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Akt1 Gene Deletion and Stroke

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Abstract

Activation of Akt has been implicated as a major contributor to neuronal survival after an ischemic insult. Numerous neuroprotective agents have been shown to augment Akt activity, suggesting that this protein represents a major mechanism of cellular salvage after injury. Estrogen is known to augment Akt, but the possibility that Akt plays a differential role in the male and female brain has yet to be evaluated. In this study, we employed both pharmacological and genetic approaches to investigate the role of Akt in stroke. Utilizing a focal stroke model we show that deletion of the Akt1 isoform does not affect stroke outcome in either male or female mice. Akt1 deficient mice had equivalent levels of phosphorylated Akt (p-Akt) when compared to their WT controls following stroke suggesting that alternative isoforms can compensate for Akt1 loss. Secondly, estrogen's neuroprotective effect is maintained in Akt1^{-/-} mice and estrogen exposure did not enhance p-Akt levels in WT female mice. Thirdly, we show that inhibiting Akt using the direct pan-Akt inhibitor triciribine has no effect on stroke outcome despite dramatic reductions in p-Akt. Our study demonstrates the limitations of genetic mouse models and suggests that the importance of Akt to ischemic outcome remains unclear.

Keywords

Akt; triciribine; estrogen; stroke

Introduction

Akt (Protein kinase B) is a subfamily of serine/threonine protein kinase with oncogenic and antiapoptotic activities [1–3]. Three isoforms of Akt, Akt1, Akt2 and Akt3 have been identified [4]. Akt is activated by extracellular stimuli in a phosphatidylinositol 3-kinase (PI3k)-dependent manner. Activated Akt phosphorylates a variety of downstream proteins, including several associated with cell death pathways such as BAD, caspase-9, Forkhead, CREB and MDM2, leading to diminished apoptotic cell death [1,5].

Akt is activated via phosphorylation. It has been suggested that Akt activation is important to neuronal survival in ischemic brain. Levels of p-Akt (serine-473) transiently increases within hours after ischemia [1,6–9], returning to baseline within 24 hours. Increased Akt activation may represent a major mechanism by which a variety of neuroprotective agents protect

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ischemic brain [8,10,11] as diminished p-Akt levels are associated with cell death [1]. Administration of neuroprotective agents restore Akt activity as measured by elevations in p-Akt levels, however this may simply reflect non-specific effects of tissue salvage.

Akt is activated by PI3-Kinase [5]. Consistent with p-Akt's proposed pro-survival role, treatment with the PI3-Kinase inhibitors Wortmannin or LY294002, led to significant reductions in p-Akt levels and enhanced cell death *in vitro* [1] and *in vivo* [12]. However, PI3-kinase inhibitors may have additional targets beyond Akt making these studies difficult to interpret [13].

The strongest evidence that p-Akt plays a direct rather than correlative role in cell death comes from *in vitro* studies that demonstrated a reduction in cell death after transfection with constitutively active Akt and increased cell death after transfection with a dominant negative form of Akt. Interestingly, introduction of a dominant negative Akt increased basal, but not NMDA-induced, cell death [1]. Consistent with the hypothesis that Akt activation is involved in ischemic neuroprotection, mice overexpressing neuronal Akt showed significant reductions in infarct volumes compared to wild-type (WT) controls [14]. However, limited data are available on mice lacking Akt, which would be expected to lead to an exacerbation of injury due to the loss of p-Akt mediated neuroprotection. A recent study utilizing Akt1 deficient mice found that deletion protected male mice from stroke damage, and had no effect in female mice [15]. Hormonal effects on Akt have been documented [16] and the contribution of estrogen to this observed gender dichotomy has not yet been investigated.

