PIKE Regulates Neuronal Dendritogenesis and Survival in Neocortex

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Abstract

Phosphoinositide 3-kinase enhancer (PIKE) binds and enhances PI3K/Akt activities. However, its physiological functions in brain have never been explored. Here we show that PIKE is important in regulating the neuronal survival and development of neocortex. During development, enhanced apoptosis is observed in the ventricular zone of PIKE knockout (PIKE −/−) cortex. Moreover, PIKE −/− neurons show reduced dendritic complexity, dendritic branch length and soma size. These defects are due to the reduced PI3K/Akt activities in PIKE −/− neurons, as the impaired dendritic arborization can be rescued when PI3K/Akt cascade is augmented in vitro or in PIKE−/−PTEN−/− double knockout mice. Interestingly, PIKE −/− mice display behavioral abnormality in locomotion and spatial navigation. Because of the diminished PI3K/Akt activities, PIKE −/− neurons are more vulnerable to glutamate or stroke-induced neuronal cell death. Together, our data established the critical role of PIKE in regulating neuronal survival and development by substantiating the PI3K/Akt pathway.

INTRODUCTION

Normal cortical development is a highly orchestrated process that involves proper proliferation, migration and differentiation of newly formed neurons to achieve a functional network (Chan et al., 2002). To control the number of neurons during development, apoptosis regulated by neurotrophic factors is an effective mechanism to eliminate differentiated neurons projected to an inappropriate target (Raff et al., 1993). However, our understanding on the molecular detail of this “survive or death” determination is incomplete. It is implicated that the integrity of phosphatidylinositol 3-kinase 3-kinase enhancer (PIKE) binds and enhances PI3K/Akt pathway. During development is necessary for preserving an intact nervous system (Backman et al., 2001; Kwon et al., 2001; Pimentel et al., 2002; Peng et al., 2004; Easton et al., 2005). It is also the central pathway for dendritogenesis that an intact PI3K/Akt pathway is essential for maintaining the complexity of dendritic arbor (Jaworski et al., 2005; Kumar et al., 2005). As such, any defect in molecules mediating the activation of PI3K/Akt might result in dwindled dendritic expansion. For example, conditional brain-derived neurotrophic factor (BDNF)

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knockout mice have reduced neuron survival, dendritic arborization and memory formation (Alcantara et al., 1997; Xu et al., 2000; Gorski et al., 2003a; Gorski et al., 2003b).

PIKE (phosphatidylinositol 3-kinase enhancers) are a family of GTPases with 3 isoforms, namely PIKE-L, PIKE-S and PIKE-A. In cultured neurons, PIKE-L interacts with various transmembrane receptors to trigger PI3K activation. Activation of mGluR-I by its agonists enhances formation of an mGluR-I-Homer-PIKE-L complex, leading to activation of PI3K and prevention of neuronal apoptosis (Rong et al., 2003). We also showed that netrin-1, a secreted laminin-related protein that plays a major role in mediating chemotaxis and chemorepulsion of axons/neurons, induces the interaction of its receptor UNCB with PIKE-L, which antagonizes the UNC5B’s pro-apoptotic activity by enhancing PI3K activity (Tang et al., 2008). In addition, PIKE-L exerts its neuroprotective actions through protecting neuronal DNase inhibitor SET from degradation by AEP (asparagine endopeptidase or legumain) during stroke or kainic acid treatment (Liu et al., 2008). On the other hand, PIKE-S (the C-terminal truncate of PIKE-L) is a nuclear PI3K activator in response to nerve growth factor (NGF) to exert its nuclear function (Ye et al., 2000; Ahn et al., 2004a). Hence, these findings support that PIKE GTPases are important signaling molecules in protecting neurons from neurotoxic insults. However, the functions of neuronal PIKE in a physiological context have never been explored.

The aim of the present study is to functionally establish the regulatory role of PIKE on PI3K
in vivo. Here, we found the PIKE knockout (PIKE −/−) mice have smaller brain with reduced neuronal size and density in neocortex. Arborization of cortical neuron is also severely impaired, leading to memory defect and locomotion hyperactivity. These defects could be rescued by amplifying the activity of PI3K/Akt through introducing activated PI3K/Akt in vitro, or depleting PTEN in vivo. Moreover, the reduced PI3K/Akt activities in PIKE −/− lead to enhanced neuronal apoptosis during development or under neurotoxic insults. Therefore, PIKE is a critical factor in mediating neuronal survival and dendritogenesis by substantiating the PI3K/Akt pathway.

**MATERIALS AND METHODS**

**Materials**

All chemical were purchased from Sigma-Aldrich (USA) unless specifically stated. Human recombinant BDNF was obtained from PeproTech (USA). In Situ Cell Death Detection (Fluorescein) Kit was from Roche Diagnostic (USA). Anti-caspase 3 (active), phospho-Akt, phospho-ERK, ERK, phospho-TrkB, TrkB, phospho-mTOR, mTOR, phospho-CREB and CREB antibodies were purchased from Cell Signaling (USA). Anti-p110α and anti-PTEN antibodies were obtained from Santa Cruz Biotechnology (USA). Ant-Ki67 antibody was from BD Biosciences (USA). Anti-Nestin antibody was from Covance (USA). Anti-BrdU-FITC antibody was from Abcam (USA). BDNF ELISA kit was purchased from Promega (USA).

