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Combined anti-apoptotic and anti-oxidant approach to acute neuroprotection for stroke in hypertensive rats

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ABSTRACT

We hypothesized that targeting key points in the ischemic cascade with combined neuroglobin (Ngb) overexpression and c-jun N-terminal kinase (JNK) inhibition (SP600125) would offer greater neuroprotection than single treatment following *in vitro* hypoxia/reoxygenation and in a randomised, blinded *in vivo* experimental stroke study using a clinically relevant rat strain. Male spontaneously hypertensive stroke-prone rats underwent transient middle cerebral artery occlusion (tMCAO); groups: tMCAO; tMCAO+control GFP-expressing canine adenovirus-2, CAVGFP; tMCAO+Ngb-expressing CAV-2, CAVNgb; tMCAO+SP600125; tMCAO+CAVNgb+SP600125 or sham procedure. Rats were assessed out to day 14 for neurological outcome before infarct determination. *In vitro*, combined lentivirus-mediated Ngb overexpression+SP600125 significantly reduced oxidative stress and apoptosis compared to single treatment(s), following hypoxia/reoxygenation in B50 cells. *In vivo*, infarct volume was significantly reduced by CAVNgb, SP600125 and further by CAVNgb+SP600125. The number of neuroglobin positive cells in the peri-infarct cortex and striatum was significantly increased 14 days post-tMCAO in animals receiving CAVNgb. Neurological outcome, measured using a 32 point neurological score, significantly improved with CAVNgb+SP600125 compared to single treatments, 14 days post-tMCAO. Combined Ngb overexpression with JNK inhibition reduced hypoxia/reoxygenation-induced oxidative stress and apoptosis in cultured neurons and reduced infarct and improved neurological outcome more than single therapy following *in vivo* experimental stroke in hypertensive rats.

KEYWORDS

Anti-apoptotic, anti-oxidant, combined therapy, hypertensive rat, transient focal ischemia.

INTRODUCTION

Currently reperfusion therapy with recombinant tissue plasminogen activator (rt-PA) is the cornerstone of treatment for acute ischaemic stroke, yet therapy can only be delivered to a fraction of patients due to a narrow therapeutic window and risk of cerebral haemorrhage [1]. Extensive research has identified excitotoxicity, oxidative stress, inflammation and cell death (necrosis and apoptosis) as key contributory pathways underlying lesion progression [2]. These diverse injury mechanisms may explain why pharmacotherapy clinical trials geared to manipulate single pathways have failed. A polytherapy approach targeting distinct pathways may be more effective and has shown promise in pre-clinical models [3-4] with the majority assessing the potential to extend the therapeutic window of rt-PA. Of these, 97% used young, healthy animals [3]. The revised STAIR [5] and other guidelines covering pre-clinical stroke studies [6] have highlighted the need to include co-morbidities in pre-clinical stroke models to better model the clinical situation and improve assessment of new therapies. The most significant risk factors (hypertension, abdominal obesity, diet, physical inactivity and current smoking) account for 80% of the global stroke risk with self-reported history of hypertension being the strongest of these [7]. This study used the spontaneously hypertensive stroke-prone rat (SHRSP) which displays stroke co-morbidities of hypertension, insulin resistance and inflammation.

We chose to target two key pathways underlying stroke pathogenesis (oxidative stress and apoptosis) through neuroglobin upregulation in combination with JNK inhibition. Neuroglobin, a neuronal specific O_2 binding protein, is generally considered to mediate neuroprotection in pre-clinical stroke studies following overexpression [8-11], however, it is worth noting that to date, neuroprotection has been demonstrated in normal rodent strains but

not in any strain exhibiting co-morbidity. Administration of adeno-associated virus (AAV) overexpressing neuroglobin significantly reduced lesion size and improved Bederson's 5-point neurological score in young, normotensive rats post-MCAO [10]. Transgenic neuroglobin overexpression in mice reduced infarct volume following tMCAO [9, 11], however, sensorimotor function remained unchanged [11]. Intravenous delivery of neuroglobin linked to a fusion protein, 2 hours pre-tMCAO reduced infarct volume and neurological deficit score in mice [8]. Adenovirus, AAV and lentivirus have been used as viral vectors to overexpress neuroprotective genes which has resulted in reduced infarct volume and improved functional outcome in permanent and tMCAO models [12]. Here, canine adenovirus type 2 (CAV-2 vectors), a vector that preferentially transduces neurons and has been shown to transduce a greater volume of brain tissue through retrograde transport from the site of injection [13-14], was used.

To provide anti-apoptotic protection we targeted JNK, a downstream mediator of extrinsic and intrinsic apoptosis. A peptide inhibitor of JNK administered 6 hours post-occlusion reduced lesion volume and improved functional outcome following tMCAO in mice [15] and reduced infarct volume when delivered 3 hours after permanent MCAO in mice [16]. Following tMCAO in spontaneously hypertensive rats, however, no such neuroprotection was seen [17]. This was attributed, in part, to the predominance of AMPA rather than NMDA activated ischemic brain injury in the SHR [17]. One further reason may be as a result of the greater ischemic insult achieved in SHR (or SHRSP) as the contribution of necrotic to apoptotic cell death may be greater and this is reflected in reduced efficacy targeting JNK signalling, highlighting, once again, the importance of including stroke-associated co-morbidities in pre-clinical studies. We used SP600125, an ATP-competitive inhibitor of JNK, shown previously to dose-dependently reduce infarct volume when given intravenously

less than 2 hours post-tMCAO in mice [18]. The efficacy of these agents alone has not previously been determined in pre-clinical stroke models displaying clinically relevant co-morbidities and the novel combination of an anti-apoptotic and anti-oxidant represents a further advance.

