PI3K/Akt and CREB Regulate Adult Neural Hippocampal Progenitor Proliferation and Differentiation

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ABSTRACT: **The phosphoinositide 3-OH kinase (PI3K)/Akt pathway has been implicated in regulating several important cellular processes, including apoptosis, survival, proliferation, and metabolism. Using both pharmacological and genetic means, we demonstrate here that PI3K/Akt plays a crucial role in the proliferation of adult hippocampal neural progenitor cells. PI3K/Akt transduces intracellular signals from multiple mitogens, including basic fibroblast growth factor (FGF-2), Sonic hedgehog (Shh), and insulin-like growth factor 1 (IGF-1). In addition, retroviral vector-mediated over-expression of wild type Akt increased cell proliferation, while a dominant negative Akt inhibited proliferation. Furthermore, wild type Akt over-expression reduced glial (GFAP) and neuronal (β-tubulin III) marker expression during differentiation, indicating that it inhibits cell differentiation. We also show that activation of the cAMP response element binding protein (CREB), which occurs in cells stimulated by FGF-2, is limited when Akt signaling is inhibited, demonstrating a link between Akt and CREB. Over-expression of wild type CREB increases progenitor proliferation, whereas dominant negative CREB only slightly decreases proliferation. These results indicate that PI3K/Akt signaling integrates extracellular signaling information to promote cellular proliferation and inhibit differentiation in adult neural progenitors.** © 2007 Wiley Periodicals, Inc. Develop Neurobiol 67: 1348–1361, 2007

Keywords: **adult neural hippocampal progenitor; proliferation; differentiation; PI3K/Akt; CREB**

INTRODUCTION

Since the first evidence for adult mammalian neurogenesis (Altman, 1962; Kaplan and Hinds, 1977) and the initial isolation of adult hippocampal neural progenitor cells (AHNPCs) from the dentate gyrus of the hippocampal formation (Ray et al., 1993; Palmer et al., 1997), considerable work has advanced our understanding of their function from a systems to a molecular level. Neurogenesis may play roles in learning and memory (Kempermann et al., 1997; Kempermann and Gage, 1999; van Praag et al., 1999a), the effects of exercise on learning (van Praag et al., 1999a), stress and depression (Gould et al., 1997; Santarelli et al., 2003), response to injury (Liu et al., 1998; Takagi et al., 1999), and aging (Gould et al., 1999b). At a cellular level, neurons generated from AHNPCs differentiate to form functional connections with existing neurons in the adult brain (Palmer et al., 1997), and the resulting mature neurons can persist for years after differentiation (Kempermann et al., 2003). Furthermore, a number of extracellular molecules that regulate several stages in the process of adult neurogenesis have been identified, and the proliferation or amplification of progenitors prior to differentiation appears to be a significant regulatory point (Takagi et al., 1999; van Praag et al., 1999b; Arvidsson et al., 2001).

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In particular, basic fibroblast growth factor (FGF-2) (Palmer et al., 1999; Yoshimura et al., 2001), vascular endothelial growth factor (VEGF) (Fabel et al., 2003), insulin-like growth factor 1 (IGF-1) (Aberg et al., 2003), epidermal growth factor (EGF) (Palmer et al., 2001), Sonic hedgehog (Shh) (Lai et al., 2003), and glucose-dependent insulinotropic polypeptide (Nyberg et al., 2005) have been discovered to regulate AHNPC proliferation. In contrast to this increasing knowledge of extracellular mitogens, however, comparatively little is known about the intracellular signaling cascades that transduce these signals to modulate cell proliferation. Nakagawa et al. demonstrated that cAMP plays a role in AHNPC proliferation *in vivo* (Nakagawa et al., 2002), and Mantamadiotis et al. found that conditional knock-out of the cAMP response element binding protein (CREB) impairs *in vivo* proliferation (Mantamadiotis et al., 2002). It has also been demonstrated that opioids and IGF-1 potentially act through activation of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-OH kinase (PI3K) pathways (Aberg et al., 2003; Persson et al., 2003). In addition, AHNPCs in a $Sox2^{-/-}$ mutant mouse exhibited reduced proliferative capability (Ferri et al., 2004). However, it is unclear whether numerous intracellular signaling pathways transduce and process the known extracellular mitogenic signals in parallel, or whether a common pathway integrates numerous upstream mitogenic signals. We have therefore investigated the intracellular pathways that mediate FGF-2 and Shh signals *in vitro*.

The FGF-2 signaling cascade begins when FGF-2 binds to the cell surface receptor FGFR-1 (Boilly et al., 2000), a member of the receptor tyrosine kinase superfamily specific for FGF-2. Upon ligation, FGFR-1 is capable of initiating intracellular signal cascades through several downstream mechanisms, including the Ras/MAPK, p38 MAP, protein kinase C (PKC), phospholipase C, and PI3K/Akt pathways [as reviewed (Eswarakumar et al., 2005)]. These cascades can be simultaneously stimulated, and crosstalk may occur between them, such as Akt mediated phosphorylation of Raf (Zimmermann and Moelling, 1999) and MAPK-activated protein kinase-2 (MAPKAPK2) mediated phosphorylation of Akt (Rane et al., 2001). By contrast, the mitogen Shh, binds to the receptor Patched (Ptch), which relieves its inhibition of the membrane protein Smoothened (Smo). Smo then activates the Gli family of transcription factors. Recent work has shown that Shh is also capable of activating PI3K/Akt in endothelial cells, and that PI3K/Akt act synergistically with Shh to activate Gli (Kanda et al., 2003).

Here, we investigate this complex signaling landscape using pharmacological and genetic techniques and demonstrate that the PI3K/Akt pathway, often associated with antiapoptotic and survival functions (Datta et al., 1999), is a key transducer of mitogenic signals that drive proliferation and inhibit differentiation of adult hippocampal neural progenitors. Additionally we show that Shh stimulation activates Akt but in a novel, translationally mediated manner.

Upon activation of PI3K by a given cell surface receptor, the kinase increases the membrane concentration of phosphatidylinositol-3,4,5-trisphosphate, which in turn recruits Akt, as well as kinases that act on Akt, to the cell membrane (Downward, 2004). Once activated via phosphorylation, the three Akt isoforms (Akt1, Akt2, Akt3) are collectively capable of phosphorylating numerous downstream signaling proteins, including members of the Bad apoptosis machinery (Datta et al., 1997), the forkhead/FOXO (Brunet et al., 1999), NFκB (Kane et al., 1999; Romashkova and Makarov, 1999), p53 (Zhou et al., 2001), and glycogen synthase kinase 3β (GSK3β) (Cross et al., 1995) pathways, as well as the mammalian target of rapamycin (mTOR), which modulates protein translation machinery (Scott et al., 1998; Sarbassov et al., 2004). In addition, *in vitro* kinase assays have demonstrated that Akt is capable of phosphorylating and activating CREB (Du and Montminy, 1998), though no direct evidence of a direct intracellular link has yet been reported.

