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ORIGINAL CONTRIBUTION

Glucagon-like peptide-1 (GLP-1) receptor activation dilates cerebral arterioles, increases cerebral blood flow, and mediates remote (pre) conditioning neuroprotection against ischaemic stroke

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Abstract

Stroke remains one of the most common causes of death and disability worldwide. Several preclinical studies demonstrated that the brain can be effectively protected against ischaemic stroke by two seemingly distinct treatments: remote ischaemic conditioning (RIC), involving cycles of ischaemia/reperfusion applied to a peripheral organ or tissue, or by systemic administration of glucagon-like-peptide-1 (GLP-1) receptor (GLP-1R) agonists. The mechanisms underlying RIC- and GLP-1-induced neuroprotection are not completely understood. In this study, we tested the hypothesis that GLP-1 mediates neuroprotection induced by RIC and investigated the effect of GLP-1R activation on cerebral blood vessels, as a potential mechanism of GLP-1-induced protection against ischaemic stroke. A rat model of ischaemic stroke (90 min of middle cerebral artery occlusion followed by 24-h reperfusion) was used. RIC was induced by 4 cycles of 5 min left hind limb ischaemia interleaved with 5-min reperfusion periods. RIC markedly (by ~ 80%) reduced the cerebral infarct size and improved the neurological score. The neuroprotection established by RIC was abolished by systemic blockade of GLP-1R with a specific antagonist Exendin(9–39). In the cerebral cortex of GLP-1R reporter mice, ~ 70% of cortical arterioles displayed GLP-1R expression. In acute brain slices of the rat cerebral cortex, activation of GLP-1R with an agonist Exendin-4 had a strong dilatory effect on cortical arterioles and effectively reversed arteriolar constrictions induced by metabolite lactate or oxygen and glucose deprivation, as an ex vivo model of ischaemic stroke. In anaesthetised rats, Exendin-4 induced lasting increases in brain tissue PO_2, indicative of increased cerebral blood flow. These results demonstrate that neuroprotection against ischaemic stroke established by remote ischaemic conditioning is mediated by a mechanism involving GLP-1R signalling. Potent dilatory effect of GLP-1R activation on cortical arterioles suggests that the neuroprotection in this model is mediated via modulation of cerebral blood flow and improved brain perfusion.

Keywords Brain arterioles · Brain capillaries · Cerebral blood flow · Glucagon-like peptide-1 · Ischaemic stroke · Middle cerebral artery occlusion · Neuroprotection · Remote ischaemic preconditioning

Introduction

Stroke remains one of the most common causes of death and disability worldwide [42]. Interventions to restore cerebral blood flow to the viable tissue surrounding the infarct core, called the penumbra, are efficient if applied within hours after the stroke onset and limited to a large artery clot removal by intravenous thrombolysis or invasive mechanical thrombectomy [74]. Many smaller vessels, however, remain constricted and contribute to the no-reflow phenomenon after stroke. This highlights the need for the development of novel therapies that can effectively

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improve/restore the microvascular flow and salvage viable brain tissue from permanent ischaemic damage [4].

Our body is capable of recruiting powerful innate mechanisms of inter-organ protection against ischaemia/reperfusion injury. These mechanisms effectively protect the heart and the brain and can be activated by cycles of ischaemia/reperfusion applied to an organ or tissue remote to the organ being protected. This well-characterized phenomenon is called remote ischaemic conditioning (RIC) [23, 31, 33, 39, 47]. More than 40 preclinical studies demonstrated that RIC can protect the brain against ischaemic stroke [14, 49, 72, 84]. Results of clinical studies [37] demonstrated that RIC improves brain perfusion, as well as visual-spatial and executive functions in patients affected by cerebral small vessel disease [83]. However, the mechanisms of RIC-induced neuroprotection are not fully understood and have been proposed to include reduction of astrogliosis, maintenance of blood–brain barrier integrity, the opening of K_{ATP} channels, prevention of let-7a and miR-43 overexpression, and other mechanisms [15, 20, 48, 69, 70, 77, 82].

Previous studies on the mechanisms of RIC-induced cardioprotection against ischaemia/reperfusion injury suggested that RIC recruits interacting neuronal and humoral mechanisms [8, 24, 26, 52, 75]. Our studies showed that these include sensory (afferent) innervation of the peripheral tissue undergoing RIC, autonomic parasympathetic (vagal) pathways, and the actions of an incretin hormone glucagon-like-peptide-1 (GLP-1), as a likely humoral mediator of RIC [5, 6, 62, 63]. We hypothesised, therefore, that GLP-1 may also mediate RIC-induced neuroprotection against ischaemic stroke.

GLP-1 is an important hormone that has multiple functions from the regulation of insulin secretion to the control of satiety and modulation of autonomic nervous system activity [2, 34–36]. GLP-1 is released by the enteroendocrine cells in the gut and also by the groups of specialized CNS neurons located in the brainstem [35, 66]. Experimental studies conducted in gerbils, mice and rats demonstrated that GLP-1 receptor (GLP-1R) agonists Exendin 4 (Ex4), liraglutide or semaglutide, administered either systemically or centrally, are highly effective in protecting the brain against ischaemic stroke induced by bilateral carotid artery occlusion or middle cerebral artery occlusion [9, 21, 50, 59, 78]. In at-risk diabetic patients, semaglutide was shown to significantly decrease the incidences of non-fatal strokes [60, 61]. However, the mechanisms underlying GLP-1R-mediated neuroprotection remain largely unknown [19, 59].

In this study, we tested the hypothesis that GLP-1 mediates neuroprotection induced by RIC. First, we determined the effect of GLP-1R blockade on RIC-induced neuroprotection, and then investigated the expression of GLP-1R and the effects of GLP-1R activation on cerebral blood vessels and brain blood flow, as a potential mechanism of GLP-1R-mediated neuroprotection against ischaemic stroke.

Methods

All animal experiments were performed in accordance with the European Commission Directive 2010/63/EU (European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes) and the UK Home Office (Scientific Procedures) Act (1986) with project approval from the University College London Institutional Animal Care and Use Committee. The study was designed and performed, the data analysed and reported in accord with the ARRIVE guidelines. Animals were maintained on a 12-h light/dark cycle and had ad libitum access to food and water.