MATERIALS & METHODS

Virus production

As expression of the coxsackievirus and adenovirus receptor, the primary receptor for CAV-2, was negligible on B50 rat neuronal cells used for *in vitro* studies (Supplementary Figure S1A), a neuroglobin-expressing lentivirus was generated with functional overexpression (mRNA and protein) confirmed (Figures 1A & 1B). Lentiviral vectors were produced by triple transient transfection of HEK293T cells with a packaging plasmid (pCMVΔ8.74), a plasmid encoding the envelope of vesicular stomatitis virus (Plasmid Factory, Germany) and pHR-SIN-SFFV-Ngb, employing polyethylenimine (Sigma-Aldrich, UK) as previously described [19]. For CAVNgb production, the neuroglobin cDNA was cloned from pET3a_Ngb into pTCAV-12VK and recombined with pTG5412 as previously described [20] and propagated in DKZeo cells [21]. CAVGFP [20], a CAV-2 vector overexpressing green fluorescent protein as a reporter gene, was used as a control.

Quantitative real time PCR (qRT-PCR)

Total RNA was extracted from lysed cells 3 days post-virus transduction using the Qiagen® miRNeasy kit as per manufacturer's instructions. Qiagen® RNAse-free DNAse was added during the on-column phase. RNA concentration and purity was determined by NanoDrop

spectrophotometry (NanoDrop Technologies, Wilmington, DE) and diluted to 200 ng/μL. cDNA was synthesised from 1 μg total RNA using the Taqman™ mRNA reverse transcription kit (Applied Biosystems, UK) with random hexamer primers. The reactions underwent sequential incubation in a 96 well plate at 25 °C (10 min), 48 °C (30 min), 95 °C (5 min) and held at 12 °C. Simplex reactions were performed for qRT-PCR with a specific neuroglobin expression probe (Rn 00583724_m1, FAM-labelled, Applied Biosystems) or appropriate housekeeper expression probes (GAPDH or 18S, VIC-labelled, Applied Biosystems). Duplicates of each sample were incubated at 95 °C (10 min) followed by 40 cycles of 95 °C (15 s) and 60 °C (1 min) using a Taqman™ 7900HT Fast Real-time PCR System (Applied Biosystems). Samples were normalised to housekeeper and relative quantification (RQ) calculated from ΔΔCt (cycle threshold) vs GFP-virus treated cells.

Immunocytochemistry to detect neuroglobin expression

Neuroglobin immunocytochemistry (ICC) was performed on 4% (w/v) paraformaldehyde-fixed cells 3 days post-virus transduction. Briefly, cells were permeabilised in 0.1% (v/v) Triton / PBS for 15 min before incubation with primary antibody (10 μg/mL; 13C8, Abcam, UK) for 1 h at room temperature (RT). Secondary antibody (4 μg/mL; goat anti-mouse Alexa Fluor 546, Invitrogen, UK; A21424) was added for 1 h at RT. Cells were mounted using Prolong Gold® with DAPI (Invitrogen, UK).

Hypoxia/reoxygenation (H/R)

Rat B50 neuroblastoma cells (ECACC, UK) were maintained in Dulbecco's minimal essential medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mmol/L L-

glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 humidified atmosphere. Cells receiving lentivirus were transduced with 5 virus particles /cell (vp/cell) lenti-GFP or lenti-Ngb for 4 h one day after plating. Forty-eight hours later cells were switched to FBS-free medium and incubated in a hypoxic chamber (1% O_2, 5% CO_2, balance N_2) for 9 h. Ten min prior to hypoxia, cells receiving SP600125 were incubated with 20 μmol/L or volume matched DMSO vehicle control. Cells were reoxygenated in complete media, with readministration of SP600125 or DMSO where necessary, for 24 h before lysis.

In vitro oxidative stress assays

Electron paramagnetic resonance (EPR) spectroscopy for reactive oxygen species (ROS) detection (e-scan R; Bruker BioSpin GmbH, Germany) utilised the spin probe 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine (CPH; Noxygen, Germany) as previously described [22]. Cells were incubated *in situ* with Krebs buffer and 1 mmol/L CPH in a total volume of 1 mL for 60 min at 37 °C for the last hour of the 24 h reoxygenation period. Instrument settings: centre field of 3392 G, modulation amplitude of 5.08 G, sweep time of 10.49 s, sweep width of 120 G and 30 scans. In the presence of ROS, CPH is oxidised to the nitroxide CP radical and the triple-line spectrum is read giving the EPR amplitude in proportion to the amount of CP• reflecting the interaction of ROS with CPH after 60 min giving a rate of ROS production calculated in counts per min. All readings were normalised for input protein using a BCA protein assay kit (Pierce, UK).

A spectrophotometric assay (Tebu-Bio, France) for malondialdehyde (MDA) was used to determine lipid peroxidation levels following H/R as per manufacturer's instructions. The

MDA and hydroxyalkenal determination protocol was used with 200 µl cell lysate (in PBS + 5 µmol/L butylated hydroxytoluene, BHT). Reaction mixtures were incubated for 60 minutes at 45 °C before absorbance was measured at 586nm in a spectrophotometer. All readings were normalised for input protein using a BCA protein assay kit (Pierce, UK).

