LETTER

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Treatment of stroke with a PSD–95 inhibitor in the gyrencephalic primate brain

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All attempts at treating strokes by pharmacologically reducing the human brain's vulnerability to ischaemia have failed, leaving stroke as a leading cause of death, disability and massive socio-economic loss worldwide[1]. Over decades, research has failed to translate over 1,000 experimental treatments from discovery in cells and rodents to use in humans[2–4], a scientific crisis that gave rise to the prevailing belief that pharmacological neuroprotection is not feasible or practicable in higher-order brains. To provide a strategy for advancing stroke therapy, we used higher-order gyrencephalic non-human primates, which bear genetic, anatomical and behavioural similarities to humans[5,6] and tested neuroprotection by PSD-95 inhibitors—promising compounds that uncouple postsynaptic density protein PSD-95 from neurotoxic signalling pathways[7–10]. Here we show that stroke damage can be prevented in non-human primates in which a PSD-95 inhibitor is administered after stroke onset in clinically relevant situations. This treatment reduced infarct volumes as gauged by magnetic resonance imaging and histology, preserved the capacity of ischaemic cells to maintain gene transcription in genome-wide screens of ischaemic brain tissue, and significantly preserved neurological function in neurobehavioural assays. The degree of tissue neuroprotection by magnetic resonance imaging corresponded strongly to the preservation of neurological function, supporting the intuitive but unproven dictum that integrity of brain tissue can reflect functional outcome. Our findings establish that tissue neuroprotection and improved functional outcome after stroke is unequivocally achievable in gyrencephalic non-human primates treated with PSD-95 inhibitors. Efforts must ensue to translate these findings to humans.

All stroke neuroprotectants have failed to be translated to clinical human use[3], though many were seemingly effective in rodents. To bridge this biological gap, we conducted experiments in cynomolgus macaques. These non-human primates (NHPs) possess higher-order brains similar to humans and a rich behavioural repertoire amenable to assessments using standardized tests[11] (see Supplementary Discussion for choice of primate species). They are amenable to magnetic resonance imaging (MRI), and to the quantification of the ischaemic penumbra, the portion of ischaemic brain that is potentially salvageable if an appropriate treatment is given[12].

Although the MRI definition of a penumbra is controversial[12,13], we operationally defined it as that brain region in which the perfusion- and diffusion-weighted MR images are mismatched (PWI/DWI mismatch). We then developed two experimental models of surgical middle cerebral artery occlusion (MCAO) in the NHPs in which the PWI/DWI mismatch evolves at different rates (Supplementary Methods). In the most severe model, the MCAO is performed distally to the orbito-frontal branch of the MCA (Supplementary Fig. 1a, b, d, f). This isolates the ischaemic brain from the collateral circulation to create a large, rapidly evolving stroke with a rapidly shrinking penumbra such that, by 3 h, more than 80% of the MRI perfusion defect exhibits a DWI signal ($MCAO^{(-)P}$; Supplementary Fig. 1g, lower panels). In the second model, the MCAO is proximal to the orbito-frontal branch, allowing a small collateral circulation to slow the evolution of the DWI signal such that, at 3 h after MCAO, less than 30% of the perfusion defect exhibits a DWI signal, signifying a stroke with an ischaemic penumbra ($MCAO^{(+)P}$; Supplementary Fig. 1c, e). However, as in humans, this penumbra evolves to a completed stroke in the absence of treatment (Supplementary Fig. 1g, upper panels).

To test the feasibility of neuroprotection in the high-order brain we used the 20-mer peptide termed Tat-NR2B9c, comprising the nine carboxy-terminal amino acids of the *N*-methyl-D-aspartate receptor (NMDAR) NR2B subunit fused to the 11-mer HIV-1 Tat protein transduction domain[7]. This agent meets recommendations for preclinical stroke drug development established by the Stroke Academic Industry Roundtable (STAIR) committee[14,15]. It acts by perturbing the protein–protein interactions of PSD-95 (ref. 16), a synaptic scaffolding protein that links NMDARs to neurotoxic signalling pathways[8,17]. Treating rats subjected to MCAO with Tat-NR2B9c is neuroprotective in multiple rodent stroke models[7,10,18], making it a promising candidate for testing in NHPs. Our overall rationale for using Tat-NR2B9c is further described in the Supplementary Discussion and Supplementary Table 1.

We first evaluated Tat-NR2B9c in NHPs subjected to $MCAO^{(-)P}$, rationalizing that efficacy in this stroke model, though it produces more severe strokes than are usually seen in humans, might maximize the chance of ultimate clinical use. The primary outcome measure was infarct volume at 30 days measured from a T_2-weighted MRI study. Anatomical secondary outcomes were infarct volumes at 4 h and 24 h by DWI MRI, at 24 h by T_2 MRI and at 30 days by T_2 MRI and histology (Supplementary Fig. 2a). Neurobehavioural outcomes were measured throughout the 30-day observation period using the non-human primate stroke scale (NHPSS)[11] and a sensorimotor battery of tasks comprising the hill and valley task, two-tube task and six-well task (Supplementary Fig. 2b, c and Supplementary Methods)[19].