Estrogen is a female hormone that has potent neurotrophic and neuroprotective roles in immature and adult brains [17]. There is good evidence to suggest that Akt plays a role in estrogen-mediated protection. Estrogen increases Akt phosphorylation *in vivo* and *in vitro* [16] and prevents injury-induced decrease of p-Akt in focal ischemia models [18]. P-Akt levels are reduced in ovariectomized mice, an effect that is reversed by estrogen replacement. However, infarct volumes are strikingly higher in oiled-treated animals, and the loss of p-Akt may simply represent a surrogate marker of increased ischemic damage rather than an estrogen-mediated neuroprotective mechanism [19].

The role that Akt plays in the response to cerebral ischemia remains unclear. In addition, the possibility that Akt plays a differential role in the male and female brain, or is related to hormonal exposure has yet to be evaluated. In this study, we employed both pharmacological and genetic approaches to assess 1) the role of Akt1 deficiency in stroke 2) the effect of Akt deficiency in male and female mice 3) the role of estrogen on Akt deficiency 4) the effect of inhibition of Akt with the direct and specific pan-Akt inhibitor; triciribine [20] on stroke outcome.

Materials and Methods

Akt1 KO mice

The present study was conducted in accordance with National Institutes of Health guidelines for the care and use of animals in research and under protocols approved by the Center for Lab Animal Care of University of Connecticut Health Center. The Akt1 knockout mice were bred in house from strains previously described [21]. All genetically modified mice were compared to their appropriate WT littermates. The animals used in all studies were age and weight matched (21–25 grams, 10–12 weeks of age).

Animal genotyping

Generation of Akt1-targeted mice is as previously described [21] backcrossed to C57BL/6 for at least 10 generations. Akt1^{+/-} (heterozygote) breeder pairs were obtained from Dr Morris J

Birnbaum at the University of Pennsylvania. Animals were genotyped by PCR using the following primers in a single reaction: 851, 5'-AGATCTTCTTCCACCTGTCTC-3'; 852, 5'-GCTCCATAAGCACACCTTCAGG-3'; and 853, 5'-GTGGATGTGGAATGTGTGCGAG-3' [21]. Phenotypically, Akt1^{-/-} mice of both genders are distinguishable from WT mice because of their smaller size after weaning and throughout adulthood [21].

Drug treatment

For estrogen treatment, WT or Akt1^{-/-} female mice were ovariectomized and were implanted subcutaneously with 17 β -estradiol (E2) pellets containing 180 μ g/ml of E2 in sesame oil (0.062-in. ID/0.125-in. OD) or oil-only control pellets 7 days prior to MCAO as previously described [22,23].

The Akt inhibitor triciribine (2 μ l, 3.25mM, dissolved in 20% DMSO) was injected intracerebroventricularly (ICV) to male WT mice at the coordinates (From bregma; -0.9mm lateral, -0.1mm posterior, -3.1mm deep) 1 hour prior to the onset of MCAO. Control animals were injected with the equal amount of 20% DMSO.

Focal cerebral ischemia model

Focal transient cerebral ischemia (90 mins MCA occlusion) was induced in WT or Akt1^{-/-} mice followed by reperfusion as described previously [23]. At the end of ischemia, the animal was briefly re-anesthetized, and reperfusion was initiated by filament withdrawal. In separate cohorts of Akt1^{-/-} male and Akt1^{-/-} female mice or WT control, as well as the triciribine/vehicle treated animals (n = 4 p/g), femoral arterial blood pressure and physiological measurements including blood pH, pO₂, pCO₂, and blood glucose, were obtained. Cortical perfusion using Laser Doppler Flowmetry was evaluated throughout MCAO and early reperfusion as described previously [23].

Behavioral scoring

At 24 hours after stroke, animal behavior were scored using the neurological deficits score system as follows: 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side; and 4, no spontaneous locomotor activity as described previously [23].

Histological assessment

At 24 hours after stroke, histological assessment was done as follows. Briefly, the animals were killed; their brains were immediately removed, and cut into 5 individual 2-mm slices using a surgical blade. The brain slices were stained with 1.5% 2,3,5-triphenyltetrazolium (TTC) at 37 °C for 30 mins and were fixed with 4% formalin. Images were digitalized, and the infarct volumes (corrected for edema) were analyzed using computer software (Sigmascan Pro5) as previously described [23].