Apoptosis assays

Apoptosis was measured with a cell death ELISA (Roche, UK) as per manufacturer's instructions. Caspase-3 ICC was performed on 4% (w/v) paraformaldehyde-fixed cells. Briefly, cells were permeabilised in 0.1% (v/v) Triton / PBS for 15 min before incubation with primary antibody (1:30 dilution; AB32351, Abcam, UK) for 1 h at RT. Secondary antibody (4 µg/mL, goat anti-rabbit Alexa Fluor 488, Invitrogen, UK; A11008) was added for 1 h at RT. Cells were mounted using Prolong Gold® with DAPI (Invitrogen, UK).

Animals

Animal experiments were carried out in accordance with the Animals Scientific Procedures Act 1986 and approved by the University of Glasgow's Ethics Review Committee. Male, SHRSP (n = 65, 270–310 g) were housed separately under a 12:12 h light:dark cycle with food and water *ad libitum*. Studies were randomised and blinded preventing bias in selection and data analysis in accordance with STAIR and other guidelines [5-6]. For randomisation, treatment groups were given a number (1-6) and animals randomly allocated to a treatment group by a colleague not involved in performing the study using R (www.r-project.org). Subject groups were: sham (*n*=6), control tMCAO (*n*=9), tMCAO+CAVGFP (*n*=9), tMCAO+CAVNgb (*n*=9), tMCAO+SP600125 (*n*=9), tMCAO+CAVNgb+SP600125 (*n*=8).

The design of the *in vivo* intervention study is shown in Supplementary Figure S2. Investigators and animal unit staff caring for the animals were blinded to group allocation. Findings are reported in accordance with ARRIVE guidelines [23].

Virus and drug administration

Anaesthetic was induced with 5% isoflurane in oxygen and animals intubated and ventilated throughout surgery (~2.5% isoflurane/oxygen). Body temperature was maintained at 37 ± 0.5 °C. Animals undergoing tMCAO had cranial burrhole surgery 5 days before tMCAO for virus administration or as a sham procedure. Interestingly, this pre-stroke surgery reduces subsequent stroke-related mortality [24]. Briefly, the head was secured in a stereotactic frame, a 1 mm cranial burrhole made and a 24G needle connected to a Hamilton syringe used to pierce the dura and administer virus into the cortex. Following a 2 min rest period 2.1 μL virus suspension was injected over 5 min, with a subsequent 2 min rest period before slow needle retraction. A reporter gene-expressing lentivirus and CAV-2 were compared for transduction levels to determine which was the most efficient vector for this study. CAV-2 vectors (3 x 10^9 physical particles) were injected at co-ordinates: AP + 1.2mm, ML + 3mm, DV – 2mm relative to bregma or 2 injections of 2 x 10^7 vp lentivirus injected at co-ordinates: AP – 0.4mm, ML + 3mm DV - 2mm and AP – 2.4mm, ML + 4mm DV - 2mm, relative to bregma. For the intervention study, the site of CAV-2 injection (3 x 10^9 physical particles) was AP – 0.7 mm, ML + 3mm, DV – 2mm relative to bregma. The burrhole was then sealed with dental cement (Wright Cottrell). Animals randomised to SP600125 received an intravenous injection [1 mg/kg in PPCES vehicle - 30% (w/v) PEG–400, 20% (v/v) polypropylene glycol, 15% (v/v) cremophor EL, 5% (v/v) ethanol, 30% (v/v) saline] 15 min pre- and 3 h post-tMCAO as previously described in mice [18].

Neurological assessments

Each animal was trained on the neurological assessments prior to MCAO to ensure reproducibility at performing tasks. Animals were assessed on a single occasion 3 days prior to tMCAO to ascertain a baseline score. The 32-point neurological score used was developed from a series of 10 tests which assess limb function, mobility and general health, the lower the score the greater the neurological deficit. Animals were further assessed by the tapered beam walk test, quantifying the average number of footfalls as a percentage of the total number taken from 3 crossings of a 130 cm tapered beam. The animals were assessed at baseline 3 days prior to tMCAO and longitudinally on days 1, 2, 3, 7, 10 and 14 post-MCAO. Where possible, behavioural measures were completed blinded to surgery and treatment groups and were video recorded for assessment of footfalls and to allow for further validation of scores by a 2nd blinded observer.

Tail cuff plethysmography

Conscious systolic blood pressure monitoring was carried out by non-invasive computerised tail-cuff, based on the plethysmographic method. Rats were preheated to ~ 39 ºC for ~ 20 minutes and restrained by wrapping in a surgical sheet, before a pneumatic pressure sensor was attached to the tail distal to a pneumatic pressure cuff, both under the control of a Programmed Electro-Sphygmomanometer. Systolic blood pressure values from each animal were determined by averaging a minimum of six separate indirect pressure measurements.

Stroke model

Animals were anesthetised as before and underwent tMCAO (45 min) by advancing a silicone-coated monofilament (Doccol Corporation, USA) through the internal carotid artery, blocking the origin of the MCA [25]. Animals were maintained under anaesthesia during the ischemia and then anaesthetic withdrawn and the animals allowed to recover consciousness after removal of the filament. The duration of occlusion was determined to reflect a reproducible infarct volume and accompanying neurological deficit but with an acceptably low mortality rate (60 min MCAO resulted in 100% mortality in SHRSP within 2-3 days of experimental stroke, unpublished observation).