Twenty macaques were randomized to receive a 10-min intravenous infusion of Tat-NR2B9c (2.6 mg kg^{-1}) or placebo (0.9% saline) beginning 1 h after the onset of a 90-min $MCAO^{(-)P}$ (Supplementary Methods and Supplementary Fig. 2a). The dose selected for NHPs was approximated from calculations of a 'primate equivalent dose' extrapolated from previous doses used in rat studies[10] and was based on normalization to interspecies differences in body surface area[20].

Animals were transferred to the MRI scanner within 15 min of MCAO and all underwent perfusion imaging to quantify the brain volume deprived of blood flow during MCAO (tissue-at-risk[13]). Additionally, MR angiography was conducted to confirm MCAO (Supplementary Methods and Supplementary Fig. 2a). A second MR angiography was performed to confirm reperfusion after the 90-min MCAO, followed by diffusion imaging at 4 h. Animals were then awakened and allowed to recover. They were re-anaesthetized and re-imaged at 24 h and at 30 days (Supplementary Fig. 2a). NHPSS scores were assigned within 8 h of MCAO and up to 30 days, and the remaining neurological tests were conducted on days 7 and 30 (Supplementary Fig. 2b, c).

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Four of ten animals receiving placebo died within 48 h of their strokes owing to brain swelling and uncal herniation. Three animals treated with Tat-NR2B9c died as a result of surgical/anaesthetic complications unrelated to stroke or to drug (Supplementary Discussion). None were excluded from the 'intent-to-treat' analysis (see Supplementary Discussion for impact of the intent-to-treat approach). All missing data due to early mortalities were imputed to reflect the largest possible infarct volumes and worst neurological scores. Although this approach biases against detecting a significant treatment effect, it is the most conservative, and the most reflective of that used in human clinical trials.

There were no differences between the drug and placebo groups in physiological parameters (Supplementary Table 2a) or in the volume of tissue at risk as determined by perfusion imaging within 15 min of MCAO (Fig. 1a). However, by 24 h, animals treated with Tat-NR2B9c exhibited a significant reduction in infarct volume compared with placebo by DWI imaging (44.0% reduction; $P = 0.039$; Fig. 1b) and by T_2-weighted imaging (37.4% reduction; $P = 0.010$; Fig. 1b, c). This reduction in infarct volume persisted as reflected by the 30-day T_2-weighed MRI scans (38.7% reduction; $P = 0.013$; Fig. 1c) and by histological evaluation at 30 days (Fig. 1d; 59.3% and 73.6% reduction in infarct volume when evaluated by intent-to-treat and with early mortalities removed, respectively; $P < 0.001$). Because NHPs, like humans, may have variable infarcts after MCAO, the infarct volume of each animal was normalized to its MRI perfusion defect measured within 15 min of MCAO. This normalization revealed that treatment with Tat-NR2B9c reduced infarcts by 55% of the volume at risk by 24 h as gauged by DWI imaging, and by 70% at 30 days as measured with T_2-weighed MRI (Fig. 1e). Infarct volumes calculated from the 24 h DWI MRI correlated well with those obtained from the histological analysis at 30 days ($R = 0.691$, $P < 0.01$).

We conducted neurological assessments throughout the 30-day observation period using the NHPSS[11] and a sensorimotor battery of tasks including the hill and valley task, two-tube choice task and six-well task[19] (Supplementary Methods). The NHPSS is a composite of ratings analogous to the National Institutes of Health Stroke Scale used in human stroke trials[21]. A score of 41 points represents severe bilateral neurological impairment and 0 is normal. The remaining tests measure a combination of overall strength of the extremity, fine motor function and the influence of a hemi-neglect or visual field defect (Supplementary Methods and Supplementary Fig. 2b, c).

Animals treated with Tat-NR2B9c exhibited improved NHPSS scores from the earliest assessment at 8 h after ischaemia onset and throughout the 30-day observation period ($P = 0.018$, two-way repeated-measures analysis of variance (ANOVA); Fig. 1f). Performance in the two-tube choice task returned to pre-stroke levels in animals treated with Tat-NR2B9c, but remained completely impaired in the placebo group (Supplementary Fig. 3a), suggesting that brain salvage prevented 'extinction', the tendency for attention to items in ipsilesional hemispace to overshadow attention to items in contralesional hemispace[19]. Treatment with Tat-NR2B9c also significantly improved the performance of animals in the six-well (Supplementary Fig. 3b) and the hill and valley staircase tasks in the left upper extremity (Supplementary Fig. 3c, d). Right upper extremity performance also showed improvements, suggesting overall improved attention and perceptual ability (Supplementary Fig. 3e, f).

In human studies, CT or MRI measurements of infarct volumes have demonstrated no or modest correlations with clinical outcomes[22,23]. Thus, the intuitive dictum that infarct size reduction is beneficial is unproven. In the current study, the 24-h DWI and 30-day T_2-weighed MRI correlated highly with the 30-day NHPSS (Supplementary Fig. 3g, h). This suggests that infarct volumes measured by MRI of the high-order brain predict neurological performance after stroke, and may comprise a reasonable surrogate measure of clinical outcome.

