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Amine-modified single-walled carbon nanotubes protect neurons from injury in a rat stroke model

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Abstract

Stroke results in the disruption of tissue architecture and is the third leading cause of death in the United States[1]. Transplanting scaffolds containing stem cells into the injured areas of the brain has been proposed as a treatment strategy[2], and carbon nanotubes show promise in this regard, with positive outcomes when used as scaffolds in neural cells[3,4] and brain tissues[5]. Here, we show that pretreating rats with amine-modified single-walled carbon nanotubes can protect neurons and enhance the recovery of behavioural functions in rats with induced stroke. Treated rats showed less tissue damage than controls and took longer to fall from a rotating rod, suggesting better motor functions after injury. Low levels of apoptotic, angiogenic and inflammation markers indicated that aminemodified single-walled carbon nanotubes protected the brains of treated rats from ischaemic injury.

We initially investigated the use of carbon nanotubes as a scaffold for stem cell therapy because of their favourable electrical properties. Our preliminary results showed that nanotubes alone (without stem cells) could reduce the size of the infarct area in the brains of rats with induced stroke without any neuronal damage (Supplementary Fig. S1). This suggested that the nanotubes could improve the tolerance of neurons to ischaemic injury.

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Author contributions

H.J.L. and S.S.K. conceived and designed the experiments. H.J.L. and J.P. performed the experiments. H.J.L., O.J.Y., N.-E.L., W.B.L. and S.S.K. analysed the data. H.J.L., J.P., O.J.Y. and H.W.K. contributed to materials/analysis tools. H.J.L., O.J.Y., D.Y.L., D.H.K., N.-E.L., J.V.B. and S.S.K. evaluated the data and co-wrote the paper.

Additional information

The authors declare no competing financial interests. Supplementary information accompanies this paper at www.nature.com/naturenanotechnology. Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/. Correspondence and requests for materials should be addressed to S.S.K.

Following this unexpected observation, we pretreated rats with nanotubes before inducing a stroke and found that treated rats suffered less neuronal and motor damage.

Amine-modified single-walled carbon nanotubes (*a*-SWNTs) were prepared by treating commercially available carboxyl SWNTs (*c*-SWNTs) with N_2/H_2 plasma (see Supplementary Information). The right lateral ventricles of Sprague–Dawley rats were injected with *a*-SWNTs or phosphate-buffered saline (PBS, vehicle) 1 week before induction of ischaemic brain injury by middle cerebral artery occlusion (MCAO) surgery. *a*-SWNT-treated animals had significantly smaller infarct regions than PBS-treated animals 1 day after 90 min of MCAO (Fig. 1a,b).

Behavioural evidence of neurological damage was assessed by animal performance on a Rota-Rod treadmill and by determining their latency times to fall from the rotating rod (see Fig. 1c for treatment schedule). Latency times in PBS- and *a*-SWNT-treated animals were similar (180 s) before MCAO (‘Pre’ in Fig. 1d). One day after MCAO, latency times for both groups were reduced, but that of *a*-SWNT-treated animals was significantly longer than that of PBS-treated animals (150.6 s versus 114.5 s) (‘MCAO’ in Fig. 1d). This protective effect lasted up to 7 days after MCAO, with the latency time for *a*-SWNT-treated animals returning to 180 s (‘Post’ in Fig. 1d). These results demonstrate that *a*-SWNT preinjection improved motor functions after brain injury, and this is consistent with less neurological damage.

Compared to other nanotube formulations (raw and *c*-SWNTs; see Supplementary Information), *a*-SWNTs displayed the greatest protective effect in the Rota-Rod treadmill test (latency time of 150.6 s versus 137.5 s and 135.33 s for raw and *c*-SWNTs, respectively; Supplementary Fig. S2c). Rats treated with known neuroprotective peptides, humanin and BF-7, served as positive controls[6,7], and both had latency times to falling of 156.6 s and 157.75 s, respectively, 1 day after MCAO. Histopathological examination revealed that raw and *c*-SWNT-treated animals had larger infarct areas than *a*-SWNT-treated animals, suggesting that amine modification is important for neuroprotection (Supplementary Fig. S2a,b). These results are consistent with previous findings that positively charged modifications such as amine ($–NH_2$) groups can stimulate neurite outgrowth[8]. Additionally, plasma treatment may have increased surface defects on the nanotubes, resulting in greater surface energy and polarity, which favour the growth of neural tissue[9].