PI3K activity has been implicated in the function of stem and neural progenitor cells during development. The phosphatase and tensin homologue deleted on chromosome 10 (PTEN), a phosphatase that antagonizes PI3K's activity, has been shown to limit the proliferative capabilities of embryonic neural stem cells (Groszer et al., 2005). Akt has also been shown to play a role in IGF-1 signaling in fetal cerebellar precursors (Lin and Bulleit, 1997), in the self-renewal of embryonic stem cells (Paling et al., 2004; Watanabe et al., 2006) and fetal neural progenitors (Sinor and Lillien, 2004), and in the survival of terminally differentiated neurons (Dudek et al., 1997). Also, $Akt3^{-/-}$ deficient mice have reduced brain size and weight, further indicating its crucial role in the development of the central nervous system (Tschopp et al., 2005). However, there is limited evidence for an Akt role in neural stem or progenitor cells of adult organisms.

CREB is a transcription factor activated in part by the phosphorylation of serine 133, after which it is capable of inducing the transcription of many classes of genes under the control of the cAMP response element (CRE). Several kinases have been shown to

phosphorylate CREB at this site, including protein kinase A (PKA) (Enslen et al., 1994), PKC (Manier et al., 2001), MAPKAPK (Xing et al., 1998), potentially Akt (Du and Montminy, 1998), and other kinases under various conditions (Shaywitz and Greenberg, 1999; Mayr and Montminy, 2001).

Here, we demonstrate that Akt is activated in AHNPCs downstream of FGF-2 and Shh and is involved in transducing the mitogenic activity of these factors, adding evidence to a developing hypothesis that Akt may play a general role in stem cell and progenitor proliferation or self-renewal (Paling et al., 2004; Sinor and Lillien, 2004; Watanabe et al., 2006). Additionally, Akt over-expression inhibits progenitor differentiation into glial and neuronal lineages. Furthermore, Akt signaling is mediated in part through CREB. These results demonstrate an important mechanistic link between mitogens, PI3K/Akt, CREB, and ultimately, adult neural progenitor proliferation and differentiation.

METHODS

Cell Culture

Adult neural progenitor cells were isolated from the hippocampi of 6-week-old female Fisher 344 rats as described (Palmer et al., 1999). Cells were cultured on tissue culture polystyrene coated with poly-ornithine and 5 μg/mL of laminin (Invitrogen) and grown in Dulbecco's modified Eagle medium (DMEM)/F-12 (1:1) high-glucose medium (Invitrogen) containing N-2 supplement (Invitrogen) and 20 ng/mL recombinant human FGF-2 (Peprotech).

For inhibitor studies, adult neural progenitor cells were plated at low density (1000 cells/well) on 96-well poly-ornithine/laminin-coated tissue culture plates with or without 20 ng/mL FGF-2, 100 n*M* recombinant rat Shh produced as described (Lai et al., 2003), or 1 ng/mL FGF-2 plus 100 ng/mL IGF-1 (Sigma). In some cases, the following compounds were included (all from Calbiochem, unless otherwise noted): LY-294002 (10 μM), PD-98059 (10 μM), rapamycin (0.5 μM), SB-203580 (10 μM), API-2 (1 μM), 2-naphthol-AS-E-phosphate (25 μM, Fluka), GSK3β Inhibitor II (5 μM), z-VAD-FMK Caspase Inhibitor V (20 μM), and cycloheximide (0.1 mg/mL, Sigma). Fifty percent media changes were conducted daily. After 5 days in culture, cell number was quantified using the WST-1 assay following the manufacturer's instructions (Roche) and utilizing a standard curve generated with known cell numbers.

Akt and CREB Cell Lines

Progenitor cells constitutively expressing mutant and wild type proteins of Akt and CREB were generated by retroviral infection. cDNA encoding the following proteins were kind gifts: wild type murine CREB [K. Saeki, International Medical Center of Japan (Saeki et al., 1999)], dominant negative murine $CREB_{S133A}$ [M. Montminy, Salk Institute (Lamph et al., 1990)], wild type murine Akt1 (S. Ferguson, Robarts Research Institute, London, Ontario Canada), and dominant negative (Akt-AAA) bovine Akt1 [from J. Woodgett, Ontario Cancer Institute (Wang et al., 1999)]. PCR products were subcloned into the MMLV retroviral vector CLPIT (Yu and Schaffer, 2006) containing the tetracycline-repressor element (Gossen et al., 1993) and a puromycin selection gene. Correct products were confirmed by sequence analysis. Retroviral vectors were packaged using CMV gag-pol and CMV VSV-G envelope helper plasmids by calcium phosphate transfection as described (Yu and Schaffer, 2006). Vectors were harvested, concentrated by ultracentrifugation, and titered on HEK 293Ts. Progenitor cells were infected at a multiplicity of infection of 1 IU/cell and were selected with 1 μg/mL puromycin (Sigma). To assay proliferation, cells were grown in 0 or 1 ng/mL FGF-2 in DMEM/F-12 + N-2 medium, in doses of tetracycline (tet) of 0, or 0.1 μg/mL. Fifty percent media changes were conducted daily, and proliferation was quantified using the WST-1 assay after 5 days in culture.

Immunoblotting

After 24-h of FGF-2 starvation, 20 ng/mL FGF-2 was added to progenitor cells, which were then lysed at various time intervals (0–1440 min) after addition. In some cases, samples were preincubated with small molecule inhibitors (listed in text and described earlier) for 2 h prior to FGF-2 addition. Lysis solution contained IGEPAL (1%, Sigma), sodium dodecyl sulfate (SDS, 0.1%), phenylmethanesulfonylfluoride (0.1 mg/mL, Sigma), aprotinin (0.03 mg/mL, Sigma), and sodium orthovanadate (1 m*M*, Sigma) in PBS. Lysate protein concentrations were quantified by BCA Protein Assay Kit (Pierce). Fifteen μg of protein from each lysate were electrophoretically separated by 12% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories). In cases where blots were stripped and reprobed, phosphorylated epitopes were probed first, as previously described (Funakoshi et al., 2002). Primary antibodies included: rabbit anti-CREB (1:1000), mouse anti-phospho-CREB (Ser133) (1:2000), rabbit anti-Akt (1:1000), and rabbit anti-phospho-Akt (Ser473) (1:500), all from Cell Signaling Technology. Secondary antibodies included donkey anti-rabbit IgG-HRP (1:5000) and goat anti-mouse IgG-HRP (1:5000) from Santa Cruz Biotechnology.

