed discrete profiles, with PKC ϵ showing a stronger band than PKC α at P6. However, the actual levels of the two enzymes could not be compared from their Western blotting profiles, because the widely different avidities of the antibodies for the respective proteins could have played an important role in determining the band intensities for the proteins. We addressed this problem by resolving and probing purified PKC ϵ and PKC α parallel to the hippocampal lysates on the same membrane. As shown in supplementary figure 1, using standard curves constructed from 0.5, 1, 2, and 5 ng of PKC ϵ and 1, 3, 5, 7, and 10 ng of PKC α resolved parallel to 60 μ g of total hippocampal lysate proteins from P6 and P15, we determined that the P6 lysate contained 1.75 ng PKC ϵ and only 160 pg of PKC α . Before using the standard curve equations, the intensity of each PKC band was normalized to the intensity of the corresponding β -actin band. The P15 lysate contained 7.36 ng of PKC ϵ and 1.187 ng of PKC α . Thus, at P6, PKC ϵ was 10.94-fold more abundant than PKC α . Between P6 and P15 PKC α underwent a 7.38-fold increase and finally, at P15, the PKC ϵ /PKC α ratio decreased to 6.

Regional and intracellular distribution of PKC α and ϵ

It is believed that subcellular localization of PKC isozymes correlates with their intracellular roles [27]. Therefore, we used immunohistochemistry to study the regional distribution of PKC α and PKC ϵ at two important time points in mouse hippocampal development, P6 and P15. At an early postnatal stage (P6), a low immunoreactivity for PKC α was observed in the dentate gyrus (DG) (Fig. 7), but at P15, the PKC α protein was expressed in abundance in the neuronal processes including the apical dendritic regions and the thin axonal processes in the hilus of the DG (Fig. 7). This suggested that PKC α could be limited to synaptogenesis and is not involved in neurogenesis. Further, its expression in cells harbored within the hilus strongly indicated that PKC α could play an important role in the maturation of the interneurons.

Our studies also revealed that at the early postnatal stage of P6, the PKC ϵ protein was expressed in the soma of the cells that line the granular layer, the subgranular zone (SGZ) of the DG, and around the hilus as well as in the CA3 layer (Fig. 8). Like the ventricular zone, the granular cell layer is the region that harbors dividing cells, which yield the DG neurons, the SGZ cells and the interneurons of the hilus. The granular layer of the DG also produce cells later in development [26,29]. All these observations, in conjunction with the pattern of expression of PKC ϵ , raise the possibility that this protein could have the potential of participating in neurogenesis during various postnatal periods. Importantly, co-localization of PKC ϵ in the nuclei along with the proliferation marker Ki67 (Fig. 9) further suggests that PKC ϵ is involved in neural proliferation during early postnatal hippocampal development. Other experiments have indeed shown that 5-HT $_1A$ -R mediated increase in cell proliferation in the hippocampal slices is ablated in the presence of a selective PKC ϵ inhibitor (Supplementary figure 2).

As mentioned earlier, the expression of PKC ϵ increased further in late postnatal stages, such as P15. Curiously, at P15, the pyramidal cell layers in CA3, CA1, the cell layers in the DG and

the interneurons of the hilus were intensely stained, and unlike PKC α , PKC ϵ was always localized to the cell body. Additionally, at P15, PKC ϵ was no longer colocalized with Ki67 in the nuclei of the cells. Rather, the staining was observed only in the cytoplasm and mostly in the Ki67(–) cells (Fig. 10).

3. Discussion

PKC isozymes participate in diverse signaling pathways that critically regulate brain development and function. In rat brain, the activity of PKC has been shown to increase as a function of age [13]. Yoshida and coworkers have shown that the expressions of various PKC isozymes are differentially controlled during rat development [44]. In rat brain, both type II (PKC β) and III (PKC α) enzymes were found to increase progressively from 3 days before birth up to 2–3 weeks of age and remained constant thereafter [14]. However, the authors also observed that the expression of PKC type I (PKC γ), which is found solely in the brain, was very low within one week, but then it showed an abrupt increase between 2 and 3 weeks of age [44].

