Learning Deficits and Agenesis of Synapses and Myelinated Axons in Phosphoinositide-3 Kinase-Deficient Mice

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Key Words

Phosphoinositide-3 kinase • Myelinated axon • Synapse • Restlessness • Motivation

Abstract

Although previous studies have reported a role for phosphoinositide-3 kinase (PI3K) in axonal definition and growth in vitro, it is not clear whether PI3K regulates axonal formation and synaptogenesis in vivo. The goal of the present study was to clarify the role of PI3K in behavioral functions and some underlying neuroanatomical structures. Immunohistochemistry, an electron-microscopic analysis and behavioral tests were carried out. Knockout mice lacking the p85 α regulatory subunit of PI3K (p85 α –/– mice) significantly showed learning deficits, restlessness and motivation deficit. Expression of phosphorylated Akt, which indirectly shows the activity of PI3K, was high in myelinated axons, especially in axonal bundles in the striatum of wild-type mice, but was significantly low in the striatum, cerebral cortex and the hippocampal CA3 of p85 α –/– mice. The axonal marker protein level decreased mainly in the striatum and cerebral cortex of p85 α –/– mice. In these two regions, myelinated axons are rich in the wild-type mice. However, the density of myelinated axons and myelin thickness were significantly low in the striatum and cerebral cortex of p85 α –/– mice. Synaptic protein level was clearly decreased in the striatum, cerebral cortex, and hippocampus of p85 α –/– mice when

compared with wild mice. The present results suggest that PI3K plays a role in the generation and/or maintenance of synapses and myelinated axons in the brain and that deficiencies in PI3K activity result in abnormalities in several neuronal functions, including learning, restlessness and motivation.

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Introduction

Phosphoinositide-3 kinase (PI3K) is a key enzyme of various signal transduction pathways that regulate cell survival and growth, immune function, metabolism, and cardiac function. Several groups reported that local activation of PI3K and accumulation of phosphatidylinositol 3,4,5-triphosphate at the tip of one of the immature neurites were important for axon specification and elongation in cultured hippocampal neurons [22, 27]. We also found that axonal elongation of cortical neurons was inhibited by downregulation of PI3K activity [33].

By in vivo experiments, the relation of PI3K to learning and memory has been suggested. Infusion of a PI3K inhibitor, LY294002 or wortmannin into hippocampal CA1 temporarily impairs retrieval and extinction of contextual memory [1, 4]. Intracerebroventricular (i.c.v.) injection of wortmannin inhibits spatial memory in rats [19], and intra-amygdala infusion of wortmannin inter-

feres with long-term fear memory [18]. These studies using PI3K inhibitors investigated animal behaviors, but did not evaluate the neurite morphology and synaptic density. However, phosphatase and tensin homolog on chromosome ten (PTEN) mutation in the cerebral cortex and hippocampus of mice, which activates the PI3K pathway leads neuronal hypertrophy, ectopic dendrites and axonal tracts with increased spine density [17]. The mutant mice show an increase in locomotor activity, anxiety-like behavior, and exaggerated reaction to sensory stimuli, seizures, and social interaction deficits. We interested whether the change of PI3K activity related to the behavioral abnormality and axonal and synaptic densities in the brain. Therefore, the goal of the present study was to clarify the role of PI3K in axonal and synaptic densities in the brain, and in behavioral functions using gene knockout mice lacking the class IA PI3K, p85 α regulatory subunit-deficient (p85 α -/-) mice. Class IA PI3K is a heterodimer consisting of a p85 regulatory subunit and a p110 catalytic subunit. The p85 regulatory subunit is essential for the stability of the p110 catalytic subunit [3, 36]. In addition, p85 also mediates the activation and membrane recruitment of the p85-p110 heterodimer through the binding of its two Src-homology 2 (SH2) domains to phosphotyrosine residues on activated growth factor receptors or their adaptor molecules such as IRS-1 and IRS-2 [35]. In mammals, multiple isoforms of p85 and p110 exist. The major p85 isoforms p85 α , p55 α , and p50 α are encoded by a single gene *pik3r1* through alternative transcription initiation sites. Two other minor isoforms, p85 β and p55 γ , are encoded by the genes *pik3r2* and *pik3r3*, respectively. The p85 α /p55 α /p50 α and p85 β isoforms are expressed ubiquitously whereas p55 γ is enriched in the brain and the testis. Three genes, *pik3ca*, *pik3cb*, and *pik3cd*, encode the three class IA PI3K catalytic subunit isoforms, p110 α , p110 β , and p110 δ , respectively [12]. The p85 α -/- mice were originally generated for studies of diabetes [32] and the immune system [30], and their use has helped to elucidate important physiological functions of PI3K. However, the role of PI3K in the central nervous system has never been investigated using p85 α -/- mice. The present study utilized a water maze test for learning ability and used histological evaluation to compare axons, synapses, and myelin in the brains of control and experimental mice. Subsequent results provide the first evidence that the absence of p85 α -/- results in abnormalities in learning- and motivation-related behaviors and that PI3K plays a role especially in the formations of myelinated axons and synapses.

