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Regional hippocampal differences in AKT survival signaling across the lifespan: Implications for CA1 vulnerability with aging

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Abstract

Distinct neuronal populations differ by the degree of damage caused from cellular stress. Hippocampal neurons of area CA1 are especially vulnerable to several stressors that increase with advanced age. We show here that survival signaling, as measured by activated AKT, was significantly reduced in the nuclear CA1 region across the lifespan compared to CA3. In agreement with these findings the pro-apoptotic protein, and AKT nuclear substrate, FOXO3a was significantly higher in CA1. Further, regional differences in PHLPP1, a recently discovered inhibitor of AKT, inversely correlated with nuclear phosphorylated AKT at Ser473. Together our data suggest that regional differences in nuclear levels of activated AKT may contribute to regional differences in hippocampal vulnerability, and implicate PHLPP1 as a potential target for therapeutic intervention to improve hippocampal health.

INTRODUCTION

Aging is associated with increased vulnerability to hippocampal cell death1. Furthermore, aging-dependent diseases can target vulnerable neuron populations. Under these conditions loss of select hippocampal neuron populations can compromise hippocampal function, contributing to age associated memory impairments.

Despite the similarity in cell type between the CA1 and CA3 regions, evidence suggests that these regions show different vulnerabilities to cell death depending on the stressor2. For example aging-dependent complications such as cardiovascular disease3, stroke4, and decreased cerebral blood flow5 can increase neuronal ischemic damage in the hippocampus. However, area CA1 is the most affected hippocampal region to ischemic insult6. Numerous *in vivo* studies using rodent models of hippocampal acute/severe ischemia7 or chronic/mild hypoperfusion8, showed targeted damage to area CA1 while sparing area CA3.

In addition to ischemic insult, differences in hippocampal vulnerability to stressor induced damage are observed in debilitating age-dependent diseases such as Alzheimer's disease. Studies in human brains show that area CA1 of the hippocampus is one of the earliest brain

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regions to develop the pathological markers associated with Alzheimer's disease9, and rodent models have correlated disease pathology to CA1 neuronal loss10. Understanding what mediates regional differences in hippocampal vulnerability may provide novel solutions for treating aging-dependent decline in hippocampal function caused by decreased neuronal health and survival.

Regional differences in hippocampal vulnerability might result from intrinsic CA1/CA3 differences in the regulation of cell survival pathways. The phosphoinositide kinase-3 (PI3-Kinase) pathway, through the activation of protein kinase B (AKT), is particularly important for neuron survival and has been shown to protect neurons against a vast variety of stressors including: ischemia11, β-amyloid12, and tau pathology13. Because AKT activation can protect neurons against stressors known to increase with aging, we sought to determine whether measures of AKT activity differed between areas CA1 and CA3 of the hippocampus across the lifespan.

RESULTS

Nuclear Active AKT differs between CA3-CA1 regions

Accumulation of nuclear phosphorylated AKT (pAKT) is critical to AKT's anti-apoptotic effects. To determine regional hippocampal differences in both nuclear pAKT levels and regulators of pAKT, nuclear and cytoplasmic enriched fractions were prepared from CA1 and CA3 hippocampal homogenates and used for western blot analysis. Cytoplasmic and nuclear fractions were probed for the nuclear protein, tata box binding protein (TBP), to verify separation. As expected, TBP was observed primarily in the nuclear protein enriched fractions (Fig. 1A) and served as a nuclear loading control in subsequent analyses. Nuclear CA1 and CA3 samples were then probed for AKT phosphorylated at Ser473 (pAKT473, ~60 KDa; Fig. 1B), AKT phosphorylated at Thr308 (pAKT308, ~75 KDa; Fig. 1C), and total AKT (Fig. 1D). No regional or aging differences were observed in nuclear total AKT levels. In marked contrast, both pAKT473 and pAKT308 were significantly higher in nuclear samples from CA3 when compared to CA1 (Fig. 1E & 1G). Further, while age had no effect on nuclear pAKT473 in either region (Fig. 1F), pAKT308 levels were reduced in area CA1 and increased in area CA3 of older animals (Fig. 1H). Together the data show regional differences in pAKT with higher levels in area CA3.

Regional differences in hippocampal nuclear FOXO3a

Activated AKT selectively phosphorylates the pro-apoptotic transcription factor FOXO3a at Ser25314, leading to nuclear exclusion, and enhanced cell survival of hippocampal neurons15. Consistent with the decrease in activated AKT in nuclei of area CA1, immunofluorescent staining of hippocampal sections from a 28 month old animal indicated lower pFOXO3a253 (red) in nuclei (blue) in area CA1 (Fig. 2A, 2B, 2C) relative to CA3 (Fig 2D, 2E, 2F) Moreover, cells which were negative for the neuronal marker tubulin III (green) exhibited little or no nuclear pFOXO3a253. To determine whether the increase in pFOXO3a253 was associated with lower nuclear FOXO3a levels, hippocampal sections were stained for FOXO3a (red). Again total FOXO3a was primarily localized to neurons (green), however; more staining was observed in the nuclei (blue) and perinuclear areas

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from CA1 (Fig. 3A, 3B, 3C) relative to CA3 (Fig. 3D, 3E, 3F). To examine total nuclear FOXO3a levels across the lifespan, western blots of nuclear CA1 and CA3 samples were probed with a polyclonal antibody against FOXO3a and a single band was detected at ~70 KDa (Fig. 3G). The results confirmed the FOXO3a immunofluorescence, exhibiting significantly higher levels of total FOXO3a in CA1 compared to CA3, consistent with the ability of pAKT to exclude FOXO3a from the nucleus (Fig. 3H). No significant aging effects on nuclear FOXO3a were observed (Fig. 3I).

Cytoplasmic factors contributing to regional differences in nuclear pAKT

Activation of AKT in the cytoplasm promotes nuclear pAKT translocation. To determine whether cytoplasmic pAKT showed similar regional differences compared to nuclear pAKT, cytoplasmic samples were probed for pAKT473 (Fig. 4A), pAKT308 (Fig. 4B), and total AKT (Fig. 4C). No significant regional differences were observed for cytoplasmic AKT total or pAKT308. Only cytoplasmic pAKT473 showed significant regional differences (Fig. 4D, 4E), and was higher in the CA3 compared to CA1. No age dependent changes were observed for either pAKT473 or pAKT308 (Fig. 4F, 4G). Next we sought to determine if known regulators of cytoplasmic pAKT might show regional hippocampal differences; suggesting a mechanism for increased cytoplasmic CA3 pAKT levels.

The insulin-like growth factor 1 (IGF-1) receptor tyrosine kinase is a robust activator of AKT signaling in hippocampal neurons. Cytoplasmic activation of PI3 Kinase by IGF-1 promotes AKT phosphorylation and enhances its nuclear translocation16. To investigate whether expression of IGF-1 receptors contribute to the differential activation of AKT, cytoplasmic CA1 and CA3 samples were probed for IGF-1Rα, and a band detected at ~150 KDa (Fig. 5A). No region or age differences in IGF-1Rα were observed suggesting that total receptor expression does not underlie regional differences in cytoplasmic pAKT (Fig. 5C).