Infarct analysis

14 days following tMCAO rats were killed by transcardiac perfusion fixation. Formalin-fixed, paraffin-embedded tissue sections (6 µm) were stained with hematoxylin and eosin. Infarct volume was assessed at 7 coronal levels throughout the MCA territory [26]. Briefly, areas of tissue infarction were identified and the location transcribed onto scale line diagrams from the rat brain atlas [27]. Line diagrams were then scanned and infarct areas were measured by image analysis (Image J, NIH, USA). The infarct volume (mm^3) for each brain was calculated by plotting the area (mm^2) of damage at each coronal level against the known anterior / posterior stereotaxic coordinates from Bregma and calculating the area under the curve. Infarct measures were made blinded to group allocation.

Immunohistochemistry for virus transduction

For determination of virus transduction efficiency and spread from the injection site, rats were killed by transcardiac perfusion fixation. Brains were then placed in a 30% (w/v) sucrose gradient and subsequently embedded in OCT. Frozen sections (40 µm) were permeabilised in 0.1% (v/v) Triton-X for 10 min and blocked for 1 h at RT. Primary antibody or IgG control were diluted (1:500, GFP Abcam Ab6556; 1:500, NeuN Millipore, MAB377) and incubated on sections overnight at 4 °C. Fluorescent secondary antibody [4 µg/mL; goat anti-rabbit (GFP) or goat anti-mouse (NeuN) Alexa Fluor 488, Invitrogen, UK; A11008 or A11001 respectively] was incubated on the slides for 1 h at RT before mounting with ProLong® Gold with DAPI. Areas of virus transduction (GFP expression) were identified on brain sections and their location transcribed onto scale line diagrams at 7 pre-determined coronal levels. The area of GFP transduction of 3 sections at each coronal level were delineated onto the appropriate coronal line diagrams, and measured using image analysis (Image J, NIH, USA).

To determine levels of neuroglobin overexpression following virus delivery pre-tMCAO, formalin-fixed, paraffin-embedded tissue sections (6 µm) were rehydrated, endogenous peroxidise quenched [30 minutes in 3% (v/v) H_2O_2 in methanol] followed by citrate buffer antigen retrieval. Sections were blocked for 1 h at RT and incubated overnight at 4 °C in primary antibody (1:200, Ngb, Sigma; 1:200 NeuN, Abcam) or IgG control. Fluorescent secondary antibody (4 µg/mL; goat anti-mouse Alexa Fluor 546, Invitrogen, UK; A21424) was incubated on the slides for 1 h at RT before mounting with ProLong® Gold with DAPI. Images were taken on a Zeiss confocal imaging system LSM 510 (Carl Zeiss, UK) using settings DAPI (405) pinhole 61 µm and TRITC (546) pinhole 71 µm and objective C-apochromat 40x/1.2W at 5 matched locations across the cortex and striatum at two coronal

levels [coordinates (mm): 5 sites at AP -0.12 from Bregma - ML +3.5, DV +1; ML+1, DV +1; ML +2, DV +4; ML +4, DV +4; ML +3.5, DV +6 and 5 sites at AP -2.28 from Bregma – ML +4.5, DV +1; ML +1, DV +1; ML +4, DV +4; ML +4.5, DV +6; ML 1.5, DV +4] to reflect the areas of high virus transduction and infarct. To quantify the extent of neuroglobin expression Image Pro (Media Cybernetics, Basingstoke, UK) was used. The area for quantification of neuroglobin cell count in peri-infarct tissue was selected using the Area of Interest Macro and pixel values were transformed to optical density units. The number of cells positive for neuroglobin was then determined as a percentage of the total number of DAPI stained nuclei within each given field. Image acquisition and subsequent quantifications were performed blinded to group allocation.

Statistics

Data are presented as mean ± s.e.m. *In vitro* experiments were performed in triplicate on ≥3 independent occasions and analysed by unpaired Students t-test. *In vivo* groups were compared using repeated measures ANOVA. Survival rates were compared using Fisher's exact test. Bonferroni's or Tukey's post-hoc test was used for multiple comparisons.

RESULTS

Neuroglobin overexpression combined with JNK inhibition protects against H/R *in vitro*

B50 neuronal cells subjected to 9 h hypoxia and 24 h reoxygenation produced significant increases in ROS generation (Figure 2A), malondialdehyde (MDA; Figure 2B), DNA fragmentation (Figure 2C) and caspase-3 nuclear translocation (Figure 2D). Lenti-Ngb pre-treatment significantly reduced ROS generation versus controls (Figure 2A). SP600125

treatment alone did not significantly reduce ROS levels but potentiated the lenti-Ngb mediated reduction (Figure 2A). MDA levels appeared maximally reduced in cells pre-treated with lenti-Ngb or SP600125. Consequently, no further benefit of combined therapy was observed (Figure 2B). Lenti-Ngb or SP600125 pre-treatment significantly reduced levels of apoptosis, with a further significant reduction from that seen with lenti-Ngb following combined intervention (Figure 2C). In all assays those cells pretreated with the Lenti-Ngb or SP600125 and the appropriate control treatments (DMSO or Lenti-GFP) the same effect as the single active agent (either lenti-Ngb or SP600125) was found demonstrating no confounding effect attributable to either vehicle or virus. Caspase-3 nuclear translocation was assessed qualitatively by ICC (Figure 2D). Lenti-Ngb or SP600125 pre-treatment reduced the extent of nuclear translocation of caspase-3 with combined treatment returning the cellular distribution pattern of caspase-3 to that of normoxic cells (Figure 2D).