PKCs also regulate MARCKS (myristoylated alanine-rich C kinase substrate), which is a key controller of embryonic cortical neurogenesis [12]. In his study, Hamada and coworkers have shown that despite negligible MARCKS phosphorylation, embryonic day-16 (E16) rat brain extracts contained both MARCKS as well as PKC γ , δ , ϵ and λ . While PKC γ and ϵ were present at very low levels in embryonic rat brains, the α and β isoforms were undetectable. Along with MARCKS, the other regulator neuronal differentiation, GAP-43, is also activated by the PKCs. McNamara and Lenox have shown that the mRNAs for the various PKC substrates (MARCKS, MLP, GAP-43, RC3), which have different subcellular and regional localizations in the hippocampus at several time points (6 hr, 12 hr, 18 hr, 24 hr, 48 hr, 5 days, or 15 days), exhibit unique expression profiles and regulation in the different cell fields of the mature hippocampus following kainic acid seizures and during subsequent synaptic reorganization [22].

nPKC δ has been shown to negatively regulate polysialyltransferase activity in the rat brain during development and also in the hippocampus during memory consolidation. In the Golgi membrane fraction, a downregulation of PKC δ is observed with concurrent, transient increase in NCAM PSA. However no change was observed in another nPKC (ϵ) or the cPKCs - α , β and γ [7]. PKC δ plays a pivotal role in the genotoxic stress response to DNA-damaging agents that lead to the induction of apoptosis [43]. More directly, expression of a constitutively active mutant of PKC δ causes apoptosis [6].

Nicotinic cholinergic modulation of synaptic transmission in the hippocampus is also found to be mediated by PKCs [2]. Additionally, PKCs have been implicated to play important roles in signaling for various growth factors, cytokines, and hormones [34]. So it is evident that PKCs control numerous signaling cascades by the virtue of their ability to phosphorylate target proteins that may include other kinases and such signaling cascades often control the electrical activity, which has been found to play an essential role in early development of nervous system [36]. The extensive involvement of PKC-mediated activity makes it likely that it is required at all developmental stages as a necessary partner with transcriptional genetic controls. This also suggests that posttranslational modification of the kinases are extremely important for every stage of neuronal development, from initial proliferation and differentiation of progenitor cells to path-finding of neurites and formation of synapses.

Activation of the PKCs is critical for neuronal cell survival [19], and is essential for proper neural tube closing. Although all known PKC isoforms were detected in the closing neural tube, use of chemical PKC inhibitors identified a particular requirement for ‘conventional’ PKC isoforms. Intriguingly, the neurulation stage is critically dependent upon PKC β I and γ ,

whereas other PKCs (α , β II, δ , and ϵ) were found to be dispensable [4]. These experiments reveal the importance of specific isoforms of PKCs during neuronal ontogenic development and call for a detailed study of the developmental regulation of the PKC isozymes.

Except for PKC ϵ and PKC θ , all the other PKC isozymes studied here (α , β , γ , δ) underwent a pronounced increase between P6 and P15. In contrast, both PKC ϵ and PKC θ bands were already quite intense at P6 and the intensity of these bands underwent only a modest increase between P6 and P15. Since, PKC ϵ at P6 and PKC α at P15 are likely to mediate neurogenesis and synaptogenesis, respectively, in this report we have proceeded to determine the absolute amounts of these two isozymes at the two developmental stages by comparing the Western blotting bands for these isozymes with those obtained from pure PKC ϵ and PKC α , respectively.