Next we investigated whether the activity of IGF-1 receptors might reflect regional differences in pAKT. Activation of IGF-1 receptors requires ligand dependent autophosphorylation of the IGF-1Rβ (95KDa) subunit. CA1 and CA3 cytoplasmic samples were probed for phosphorylated IGF-1Rβ(Tyr1135/1136) and a dominant band detected at ~95KDa (Fig. 5B). The existence of multiple bands above 95KDa may be due to additional posttranscriptional modification of IGF-1Rβ. Though no significant regional differences in pIGF-1Rβ were observed, there was a significant interaction (p=.016) between region and aging (Fig. 5D). Activated IGF-1 receptors remained stable across the lifespan in CA1. However in CA3, activated IGF-1 receptors were much higher compared to CA1 in young animals and decreased significantly across the lifespan, falling well below CA1 levels at 37months of age.

Phosphatase and Tensin Homolog (PTEN) can act as a negative regulator of AKT at the cell membrane17. Total cytoplasmic PTEN was detected as a single band at ~50 KDa and levels were not different across regions (Fig. 6A). In contrast, PTEN levels varied as a function of age ($p < 0.001$) with the highest levels observed between 17–28 months (Fig. 6C). PTEN is regulated by multiple phosphorylation sites including ser370, such that the phosphorylated form (pPTEN370) is less active than the dephosphorylated form. Therefore, cytoplasmic samples were probed with an antibody specific for pPTEN370, and a band detected at

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~55KDa (Fig. 6B). The level of pPTEN370 was not influenced by age (Fig. 6D), however; pPTEN370 was significantly higher in cytoplasmic CA1 samples relative to region CA3 (Fig. 6E). Since pPTEN370 should enhance the level of pAKT, the activity of PTEN does not appear to explain the reduced level of nuclear pAKT in region CA1.

Phosphatases directly acting on AKT contribute to regional differences in nuclear pAKT

The serine/threonine phosphatase PP2A turns off AKT activity by dephosphorylation of AKT at Thr30818. CA1 and CA3 nuclear fractions were probed with an antibody against the PP2A-A subunit, and a single band was detected at ~60KDa (Fig. 7A). Comparing CA1 and CA3 hippocampi across age groups revealed no significant regional or age differences in total PP2A-A, suggesting nuclear PP2A-A levels do not underlie the robust regional differences in active AKT (Fig. 7C).

The PH domain and leucine-rich repeat protein phosphatase 1 (PHLPP1) dephosphorylates AKT at Ser473 in a highly specific fashion19. Examination of nuclear fractions for total PHLPP1 (Fig. 7B) indicated that PHLPP1 tended ($p=.096$) to increase with age from 17 months to 38 months (Fig. 7D). Furthermore, significantly higher levels of PHLPP1 were observed in CA1 nuclear samples compared with CA3 (Fig. 7E). Consistent with recent reports that PHLPP1 dephosphorylates pAKT473, the variability in the level of nuclear pAKT473 in region CA1 and CA3 across the lifespan was negatively correlated with levels of nuclear PHLPP1 ($p<.001$) (Fig. 7F). In agreement with western analysis, immunofluorescent staining from a 28 month old animal indicated more CA1 PHLPP1 (Red) compared to area CA3 (Fig. 7G & 7H). In order to verify antibody specificity, nuclear enriched fractions were probed with the same PHLPP1 antibody used for immunofluorescence, and a single dominant band observed at approximately 145 KDa (Supplementary Fig. 1).

Cytoplasmic pAKT473 was reduced in CA1 across the lifespan, and immunofluorescence showed higher cytoplasmic CA1 PHLPP1 (in addition to nuclear). Therefore we examined whether cytoplasmic PHLPP1 levels were higher in area CA1 across lifespan (Fig. 8A). Collapsed across ages, cytoplasmic CA1 PHLPP1 was significantly higher compared to CA3 (Fig. 8B). However, there was a significant interaction ($p=.036$) between aging and region. Though CA3 cytoplasmic PHLPP1 remained low across the lifespan, CA1 PHLPP1 showed more variation; significantly dropping from 4 to 17 months of age, and significantly rising from 17 to 28 months of age (Fig. 8C).

To support our conclusion that PHLPP1 contributes to the regional differences in hippocampal nuclear pAKT across lifespan, we were interested in probing an additional signaling protein known to be dephosphorylated by PHLPP1, but independent from the AKT pathway. Recently, Gao et al. (2008) demonstrated that knock down of PHLPP increased phosphorylation of PKCα *in vitro*20. Therefore, we probed for regional nuclear differences in total PKCα (Fig. 9A) and PKCα phosphorylated at Ser 657 (pPKCα657; Fig. 9B). Both showed a prominent band at ~75KDa. No change in nuclear total PKCα was observed for region or age. However, pPKCα657 was significantly higher in area CA3 consistent with our observations that PHLPP1 was lower in this region (Fig. 9C). No effect of age was observed for pPKCα657 (Fig. 9D).

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DISCUSSION

REGIONAL DIFFERENCES IN AKT SURVIVAL SIGNALING

The main finding of the current study was an increase in the activity of the AKT pathway in CA3 relative to CA1. AKT is activated by phosphorylation at thr308 and ser473 and we observed increased levels of phosphorylation AKT in region CA3 in the absence of regional differences in total AKT expression. Nuclear pAKT473 was approximately 50% higher in area CA3 compared to CA1, and cytoplasmic pAKT473 was approximately 25% higher in area CA3 compared to cytoplasmic CA1. Further, nuclear pAKT308 was increased in area CA3. In addition, regional differences in the AKT nuclear target substrate, FOXO3a, were consistent with differences in the level of pAKT. FOXO proteins have been shown to play a pro-apoptotic role in the hippocampus, and are activated under ischemic conditions21. AKT negatively regulates the pro-apoptotic protein FOXO3a through the shuttling and degradation of FOXO3a22. This regulation involves AKT mediated phosphorylation of FOXO3a at Ser25323, and the level of pFOXO3a253 provides a measure of AKT activity. Examinations of pFOXO3a253 with immunofluorescence indicated increased phosphorylation in region CA3, confirming that AKT signaling is particularly high within the nuclei of CA3 pyramidal neurons. The phosphorylation of FOXO3a is the primary step in translocation of FOXO3a out of the nucleus for degradation in the cytoplasm24. Thus, despite the increase in pFOXO3a253, total FOXO3a was significantly reduced in nuclear fractions from hippocampal CA3 compared to CA1. The results on pAKT and FOXO3a are consistent with the increase in the level of AKT activity in region CA3 and suggest that the differential regional vulnerability is related to decreased AKT signaling in region CA1.

In addition to AKT/FOXO3a protein levels, robust regional differences were observed for phosphorylated PKCα. Studies suggest that activated PKCα can also play an important role in cell survival and growth25. Moreover, marked down regulation of PKCα and other PKC isoforms in the vulnerable CA1 hippocampal region has been observed after hippocampal ischemic injury26. Down regulation of PKCα after ischemia was not seen in damage resistant hippocampal regions. Therefore, the increase of phosphorylated nuclear PKCα in region CA3 may also help to protect against ischemic damage across the lifespan.