Akt Immunostaining

AHNPCs were seeded at 10,000 cells/well in Falcon 8-well chamber slides and starved of FGF-2 for 24 h. Medium containing 20 ng/mL FGF-2 was added to each well, and cells were fixed in 4% paraformaldehyde. Samples were blocked as previously described (Lai et al., 2003) and incubated overnight with rabbit anti-phopho-Akt (Ser473) antibody (Cell Signaling, 1:100). Slides were then washed and incu-

bated with Alexa 488 donkey anti-rabbit antibody and TO-PRO nuclear stain (Molecular Probes) for 2 h at room temperature. Slides were mounted with Pro-long Anti-Fade reagent and imaged on a Leica TCS confocal microscope.

Quantitative RT-PCR

AHNPCs were seeded at 500,000 cells per plate on 6 cm culture dishes (Falcon) in DMEM/F-12 + N2 medium containing either 20 ng/mL FGF-2 or 1% fetal bovine serum (Invitrogen) plus 1 μM retinoic acid (Biomol) to induce differentiation. Medium was replenished on day 2, and on day 4 RNA was isolated by TRIzol® (Invitrogen) according to manufacturer's instructions. cDNA's were then generated using Invitrogen's Thermoscript™ RT-PCR kit according to manufacturer's instructions. Using a BioRad iCycler, Taqman® probe QPCR was performed for the astrocytic marker GFAP and the neuronal marker β-tubulin III with the 18S ribosomal subunit as an internal control. GFAP and β-tubulin III probes from Biosearch Technologies contained FAM490 fluorophore with Black Hole Quencher® (BHQ), while the 18S rRNA probe contained CAL610 fluorophore with BHQ. Primer and probe sequences were as follows: GFAP: 5′-GACCTGCGACCTTGAGTCCT-3′, 5′-TCTCCTCCTTGAGGCTTTGG-3′, probe 5′-FAM490-TCCTTGGAGAGGCAAATGCGC-BHQ-3′; β-tubulin III: 5′-GCATGGATGAGATGGAGTTCACC-3′, 5′-CGACTCCTCGTCGTCATCTTCATAC-3′, probe 5′-FAM490-TGAACGACCTGGTGTCTGAG-BHQ-3′; 18S rRNA: 5′-GTAACCCGTTGAACCCCATTC-3′, 5′-CCATCCAATCGGTAGTAGCGA-3′, probe 5′-CAL610-AAGTGCGGGTCATAAGCTTGCG-BHQ-3′.

RESULTS

PI3K/Akt Mediate the Proliferative Signal from Multiple Mitogens

FGF-2 (Palmer et al., 1999), Shh (Lai et al., 2003), and IGF-1 (Aberg et al., 2003) are adult hippocampal neural progenitor mitogens. To determine which of the many possible downstream effectors of these factors are important for AHNPC proliferation, we utilized a series of established small molecule inhibitors. Cells were grown with 20 ng/mL FGF-2 or 100 nM Shh and inhibitors of PI3K (LY-294002), Akt (API-2), mTOR (rapamycin), MEK (PD-98059), p38 MAP (SB-203580), and GSK3β (GSK3β Inhibitor II). Results are shown in Figure 1(A) (FGF-2) and Figure 1(B) (Shh), normalized to the vehicle control.

Although the inhibitors of MEK, p38 MAP, and GSK3β did significantly alter proliferation compared to the DMSO + FGF-2 control [Fig. 1(A)], the inhibitors of known transducers in the PI3K pathway (LY-294002, API-2, and rapamycin) dramatically reduced cell proliferation [Fig. 1(A)]. In fact, addition of these drugs to cultures containing 20 ng/mL FGF-2 reduced proliferation levels to those seen in carrier control cultures without FGF-2.

As PI3K and Akt are known to function as antiapoptotic signals (Datta et al., 1999; Downward, 2004), the decreased cell numbers observed in the presence of PI3K pathway inhibitors could be due to either decreased cell proliferation or increased cell death. FGF-2 proliferation experiments were therefore also conducted in the presence of the caspase inhibitor z-VAD-FMK. The caspase inhibitor did not result in increased cell numbers, indicating that apoptosis did not play a role in the apparent anti-proliferative effects of these inhibitors [Fig. 1(A)]. Similarly, it should be noted that for all the PI3K pathway inhibitors with FGF-2, more cells were present after 5 days than were initially plated. Collectively, these data indicate that PI3K/Akt activity is important for robust cell proliferation in response to FGF-2. In addition, these results motivated a thorough analysis of the role of the PI3K/Akt pathway in AHNPC proliferation and FGF-2 signal transduction.

Accordingly, we investigated whether PI3K/Akt were equally important for other known AHNPC mitogens. Inhibitors of the PI3K/Akt pathway also caused a reduction in proliferation in Shh-stimulated cells [Fig. 1(B)], indicating that this pathway may transduce proliferative signals from mitogens not normally thought to act through PI3K/Akt. Interestingly, in contrast to FGF-2 result, the MEK inhibitor PD-98059 inhibited Shh-stimulated AHNPC proliferation to a similar extent as the PI3K/Akt inhibitors, representing a possible novel mechanism of Shh action.

To further confirm the importance of PI3K/Akt in AHNPC proliferation, we tested its role in the transduction of other mitogenic signals. Cells were grown in 1 ng/mL FGF-2 plus 100 ng/mL IGF-1, or 1 ng/mL FGF-2 plus 100 ng/mL VEGF. IGF-1 induced a significant increase in AHNPC proliferation over 1 ng/mL FGF-2 alone [Fig. 1(C)], whereas IGF-1 alone was unable to appreciably enhance cell proliferation (data not shown). Additionally, in this assay VEGF did not induce a significant increase in proliferation (data not shown). Importantly, proliferation in FGF-2/IGF-1 was significantly attenuated by the presence of LY-294002 [Fig. 1(C)]. However, PI3K inhibition also attenuated cell proliferation in the 1 ng/mL FGF-2 control, such that the 1 ng/mL FGF-2-only and 1 ng/mL FGF-2 plus 100 ng/mL IGF-1 samples were statistically similar when grown in the presence of inhibitor. This result therefore demonstrates a PI3K pathway dependence for the proliferative effect of IGF-1 over FGF-2 alone.

