ORIGINAL ARTICLE

Protein kinase C delta accumulates in neurofibrillary tangles in Alzheimer's disease

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Abstract

The protein kinase C (PKC) family is associated with tau pathology in Alzheimer's disease (AD). PKCδ, a novel PKC isoform, has universal, rather than cell-type-specific, roles in the human brain and may be critical for cellular functions, such as control of growth, differentiation, and apoptosis. However, little information is available regarding the localization and function of PKCδ in the AD brain. In this study, we investigated PKCδ localization in postmortem human brain tissues from elderly individuals (controls) and patients with AD using immunohistochemistry. In control brains, punctate staining of PKCδ was observed in the cytoplasm, nuclei of granules and pyramidal neurons in the hippocampus. In AD brains, PKCδ expression in neuronal cell bodies was lower than that in controls. Numerous neurofibrillary tangles (NFTs) and degenerative neurites within senile plaques were strongly immunopositive for PKCδ in the AD hippocampus. Residual neurons were weakly positive in the AD hippocampi compared to the control, and many intracellular NFTs were strongly positive for PKCδ in the CA1 subfield and the subiculum. Immunoelectron microscopy of the AD hippocampus showed that PKCδ was localized in the paired helical filaments of NFTs, nuclei, small vesicles, and lysosomes. Double immunostaining showed that PKCδ was extensively colocalized with glycogen synthase kinase 3β (GSK3β), which is involved in tau pathology. These results suggest that PKCδ is localized to paired helical filaments in intracellular NFTs in the AD brain and plays an important role in tau pathology.

Keywords: protein kinase C delta, Alzheimer's disease, neurofibrillary tangle, immunohistochemistry, human brain

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive memory loss and cognitive impairment. AD is characterized by the presence of extracellular senile plaques (SPs), intracellular neurofibrillary tangles (NFTs), neuronal loss, and chronic inflammation involving the activation of glia [1, 2]. Senile plaque formation occurs approximately 20 years before symptom onset. Conversely, the location and number of NFTs correlate directly with the severity of AD dementia and duration of illness [3]. NFTs are composed of straight and paired helical filaments consisting of an aberrantly hyperphosphorylated form of the microtubule-associated protein (MAP) tau [4–6]. Tau protein is normally expressed in the cytoplasm of cell bodies and neurites and is distributed to axons, where tau assembles and stabilizes microtubules. These functions of tau can be modulated by phosphorylation of serine (S), threonine (T), or tyrosine residues [7–9].

Abnormal tau hyperphosphorylation in AD brains may result from an altered balance between tau kinase and phosphatase activities [10]. Tau phosphorylation is mediated by multiple kinases such as glycogen synthase kinase 3 beta (GSK3β), cyclin-dependent kinase 5 (CDK5), microtubule-affinity-regulating kinase (mitogen-activating protein kinase, MARK), extracellular signal-regulated kinase (ERK)1/2, and p70 S6K [11–13]. These kinases are activated by several other kinases, including Akt (also known as protein kinase B, PKB) [14] and protein kinase C (PKC) family [15–17]. These kinases (Akt/PKB [14], PKCι/λ [15], PKMζ [16], and PKN [17]) accumulate in the NFTs in the AD brain. These members are activated by phosphoinositide-dependent

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kinase-1 (PDK1) in the phosphoinositide 3-kinase (PI3K) signaling pathway [18, 19].

The PKC family consists of at least ten isozymes, and are divided into three subfamilies based on their second-messenger requirements: conventional (or classical), novel, and atypical PKCs. Conventional PKCs contain isoforms α, β_I, β_{II}, and γ. For activation, these require calcium ion (Ca^{2+}), diacylglycerol (DAG: a degradation product of phosphatidylinositol 4,5-bisphosphate, PIP2), and phospholipids such as phosphatidylserine. Novel PKCs contain isoforms δ, ε, η, and θ. These require DAG, but not Ca^{2+} for activation. Atypical PKCs require neither Ca^{2+} nor DAG for activation [20]. PKCs are involved in critical cell signaling pathways in many disorders, such as acquired immunodeficiency syndrome (AIDS), cancer, and AD. In the AD brain, PKCs are involved in memory [21, 22], amyloid pathology [23], tau pathology [24], and inflammation [25]. PKCs directly participate in associative memory storage. In addition, systemic deficiency in the PKC–MAP kinase (MAPK)/ERK1/2 pathway could explain the symptomatic memory loss in AD and many other aspects of AD pathology [25, 26]. In amyloid pathology, PKCs phosphorylate the C-terminus of amyloid precursor protein (APP) and modulate APP metabolism [25, 27, 28]. In tau pathology, PKCs phosphorylate ERK1/2 and GSK3β and increase the levels of phosphorylated tau (p-tau), resulting in neuritic pathology in AD [25, 29]. Furthermore, AD might exhibit early inflammation signaling triggered by inflammatory mediators such as bradykinin, which causes PKC activation, as well as tumor necrosis factor-alpha (TNF-α) and interleukins, which cause ERK1/2 phosphorylation [25, 30].

PKC delta (PKCδ) is a novel PKC isoform and a serine/threonine protein kinase that plays a key role in growth regulation and tissue remodeling. Traditional models of PKC activation have focused on lipid cofactors and anchoring proteins that localize the active conformation of PKCδ to membranes in close proximity to their target substrates [31]. PKCδ is suggested to have universal rather than cell-type-specific roles in the human brain and play critical roles in cellular functions such as the control of growth, differentiation, and apoptosis [32]. However, little information is available regarding PKCδ localization in the AD brain. In this study, we investigated the localization of PKCδ in postmortem human brain tissues from elderly individuals and patients with AD using immunohistochemistry.

Materials and Methods

Human brains

Postmortem human brain tissues from 10 individuals (control) without neurological disorders (sex: 7 men and 3 women; age range: 56–88 years; mean ± SD: 67.5 ± 11.7 years) and 13 patients with sporadic AD (sex: 7 men and 6 women; age range: 56–89 years; mean ± SD: 80.3 ± 8.9 years) were used in this study (**Table 1**). No significant difference was seen in postmortem delay (PMD) between control and AD cases (mean ± SD, 4.9 ± 1.6 vs. 4.9 ± 1.3 h). Two AD cases were of intermediate likelihood according to the National Institute on Aging-Reagan criteria, in which the Consortium to Establish a Registry for Alzheimer's disease (CERAD) SP score was moderate and Braak and Braak's NFT score was IV (mild AD). The other 11 cases had a high likelihood, with a high CERAD score and an NFT score of V or VI (severe AD) [33–35]. All procedures in this study were performed strictly according to the principles of the Declaration of Helsinki and the local Ethics Committee's Clinical Study Guidelines and were approved by the internal review board (Shiga University of Medical Science: 29–115).