Cerebral ischaemia has long been known to inhibit global protein synthesis and gene transcription profoundly[24], suggesting that impaired

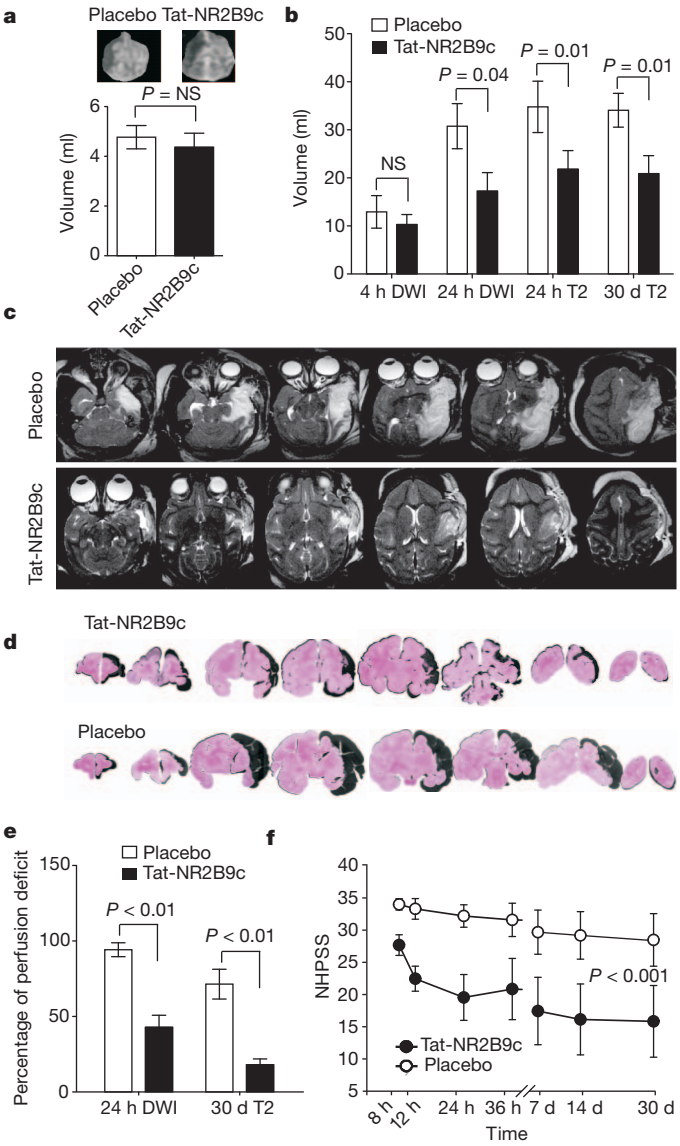

Figure 1 | Treatment with Tat-NR2B9c attenuates infarct volume in NHPs subjected to MCAO$^{(-)P}$. **a**, Volumes of perfusion defects (Methods) at baseline. NS, not significant. **b**, Analysis of stroke volumes as measured by DWI and T_2 MRI over 30 days. **c**, Representative T_2-weighted images of strokes incurred in placebo and drug-treated animals 24 h after MCAO$^{(-)P}$. **d**, Representative serial histological sections were stained with haematoxylin and eosin taken 30 days after MCAO$^{(-)P}$ from Tat-NR2B9c- and placebo-treated animals. Shaded areas represent the anticipated brain area based on mirroring the contralateral side. **e**, Stroke volumes calculated using 24-h DWI and 30-day T_2-weighted MRI scans normalized to the initial perfusion defects in each animal. **f**, NHPSS over the 30-day observation period. Symbols, bars and error bars in **a**, **b**, **e**, **f** indicate means of $n = 10$ per group ± s.e.m.

transcriptional capacity is a measure of dysfunction in affected cells. To examine whether neuroprotection by Tat-NR2B9c may prevent such dysfunction, we examined the transcription response on a genome-wide level in macaques subjected to stroke. Six additional macaques were subjected to a permanent MCAO$^{(-)P}$, and were treated either with Tat-NR2B9c or with placebo beginning at 5 min after ischaemia onset. The animals were placed in the MRI scanner, and DWI MRI scans were obtained every 15 min. The volume of brain in which DWI hyperintensity was detectable increased over time in both groups. However, treatment with Tat-NR2B9c attenuated the rate of this increase by about twofold (Fig. 2a; time constants = 2.20 ± 0.28 h and 4.50 ± 0.54 for control and Tat-NR2B9c, respectively; $P = 0.019$). Moreover, within the ischaemic volume, the DWI intensity in brains of