**Knockout mice generation**

Heterozygous whole body PIKE knockout C57BL/6 mice with a targeted deletion of exon 3 to 6 of CNTG1 were generated under contract by Ozgene (Australia). Mice were then bred to homozygosity by heterozygous mating. PIKE/PTEN double knockout mice were generated by crossing the Cre-recombinase overexpressed PIKE −/− mice with PTEN flox/ flox mice (The Jackson Laboratory, USA) until homozygosity. Identities of the mice were examined by PCR using genomic DNA extracted from the tail as previously reported (Lesche et al., 2002; Chan et al., 2010a). All animal experiments were performed according
to the care of experimental animal guideline and approved by the Institutional Animal Care and Use Committee (IACUC) from Emory University.

**DNA and protein content in mouse brain**

Brain DNA and protein content from male mice was deduced as previously described (Easton et al., 2005). Briefly, the brains of age-matched male mutant and control mice were weighed and homogenized in TNE buffer (10 mM Tris base, 10 mM EDTA, 200 mM NaCl, pH 7.4). Samples were then diluted in TNE buffer containing 0.1 μg/ml Hoechst 33258 for measurement of the DNA content using fluorescence plate reader (BMG Labtech, USA) at an excitation of 365 nm and an emission of 460 nm. A standard curve was generated using salmon sperm DNA. NP-40 was added to the remaining brain homogenate to a final concentration of 1%, and the lysate was clarified by centrifugation at 16,000 × g at 4°C. The protein concentration of the supernatant was determined by a BCA protein assay (BioRad, USA).

**Cortical neuron culture**

Cortical primary neurons were dissected from E18 embryo and cultured as described (Tang et al., 2008). DIV 3 or 7 neurons were infected with various adenoviruses as indicated. Three days after infection, pictures of the neurons were taken by fluorescence microscopy and dendritic complexity was scored using computer software ImageJ (National Institute of Health, USA). For BDNF treatment, neurons of DIV 3 were stimulated with BDNF (100ng/ml) for 3 days. The neurons were then fixed in 4% formaldehyde, permeabilized and staining with anti-MAP2 antibody. Dendritic arborization was scored as aforementioned.

**Immunohistochemical/Immunofluorescent staining**

E12.5 embryo or brain tissues were fixed in 4% paraformaldehyde, paraffin embedded and sectioned in standard procedures. After serial rehydration and permeabilization in 0.1% TBST, sections were immunostained using specific antibodies as indicated and counterstained with hematoxylin using Zymed Histostain SP kit (Invitrogen, USA) or DAPI. For BrdU incorporation, pregnant mice (E12.5) were injected with BrdU (Sigma) (50 mg/kg, intraperitoneal) to label the dividing neuronal cells and were sacrificed 2 h later. The embryos were fixed in 4% paraformaldehyde, paraffin embedded and sectioned in standard procedures. DNA was denatured in 2 M HCl and stained using anti-BrdU-FITC antibody.

**In vitro PI3K assay**

P110α was immunoprecipitated with anti-p110α antibody from 1 mg lysate (male brain) and washed with the following buffers: 3 times with buffer A (PBS, 1% NP-40, 1 mM DTT); 2 times with Buffer B (PBS, 0.5 M LiCl, 1 mM DTT); 2 times with Buffer C (10 mM Tris-HCl pH 7.4, 0.1 M NaCl, 1 mM DTT) at 4 °C. PI3K activities were determined using an in vitro PI3K assay as described previously (Ye et al., 2000). The 32P-labeled phosphatidylinositol lipids were resolved on TLC.

**Stroke experiment**

The middle cerebral artery occlusion (MCAO) induced stroke was performed as previously reported (Liu et al., 2008). The animals (male) were anesthetized with 4% chloral hydrate. The rectal and masseter muscle temperatures were controlled at 37°C with a homeothermic blanket. Cerebral perfusion (CP) in the distribution of the middle cerebral artery was monitored throughout the surgical procedure with a laser Doppler (Perimed Inc., USA), and only animals with a >80% decrease in CP were included in this study. After 48 h, MCAO mice were sacrificed and brains were cut onto 5 μm sections and stained with TUNEL assay. TUNEL assay was performed using In Situ Cell Death Detection (Fluorescein) Kit.
**Behavioral tests**

The water maze test was performed in a circular swim arena (diameter of 116 cm, height of 75 cm) surrounded by extra-maze visual cues that remained in the same position for the duration of training and filled to cover the platform by 1 cm at 22 °C. Water was made opaque with nontoxic, white tempera paint. The escape platform was a circular, nonskid surface (area 127 cm²) placed in the NW quadrant of the maze. Acquisition training consisted of 5 tests d with four daily trials. Male mice entered the maze facing the wall and began each trial at a different entry point in a semirandom order. Trials lasted 60 s or until the animal mounted the platform with a 15-min intertrial interval. A probe trial was conducted on day 6 wherein the platform was removed, and the animal swam for 60 s and the time spent in the target quadrant (NW) versus the adjacent and opposite quadrants was recorded. A video camera mounted above the swim arena and linked to TopScan software recorded swim distance, swim speed and time to platform and was used for tracking and analysis.

The Y-maze was performed that each mouse (male) was placed at the end of one arm and allowed to move freely in the maze for 8 min. The total number of arm entries and the alternation behavior were recorded by video camera and scored. Spontaneous alternation was calculated as (Total of alternation/total arm entries-2).

Metabolic cage studies using female mice were performed as previous described (Chan et al., 2010a).

**Statistics**

Results are expressed as mean ± S.E.M. Statistical analysis was performed using computer software Prism (GarphPad Software, USA). Data were considered significant when P<0.05.