Models of ischaemic stroke and remote ischaemic conditioning in rats

The experiments were conducted in male Sprague–Dawley rats (220-250 g) ($n = 39$). The animals underwent surgery to allow transient middle cerebral artery occlusion (MCAO) to induce cerebral ischaemia, as an experimental model of stroke [76]. Rats were anaesthetised with isoflurane (4% induction, 2% maintenance) and the right common carotid artery was exposed. A size-matched silicon-coated monofilament (Doccol Corporation) was advanced through the right common and internal carotid arteries towards the middle cerebral artery junction until the resistance was met (distance ~ 2 cm). The wound was closed by removable stitches, analgesia was administered (Vetergesic, 0.01 mg kg^{-1}, i.p.), and the animals were placed in a heated recovery chamber. The animals were allowed to recover from anaesthesia to determine the presence of the functional signs of cortical ischaemia, including walking towards the contralateral side, left forelimb flexion and body rotation to the left when the animal was held by the tail. Animals not displaying these signs were excluded from the study. After 75 min of MCAO, the animals were re-anaesthetised with isoflurane and placed on a servo-controlled heating pad (body temperature maintained at 37 ± 0.5 °C). After 80 min of MCAO, sham-RIC or RIC was applied. The wound was re-opened to expose the carotid arteries, the MCA occluder was withdrawn 90 min after the onset of ischaemia, the common carotid artery was ligated, and the wound was closed by suturing. The animals were placed in a recovery chamber until their complete recovery, before returning to their home cages.

The animals were randomized into four experimental groups: (1) rats with MCAO/sham-RIC; (2) rats subjected to RIC starting 10 min before the onset of reperfusion; RIC protocol involved 4 cycles of 5 min left hind limb ischaemia

interleaved with 5-min reperfusion periods [10, 29], applied using an inflatable 12-mm cuff (IVM, USA). The cuff was inflated to 200 mmHg to stop the blood flow through the limb, as reported previously [12]; (3) rats with MCAO treated with a competitive GLP-1R antagonist Exendin (9–39) (Ex9, 50 µg kg^{-1}, intravenously) 20 min before the onset of reperfusion; (4) rats subjected to the RIC protocol starting 10 min before the onset of reperfusion and treated with Ex9 10 min prior to the first episode of RIC. Experimental timeline is illustrated by Fig. 1a.

Twenty four hours after MCAO, all animals underwent behavioural neurological assessment using the 0–22 scale, as described in detail previously [9, 79]. The principal points of the functional status evaluation were spontaneous activity, gait, postural signs, lateral resistance, limb placing, and parachute reflex. Higher neuroscores indicated more severe neurological deficits. The rats were then euthanized with sodium pentobarbital overdose (200 mg kg^{-1}, i.p.). The brains were immediately removed, sectioned at 1.25-mm thickness, stained with 1% triphenyl tetrazolium chloride (TTC) and fixed in formalin. The sections were photographed, and the infarct areas were determined by computerized planimetry (Image J). Infarct sizes (IS) are presented as percentages of the hemispheric volume (%HLV). Functional status evaluation and IS measurements were performed by the investigator blinded to the experimental animal group allocation.

Acute brain slice preparation

Young Sprague–Dawley rats (P21 of either sex) were humanely killed by cervical dislocation, the brains were removed and placed in ice-cold artificial cerebrospinal fluid (aCSF) containing 124 mM NaCl, 26 mM $NaHCO_3$, 3 mM KCl, 2 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 11 mM $MgSO_4$, 10 mM glucose saturated with 95% O_2/5% CO_2 (pH 7.4). Coronal cortical slices (thickness 300 µm) were cut using a vibratome and then incubated at room temperature for 1 h in a standard aCSF solution containing 1 mM Mg^{2+} saturated with 95% O_2/5% CO_2.

Recordings of cerebrovascular responses

Recordings were made from slices placed on an elevated grid in a flow chamber at ~ 32 °C (flow 2 ml min^{-1}). Cortical arterioles and capillaries were identified by the diameter of the vessel (arterioles > 10 µM; capillaries < 10 µM) and the presence of smooth muscle, as described [64]. Brightfield imaging recordings of cerebrovascular responses induced by experimental treatments were performed using a Zeiss Axioskop 2 upright microscope with a 40 × water immersion objective and a Hamamatsu CCD camera. Images were acquired every 30 s and vessel diameter changes were measured using ImageJ. The percentage change in vessel diameter from the baseline was calculated and the last 5 min of vessel diameter recordings from each group was averaged for comparison. Lactate (5 mM) was added to the perfusate to constrict the vessels. Lactate was used as a constricting agent because the release of this metabolite markedly increases in hypoxic and ischaemic conditions [43, 44, 57]. The model of brain ischaemia was induced by oxygen/glucose deprivation (OGD). OGD was induced by exposing the slices to aCSF saturated with 95% N_2/5% CO_2 gas mixture containing 7 mM sucrose, replacing glucose. The rate of aCSF flow through the chamber was increased to 4 ml min^{-1} (to reduce the time for gas exchange between the media and the atmosphere) and the period of OGD lasted for 25 min. Exendin-4 (Ex4; 100 nM, Tocris) was used as GLP-1R agonist. Ex9 (1 µM, Cohesion Biosciences) was used to block GLP-1Rs. *N*(gamma)-nitro-L-arginine methyl ester (L-NAME; 100 µM, Sigma) was used to inhibit nitric oxide synthesis. Adenylate cyclase was blocked with SQ22536 (100 µM, Tocris).

Measurements of brain tissue PO_2 in vivo

Young male rats (200–250 g; $n = 11$) were anaesthetised with urethane (initial dose, 1.3 g kg^{-1}, i.p.; then 10–25 mg kg^{-1} h^{-1}, i.v.). Adequate anaesthesia was ensured by maintaining stable levels of arterial blood pressure and heart rate, showing lack of responses to a paw pinch. The body temperature was maintained at 37 ± 0.5 °C. The femoral artery and vein were cannulated for measurement of the arterial blood pressure and administration of anaesthetic, respectively. The trachea was cannulated, and the animal was ventilated with room air using a positive pressure ventilator with a tidal volume of ~1 ml 100 g^{-1} and a ventilator frequency similar to the resting respiratory rate (~60 strokes min^{-1}). The animal was then placed in a stereotaxic frame and a small craniotomy (~ 1 mm in diameter) was made in the parietal bone. Brain tissue PO_2 was recorded in the left cerebral cortex using optical probes (250 µm tip diameter; OxyLite system; Oxford Optronix) placed ~ 2 mm below the surface of the brain, as described previously [58]. The operation of the sensor is based on fluorescence technology that allows real-time recordings of tissue PO_2. In urethane-anaesthetised rats, changes in brain tissue PO_2 parallel changes in cerebral blood flow [18], and were used in this study as a robust proxy measure of brain perfusion.