Immunoblotting

The neocortices of two AD cases and a control case were used for immunoblotting. The tissue samples were homogenized in five volumes of 50 mmol/L Tris-HCl (pH 7.4) con-

Table 1 Cases used in the present study

	N	Sex Man/Woman	Age (years) M ± SD	PMD (hours) M ± SD	NIA-Regan	CERAD Aβ
Control cases	10	7/3	67.5 ± 11.7	4.9 ± 1.6	N/A	N/A
AD cases	13	7/6	80.3 ± 8.9	4.9 ± 1.3		
(Braak stage for NFT)						
IV	2	1/1	83.0 ± 5.7	4.5 ± 2.1	Probable	Moderate
V	6	3/3	82.2 ± 6.9	4.7 ± 1.5	Definite	Frequent
VI	5	3/2	77.0 ± 12.1	5.4 ± 0.9	Definite	Frequent

AD, Alzheimer disease; NFT, neurofibrillary tangle; M ± SD, mean and standard deviation; PMD, postmortem delay; NIA-Regan, National Institute on Aging-Reagan Institute criteria for the neuropathological diagnosis of Alzheimer disease; CERAD, Consortium to Establish a Registry for Alzheimer's Disease; Aβ, amyloid beta; N/A, not applicable

taining 1 mmol/L EDTA, 1 μg/mL pepstatin, and protease inhibitors (Complete Mini; Roche Diagnostics, Mannheim, Germany; 1 tablet/10 mL).

Twenty micrograms of the total homogenates and prestained precision protein standards (Bio-Rad, Hercules, CA) were subjected to 5–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and then transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Tokyo, Japan). The membrane was incubated with 5% Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 h at room temperature and further incubated overnight with a polyclonal antibody against PKCδ (Santa Cruz Biotechnology, Dallas, TX) diluted at 1:1,000 in 25 mmol/L Tris-buffered saline (TBS) containing 5% Blocking One at 4°C. After washing with 25 mmol/L TBS containing 0.1% Tween-20 (Bio-Rad), the membrane was incubated for 2 h with peroxidase-labeled anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted at 1:50,000. Peroxidase labeling was detected by chemiluminescence using a SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Immunohistochemistry

The fixation, processing, and immunohistochemical examination of frozen human brain tissues, including cerebellum, hippocampus, entorhinal cortex, amygdala, superior/middle/inferior temporal gyrus, and angular gyrus, were performed as previously described [36]. In brief, small blocks were immersed in 4% formaldehyde fixative dissolved in 0.1 mol/L phosphate-buffered saline (PBS; pH 7.4) for 2 days at 4°C and then immersed in 10 mmol/L PBS (pH 7.4) containing 15% sucrose and 0.1% sodium azide at 4°C. Sections (20 μm thick) were cut using a microtome, collected in maintenance solution, and stored at 4°C until use.

In the present study, tissue sections from two AD cases (mild and severe) and two control cases were used for immunohistochemical analysis. For immunohistochemistry, the sections were treated with 0.5% hydrogen peroxide in 10 mmol/L PBS containing 0.3% Triton-X100 (PBST) for 30 min to inactivate endogenous peroxidase activities. The sections were then incubated with PBST containing 10% skim milk to block non-specific protein binding for 2 h, followed by incubation with primary antibodies for 3 days at 4°C. Antibodies used in this study are listed in **Table 2**. After the sections were washed three times with PBST, they were incubated with biotinylated goat anti-rabbit IgG antibody (1:1,000 dilution; Vector Laboratories, Burlingame, CA) for 1 h at room temperature. After washing three times with PBST, the sections were finally incubated with an avidin-biotin-peroxidase complex (ABC; 1:1,000 dilution; VECTASTAIN Elite ABC, Vector Laboratories) for 1 h at room temperature. Peroxidase-labeled sections were developed by incubation in 50 mmol/L Tris-HCI (pH 7.6) containing 0.01% 3,3'-diaminobenzidine tetrahydrochloride, 0.6% nick-

Table 2 Antibodies used in the present study

Antigen	Antibody	Source	Type	Dilution
PKCδ	C-20	Santa Cruz*	Rb	1:300 (IB)
				1:500 (IHC)
tau	HT7	Innogenetics	Ms	1:5000
p-tau	AT8	Innogenetics	Ms	1:10000
abnormal tau	Alz50	Dr. Davies†	Ms	1:5000
C4d		Quidel	Ms	1:500
pMEK	pS222	Biosource	Rb	1:2000
GSK3β	beta	Millipore	Ms	1:1000
PTEN		R&D	Rb	1:10000
p70 S6K	pT421/S424	Cell Signaling	Rb	1:1000
	pT389	Cell Signaling	Ms	1:500
pNF-H	SMI-31	Calbiochem	Ms	1:5000
GFAP	GA5	Millipore	Ms	1:5000
HLA-DR/DP/DQ	CR3/43	Abcam	Ms	1:300

IB, immunoblotting; IHC, immunohistochemistry; p, phosphorylation; T, threonine; S, serine; Rb, rabbit polyclonal antibody; Ms, mouse monoclonal antibody

* PKCδ (C-20) has been discontinued and replaced by PKCδ (G-9).

† Generously gifted by Dr. P. Davies (Albert Einstein College of Medicine, NY, USA)

el ammonium sulfate and 0.00015% hydrogen peroxide.

Immunohistochemical analysis of mirror image sections from the amygdala in AD cases were performed by incubation with antibodies against PKCδ, phosphatase and tensin homolog (PTEN), ERK kinase phosphorylated at S222 (pMEK), and p70 S6 kinase phosphorylated at T389 or T421/S424 (**Table 2**), followed by ABC method as described above.

Immunoelectron microscopy was performed using the pre-embedding immunogold labeling method in the AD hippocampus. Sections were incubated with a polyclonal antibody against PKCδ in PBS containing 5% bovine serum albumin for 3–7 days at 4°C. After washing three times with PBS for 10 min, the sections were incubated overnight with nanogold-labeled goat anti-rabbit IgG antibody (1:500 dilution; Nanoprobes, Yaphank, NY) at 4°C. After washing three times with PBS for 10 min, the sections were fixed with 1% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 10 min. The sections were washed three times with 0.1 mol/L phosphate buffer (pH 7.4) containing 50 mmol/L glycine for 5 min and then three times with PBS for 10 min. After washing twice with distilled water (DW) for 5 min, the sections were treated using a GoldEnhance EM (Nanoprobes) according to the manufacturer's instructions. After washing with DW, the stained sections were post-fixed with 1% osmium tetroxide, followed by washing with DW, dehydration in a graded alcohol series and flat embedding in Epon WE812 (Wako, Osaka, Japan). Ultrathin sections were examined using a model H-7100 transmission electron microscope (Hitachi, Tokyo, Japan) at 75 kV.