Materials and Methods

Animals

Mice that lack the p85 α regulatory subunit of PI3K (p85 α -/- mice) were kindly supplied by Dr. T. Kadowaki (University of Tokyo) and Dr. S. Koyasu (Keio University). This p85 α -/- line was generated by backcrossing to BALB/c mice for twelve generations before intercrossing heterozygous mice. p85 α -/- mice were born from breeding between male p85 α -/- and female p85 α +/- mice. Male or female PI3K-/- mice (BALB/c background) and separately bred wild-type BALB/c mice were used. All mice were maintained under specific pathogen-free conditions in our animal facilities. All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Sugitani Campus of University of Toyama.

Water Maze

The Morris water maze test was performed. Purple-colored water was poured into a round tank (diameter, 122 cm; height, 28 cm), and a purple platform (diameter, 12 cm) was placed 1.2 cm below the water level in the middle of a fixed quadrant. The water temperature was adjusted to 21–23°C. Memory acquisition test was performed four times daily (15-min intervals between tests) for 5 days. The mice were allowed to swim freely (time limit; 60 s) to seek an invisible platform and were left for an additional 30 s on the platform. Time spent to reach to the platform was defined as the escape latency. The platform position was not moved during all trials. The start position for each trial was kept within a day, but was changed daily. Mice failing to find the platform after 60 s were manually placed on the platform for 30 s. A memory retention test was performed 3 days after the last session of acquisition test. The platform was removed, and each mouse was allowed a free 60-second swim. The number of crossings over the point where the platform had been located was counted.

In a reference test using a visible platform, the water level was 1.2 cm below the platform, and a landmark (a toy block) was placed on the platform. Using this visible platform, water maze trials were carried out.

All swimming performances (escape latency, turn number, mobility, distance traveled, swimming velocity, swimming trace and crossing numbers over the platform position) were recorded by a digital camera and analyzed by a tracking system, EthoVision 3.0 (Noldus Information Technology, Wageningen, The Netherlands). Distance traveled within a radius of 25 cm of the start position or the platform was measured, and was divided by the total duration in each trial (fig. 1g).

Immunohistochemistry

Immediately after the water maze test, mice were anesthetized by chloral hydrate and decapitated. Brains were quickly harvested and frozen in powdered dry ice. Brains were subsequently cut into 12- μ m coronal sections using a cryostat (CM3050S, Leica, Heidelberg, Germany). Slices of the whole brain area were fixed with 4% paraformaldehyde and stained with an antibody against phosphorylated-Akt at Ser473 (P-Akt; R&D Systems, Minn., USA; 1:500; a marker of PI3K activation), phosphorylated neurofilament-H (P-NF-H, Clone SMI35; Sternberger Monoclonals, Lutherville, Md., USA; 1:1,000; an axonal marker), synaptophysin (Clone SY38; Chemicon; 1:500; a synaptic marker), or myelin basic protein (MBP; Chemicon; 1:100; a myelin marker). Alexa Flu-