CAV-2 vectors more efficiently transduce the adult rat cortex than lentivirus

Different levels and distribution of reporter gene, GFP, expression were found for the reporter gene-expressing lentivirus and CAV-2 (Figures 3A & 3B). One week after a single cortical injection of CAV-2 the rostra-caudal spread from the injection site was in excess of 7 mm with spread through the cortical parenchyma. In contrast, following double cortical injection of lentivirus spread was minimal (< 2.5 mm) from the site(s) of injection (Figures 3A & 3B). Determination of the tissue volume expressing the reporter gene, GFP, demonstrated that seen with the CAV-2 vector was significantly greater than that resulting from a double cortical injection of lentivirus (156 ± 11.1* vs $20 \pm 3.8\ mm^3$; ***$p<0.0002$ vs lentivirus Figure 3C). Therefore, a Ngb-expressing CAV-2 virus was generated for *in vivo* studies and functional Ngb overexpression (mRNA and protein) confirmed *in vitro* (Figure

1C & 1D, respectively). Expression of CAR mRNA, the primary receptor for CAV-2 virus, in the brain of SHRSP was confirmed (Supplementary Figure S1B).

Neuroglobin overexpression combined with JNK inhibition protects *in vivo* against cerebral ischaemia/reperfusion

Virus-mediated overexpression of neuroglobin

Neuroglobin expression was determined 14 days post-tMCAO across all groups (Figure 4A), 19 days after the CAV-2 vector was administered. In control tMCAO animals there was a small, non-significant increase in the number of cells expressing neuroglobin in the peri-infarct cortical and striatal regions (9.9 ± 1.3 %) compared to sham animals (3.1 ± 0.5 %) (Figure 4B). Similarly, no significant change in the number of cells positive for neuroglobin expression was seen in those animals randomly allocated to receive SP600125 (5.8 ± 1.1 %) (Figure 4A & 4B). The endogenous expression of neuroglobin in these groups was largely restricted to the peri-infarct region (Figure 4A). In animals receiving CAVNgb alone (25 ± 3.1 %) or in combination with SP600125 (24.6 ± 2.2 %) there was a marked and significant increase in the number of cells positive for neuroglobin expression within the peri-infarct cortex and striatum 19 days after virus administration (Figure 4A) compared to sham, control tMCAO or SP600125 treated animals (Figure 4B). The increased neuroglobin expression in CAVNgb treated groups was evident in both cortical and striatal regions around the infarct (Figure 4A). The rostro-caudal spread in enhanced neuroglobin expression following tMCAO appeared consistent with that seen for CAVGFP (Figure 3) with increased expression evident over a rostro-caudal distance of approximately 7 mm from the site of injection. Neuroglobin expression was primarily co-localized with NeuN-positive cells

indicating a neuronal expression. Note, not all NeuN positive cells appear positive for Ngb (Figure 4C).

Survival and Systolic blood pressure

No intervention affected SBP at 7 (data not shown) or 14 days post-tMCAO (Table 1). Furthermore, there was no significant difference in the starting/final SBP values between groups. Post-tMCAO associated weight loss was similar between groups (Table 1). Survival over the duration of the study was 100% in the sham (6/6) and tMCAO with combined intervention (8/8) groups only. Although mortality occurred in the other groups, survival rates were not significantly different between groups: tMCAO 9/13; tMCAO+CAVNgb 9/10; tMCAO+SP600125 9/13; tMCAO+CAVGFP 9/11 (Figure 5A).

Infarct volume

Infarct volumes were comparable in untreated tMCAO control (324 ± 16.4 mm^3, *n*=9) and tMCAO+CAVGFP (314 ± 22.2 mm^3, *n*=9) rats (Figure 5B). SP600125 and CAVNgb treatment (*n*=9 per group) significantly reduced infarct volume by 33.8% (215 ± 34.2 mm^3*) and 38.6% (199 ± 14.4 mm^3*), respectively compared to control tMCAO (*p<0.01 vs tMCAO). Importantly, combined intervention (*n*=8) produced a greater reduction in infarct volume than single treatments (57%; 137 ± 20.7 mm^3*†; p<0.01 vs *tMCAO or †SP600125 and CAVNgb). The rostro-caudal extent of infarction in each group is shown in Figure 5C. Morphological analysis demonstrated that those animals receiving CAVNgb, SP600125 or CAVNgb+SP600125 the extent of infarct in the primary motor cortex and striatum was reduced vs control tMCAO or CAVGFP (Figure 5D). However, there was a region in the

primary somatosensory forelimb and hindlimb cortex which was consistently infarcted irrespective of intervention (Figure 5D, middle panel).

Neurological deficit

All tMCAO groups exhibited a significant reduction in neurological score from baseline at 24 hours post-tMCAO (Figure 6A). No significant difference in neurological score was observed between groups over days 1-3. At day 7, a significant improvement in neurological recovery was observed with combined therapy compared to control tMCAO. However, at day 10 there was a significant improvement with single treatment groups compared to control tMCAO, but no further improvement with combination therapy. By day 14, single treatments significantly improved neurological score with combined therapy producing a further significant improvement compared to single treatments (Figure 6A). All tMCAO groups exhibited a significant increase in percentage footfalls 24 hours post-tMCAO on the tapered beam walk test (Figure 6B). By day 14, a significant improvement in total footfalls was evident in all treatment groups versus the control group. However, no additional improvement with combined therapy was observed (Figure 6B).