To elucidate the colocalization of PKCδ with pathological markers, the double immunofluorescence labeling of neuritic pathology for PKCδ and either tau, p-tau, GSK3β, phosphorylated neurofilament, astrocytic filament (glial fibrillary acidic protein, GFAP), or complement protein (C4d), the sections were incubated with each combination of the primary antibodies (**Table 2**). Immunostaining was followed by incubation with appropriate secondary antibodies coupled either to fluorescein or rhodamine isothiocyanate (1:500–1:1,000 dilution; Millipore). The sections were analyzed using a Fluoview FV 500 confocal scanning laser microscope (Olympus, Tokyo, Japan).

Results

Characterization of anti-PKCδ antibody

Immunoblot analysis using anti-PKCδ antibody showed a single band at 78 kDa in total homogenates of neocortices from control and AD cases (**Fig. 1**), which corresponds to the molecular weight of PKCδ. The expression level of PKCδ showed no significant differences between control and AD brains (**Fig. 1**). These results consistent with those reported by Matsushima et al. [37].

Cellular localization of PKCδ in the control and AD brains

Cellular localization of PKCδ in the control and AD brains was analyzed by light-microscopic immunohistochemistry. In the control brains, PKCδ immunoreactivity was localized in neurons (**Fig. 2a–c**). The cell bodies of hippocampal pyramidal cells, and fine or coarse granules with torpedoes-shape in cerebellar Purkinje cells are positive for PKCδ (**Fig. 2a, b**). In the control temporal lobe, a few varicose fibers with axon-like profiles were also stained in the cerebrum (black arrows in **Fig. 2c**). In addition, large PKCδ-positive granules were observed in the white matter of the temporal lobe (**Fig. 2d**). On the other hand, in mild (**Fig. 2e**) and severe (**Fig. 2f**) AD brains, neuronal cell bodies were weakly stained for PKCδ compared to the control (**Fig. 2a, e**). In the AD hippocampal cortices, numerous NFTs (white arrows in **Fig. 2e** and **f**) and degenerative neurites within SPs (arrowheads in **Fig. 2f**) are strongly immunopositive for PKCδ, whereas the expression of PKCδ in AD neuronal cell bodies is lower density than that in the control (**Fig. 2e, f**). Similar staining was observed in the

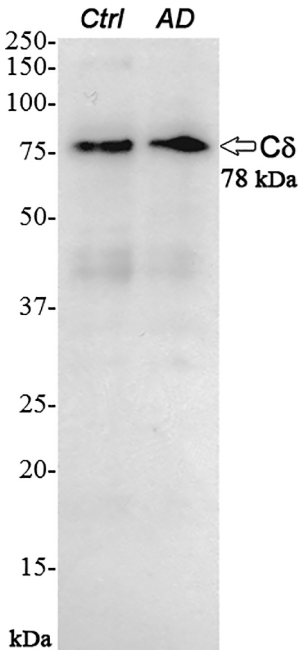

Fig. 1 Immunoblots for PKCδ

Total homogenates (20 μg protein / lane) of neocortices from aged control (left lane) and AD cases (right lane) were subjected to SDS-PAGE followed by immunoblotting. Immunostaining was carried out with a rabbit anti-PKCδ antibody.

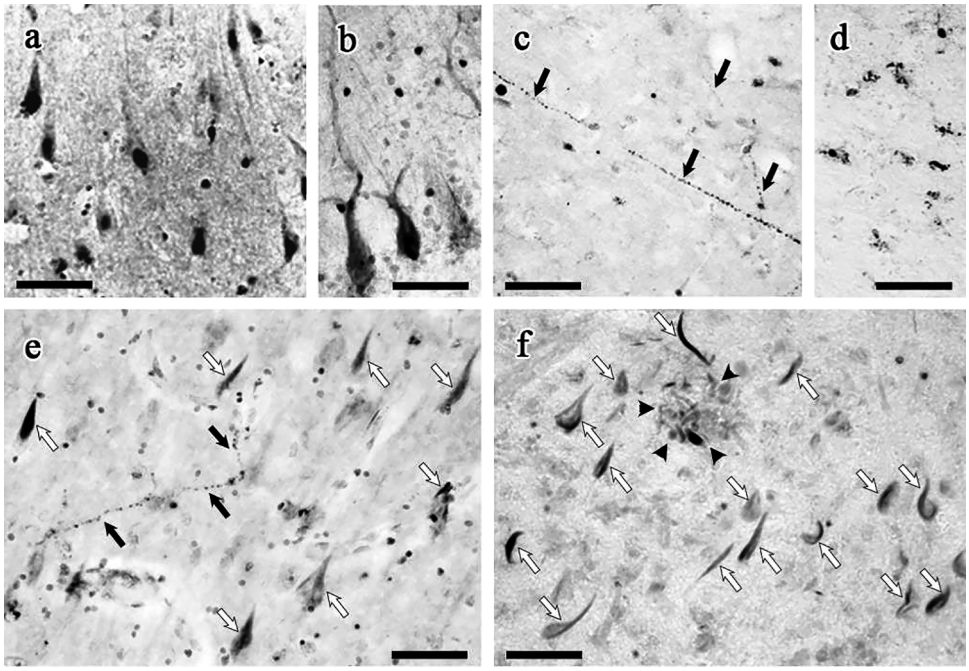

Fig. 2 Cellular localization of PKCδ in the human brains

The cellular localization of PKCδ in control hippocampus (a), cerebellum (b), temporal lobe (c, d), and AD hippocampal cortices (e, f) was examined by immunohistochemistry using a PKCδ-specific antibody and light microscope. A few varicose fibers with axon-like profiles are positive in the control temporal lobe and AD hippocampal cortices (arrows in c, e) are positive. In the AD brains, numerous NFTs (white arrows) and degenerative neurites within SPs (arrowheads in f) are strongly immunopositive for PKCδ. Scale bars: 50 μm.

entorhinal cortex, amygdala, temporal gyrus, and angular gyrus. Positive varicose fibers (**Fig. 2e**) and granules were also observed. The residual neurons were weakly positive for AD.

Distribution of PKCδ in hippocampal formations

Distribution of PKCδ in the hippocampal formation of control and severe AD brains was examined. In the control, the expression of PKCδ was observed in all subfields of the hippocampus (**Fig. 3a–d**). Punctate staining for PKCδ was found in the cytoplasm and the nuclei of granules and pyramidal neurons. The apical dendrites were also immunolabeled in CA1 pyramidal and subicular neurons (**Fig. 3d**). In the AD hippocampus, numerous NFTs and punctate structures were positive for PKCδ (**Fig. 3e–h**). Residual neurons were weakly stained for PKCδ, compared to those in the control. Positive granules and axon-like structures were observed in both AD and control brains.

Subcellular localization of PKCδ in the AD hippocampus

Immunoelectron microscopic analysis demonstrated the precise localization of PKCδ in the hippocampus with severe AD. PKCδ was localized in the nuclei (thick arrows in **Fig. 4a**), small vesicles (thin arrows (black: within vesicle, white: on membrane) in **Fig. 4a**), and lysosomes (arrowheads in **Fig. 4a**) around paired helical filaments in AD neurons (**Fig. 4a–c**). Additionally, PKCδ immunoreactivity was detected in the paired helical filaments (**Fig. 4b, c**).