Fig. 1f. Learning deficit and characteristic behaviors in $p85\alpha^{-/-}$ mice. Memory acquisition ability was tested in a water maze for 5 days. Overlaid traces of swimming performances (test A) of 5 mice each in wild-type and $p85\alpha^{-/-}$ mice on days 1–5 (4 trials/day) are shown. The large circle shows the frame of the pool, and the small blue circle indicates the platform. A black circle is a starting position of each trial. No goal percentage means a failure rate of seeking the platform in total trials per day. Male, 14 weeks old, wild type; $n = 5$, $p85\alpha^{-/-}$; $n = 5$.

Fig. 1. Learning-deficit and characteristic behaviors in p85 $\alpha^{-/-}$ mice. Memory acquisition ability was tested in a water maze for 5 days. **g** Time spent around (within 25 cm from) the start position or around the platform (within 25 cm) was shown as ratio to total duration of swimming to reach the platform in each trial. **h** Three days after the last session of the acquisition test, memory retention was tested. The number of crossings over the point where the platform had been located was counted. **i** Escape latencies to a visible platform in wild-type and p85 $\alpha^{-/-}$ mice are shown. **g, h** Male, 14 weeks old, wild type; $n = 7$, p85 $\alpha^{-/-}$; $n = 5$. **i** Male, 11 weeks old, wild type; $n = 8$, p85 $\alpha^{-/-}$; $n = 7$. Values are means $\pm$ SEM. * $p < 0.05$ (RM 2-way ANOVA).

or 488- and Alexa Fluor 568-conjugated goat anti-mouse IgGs (Molecular Probes, Eugene, Oreg., USA; 1:200) were used as secondary antibodies. The fluorescent images in 5 brain regions (cerebral cortex, striatum, hippocampal CA1, CA3, and dentate gyrus) were captured using fluorescent microscopy (AX-80, Olympus, Tokyo, Japan) at $666 \times 880 \mu\text{m}$ (the hippocampus) or $333 \times 440 \mu\text{m}$ (other areas). To evaluate the expression level of each protein in each region, 2–4 serial slices per mouse were semiquantified. The measuring points were selected with 10–25 squares (fixed size of a square: $50 \times 50 \mu\text{m}$) to cover the whole area of each region or subregion (e.g. the molecular layer of dentate gyrus). Fluorescence intensities of immunopositive areas (after subtracting the background intensity) in those squares were quanti-

fied using ATTO densitography (ATTO, Tokyo, Japan). The background intensity was determined by staining slices without each first antibody.

Protein Extraction and Western Blotting

Brain sections were homogenized with ice-cold lysis buffer, pH 7.5, containing 137 mM NaCl, 20 mM Tris-HCl, 1% Tween 20, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail. Samples were then centrifuged at $2,000 g$ for 10 min at 4°C . To ensure equal loading of the lanes, the protein concentration of each tissue was determined using a Bio-Rad protein assay kit with BSA as a standard, and then immunoblotting was carried out. For determination of P-Akt (R&D Systems, Minn.,

USA; 1:1,000), Akt1/2 (Santa Cruz Biotechnology, Santa Cruz, Calif., USA; 1:200) and β -actin (Sigma-Aldrich, Saint Louis, Mo., USA; 1:2,000) protein expressions, each sample (30 μ g of protein) was electrophoresed through 8% SDS-PAGE. Separated proteins were transferred electrophoretically to a nitrocellulose membrane, blocked with 5% bovine serum albumin solution for 1 h, and then incubated with the corresponding primary anti-P-Akt, Akt1/2 or β -actin antibody overnight at 4°C. After the blots were washed, those were incubated with goat anti-rabbit IgG HRP-conjugated secondary antibodies for 90 min at room temperature. Each antigen-antibody complex was visualized using ECL Plus Western blotting system (Amersham Bioscience, Piscataway, N.J., USA) and detected by chemiluminescence with LAS-1000 Plus (Fujifilm, Tokyo, Japan). Band densities were quantified by ATTO densitography (ATTO).