The regulation profiles generally suggest that the signaling activity of the isozymes α , β , γ , δ is likely to be essential during the later stages of neonatal development, whereas the isozymes ϵ and θ are probably required at both early as well as later stages of hippocampal development. Our results in mouse hippocampus are consistent with many of the earlier studies either in total brain [13], [44], or in cerebellum [15], however contrary to the observation of Jiang and coworkers of non-postnatal regulation of PKC δ [16], we found definite postnatal change in expression of the isozyme. This difference may be attributed to unique species variance. Like PKC ϵ and θ , PKC δ is activated not by Ca^{2+} but by diacylglycerol and PS, whereas the conventional PKC isozymes α , β , and γ require Ca^{2+} , diacylglycerol, and PS for their activation. The role of Ca^{2+} in synaptic transmission, which in turn is pivotal for synaptogenesis, becomes increasingly crucial as more synaptic connections are made and therefore, it is expected that Ca^{2+} -sensitive PKC α will be involved critically at P15 when the synaptic connections are established. In support of this speculation, immunohistochemical data showed expression of PKC α in the neuritic processes that form a network in the hippocampus at P15 (Fig. 7).

In contrast to the supportive role of PKC α , PKC δ is known to mediate signals that cause apoptosis [38], which is required for the elimination of neurons that fail to establish connections with their partners during synaptogenesis. Therefore, the dramatic induction of PKC δ between P6 and P15 could serve the specific purpose of preparing the neurons for apoptosis in case their effort to establish synaptic connections with appropriate targets fail. An apoptotic signal is triggered on in such unsuccessful synapses, which activates the available PKC δ molecules to promote axonal retraction and apoptosis.

Finally, of the two novel PKC isozymes that are present at a relatively higher level even at P6 (PKC ϵ and θ), PKC ϵ is the likely molecule that links the 5-HT $_{1A}$ -R to the MAPK pathway and proliferation of preneuronal cells at P6 (Supplementary figure 2) [23]. Furthermore, Wheeler and coworkers demonstrated the involvement of PKC ϵ in proliferation by showing that overexpression of this isozyme in mouse epidermis results in a spontaneous myeloproliferative-like disease [41]. In line with this observation, PKC ϵ is expressed in the nuclei of neuroblasts in the DG layers and also in the interneurons of the hilus (Fig. 9). Thus, the abundant PKC ϵ molecules may couple to the 5-HT $_{1A}$ -R to boost proliferation at P6 and then, at P15, link to other signaling cascades to promote other functions (5-HT $_{1A}$ -R signaling does not stimulate cell division in the DG at P15) [23]. However, its expression in the CA3 and CA1 cell layers as well as in the interneurons of the *stratum radiatum* may serve some function (Fig. 8), which has yet to be determined.

While studying the changes in 5-HT $_{1A}$ -R-mediated signaling in early post-natal hippocampus, we discovered a differential, stage-specific involvement of PKC isozymes [24]. This prompted us to consider a stoichiometric control of events by the relative concentrations of the PKC isozymes involved. Results reported in this study will not only help explain our earlier

observations, but they will also provide valuable clues to many other unexplained mechanisms in brain development.

4. Experimental Procedure

Mouse monoclonal antibodies for PKC β , γ , θ and δ were purchased from BD Transduction Laboratories, BD Biosciences (San Diego, CA). Antibodies to PKC α (mouse monoclonal), PKC ϵ (rabbit polyclonal) and horse radish peroxidase-labeled secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and mouse monoclonal anti- β actin antibody was purchased from Sigma (St. Louis, MO). The Alexafluor-labeled fluorescent secondary antibodies were obtained from Molecular Probes (Eugene, OR). Pure PKC ϵ was obtained from Calbiochem (Gibbstown, NJ) and PKC alpha was purchased from Sigma. The Vectastain ABC kit was obtained from Vector laboratories (Burlingame, CA) and 3,3'-Diaminobenzidine was procured from Sigma. Platinum Taq DNA polymerase High Fidelity was obtained from Invitrogen (Carlsbad, CA). The Supersignal luminol kit was procured from Pierce (Rockford, IL). The Vibratome cryostat was from Global Medical Instrumentation (Ramsey, MN). The protease inhibitor cocktail was obtained from Roche Diagnostics GmbH, Mannheim, Germany. The Tissue-Tek O.C.T. compound was obtained from Electron Microscopy Sciences (Hatfield, PA). Permunt was procured from Fisher Scientific (Pittsburg, PA), Prolong gold antifade reagent was obtained from Invitrogen, the GeneAmp PCR system 2700 was purchased from Applied Biosystems (Foster City, CA), and RNAeasy mini kit was obtained from Qiagen (Valencia, CA)