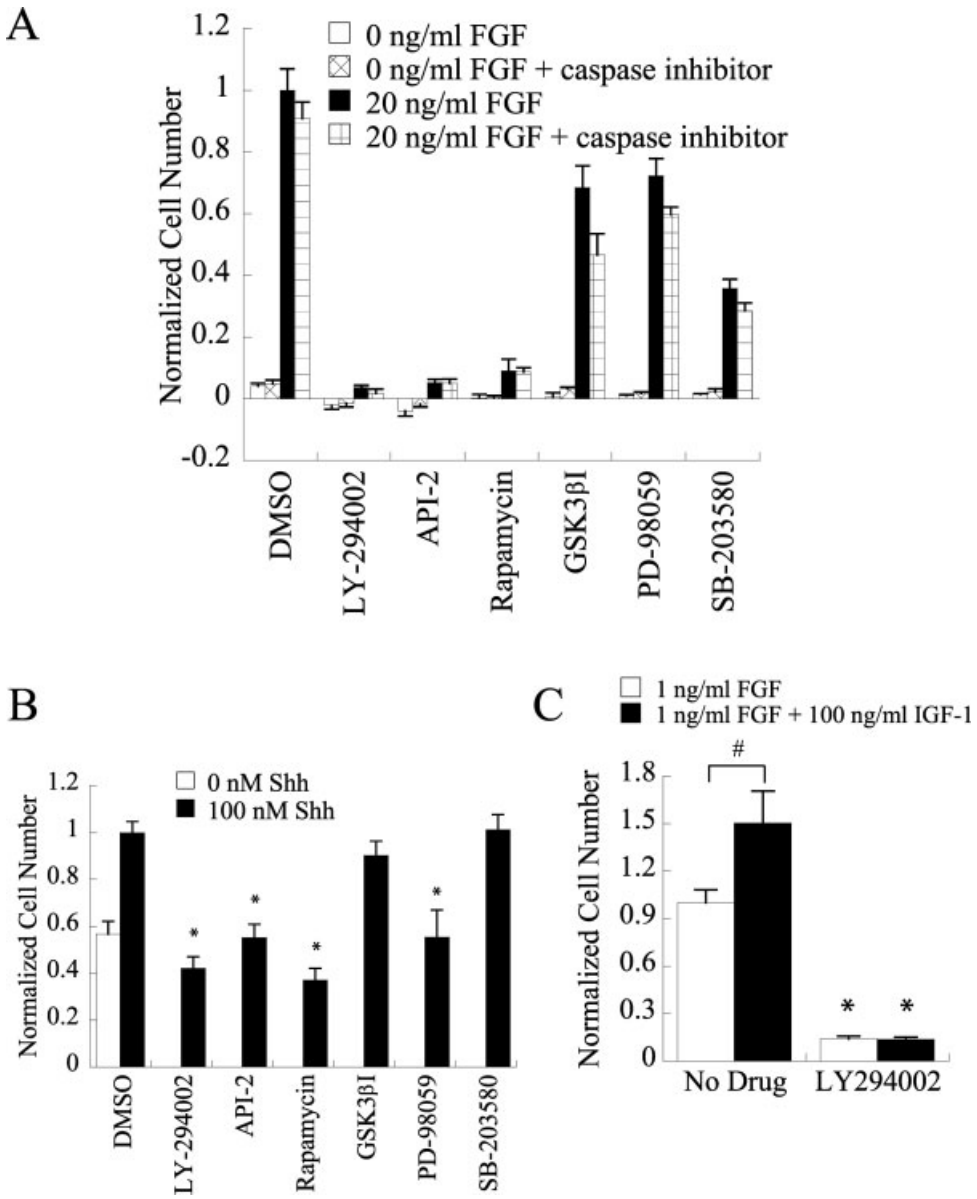

Figure 1 (A) Cells were seeded in quintuplicate in 96-well laminin-coated plates at a density of 1000 cells/well in indicated media. Cells were counted by WST-1 assay after 5 days in culture. Drugs added include: LY-294002 (PI3K antagonist, 10 μM), API-2 (Akt antagonist, 1 μM), rapamycin (mTOR antagonist, 500 nM), GSK3β Inhibitor II (GSK3βI, 5 μM), PD-98059 (MEK inhibitor, 10 μM), SB-203580 (p38 MAP inhibitor, 10 μM), and z-VAD-FMK (caspase inhibitor, 20 μM), all dissolved in DMSO. Data are represented as cell number normalized to a DMSO carrier control sample with FGF-2 (FGF). Media conditions include: no mitogen, 20 ng/mL FGF-2, and 20 ng/mL FGF-2 with caspase inhibitor. (B) Cells were cultured as described in (A); however, media conditions instead include: no mitogen and 100 nM Shh. Data are represented as normalized to a DMSO carrier control with 100 nM Shh. (C) Cells were cultured as in (A) and (B) in medium containing 1 ng/mL FGF-2 or 1 ng/mL FGF-2 plus 100 ng/mL IGF-1. Data are represented as normalized to the 1 ng/mL FGF-2 only sample. For all panels, error is the 95% confidence interval, and asterisks indicate a statistically significant difference from appropriate control ($p < 0.05$), and (#) denotes a statistically significant difference ($p < 0.05$) between the indicated conditions.

Akt is Activated upon AHNPC Stimulation by FGF-2 and Shh

In the initial pharmacological screen, PI3K/Akt pathway inhibition reduced progenitor proliferation in response to several mitogens. On the basis of these results, we sought to confirm the importance of Akt in FGF-2 signaling and AHNPC proliferation. Immunoblotting was performed to determine Akt activation. Cells were deprived of FGF-2 for 24 h, and at various time points after 20 ng/mL FGF-2 addition, cell lysate was collected and analyzed with antibodies against the phosphorylated, active form of Akt and total Akt [Fig. 2(A)]. Akt activation above baseline levels is observed within 10–15 min after FGF-2 addition, consistent with prior observations in murine fibroblasts (Park et al., 2003). This activation increased over the first hour of FGF-2 administration and peaked at 1–2 h. Subsequently, at 24 h post-simulation the phospho-Akt signal decreased to a level greater than the baseline activation in the absence of FGF-2. Cells were also preincubated with LY-294002 and rapamycin prior to FGF-2 addition. As anticipated, PI3K inhibition decreased Akt activation at all time points relative to the corresponding inhibitor-free condition [Fig. 2(A)]. Conversely, Akt signaling was unaffected by the presence of rapamycin (data not shown).

Interestingly, we also found that Akt is activated in response to Shh [Fig. 2(B)]. However, the time course of activation is delayed compared to FGF-2. As the slower response suggested that protein synthesis may be involved in this Akt activation, the experiment was also performed in the presence of cycloheximide. The protein synthesis inhibitor intriguingly substantially reduced Shh-induced activation of Akt [Fig. 2(B)].

To confirm immunoblotting results, we next immunostained for phospho-Akt in culture. Cells were again deprived of FGF-2 for 24 h. Growth factor (20 ng/mL) was then added, and the cells were stained for phospho-Akt. Representative cells are shown in Figure 2(C) without FGF-2 and after 10 min of FGF-2 exposure, where the increased fluorescence and characteristic membrane localization of phosphorylated Akt are clearly visible at the later time point.