**RESULTS**

**PIKE −/− mice display smaller brain size with reduced cortical thickness**

In order to study the functions of PIKE in a physiological manner, we generated whole body PIKE −/− mice with all PIKE isoforms deleted using the loxP/Cre system (Chan et al., 2010a). In peripheral tissues, PIKE-A, the third PIKE isoform that binds Akt and elevates its kinase activity (Ahn et al., 2004b; Ahn et al., 2004c), regulates mammary gland development and obesity onset (Chan et al., 2010b; Chan et al., 2010a). We also observed a noteworthy reduction in the brain size of adult PIKE −/− mice without alternations in the body length (7.84 ± 0.11 cm in wild-type vs 7.72 ± 0.17 cm in knockout). Measurement of the adult brain mass confirmed the weight of PIKE −/− brain was significantly lower (0.47 ± 0.01 g in wild-type vs 0.41 ± 0.01 g in knockout, P<0.001, Student’s t-test, n=5). To clarify how PIKE controls the brain size, we measured the total DNA and protein content in wild-type and PIKE −/− brain. Both DNA (60.4 ± 1.6% of wild-type control) and protein contents (74.1 ± 5.6% of wild-type control) (Fig 1A) were notably decreased in PIKE −/− brain, suggesting the number of cells in PIKE −/− brain is reduced. Also, a decrease of DNA-to-protein ratio (65.2 ± 8.5% of control mice) was also recorded, which suggests that the decrease of brain size in PIKE −/− mice is a combined result of diminished cell number and the cell size.

To further confirm that the diminution of brain mass in PIKE −/− mice is caused by a reduction of neuron size, we quantified the soma area of neurons in somatosensory cortex, hippocampus and cerebellum (Fig 1B). We detected no significant changes in morphology and soma area of neurons in hippocampus CA3 region and cerebellum. In contrast, a significant reduction (76.9% of wild-type mice) of cell body size was detected in cortical...
neurons. To assess whether this defect alters the architecture of the brain, we examined the structure of somatosensory cortex histologically. Compared with the age-matched littermate control, the cortical layers are not well defined in PIKE −/− mice with a reduction of layer II/III thickness in both infant (Fig 1C) and adult brains (Fig 1D). Moreover, lower neuronal density was scored in layer II/III of PIKE −/− adult brain (169.0 ± 10.5 neuron per field in wild-type vs 138.1 ± 10.2 neuron per field in knockout, P<0.05, Student’s t-test, n=4). These histological findings further demonstrate that the reduced brain size in PIKE −/− mice is attributed to the decreased cell number and soma size in the cortex.

PIKE depletion ablates neuronal survival but not proliferation

To determine if the decreased neurons in PIKE −/− brain is resulted from dysregulation of cell proliferation or excessive cell death during cortical development, we determined the proliferation of cortical neurons of E12.5 embryo using 5-bromodeoxyuridine (BrdU) labeling. A comparable density of proliferating cells was detected between wild-type and PIKE −/− cortex (Fig 2A), suggesting neurogenesis is not affected in PIKE −/− mice. To further examine cell proliferation, we conducted Ki67 and nestin co-staining (Fig 2B). No difference was observed between wild-type control and PIKE −/− mutants, further indicating normal cortical neurogenesis. We next sought to assess the degree of cell death in PIKE −/− embryo. Markedly enhanced apoptosis was observed in the nestin-positive progenitor cells in the ventricular zone of PIKE −/− neocortex at E12.5, as revealed by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay (Fig 2C). The apoptosis in cortical neurons was sustained beyond the developmental stages as we observed positive caspase-3 staining in the somatosensory cortex of adult PIKE −/− mice but not in wild-type controls (Figure 2D). Together, these data support that PIKE is essential for cortical neuronal survival but dispensable for cortical neurogenesis.

PIKE is essential for dendritic branching

We next examined if PIKE ablation causes any morphological defect to neurons. We prepared cortical neuronal cultures from wild-type and PIKE −/− embryo (E18) and infected the neurons with adenovirus expressing GFP at DIV 7 (Fig 3A). Twenty four h after infection, dendritic morphology was monitored by fluorescent microscopy. PIKE −/− neurons displayed significantly reduced dendritic branching as revealed by Sholl analysis (Fig 3B). Total dendritic branch length (414.6 ± 31.1 µm in wild-type vs 163.8 ± 14.7 µm in knockout, P<0.001, Student’s t-test, n=10) and terminal tip number (23.4 ± 1.7 in wild-type vs 9.9 ± 1.1 in knockout, P<0.001, Student’s t-test, n=10) were also curtailed, underscoring PIKE plays a critical role in controlling the overall development of dendritic morphology. However, no significant change in the number of primary dendrite was observed in PIKE −/− neurons (data not shown). In agreement with the reduced soma size of cortical neurons in brain section, the soma of cultured cortical neurons from PIKE −/− embryo was also smaller (47.0 ± 6.0 wild-type vs 29.2 ± 1.5 in knockout, P<0.05, Student’s t-test, n=10). We have performed identical analysis on hippocampal neurons isolated from PIKE −/− mice, but no significant difference was found (data not shown). Therefore, deletion of PIKE might selectively affect cortical but not hippocampal neuronal morphogenesis.