DISCUSSION

We describe, for the first time, an improved beneficial effect (both reduced infarct volume and improved neurological recovery determined longitudinally) through combined anti-apoptotic and anti-oxidant intervention in a clinically relevant stroke model displaying stroke-associated co-morbidities. Combined intervention mediated greater neuroprotection than either therapy alone both *in vitro* using a model of H/R and *in vivo* after tMCAO.

Furthermore, this study introduces neurotrophic CAV-2 vector as a platform that will allow better understanding of stroke pathophysiology through high levels of brain transduction. Together, this supports the potential for combined intervention strategies, in this case targeting excessive oxidative stress and apoptosis, following cerebral ischemia.

In vitro studies demonstrated that neuroglobin overexpression combined with JNK inhibition mediated greater neuroprotection, less oxidative stress and apoptosis after H/R injury compared to individual treatments. While each single treatment has been studied previously in neuronal cells *in vitro*, combination therapy has not. Immortalised HN33 hippocampal neurones transfected to overexpress neuroglobin exhibited improved cell viability, measured by MTT assay, following 8 or 24 hours of anoxia [28]. Additional mechanistic studies determined that there was no increase in oxygen consumption in neuroglobin-overexpressing cells compared to controls and an involvement of nitric oxide scavenging was further ruled out through the failure of neuroglobin to protect against sodium nitroprusside-induced toxicity [28]. It has since been demonstrated that neuroglobin elicits protection from H/R insult *in vitro* through multiple mechanisms including improving mitochondrial function, maintenance of intracellular ATP levels, inhibition of calcium influx, binding to cytochrome *c* and reduced caspase 3/7 and 9 activity in human neuroblastoma cells overexpressing neuroglobin [29-31]. In the present study, vector-mediated neuroglobin overexpression resulted in reduced ROS generation (Figure 2). Although neuroglobin was initially believed to act purely as an anti-oxidant, studies [29, 31] including this study suggest anti-apoptotic effects through an interaction with cytochrome *c*, reduced caspase 3/7 or 9 activity/nuclear localisation and DNA fragmentation (reviewed in [32]). It is impossible to distinguish unequivocally between apoptotic and oxidative stress pathways as they are inextricably linked with extensive crosstalk between. However, neuroglobin directly activates the

PI3K/AKT/mTOR pathway of cell survival [33]. Furthermore, JNK may be involved in pro-oxidant pathways since the N-terminal Von Hippel-Lindau recognition site of hypoxia inducible factor-1α contains a JNK binding domain [34].

Notably, translation to an *in vivo* stroke model confirmed that combined neuroglobin upregulation and JNK inhibition was better than single treatment using infarct size and neurological deficit as outcome measures (Figures 4-6). The greatest reduction in infarct size, detected in the combination treatment group, was accompanied by an improved neurological score and reduction in footfalls at day 14. The tapered beam walk test failed to demonstrate any additional improvement with combined treatment which likely reflects the conserved region of infarct within the primary somatosensory forelimb/hindlimb cortex in all groups (Figure 5D). There was a trend towards an increase in endogenous neuroglobin levels 14 days following tMCAO in the SHRSP although this was not significantly enhanced compared to sham (Figure 4). We cannot exclude the possibility that peak neuroglobin levels occurred earlier and returned toward basal levels 2 weeks after cerebral ischemia. Endogenous upregulation of neuroglobin in cells in the peri-infarct region agrees with other preclinical [28] and clinical stroke studies [35] where increased neuroglobin levels were reported. Importantly, virus-mediated overexpression of neuroglobin was sustained to 14 days after tMCAO (19 days post-administration) in those animals randomised to receive CAVNgb or the combined treatment. Interestingly, in SP600125 treated animals, which had a similar infarct volume to the CAVNgb single intervention group, there was no difference in the number of neuroglobin positive cells compared to sham. This may reflect that with the smaller infarct resulting from SP600125 treatment, the stimulus for endogenous neuroglobin upregulation following tMCAO was reduced. Interestingly, in the intervention study, increased neuroglobin expression was detected in the striatum and cortex following virus

delivery and tMCAO. Striatal gene expression was not evident from the CAVGFP administration in naive rats (Figure 3). While we cannot distinguish between endogenous or virus-mediated upregulation in neuroglobin expression within the striatum, we can postulate that following tMCAO transduction profiles may differ or that there is an active transport process occurring similar to what has previously been described with CAV-2 vectors [36].