Western blots

Western blots were done as described previously [23] with modification. Samples were obtained by removal of the brain from the skull followed by immediate removal of the cerebellum and occipital pole (posterior 2mm of brain) and olfactory/frontal pole (1st mm of brain) to focus on the area supplied by the MCA (Animal number: n = 5 for stroke groups and n = 3 for sham groups). Brains were the dissected into the right (ischemia) and left (non-ischemia) hemispheres. Briefly the samples were homogenized using lysis buffer and protein was loaded on a 4–15% gradient SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Akt (1:500) Akt1 (1:500), Akt2 (1:1000), Akt3 (1:500) and

phosphorylated-Akt (1:1000; Serine-473) were detected using corresponding antibodies from Cell Signaling. Beta-actin (1:5000; Sigma) were used as loading controls. The p-Akt antibody detected phosphorylated Akt1, Akt2 and Akt3 at ser-473. The lower band in the p-Akt blots is the p-Akt band. All blots were incubated overnight in primary antibody at 4 °C in TBS buffer containing 4% bovine serum albumin and 0.1% Triton X-100. The secondary antibodies (1:5000 for Mouse IgG, Amersham) were diluted and ECL (pico) detection kit (Amersham Biosciences) was used for signal detection.

Statistics

Data from individual experiments were presented as mean $\pm$ standard error of mean. One-way ANOVA (with Tukey post-hoc correction, when appropriate) was used for the comparison of the means between the experimental groups except the neurological deficit scores which were done by Mann-Whitney U-test. $P < 0.05$ was considered statistical significant. Induction of ischemia, behavioral and histological assessments were done by an investigator blinded to genotype/drug treatment.

Results

Stroke outcome in Akt1^{-/-} mice

To exam the role of Akt1 in cerebral ischemia, we compared the stroke outcome of male and female Akt1^{-/-} mice (90 min MCAO; 24 hours survival) to their corresponding WT littermates. Male Akt1^{-/-} deficient mice ($n = 8$) showed no difference in infarction volume compared to WT controls ($n = 8$) (cortex: KO $35.8 \pm 2.7\%$ versus WT $37.7 \pm 6.6\%$; striatum: KO $56.3 \pm 6.7\%$ versus $44.1 \pm 7.6\%$; total KO $41.1 \pm 3.0\%$ versus WT $40.5 \pm 5.1\%$) (Figure 1a). Examination of gonadally intact female mice showed similar effects. There was no difference in the infarct volume between female Akt1^{-/-} ($n = 7$) and female WT control ($n = 7$) mice in total (KO $28.0 \pm 5.3\%$ versus WT $28.6 \pm 4.4\%$), striatal, or cortical infarct volumes (Figure 1b). As expected, females had smaller strokes than age-matched males in each genotype secondary to the protective effect of estrogen.

No differences in mean arterial pressure, pH, pO₂, pCO₂, or blood glucose were seen between the male and female Akt1^{-/-} and their corresponding WT controls. Laser Doppler Flow (LDF) was equivalently reduced during ischemia ($12.7 \pm 2.6\%$ in male Akt1^{-/-} versus $10.9 \pm 0.4\%$ in male WT, $n = 4$ /gp) and was restored equally in early reperfusion ($84.2 \pm 7.8\%$ Akt1^{-/-} versus $79.3 \pm 5.3\%$ in male WT, $n = 4$ /gp) as was LDF in female Akt1^{-/-} and female WT littermates (see table 1).

We examined the time course of activation of Akt after stroke in WT mice (female) as measured by phosphorylation status (Luo, 2003). Stroke-induced Akt phosphorylation peaked at 8 hours after stroke, an effect that was no longer present at 24 hours - (figure 1c,1d) as has been described by others [6]. To investigate how Akt1^{-/-} deletion affects the level of p-Akt after an ischemic insult, we examined the level of p-Akt in Akt1^{-/-} and WT mice seven hours following induction of MCAO. Despite the complete absence of Akt1, there was no reduction in baseline or ischemia-induced p-Akt in the Akt1^{-/-} mice when compared to WT (Figure 1e,1f). There was a small compensatory increase in the protein levels of Akt3 in the Akt1^{-/-} mice (Figure 1e; $n = 5$ for stroke and 3 for sham).