Overexpression of active PI3K/Akt signaling rescue dendritic arborization defects in PIKE −/− neurons

The reduced dendritic arborization in PIKE −/− neurons fits with previous finding that chronic inhibition of PI3K reduces soma size and dendritic complexity (Kumar et al., 2005). Given that PIKE proteins are PI3K/Akt enhancers, it is anticipated that PI3K and Akt activity in PIKE −/− brain might be reduced. As expected, we observed a reduction of PI3K activity in both cytosolic and nuclear fractions of PIKE −/− cortex (Fig 4A). Conceivably, the decreased PI3K activity in PIKE −/− neurons might account for the reduced
dendritogenesis. To test this possibility, we infected \( PIKE^{-/-} \) cortical neurons (DIV 3) with adenovirus overexpressing wild-type PIKE-L, dominant-negative PIKE-L KS (PIKE-L K413AS414N), which prevents the stimulation of PI3K (Rong et al., 2003), constitutively active PI3K (PI3K-CA) and Akt (Akt-CA), respectively. Three days after infection, overexpression of PIKE-L, PI3K-CA or Akt-CA but not PIKE-L KS prominently increased dendritic complexity and significantly augmented spine morphology (Fig 4B and C). Quantitative analysis revealed that the number of terminal tips (Fig 4D) and total dendritic length (Fig 4E) were markedly increased in PIKE-L, PI3K-CA and Akt-CA virus infected \( PIKE^{-/-} \) neurons.

To confirm that the impaired arborization in \( PIKE^{-/-} \) neurons is PI3K/Akt-dependent in vivo, we generated the whole body \( PIKE^{-/-}\PTEN^{-/-} \) double knockout mice and examined the morphology of the cultured cortical neurons. PTEN is a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase that negatively regulates the PI3K/Akt pathway (Myers et al., 1998). Therefore, it is anticipated that PTEN ablation in \( PIKE^{-/-} \) neurons should restore the PI3K/Akt function, thus rescuing the defective arborization. As expected, Akt phosphorylation was increased in \( PIKE^{-/-}\PTEN^{-/-} \) neurons as revealed by immunofluorescence staining (Fig 5A) and immunoblotting (Fig 5B, 1st panel). Arbor complexity (Fig 5C), total dendritic length (165.3 ± 12.3 µm in \( PIKE^{-/-} \) vs 333.4 ± 37.7 µm in \( PIKE^{-/-}\PTEN^{-/-} \), \( P<0.001 \), Student’s t-test , \( n=10 \)) and the number of total terminal tip (16.9 ± 0.8 in \( PIKE^{-/-} \) vs 29.3 ± 3.5 in \( PIKE^{-/-}\PTEN^{-/-} \), \( P<0.01 \), Student’s t-test , \( n=10 \)) were also significantly increased in \( PIKE^{-/-}\PTEN^{-/-} \) neurons than the \( PIKE^{-/-} \) mutants. Most importantly, dendritic arborization was rescued in \( PIKE^{-/-}\PTEN^{-/-} \) mice in vivo as demonstrated by the Golgi staining of neuron in the somatosensory cortex (Fig 5D). Hence, PIKE regulates neuronal dendritic branching through mediating PI3K signaling cascade.

\( PIKE^{-/-} \) mice display impaired spatial navigation and locomotor activity

Cortex is involved in navigation and working memory formation (Hoh et al., 2003). To test if PIKE depletion causes any behavioral or learning ability change, we performed T-maze and Morris water maze tests on \( PIKE^{-/-} \) mice. \( PIKE^{-/-} \) mice demonstrated comparable learning ability with the wild-type control, as similar latencies to find the submerged platform in the water maze were recorded between these two genotypes (Fig 6A). In the probe test, however, the mutant animals spent significantly less time (47.6 ± 4.2 % in wild-type vs 35.8 ± 4.9 % in \( PIKE^{-/-} \), \( P<0.001 \), Student’s t-test , \( n=10 \)) in the quadrant where the platform used to be (Fig 6B), suggesting \( PIKE^{-/-} \) mice are disabled to perform tasks involving long-term memory (Gunnerson et al., 2007). Similar observation was obtained in another working memory test. \( PIKE^{-/-} \) mice also exhibited a reduction in percentage alternation in the Y-maze test (61.1 ± 1.9 % in wild-type vs 54.0 ± 1.7 % in knockout, \( P<0.05 \), Student’s t-test, \( n=7 \)), which further suggests the mutant mice have impaired short term memory (Ma et al., 2007). Interestingly, we found that \( PIKE^{-/-} \) mice are hyperactive in both water maze and Y-maze tests. In the water maze test, both swim speed and swim distance are substantially higher in \( PIKE^{-/-} \) mice (Fig 6C and D). Similarly, significantly faster speed (48.4 ± 2.6 mm/s in wild-type vs 61.0 ± 4.8 mm/s in knockout, \( P<0.05 \), Student’s t-test , \( n=7 \)) and longer distance travelled (23227 ± 1264 mm in wild-type vs 29276 ± 2304 mm in knockout, \( P<0.05 \), Student’s t-test , \( n=7 \)) in the Y-maze were recorded in the \( PIKE^{-/-} \) animals. Open field test also revealed a higher activity in \( PIKE^{-/-} \) mice (Chan et al., 2010a). To eliminate the possibility that the mutant animals have higher curiosity towards novel environment that causes the enhanced activity, we single housed the animals in computerized metabolic cage for 3 days. At day 4, the activity was measured over a 24 h period. As anticipated, enhanced activity was observed in \( PIKE^{-/-} \) mice during dark phase with normal circadian rhythm (Fig 6E).
BDNF-induced dendritogenesis is impaired in PIKE −/− neurons