REGULATION OF AKT SURVIVAL SIGNALING

We were interested in determining whether differences in AKT regulators might help reveal the mechanism responsible for regional differences in hippocampal nuclear pAKT. The IGF-1/PI3 Kinase pathway is a classic activator of AKT. IGF-1 binding to the IGF-1α receptor subunit leads to autophosphorylation/activation of IGF-1Rβ receptor subunit, and downstream phosphorylation of AKT. It has been reported that IGF-1 receptor levels are higher in CA3 relative to CA1 in young and old animals27. Therefore, we expected to see higher levels of CA3 IGF-1Rα expression across the lifespan. However, we found no regional variation in IGF-1Rα total, suggesting IGF-1 receptor levels are not responsible for robust increases in CA3 pAKT. Differences in our results may be explained by the aforementioned study using radioligand binding assays, in which IGF-1 ligand was used as the probe to evaluate receptor totals. Because the brain and hippocampus express IGF-1

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binding proteins and receptors, studies using IGF-1 binding do not differentiate the IGF-1 receptor from IGF-1 binding proteins.

We next determined whether regional differences in IGF-1 receptor activity, as measured by IGF-1Rβ phosphorylation, might explain regional differences in pAKT. Intriguingly, though no change in CA1 IGF-1Rβ phosphorylation was observed, there was an age dependent decline in CA3 IGF-1Rβ phosphorylation. However, decreased CA3 IGF-1Rβ phosphorylation did not reflect differences in either CA3 pAKT473 or pAKT308 levels. The data suggest that 1) IGF-1 does not mediate the observed increase in CA3 pAKT levels and 2) aging differentially impacts CA1 and CA3 IGF-1 receptor activity. In support for this conclusion, previous studies in old rats showed intraventricular perfusion of IGF-1 failed to correct an aged-dependent reduction in glucose metabolism in area CA3, however, did improve glucose metabolism in area CA128.

The phosphatases PTEN and PP2A also regulate pAKT levels. PTEN works upstream to prevent AKT activation in the cytoplasm, while PP2A works downstream to dephosphorylate nuclear pAKT. However, no regional differences in cytoplasmic PTEN or nuclear PP2A were observed. Unexpectedly, phosphorylated (i.e. inhibited) PTEN was increased in cytoplasmic samples from region CA1. Initial findings suggest PTEN and PP2A do not mediate the observed regional differences in nuclear pAKT across the lifespan. Nevertheless, it should be noted that many additional post-transcriptional modifications can alter phosphatase activity of PTEN29 and PP2A30. As we did not measure phosphatase activity directly, the potential contribution PTEN and PP2A activity to pAKT levels cannot be addressed by the current experiments. The role of phosphatase activity deserves future investigation; particularly because PTEN and PP2A play a direct role in the regulation of pAKT308.

In contrast to PTEN and PP2A, regional differences were observed for the expression of the phosphatase PHLPP1. Current knowledge of how PHLPP phosphatase activity is regulated remains limited. PHLPP exists as two isoforms, PHLPP1 and PHLPP2, of which the first isoform has two splicoforms PHLPP1α ~135KDa and PHLPP1β ~190KDa31. Studies by Gao et al. (2005) revealed that PHLPP1α directly binds and dephosphorylates pAKT at Ser473. These researchers found that knockdown of PHLPP1 in 293T cells caused an upregulation of endogenous phosphorylated PKCα. To date no phosphorylation sites or other post-transcriptional modifications of PHLPP1 have been discovered, though they likely exist. However, Shimizu et al. (2007) showed calpain mediated cleavage of PHLPP could downregulate phosphatase activity on target substrates in area CA1 of the hippocampus, and prevent nuclear activation of MAPK/CREB32. These results suggest that proteolytic degradation of PHLPP1 is one important regulatory mechanism of phosphatase activity.

Our results are consistent with the idea that changes in total hippocampal PHLPP1 levels are important in regulating the degree of pAKT473 phosphorylation. Thus, hippocampal nuclear PHLPP1 levels matched regional differences in nuclear pAKT473 such that PHLPP1 was elevated in region CA1, and CA1 exhibited the lowest level of nuclear pAKT473. Furthermore, nuclear PHLPP1 levels were inversely correlated with pAKT473 levels across

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the lifespan. Finally, the observed decrease in CA1 nuclear pPKCα657, another substrate for PHLPP1, supported our conclusion that the level of nuclear PHLPP1 is important in the regulation of target nuclear substrates.

In addition to nuclear PHLPP1, cytoplasmic levels of PHLPP1 were reduced across the lifespan in area CA3 and cytoplasmic pAKT473 levels were higher across the lifespan in area CA3. Alternatively, collapsed across age, cytoplasmic PHLPP1 was significantly higher in region CA1. Together the nuclear and cytoplasmic data suggest cellular levels of PHLPP1 are higher in region CA1 and correspond to reduced cellular levels of pAKT473. Notably, regional differences in cytoplasmic pAKT308 were not observed. These findings support our conclusion that PHLPP1 is important in selectively regulating pAKT473 levels across the lifespan, and suggest regional differences in nuclear CA1/CA3 pAKT308 depend upon regulation (or dysregulation) by mechanisms distinct from PHLPP1.

Despite our data supporting PHLPP1's role in mediating regional differences in hippocampal pAKT473, additional regulation seems very likely; primarily because CA1 pAKT473 levels were remarkably stable across the lifespan in both the nucleus and the cytoplasm while CA1 PHLPP1 showed much more variability. One possibility is that regional differences in growth factor mediated AKT activation, other than IGF-1, help to regulate AKT across the lifespan. Immunohistochemical studies in rat brain showed BDNF levels are extremely high in area CA3 and extremely low in area CA133. Further, these differences are maintained across the lifespan34. Because BDNF can activate AKT, and has been shown to rapidly inhibit FOXO3a nuclear localization35, BDNF signaling may also contribute to the observed maintenance of pAKT across the lifespan. However, comparative studies of growth factor mediated survival signaling in hippocampal cell culture show IGF-1 strongly induces AKT though weakly induces ERK, while BDNF strongly induces ERK and only weakly induces AKT36. Therefore whether BDNF contributes to sustained AKT activity in vivo, and whether its contribution is large or small, remains unknown but deserves further investigation.

AKT ACTIVITY ACROSS THE LIFESPAN

Aging-dependent dysregulation of proteins that mediate survival signaling may contribute to increased hippocampal vulnerability during aging. The results of the current study indicate that the level of nuclear pAKT308 decline in region CA1 with advanced age. Alternatively CA1 pAKT473 was stable across the lifespan. This raises the question of whether the loss in dual phosphorylated AKT exacerbates vulnerability of region CA1 with advanced age. Recent *in vitro* studies examining the regulation of AKT phosphorylation and AKT activity provided evidence that dual phosphorylation of pAKT308 and pAKT473 are temporally associated/coordinated, and together predicted AKT activity37. In addition, studies in NIH3T3 cells showed that a pAKT308 mutant failed to activate when treated with growth factor, while a pAKT473 mutant showed reduced activity; thus dual phosphorylation appears to be required for full AKT kinase activity38. In aged animals, decreased CA1 pAKT308 levels would predict reduced total AKT activity even though pAKT473 remained stable.