Estrogen-mediated protection is present in Akt1^{-/-} mice

To exam if part of estrogen's protective effect is mediated by Akt1, we directly investigated the effect of estrogen in the Akt1^{-/-} mice following MCAO. Estrogen treatment ($n = 6$) significantly reduced the infarct volume in female ovariectomized Akt1^{-/-} mice in comparison to oil treated mice ($n = 7$) (cortex: E2 $14.5 \pm 4.5\%$ versus oil $47.2 \pm 4.3\%$ $p < 0.05$; stratum:

E2 $39.1 \pm 8.8\%$ versus oil $66.9 \pm 5.2\%$ $p < 0.05$; total: E2 $16.3 \pm 1.8\%$ versus oil $46.3 \pm 5.4\%$ $p < 0.05$) (figure 2a). We also assessed the effect of estrogen in ovariectomized WT female mice to serve as a control for this study. Infarct volume in ovariectomized WT mice was significantly decreased by the treatment of estrogen ($n = 6$) when compared to control group treated with oil ($n = 7$) (cortex: E2 $17.8 \pm 5.5\%$ versus oil $44.7 \pm 6.0\%$ $p < 0.05$; striatum E2 $44.2 \pm 13\%$ versus oil $74.4 \pm 3.9\%$ $p < 0.05$, total E2 $23.3 \pm 5.8\%$ versus oil $49.4 \pm 5.9\%$ $p < 0.05$) (figure 2b). There was no effect of gene deletion on stroke outcomes consistent with intact female mice (figure 1). Estrogen treatment also led to improved short-term behavioral outcomes as measured by the neurological deficits score in both Akt1^{-/-} (E2 1.8 ± 0.3 versus oil 3.0 ± 0.2 $p < 0.05$) and WT mice (E2 1.5 ± 0.2 versus oil 2.4 ± 0.2 $p < 0.05$) (table 2).

In addition, p-Akt levels were also examined in the E2 treated female ovariectomized WT mice 7 hours following the onset of MCAO. E2 treatment did not significantly enhance p-Akt levels following ischemia (Figure 2c, 2d, band 2 and band 5) or in sham-treated mice (band 1 versus band 4).

Pan-Akt inhibitor triciribine treatment and the stroke outcome

To ensure adequate delivery of the agent to the brain, and to increase specificity, we injected triciribine directly into the ipsilateral ventricle one hour prior to stroke. Direct CNS application of the AKT inhibitor had no effect on infarct outcome (Cortex: Triciribine 51.3 ± 6.0 versus vehicle 42.7 ± 8.4 ; Striatum: Triciribine 45.2 ± 15 versus vehicle 51.2 ± 9.4 ; Total: 46.0 ± 7.6 versus 44.2 ± 5.5 , $n = 5/pg$) (Figure 3a) however dramatic reductions in p-Akt levels were seen in a separate cohort (seven hours after MCAO) suggesting a potent inhibition of p-Akt with triciribine administration (Figure 3b and 3c, band 1 versus band 4).