Dendritic arborization is regulated by numerous external clues and cooperation of various intracellular signaling molecules. BDNF has been well-recognized as a critical factor in controlling cortex development and dendritic patterning (Cohen-Cory and Fraser, 1995; Alcantara et al., 1997; Xu et al., 2000; Gorski et al., 2003b). Given that PIKE-S is a downstream mediator of another neurotrophin NGF (Ye et al., 2000; Ye et al., 2002), we hypothesized that PIKE might be involved in the BDNF signaling. To test this hypothesis, we cultured cortical neurons from wild-type and PIKE −/− embryo (E18), and examined the signaling pathways induced by BDNF stimulation. As shown in Fig 7A, BDNF triggers comparable TrkB phosphorylation in both genotypes (1 panel). However, both BDNF-induced PI3K activities (3rd panel), Akt and mTOR phosphorylations (4th and 8th panels) were markedly decreased in PIKE −/− neurons. On the other hand, ERK and CREB phosphorylations were not affected in the absence of PIKE (6th and 10th panels), suggesting that PIKE selectively mediate BDNF-triggered PI3K/Akt signaling but not the Ras/ERK cascades.

Next, we tested if PIKE are critical for BDNF-induced dendritogenesis. Stimulation of wild-type neurons with BDNF increased the number of primary dendrite and dendritic arborization (Fig 7B to D). However, no significant increase of these parameters occurred in BDNF-stimulated PIKE −/− neurons. On the other hand, BDNF was able to increase the total dendritic length and terminal tip number in both wild-type and PIKE −/− neurons, but the absolute value of increment was significantly smaller in PIKE −/− neurons (Fig 7E and F).

PIKE is necessary for protecting neurons from glutamate and ischemia attacks

BDNF plays an essential role in promoting neuronal survival in addition to dendritogenesis. Interestingly, we found that the neuroprotective function of BDNF in PIKE −/− neuron was also compromised. In the cultured wild-type cortical neurons (DIV 7), glutamate stimulation caused 20.6 ± 2.0 % wild-type neurons to undergo apoptosis. Pre-incubation of BDNF reduced the number of cell death to 12.0 ± 1.2 %. In contrast, glutamate challenge resulted in 31.2 ± 2.1 % cell death in PIKE −/− neurons, which is significantly higher than the wild-type control. Although BDNF stimulation reduced the number of cell death to 25.1 ± 2.4 % in PIKE-ablated neurons, it is still significantly higher than the wild-type cells under the same treatment (Fig 8A). Thus the neuroprotective function of BDNF towards excitotoxicity challenge is partially impaired in the crippled of PIKE proteins.

To test if PIKE is also critical in protecting cells from neurotoxic insult in vivo, we conducted ischemic experiments using transient middle cerebral artery occlusion (MCAO) model. Ischemic stroke is one of the major causes leading to neuronal cell death and represents a common model of apoptotic insult to neurons. It is reported that cortical BDNF expression is increased to protect the cells from further damage after MCAO (Kokaia et al., 1995; Kokaia et al., 1998; Ferrer et al., 2001). Thus, it is anticipated that brain tissue with PIKE depletion would be more vulnerable to cell death in the affected region. After ischemic insult, damage in cortical area was observed in both wild-type and knockout animals (Fig 8B). The infarct volume, however, was significantly larger in PIKE −/− brain (22.0 ± 2.1 mm³ in wild-type vs 32.5 ± 1.3³ in knockout, P<0.01, Student’s t-test , n=4), suggesting that PIKE −/− brains are more vulnerable to the ischemic damage. Nissl staining of the infarcted cortical area revealed extensive neuronal cell loss in PIKE −/− mice (Fig 8C). Necrosis is the major cause of neuronal cell death after stroke, but apoptosis also plays a role in triggering the cell death. More DNA degradation in infarcted brain area was detected by TUNEL assay in PIKE −/− brain than that in wild-type control (Fig 8D), implicating that PIKE is indispensable for suppressing stroke-induced apoptosis.
revealed that fragmentation of PARP spontaneously occurred in PIKE knockout brain and was strongly increased after stroke (Fig 8E, 2nd panel). As expected, more pronounced SET degradation, which coupled to AEP activation, was observed in PIKE −/− mice than control mice (Fig 8E, 4th and 5th panels). Furthermore, degradation of UNC5B was substantially enhanced in PIKE knockout brain after stroke than wild-type mice (Fig 8F, 3rd panel). Hence, our results support that PIKE is critical for preventing neuronal cell death upon glutamate challenge or ischemic stroke.

**DISCUSSION**

In the present study, we report that PIKE GTPases are important in controlling neuronal survival and dendritic arborization by maintaining the integrity of the PI3K/Akt cascade. PIKE −/− brain has thinner cortex, fewer neuronal density and smaller neuronal soma size (Fig 1). This reduction in neuronal number is a result of enhanced apoptosis during development as apoptotic neurons were readily detectable in both infant and adult brains (Fig 2). Moreover, dendritic arborization is impaired in PIKE −/− neurons (Fig 3). All these morphological defects in PIKE −/− neurons is caused by reduced PI3K/Akt signaling (Fig 4 and 5), which is partially incited by BDNF resistance (Fig 6). Consequently, mice lacking PIKE are retarded in performing tasks that involve working memory (Fig 7) and more vulnerable to neurotoxic insults like stroke-induced cell death (Fig 8). Therefore, our results suggest that PIKE are important factors for neuronal development, survival and function by substantiating the PI3K/Akt cascade both *in vitro* and *in vivo*.