Colocalization of PKCδ in NFTs and glial cells in AD hippocampus

To elucidate the colocalization of PKCδ in NFTs in the hippocampus with mild AD, we performed double immunofluorescence labeling for PKCδ. **Fig. 5a** and **5b** show the double immunofluorescence staining for PKCδ (red) and tau (green) recognized by HT7. Some tangles were double-positive for PKCδ and HT7 (yellow). **Fig. 5c** shows the double immunofluorescence staining for PKCδ (red) and p-tau (green) recognized by AT8 in tangle-bearing neurons. Interestingly, most PKCδ-positive NFTs were strongly positive for GSK3β (**Fig. 5d**). PKCδ was also present in intracytoplasmic vesicles where no (or very weak) staining for Alz50 was present. Most PKCδ-positive NFTs were negative for abnormal tau as recognized by Alz50 (**Fig. 5e**). The C4d-positive extracellular ghost tangles were negative for PKCδ (**Fig. 5f**).

The association of PKCδ (red) with glial fibrils in fibrillary astrocytes (green) were examined in AD angular gyrus. Interestingly, many vesicles were labeled for PKCδ within the cell bodies (**Fig. 5g**), and some of them were associated with glial fibrils in reactive astrocytes, expressing glial fibrillary acidic proteins (GFAP) surrounding SPs in the temporal

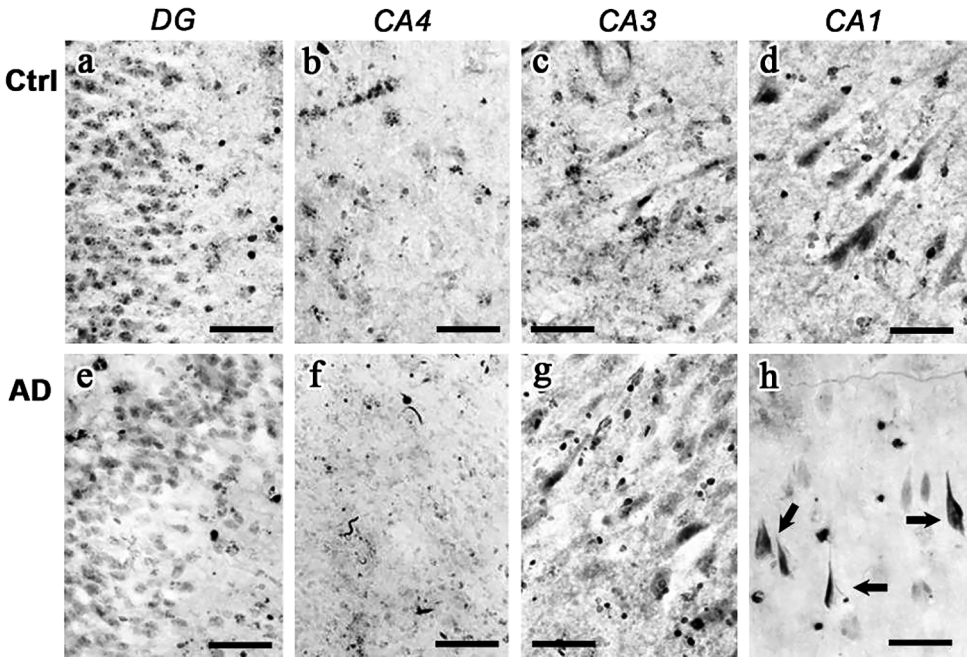

Fig. 3 Distribution of PKCδ in hippocampal formations

The subfields of hippocampus, such as DG (a, e), CA4 (b, f), CA3 (c, g), and CA1 (d, h) were immunostained for PKCδ. AD hippocampal neurons (e–f) showed remarkably reduced PKCδ accumulation, and residual neurons were weakly positive whereas punctate staining for PKCδ was seen in the cytoplasm or the nuclei of granule or pyramidal neurons in control hippocampi (a–d), and many intracellular NFTs were strongly labeled for PKCδ in CA1 subfield (arrows in h). DG; dentate gyrus. Scale bars: 50 μm.

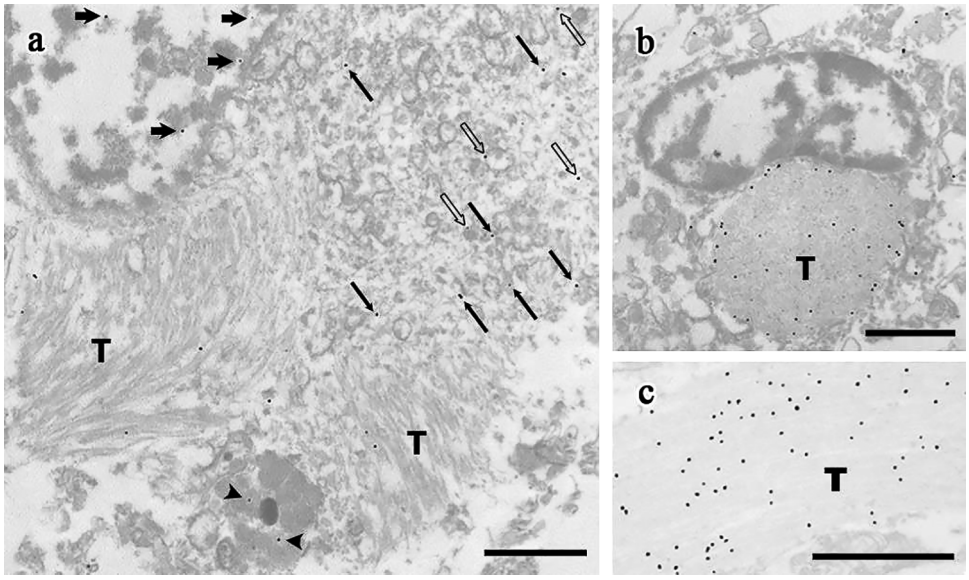

Fig. 4 Immunoelectron microscopy for localization of PKCδ in the AD hippocampus

Immunoelectron microscopic analysis demonstrated the subcellular localization of PKCδ (a) in the nuclei (thick arrows), small vesicles (thin arrows, indicating within the vesicle in black and on the membrane in white), and lysosomes (arrowheads), as well as (b, c) in NFTs (T) in the AD hippocampus. Scale bars: 2 μm.

lobe with mild AD (**Fig. 5h**). PKCδ-positive vesicles were engulfed in reactive microglial cells, which were labeled green for human major histocompatibility antigen class II, HLA-DR/DP/DQ, in the grey matter or in the white matter of AD middle temporal gyrus (**Fig. 5g**). PKCδ-positive varicose fibers were negative for pNF-H, a marker of phosphorylated neurofilaments (**Fig. 5i**).