Discussion

This study demonstrates several important findings that specifically relate to the role of Akt in ischemic brain. Firstly, deleting the Akt1 gene does not affect stroke outcome in either male or female mice. Additionally, the levels of p-Akt were not affected by the loss of the Akt1 isoform in mice and were equivalently elevated after injury in WT and Akt1^{-/-} mice. Secondly, estrogen-mediated neuroprotection is maintained in Akt1^{-/-} mice, suggesting that the Akt1 isoform may not be an important mediator of estrogen-induced protection. Estrogen exposure did not enhance activated Akt levels (as measured by p-Akt) in WT female mice. Thirdly, the Akt inhibitor triciribine when injected directly into the brain dramatically reduced p-Akt levels but had no effect on the stroke outcome. These studies also highlight some general issues cautioning against over-reliance on either genetic models or pharmacological agents in the evaluation of molecular events in stroke. The importance of examining compensatory changes in other systems, as well as the examination of downstream effects of gene deletion should be routinely evaluated. Deletion of the Akt1 isoform led to complete absence of Akt1 protein (Figure 1e), however a compensatory increase in basal levels Akt3 were seen. As a result, total Akt levels are unchanged in the Akt1^{-/-} mice, and most importantly, stroke-induced increases in the activated form of Akt, p-Akt, are unchanged. It is not surprising that these mice have no significant stroke phenotype, if p-Akt is the important mediator of stroke outcome. However, the importance of p-Akt as a mediator of neuroprotection is not completely consistent with data showing a dramatic reduction in p-Akt levels in the brains of WT mice administered triciribine icv (figure 4b and c) which showed no increase in stroke damage as would be expected by inhibition of anti-apoptotic p-Akt.

Changes in p-Akt levels have been well documented in numerous ischemia models including both global [24–26] and focal cerebral ischemia [9,27]. These reports showed a temporal increase of p-Akt at serine-473 after injury [9,24–26]. Our results are consistent with these previous reports, with an early deactivation of Akt (see band 1 in figure 1c) followed by a

sustained increase at four and eight hours after stroke. This is the first report confirming that p-Akt elevations also occur after stroke in the WT female brain. As we have previously described striking gender dichotomies in other cell death pathways [23] we examined the effect of Akt1 gene deletion in both genders. Previous studies in mouse hearts have demonstrated a greater recovery in cardiac function and higher levels of p-Akt after preconditioning in females compared to males. Additionally, p-Akt is induced by E2 in numerous experimental models [28,29], suggesting a possible effect of gonadal hormones. We examined both of these possibilities in our studies.

In this case, neither male nor female Akt1^{-/-} mice showed any response to the loss of Akt1. This is in contrast to a recent study by Kitano et al., which demonstrated a modest decrease in stroke damage in male but not female Akt1^{-/-} mice. There are several explanations for these differences. In our studies, a milder insult was used (90 minutes instead of 2 hours), with correspondingly smaller infarcts. Indeed at 90 minutes of occlusion, stroke outcome in our male WT and Akt1^{-/-} mice were similar to that of the male Akt1^{-/-} mice in Kitano's study suggesting that the level of damage may play a role in stroke outcome in this strain. Indeed, as the females used in that study had intact gonadal function, they had much smaller infarcts compared to males, an effect seen in both WT and knockout strains, perhaps masking the female effects. We examined both ovariectomized and E2-replaced Akt1^{-/-} female mice in our study, to directly assess hormonal effects. As expected, ovariectomized females had significantly larger infarcts than either E2-treated or intact females, a finding that was seen in both WT and Akt1^{-/-} mice. This suggests that the Akt1 isoform does not mediate the protective effect of estrogen. Indeed Akt1^{-/-} mice maintained their sensitivity to estrogen's protective effect, and E2 treatment did not enhance stroke-induced p-Akt levels in WT females (Figure 2c).

Previous work has suggested a major role of Akt in estrogen-mediated neuroprotection. Estrogen increases Akt phosphorylation in both *in vivo* and *in vitro* models of ischemic injury [16]. However as there is an almost universal reduction in injury with E2 treatment enhanced neuronal survival could account for this apparent "up-regulation" in p-Akt [18]. Both p-Akt and phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2) pathways are activated within minutes of E2 exposure [28,29]. E2-induced Akt activity may be secondary to non-genomic effects in which E2 binds to the plasma membrane estrogen receptor, subsequently activating Akt through the classic Akt/PI3-K pathway [19].