Immunohistochemistry

Mice expressing fluorescently tagged GLP-1R (Glp1r-cre/tdRFP) [16, 71] were used to determine the expression of the receptor by the brain blood vessels. The animals were terminally anaesthetised with sodium pentobarbital overdose (200 mg kg^{-1}; i.p.) and perfused through the heart with 0.01 M phosphate-buffered saline. The brains were removed

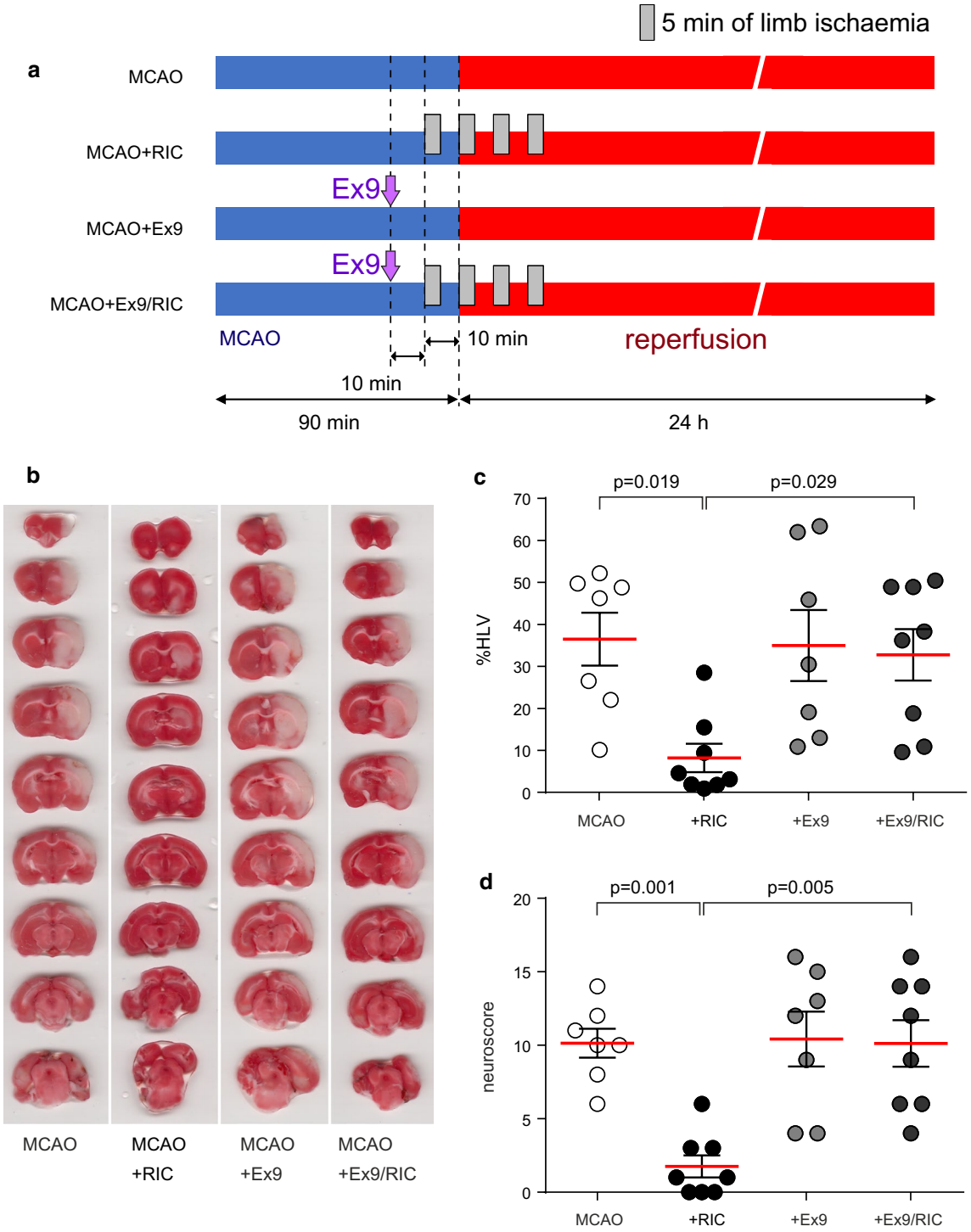

Fig. 1 GLP-1 receptors mediate neuroprotection against ischaemic stroke established by remote ischaemic conditioning. **a** Illustration of the experimental protocols. In rats, ischaemic stroke was induced by 90 min of middle cerebral artery occlusion (MCAO) followed by 24 h of reperfusion. Remote ischaemic conditioning (RIC) was established following 4 cycles of 5-min left hind limb ischaemia interleaved by 5-min reperfusion periods. Arrows indicate the time (20 min before the onset of reperfusion and/or 10 min prior to the application of the first RIC cycle) of intravenous administration of GLP-1 receptor antagonist Exendin(9–39) (Ex9). **b** Representative images of TTC-stained coronal brain sections from four experimental groups 24 h after MCAO. **c**, **d** Summary data illustrating the effect of RIC on brain infarct size and behavioural neurological traits 24 h after MCAO. Neuroprotection induced by RIC was prevented by systemic GLP-1 receptor blockade with Ex9. Infarct size is presented as a percentage of the hemispheric lesion volume (HLV). MCAO group, $n=7$; MCAO + RIC group, $n=8$; MCAO + Ex9 group, $n=7$; and MCAO + Ex9/RIC group, $n=8$ rats. Individual data and means ± SEM are shown. p values—ANOVA followed by Sidak's correction for multiple comparisons

and fixed overnight in 4% paraformaldehyde. Free-floating sections (20 µm thickness) were incubated (overnight at 4 °C) with anti-DsRed antibody (1:500) (Takara Bio) to detect GLP-1R expressing cells and anti-smooth muscle actin (SMA) antibody conjugated to FITC (1:350) (Sigma) for the detection of arteries and arterioles. Incubation with anti-rabbit AlexaFluor 568 (1:200, 2 h) was performed on the next day, followed by endothelial cell labelling using biotinylated lectin (1:250, 30 min) (Vector Biolabs), visualised using AMCA streptavidin (1:100, 1 h) (Vector Biolabs). Tiled images of coronal cortical cross-sections were obtained using Zeiss LSM 800 confocal microscope.