Colocalization of PKCδ with marker proteins in PI3 pathway in AD

Mirror image sections from the amygdala with mild AD were immunostained for PTEN (**Fig. 6a**), PKCδ (**Fig. 6b**), and pMEK (**Fig. 6c**). As shown in **Fig. 6a–c**, the number of PKCδ-positive NFTs was less than that of PTEN-positive NFTs and more than that of pMEK-positive NFTs.

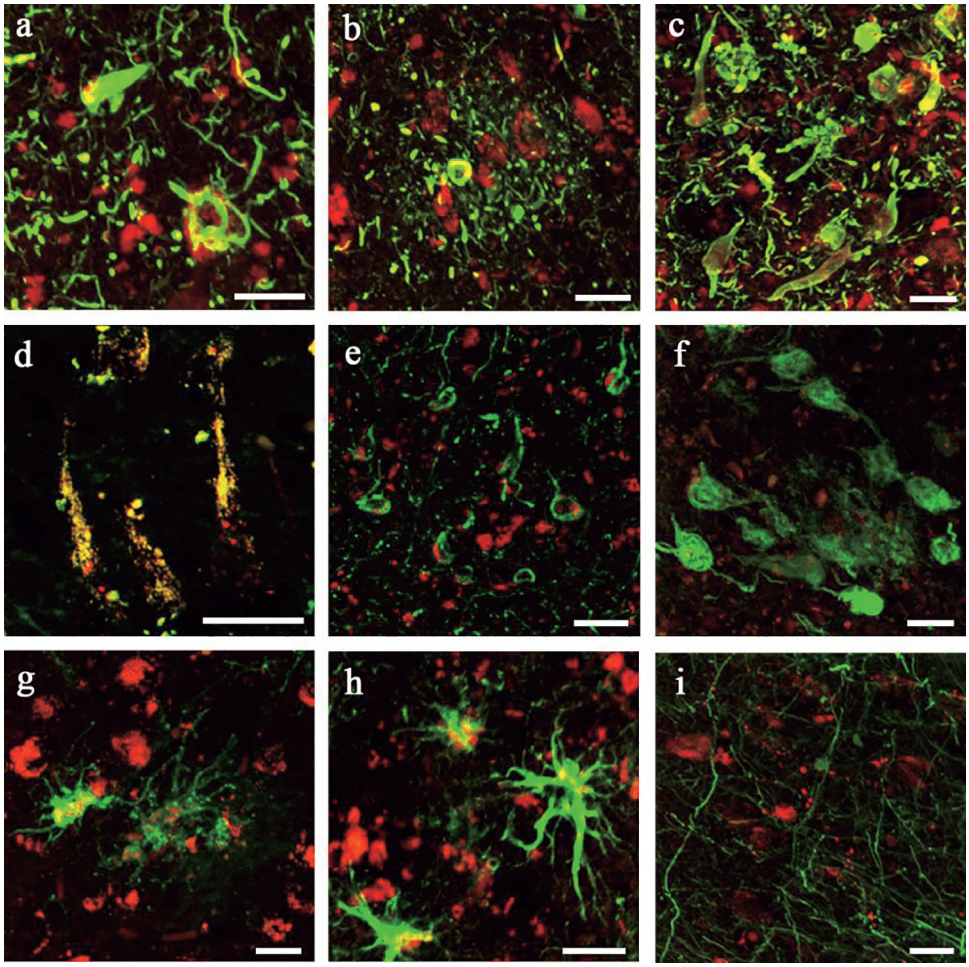

Fig. 5 Colocalization of PKCδ in NFTs and glial cells in AD hippocampus

Colocalizations of PKCδ (red) with tau (a, b) p-tau (c), GSK3β (d), abnormal tau (e), C4d (f), or pNF-H (i) in the AD hippocampus NFTs were analyzed by the double immunofluorescence staining. PKC immunoreactivity was analyzed in microglia (g) and astrocytes (h) by immunostaining in AD middle temporal gyrus. Scale bars: 20 μm.

Some NFTs were double-stained for PTEN and PKCδ (black arrows). Although a few neurons were double-stained with PKCδ and pMEK (white arrows), most PKCδ-positive NFTs were not labeled with pMEK. The distribution pattern of PKCδ-positive tangles (**Fig. 6e**) differed from that of tangles that were positive for p70 S6K phosphorylated at T389 (**Fig. 6d**) or T421/S424 (**Fig. 6f**).

Discussion

The present study is the first to demonstrate the localization of PKCδ in the human brain. In the control brain, PKCδ immunoreactivity was mainly observed in the neuronal cell bodies. In AD brains, PKCδ immunoreactivity was detected in NFTs. Immunoelectron microscopy confirmed that PKCδ was localized to the paired helical filaments in neurons.

Several studies have reported a relationship between PKC members and tau pathology. A previous study demonstrated that decreased levels of inositol 1,4,5-trisphosphate (IP3) receptor and PKC in the entorhinal cortex-hippocampal formation correlated with the progression of AD-related neurofibrillary changes [38]. Simultaneous inhibition of both PI3K and PKC reportedly induced stronger overactivation of GSK3β and hyperphosphorylation of tau in hippocampal slice cultures [39]. *In vivo*, injection of PKC inhibitors into the lateral ventricles of rat brains led to the overactivation of GSK3β, hyperphosphorylation of tau, and impaired spatial memory [40]. However, no study has examined PKCδ in the brains of healthy individuals and patients with AD.

The present study suggests that PKCδ may be involved in tau pathology. To clarify the types of NFT subpopulations that were positive for PKCδ, we performed double immunostaining for PKCδ and several marker proteins. PKCδ-positive tangles were a subpopulation stained with HT7 and AT8 that recognize tau and p-tau, respectively, whereas PKCδ immunoreactivity was not detected in Alz50-positive tangles. Alz50-positive abnormal tau is found

