Induction of the cell survival kinase Sgk1: a possible novel mechanism for α-phenyl-N-tert-butyl nitrone (PBN) in experimental stroke. Supplementary File

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Generation of Sgk1$^{-/-}$ mice.

The entire *Sgk1* gene (obtained from Genome Systems, St. Louis, MO) was subcloned into pSL301[1]. Three loxP sites were introduced into the introns in the *Sgk1* locus. The first was within intron 1, using a BsaHI restriction site, while the second and third loxP sites were inserted into intron 6 at the XcmI site bracketing a neo expression cassette. Two FRT sites were introduced, flanking the neo cassette close to the second and third loxP sites[1]. These sites allow removal of the neo cassette in vivo by crossing mice with the floxed SGK1 and neo cassette with FLPe deleter mice. After linearization, the targeting construct was electroporated into R1129/Sv x 129/Sv-CP embryonic stem (ES) cells. Recombinant clones with the desired recombination were identified by Southern blotting.

Positive ES clones were microinjected into C57BL6-derived blastocysts and implanted into pseudo-pregnant foster females. Progeny were identified by PCR[1] and Southern blot analysis was used to verify integration of the transgene. After germ line transmission of the modified allele, heterozygous floxed Sgk1 mice were inbred to generate mice homozygous for the floxed Sgk1 allele. To generate Sgk1$^{-/-}$ mice, homozygous floxed Sgk1 mice were crossed with global Cre-deleter mice (BALB/c-Tg(CMVcre)1Cgn/J, from Jackson Laboratory, Bar Harbor, ME). In the offspring, nucleotides 121–1615 (encompassing exons 2–6) of *Sgk1* were eliminated. Progeny were screened by PCR[1]. Sgk1$^{-/-}$ mice were then backcrossed to 129/SvJ wild-type mice for five generations.

Sgk1$^{+/-}$ heterozygotes were transferred to St George's University of London and crossed with C57/BL6 for five generations, then maintained on this background throughout the experimental series. Litters were genotyped by PCR using three primers: SGK1F1: 5'-TTCACTGCTCCCCTCAGTCT-3'; SGK1wtR : 5'- AAGAGAGGAAAAGGGGAGATGG-3'; SGK1R2: 5'-TCAAACCCAAACCAAGCAAT-3'. See Fig S1. Offspring from all subsequent matings were routinely genotyped. On mating of heterozygous breeding pairs the proportion of Sgk1$^{-/-}$ offspring was low (non-Mendelian) as reported by others for Sgk1 null mice on a C57/BL6 background[2, 3]. For this reason sister colonies of Sgk1$^{-/-}$ and Sgk1$^{+/+}$ breeding pairs were established.

Supplementary Methods for RT-PCR

RT-qPCR was used to quantify the expression of Sgk3 in the mouse brain lysates using a Taqman Gene Expression Assay (Applied Biosystems-Life Technologies, Paisley, UK). Gapdh, the house regulating gene was also used as a normalising control. A standard curve of cDNA was run from a local standard mouse brain sample, with a known cDNA concentration (200ng/µl). The standard curve was generated by having 4 serial dilutions of our standard sample cDNA: 0.1, 1.0, 10 and 100 ng/µl. The following were added into each reaction: 10µl of master mix, 1µl of the corresponding probe (either Sgk3 or gapdh) and 10µl of cDNA of each serial dilution. RT-qPCR was performed using the CFX96 Touch Real-Time PCR Detection System at the optimal cycling temperatures specified by the manufacturers for the Taqman assay. Analysis of RT-qPCR using Biorad CFX Manager 3.0 software allowed the cycle threshold (Cp) to be obtained, see Figure S2. For each cDNA sample, the Cp value for Sgk3 was subtracted from the Cp value for gapdh, to give the ΔCp value. The expression of the target gene, relative to gapdh, was obtained as $E = 2^{-\Delta Cp}$.

Supplementary File Reference List

1. Fejes-Toth G, Frindt G, Naray-Fejes-Toth A, Palmer LG. Epithelial Na+ channel activation and processing in mice lacking SGK1. *Am J Physiol Renal Physiol* 2008; 294: F1298-F1305.

2. Catela C, Kratsios P, Hede M, Lang F, Rosenthal N. Serum and glucocorticoid-inducible kinase 1 (SGK1) is necessary for vascular remodeling during angiogenesis. *Dev Dyn* 2010; 239: 2149-2160.