Data analysis

Differences between the experimental groups were analysed using GraphPad Prism 6 software. Comparisons were made using analysis of variance (ANOVA) followed by Sidak's *p* value correction for multiple comparisons or Student's *t* test, as appropriate. Data are reported as individual values and means ± standard error of the means (SEM).

Results

In rats, MCAO lasting 90 min led to the development of cerebral infarcts averaging 36.5 ± 6.3% ($n = 7$) of the hemisphere volume (Fig. 1b). RIC induced by 4 episodes of unilateral femoral ischaemia/reperfusion markedly reduced the cerebral infarct size (8.2 ± 3.4%; $p = 0.019$) (Fig. 1b) and improved the neurological score (from 10 to 2 on the 22-point scale; $p = 0.001$) (Fig. 1c). The neuroprotective effect of RIC was abolished by systemic GLP-1R blockade with Ex9 (Fig. 1b, c). In this experimental group (MCAO + Ex9 + RIC) the cerebral infarct size was 32.8 ± 6.1% ($p > 0.9$ vs 36.5 ± 6.3% in the MCAO + sham-RIC group) and the neuroscore was 10 ± 2 ($p > 0.9$ vs 10 ± 1 in the MCAO + sham-RIC group). Ex9 had no effect on the infarct size and behavioural neurological traits in animals subjected to MCAO and sham-RIC procedure (Fig. 1b, c). These data suggested that signalling via GLP-1Rs is critically important for RIC-induced neuroprotection against ischaemic stroke.

GLP-1 may act on brain vessels and aid neuroprotection by improving/maintaining cerebral blood flow in the regions surrounding the affected brain area. To test this hypothesis, we next determined the expression of GLP-1R by the brain vasculature and investigated the effects of GLP-1R activation on cerebral arterioles and capillaries. In the cerebral cortex of the GLP-1R reporter mice, expression of GLP-1R was found to be largely confined to arteriolar smooth muscle and endothelial cells (Fig. 2a, b). Patchy GLP-1R expression was detected in cells associated with isolated capillaries and veins (Fig. 2c, d). It was found that in the mouse brain 69 ± 17% of cortical arterioles, 2 ± 0.2% of capillaries and 6 ± 6% of venules express the GLP-1R.

Next, the effect of GLP-1R activation on cortical arterioles and capillaries was determined in acute brain slices. Vessels in brain slices are lacking tone and are typically fully dilated (due to the absence of the perfusion pressure), therefore, studies of cerebrovascular responses ex vivo require the application of the constricting agents [65]. We used lactate as a constricting agent because the release of this metabolite increases in hypoxic and ischaemic conditions [43, 44, 57]. In a series of preliminary trials, we found that lactate in concentrations of 5 mM and 20 mM in osmolarity-controlled aCSF reduced the diameter of cortical arterioles after 20 min of application by 24 ± 4% and 23 ± 1%, respectively ($p < 0.001$) (Fig. 3a, b). Therefore, in all the subsequent experiments we used 5 mM lactate to constrict the cortical arterioles.

In the presence of 5 mM lactate, bath application of GLP-1R agonist Ex4 (100 nM) caused significant dilations of cortical arterioles ($p = 0.031$), effectively reversing lactate-induced constrictions (Fig. 3a, c). The effect of Ex4 on cortical arterioles was not affected by L-NAME (100 µM) (Fig. 3d), suggesting that GLP-1R-mediated cerebrovascular responses are not mediated by the release and actions of nitric oxide. However, arteriolar dilations induced by Ex4 were prevented by the broad-spectrum adenylate cyclase inhibitor SQ22536 (100 µM) (Fig. 3e), indicating that the vascular effects of GLP-1R activation are mediated by a cAMP/PKA signalling pathway.

Next the effect of Ex4 on cerebral blood vessels was investigated in the ex vivo model of ischaemic stroke, induced by oxygen and glucose deprivation of cortical brain slices. Similarly to the effect of lactate, OGD led to strong constrictions of cortical vessels (Fig. 4a, b). OGD reduced cortical capillary diameter by 13 ± 6% ($p = 0.019$) and arteriolar diameter by 27 ± 4% ($p < 0.001$) 10 min after the stimulus onset. Ex4 had no effect on OGD-induced reduction in capillary diameter ($p = 0.74$; Fig. 4d), but reversed the effect of OGD on cortical arterioles ($p < 0.001$; Fig. 4a, c). The dilatory effect of Ex4 on cortical arterioles in conditions of OGD was blocked by Ex9 (1 µM; Fig. 4c), confirming that the effect of Ex4 is specific and mediated by GLP-1Rs.