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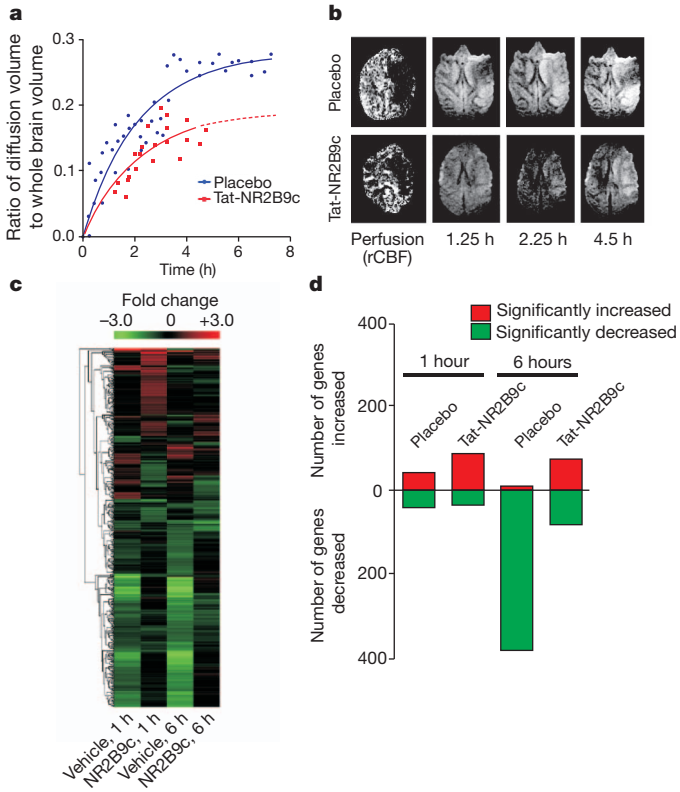

Figure 2 | Treatment with Tat-NR2B9c preserves capacity for transcription in NHPs subjected to stroke. Animals were subjected to MCAO$^{(-)P}$ and treated within 5 min with Tat-NR2B9c ($n = 3$) or placebo ($n = 3$). **a**, Time course of increase in DWI hyperintensity after MCAO$^{(-)P}$ in treated and control animals. **b**, Representative DWI images from **a**. **c**, Hierarchical cluster analysis of whole-genome response to MCAO$^{(-)P}$ from ischaemic penumbras of control and Tat-NR2B9c-treated animals. **d**, Plot of significantly differentially regulated genes in the drug- and placebo-treated NHP penumbras at 1 and 6 h after stroke.

Tat-NR2B9c-treated animals remained lower than that of untreated controls, suggesting that tissue within the infarct volume maintained better integrity (Fig. 2a, b).

We collected tissue from the ischaemic penumbras (adjacent to infarcted tissue) and from corresponding sites in the contralateral, non-ischaemic hemisphere at 1 h and 6 h after ischaemia onset (Supplementary Methods and Supplementary Fig. 4). We selected these after-stroke intervals as they correspond to timeframes during which most neuroprotectants are administered. RNA was extracted and hybridized to whole-genome macaque arrays (Supplementary Methods). Differential gene expression analysis revealed that treatment with Tat-NR2B9c resulted in a lower proportion of downregulated genes and preservation of the capacity to upregulate genes at both 1 h and 6 h after stroke (Fig. 2c, d and Supplementary Table 3). Specific genes and pathways that were differentially regulated in placebo and drug-treated animals included several that are associated with endogenous cytoprotective responses to ischaemia and cell stress (Supplementary Data, Supplementary Fig. 5 and Supplementary Tables 3 and 4). Overall, this genome-wide survey suggests that neuroprotection with Tat-NR2B9c preserved cellular functionality as gauged by the capacity for gene transcription in ischaemic brain tissue.

Our experiments demonstrate the feasibility of neuroprotection in the higher-order brain when treatment with a PSD-95 inhibitor is initiated 60 min after the onset of a 90-min MCAO$^{(-)P}$. However, only a small proportion of stroke victims may fit this pattern. Consequently, we next evaluated the use of treatment with Tat-NR2B9c in experiments having a broader clinical applicability. Currently, the only widely approved treatment for acute ischaemic stroke is reperfusion of occluded brain arteries using the intravenous infusion of the fibrinolytic agent, recombinant tissue plasminogen activator (rt-PA). Though Tat-NR2B9c is effective in rodents in the absence of reperfusion[10,18], the clinical use of rt-PA suggests that building neuroprotection onto a reperfusion setting may be the most practical means to maximize its use. Reperfusion with intravenous rt-PA is most beneficial in improving clinical outcomes when administered within 90 min after stroke onset, and benefit decreases thereafter until it is marginal or nil at 4.5 h (refs 25, 26). This narrow window for the use of reperfusion limits the number of patients who might benefit. Thus one potential application of early treatment with a neuroprotectant is to extend the interval during which clinical benefit may be obtainable from reperfusion therapy. To examine this, we evaluated in the NHPs whether administration of Tat-NR2B9c 60 min after MCAO$^{(-)P}$ onset might improve stroke outcome when reperfusion is delayed until the 4.5-h time point, at which intravenous rt-PA is no longer of significant benefit in humans.

Twelve macaques were randomized to receive a 10-min intravenous infusion of Tat-NR2B9c (2.6mg/kg) or placebo (0.9% saline) beginning 1 h after the onset of a 4.5-h MCAO$^{(-)P}$. Otherwise, methods were similar to our first study except for the timing of MRI scans and that final imaging and neurological assessments were conducted at 7 days.