Estrogen is a pleiotropic hormone and it produces neuroprotection through multiple mechanisms such as anti-inflammation or preserving cerebral blood flow ([16,30]. In our studies, no enhancing effects of E2 were seen on p-Akt levels in WT female brains, but we only examined one time-point. We chose seven hours as it represents a peak of Akt activity. Certainly this does not preclude direct effects of E2 on p-Akt at other time-points, especially if effects are rapid and non-genomic. It is possible that E2 reduces the early apparent deactivation of Akt (see 2 hours; band 1 Figure 1c) which was not evaluated in these studies. Our studies also assayed the effect of p-Akt on brain homogenates, and it is possible that effects are seen only in one cell type (ie. neurons, or astrocytes), or that the effects are only limited to one area of brain. In any case, the Akt1 isoform does not appear to mediate any putative E2 effect, as Akt1^{-/-} mice were equally responsive to its protective action.

Most of the studies examining activation of p-Akt have focused on changes in phosphorylation of Ser473 as this is consistently elevated at early time points after reperfusion. However, it is well described that complete activation of Akt requires phosphorylation of both a threonine and serine residue [20]. Previous works suggests that the regulation of these sites is uncoupled in ischemic brain. Unlike the well documented increase in Ser 473 phosphorylation with ischemia, phosphorylation of Akt1 on Thr-308 is reduced after reperfusion [9] and remains consistently down-regulated. It is possible that changes in the Thr308 site are important, but

during later recovery. In our experience, the infarction is completed within 24 hours and by then p-Akt (ser) levels are returning to baseline therefore, we specifically chose to evaluate both a 24 hour end-point and focused on the Ser-473 site in these early studies.

Three isoforms of AKT have been described. Our work clearly demonstrates that the selective deletion of the Akt1 isoform does not change p-Akt levels or stroke outcome. Deletion of one isoform of the Akt gene family may lead to compensation by the other isoforms which would explain the lack of downstream effects (p-Akt levels). Our data show that Akt2 protein levels are equivalent in WT and Akt1^{-/-} mice but Akt3 protein level are increased in the Akt1^{-/-} mice, especially in stroke brains (figure 1e). The significance of this is not known. Data from cell cultures suggest that only a small amount of any of the isoforms is sufficient for cell survival. Utilizing both Akt1^{-/-}, Akt2^{-/-} and Akt1^{-/-} + Akt2^{-/-} mouse embryonic fibroblasts (MEFs), Liu and colleagues found that levels of Akt3 protein and p-Akt are unchanged by deletion of one or both of the other Akt isoforms [31]. Addition of Akt3 siRNA to the Akt1^{-/-} + Akt2^{-/-} MEF's led to a dramatic reduction in Akt, and enhanced sensitivity to cellular stressors. The levels of p-Akt were not directly assessed in these studies but it would appear by both these *in vitro* studies and our *in vivo* work that cells need minimal levels of Akt to survive. It is likely that only near complete loss of all Akt isoforms would significantly reduce p-Akt levels or have noticeable effects on stroke outcome. This is consistent with our pharmacological data that showed striking reductions in p-Akt levels after icv triciribine administration, with no exacerbation in stroke outcome.