Electron Microscopy

Mice were anesthetized with chloral hydrate, and tissues were fixed via transcardial perfusion of 2.5% glutaraldehyde in 0.1 M phosphate buffer. Ultrathin sections (0.13 μ m thick) of the striatum were cut and electron-stained. Micrographs were taken with a JEM-200CX (JEOL, Tokyo, Japan). Axon diameter, myelin thickness and distance between adjacent axons were measured using Image J software (NIH, <http://rsb.info.nih.gov/ij/>).

Statistical Analysis

Statistical comparisons were performed with the Student's *t*-test or with repeated-measures two-way analysis of variance (RM 2-way ANOVA) followed by Dunnett's post hoc test. $p < 0.05$ was considered statistically significant. Data are represented as means with SEM.

Results

Behavioral Characterization of PI3K Knockout Mice

In water maze tests, male mice (14 weeks old) were used. The time to reach the invisible platform decreased with each trial day in wild-type mice. By contrast, the escape latency did not decrease in $p85\alpha^{-/-}$ mice, even after 5 trial days (fig. 1a). Repeated measures two-way ANOVA revealed significant group $\times$ time interaction ($F(4, 40) = 3.46$, $p = 0.0162$). However, $p85\alpha^{-/-}$ mice displayed restless movement and changed head direction very frequently while swimming. The frequency of changing direction (turn numbers) during swimming trials was significantly higher in $p85\alpha^{-/-}$ mice than in wild-type mice (fig. 1b). Mobility shows how much the mouse's body moves independent of spatial displacement. The mobility value was significantly higher in $p85\alpha^{-/-}$ mice than in wild-type mice (group $\times$ trial interaction: $F(4, 40) = 2.75$, $p = 0.0411$) (fig. 1c). A distance traveled to the platform decreased with each trial day in wild-type mice. By contrast, distances traveled in $p85\alpha^{-/-}$ mice weren't shortened (group $\times$ trial interaction: $F(4, 40) = 2.65$, $p =$

0.0470) (fig. 1d). Swimming velocity in $p85\alpha^{-/-}$ mice was significant lower than that in wild-type mice (group effect: $p < 0.0001$, no group $\times$ trial interaction) (fig. 1e). However, it is certain that $p85\alpha^{-/-}$ mice neither float nor move slowly in the water maze. Rather than, $p85\alpha^{-/-}$ mice moved their bodies bit by bit even not to swim ahead (fig. 1c), and swam with frequent turning direction (fig. 1b). These movements may reflect to the slower swimming velocity in $p85\alpha^{-/-}$ mice. Traces of swimming/platform-seeking performances demonstrated that wild-type mice searched the goal at early trial days, and gradually became to go straight on the goal (fig. 1f). Rates of failure to reach the goal were very low in wild-type mice. By contrast, $p85\alpha^{-/-}$ mice did not show active goal seeking and spent the majority of the swimming time in a limited area where is near a starting position. The rate of failure to reach the goal did not decrease during trials for 5 days in $p85\alpha^{-/-}$ mice. The rate of duration around the start position to total swimming duration in $p85\alpha^{-/-}$ mice was significant higher than that in wild-type mice (group effect: $p < 0.00001$, no group $\times$ trial interaction) even in early trials (fig. 1g). On the other hand, the rate of duration around the platform to total swimming duration in $p85\alpha^{-/-}$ mice was significant lower than that in wild-type mice (group effect: $p < 0.00005$, no group $\times$ trial interaction). After the memory acquisition test was completed, the mice were subjected to a memory retention test, wherein the number of crossings over the platform position was counted. The number of crossings was significantly lower in $p85\alpha^{-/-}$ mice than wild-type mice (fig. 1h). Escape latencies to a visible platform were measured before all tests, and were not significantly different when comparing the two groups (fig. 1i).