There were no mortalities and no differences between the drug and placebo groups in physiological parameters (Supplementary Table 2b) or in the volume of tissue at risk upon MCAO$^{(-)P}$ (Fig. 3a). However, despite the prolonged ischaemic interval, animals treated with Tat-NR2B9c exhibited a significant reduction in infarct volumes compared with placebo when evaluated by T_2 and DWI imaging at 48 h and by T_2-weighted imaging at 7 days (Fig. 3b–e). Moreover, animals treated with Tat-NR2B9c exhibited improved NHPSS scores from the earliest assessment at 12 h after ischaemia onset and throughout the 7-day observation period ($P = <0.001$, two-way repeated-measures ANOVA; Fig. 3f), and trended to better performance in the six-well and the valley staircase tasks (Supplementary Fig. 6a). These results suggest that early treatment with Tat-NR2B9c may increase the window during which reperfusion may have functional benefits, even in the model of severe MCAO$^{(-)P}$ in which collateral circulation is limited and the penumbra is small (Supplementary Fig. 1). The size of the benefit of treatment at 4.5 h after stroke as gauged by MRI and by neurological evaluations suggests a potential for use of early neuroprotection to extend the benefits of reperfusion therapy even beyond the 4.5-h window.

Although treatment with a neuroprotectant within 60 min of stroke onset is feasible in a small subset of patients[27], extending the therapeutic window of administration would benefit a much greater proportion of stroke victims. Thus we determined whether administering Tat-NR2B9c at 3 h after stroke onset is beneficial in the setting of a prolonged MCAO. In humans, reperfusion with intravenous rtPA administered 3 h after stroke onset is beneficial even in the absence of neuroprotection[28]. This attests to the existence of a salvageable penumbra at this time in many patients. We reproduced this clinical situation experimentally by using the MCAO$^{(+)P}$ model in which the NHPs exhibit a significant PWI/DWI mismatch (penumbra) at 3 h. Like in humans, such a mismatch progresses to infarction in the absence of treatment (Supplementary Fig. 1).

Twenty-four macaques were randomized to receive a 10-min intravenous infusion of Tat-NR2B9c (2.6 mg kg^{-1}) or placebo (0.9% saline) beginning 3 h after the onset of a 3.5 h MCAO$^{(+)P}$. Other methods were unchanged, except that final imaging and neurological assessments were conducted at 14 days. There were no mortalities and no differences in physiological parameters (Supplementary Table 2c) or in the volume of tissue at risk upon MCAO$^{(+)P}$ between the groups (Fig. 4a). However, despite both the prolonged ischaemic interval and the delayed treatment with Tat-NR2B9c, drug-treated animals exhibited significant reductions in infarct volumes compared with placebo as evaluated by anatomical (MRI) criteria at 48 h and 14 days (Fig. 4b–e). The NHPs treated with Tat-NR2B9c also exhibited improved NHPSS

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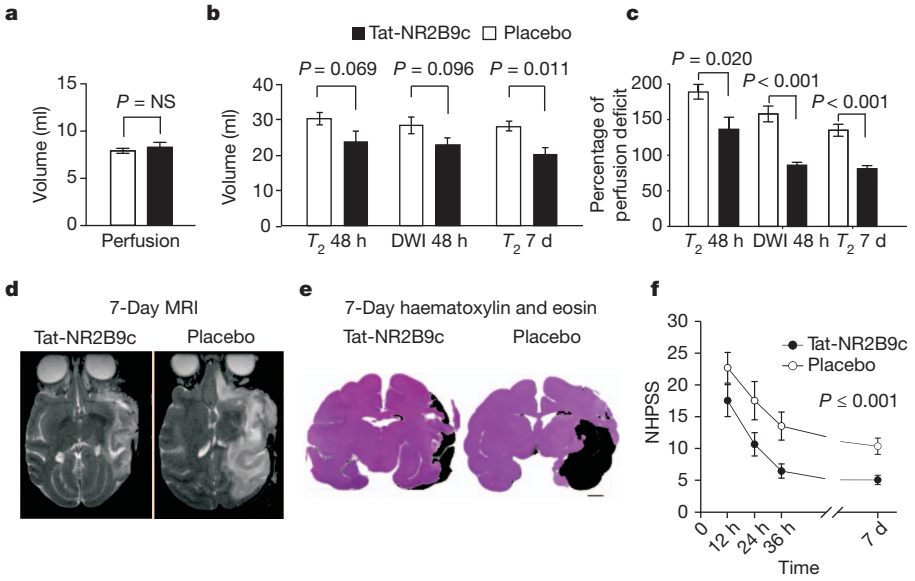

Figure 3 | Treatment with Tat-NR2B9c at 60 min improves MRI and functional outcome after a 4.5-h MCAO$^{(-)p}$. **a**, Volumes of perfusion defects (Methods) at baseline. **b**, Stroke volumes as measured by DWI and T_2 MRI over 7 days. **c**, Stroke volumes from the 48-h DWI and T_2- and 7-day T_2-weighted MRI scans normalized to each animal's initial perfusion deficit. **d**, Representative 7-day MRIs. **e**, Representative 7-day histology. **f**, NHPSS scores over the 7-day observation period. Symbols, bars and error bars in **a**, **b**, **c**, **f** indicate means of $n = 6$ per group ± s.e.m.

scores throughout the 14-day observation period ($P = 0.004$, two-way repeated-measures ANOVA; Fig. 4f), and tended to better performance in the six-well and the valley staircase tasks (Supplementary Fig. 6b). Thus, treatment with Tat-NR2B9c 3 h after stroke onset is effective in reducing stroke damage in NHPs. As this therapeutic window is practical in stroke victims[28], treatment with a PSD-95 inhibitor may constitute a clinically practicable therapeutic strategy.