Animals

Experiments were carried out using Wild-type C57/B6 mouse pups of either sexes. All procedures complied with the standards for care and use of animal subjects as stated in the "Guide for the care and use of laboratory animals", US Public Health Services and were approved by the IACUC at the College of Staten Island.

Western blotting

Hippocampi at different postnatal days (P2, P4, P6, P8, P10, P15, P20) were collected and lysed in phosphate buffered saline (PBS) containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mmol/L Na₃VO₄ (RIPA buffer) plus freshly added protease inhibitor cocktail. Tissue lysates were briefly sonicated and protein concentration was measured according to the method of Lowry, using BSA as the standard. Purified proteins PKC α (1, 3, 5, 7, 10 ng) and PKC ϵ (0.5, 1, 2, 5 ng) as well as proteins from tissue lysates were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were successively incubated in blocking buffer (5% fat-free milk in TBST [20 mmol/L Tris-HCl, pH 7.4, 0.8% NaCl, 0.1% Tween 20]), and then probed with different primary antibodies, followed by treatment with HRP-linked respective secondary antibodies. Detection was carried out with the Supersignal luminol kit and bands were densitometrically quantified using a Fluorchem FC2 imaging system (Alpha Innotech, San Leandro, CA). All primary antibodies were used at 1:1000 dilution, except for anti-PKC ϵ (1:5000) and anti- β -actin (1:10,000). Standard curves were obtained from different dilutions of the purified proteins PKC α and PKC ϵ , and the absolute protein content of PKC alpha and epsilon per 60 μ g of hippocampal lysate was quantified from these standard curves.

Immunohistochemistry

P6 and P15 mice were anesthetized and perfusion-fixed via the left ventricle with PBS (pH 7.3) followed by 4% paraformaldehyde in PBS. Brains were dissected and placed in 4% paraformaldehyde and 30% sucrose for 24 hours and 48 hours respectively. Following this, brains were mounted in Tissue-Tek O.C.T. compound and 30- μ thick cryosections were made

with a Vibratome cryostat. Free-floating cryosections were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 30 min, rinsed three times with PBS, then blocked for 1 hour in Tris-buffered saline containing 0.1% Triton X-100 (TBS-T) and 3% serum from the animal used to raise the 2° antibody that was going to be used later. Primary antibody (anti- PKC α and PKC ϵ , 1:250) incubation was carried out overnight at 4°C with gentle shaking. After several washings with TBS-T, cells were incubated with diluted (1:200) biotinylated 2° antibody solution (Vectastain ABC kit) for 1 hour at room temperature, washed several times with TBS-T, treated with vectastain ABC reagent for 30 min. Slices were next washed three times with PBS, incubated with 0.1% diaminobenzidine and 0.02% H₂O₂ for 2.5 min, and then the sections were washed with PBS and mounted with Permount. A negative control was obtained by treatment of parallel slices with 2° antibody without primary antibody. Fluorescence immunohistochemistry was performed using anti-PKC ϵ and anti-Ki67 (1:250; Vector Laboratories). Secondary antibodies used were labeled with Alexa Fluor 568 (1:200) or Alexa Fluor 488 (1:200). The sections were mounted with prolong gold antifade reagent for visualization and photography using confocal laser scanning microscope (Nikon Eclipse 90i).