Downregulation of PI3K Activity Is Not Ubiquitous in Brain Regions of $p85\alpha^{-/-}$ Mice

The serine-threonine kinase, Akt, is the major downstream target of PI3K in many signal transduction pathways, and two major phosphorylated sites are Thr308 by PDK1 and Ser 473 by PDK2. In B lymphocytes of the $p85\alpha^{-/-}$ mice, phosphorylations of Akt on the Thr308 and Ser473 residues were severely blocked [30]. Phosphorylation of Akt on the Ser473 was more sensitively increased by PI3K stimulation in cultured cortical and hippocampal neurons [14]. Therefore, we measured P-Akt (Ser473) as an index of the brain PI3K activity. P-Akt levels in the striatum were significantly higher in wild-type mice when compared with $p85\alpha^{-/-}$ mice (fig. 2, 5, 6). There are numerous axonal projections to the striatum, where they form thick bundles and are myelinated

Fig. 2. PI3K activity. Downregulation of phosphorylated Akt level in certain brain regions. Brain slices were immunostained with phosphorylated Akt (P-Akt) antibody (**a–e**). P-Akt-positive areas were measured in the cerebral cortex (**a**), striatum axonal bundles (**b**), hilus and molecular layer in the dentate gyrus (**c**), striatum oriens and striatum radiatum in CA1 (**d**), and striatum oriens, striatum lucidum, and striatum radiatum in CA3 (**e**). Expression levels of P-Akt,

Akt1/2 and β -actin in the cerebral cortex, striatum and hippocampus were quantified by Western blotting (**f**). Ratios of P-Akt to Akt1/2 were shown. **a–e** Female, 11 weeks old, wild type; $n = 4$, p85 $\alpha^{-/-}$; $n = 3$. In case of the striatum (**b**), the expression of P-Akt was measured in every axonal bundle ($n = 44–90$). **f** Male, 14 weeks old, wild type; $n = 3$, p85 $\alpha^{-/-}$; $n = 3$. Values are means $\pm$ SEM. * $p < 0.05$ when compared with wild-type mice (Student's t -test).

Fig. 3. Downregulation of axonal formation in certain brain regions. Brain slices were immunostained with phosphorylated neurofilament-H (P-NF-H) antibody. P-NF-H-positive areas were measured in the cerebral cortex (**a**), the striatum (**b**), hilus and molecular layer in the dentate gyrus (**c**), stratum oriens and stratum radiatum in CA1 (**d**), and stratum oriens, stratum lucidum, and stratum radiatum in CA3 (**e**). **a–e** Female, 11 weeks old, wild type; $n = 4$, p85 $\alpha^{-/-}$; $n = 3$. Values are means $\pm$ SEM. * $p < 0.05$ when compared with wild-type mice (Student's *t*-test). In case of the striatum (**b**), the expression of P-NF-H was measured in every axonal bundle ($n = 80$ – 94). * $p < 0.05$ when compared with wild-type mice (Student's *t*-test).

(fig. 6, 7). In wild-type mice, P-Akt was distributed mainly in the axonal tracts. By contrast, the P-Akt level was very low in the striatum of p85 $\alpha^{-/-}$ mice, and axonal tracts were comparatively thin (fig. 6, 7). Furthermore, P-Akt levels were also markedly decreased in the stratum oriens, stratum lucidum, and stratum radiatum of hippocampal CA3 (fig. 2e) and the cerebral cortex (fig. 2a) of p85 $\alpha^{-/-}$ mice when compared with wild-type mice. Further, P-Akt expression tended to be lower in other regions of the hippocampus in p85 $\alpha^{-/-}$ mice when compared

with wild-type mice, particularly in the dentate gyrus (fig. 2c, d).

Western blotting showed decreases in P-Akt in p85 $\alpha^{-/-}$ mice especially in the cerebral cortex and striatum (fig. 2f). Levels of the nonphosphorylated form of Akt were not decreased in the cerebral cortex, striatum and hippocampus of p85 $\alpha^{-/-}$ mice compared with wild-type mice. Ratio of P-Akt to Akt was significantly decreased in the cortex and especially in the striatum of p85 $\alpha^{-/-}$ mice.