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It is unclear what mechanism(s) underlie the age-related decline in nuclear CA1 pAKT308. One possible explanation may be that aging differentially effects nuclear CA1/CA3 membrane phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) levels. AKT can be activated in the nucleus by identical signaling mechanisms observed at the cell membrane39. However, before AKT can be phosphorylated at thr308, PI3 Kinase must be activated upstream. When activated, PI3 Kinase will phosphorylate membrane bound PIP_2 to PIP_3. PIP_3 will then allow both AKT and PIP_3–dependent kinase 1 (PDK-1) to bind the inner membrane via their PH domains. PDK-1 is responsible for phosphorylating AKT at thr308, and binding of both enzymes to PIP_3 is critical to allowing PDK-1 access to AKT's active site at thr308. Therefore, changes in nuclear CA1 PIP_3 levels across the lifespan could alter nuclear CA1 AKT308 levels. However little is known about the regulation and control of nuclear PIP_3, let alone how aging might influence nuclear PIP_3 levels.

Alternatively, Ca^{2+}/calmodulin-dependent protein kinase kisase (CaM-KK) can selectively and directly phosphorylate AKT at thr308 but not on ser47340. Intriguingly CaM-KKα is found only in the nucleus where it acts to regulate its better known substrate Ca^{2+}/calmodulin-dependent protein kinase IV. Because Ca^{2+} imbalance plays an important role in hippocampal CA1 loss of function with aging, altered hippocampal CaM-KK activity with age may also play a role in selectively altering nuclear pAKT308 phosphorylation across lifespan in area CA1. This idea remains to be tested.

Together the results suggest regional hippocampal differences in the AKT pathway may contribute to regional differences in neuronal vulnerability to certain stressors, and help protect CA3 neurons from pathology associated with age-dependent disease. In support for these conclusions, the pro-apoptotic protein FOXO3a was higher in area CA1 across lifespan and actively inhibited in pyramidal neuron nuclei of region CA3. Further nuclear PHLPP1 was higher in region CA1 and correlated with levels of nuclear pAKT473. To the best of our knowledge these findings are novel and suggest a new hypothesis to explain the mechanisms for CA1 vulnerability to certain kinds of stressors.

MATERIALS & METHODS

Aged Animals

NIA Fischer 344- Brown Norway rats were kept in specific pathogen free (SPF) housing and maintained on a 12 hour light/dark cycle. Animals were aged 4 (n=4), 17 (n=3), 28 (n=4), and 37 (n=4) months, and fed a standard ab libitum (AL) diet. Animals were euthanized by CO2 asphyxiation and decapitated. Hippocampi were removed, separated into CA1 and CA3 sections, and flash frozen in liquid nitrogen. Samples were stored at −80°C until further processing.

Homogenization & Nuclear Enrichment

Tissues were homogenized using NER nuclear separation kit (PIERCE) according to the manufacturer's instructions. Briefly, frozen samples (~30–40 mg of tissue/sample) were removed from −80 freezer and immediately placed in a 2mL glass dounce homogenizer (Kimble-Kontes) containing ice cold CERI buffer (for cytoplasmic fraction) with 1.5X

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protease inhibitors, 1X EDTA, and 2X phosphatase inhibitors (PIERCE). Samples were then homogenized using 11 strokes with pestle B and 10 strokes with pestle A, and transferred to .5mL tubes. After sitting on ice for 10 minutes, samples were briefly vortexed, and spun at 16,000g's/4°C/5mins. Samples were then placed back on ice and supernatant was collected and saved for cytoplasmic protein analysis. Next NERI buffer (for nuclear fraction), containing 1.5X protease inhibitors, 1X EDTA, and 2X phosphatase inhibitors (PIERCE) was added to the cell pellet. Samples were vortexed every 10 minutes for 40 minutes and spun at 16,000g's/4°C/10mins. The supernatant (containing nuclear enriched protein fraction) was collected and stored for later analysis.

Western Blot

Protein concentrations were determined using BSA method (PIERCE). Kaleidoscope protein standards (Bio Rad) and CA1/CA3 samples (20ug/lane) were loaded on 4–15% gradient gels (Bio Rad) and run for 1 hour at 120V. Proteins were then transferred to PDVF membranes (Amersham) overnight at 50V/4°C. Blots were then stained with Ponceau S and photocopied. Blots were washed 5mins in T-BST and subsequently blocked in T-BST (7%milk) for 1 hour. Primary antibodies were then applied to blots overnight at 4°C (Table 1), washed 3 times with TBS, and secondary antibodies applied for 2 hours at room temperature. Blots were then developed using ECL Plus Western Blot Detection Kit (Amersham) on biomax film (Kodak). Blots were scanned using 6500 scanner (Bio Rad), and densitometry determined using UNSCAN IT software (silk scientific).

Immunofluorescence

A 28 month old Fisher 344/BN rat was anesthetized and perfused with 4% paraformaldehyde. The brain was removed, placed in paraformaldehype for 1 hour, and transferred to 30% sucrose solution for 72 hours. Following fixation the brain was embedded in optimal cutting compound (OCT) and 8μm coronal sections were made using a cryostat. Slices were collected on Superfrost Plus glass slides (Fisher), and allowed to air dry for 1hour. Slides were then washed with PBS and permeabilized with .05% TritonX-100/PBS for 15mins. Slides were washed again in PBS and treated with1% SDS/PBS for 5mins for mild antigen retrieval. Sections were the washed in PBS, blocked with 20% goat serum (in 1% BSA PBS) for 2 hours, and incubated overnight (at 4°C) in 3% goat serum PBS containing (rabbit anti-phospo or total FOXO3a + mouse anti-neuronal tubulin III) or (rabbit anti-PHLPP1). Slides were then washed with PBS and incubated with secondary (Alexa Fluor goat anti-rabbit 594/goat anti-mouse 488; Invitrogen) for 1.5 hours. Slides were then stained with .3% Sudan Black B in 70% ethanol for 10mins to remove lipofuscin autofluorescence. Finally slides were mounted (Prolong Gold Anti-Fade with DAPI; Invitregen) and images taken on an Axiovert 40 CFR fluorescent microscope (Zeiss).

Statistical Analysis

Western blot densitometry was analyzed using 3-Way-ANOVA (NCSS Statistical Software); data significant at $p<.05$. Post-hoc analysis was performed using Fisher LSD test. All graphs were produced using Prism software (Silk Scientific).