3. Wulff P, Vallon V, Huang DY, Volkl H, Yu F, Richter K et al. Impaired renal Na(+) retention in the sgk1-knockout mouse. *J Clin Invest* 2002; 110: 1263-1268.

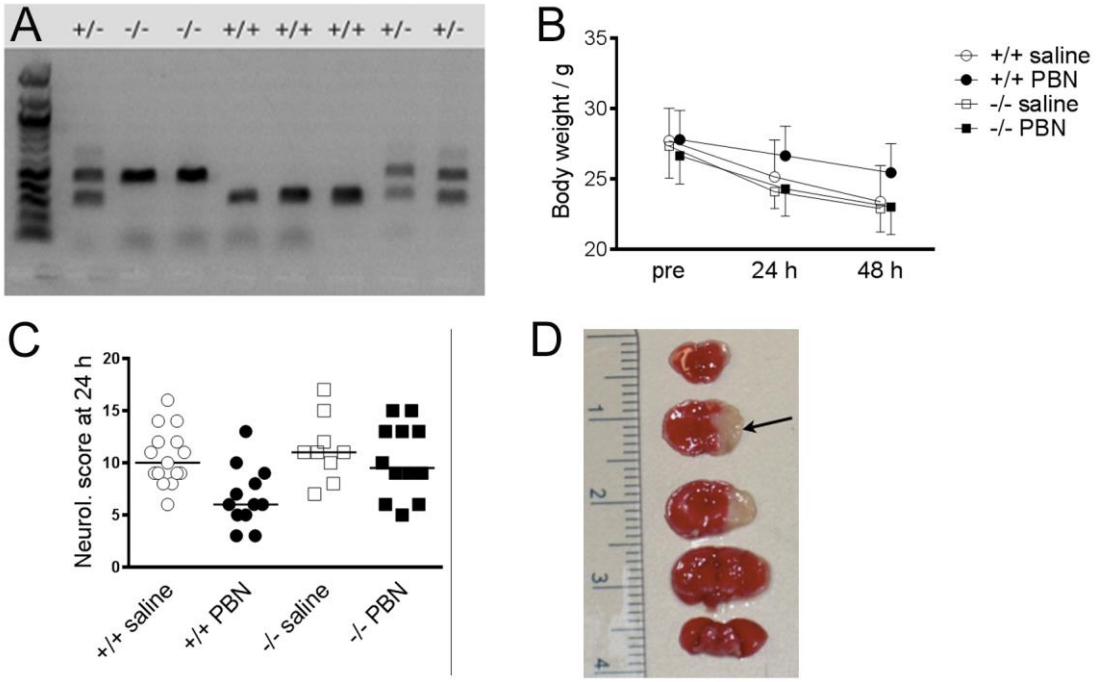

Figure S1. A, genotyping of $Sgk1^{+/+}$, $Sgk1^{-/-}$ and heterozygous $Sgk1^{+/-}$ mice. Ear-punch derived tissue from 8 week old mice were lysed and DNA isolated. DNA samples were amplified by PCR using the 3 primers listed in supplementary methods, and products separated by gel electrophoresis. B, mice showed a decline in body weight at 24 h and 48 h following transient MCAo. Symbols show the mean and 1 SD. C, at 24 hours post-MCAo PBN-treated $Sgk1^{+/+}$ mice (+/+ PBN) exhibited less neurological deficit than saline-treated WT (+/+ saline; n=12, n=15, respectively; $p<0.01$). PBN-treated $Sgk1^{-/-}$ mice (-/- PBN) had no difference in neurological deficit relative to saline-treated $Sgk1^{-/-}$ mice (-/- saline; n=12, n=9 respectively). Horizontal bars show the median value for each group. D, an example of TTC staining in coronal slices from an $Sgk1^{+/+}$ mouse 48 h after transient MCAo. Living tissue is stained red on TTC conversion by active mitochondria. Ischaemic infarct fails to stain red (see arrow). Numerical scale at the left side shows centimetres.