Fig. 4. Synaptic density was reduced in all regions in p85 $\alpha^{-/-}$ mice. Brain slices were immunostained with synaptophysin (**a–e**) or myelin basic protein (MBP) antibodies (**f, g**). Synaptophysin-positive areas were measured in the cerebral cortex (**a**), striatum matrix (**b**), hilus and molecular layer in the dentate gyrus (**c**), stratum oriens and stratum radiatum in CA1 (**d**), and stratum oriens, stratum lucidum, stratum radiatum in CA3 (**e**). MBP-positive areas were measured in the cerebral cortex (**f**) and striatum axonal bundles (**g**). **a–g** Female, 11 weeks old, wild type; $n = 4$, p85 $\alpha^{-/-}$; $n = 3$. In the striatum (**b, g**), expression of synaptophysin and MBP was measured in every axonal bundle ($n = 63–94$ and $n = 44–88$, respectively). Values are means $\pm$ SEM. * $p < 0.05$ when compared with wild-type mice (Student's t -test).

Formation of Axons Is Inhibited in a Region-Specific Manner by PI3K Deficiency

Axonal density was evaluated by immunohistochemistry for phosphorylated NF-H (P-NF-H) as P-NF-H is expressed specifically in axons [15, 16, 26, 29]. P-NF-H levels were significantly lower in the cerebral cortex and striatum of p85 α -/- mice than in that of wild-type mice (fig. 3a, b, 5). Further, in the striatum of wild-type mice, P-NF-H-positive axons were seen in both thick axonal bundles and thin axons, which projected in a mesh-like fashion into the space between the bundles (fig. 6). By contrast, axonal bundles were thin, and the axonal filament density was very low in p85 α -/- mice (fig. 5, 6). Electron-microscopic observation showed significant decrease in diameters in striatum myelinated axons of p85 α -/- mice ($0.72 \pm 0.02 \mu\text{m}$) when compared with wild-type mice ($1.02 \pm 0.03 \mu\text{m}$) (fig. 7b). Interspace of axons in a bundle of p85 α -/- striatum was larger than in wild-type striatum. By contrast, axons in a striatum bundle of wild-type mice were tightly arranged. Distances of adjacent axons were 0.06 ± 0.01 and $0.22 \pm 0.02 \mu\text{m}$ in wild-type and p85 α -/- mice, respectively (fig. 7d). Unexpectedly, P-NF-H levels in the hippocampus were not different when comparing wild-type and p85 α -/- mice (fig. 3c–e, 5).

Synaptic Loss and Myelin Hypoplasia in p85 α -/- Mice

Expression levels of synaptophysin, a presynaptic marker, were significantly decreased in the cerebral cortex, all regions of the hippocampus, and particularly in the striatum in p85 α -/- mice when compared with wild-type mice (fig. 4a–e, 5). In the striatum, synaptophysin-positive puncta were rich in the matrix space between the bundles in wild-type mice (fig. 6). By contrast, these synaptic sites were decreased in the striatum matrix of p85 α -/- mice.

Generally, dense myelin formations are present in various areas of the white matter, including in the regions around axonal tracts in the striatum and in the cerebral cortex. Characterization of myelin basic protein (MBP) levels in the cerebral cortex and the striatum revealed that MBP levels were significantly lower in the striatum of p85 α -/- mice when compared with wild-type mice (fig. 4, 5). Further, MBP-positive myelin clearly surrounded axonal bundles in the striatum of wild-type mice, but the myelin was much less dense in similar areas in p85 α -/- mice (fig. 6).

The decrease in myelination of p85 α -/- axons was confirmed by electron microscopy (fig. 7a). Thicknesses of myelins were 0.13 ± 0.004 and $0.11 \pm 0.003 \mu\text{m}$ in wild-type and p85 α -/- mice, respectively (fig. 7c).