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Supplementary Material

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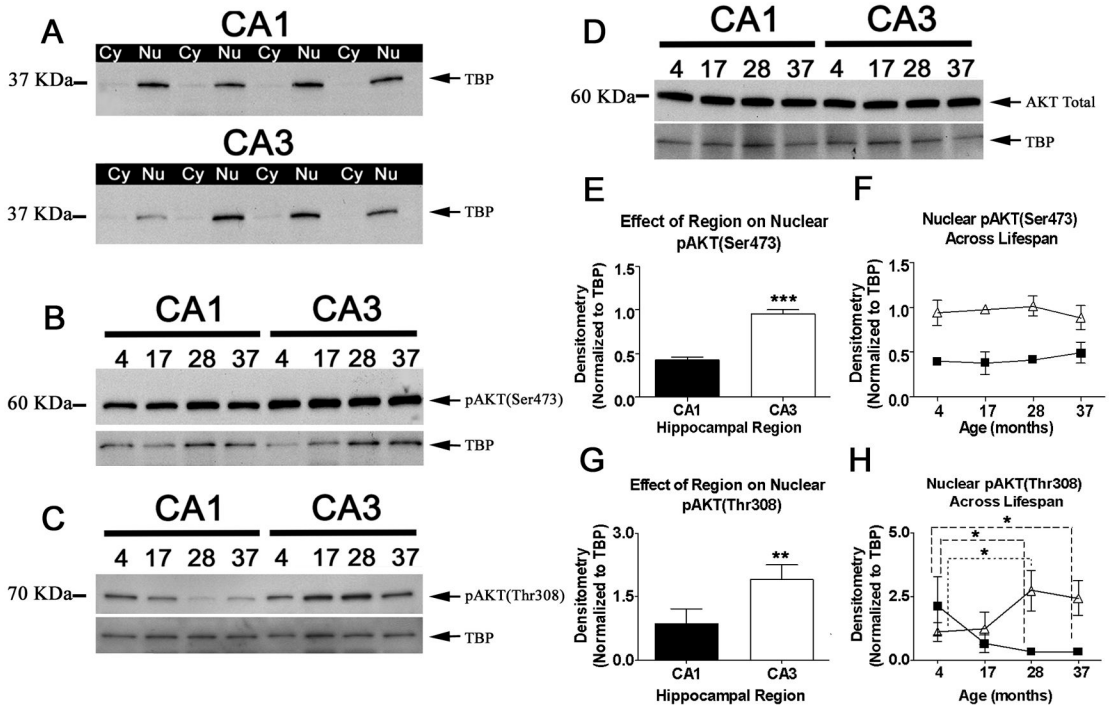

Fig. 1.

Activated AKT is increased in nuclear fractions from region CA3 compared to region CA1. (**A**) Control blot showing TBP is localized to nuclear enriched fractions (Nu) relative to cytoplasmic enriched fractions (Cy). (**B, C, D**) Representative blots of nuclear CA1/CA3 pAKT473, pAKT308, and AKT total, from animals aged 4, 17, 28, and 37 months (Top blots) and TBP nuclear loading control (Bottom blots). (**E & G**) Mean nuclear pAKT averaged across ages (n=15) for CA1 (filled bars) and CA3 (open bars). (**F & H**) Effect of age on nuclear pAKT in area CA1 (filled squares) and CA3 (open triangles) for animals aged 4 (n=4), 17 (n=3), 28 (n=4), and 37 (n=4) months. Dashed lines indicate significant aging effects in CA1 (---) and CA3 (·····). In this figure and subsequent figures, asterisks indicate significant differences *p<.05, **p<.01, ***p<. 0001.

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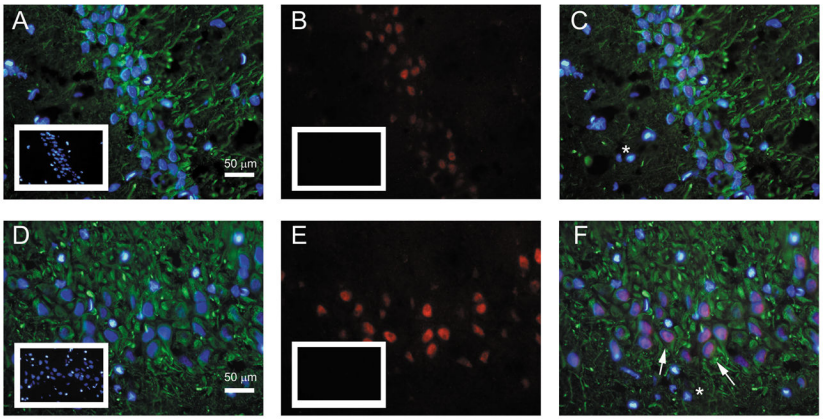

Fig. 2.

Nuclear pFOXO3a253 is higher in region CA3 and lower in region CA1. (**A & D**) Immunofluorescence showing CA1 and CA3 neuronal marker tubulin III (green), and nuclei (blue). White boxes in lower left indicate secondary antibody only controls. No green staining was observed. (**B & E**) Immunofluorescence showing CA1 and CA3 pFOXO3a253 (red). More staining is observed in region CA3 compared to CA1. No staining was observed in secondary antibody only controls. (**C & F**) Merged images of **(A/B & D/E)** showing pFOXO3a is localized to nuclei of CA1 and CA3 pyramidal neurons. White arrows indicate intense nuclear pFOXO3a253 CA3 staining. White asterisks indicate non-neuronal nuclei.

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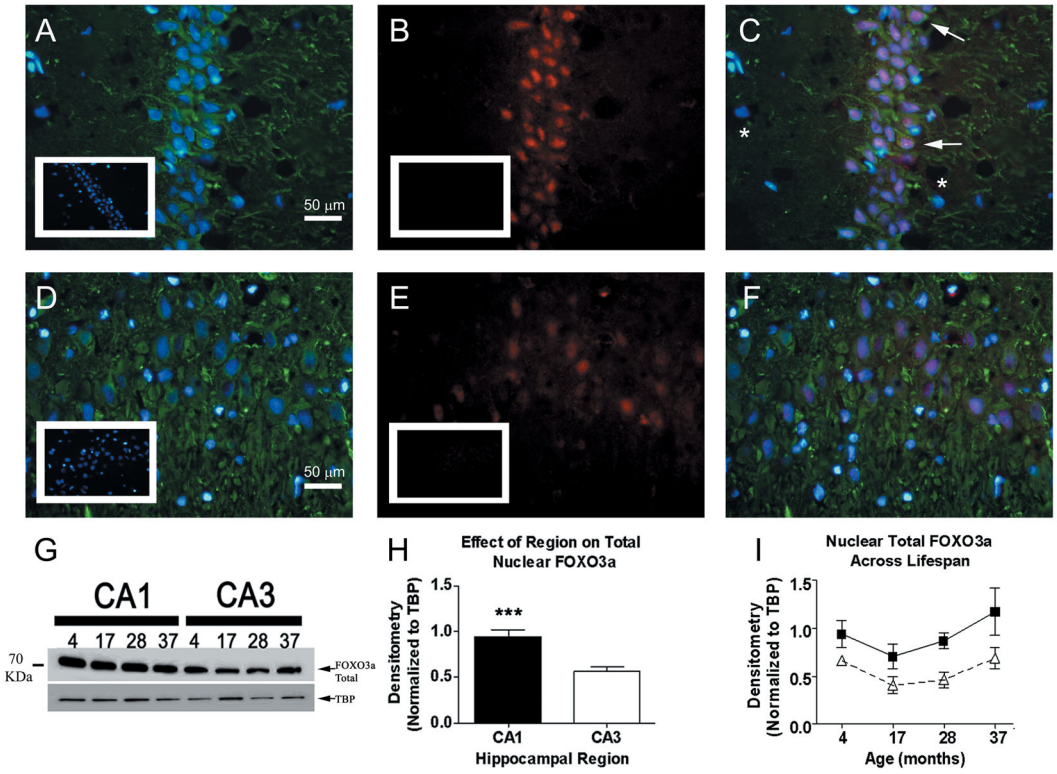

Fig. 3.