After the failure of all past neuroprotectants in clinical trials, the feasibility of a pharmacological strategy to lessen the damaging effects of a stroke in the higher-order brain was in doubt[4,29]. The current prevailing belief, that neuroprotection in humans is not achievable, arose in response to these failures. However, there has never been concrete evidence that stroke mechanisms in rodents and higher-order brains differ to an extent that would preclude neuroprotection in primates or humans. Moreover, it is acknowledged that failures of clinical trials may have been related to trial design[29] and to shortcomings of past preclinical studies[30]. We have provided four distinct studies illustrating relevant situations in which neuroprotection with PSD-95 inhibitors is feasible in gyrencephalic Old World primates as gauged by measures of improved functional outcomes and reduced tissue damage by MRI, by histology and by retained capacity for transcription. Our findings using PSD-95 inhibitors may not be generalizable to other neuroprotective strategies. However, they defeat the current pessimistic belief by demonstrating that pharmacological neuroprotection of the high-order brain of gyrencephalic primates is unequivocally possible. Unless there exist fundamental, as yet unknown, relevant differences between such primates and humans, neuroprotection in humans using PSD-95 inhibitors should also be feasible.

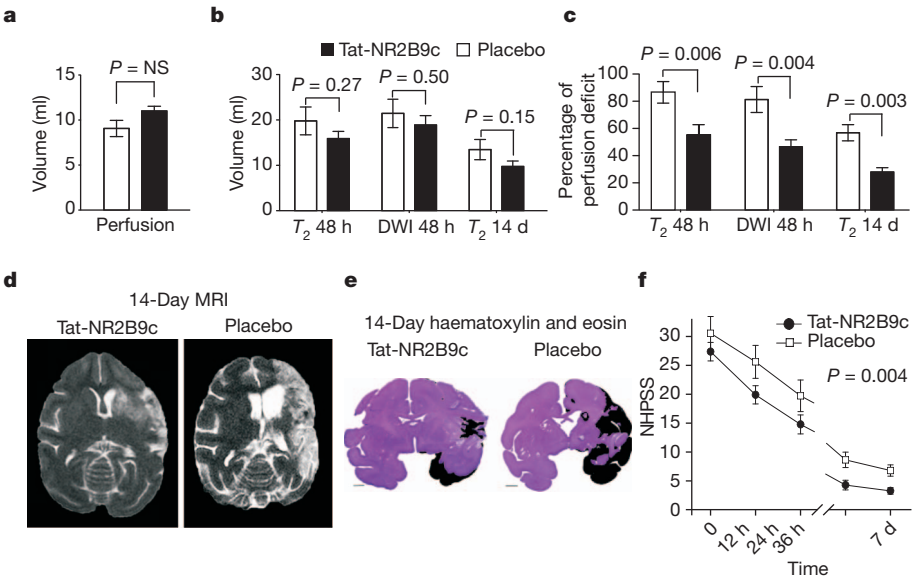

Figure 4 | Treatment with Tat-NR2B9c at 3 h improves MRI and functional outcome after a 3.5-h MCAO$^{(+)p}$. **a**, Volumes of perfusion defects (Methods) at baseline. **b**, Stroke volumes as measured by DWI and T_2 MRI over 14 days. **c**, Stroke volumes from the 48-h DWI and T_2 and 14-day T_2-weighted MRI scans normalized to each animal's initial perfusion deficit. **d**, Representative 14-day MRI and **e**, representative 14-day histology. **f**, NHPSS scores over the 14-day observation period. Symbols, bars and error bars in **a**, **b**, **c**, **f** indicate means of $n = 12$ per group ± s.e.m.

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METHODS SUMMARY

All experiments were conducted in male captive-bred cynomolgus macaques (*Macaca fascicularis*; 2.4–5.0 kg). They were approved by the Animal Care Committee of the University Health Network and complied with the relevant guidelines and regulations of the Canadian Council on Animal Care and with the 'recommendations for ensuring good scientific enquiry' of the STAIR committee[14], including allocation concealment and blinded assessment of all outcomes. MCAO was achieved under general anaesthesia through a right pterional craniotomy using a 5 mm titanium aneurysm clip. Animals were imaged on a 7 T Bruker BioSpec system running Paravision 4.0 software and using a B-GA20S gradient coil. RNA isloated from tissue samples (Qiagen Kit) and having an RNA integrity number greater than 7.0 was hybridized to Agilent whole-genome microarrays of rhesus macaque (part G2519F) according to the manufacturer's instructions.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions D.C. and M.T. performed the experimental procedures, collected and analysed the data and drafted the manuscript. L.T. performed experimental procedures and data collection.