To determine the effect of GLP-1R activation on cerebral blood flow in vivo, changes in brain tissue PO_2 induced by systemic administration of Ex4 (10 µg kg^{-1}, intravenously) were recorded in anaesthetised and artificially ventilated rats. It was found that Ex4 induces lasting increases in brain tissue PO_2 (Fig. 5). Ex4 increased PtO_2 in the cerebral cortex from 22.0 ± 1.6 mmHg to 30.4 ± 2.9 mmHg (38% increase; $p = 0.007$, $n = 6$) 30 min after the injections, and this effect was sustained for at least 3 h (Fig. 5). Since in this model changes in brain tissue PO_2 parallel changes in brain

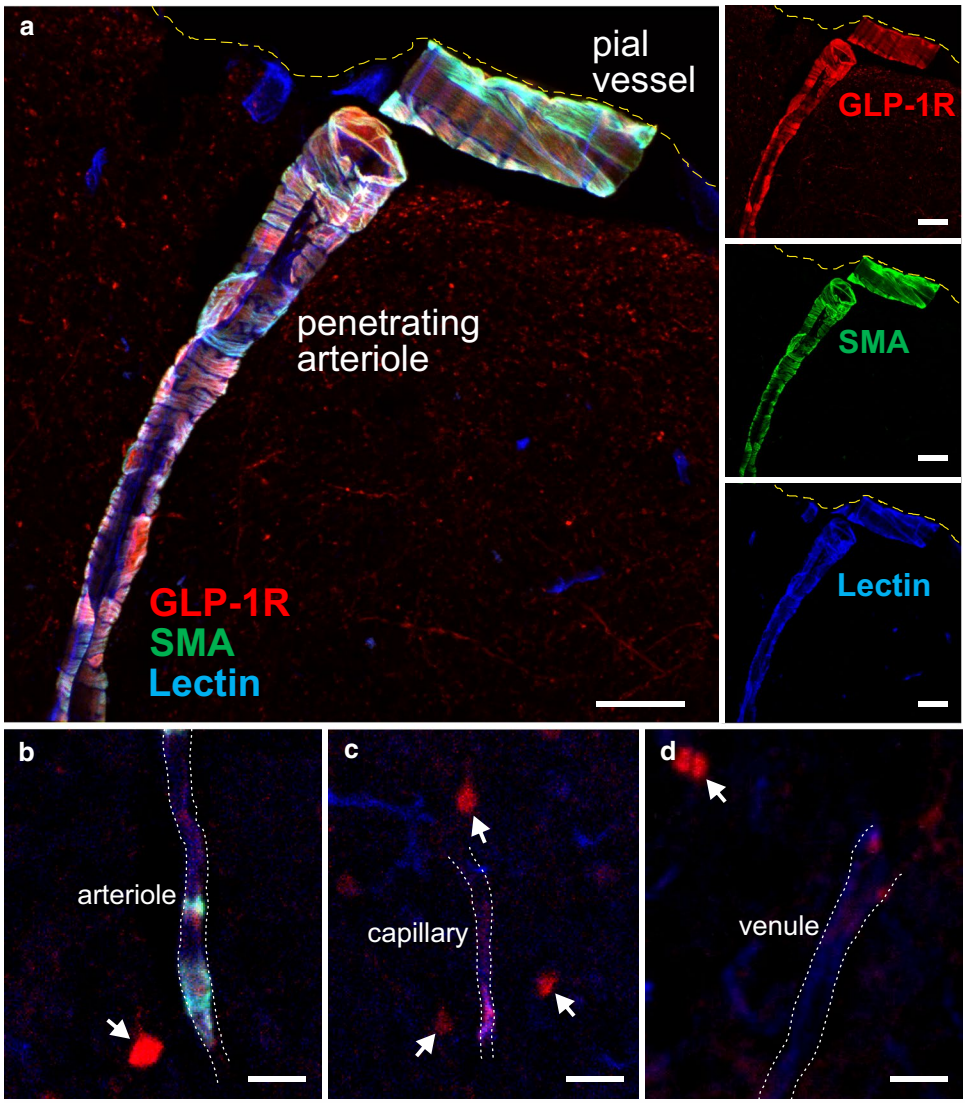

Fig. 2 GLP-1 receptor expression in cortical blood vessels. Representative images of coronal sections of the cerebral cortex of glp1r-Cre/ROSA26-tdRFP mouse, immunostained for red fluorescent protein (red), illustrating GLP-1 receptor expression associated with penetrating cortical arteriole (**a**), parenchymal arteriole (**b**), capillary (**c**), and venule (**d**). Arteriole smooth muscle cells were labelled by immunohistochemical detection of smooth muscle actin (SMA, green). Endothelial cells were labelled with lectin (blue). Arrows point to other cortical GLP-1 receptor-positive cells. Scale bars = 20 µm

perfusion [18], these data indicate that activation of GLP-1Rs increases cerebral blood flow.

Discussion

Powerful innate mechanisms of inter-organ protection are activated by remote ischaemic conditioning which can be established by cycles of ischaemia/reperfusion applied to an organ/tissue distant from the organ being protected [13]. Numerous experimental studies demonstrated the efficacy of RIC in protecting the brain and the heart against ischaemia/reperfusion injury. We previously proposed that the signalling mechanisms from the remote organ to the heart involve sensory (afferent) innervation of the peripheral tissue undergoing RIC, autonomic parasympathetic innervation of the visceral organs, and the release and actions of GLP-1 [5, 6, 62, 63]. The results of the present study suggest that RIC-induced neuroprotection against ischaemic stroke is mediated by a similar mechanism involving GLP-1R-mediated signalling. This conclusion is supported by the central finding of the present study that the highly selective GLP-1R antagonist Ex9 blocks the neuroprotective effect of RIC.

Several studies reported the neuroprotective effects of GLP-1 and GLP-1R agonists in preclinical models of stroke [9, 21, 45]. Yet, the mechanisms underlying the GLP-1R-mediated neuroprotection are not fully understood. There is evidence that treatment with GLP-1R agonists increases GLP-1R expression, increases the level of the neurotrophic factor BDNF, protects the blood–brain barrier through MMP-9 regulation, decreases microglial activation, oxidative stress and the release of apoptotic factors [41, 48, 50, 51, 78, 85]. Here we tested the hypothesis that GLP-1 actions reduce ischaemic brain tissue damage via activation of GLP-1R on cerebral vasculature leading to the improvement of brain blood flow. The data obtained support this hypothesis by