Nuclear total FOXO3a is lower in region CA3 and higher in region CA1. (**A & D**) Immunofluorescence showing CA1 and CA3 neuronal marker tubulin III (green), and nuclei (blue). White boxes in lower left indicate secondary antibody only controls. No green staining was observed. (**B & E**) Immunofluorescence showing CA1 and CA3 total FOXO3a (red). More staining is observed in region CA1 compared to CA3. No staining was observed in secondary antibody only controls. (**C & F**) Merged images of **(A/B & D/E)** showing total FOXO3a is localized to nuclei and perinuclear areas of CA1 and CA3 pyramidal neurons. White arrows indicate intense nuclear total FOXO3a CA1 staining. White asterisks indicate non-neuronal nuclei. (**G**) Representative blot showing nuclear CA1/CA3 total FOXO3a (Top blot) and TBP (Bottom blot) from animals aged 4, 17, 28, and 37 months. (**H**) Mean normalized density averaged across ages (n=15) in CA1 (filled bars) and CA3 (open bars). (**I**) Nuclear FOXO3a plotted as a function of age for CA1 (filled squares) and CA3 (open triangles) for animals aged 4 (n=4), 17 (n=3), 28 (n=4), and 37 (n=4) months.

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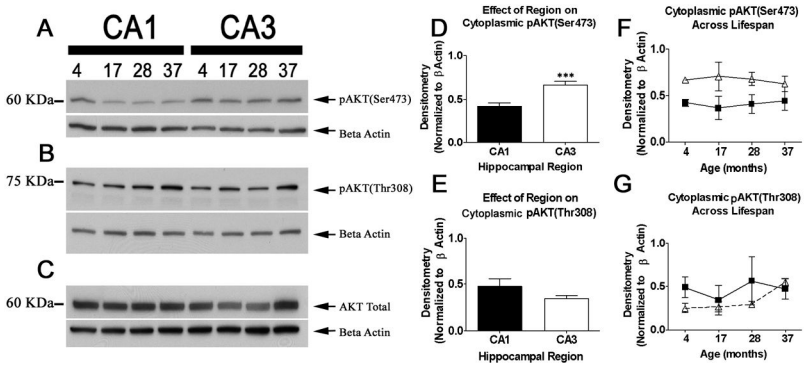

Fig. 4.

Only AKT phosphorylated at Ser473 is increased in cytoplasmic fractions from region CA3 compared to region CA1. (**A, B, C**) Representative blots of cytoplasmic CA1/CA3 pAKT473, pAKT308, and AKT total, from animals aged 4, 17, 28, and 37 months (Top blots) and β-Actin cytoplasmic loading control (Bottom blots). (**D & E**) Mean cytoplasmic pAKT averaged across ages for CA1 (n=14; filled bars) and CA3 (n=15; open bars). (**F & H**) No effect of age on cytoplasmic pAKT in area CA1 (filled squares) and CA3 (open triangles) for animals aged 4 (n=4), 17 (n=3), 28 (n=4), and 37 (CA1 n=3/CA3 n=4) months.

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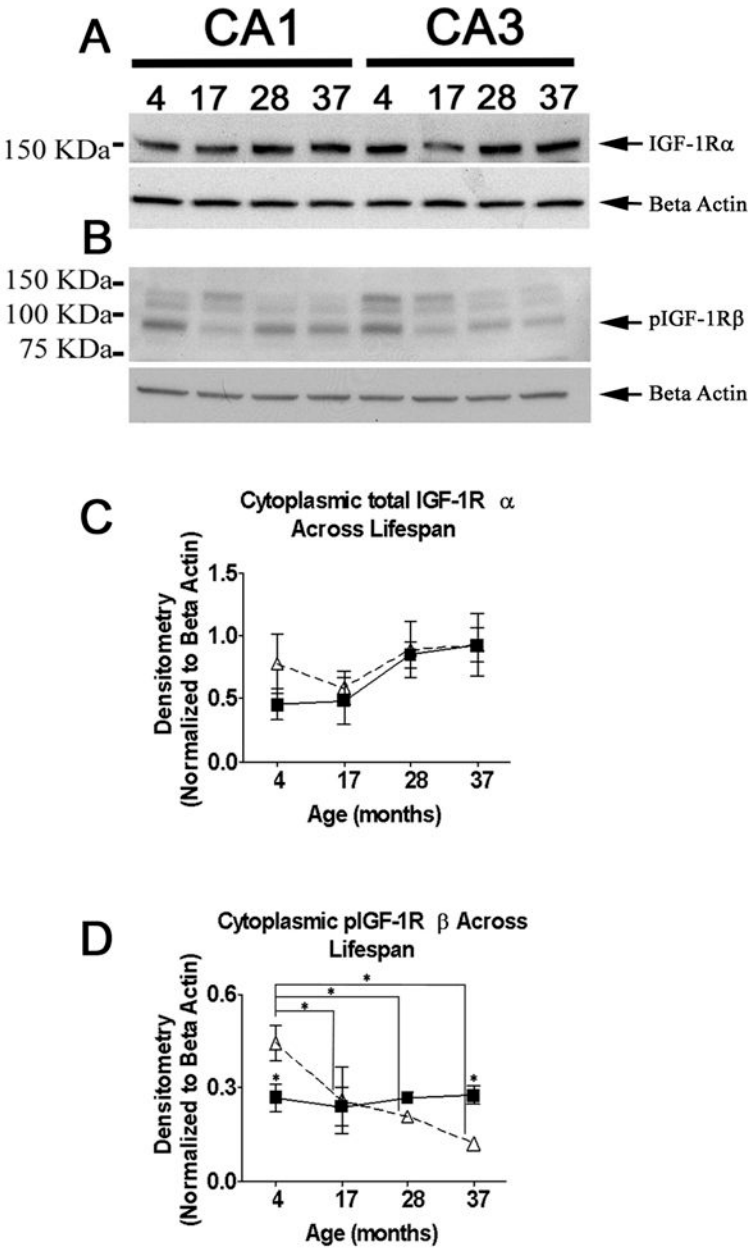

Fig. 5.

IGF-1 receptors do not predict regional differences in pAKT. (**A & B**) Representative blots showing total cytoplasmic IGF-1Rα and cytoplasmic pIGF-1Rβ from animals aged 4, 17, 28, and 37 months (Top blots) and cytoplasmic loading control β-Actin (Bottom blots). (**C**) No effect of age or region for cytoplasmic IGF-1Rα in area CA1 (filled squares) and CA3 (open triangles) in animals aged 4 (n=4), 17 (n=3), 28 (n=4), and 37 (n=4) months. (**D**) No effect of age for cytoplasmic pIGF-1Rβ in area CA1 (filled squares). However, there is a significant age-dependent decline in area CA3 (open triangles) for animals aged 4 (n=4), 17 (n=3), 28 (n=4), and 37 (n=4) months. Solid lines indicate aging differences in CA3 pIGF-1Rβ.