Due to the limitations in our genetic studies we utilized the pan-Akt inhibitor, triciribine, locally (icv) as an alternative strategy to reduce p-Akt. Our results show that although triciribine potentially reduced stroke-induced activation of p-Akt after icv administration, it had no effect on stroke outcome. To our knowledge, this is the first report investigating the effect of a direct pan-inhibitor of Akt in an ischemia model. This drug was shown to be a non-isoform-specific Akt inhibitor at much lower doses than previously tested [20]. Triciribine is highly selective for Akt and has no known effects on upstream activators of Akt such as PI3-kinase and PDK1 [20] making it more selective than PI3K inhibitors such as Wortmannin and LY294002 which compete at the ATP binding site of the lipid kinase catalytic domain of all PI3Ks leading to broad specificity [32]. Earlier studies utilizing LY294002 demonstrated a reduction in p-Akt (Ser-473) levels and an increase in DNA damage after focal stroke [6,15]. The specificity of these upstream inhibitors for the Akt pathways is poor and conflicting data in *in vitro* models has been documented as LY294002 had a protective effect in PC12 cells exposed to oxygen-glucose deprivation [33]. However, the exact mechanism by which triciribine changes pAkt levels is unknown. It has been suggested that triciribine neither functions as ATP competitor nor as the substrate competitor [20]. It has been shown that triciribine does not inhibit the known upstream activators of Akt such as PI3 kinase and PDK1 although triciribine inhibits numerous down stream target of Akt [20]. From our data, it appears that triciribine inhibited Akt through, at least in part, lowering the total Akt protein level in the ischemic brain. Triciribine can act as a purine analog leading to the inhibition of DNA synthesis and subsequent protein production which may explain the reduction in the ischemic brain [reviewed in 34]. It has been well documented that ischemia leads to translation arrest and perhaps the reduction in pAkt is only seen under stress conditions such as stroke. Indeed, triciribine has a much more potent effect on pAkt inhibition in cell lines that have high level of pAkt expression (making it an attractive cancer target). Perhaps the high levels of pAkt seen in stroke brain are necessary for full activation of this agent. In any case, this agent did not lead to any changes in infarct outcome despite its dramatic effects on pAkt levels.

Conclusion

Akt1 gene deletion does not affect stroke outcome. The neuroprotective effect of estrogen is independent of Akt1. Our study highlights it is difficult to draw specific conclusions with a single gene deletion when multiple isoforms of a protein are expressed. Compensatory effects on other pathways and isoforms should always be examined and studies using knockout animals should be interpreted with caution. We have also shown that inhibiting Akt using a pan-inhibitor had no effect on the stroke outcome despite dramatic reduction in activated Akt in the ischemic hemisphere. The contribution of Akt to neuroprotection is not clear as the elevations in p-Akt seen after in vivo stroke injury may be simply related to the degree of injury. Augmentation of this pathway may not be a feasible approach to neuroprotection in stroke.

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Figure 1.

Akt1 deletion did not affect the stroke outcome in mice (figure 1a for male and figure 1b for female) subject to 90 min MCAO with 24 hours survival. Cortical, striatal, and total infarct volumes (shown as % of the non-ischemic hemisphere). Mean $\pm$ sem (* $p < 0.05$, One-way ANOVA, compared to corresponding wt control). ST: stroke hemisphere; NS: non-stroke hemisphere of the stroke mice brain; SH: sham-operated mice brain; Figure 1c and d: the time course (2, 4, 8, and 24 hours after the onset of MCAO) of p-Akt level following MCAO in female wt mice. Figure 1e: Akt1, Akt2, Akt3, and p-Akt profiles 7 hours following MCAO in male Akt1 ko and WT mice. Figure 1f: p-Akt blots were expressed as the percentage of control band (β -actin). Antibodies detecting either unphosphorylated Akt, Akt1, Akt2 and Akt3, or phosphorylated Akt (ser-473) and β -actin, which serves as a loading control (*bottom panel*), were used.

Figure 2.

The robust neuroprotective effect of estrogen was maintained in ovariectomized female Akt1^{-/-} mice subject to 90 min MCAO with 24 hours survival. Cortical, striatal, and total infarct volumes were measured (% of the non-ischemic hemisphere). 2a: ovariectomized female Akt1^{-/-} mice; 2b: ovariectomized female WT mice. Mean ± sem (*p < 0.05, One-way ANOVA, compared to vehicle-treated mice control). Figure 2c and 2d: estrogen treatment did not enhance the p-Akt levels in female ovariectomized wt mice at 7 hours following stroke. ST: stroke hemisphere; NS: non-stroke hemisphere of the stroke mice brain; SH: sham-operated mice brain; p-Akt blots were expressed as the percentage of control band (β-actin). Antibodies detecting either nonphosphorylated Akt, Akt1, Akt2 and Akt3, or phosphorylated Akt (ser-473) and β-actin, which serves as a loading control (*bottom panel*), were used. Estrogen pellets were implanted into (s.c.) the Akt1^{-/-} (figure 2a) and WT (figure 2b) female ovariectomized mice 7 days prior to the MCAO. Controls were implanted with vehicle pellets.