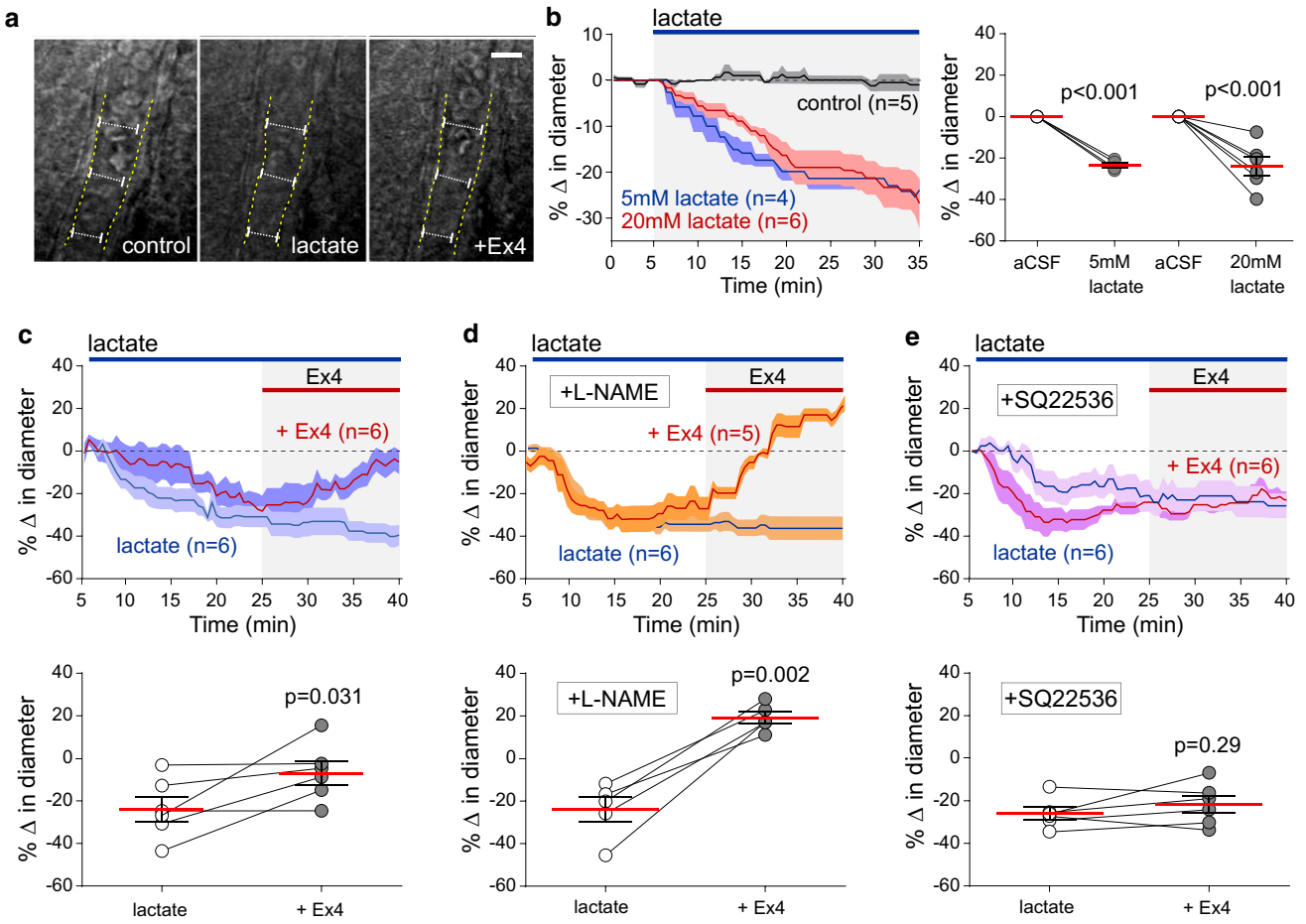

Fig. 3 GLP-1 receptor activation dilates cortical arterioles. **a** Representative DIC images illustrating a cortical arteriole response to 5 mM lactate and GLP-1 receptor agonist Exendin-4 (Ex4; 100 nM), applied in the presence of lactate, recorded in a coronal slice of a rat cerebral cortex. The yellow dashed line outlines the edge of the vessel; the length of the white dotted line illustrates the smallest diameter of the vessel recorded during the course of the experiment. Scale bar = 10 µm. **b** Summary data illustrating changes in the diameter of cortical arterioles recorded over time and peak arteriolar responses induced by lactate applied in concentrations of 5 or 20 mM. **c** Summary data illustrating the effect of Ex4 on the diameter of cortical arterioles pre-constricted with lactate (5 mM). Lactate-evoked arteriolar constrictions were effectively reversed by Ex4. **d** Summary data illustrating the effect of Ex4 on the diameter of cortical arterioles pre-constricted with lactate (5 mM) in the presence of nitric oxide synthase inhibitor L-NAME (100 µM). L-NAME had no effect on Ex4-induced arteriolar dilations. **e**, Summary data illustrating the effect of Ex4 on the diameter of cortical arterioles pre-constricted with lactate (5 mM) in the presence of adenylate cyclase inhibitor SQ22536 (100 µM). SQ22536 prevented the arteriolar dilations induced by Ex4. Numbers in parentheses indicate the numbers of slices obtained from the same number of animals (biological replicates). Individual data and/or means ± SEM are shown. *p* values—paired *t* test

showing that GLP-1R are expressed by the cells lining cortical arterioles and that the GLP-1R agonist Ex4 effectively reverses the constriction of these arterioles induced by lactate or simulated ischaemia (OGD) ex vivo and increases the cerebral blood flow in vivo. Collectively these data suggest that vascular mechanisms are likely to mediate the neuroprotective effects downstream of GLP-1R activation. The importance of preserving microvascular flow in the context of cardioprotection against myocardial ischaemia/reperfusion injury had been recently highlighted [32].

GLP-1Rs have been shown to be expressed by the arterial smooth muscle cells in the peripheral vasculature [71]. The vasodilatory effect of GLP-1 on the aorta was shown to be mediated through glucagon signalling, K_{ATP} channels, PKA phosphorylation [27, 30, 73] and increased NO production [3]. A recent study demonstrated that Ex4 causes dilation of retinal capillaries and this effect is mediated by NO [86]. The data obtained in this study show that the vasodilatory effect of GLP-1R activation on cortical arterioles is mediated by cAMP and is independent of NO production. That cAMP mediates the effects of a GLP-1 analogue is not surprising considering that GLP-1R is a well-characterized G_s-coupled receptor [17].