Previous studies determining the neuroprotective effect of neuroglobin *in vivo* demonstrated neuroglobin overexpressing transgenic mice had lower levels of lipid peroxidation measured by MDA assay in the ipsilateral hemisphere following tMCAO [11]. In addition, reduced numbers of TUNEL-positive cells and increased numbers of neurones with a normal morphology (from cresyl violet histology) were seen following delivery of neuroglobin linked to a fusion protein prior to tMCAO in C57BL/6J mice [8]. The current study demonstrates that in spite of the multifaceted mechanism of action of Ngb (anti-oxidant and anti-apoptotic) that further protection can be afforded through combined JNK inhibition. All previous experimental stroke reports of neuroprotection afforded by neuroglobin have been determined in normal mice or rats or using transgenic overexpression [8-11]. In using SHRSP, which exhibit a number of stroke-related co-morbidities, we have addressed STAIR guidelines [5] as hypertension was reported as the strongest risk factor for stroke in a global case-study [7]. Furthermore, all analyses were performed under blinded conditions with animals randomly assigned to experimental groups and blood pressure monitoring throughout, in accordance with the pre-clinical stroke study guidelines [6]. Of 142 publications describing combined therapy for stroke only 3% have used hypertensive rats [3]. The majority of these determined the potential for adjuvant therapy to extend the therapeutic window for rt-PA [4]. Only one combination treatment study (anti-oxidant tempol and/or cannabinoid/NMDA antagonist dexanabinol following permanent MCAO) used hypertensive

rats and this failed to identify any additional benefit of combined treatment, attributing this to a ceiling neuroprotection achieved by either single treatment [37]. We designed our study to limit this possibility. The dose of SP600125 used (1 mg/kg) was selected based on previous studies in mice demonstrating a significant, but sub-maximal (seen at 3 mg/kg), reduction in infarct volume after tMCAO [18] in an attempt to avoid a similar ceiling effect. A recovery period of 14 days was set to ensure capture of final infarct size (shown previously to continue to evolve 48-72 hours post-tMCAO in rats [38]) and allow serial assessment of neurological outcome measures.

The utility of CAV-2 vectors for neurodegenerative diseases has previously been demonstrated in dopamine-deficient mice where CAV-2 transduced neurons expressed tyrosine hydroxylase for >1 year from delivery [39]. More recently in a lysosomal storage disorder, neonatal delivery led to persistant transgene expression and normalization of the functional deficit for up to 20 weeks [40]. A single cortical CAV-2 injection efficiently transduced neurons and resulted in an 8-fold greater transduction volume and covered a greater rostral-caudal distance than lentivirus in the present study. In this, the first study using CAV-2 in a cerebral ischemia model, increased levels of neuroglobin transduction remained in neurons of peri-infarct cortical and striatal regions 2 weeks after tMCAO and 19 days post-CAV delivery, demonstrating sustained and selective transgene expression. Gene and cell based-therapies are now being clinically tested for a wide range of diverse disease pathologies. While the success of the CAV-2 vector to directly deliver neuroglobin to the brain parenchyma overcomes limitations relating to the ability of pharmacological anti-oxidants to reach the brain by failure to cross the blood-brain barrier, translation of novel gene delivery vectors, such as CAV-2, will require further extensive pre-clinical research. A more feasible clinical prospect may be through pharmacological upregulation of neuroglobin

to similar levels achieved with CAV-2 vectors. A recent study described the use of the prolyl hydroxylase inhibitor (PHI) deferoxamine and the short-chain fatty acids cinnamic and valproic acids to pharmacologically increase neuroglobin expression in HN33 cells *in vitro* [41]. However, whether such an effect on neuroglobin expression is also evident *in vivo* has yet to be described. In addition, these agents are likely to affect a number of pathways in addition to merely upregulating neuroglobin – the PHIs, for example, enhance levels of hypoxia inducible factor-1α and -2α resulting in neuroprotection through a plethora of target pathways [42].

In conclusion, the combination of anti-apoptotic JNK inhibition and anti-oxidant neuroglobin upregulation demonstrates a significant neuroprotective potential when administered prior to tMCAO. This study demonstrates that targeting these pathways in combination improves efficacy compared to a single target using an animal model exhibiting stroke-associated co-morbidities. Further use of such a combined approach targeting excessive oxidative stress and apoptosis may be applicable in disease settings where these pathways are implicated, such as neurodegenerative disorders (Alzheimer's disease, Parkinson's disease), traumatic brain injury or hypoxic insult (such as hypoxic-ischemic neonatal encephalopathy, a problem in perinatal medicine). The next step in stroke translation will be to assess if neuroprotection and improved outcome can be achieved when treatment is delivered in the acute post-stroke period and if it can act additively or synergistically with thrombolysis. However, although many single and combination therapeutic approaches have proven successful in pre-clinical models, this has not led to successful translation in stroke patients and thrombolysis within a tight time frame of the onset of acute cerebral ischemia remains the primary intervention at this time.

DISCLOSURE/CONFLICT OF INTEREST

None.

Supplementary information is available at the Journal of Cerebral Blood Flow & Metabolism website – www.nature.com/jcbfm

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Figure 1: Confirmation of neuroglobin-overexpression from viral vectors. Functional overexpression of Ngb was assessed by TaqMan™ qRT-PCR (mRNA) and ICC (protein) in B50 neuronal (lenti-Ngb) or HepG2 (CAVNgb) cells 3 days post-transduction. (A, C) Ngb mRNA levels were normalised to GAPDH (B50 cells, lenti-Ngb) or 18S (HepG2 cells, CAVNgb) following viral transduction. Relative quantification (RQ) was calculated from ΔΔCt (cycle threshold) and compared to GFP-expressing virus levels. $RQ \pm RQ_{max}/RQ_{min}$ shown and analysed by Student's unpaired t-test with Bonferroni's post-hoc correction. *$p<0.01$ and **$p<0.001$ vs GFP-expressing virus, $n=3$. (B, D) Representative photomicrographs of Ngb protein expression determined by ICC in cells transduced with Ngb-expressing viruses using an α-Ngb antibody (B: nuclei=blue, DAPI; both: Ngb=red, TRITC). Negative staining in isotype-matched IgG controls shown for lenti-Ngb, untreated cells represent basal expression levels of neuroglobin expression. Scale bar = 100 µm.