Discussion

p85 α -/- mice displayed several peculiar behaviors in this study. Learning behavior was significantly impaired in p85 α -/- mice in water maze tests (fig. 1a, h). Since the p85 α -/- mice and wild-type mice could reach the visible platform with comparable latencies (fig. 1i), motor function and eyesight must not be impaired in p85 α -/- mice. Swimming velocity in learning test using an invisible platform was lower in p85 α -/- mice than in wild-type mice (fig. 1e). However, this result does not necessarily indicate low locomotor activity of p85 α -/- mice in swimming since p85 α -/- mice didn't show floating behavior, and changed the body direction so frequently (fig. 1b, c). In addition, the distance traveled in p85 α -/- mice was longer than that in wild-type mice (fig. 1d). Therefore, swimming velocity, that is moved distance per time, is seemingly smaller in p85 α -/- mice than in wild-type mice. Swimming traces (fig. 1f) and time spent around the start position (fig. 1g) showed that p85 α -/- mice did not swim actively to seek the platform, and tend to swim near the start position (fig. 1g) during the whole trials. p85 α -/- mice may have insufficient opportunity to learn and memorize the platform position and surrounding information. The failure percentage of reaching the platform was decreased in wild-type mice for 5 days (2.9–0%) whereas it remained comparatively high rate in p85 α -/- mice (22.9%). These data suggest that p85 α -/- mice have impairment in learning task, motivation deficit and an increase in restlessness.

In p85 α -/- mice, PI3K activity was decreased mainly in the striatum, cerebral cortex and CA3 and was slightly decreased in the dentate gyrus and CA1 (fig. 2). The regulatory subunits of class IA PI3K, p85 α , p55 α , p50 α , p85 β and p55 γ , are distributed widely throughout the whole region of the brain, and expression of p85 α is highest among all the subtypes [13]. In p85 α -/- mice, increased expression of p55 α and p50 α was observed in adipocytes and skeletal muscle [32], and a slight increase in p50 α expression was observed in B cells [30]. This isoform switch was associated with an increase in adipocytes PI3K activity in p85 α -/- mice, whereas the PI3K activity of B and T cells from p85 α -/- mice were 5 and 60%, respectively, of those seen in similar cells from wild-type mice [30]. Although it is not clear whether other subunits, such as p55 α and p50 α , regulate PI3K activity in various brain regions of the p85 α -/- mice, the present results demonstrated that PI3K activity was lowered in specific brain regions. Recent studies using cultured cells suggest that PI3K is a key upstream regulator of axonal formation [22, 33, 34]. However, P-NF-H-positive axonal

Fig. 5. Immunohistochemistry for P-Akt, P-NF-H, synaptophysin, or MBP in wild-type and p85 $\alpha^{-/-}$ mice. Represented slice images immunostained by P-Akt, P-NF-H, synaptophysin or MBP were shown in wild-type and p85 $\alpha^{-/-}$ mice. The cerebral cortex, striatum and hippocampus were observed.

Fig. 6. Phosphorylated Akt level, synaptogenesis, and axonal density and thickness were decreased in the striatum of $p85\alpha^{-/-}$ mice. Representative images of axons in the striatum are shown in wild-type and $p85\alpha^{-/-}$ mice.

Fig. 7. Axonal diameter and thickness of myelin were reduced in the striatum of p85 α ^{-/-} mice. **a** Electron micrographs of striatum axonal bundles from wild-type and p85 α ^{-/-} mice. Diameters of axons (**b**) and thickness of myelins (**c**) were measured in the striatum of wild-type (n = 212) and p85 α ^{-/-} (n = 221) axons. **d** Distances of adjacent axons were measured in the striatum of wild-type (n = 246) and p85 α ^{-/-} (n = 286) axons. * p < 0.0001 when compared with wild-type mice (Student's t-test).

density in hippocampus was maintained in p85 α ^{-/-} mice (fig. 3). By contrast, the axon density was disrupted in the cerebral cortex and striatum in p85 α ^{-/-} mice. One major difference of hippocampal axons against cortical and striatal axons is less myelination. For example, immunostaining experiments have demonstrated abundant myelin in axonal bundles in the striatum and cerebral cortex but not in axon filaments in the hippocampus. Ultrastructures of axonal bundles in the striatum clearly demonstrated that axons in p85 α ^{-/-} mice were thin and were surrounded by comparatively thin myelins (fig. 7). In addition, density of axons in a bundle was low in p85 α ^{-/-} mice when compared with wild-type mice.