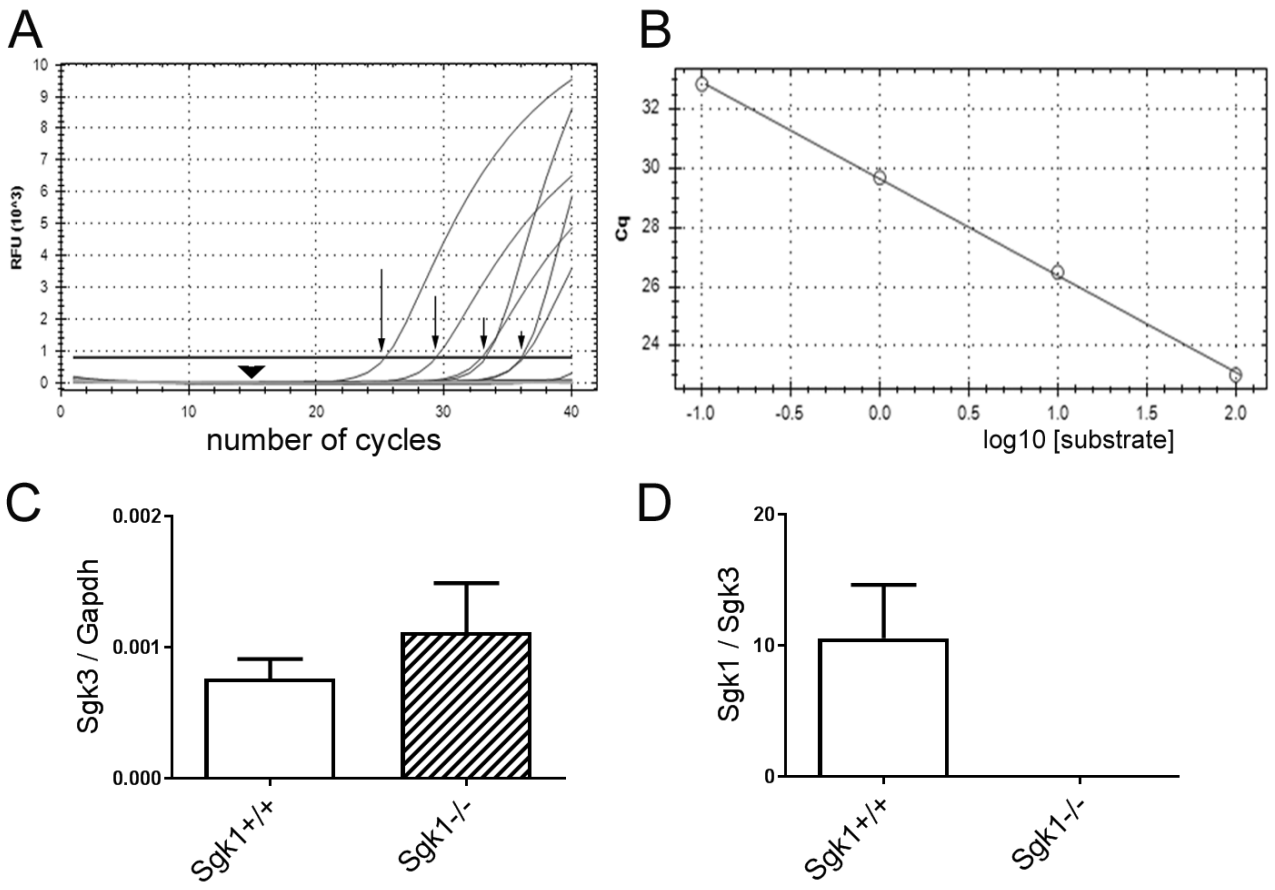

Figure S2. Sgk3 mRNA expression in $Sgk1^{+/+}$ and $Sgk1^{-/-}$ mice.

A, example of Taqman amplication curves for the housekeeping gene Gapdh. Samples represent a standard curve for known concentrations of cDNA. The solid horizontal line is used to derive Cp values for cDNA concentrations of 100, 10, 1.0 and 0.1 nanograms per microliter (Cp values indicated by successively shorter arrows). As a negative control, an identical 100 ng/µl sample where reverse transcriptase was omitted from the reaction mixture showed no amplification (grey line, marked with a large arrowhead). Standard curve derived from data in A. Cq values plotted against cDNA concentration (logarithmic scale). The straight line of best fit is shown ($Y = -3.27*X + 29.7$, $R^2 > 0.99$). C, abundance of Sgk3 mRNA, relative to the housekeeper Gapdh, in brain lysates from $Sgk1^{+/+}$ (n=9) and $Sgk1^{-/-}$ mice (n=6). D, abundance of Sgk1 mRNA, relative to Sgk3 mRNA, in brain lysates from $Sgk1^{+/+}$ (n=4) and $Sgk1^{-/-}$ mice (n=4). Sgk1 mRNA was undetectable in $Sgk1^{-/-}$ mice. In B and C, bars show mean + 1 SD.