Isolation of RNA and measurement of mRNA levels by reverse transcriptase-PCR (RT-PCR)

Mouse hippocampi at postnatal days 2, 4, 6, 8, 10, 15 and 20 were obtained and stored in RLT buffer. Total RNA was isolated from the tissue using the RNeasy mini kit and cDNA was synthesized from 3 μ g RNA using an oligo(dT) primer provided in the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen). A 2- μ l aliquot of the cDNA synthesized was then used in a 50- μ l PCR reaction with 10x High Fidelity PCR buffer, 50 mM MgSO₄, 10 mM dNTP mix, 10 μ M of each primer (Table 1) and 1.5 U of Platinum Taq DNA polymerase High Fidelity. The cDNA sequences were amplified using a GeneAmp PCR system 2700 and the following cycles: 30 s at 94 °C; 30 s at the specific annealing temperature (T_A) for each set of primers; 1 min at 68 °C; 10 min at 72 °C. For control reactions to amplify the β -actin gene, the cycles were: 2 min at 94 °C, 30 s at 94°C, 1 min 30 s at 68 °C, 10 min at 72 °C. PCR fragments were analyzed by electrophoresis on a 2% agarose gel for PKC α , β , δ , ϵ , γ , β actin and a 3 % gel for PKC θ . The PCR was performed in such a way that the amount of products formed remained within the linear (pre-saturation) range. To this end, we conducted PCR for increasing number of cycles (15,20,25,30,35,⁴⁰, 45) to rigorously establish the midpoint of the linear segment within the logarithmic amplification profile of each message. The final number of cycles used and the annealing temperatures (T_A) for the different messages are shown in Table 2.

Statistical analysis

For both protein as well as mRNA levels, all comparisons between P6 and P15 were performed using students' t-test among unpaired data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

PBS	phosphate buffered saline
5-HT_{1A}-R	serotonin 1A receptor
PKC	Protein kinase C
PCR	Polymerase chain reaction
RT-PCR	reverse transcriptase-PCR

PMSF	Phenylmethylsulfonyl fluoride
EGTA	Ethyleneglycol tetraacetic acid

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Figure 1. PKCα protein level increases sharply during neonatal hippocampal development and it is regulated at the transcriptional level

The bar graphs with statistical analysis were obtained after normalization to β-actin on each Western blotting analysis of proteins (a) or agarose gel electrophoresis of RT-PCR products (b). The Western blotting profile is representative of three independent experiments and the agarose gel image represents five discrete RT-PCR experiments.

Figure 2. PKCβ protein as well as mRNA levels increase during neonatal hippocampal development
 The bar graphs with statistical analysis were obtained after normalization to β-actin on each Western blotting analysis of proteins (a) or agarose gel electrophoresis of RT-PCR products (b). The Western blotting profile is representative of three independent experiments and the agarose gel image represents six discrete RT-PCR experiments.

Figure 3. PKC γ protein and mRNA increase during neonatal hippocampal development

The bar graphs with statistical analysis were obtained after normalization to β -actin on each Western blotting analysis of proteins (a) or agarose gel electrophoresis of RT-PCR products (b). The Western blotting profile is representative of three independent experiments and the RT-PCR profile represents four independent experiments.

Figure 4. PKC δ protein level increases sharply during neonatal hippocampal development and it could be partially regulated at the transcriptional level

The bar graphs with statistical analysis were obtained after normalization to β -actin on each Western blotting analysis of proteins (a) or agarose gel electrophoresis of RT-PCR products (b). The Western blotting profile is representative of three independent experiments and the agarose gel image represents five discrete RT-PCR experiments.

(a)

(b)

Figure 5. PKC ϵ is relatively high at an early stage and its mRNA as well as protein undergoes a modest increase during neonatal development

The bar graphs with statistical analysis were obtained after normalization to β -actin on each Western blotting analysis of proteins (a) or agarose gel electrophoresis of RT-PCR products (b). The Western blotting profile is representative of three independent experiments and the agarose gel image represents six discrete RT-PCR experiments.

Figure 6. PKC θ is moderately high at an early stage and it undergoes a two-fold increase during neonatal development, but no significant increase in mRNA is observed

The bar graphs with statistical analysis were obtained after normalization to β -actin on each Western blotting analysis of proteins (a) or agarose gel electrophoresis of RT-PCR products (b). The Western blotting profile is representative of three independent experiments and the RT-PCR profile is also representative of three discrete experiments.