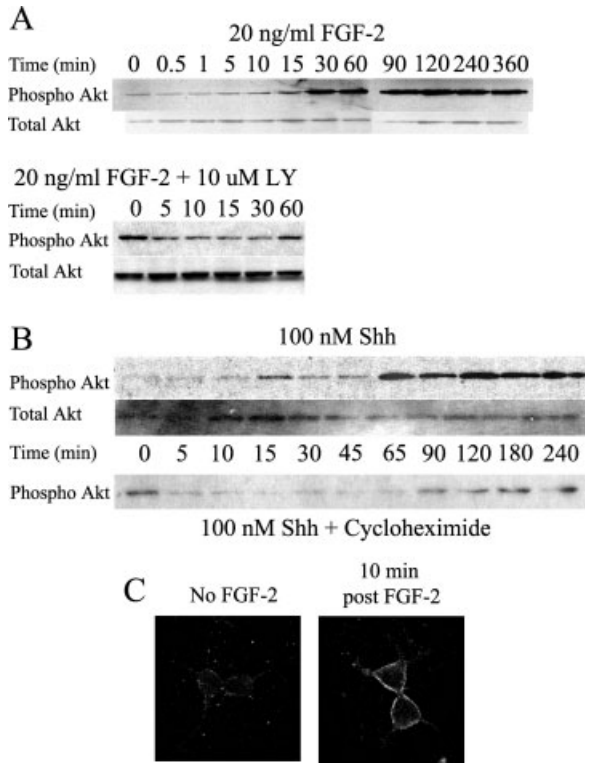

Figure 2 (A) Cell lysates were analyzed for activated Akt levels in cultures preincubated with or without LY-294002 (10 μM, PI3K inhibitor). Lysates were collected at indicated times (in minutes) after addition of FGF-2, and immunoblots were performed for Ser473 phospho-Akt and total Akt. All samples were exposed to 20 ng/mL FGF-2. (B) Shh immunoblots were performed as in (A). Conditions include 100 nM Shh with or without 0.1 mg/mL cycloheximide. (C) AHNPCs were fixed after 24 h of FGF-deprivation (No FGF-2) or after 10 min of exposure to 20 ng/mL FGF-2. Samples were stained with an antibody to Ser473 phosphorylated Akt and imaged by confocal microscopy.

Akt Promotes AHNPC Proliferation and Inhibits Differentiation

To provide genetic evidence to complement the pharmacological and immunoblotting data, we over-expressed wild type (wt) Akt. The cDNA was inserted into a retroviral vector under the control of a tetracycline-regulated promoter [where transgene expression decreases with increasing tetracycline concentration (Yu and Schaffer, 2006)]. Akt over-expression was confirmed by Western blot analysis [Fig. 3(A)], and proliferation of the mutant cell line, along with a cell line created with an empty control vector, was tested in a 5-day growth assay. Cells were cultured with or without 0.1 μg/mL tetracycline (tet) [Fig. 3(B)]. Even in the complete absence of FGF-2 these Akt over-expressers exhibited a dramatic increase in proliferation compared to cells infected with an empty vector control. Furthermore, the addition of tetracycline mitigated the proliferative effect.

We also over-expressed a dominant negative (dn) version of Akt containing alanine substitutions in the kinase domain and at sites of enzymatic activation by