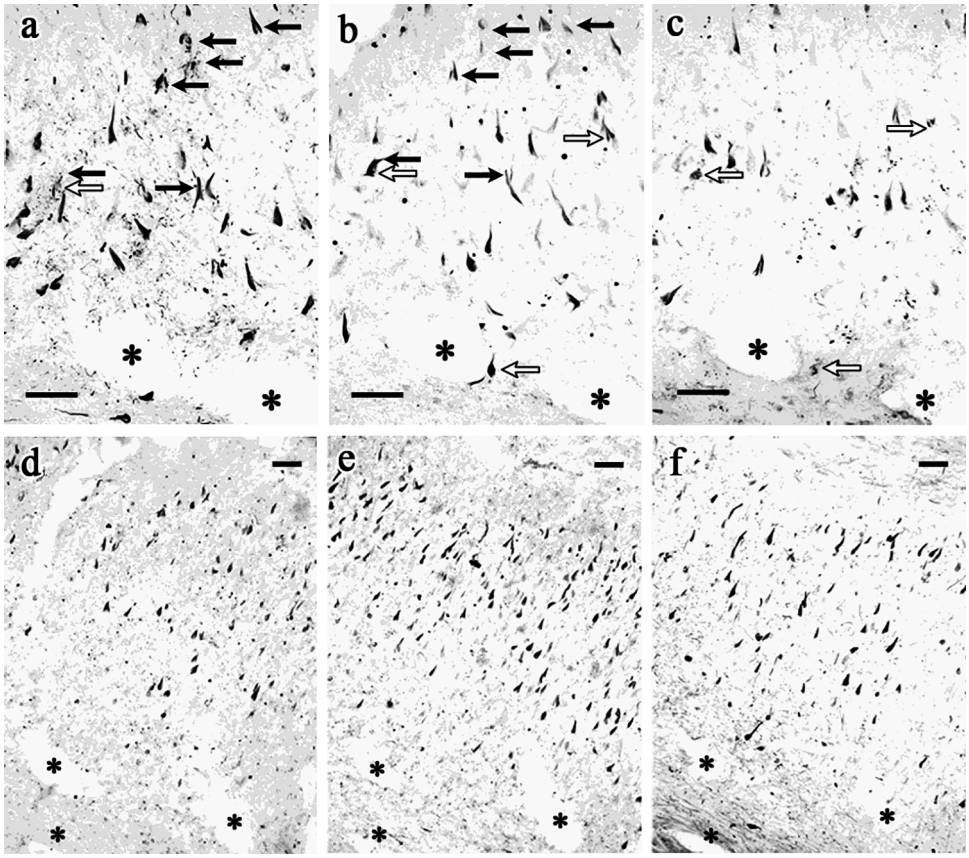

Fig. 6 Colocalization of PKCδ with marker proteins of the PI3 pathway in AD amygdala

Immunohistochemical analysis of mirror image sections from the amygdala in AD was performed by incubation with antibodies against PTEN (a), PKCδ (b or e), pMEK (c), and p70 S6K phosphorylated at T389 (d) or T421/S424 (f). Some NFTs were double-stained for PKCδ and PTEN (black arrows in a and b) or for PKCδ and pMEK (white arrows in b and c) Asterisk indicate the same vessel. Scale bars: 50 μm.

at a relatively early stage. Furthermore, PKCδ was not colocalized with C4d-positive extracellular tangles. Taken together, these findings indicate that PKCδ is localized in matured intracellular NFTs. Interestingly, PKCδ is also localized in intracellular NFTs in astrocytes and microglia. These results are consistent with those of previous studies. In reactive astrocytic and microglial processes, PKCδ colocalized with GFAP and HLA-DR/DP/Dq, which is associated with some kinases in the PI3K signaling pathway [7, 41]. Furthermore, another study reported that reactive astrocytes were expressed in the hippocampal CA1 region of mice with status epilepticus, similar to PKCδ and reactive astrocytes in the brains of patients with AD [42].

PKCδ-induced tau phosphorylation may be mediated by the PKCδ-GSK3β and/or PKCδ-MEK/ERK pathways [11, 43–45]. In the present study, PKCδ co-localized with GSK3β, but not with pMEK, suggesting that the PKCδ-GSK3β pathway may be primarily involved in PKCδ-induced tau phosphorylation in the AD brain. PKCs including PKCδ, are upregulated by PI3K-dependent PDK1 activation [46, 47]. PI3K-dependent PDK1 activation is negatively regulated by the tumor suppressor protein, PTEN [48]. We previously reported the association of PTEN with GSK3β and pMEK in intracellular NFTs in the AD brain [49]. In the present study, PKCδ was found to colocalize with PTEN. These results suggest that the PTEN/PI3K-PDK1-PKCδ pathway may lead to the modulation of GSK3β signaling and facilitate tau pathology in the AD brain. However, the precise mechanisms for the modulation and the involvement in tau pathology remain to be elucidated.

On the other hand, the alternative PI3K-PDK1 pathway directly activates the mammalian target of rapamycin (mTOR) [50]. Activated mTOR phosphorylates p70 S6K at T389, which induces tau phosphorylation. PKCδ is required during PI3K-mediated mTOR/S6K activation, as observed during insulin stimulation [51]. Phosphorylation of p70 S6K at T421/S424 is mediated by the PI3K-PD1-PKC pathway, which is complemented by the ERK1/2 and p38 MAPK pathways [52]. Previous studies have demonstrated that the levels of p70 S6K phosphorylated at T421/S424 and T389 are increased in AD brains [53]. In addition, we reported that p70 S6K phosphorylated at T421/S424 and T389 accumulates more in intracellular NFTs than p70 S6K phosphor-

ylated at T229 [54]. The present data showed that PKCδ did not colocalize with p70 S6K phosphorylated at T421/S424 and T389. These results suggest that PKCδ may not be involved in PI3K-mediated mTOR/S6K or ERK/MEK pathways in tau pathology.

Conclusion

We investigated the distribution of PKCδ in postmortem brain tissues from elderly individuals and patients with AD using immunohistochemistry. In control brains, PKCδ expression was observed in the neurons. Punctate staining of PKCδ was observed in the cytoplasm and nuclei of granules as well as pyramidal neurons in the control hippocampus and apical dendrites. In the brains of patients with AD, PKCδ expression in neuronal cell bodies was lower than that in controls. NFTs and degenerative neurites in SPs were strongly immunopositive for PKCδ. Using electron microscopy, PKCδ immunoreactivity was detected in the paired helical filaments, as well as in nuclei, small vesicles, and lysosomes. Double immunostaining showed that PKCδ was extensively colocalized with GSK3β compared to pMEK or p70 S6 kinase. Thus, PKCδ may play a role in modulating the GSK3β signaling pathway in tau pathology.

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The authors declare no conflict of interest.

References

[1] Serrano-Pozo A, Mielke ML, Gómez-Isla T, Betensky RA, Growdon JH, Frosch MP, Hyman BT (2011) Reactive glia not only associates with plaques but also parallels tangles in Alzheimer's disease. Am J Pathol 179: 1373–1384

[2] Takata K, Kitamura Y (2012) Molecular approaches to the treatment, prophylaxis, and diagnosis of Alzheimer's disease: tangle formation, amyloid-β, and microglia in Alzheimer's disease. J Pharmacol Sci 118: 331–337

[3] Arriagada PV, Growdon JH, Hedley-White ET, Hyman BT (1992) Neurofibillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. Neurology 42: 631–639

[4] Avila J (2006) Tau phosphorylation and aggregation in Alzheimer's disease pathology. FEBS Lett 580: 2922–2927

[5] Billingsley ML, Kincaid RL (1997) Regulated phosphorylation and dephosphorylation of tau protein: effects on microtubule interaction, intracellular trafficking and neurodegeneration. Biochem J 323: 577–591