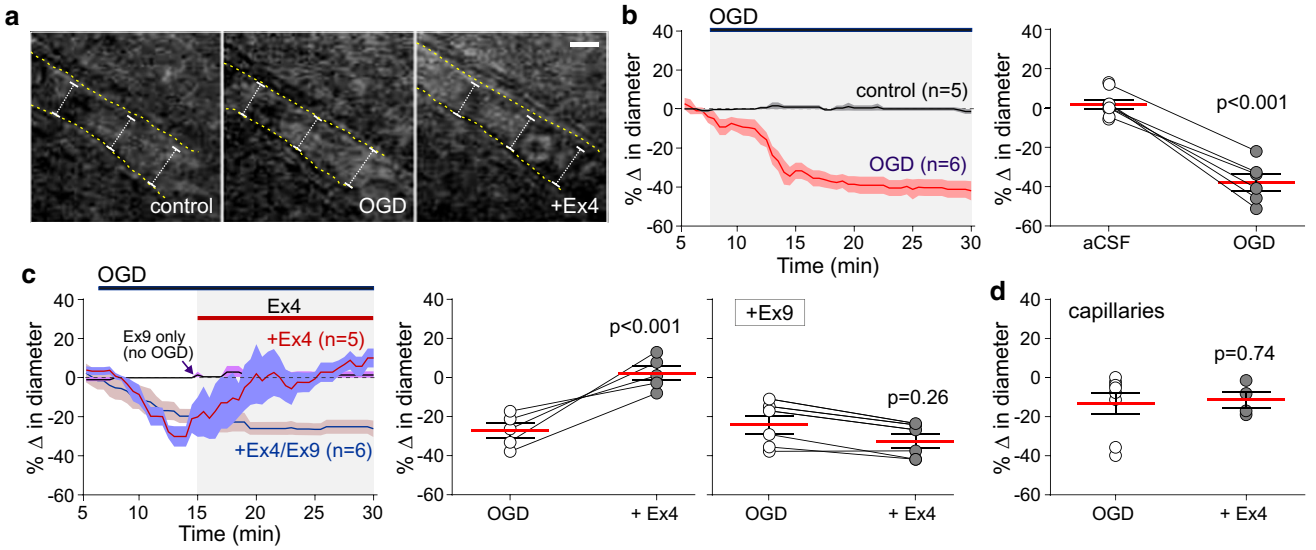

Fig. 4 GLP-1 receptor activation dilates cortical arterioles in the ex vivo model of ischaemic stroke. **a** Representative DIC images illustrating a cortical arteriole response to oxygen and glucose deprivation (OGD) followed by GLP-1 receptor agonist Exendin-4 (Ex4; 100 nM) applied in conditions of OGD, recorded in a coronal slice of a rat cerebral cortex. Scale bar = 10 µm. **b** Summary data illustrating changes in the diameter of cortical arterioles recorded over time and peak arteriolar responses induced by OGD. **c** Summary data illustrating the effect of Ex4 on the diameter of cortical arterioles in conditions of OGD in the absence and presence of GLP-1 receptor antagonist Ex9 (1 µM). Arteriolar constrictions induced by OGD were reversed by Ex4. **d** Summary data illustrating peak changes in the diameter of cortical capillaries induced by OGD and after application of Ex4 in conditions of OGD. Numbers in parentheses indicate the numbers of slices obtained from the same number of animals (biological replicates). Individual data and/or means ± SEM are shown. *p* values—paired (**b**, **c**) or unpaired (**d**) *t* test

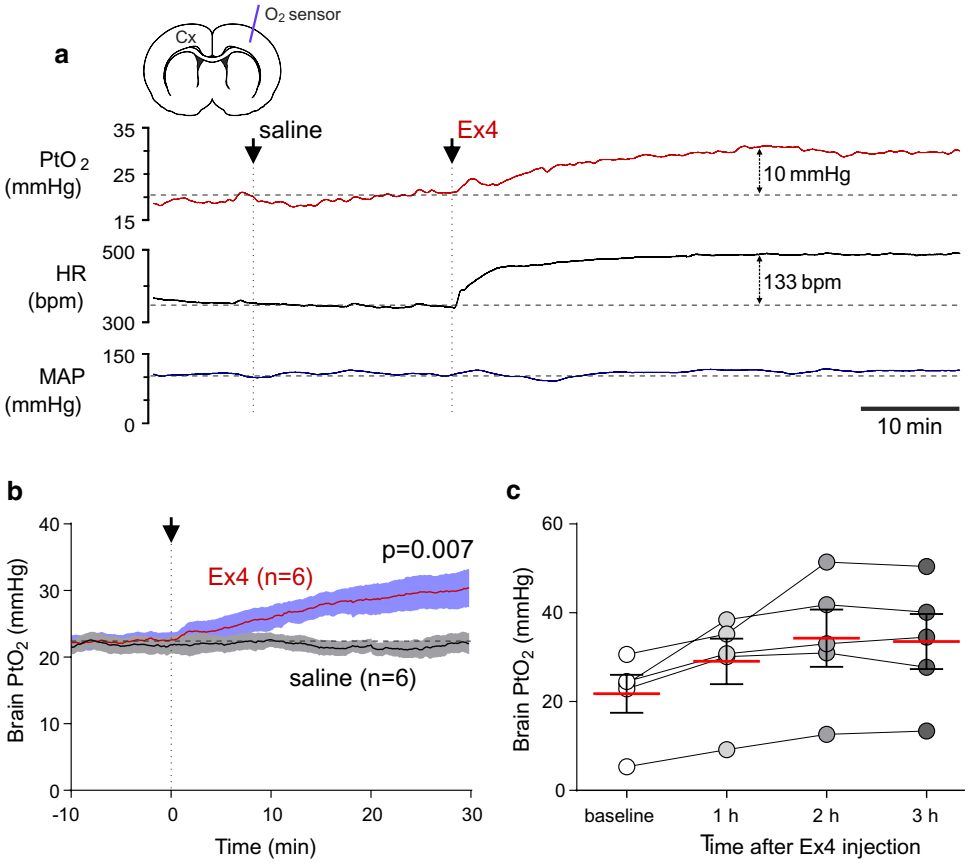

Fig. 5 GLP-1 receptor activation increases cerebral blood flow in vivo. **a** Representative raw traces of changes in brain tissue partial pressure of oxygen (PtO_2), heart rate (HR) and mean arterial blood pressure (MAP) after intravenous infusion of saline (0.1 ml) followed by GLP-1 receptor agonist Exendin-4 (Ex4; 10 µg kg^{-1}, 0.1 ml volume) in an anaesthetised and artificially ventilated rat. *Cx* cerebral cortex. **b** Summary data illustrating changes in brain PtO_2 recorded continuously for 30 min after the intravenous infusion of saline or Ex4 (10 µg kg^{-1}) in anaesthetised and artificially ventilated rats ($n = 6$). **c** Summary data illustrating the lasting effect of GLP-1R activation on brain PtO_2 in a separate cohort of animals ($n = 5$). 5 min-long recordings of PtO_2 were taken at baseline and then 1,2, and 3 h after the administration of Ex4 (10 µg kg^{-1})