Figure 7. Temporal and regional distribution of PKC α in the hippocampus during neonatal development

Expression levels of PKC α is difficult to detect in all regions of the hippocampus at P6 (B). At P15 (D and E) it is expressed strongly in the axonal processes (black white arrow) and the apical dendritic regions (white arrow) of the hilus of the DG and also in the cell body (black arrow) of the interneurons of the hilus. Representative negative controls for P6 (A) and P15 (C) are also shown.

Figure 8. Temporal and regional distribution of PKCε in the hippocampus during neonatal development

At P6, PKCε protein is present in the cell bodies (solid black arrows) throughout the granular cell layer, subgranular zone (SGZ) (B) of the dentate gyrus, around the hilus (D) as well as in the CA3 region (C). At P15, a continued elevated PKCε level was observed in the cell bodies of granular layers of the dentate gyrus, the interneurons of *stratum radiatum* (thin black arrows) and the pyramidal cell layers of the CA1 and CA2 region (F). High immunoreactivity was also observed in the cell bodies of CA3 region (G). Representative negative controls for P6 (A) and P15 (E) are also shown.

Figure 9. At P6, PKC ϵ is expressed mainly in the nuclei of the neuroblasts
Colocalization of PKC ϵ and Ki67 staining was observed in the neuroblasts in the DG, especially in the SGZ including the hilus (A–C). Higher magnifications show that PKC ϵ expression was confined only to the nuclei of the Ki67(+) cells (D–F).

Fig. 10. At P15, PKC ϵ is expressed in the cytoplasm of cells in the DG, CA1 and CA3 layers. While the Ki67 staining was still observed in the nuclei, the PKC ϵ immunoreactivity was detected only in the cytoplasm of cells. Although a few neuroblasts displayed PKC ϵ immunoreactivity, most cells stained with anti-PKC ϵ were Ki67(-)(A-C).

Table 1

PCR Primers

Name	Primer	Position	EMBL Accession
PKC-Alpha-Fwd	TGAATCCTCAGTGGAATGAGT	818–838	X52685
PKC-Alpha-Rvs	GGTTGCTTTCTGTCTTCTGAA	1122–1142	X52685
PKC-Beta-Fwd	AAGGAAGCGAGGGCAATGAA	914–933	X59274
PKC-Beta-Rvs	AGTTCATCTGTACCCTTCCGCTCT	1112–1135	X59274
PKC-Gamma-Fwd	GCTGTATGAGATGTTGGCAG	1968–1987	L28035
PKC-Gamma-Rvs	GAGATTACATGACAGGCACG	2414–2433	L28035
PKC-Delta-Fwd	CACCATCTTCCAGAAAGAACG	883–903	M69042
PKC-Delta-Rvs	CTTGCCATAGGTCCAGTTGTTG	1213–1234	M69042
PKC-Theta-Fwd	TTGATCTTTCCAGAGCCACG	1515–1534	D11091
PKC-Theta-Rvs	CTTCGCATCTCCTAGCAT	1675–1692	D11091
PKC-Epsilon-Fwd	TCGATCTCTCGGGATCAT	395–412	NM_011104
PKC-Epsilon-Rvs	CGGTTGTCAAATGACAAGGCC	1104–1124	NM_011104
Beta Actin -Fwd	GTTCGCCATGGATGACGATATCGC	73–96	NM_007393.3
Beta Actin -RVS	GACCTGGCCGTCAGGCAGCTCA	799–820	NM_007393.3

All primers were sequenced from *mus musculus* and accession numbers refer to those on the EMBL database.

Table 2

Annealing temperatures and number of cycles used for PCR.

Isozymes	T _A	Cycles
α, θ	52 °C	35
β, γ	50 °C	30
δ	52 °C	30
ϵ	48 °C	35