[6] Iqbal K, Alonso Adel C, Chen S, Chohan MO, El-Akkad E, Gong CX, Khatoon S, Li B, Liu F, Rahman A, Tanimukai H, Grundke-Iqbal I (2005) Tau pathology in Alzheimer disease and other tauopathies. Biochim Biophys Acta 1739: 198–210

[7] Burguillos MA, Deierborg T, Kavanagh E, Persson A, Hajji N, Garcia-Quintanilla A, Cano J, Brundin P, Englund E, Venero JL, Joseph B (2011) Caspase signalling controls microglia activation and neurotoxicity. Nature 472: 319–324

[8] Hanger DP, Anderton BH, Noble W (2009) Tau phosphorylation: the therapeutic challenge for neurodegenerative disease. Trends Mol Med 15: 112–119

[9] Lebouvier T, Scales TM, Williamson R, Noble W, Duyckaerts C, Hanger DP, Reynolds CH, Anderton BH, Derkinderen P (2009) The microtubule-associated protein tau is also phosphorylated on tyrosine. J Alzheimers Dis 18: 1–9

[10] Iqbal K, Grundke-Iqbal I (1992) Ubiquitination and abnormal phosphorylation of paired helical filaments in Alzheimer's disease. Mol Neurobiol 5: 399–410

[11] Mazanetz MP, Fischer PM (2007) Untangling tau hyperphosphorylation in drug design for neurodegenerative diseases. Nat Rev Drug Discov 6: 464–479

[12] Pei JJ, An WL, Zhou XW, Nishimura T, Norberg J, Benedikz E, Götz J, Winblad B (2006) P70 S6 kinase mediates tau phosphorylation and synthesis. FEBS Lett 580: 107–114

[13] Takashima A (2006) GSK-3 is essential in the pathogenesis of Alzheimer's disease. J Alzheimers Dis 9: 309–317

[14] Pei JJ, Khatoon S, An WL, Nordlinder M, Tanaka T, Braak H, Tsujio I, Takeda M, Alafuzoff I, Winblad B, Cowburn RF, Grundke-Iqbal I, Iqbal K (2003) Role of protein kinase B in Alzheimer's neurofibrillary pathology. Acta Neuropathol 105: 381–392

[15] Shao CY, Crary JF, Rao C, Sacktor TC, Mirra SS (2006) Atypical protein kinase C in neurodegenerative disease II: PKCiota/lambda in tauopathies and alpha-synucleinopathies. J Neuropathol Exp Neurol 65: 327–335

[16] Crary JF, Shao CY, Mirra SS, Hernandez AI, Sacktor TC (2006) Atypical protein kinase C in neurodegenera-

tive disease I: PKMzeta aggregates with limbic neurofibrillary tangles and AMPA receptors in Alzheimer disease. J Neuropathol Exp Neurol 65: 319–326

[17] Kawamata T, Taniguchi T, Mukai H, Kitagawa M, Hashimoto T, Maeda K, Ono Y, Tanaka C (1998) A Protein Kinase, PKN, Accumulates in Alzheimer Neurofibrillary tangles and associated endoplasmic reticulum-derived vesicles and phosphorylates tau protein. J Neurosci 18: 7402–7410

[18] Bayascas JR (2010) PDK1: the major transducer of PI 3-kinase actions. Curr Top Microbiol Immunol 346: 9–29

[19] Mora A, Komander D, van Aalten DM, Alessi DR (2004) PDK1, the master regulator of AGC kinase signal transduction. Semin Cell Dev Biol 15(2): 161–170

[20] Gschwendt M (1999) Protein kinase C delta. Eur J Biochem 259(3): 555–564

[21] Nelson TJ, Sun MK, Hongpaisan J, Alkon DL (2008) Insulin, PKC signaling pathways and synaptic remodeling during memory storage and neuronal repair. Eur J Pharmacol 585(1): 76–87

[22] Sun MK, Alkon DL (2014) The "memory kinases": roles of PKC isoforms in signal processing and memory formation. Prog Mol Biol Transl Sci 122: 31–59

[23] Olariu A, Yamada K, Nabeshima T (2005) Amyloid pathology and protein kinase C (PKC): possible therapeutics effects of PKC activators. J Pharmacol Sci 97(1): 1–5

[24] de Barry J, Liégeois CM, Janoshazi A (2010) Protein kinase C as a peripheral biomarker for Alzheimer's disease. Exp Gerontol 45(1): 64–69

[25] Alkon DL, Sun MK, Nelson TJ (2007) PKC signaling deficits: a mechanistic hypothesis for the origins of Alzheimer's disease. Trends Pharmacol Sci 28: 51–60

[26] Olds JL, Anderson ML, McPhie DL, Staten LD, Alkon DL (1989) Imaging of memory-specific changes in the distribution of protein kinase C in the hippocampus. Science 245(4920): 866–869

[27] Etcheberrigaray R, Tan M, Dewachter I, Kuipéri C, Van der Auwera I, Wera S, Qiao L, Bank B, Nelson TJ, Kozikowski AP, Van Leuven F, Alkon DL (2004) Therapeutic effects of PKC activators in Alzheimer's disease transgenic mice. Proc Natl Acad Sci U S A 101(30): 11141–11146

[28] Tagawa K, Homma H, Saito A, Fujita K, Chen X, Imoto S, Oka T, Ito H, Motoki K, Yoshida C, Hatsuta H, Murayama S, Iwatsubo T, Miyano S, Okazawa H (2015) Comprehensive phosphoproteome analysis unravels the core signaling network that initiates the earliest synapse pathology in preclinical Alzheimer's disease brain. Hum Mol Genet 24(2): 540–558

[29] Isagawa T, Mukai H, Oishi K, Taniguchi T, Hasegawa H, Kawamata T, Tanaka C, Ono Y (2000) Dual effects of PKNalpha and protein kinase C on phosphorylation of tau protein by glycogen synthase kinase-3beta. Biochem Biophys Res Commun 273(1): 209–212

[30] Khan TK, Alkon DL (2006) An internally controlled peripheral biomarker for Alzheimer's disease: Erk1 and Erk2 responses to the inflammatory signal bradykinin. Proc Natl Acad Sci U S A 103(35): 13203–13207

[31] Steinberg SF (2004) Distinctive activation mechanisms and functions for protein kinase Cdelta Biochem J 384: 449–459

[32] Kikkawa U, Matsuzaki H, Yamamoto T (2002) Protein Kinase C delta (PKC delta): Activation Mechanisms and Functions. J Biochem 132: 831–839

[33] Braak H, Braak E. Neuropathological staging of Alzheimer-related changes (1991) Acta Neuropathol 82: 239–259

[34] Hyman BT, Phelps CH, Beach TG, Bigio EH, Cairns NJ, Carrillo MC, Dickson DW, Duyckaerts C, Frosch MP, Masliah E, Mirra SS, Nelson PT, Schneider JA, Thal DR, Thies B, Trojanowski JQ, Vinters HV, Montine TJ (2012) National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease. Alzheimers Dement 8: 1–13