Author Information Microarray data are deposited in National Center for Biotechnology Information (NCBI) Gene Expression Omnibus under accession number GSE35589. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/nature. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to M.T. (mike.tymianski@uhn.ca).

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METHODS

Animals. Male captive-bred cynomolgus macaques (2.4–5.0 kg) were pair housed in an environmentally controlled and enclosed primate colony of 10–30 animals on a 12-h light–dark cycle (light on 06:00 to 18:00). Animals were housed in 115 cm × 115 cm × 200 cm home cages and had access to a common recreation space during light hours. The colony was supervised daily by an experienced team consisting of a veterinarian, two veterinary technicians, one laboratory technician and one graduate student. Animals were provided with water *ad libitum*, daily complete diet in the form of Monkey Chow (Purina) and mixed dietary enrichment in the form of nuts, fresh fruit and vegetables throughout the day. Environmental enrichment in the form of puzzles, primate-specific toys and audiovisual media was provided during light hours. Animals were fasted for 12 h before administration of anaesthesia or behavioural testing.

Stroke model. Animals were anaesthetized (isoflurane 1.0–2.5%), intubated and ventilated. Non-invasive monitoring included blood pressure by leg cuff, end-tidal CO_2, O_2 saturation, electrocardiogram and temperature by rectal probe. Temperature was maintained (37 ± 0.5 °C) by heating blanket. A femoral arterial line was used to monitor blood pressure and blood gases. MCAO in cynomolgus macaques (3.0–4.0 kg) was performed using a right pterional craniotomy and occluding the right MCA in the Sylvian fissure[31] with a 5-mm titanium aneurysm clip either proximal or distal to the orbitofrontal branch and origin of lenticulo-striate arteries, thus producing a stroke with a large penumbra ($MCAO^{(+)p}$) or small penumbra ($MCAO^{(-)p}$), respectively (main text and Supplementary Fig. 1). The final infarct volume produced in the $MCAO^{(+)p}$ model was approximately 30% smaller than that produced with $MCAO^{(-)p}$ owing to the presence of leptomeningeal collaterals. At the end of the MCAO, the aneurysm clip was removed to restore blood flow.

Defining ischaemic penumbra tissue. Penumbra tissue was operationally defined as tissue that was not yet infarcted at the time of tissue collection, but which consistently went on to later infarction. Because the penumbra might be variable in macaques, eight 2 mm × 2 mm biopsies were taken at either 1 or 6 h after MCAO from cortex across the entire MCA vascular distribution ipsilateral to the stroke and contralateral to the stroke from sites mirroring those taken from ischaemic cortex (Supplementary Fig. 4). Biopsied positions were photographed (Supplementary Fig. 4a). To determine which of the eight biopsies represent penumbral tissue, the animals were transferred to a 7 T MRI scanner and DWI, T_2 and perfusion imaging was performed within 15 min after the biopsy and at 5.75 h. Penumbra tissue at 1 h was defined as tissue devoid of infarction that progressed to infarction at 5.75 h by DWI (Supplementary Fig. 4b–d). Penumbral tissue at 6 h was defined as tissue within the confines of the MR perfusion defect but without demonstrable DWI or T_2 hyperintensity. The use of MRI to define the penumbra of NHPs is essential as the amount of salvageable tissue shrinks significantly by 6 h in the $MCAO^{(-)p}$ model.

General MRI procedures. Imaging was performed on a 7 T Bruker BioSpec system running Paravision 4.0 software and using a B-GA20S gradient coil. A 15.5-cm inner diameter quadrature transmit/receive volume coil was used for NHP scans. NHPs were intubated, ventilated and imaged prone. Physiological monitoring was maintained throughout. The protocol provides stacks of two-dimensional T_2-weighted, perfusion- and diffusion-weighted images in an axial plane. T_2-weighted imaging uses the rapid acquisition with relaxation enhancement (RARE) method, also termed fast-spin echo (echo time/repetition time (T_E/T_R) = 84/5,000 ms, rare factor = 14, 225 × 225 matrix over a 9-cm field of view for 400 μm × 400 μm × 1500 μm resolution). Diffusion-weighted imaging uses a spin-echo multi-shot echo-planar imaging (EPI) technique (T_E/T_R = 32/10,000 ms, nine EPI shots, 250 kHz bandwidth, three orthogonal diffusion directions at $b = 1{,}000\ s\ mm^{-2}$, ten averages with a 180 × 180 matrix over a 9-cm field of view for 500 μm × 500 μm × 1500 μm resolution). Perfusion imaging was performed using a dynamic, contrast-enhanced, susceptibility-weighted perfusion method (T_2*EPI, T_E = 18 ms, two EPI shots, 2-s temporal resolution and 90 repetitions, 180 × 180 matrix over a 9-cm field of view for 700 μm × 700 μm × 1500 μm resolution) over five contiguous slices. For perfusion scans, a gadolinium (0.1 mmol kg^{-1}) bolus was injected intravenously, starting on the third repetition with a total injection time of 7 s through a peripheral intravenous port. Diffusion images were post-processed in MATLAB (Natick) to generate an average image from three $b = 1{,}000\ s\ mm^{-2}$ images and to calculate an apparent diffusion coefficient map. Stroke volumes were calculated using ITK-Snap contouring software (Pittsburgh) with stacks of average diffusion images reconstructed in three dimensions. Perfusion imaging was processed using PerfTool software[32] to produce cerebral blood flow maps.