PI3K/Akt signaling has been implicated in essential aspects of axonal and dendritic morphogenesis during nervous system development, including axon/dendrite elongation, guidance and branching (Cosker et al., 2008; Luikart et al., 2008). It is also fundamental for the maintenance of appropriate architecture of individual neurons and thus influences connectivity in the adult brain (Kwon et al., 2006; van Diepen and Eickholt, 2008). Here, we show that dendritic morphology is prominently attenuated in PIKE −/− neurons (Fig 3). Introduction of wild-type PIKE-L but not GTPase-deficient mutant PIKE-L-KS evidently increased the dendritic complexity (Fig 4), indicating that GTPase activity of PIKE is critical for this process and fitting our previous studies that PIKE up-regulates PI3K/Akt signaling in a GTPase-dependent manner (Ye et al., 2000; Rong et al., 2003; Ahn et al., 2004b). Further, we show that overexpression of PI3K-CA or Akt-CA *in vitro* or deleting *PTEN in vivo* notably augments the dendritic patterning in PIKE −/− neurons (Fig 5), supporting the reduced dendritic morphology is mainly attributed to the diminished PI3K/Akt activity in PIKE −/− neurons. We also hypothesized that PIKE might be a downstream effector of extracellular clues for dendritogenesis via PI3K/Akt pathway. As expected, we find that PIKE −/− neurons are partially irresponsive towards BDNF-induced dendritic arborization but not dendritic elongation (Fig 7). Intriguingly, PIKE −/− neurons are selectively resistant towards BDNF-stimulated PI3K but not ERK activation. It is reported that dendritic growth and arborization is controlled by PI3K/Akt and Raf/ERK pathways that the PI3K/Akt cascade is mainly responsible for dendritic complexity increase, whereas the dendritic length growth is co-regulated by PI3K/Akt and Raf/ERK signaling (Jaworski et al., 2005; Kumar et al., 2005). Conceivably, it is the BDNF-induced ERK activation causes the slightly increase of dendritic length in the PI3K/Akt-defective PIKE −/− neurons.

Neurotrophins also regulate the survival of several types of neurons by provoking the PI3K/Akt pathway. We have shown previously that PIKE-S is a downstream effector of NGF signaling to mediate the anti-apoptotic actions (Ye et al., 2000; Ye et al., 2002; Ahn et al., 2004b). We show here that PIKE are also mediators of BDNF signaling. Indeed, PIKE −/− mice shared striking phenotypic similarities with cortex-specific *BDNF/Ttrb* knockout mice. Both of them have compressed cortical thickness in layer II/III, diminished soma size
of cortical neuron, decreased cortical neuron number, reduced dendritic complexity, enhanced locomotor activity and impaired memory (Xu et al., 2000; Gorski et al., 2003a). Because a detailed study on cortical cell survival in the embryonic cortex has not been performed in BDNF−/− or TrkB−/− mice, it is unknown if enhanced apoptosis, as detected in the neocortex of PIKE−/− embryo, will be found in these animals. In contrast with the severe loss of sympathetic neurons in dorsal root and superior cervical ganglia in BDNF or TrkB−/− mice (Klein et al., 1993; Jones et al., 1994), we found no such significant defects in PIKE−/− mice (data not shown), suggesting that BDNF-induced PIKE functions are mainly restricted in the CNS.

We observed significant amount of neurons in the cortex of adult PIKE−/− brains are undergoing apoptosis in both basal and neurotoxic conditions (Fig 2 and 8) although the cortical BDNF content is comparable between wild-type and PIKE−/− mice as determined by BDNF ELISA (178.8 ± 16.9 pg/mg lysate in wild-type vs 163.9 ± 24.6 pg/mg lysate in knockout, n=4). BDNF is well-known with its neuroprotective functions during various neurotoxic insults. It has been reported that BDNF protects against glutamate-induced apoptotic cell death via PI3K and ERK pathways in vitro (Almeida et al., 2005). Moreover, in pathological conditions like forebrain ischemia, infusion of BDNF shortly after stroke can effectively reduce total infarct volume (Yamashita et al., 1997). Indeed, increased BDNF expression after ischemia and seizure induction is suggested as a protective mechanism against excessive neuronal death (Tsukahara et al., 1994; Kokaia et al., 1995; Kokaia et al., 1998). Therefore, it is logical to find that PIKE−/− neurons, which are partially defective in BDNF signaling, are more vulnerable to both glutamate and stroke-induced cell death.

Dendrites are the primary sites in which neurons receive, process, and integrate inputs from multiple presynaptic partners. The functions of dendrites depend on the branching pattern and specialization (Cosker and Eickholt, 2007). The development of a highly branched dendritic tree is thus essential for the establishment of functional neuronal connections. In fact, several researches have shown that learning processes alter dendritic morphology of pyramidal cells in the cerebral cortex (Turner et al., 2003; Kolb et al., 2008; Gelfo et al., 2009). In general, experience stimulation induces an area-dependent dendritic growth related to the experience. One of these important experiences learning is the spatial navigation. Although most of the effort to study the mechanisms of spatial navigation has focused on the hippocampus, many studies suggest that cortex also plays a role in this process. For example, rat with lesions in a variety of cortical area show impaired water maze task performance (Hoh et al., 2003). It has also been shown that spatial task learning increases dendritic length and branching of neurons in layer III of occipital cortex (Kolb et al., 2008). BDNF/PIKE signaling is also involved in the spatial learning process as forebrain-specific BDNF conditional knockout mice or PIKE−/− mice are defective in the water maze test (Fig 6) (Gorski et al., 2003a). Presumably, wiring of the nervous system and the neuronal circuit in BDNF−/− or PIKE−/− brains are impaired, leading to a reduced ability in performing memory task.