[35] Mirra SS, Heyman A, McKee D, Sumi SM, Crain BJ, Brownlee LM, Vogel FS, Hughes JP, van Belle G, Berg L (1991) The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology 41: 479–486

[36] Kawamata T, Tooyama I, Yamada T, Walker DG, McGeer PL (1993) Lactotransferrin immunocytochemistry in Alzheimer and normal human brain. Am J Pathol 142: 1574–1585

[37] Matsushima H, Shimohama S, Chachin M, Taniguchi T, Kimura J (1996) Ca2+-dependent and Ca2+-independent protein kinase C changes in the brain of patients with Alzheimer's disease. J Neurochem 67: 317–323

[38] Kurumatani T, Fastbom J, Bonkale WL, Bogdanovic N, Winblad B, Ohm TG, Cowburn RF (1998) Loss of inositol 1,4,5-trisphosphate receptor sites and decreased PKC levels correlate with staging of Alzheimer's disease neurofibrillary pathology. Brain Res 796: 209–221

[39] Li X, Lu F, Tian Q, Yang Y, Wang Q, Wang JZ (2006) Activation of glycogen synthase kinase-3 induces Alz-

heimer-like tau hyperphosphorylation in rat hippocampus slices in culture. J Neural Transm 113: 93–102

[40] Liu SJ, Zhang AH, Li HL, Wang Q, Deng HM, Netzer WJ, Xu H, Wang JZ (2003) Overactivation of glycogen synthase kinase-3 by inhibition of phosphoinositol-3 kinase and protein kinase C leads to hyperphosphorylation of tau and impairment of spatial memory. J Neurochem 87: 1333–1344

[41] Wang HH, Hsieh HL, Wu CY, Yang CM (2010) Oxidized Low-Density Lipoprotein-Induced Matrix Metalloproteinase-9 Expression via PKC-delta/p42/p44 MAPK/Elk-1 Cascade in Brain Astrocytes. Neurotox Res 17: 50–65

[42] Liu JX, Liu Y, Tang FR (2011) Pilocarpine-induced status epilepticus alters hippocampal PKC expression in mice. Acta Neurobiol Exp 71: 220–232

[43] Tsujio I, Tanaka T, Kudo T, Nishikawa T, Shinozaki K, Grundke-Iqbal I, Iqbal K, Takeda M (2000) Inactivation of glycogen synthase kinase-3 by protein kinase C delta: implications for regulation of tau phosphorylation. FEBS Lett 469(1): 111–117

[44] Ueda Y, Hirai Si, Osada Si, Suzuki A, Mizuno K, Ohno S (1996) Protein kinase C activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. J Biol Chem 271(38): 23512–23519

[45] Zhu X, Lee HG, Raina AK, Perry G, Smith MA (2002) The role of mitogen-activated protein kinase pathways in Alzheimer's disease. Neurosignals 11(5): 270–281

[46] Webb PR, Wang KQ, Scheel-Toellner D, Pongracz J, Salmon M, Lord JM (2000) Regulation of neutrophil apoptosis: a role for protein kinase C and phosphatidylinositol-3-kinase. Apoptosis 5(5): 451–458

[47] Balendran A, Hare GR, Kieloch A, Williams MR, Alessi DR (2000) Further evidence that 3-phosphoinositide-dependent protein kinase-1 (PDK1) is required for the stability and phosphorylation of protein kinase C (PKC) isoforms. FEBS Lett 484(3): 217–223

[48] Raimondi C, Falasca M (2011) Targeting PDK1 in cancer. Curr Med Chem 18(18): 2763–2769

[49] Sonoda Y, Mukai H, Matsuo K, Takahashi M, Ono Y, Maeda K, Akiyama H, Kawamata T (2010) Accumulation of tumor-suppressor PTEN in Alzheimer neurofibrillary tangles. Neurosci Lett 471: 20–24

[50] O'Neill C (2013) PI3-kinase/Akt/mTOR signaling: impaired on/off switches in aging, cognitive decline and Alzheimer's disease. Exp Gerontol 48(7): 647–653

[51] Moschella PC, Rao VU, McDermott PJ, Kuppuswamy D (2007) Regulation of mTOR and S6K1 activation by the nPKC isoforms, PKCepsilon and PKCdelta, in adult cardiac muscle cells. J Mol Cell Cardiol 43: 754–766

[52] An WL, Bjorkdahl C, Liu R, Cowburn RF, Winblad B, Pei JJ (2005) Mechanism of zinc-induced phosphorylation of p70 S6 kinase and glycogen synthase kinase 3beta in SH-SY5Y neuroblastoma cells. J Neurochem 92: 1104–1115

[53] An WL, Cowburn RF, Li L, Braak H, Alafuzoff I, Iqbal K, Iqbal IG, Winblad B, Pei JJ (2003) Up-regulation of phosphorylated/activated p70 S6 kinase and its relationship to neurofibrillary pathology in Alzheimer's disease. Am J Pathol 163: 591–607

[54] Sonoda Y, Tooyama I, Mukai H, Maeda K, Akiyama H, Kawamata T (2015) S6 kinase phosphorylated at T229 is involved in tau and actin pathologies in Alzheimer's disease. Neuropathology 36: 325–332

PKCδ はアルツハイマー型認知症のタウタングルに蓄積する

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要旨

Protein kinase C（PKC）ファミリーは，アルツハイマー病（AD）のタウ病理に関連することが知られている．Novel PKC アイソフォームの一つである PKC delta（PKCδ）は，ヒトの脳において細胞特異的ではなく普遍的な役割を果たしており，成長，分化，アポトーシスの制御などの細胞機能において重要である可能性がある．しかし，AD 脳における PKCδ の局在と機能に関する情報が殆どない．本研究では，免疫組織化学法を用いて，高齢健常者および AD 患者の剖検脳における PKCδ の局在を検索した．高齢健常脳では海馬の顆粒層において，PKCδ の点状染色が錐体細胞の細胞質または核で観察された．一方，AD 脳では，錐体ニューロン細胞体における PKCδ の発現は高齢健常脳よりも低かった．さらに，AD 海馬の老人斑内，多数の神経原線維変化（NFT）および変性神経突起は，PKCδ に対して強く標識された．AD 海馬の残存ニューロンは高齢健常者と比較して PKCδ 弱陽性であり，多くの細胞内 NFT は CA1 領域および海馬台では PKCδ 強陽性であった．AD 海馬を免疫電子顕微鏡法で観察したところ，PKCδ が NFT の paired helical filaments（PHF），核，小胞，リソソームに局在していることが示された．免疫二重染色法では，PKCδ がタウ病理に関与する GSK3β と広範囲に共局在していることが明らかとなった．これらの結果は，AD 脳において，PKCδ は細胞内 NFT の PHF に局在し，タウ病理学において重要な役割を果たしていることを示唆している．