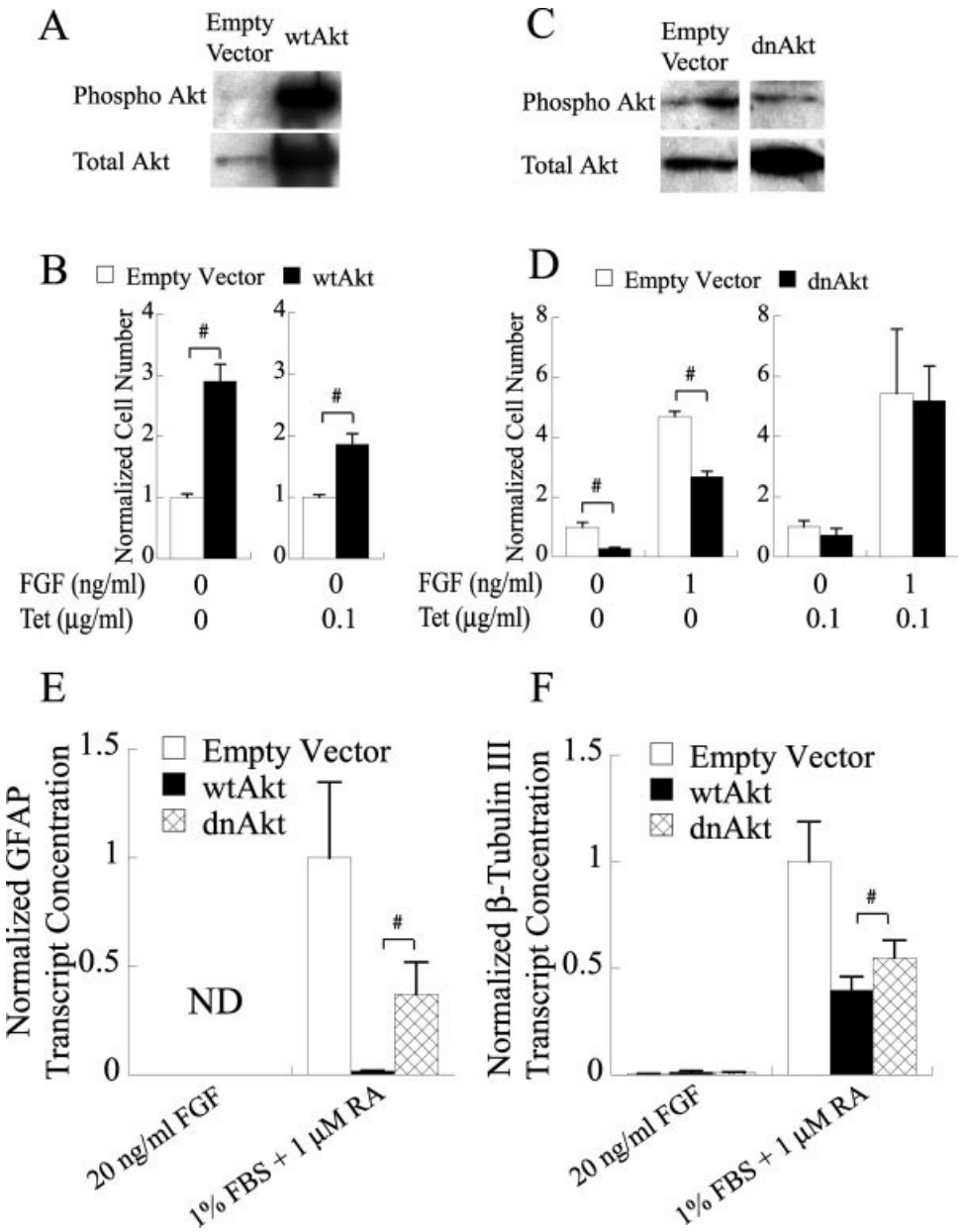

Figure 3 (A) Cells were stably infected with an empty retroviral vector or one containing wild type Akt (wtAkt), and Western blot analysis confirmed wtAkt over-expression. The blot was probed with Ser473 phosphorylated Akt antibody, stripped and reprobed for total Akt. (B) Increased proliferation was observed in cells expressing wtAkt. Cells were seeded in quintuplicate in 96-well laminin-coated plates at a density of 1000 cells/well in the indicated media and counted after 5 days in culture. Data in each panel are represented as cell number normalized to the empty vector control sample. (C) Western blot analysis confirmed over-expression of dominant negative Akt (dnAkt). The blot was probed with Ser473 phosphorylated Akt antibody, stripped, and re-probed for total Akt. All bands are from the same gel, but intervening lanes have been removed for clarity. (D) Decreased proliferation is observed in cells expressing dnAkt. Cells were stably infected with an empty retroviral vector or one containing dnAkt. Cells were seeded in quintuplicate in 96-well laminin-coated plates at a density of 1000 cells/well in indicated media and counted after 5 days in culture. Data in each panel are represented as cell number normalized to the empty vector control without FGF-2. (E, F) Cells were cultured in triplicate under proliferating (20 ng/mL FGF-2) or differentiating conditions (1% fetal bovine serum $+1$ μM retinoic acid) for 4 days. As measured by quantitative RT-PCR, GFAP (E) and β-tubulin III (F) expression are lower in cells over-expressing wild type Akt, while modestly lower GFAP and β-tubulin III expression is seen in cells expressing dominant negative Akt. Data are represented as transcript concentration normalized to the empty vector control sample. For all panels, error is the 95% confidence interval, and (#) denotes a statistically significant difference ($p < 0.05$) between the indicated conditions. ND: not detected.

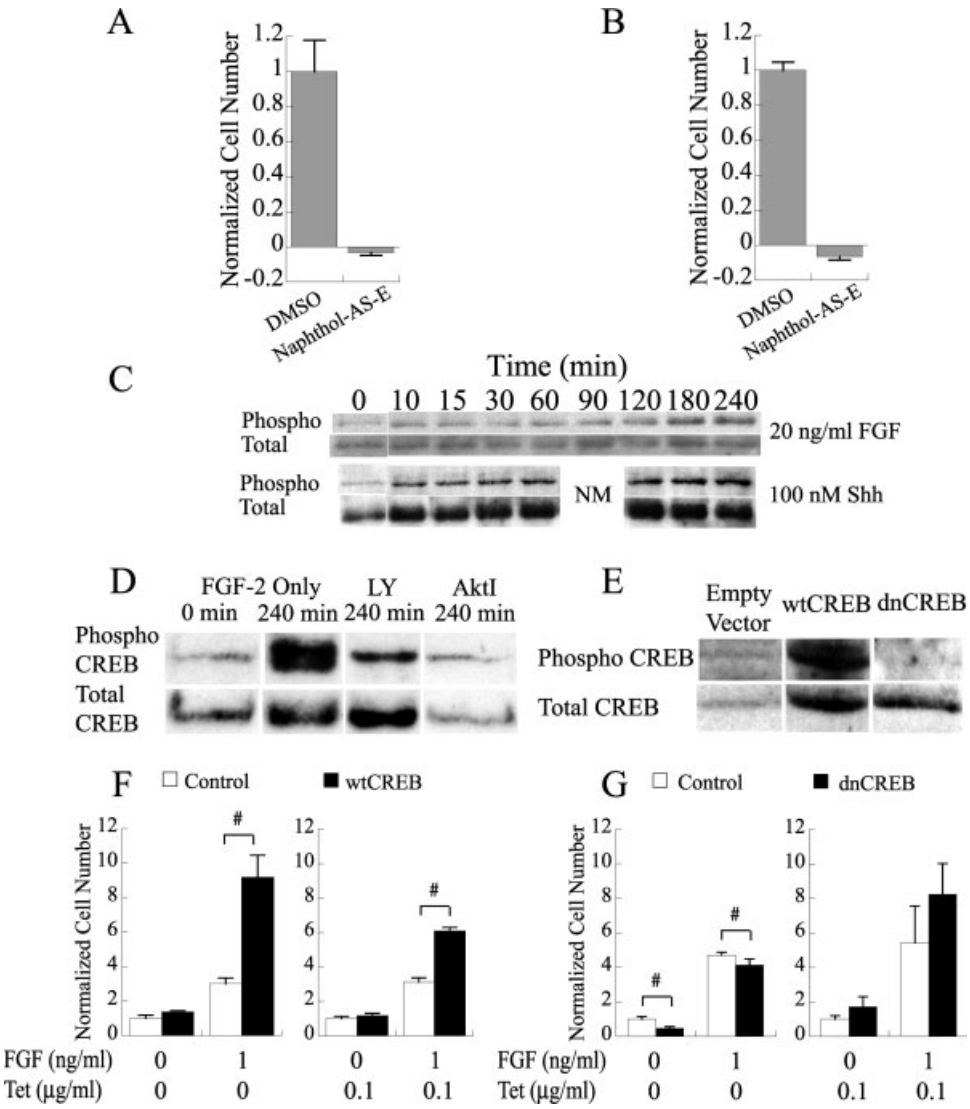

Figure 4 (A,B) Cells were seeded in quadruplicate in media containing either 20 ng/mL FGF-2 (A) or 100 nM Shh (B) and counted after 5 days in culture with 2-naphthol-AS-E-phosphate (CREB antagonist, 25 μM). Data are represented as cell number normalized to a DMSO carrier control sample. (C) Cell lysates were analyzed for levels of activated CREB. Lysates were collected at indicated times after addition of 20 ng/mL FGF-2 or 100 nM Shh, and immunoblots were performed for phospho-CREB and total CREB. NM: not measured. (D) CREB immunoblots were performed as above with cells that had been pre-incubated for 2 h with either LY-294002 (LY, 10 μM, PI3K inhibitor) or API-2 (AktI, 1 μM, Akt inhibitor). All bands are from the same gel, but intervening lanes have been removed for clarity. (E) Cells were stably infected with an empty retroviral vector, the vector containing wild type CREB (wtCREB), or the vector carrying a dominant negative CREB (dnCREB). Western blot analysis confirmed over-expression of wtCREB and dnCREB. The blot was probed with a Ser133-phosphorylated CREB antibody, stripped, and re-probed for total CREB. All bands are from the same gel, but intervening lanes have been removed for clarity. (F,G) Increased proliferation is observed (F) in cells over-expressing wtCREB, while decreased proliferation is observed in (G) cells expressing dnCREB. Cells were seeded in quintuplicate in the indicated media and counted after 5 days in culture. Data in each panel are represented as cell number normalized to the empty vector control without FGF-2. For all panels, error is the 95% confidence interval, and (#) denotes a statistically significant difference ($p < 0.05$) between the indicated conditions.