The energy metabolism is altered in PIKE−/− mice that the knockout animals preferentially utilize lipid as the energy source without changing the amount of food intake (Chan et al., 2010a). It has been reported that perinatal malnutrition reduces the brain mass, cortical and hippocampal BDNF expression in cortex and the ability of memory formation (Wang and Xu, 2007; Hernandez et al., 2008). Surprisingly, over-nutrition during the fetal development also reduces the BDNF expression and dendritic arborization in hippocampus (Tozuka et al., 2010), suggesting a balanced energy supply is necessary for proper brain development. Although we have provided evidences in the current study that the TrkB signaling defect is not caused by reduced BDNF concentration but an intrinsic impairment of the intracellular signaling component, we could not completely exclude the possibility that the change of
energy metabolism in PIKE −/− brain, if any, contributes to the reduce brain mass, behavioral abnormalities and neuronal vulnerability to insults. A detailed examination on neuronal metabolic profile of PIKE −/− mice is thus necessary to address the issue.

Collectively, our data support that PIKE are critical neurotrophin-mediated regulators to neuronal development and survival under both physiological and pathological conditions.

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REFERENCES


Fig 1. Reduction of soma size, neuronal density and cortical thickness in PIKE −/− brain
A. DNA and protein contents in brain collected from 1.5-month-old wild-type (+/+) and PIKE −/− (−/−) (n=5). Results are expressed as a percentage relative to the wild-type control (*: P<0.05, Student’s t-test).
B. Soma size of cortical neurons is significantly decreased in wild-type (+/+) and PIKE −/− (−/−) mice (1.5-month-old). Results are expressed as mean ± S.E.M of 5 mice from each genotype (*: P<0.05, Student’s t-test).
C. Nissl staining of 1-day-old wild-type (+/+) and PIKE −/− (−/−) brain. Representative results from at least three mice of each genotype were shown. Scale bar represents 50 µm.
D. Nissl staining of 1.5-month-old wild-type (+/+) and PIKE −/− (−/−) brain. Representative results from at least three mice of each genotype were shown. Scale bar represents 100 μm.
Fig 2. Enhanced apoptosis in PIKE −/− neurons in vivo
A. PIKE is not required for neurogenesis. Normal neurogenesis was detected in E12.5 neocortex in PIKE −/− (−/−) and the wild-type (+/+ ) littermates as revealed by BrdU labeling. Scale bar represents 50 µm.
B. Nestin and Ki67 co-staining in E12.5 neocortex in PIKE −/− (−/−) and the wild-type (+/+ ) littermates. Scale bar represents 20 µm.
C. PIKE ablation enhances neuronal apoptosis during brain development. Enhanced TUNEL positive signals were observed in ventricular zone of PIKE −/− (−/−) brain. TUNEL positive cells were indicated with white arrows. Scale bar represents 50 µm.
D. Immunohistochemical staining of active caspase in adult (1.5-month-old) PIKE −/− (−/−) and wild-type (+/+ ) cortex. Cells with positive signals were indicated by black arrows. Scale bar represents 50 µm.
Fig 3. Dendritic arborization is reduced in PIKE −/− neuron

A. Overall view of 7-DIV cortical neurons from wild-type (+/+ ) and PIKE −/− (−/−) mice. The cultured neurons were infected with adenovirus expressing GFP. Scar bar represents 10 µm.

B. Sholl analysis wild-type (■) and PIKE −/− (□) cortical neurons (DIV 7). Significant difference of intercrossing begins at ~ 20µm from soma (**: P<0.01; ***: P<0.001; Two-way ANOVA, n=10).
Fig 4. Dendritic arborization in PIKE −/− neurons could be antagonized by overexpression of PI3K/Akt in vitro

A. PI3K activities in cortex of PIKE −/− mice (+/+) and its control (+/+ littermate). Cytosolic and nuclear fraction of mouse brain tissues were isolated and subjected to immunoprecipitation using anti-p110α antibody followed by PI3K assay (top panel). The purity of each fraction was verified (middle and bottom panels).

B. Sholl analysis of PIKE −/− cortical neurons infected with various adenoviruses including control virus (Ad-control, □), virus overexpressing wild-type PIKE-L (Ad-PIKE-L WT, ■), GTPase mutated PIKE-L (Ad-PIKE-L KS, ◊), constitutively active PI3K subunit p110 (Ad-p110 CA, ○) or constitutively active Akt (Ad-Akt CA, ▲).

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C. Area under curve (AUC) of Sholl analysis showed in (B) (*: P<0.05; ***: P<0.001 vs Ad-control, Student’s t-test, n=8).

D. Total terminal tip number of PIKE −/− cortical neurons infected with various adenoviruses as indicated (**: P<0.01; ***: P<0.001 vs Ad-control, Student’s t-test, n=8).

E. Total dendritic branch length of PIKE −/− cortical neurons infected with various adenoviruses as indicated (**: P<0.01; ***: P<0.001 vs Ad-control, Student’s t-test, n=8).
Fig 5. *PTEN* ablation enhances dendritogenesis in *PIKE* −/− brain

A. Overall view of cortical neurons from PIKE knockout (*PIKE* −/−) and PIKE/PTEN double knockout (*PIKE*−/−*PTEN*−/−) mice. The cultured neurons were visualized by infecting with adenovirus expressing GFP. Phosphorylation of Akt was also examined by immunofluorescence staining. Scar bars represent 20 µm.

B. Verification of PTEN ablation in PIKE−/−PTEN−/− cortical neurons. Total cell lysates were collected from PIKE−/− and PIKE−/−PTEN−/− neurons and subjected to immunoblotting analysis using antibodies as indicated.