In GLP-1R reporter mice, we found that most vascular GLP-1R expression in the cortex is associated with arterioles with no significant receptor expression detected in cells associated with brain capillaries and venules. These data are fully consistent with the results of single-cell RNA sequencing of brain vasculature [80]. Dilation of cortical arterioles induced by GLP-1R activation is likely to lessen the impact of the neurotoxic effects associated with impaired cerebral perfusion in stroke, as early improved perfusion is beneficial for better functional outcomes [28]. Indeed, maintaining/restoring blood supply in the penumbra has been shown to reduce the size and severity of cerebral infarcts [4]. The conclusions of this study are also supported by the recent evidence obtained in rodent models showing a marked reduction of cerebral blood flow after MCAO and improved collateral circulation in response to RIC [46, 55]. However, one of the limitations of our study is that the effect of RIC on collateral circulation was not assessed in conditions of systemic GLP-1R blockade with Ex9.

The source of GLP-1 that acts on GLP-1Rs expressed by cortical arterioles and mediates RIC-induced neuroprotection against ischaemic stroke remains to be determined. In our previously proposed model of RIC-induced cardioprotection, we suggested that cycles of remote ischaemia/reperfusion activate tissue nociceptors [5] that project to the CNS, leading to activation of a specific group of vagal preganglionic neurons [62] that innervate the gut [63], and upon activation stimulate the release of GLP-1 into the systemic circulation, culminating in cardioprotective action of GLP-1 on the heart [6, 81]. It is plausible that the same reflex mechanism underlies RIC-induced neuroprotection: RIC stimulates GLP-1 secretion by enteroendocrine cells of the gut, leading to GLP-1 release into the systemic circulation and its action on the brain vasculature. However, most of the intestinally-derived GLP-1 is believed to be rapidly degraded and low levels of GLP-1 are usually detected in the systemic circulation. For example, in a study showing that GLP-1R activation has protective effects on the endothelium in obese rats and patients, only modest increases in plasma GLP-1 were reported [67]. Yet, there is also evidence that the lasting effects of GLP-1 may persist for some time even when the circulating levels of the hormone return back to the normal level [38].

It is also conceivable that RIC stimulates the release of GLP-1 within the brain by the preproglucagon neurons that reside in the brainstem and have projections to the forebrain [16, 40, 53]. This distinct population of GLP-1-producing neurons reside in the dorsal vagal complex [53] and in close proximity to the pool of vagal preganglionic neurones that are critically important for RIC-induced effects [62]. Conceivably, afferent inputs from the peripheral organ/tissue undergoing RIC activate both neighbouring populations of brainstem neurons leading to simultaneous increases in vagal activity and release of GLP-1 in the CNS. However, the brainstem PPG neurones have no direct projections to the cerebral cortex and GLP-1 receptors are not widely expressed by cortical neurons [16]. Therefore, it seems unlikely that RIC-induced neuroprotection is mediated by direct actions of GLP-1 on brain neurons. Collectively, the data obtained in this study point to the importance of the mechanism that modulates cerebral blood flow and is mediated by vascular GLP-1Rs located on the luminal side of the blood–brain barrier.

Despite strong pre-clinical evidence of the effectiveness of RIC in protecting the heart and the brain against ischaemia/reperfusion injury, it has failed to translate into a clinical treatment of myocardial infarction or stroke. Studies conducted in young and healthy experimental animals reported that RIC can markedly reduce cortical infarcts [10, 72, 84]. Yet, clinical data show no major beneficial effect of RIC on stroke outcomes [68], although with some suggestion of reduced recurrent strokes with intracerebral artery stenosis treatment, and decreased stroke severity with carotid stenosis [69]. We previously demonstrated that RIC is critically dependent on autonomic parasympathetic mechanisms [62] and it is plausible that its clinical efficacy is compromised by the inability of many patients to recruit vagal activity. As we reasoned in our earlier publications [7, 25, 26, 63] and reported supporting evidence [1, 56], vagal tone decreases with age and could be severely diminished or even absent in many disease states, rendering many patients unable to recruit innate mechanisms of inter-organ protection. If GLP-1 is a common mediator of cardio- and neuroprotection induced by RIC, then administration of GLP-1 stable analogues may offer a better therapeutic solution for the treatment of acute myocardial infarction and ischaemic stroke, especially in patients with autonomic dysfunction. Indeed, exenatide (synthetic Ex4) had been shown to reduce the myocardial injury in patients with myocardial infarction [54], although the results of a recent clinical trial studying the individual effects of RIC, exenatide, and their combination (COMBAT-MI trial) showed no effect of these treatments on infarct size [22]. However, as it was highlighted [11] in that study neither the dose given, nor the plasma concentration of exenatide were known to determine the bioavailability of the drug at the time of treatment.

In conclusion, this study shows that GLP-1R activation mediates neuroprotection against ischaemic stroke established by remote ischaemic conditioning. The neuroprotection induced by GLP-1 is likely to be mediated via its action on cortical arterioles and improved perfusion in the areas surrounding the infarcted brain tissue.

Author contributions AVG: conceived and directed the project; SN, MB, PC, NK, INC, AK and SMT: performed research; SN, MB, PC,

and NK: analyzed the experimental data; SMD and DY: designed the experiments in the rat model of ischaemic stroke; AVG and ST: designed the experiments in brain slices; FR: contributed unique reagents/analytic tools; AVG and SN: wrote the paper; All authors revised the article critically for important intellectual content.

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Declarations

Conflict of interest The authors declare no competing financial interests.

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