phosphorylation (Wang et al., 1999). dnAkt over-expression was confirmed by Western blot analysis of total Akt expression, while probing the same blot for Akt phosphorylated at Ser473 demonstrated reduced active Akt [Fig. 3(C)]. Cells were cultured either with or without 0.1 μg/mL tetracycline (tet) and with or without 1 ng/mL FGF-2 [Fig. 3(D)]. Proliferation of control cells cultured with FGF-2 was $\sim$4-fold higher than the FGF-2-free condition. Decreased proliferation was observed in the cells expressing dominant negative Akt, and tetracycline addition restored proliferation to levels comparable to control. It is interesting to note that dominant negative Akt expression did not completely halt proliferation, indicating a possible role of other signaling pathways.

We next analyzed Akt's effect on neural progenitor differentiation. Cells over-expressing wild type or dominant negative Akt were grown under proliferative conditions (20 ng/mL FGF-2) or stimulated to differentiate into astrocytic and neuronal lineages with 1% fetal bovine serum $+1$ μM retinoic acid, as previously described (Palmer et al., 1999). Quantitative RT-PCR of lineage markers was used to analyze cell differentiation, as we have previously reported (Abranches et al., 2006) and analogous to the use of promoter-luciferase constructs to quantify lineage marker expression (Kuwabara et al., 2004). Under proliferative conditions, GFAP, a marker highly expressed in astrocytes, was undetectable in all cell types [Fig. 3(E)]. Although the neuronal marker β-tubulin III was detected, there was no significant difference between all cell types under proliferative conditions [Fig. 3(F)]. As expected, fetal bovine serum and retinoic acid addition strongly upregulated lineage marker expression in naive cells. However, cells over-expressing wild type Akt exhibited a drastic ($\sim$50$\times$) decrease in GFAP expression and a more moderate ($\sim$2.5$\times$) decrease in β-tubulin III expression compared to empty vector control, indicating that Akt over-expression inhibits the ability of AHNPCs to differentiate into glial and neuronal lineages. Interestingly, cells over-expressing the dominant negative Akt exhibited lineage marker levels significantly greater than cells over-expressing wild type Akt, but still below empty vector control cells.

CREB Promotes AHNPC Proliferation and Acts Downstream of Akt

Akt has a number of potential downstream effectors (Cross et al., 1995; Datta et al., 1997; Scott et al., 1998; Brunet et al., 1999; Kane et al., 1999; Romashkova and Makarov, 1999; Zhou et al., 2001; Sarbassov et al., 2004; Eswarakumar et al., 2005). However, because CREB has been implicated as a regulator of progenitor proliferation (Carlezon et al., 2005), we probed potential connections between Akt and CREB. First, we found that both FGF-2- and Shh-induced proliferation were completely halted in the presence of the CREB inhibitor 2-naphthol-AS-E-phosphate [Fig. 4(A,B)]. We next analyzed the time course of CREB activation upon the administration of FGF-2 and Shh to AHNPCs [Fig. 4(C)] and found that it increased over 4 h, similar to Akt activation but with a later onset. We next measured CREB activation in FGF-2-stimulated cells in the presence of signal transduction inhibitors [Fig. 4(D)]. When cells were pre-treated with LY-294002 or the Akt inhibitor API-2, CREB activation was substantially decreased compared to drug-free control. There was no discernable long-term effect of rapamycin on FGF-2-induced CREB activation (data not shown).

To further analyze whether CREB is necessary or sufficient for cell proliferation, we also analyzed the effects of wild type and dominant negative CREB over-expression, which were confirmed by Western blot analysis [Fig. 4(E)]. Cell proliferation was assayed as in Figure 3, and the proliferation of control cells cultured with FGF-2 was $\sim$3-fold higher than the FGF-2-free condition, slightly different but consistent with the prior result [Fig. 3(D)]. In contrast to Akt, wild type CREB over-expression did not significantly alter proliferation in the absence of FGF-2. However, with just 1 ng/mL FGF-2, proliferation increased more than 3-fold relative to empty vector control [Fig. 4(F)], whereas tetracycline reversed proliferation to levels comparable with control. By contrast, the mutant CREB inhibited AHNPC proliferation by approximately half in the absence of FGF-2 [Fig. 4(G)]. However, that difference was less pronounced in the presence of 1 ng/mL FGF-2, indicating that the proliferative signal is not solely mediated by CREB. Finally, full recovery to levels indistinguishable from control cells was observed with tetracycline addition regardless of FGF-2 supplementation.

DISCUSSION

Although an increasing number of extracellular factors have been found to regulate the proliferation of adult hippocampal neural progenitors (Palmer et al., 1999, 2001; Yoshimura et al., 2001; Aberg et al., 2003; Fabel et al., 2003; Lai et al., 2003; Nyberg et al., 2005), the intracellular transducers that control AHNPC proliferation have not been extensively studied, with several exceptions (Mantamadiotis et al.,

2002; Nakagawa et al., 2002; Persson et al., 2003; Mao and Lee, 2005). Elucidating the signaling mechanisms that regulate AHNPC proliferation and differentiation will enhance our understanding of how the adult brain regulates neurogenesis, as well as lead to potential longer term exploration of modulating neurogenesis for therapeutic application [as reviewed (Lie et al., 2004)]. Our results are the first to demonstrate the importance of PI3K/Akt in adult hippocampal progenitor proliferation driven by multiple mitogens (FGF-2, Shh, and IGF-1).