Figure 2: Neuroglobin overexpression combined with JNK inhibition protects against H/R injury *in vitro*. Normoxic control cells (solid bars) received the same treatment as cells exposed to 9 hours hypoxia with 24 hours reoxygenation (hypoxic; open bars). Oxidative stress assays for H/R injury: (A) ROS generation detected by EPR; (B) lipid peroxidation levels by MDA assay and apoptosis by: (C) Apoptotic cell death ELISA and (D) caspase-3 (green) ICC (nuclei=blue, DAPI). Scale bar, 100 µm. Data presented as mean±s.e.m., analysed using unpaired Student's t-test and Bonferroni's post-hoc correction, representative of $n=3$. *$p<0.01$ vs untreated hypoxic cells, †$p<0.001$ vs. lenti-Ngb or SP600125 treated hypoxic cells, #$p<0.01$ vs. lenti-Ngb treated hypoxic cells.

Figure 3: Comparative transduction levels from CAV-2 and lentivirus in adult rat brain. (A) Rostro-caudal gene transduction (grey) following single injection of 3 x 10^9 vp CAVGFP (*n*=4; left) and double injection of 2 x 10^7 vp lenti-GFP (*n*=4; right) determined from the median animal in each virus group. Black dots represent cortical injection sites. The staining pattern of GFP expression was transcribed onto stereotaxic atlas plates [27]. (B) Representative cortical images of GFP epifluorescence from lentivirus or CAV-2 transduction 7 days after stereotactic injection; scale bar, 100 μm (top) and 20 μm (bottom). Images taken from the primary somatosensory cortex (S1FL) at coronal level Bregma -0.12mm (indicated with an X on atlas plate image). (C) Volume of tissue transduced by each viral vector quantified from area under the curve analysis across 7 coronal levels. Data presented as mean±s.e.m.; ***p<0.0002 vs lentivirus using Student's unpaired t-test.

Figure 4: CAV-mediated overexpression of neuroglobin following stereotactic delivery and subsequent tMCAO. (A) Infarct location (grey) and representative distribution of neuroglobin expression (red) for each group (top panel) determined by IHC 19 days after CAV delivery and 14 days post-tMCAO. Representative images showing levels of neuroglobin expression at 2 distinct peri-infarct sites (Bregma -0.12mm, cortical site, top panel or Bregma -2.28mm, striatal site, bottom panel) following tMCAO. Inset panels representative of isotype matched IgG control staining. Scale bar, 50 μm. Image location indicated by X on atlas plate - primary somatosensory cortex (S1FL) or striatum (Str). (B) Quantification of neuroglobin positive cells expressed as a percentage of total cells (DAPI nuclear stain) within 5 matched locations across the cortex and striatum at 2 coronal levels. Data presented as mean±s.e.m. and analysed by ANOVA using Tukey's post-hoc correction. **p<0.001 vs sham, †p<0.01 vs. control tMCAO and $^{\#\#}$p<0.001 vs. tMCAO+SP600125. (C) Representative double labelled images of immunopositive neurones (NeuN) and neuroglobin-

positive (Ngb) cells at coronal level Bregma -2.28mm in the striatum (Str). Representative dual labelled cells indicated by arrows. Scale bar, 50 µm.

Figure 5: Neuroglobin overexpression combined with JNK inhibition does not affect mortality but reduces infarct volume following ischemia/reperfusion *in vivo*. (A) Kaplan-Meier curves showing comparable survival rates between sham, tMCAO and intervention groups using Fisher's exact test. (B) Infarct volume determined 14 days post-tMCAO. Individual data points for each animal, with line representing the mean. (C) Rostro-caudal distribution of infarct. Data presented as mean±s.e.m. and analysed by one way ANOVA with Bonferroni's post-hoc correction; $p<0.01$ vs *tMCAO, †tMCAO+SP600125 or tMCAO+CAVNgb and #tMCAO+SP600125. (D) Representative transcribed infarct (top panel) and H&E images (middle and bottom panels) from the median animal in each group at coronal level Bregma -0.12mm. Middle panel - primary somatosensory cortex (S1HL & S1FL, indicated with 1 on atlas plate image) and bottom panel – striatum (Str, indicated with 2 on atlas plate image). Scale bar, 50 µm.

Figure 6: Neuroglobin overexpression combined with JNK inhibition improves neurological deficit. (A) 32-point neurological score measured general condition, righting reflex, grip strength, paw placement, circling, horizontal bar, inclined platform, visual forepaw reaching, rotation and mobility. (B) Movement on tapered beam was tracked, footfalls onto an under-hanging ledge recorded for both ipsi- and contralateral side and expressed as a percentage of footsteps taken. Data presented as mean±s.e.m. and analysed by ANOVA using Bonferroni's post-hoc correction, (n=6-9 per group). *$p<0.01$ vs. tMCAO and †$p<0.01$ vs. single treatment.

Table 1: Physiological parameters. SBP measured 3 days pre- (baseline), 7 days and 14 days post-tMCAO (pre-sacrifice) and the change in body weight over the corresponding period. Data presented as mean±s.e.m, *n*=6-9.