Microarrays. RNA isolated from tissue samples (Qiagen Kit) and having an RNA integrity number greater than 7.0 were hybridized to Agilent whole-genome microarrays of rhesus macaque (part G2519F) according to the manufacturer's instructions. Cynomolgus arrays are not commercially available, but rhesus and cynomolgus macaques are closely related on the phylogenetic tree[33] and the use of NHP- and human- specific microarrays to study related Old World monkeys is customary[34]. The rhesus genome is sequenced and is integrated into the bioinformatics packages used in our analysis[35]. Several controls are embedded. First, each array contains 43,803 gene transcripts, of which most genes are duplicated. Additionally, each array includes positive and negative controls for the hybridization reaction and control spots to confirm orientation of the array and order and position of probe sets during scanning. Two-colour arrays were hybridized with experimental samples on one channel and a common Universal RNA Reference sample on the second channel allowing additional corrections for heterogeneity in hybridization and variation in background staining within arrays as well as a means to normalize data between arrays. This provided a control for inter-array comparisons. Intra- and inter- array corrections were performed before generating a ratio of fluorescence of sample: universal control for each complementary DNA expressed sequence tag. Gene lists were generated by comparing differential gene expression (stroke versus contralateral control brain) within each group at 1 and 6 h. Significantly, differentially expressed genes were defined as those with fold change ≥ 2 and $P \leq 0.05$. Mean fold change for each differentially regulated expressed sequence tag in each treatment group was included in an unsupervised hierarchial cluster analysis (Agilent GeneSpring GX 10) to provide a graphical display of gene expression data (Fig. 3). Unique and common differentially expressed genes from each treatment group were further reviewed by constructing Venn diagrams from the lists of significantly differentially expressed genes (Venny online tool, bioinfogp.cnb.csic.es/tools/venny/index.html). Significantly differentially expressed genes were also included in a pathway analysis using Pathway-Express, a tool that accounts for gene enrichment for KEGG pathways and ranks pathways based on the number of genes from each pathway present and the relative expression of these genes[36].

Experimental design and statistical analysis. The stroke experiments were performed in compliance with the 'recommendations for ensuring good scientific enquiry' of the STAIR committee[14]. Sample size determinations were based on the desire to detect a 40% difference in infarct volumes between drug and placebo based on the final T_2-weighted MRI at a power of 0.8, alpha = 0.05 and an assumed standard deviation of 30% of group means. Primary analysis was based on an intent-to-treat approach, with no exclusions of any animals enrolled. The cynomologus macaques were block-randomized to treatment with drug or placebo (vehicle only). The investigators responsible for the induction, maintenance and reversal of ischaemia, for decisions about the care of (including the early killing of) experimental animals and for assessment of all outcomes were blinded to the experimental group to which an animal belonged. Differences between groups were measured using Student's *t*-test, or repeated-measures ANOVA, as required. Missing values due to premature death or inability to complete a task were imputed to reflect the worst score achievable on the task, or the maximum possible stroke volume as defined by largest infarct volume achieved across all animals.

Neurological assessments. These were conducted using the previously validated NHPSS[11] and a sensorimotor battery of tasks including the hill and valley task, two-tube task and six-well task[19]. The NHPSS score is a composite of ratings of state of consciousness, defence reaction, grasp reflex, extremity movement, gait, circling, bradykinesia, balance, neglect, visual field cut/hemianopsia and facial weakness, many of which are incorporated in the National Institutes of Health Stroke Scoring system in humans. From a total of 41 points, 0 corresponds to normal behaviour and 41 to severe bilateral neurological impairment. The remaining tests were modified from assays developed for the common marmoset (*Callithrix jacchus*) as described elsewhere[19,37]. In addition to evaluating finer sensorimotor functions, they also test extinction and perceptual spatial impairment/neglect[19]. In pilot experiments in five macaques subjected to a 90-min MCAO, NHPSS results demonstrated an initial peak in score (mean 36.3, SEM = 5.7) that persisted for the first 36 h and then gradually dropped to a plateau between 14 and 30 days (mean = 14.36, SEM = 3.2). Sensorimotor testing revealed that animals had severe left spatiotemporal neglect and left hemiparesis that showed minor recovery over time at 7 and 30 days after stroke. These deficits were evident as significant delays in completion of six-well (mean delay of 7.8× and 5.33× baseline) and hill and valley tasks for the left arm (mean delays of 8.2× and 6.4× baseline on valley segment and 7.6× and 5.8× baseline on hill segment).

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