IGF-1 has been shown to both stimulate AHNPC proliferation and activate PI3K/Akt (Aberg et al., 2003), and our results build upon this finding with chemical and genetic evidence that Akt is a central regulator of AHNPC proliferation and an inhibitor of cell differentiation. It has recently been shown in nonneural cells that Shh can activate a PI3K signal within 5–15 min (Kanda et al., 2003; Riobo et al., 2006); however, Shh stimulation of AHNPCs induced a delayed, protein synthesis dependent Akt activation [Fig. 2(B)], indicating a novel mechanism. For example, Shh could potentially upregulate growth factor signaling components, such as the platelet-derived growth factor receptor PDGFRα that is up-regulated by Shh stimulation of C3H10T1/2 cells (Xie et al., 2001). In addition, the Shh receptor Patched can regulate insulin-like growth factor-2 (IGF-2) expression (Hahn et al., 2000), which could potentially activate PI3K in an autocrine fashion. Since PI3K/Akt has recently been shown to act synergistically with Shh to stimulate Gli2 (Riobo et al., 2006) and N-myc (Kenney et al., 2004), this novel result of Shh transcriptional activation of Akt may represent a positive feedback loop that can further reinforce Shh effects on cells. Future work may elucidate the mechanism of Shh activation of Akt, investigate this potential positive feedback loop, and determine the specific Akt isoform(s) activated in AHNPCs.

We also found that MEK does not strongly mediate FGF-2-induced AHNPC proliferation [Fig. 1(A)], similar to results reported in embryonic carcinoma cells (Mao and Lee, 2005), but contrasting with other findings in AHNPCs (Aberg et al., 2003) and with known roles for MEK and p38 MAP pathways in the proliferation of other cell types (Kato et al., 1998; Schaeffer and Weber, 1999). Similar to our findings, primate and murine embryonic stem cell proliferation and self-renewal rely on Akt (Paling et al., 2004; Watanabe et al., 2006), and MEK pathway inhibition actually promotes murine embryonic stem cell self-renewal (Burdon et al., 1999). MEK pathway inhibition, however, did attenuate Shh activity [Fig. 1(B)], a novel result that should be explored in future work.

Akt also plays a role in AHNPC differentiation. When cultured under media conditions that strongly drive cell differentiation (Takahashi et al., 1999; Lai et al., 2003), AHNPCs over-expressing wild type Akt exhibited substantially lower ($\sim 50\times$) expression of the astrocytic marker GFAP [Fig. 3(E)]. GFAP expression was also modestly lower in cells expressing dominant negative Akt but was still below the control ($\sim 2.5\times$), perhaps consistent with the finding that Akt activation is involved in astrocytic differentiation induced by ciliary neurotrophic factor (Hermanson et al., 2002). The expression of the neuronal marker β-tubulin III was also lower ($\sim 2.5\times$) after exposing cells over-expressing Akt to differentiation conditions [Fig. 3(F)]. Again, this marker was modestly lower in cells expressing dominant negative Akt, consistent with Akt's role in neuronal differentiation and survival (Dudek et al., 1997; Vojtek et al., 2003). Together, these data support a model that high levels of Akt activation inhibit cell differentiation, whereas low Akt activation levels may be permissive or necessary for cell differentiation.

PI3K and Akt have a number of downstream effectors. mTOR functions downstream of Akt in embryonic cortical neural progenitor cells (Sinor and Lillien, 2004), consistent with our observed rapamycin inhibition of adult AHNPCs [Fig. 1(A)]. However, we also investigated CREB's role in FGF-2 induced proliferation in detail. A small molecule CREB inhibitor inhibited AHNPC proliferation, while a dominant negative form of the protein moderately decreased the cells' proliferative capacity [Fig. 4], indicating that CREB is not the sole mediator of proliferation. Moreover, wild type CREB overexpression sensitized cells to FGF-2, a result that also indicates that CREB still relies upon upstream signals to promote proliferation. Further studies will be necessary to quantitatively understand CREB's role in FGF-2 intracellular signaling and its relative importance compared to other transcription factors.

There are conflicting reports on PI3K/Akt pathway activation of CREB. An *in vitro* kinase assay suggests a direct phosphorylation of CREB by Akt (Du and Montminy, 1998), and cellular data in PC12 cells (Pugazhenthi et al., 2000), neonatal cardiomyocytes (Mehrhof et al., 2001), rat pituitary tumor cells (Hayakawa et al., 2002), and striatal neurons (Brami-Cherrier et al., 2002) imply that CREB is positively regulated by Akt. However, other studies suggest that Akt may repress CREB, since Akt phosphorylates and inactivates GSK3β (Cross et al., 1995), which may otherwise phosphorylate (at serine 129) and activate CREB (Fiol et al., 1994). However, since other reports indicate that GSK3β inhibits CREB (Grimes and Jope,

2001), it is unclear whether Akt inhibition should positively or negatively influence CREB via GSK3β. Future work will therefore be necessary to analyze the mechanism of Akt activation of CREB.

There are numerous downstream transcriptional targets of Akt, CREB, and the Shh-responsive transcription factor Gli that could influence or mediate proliferative effects. These include cell cycle components such as cyclin A (Mayr and Montminy, 2001) and cyclin D2, which has been shown to be important for adult neurogenesis (Kowalczyk et al., 2004). CREB can also activate targets such as *c-fos*, CREB itself, and more than 100 other genes [as reviewed in (Mayr and Montminy, 2001)]. In addition, since the over-expression of dominant negative forms of CREB and Akt do not completely halt AHNPC proliferation, other pathways are likely involved in proliferation.

Further work should also focus on downstream targets of Akt that inhibit AHNPC differentiation, particularly the dramatic inhibition of astrocyte differentiation [Fig. 3(E)]. This pathway may influence Sox family transcription factors such as Sox2, which is required for embryonic development and necessary for AHNPC maintenance and proliferation (Ferri et al., 2004).

Further work is required to analyze the role of Akt in adult neurogenesis *in vivo*, but these results do have several potentially intriguing implications. For instance, Robles et al. have found that Akt expression in the dentate gyrus is upregulated in rats performing spatial discrimination learning exercises (Robles et al., 2003), behavioral conditions that in another study have been shown to upregulate adult neurogenesis (Gould et al., 1999a). In addition, the PI3K/Akt pathway has a well documented role in cancer progression that makes it an attractive therapeutic target (Hennessy et al., 2005; Cully et al., 2006); however, our results indicate that Akt inhibition *in vivo* may have the unintended side effect of inhibiting the development of new neurons in the adult brain. Future work to determine the relative importance of different Akt isoforms to AHNPC proliferation may provide opportunities to target tumors without affecting neural progenitor function.

In summary, this work demonstrates that Akt is an important regulator of adult hippocampal neural progenitor cell proliferation and differentiation, as well as implicates CREB as a downstream effector. Recent work demonstrating a role for Akt in the proliferation of embryonic stem cells (Paling et al., 2004; Watanabe et al., 2006) and stem cells in the developing nervous system (Lin and Bulleit, 1997; Sinor and Lillien, 2004), in concert with our work with FGF-2, Shh and IGF-1, makes Akt an attractive potential target for understanding regulatory mechanisms of proliferation and self-renewal in various stem and progenitor cell types.

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