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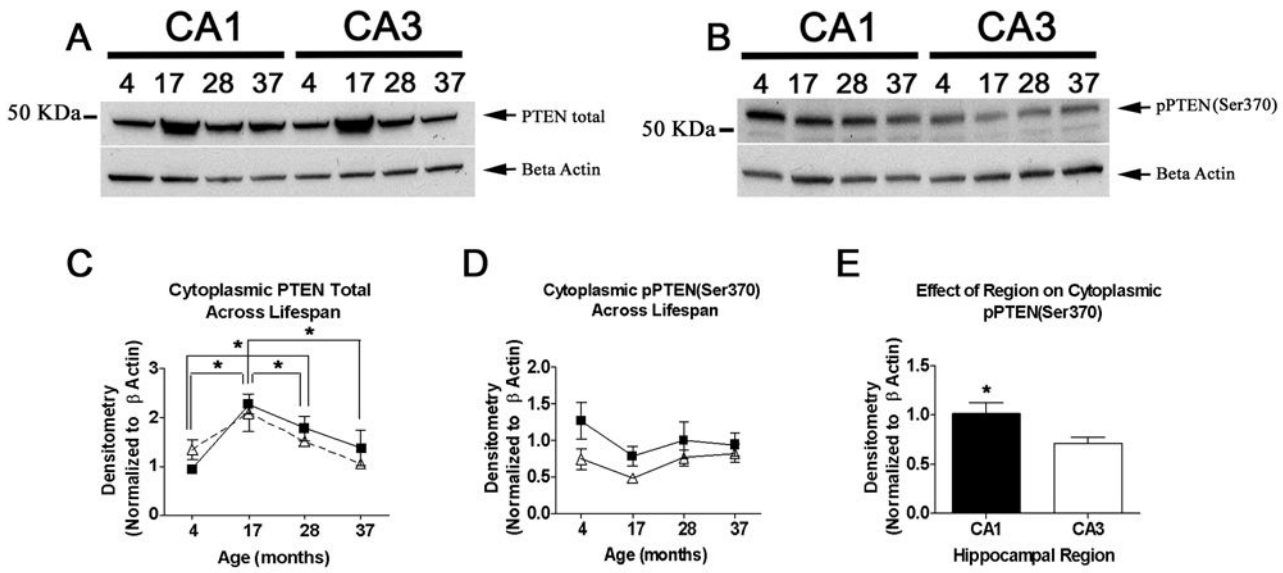

Fig. 6.

PTEN does not predict regional differences in pAKT. (**A & B**) Representative blots showing total PTEN, and pPTEN370 in animals aged 4, 17, 28, and 37 months (Top blots) and cytoplasmic loading control β-Actin (Bottom blots). (**C**) CA1 (filled squares) and CA3 (open triangles) cytoplasmic PTEN total shows no significant regional effects but significant aging effects in animals aged 4 (n=4), 17 (n=3), 28 (n=4), and 37 (n=4) months. Solid lines indicate aging differences in CA1/CA3 PTEN total. (**D**) CA1 (filled squares) and CA3 (open triangles) cytoplasmic pPTENSer370 was not different across lifespan in animals aged 4 (n=4), 17 (n=3), 28 (n=4), and 37 (n=4) months, however, (**E**) elevated levels of pPTEN370 were observed (n=15) in region CA1 (filled bars) relative to CA3 (open bars).

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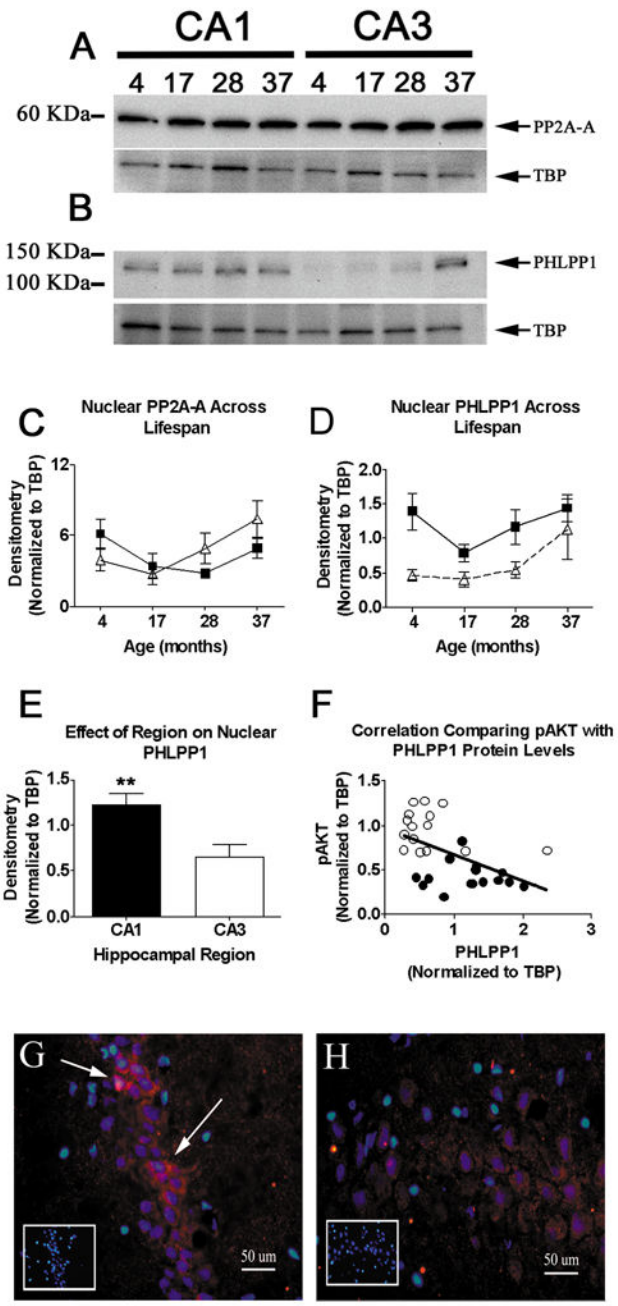

Fig. 7.

Nuclear PHLPP1 but not PP2A-A correlate with regional differences in nuclear pAKTSer473. (**A & B**) Representative blots showing nuclear total PP2A-A and PHLPP1 in animals aged 4, 17, 28, and 37 months (Top blots) and TBP (Bottom blots) (**C**) CA1 (filled squares) and CA3 (open triangles) nuclear PP2A-A across the lifespan. (**D**) CA1 (filled squares) and CA3 (open triangles) nuclear PHLPP1 had a tendency to increase with age, and (**E**) significant regional differences (n=15) were observed in CA1 (filled bars) relative to CA3 (open bars). (**F**) Linear regression of combined CA1 (dark circles)/CA3(open circles) samples across the lifespan showing PHLPP1 levels correlate with pAKTSer473 (r^2=0.27).

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(**G & H**) Immunofluorescence showing CA1 and CA3 PHLPP1 (red) and nuclei stained with DAPI (blue). White border squares at bottom left indicate secondary only controls. White Arrow heads indicate nuclear and perinuclear regions of intense PHLPP1 staining in area CA1. Animals aged 4 (n=4), 17 (n=3), 28 (n=4), and 37 (n=4) months for lifespan graphs.