C. Sholl analysis of PIKE−/− (■) and PIKE−/−PTEN−/− (□) cortical neurons (7 DIV) (**: P<0.01; ***: P<0.001, Two-way ANOVA, n=10).

D. Camera lucida drawings of cortical neurons in layer II/III of 1.5-month-old wild-type (*PIKE*+/+*PTEN*+/+), PIKE−/− and PIKE−/−PTEN−/− brain after Golgi staining. Scale bar represents 20 µm.
**Fig 6. Behavioral deficits of PIKE −/− mice**

A. *PIKE −/−* mice possess comparable learning ability. Latency of platform finding during acquisition phase of the Morris water maze test performed by 2-month-old wild-type (■) and *PIKE −/−* (□) male mice (n=7).

B. Percentage time spent in each quadrant of the water tank during the probe trial of the Morris water maze test. The tank was divided into 4 quadrants: north-east (NE), south-east (SE), south-west (SW) and the platform. The time that the animals spent on each quadrant was recorded (*: P<0.05, Student’s t-test, n=7).
C. Swim speed of the wild-type (■) and PIKE −/− (□) male mice during the acquisition phase of Morris water maze test (***: P<0.001, Two-way ANOVA, n=7).
D. Swim distance of the wild-type (■) and PIKE −/− (□) male mice during the acquisition phase of Morris water maze test (*: P<0.05, Two-way ANOVA, n=7).
E. Locomotor activity of single-housed wild-type (■) and PIKE −/− (□) mice on the computerized metabolic cage over a 24 h period. Insert is the comparison of total activity between the genotypes (*: P<0.05, Student’s t-test, n=6).
Fig 7. PIKE mediates BDNF-provoked dendritic arborization

A. Signaling cascade examination in wild-type (+/+) and PIKE −/− (−/−) cortical neurons after BDNF stimulation. Cortical neurons (7 DIV) were stimulated by BDNF (10 ng/ml) for 15 min. Total cell lysates were prepared and phospho-TrkB (1st panel), phospho-Akt (4th panel), phospho-EKR (6th panel), phospho-mTOR (8th panel) and phospho-CREB (10th panel) were examined by immunoblotting analysis. Total proteins of TrkB (2nd panel), Akt (5th panel) and ERK (7th panel), mTOR (9th panel) and CREB (11th panel) were determined to show equal loading. PI3K activity (3rd panel) was also examined by in vitro kinase assay. PIKE-L expression was checked to verify the genotype (12th panel).
B. Sholl analysis of wild-type and PIKE −/− cortical neurons (3 DIV) before (■: wild-type, □: knockout) and after (●: wild-type, ○: knockout) BDNF (10 ng/ml, 3 days) stimulation.

C. Area under curve of Sholl analysis showed in (B) (**: P<0.01; ***: P<0.001 vs different genotypes of the same treatment; c: P<0.001 vs the same genotype under different treatment, Student’s t-test, n=15).

D. Number of primary dendrite in wild-type (+/+) and PIKE −/− (−/−) cortical neurons (3 DIV) before and after BDNF treatment (*: P<0.001 vs different genotype of the same treatment; a: P<0.05 vs the same genotype under different treatment, Student’s t-test, n=15).

E. Total dendritic branch length in wild-type (+/+) and PIKE −/− (−/−) cortical neurons (3 DIV) before and after BDNF treatment (*: P<0.05; **: P<0.01 vs different genotype of the same treatment; a: P<0.05; b: P<0.01 vs the same genotype under different treatment, Student’s t-test, n=15).

F. Terminal tip number in wild-type (+/+) and PIKE −/− (−/−) cortical neurons (3 DIV) before and after BDNF treatment (*: P<0.05; **: P<0.01 vs different genotype of the same treatment; a: P<0.05; b: P<0.01 vs the same genotype under different treatment, Student’s t-test, n=15).
Fig 8. Enhanced apoptosis in PIKE −/− neurons in vitro and in vivo

A. TUNEL analysis of cortical neurons from wild-type (+/+) and PIKE −/− (−/−) mice (7 DIV) after glutamate challenge. Neurons were either pre-treated with PBS or BDNF (100 ng/ml) for 24 h followed by glutamate (50 µM) for 16 h. The number of apoptotic cells were scored under fluorescent microscope (*: P<0.05; **: P<0.01 vs different genotype of the same treatment; a: P<0.05; vs the same genotype under different treatment, Student’s t-test, n=3).
**B.** *PIKE* −/− mice were vulnerable to stroke attack. Triphenyl-tetrazolium chloride stained coronal brain sections from wild-type (+/+) and *PIKE* −/− (−/−) mice scarified after MCAO. Results are representative pictures of 4 mice from each genotype.

**C.** More profound cell death was observed in infarcted area of *PIKE* −/− brain. Notably, more densely stained cells were found in wild-type (+/+ ) sample but not in the mutant (−/−). Scale bar represents 50 µm.

**D.** TUNEL assay of infarct area after MCAO. Enhanced cell death was found in PIKE-null brain. Scale bar represents 100 µm.

**E.** Enhanced apoptotic signaling in *PIKE* −/− brain after stroke. Thirty-six hours after MCAO, the mice were scarified and the apoptotic markers in the brain were detected by immunoblotting. Increased level of apoptotic markers (PARP, 2nd panel) was detected in *PIKE* −/− brain. Cleavage of PIKE-mediated neuronal effectors including UNC5B (3rd panel), AEP (4th panel) and SET (5th panel) were also examined. The expression of PIKE-L was confirmed (1st panel).