Figure 3.

I.C.V treatment of triciribine did not affect the stroke outcome (90 min MCAO) in male WT mice (figure 3a) but dramatically reduced p-Akt level (figure 3b and 3c). Controls were given 20% DMSO. For infarct measurements, mice were subject to 24 hours survival from the start of MCAO and for protein measurements, the survival time was 7 hours. Cortical, striatal, and total infarct volumes (shown as % of the non-ischemic hemisphere). ST: stroke hemisphere; NS: non-stroke hemisphere of the stroke mice brain; SH: sham-operated mice brain; p-Akt blots were expressed as the percentage of control band (β -actin). Antibodies detecting either unphosphorylated Akt, Akt1, Akt2 and Akt3, or phosphorylated Akt (ser-473) and β -actin, which serves as a loading control (*bottom panel*), were used. Mean $\pm$ sem (* $p < 0.05$, One-way ANOVA, compared to vehicle-treated mice control).

Table 1
Physiological parameters (table 1a) and LDF (Laser Doppler Flow) (table 1b) measured in WT, and Akt1^{-/-} male and Akt1^{-/-} female mice. There were no significant differences between the Akt1^{-/-} mice and their corresponding WT controls.

Table 1a			pH	CO2 (mmHg)	O2 (mmHg)	Glucose	MABP
Pre-ischemia	WT		7.37 ± 0.032	39.6 ± 4.4	114 ± 9.2	137 ± 7.8	94 ± 3.4
	Akt1 ^{-/-} male		7.37 ± 0.018	40.2 ± 3.2	114 ± 6.3	157 ± 20	98 ± 4.7
Ischemia	WT		7.33 ± 0.018	42.1 ± 3.6	110 ± 7.4	165 ± 21	95 ± 4.0
	Akt1 ^{-/-} male		7.33 ± 0.026	43.5 ± 3.9	104 ± 5.4	145 ± 18	99 ± 5.3
Pre-ischemia	WT		7.37 ± 0.022	40.0 ± 1.2	121 ± 17	125 ± 5.4	93 ± 5.0
	Akt1 ^{-/-} female		7.36 ± 0.025	38.3 ± 1.7	126 ± 3.9	119 ± 16	96 ± 5.3
Ischemia	WT		7.30 ± 0.034	45.5 ± 4.2	115 ± 11	132 ± 16	97 ± 5.8
	Akt1 ^{-/-} female		7.33 ± 0.012	42.2 ± 2.5	114 ± 8.5	107 ± 5.4	98 ± 3.1
Table 1b			Reperfusion (%)				
Male	WT		Ischemia (%)				
	Akt1 ^{-/-}		10.9 ± 0.4		79.3 ± 5.3		
Female	WT		12.7 ± 2.6		84.2 ± 7.8		
	Akt1 ^{-/-}		12.6 ± 1.0		87.8 ± 3.4		
			13.4 ± 2.2		80.8 ± 5.6		

There were no significant differences between the Akt1^{-/-} mice and their corresponding WT controls. The physiological parameters were measured prior to and 60 min after MCAO; LDF was shown averaged over 90 minutes ischemia or 30 minutes reperfusion and expressed as the percentage of the base line.

Table 2

Estrogen reduced the neurological deficit scores in both the Akt1^{-/-} and WT ovariectomized female mice subject to 90 min MCAO with 24 hours survival (Mean $\pm$ sem; *p < 0.05 Mann-Whitney U-test).

	Oil-treated	Estrogen-treated
Akt1 ^{-/-} mice	3.0 $\pm$ 0.2	1.8 $\pm$ 0.3 *
WT mice	2.4 $\pm$ 0.2	1.5 $\pm$ 0.2 *