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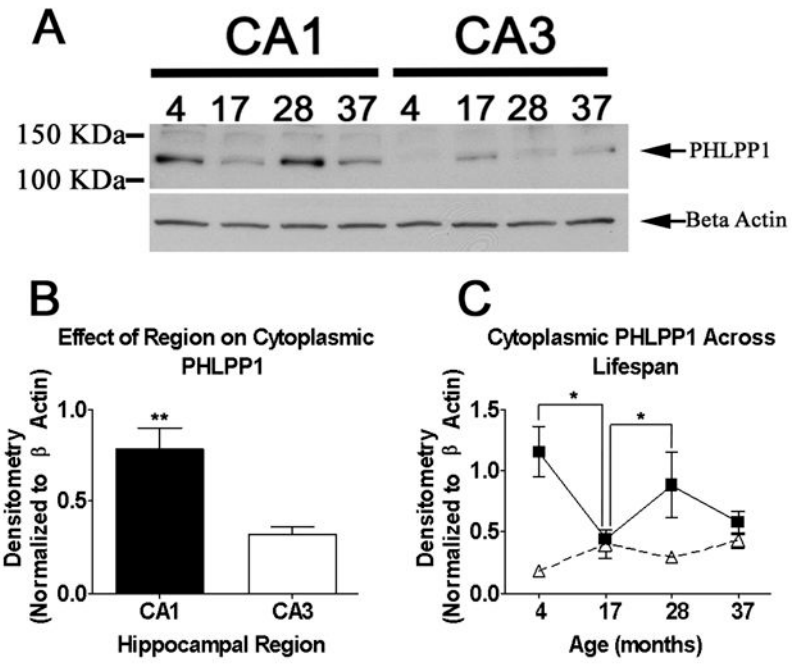

Fig. 8.

Cytoplasmic PHLPP1 is increased in area CA1. (**A**) Representative blot showing cytoplasmic total PHLPP1 in animals aged 4, 17, 28, and 37 months (Top blot) and β-Actin (Bottom blot) (**B**) Mean normalized densitometry averaged across ages (n=15) in CA1 (filled bars) and CA3 (open bars). Significant regional differences in cytoplasmic PHLPP1 (n=15) were observed in area CA1 (filled bars) relative to area CA3 (open bars). (**C**) CA1 PHLPP1 show significant variation across the lifespan (filled squares). Solid lines indicate aging differences in CA1 PHLPP1. Alternatively CA3 (open triangles) cytoplasmic PHLPP1 showed reduced and stable levels across the lifespan. Animals aged 4 (n=4), 17 (n=3), 28 (n=4), and 37 (n=4) months for lifespan graph.

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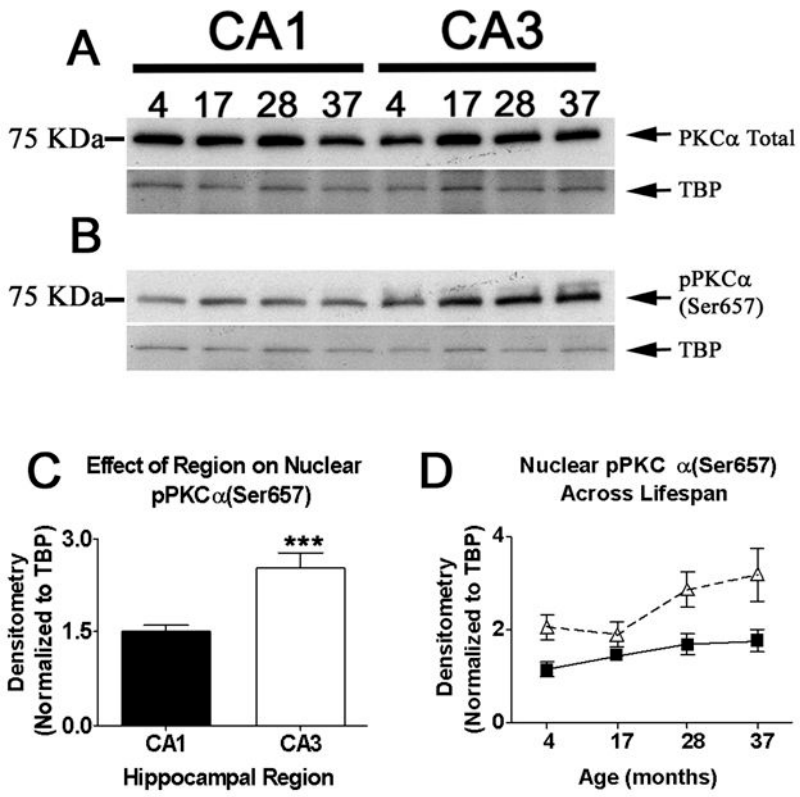

Fig. 9.

Regional differences in nuclear pPKCα657. (**A & B**) Representative blots showing nuclear CA1/CA3 total PKCα and pPKCα657 levels in animals aged 4, 17, 28, and 37 months (Top blots) normalized to TBP (Bottom blots). (**C**) Mean normalized densitometry averaged across ages (n=15) in CA1 (filled bars) and CA3 (open bars). Significantly higher levels of pPKCα657 are observed in the nucleus of area CA3. (**D**) CA1 (filled squares) and CA3 (open triangles) nuclear pPKCα657 shows no significant aging effects. Animals aged 4 (n=4), 17 (n=3), 28 (n=4), and 37 (n=4) months for lifespan graph.

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Table 1

Antibody List

Antibody	Concentration	Host	Clonality	Company
TBP	1:1000	Mouse	Monoclonal	ABR
Beta Actin	1:8000	Chicken	Polyclonal	Abcam
P(Thr308)AKT	1:1000	Rabbit	Monoclonal	Cell Signaling
p(Ser473)AKT	1:1000	Rabbit	Polyclonal	Cell Signaling
AKT Total	1:1000	Rabbit	Polyclonal	Cell Signaling
FOXO3a	1:1000	Rabbit	Polyclonal	ABR
pFOXO3a253 **Immuno-Fluo**	2μg/mL	Raddit	Polyclonal	Abcam
FOXO3a **Immuno-Fluo**	1:100	Rabbit	Monoclonal	Cell Signaling
IGF-1R alpha	1:200	Rabbit	Polyclonal	Santa Cruz
pIGF-1Rbeta	1:1000	Rabbit	Monoclonal	Cell Signaling
p(Ser370)PTEN	1:1000	Rabbit	Polyclonal	Abcam
PTEN Total	1μg/mL	Rabbit	Polyclonal	Abcam
PP2A-A	1:1000	Mouse	Monoclonal	Cell Signaling
PHLPP1	1:200	Goat	Polyclonal	Santa Cruz
PHLPP1 **Immuno-Fluo**	1:50	Rabbit	Polyclonal	Cayman
p(Ser657)PKCα	. 5μg/mL	Rabbit	Polyclonal	Upstate
PKCα Total	.4μg/mL	Mouse	Monoclonal	Upstate
Neuronal Beta Tubulin III	1:1000	Mouse	Monoclonal	Abcam