These results suggest that PI3K is mainly required for generation of myelinated axons. Indeed, previous studies have reported that the PI3K/Akt pathway was important for survival of oligodendrocytes [11], extension of myelin sheaths [6] and the axon-myelin interaction [23, 31]. Therefore, the observation of decreased axonal bundle thickness and decreased myelin expression in the striatum of p85 α ^{-/-} mice may be due to a reduction of myelin formation, which subsequently alters the interaction between axons and myelin. Although the role of PI3K for axonal formation has been suggested using cultured embryonic neurons [22, 27], differences of myelination were

almost ignored. Regardless, the present study suggests that PI3K is involved in the axonal formation and/or axonal targeting in specific regions under organized neuronal circuits in the brain and that PI3K may play a key role in axon-myelin-positive interaction.

Synaptophysin-positive synaptic density was decreased in the cerebral cortex, striatum and hippocampus of p85 α ^{-/-} mice (fig. 4), suggesting that synaptogenesis and/or synaptic maintenance may be directly regulated by PI3K, but not simply by resulting from axonal formation. Synaptogenesis is controlled by a multi-step process involving guidance of axons to targets, maturation of axons and dendrites, contact of axons and dendrites through adhesive factors, formation of presynaptic active zones, recruitment of postsynaptic receptors and scaffolding proteins, and synaptic stabilization. Studies have reported that various regulatory factors, including fibroblast growth factor [25], neural cell adhesion molecule [8], L1 [20], and thrombospondin-1 [28], interact with the PI3K cascade, and participate in this process. In addition, the p85 subunit of PI3K forms a complex with synapsin in nerve terminals, and controls synaptic vesicle delivery to the readily releasable pool from synaptic vesicle clusters [7]. Therefore, PI3K deficiency likely affects synaptogenesis and/or synaptic maintenance at many

different regulatory points during the developmental period and/or at adult period. It is not surprising that synaptic formation was severely reduced in p85 α -/- mice.

p85 α -/- mice displayed several peculiar behaviors and agenesis of axons, myelin and synapses. PTEN is a negative regulator of the PI3K pathway [21]. Conditional Pten mutation in the cerebral cortex and hippocampus induces phosphorylation of Akt and ectopic formations of dendrites, axons and synapses, resulting hypertrophy of the cerebral cortex and hippocampus [17]. The Pten mutant mice show hyperactivity, reaction to sensory stimuli, anxiety-like behaviors, seizures, and social interaction deficits. Authors speculated that the abnormalities of the mutant mouse may relate with autism spectrum disorders. Overactivation of PI3K results in hyperformation of neuronal connections in the Pten mutant mice. By contrast, the present data suggest that decreased PI3K activity may induce hypoplasia of axons and synapses. These two approaches seem to give a similar conclusion of role of PI3K for the neuronal network.

The p85 α -/- mouse shows hypoglycemia [31]. Hypoglycemia rats after the insulin administration showed low locomotor activity and no change of cognition [9]. By contrast, several lines of hyperglycemia rats as diabetic models exhibit no effect on [11] and deficits in learn-

ing and memory [2, 10]. Taken together, behaviors of p85 α -/- mice observed in the present study may not relate to mainly the blood glucose level.

Considering that the hippocampus importantly relates to learning task [24], low activity of PI3K and less density of synapses in the hippocampus of p85 α -/- mice may be related to learning deficit. By contrast, structural losses of myelinated axons and synapses in the basal ganglia-thalamo-cortico-striatal circuit of p85 α -/- mice may result in emotional abnormalities such as motivation deficit.

The data from the present study suggest that PI3K plays a key role in the generation and/or maintenance of synapses and myelinated axons in the brain and that deficiencies in PI3K activity may result in abnormalities in various neuronal functions, including learning, restlessness and motivation.

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