To determine the molecular basis for the greater protective effects of *a*-SWNTs relative to those of raw or *c*-SWNTs, *a*-SWNTs were analysed using X-ray photoelectron spectroscopy (XPS). The broad-scan XPS spectrum of *a*-SWNTs (Supplementary Fig. S3a) showed an additional N 1s peak not seen in the spectra of the raw and *c*-SWNTs[10]. Examination of the high-resolution spectra (Supplementary Fig. S3b–d) revealed salient increases in the intensity of the CO–NH peak at 288.1 eV in the C 1s spectrum and the peaks corresponding to NH_2 at 399.2 eV, CN at 400.1 eV, and C = N–OH at 401.6 eV in the N 1s spectrum due to plasma amination. These results reflect the formation and chemical bonding of amine groups on the surface of *a*-SWNTs through plasma amination; this large number of functional groups on *a*-SWNT surfaces imparts substantial charge effects and electrostatic activity[10,11].

When non-ischaemic animals were injected with *a*-SWNTs or PBS, no anatomical changes in the brain or neuronal damage were observed 3 weeks after injection, and no impairment of motor function was found after 6 weeks in *a*-SWNT-treated animals (Supplementary Fig. S4). Fourier transform Raman spectroscopy confirmed the presence of a-SWNTs in the brain, which persisted for at least 6 weeks (1,650 nm Raman shift, Supplementary Fig. S4c). Together, these results indicate that *a*-SWNTs did not damage brain tissues or cause behavioural impairment in normal, non-ischaemic rats.

To further understand the mechanisms of the *a*-SWNT-induced protection of the rat brain after MCAO, we evaluated neuronal apoptosis using terminal deoxynucleotidyl transferase biotindUTP nick end labelling (TUNEL). The numbers of TUNEL-positive cells 7 days after MCAO were similar in brain sections from *a*-SWNT-pretreated and sham-operated animals, which underwent neither *a*-SWNTs injection nor MCAO (Fig. 2a,e). Brain sections of rats pretreated with PBS (Fig. 2c) had significantly higher numbers of TUNEL-positive cells than those pretreated with *a*-SWNT (Fig. 2e) and sham-operated rats (Fig. 2a). The expression levels of apoptosis markers were also measured in the infarct region of the cortex. Western blot analysis of the cerebral cortex, including the ischaemic penumbra, 7 days after MCAO, revealed strong upregulation of proapoptotic proteins, including p53 and Bax, in PBS-treated rats and reduced expression of these proteins in *a*-SWNT-treated rats (Fig. 2g).

Inflammation and glial responses are important components of the cellular and molecular pathways involved in stroke-induced destructive responses[12,13]. The effect of MCAO on inflammation and glial activation in PBS- and *a*-SWNT-treated rats was determined by immunohistochemical staining of the ipsilateral hemispheres for glial fibrillary acidic protein (GFAP) and ionized calcium-binding adaptor molecule-1 (Iba-1). GFAP- or Iba-1-positive cells appear brown due to 3,3′-diaminobenzidine staining. To normalize for interindividual variation, the contralateral side of each rat brain was also investigated.

MCAO increased GFAP and Iba-1 expression, but, compared to rats pretreated with PBS, *a*-SWNT pretreatment showed lower expression of both markers and the staining pattern appeared similar to that in sham-operated rats (Fig. 3a–f,m,n). The contralateral hemispheres from PBS- and *a*-SWNT-treated animals, as well as from sham-operated rats, did not exhibit significant differences in glial activation (Fig. 3g–l). *a*-SWNTs were found in the dentate gyrus of the hippocampus and the surrounding area of the contralateral side, but no changes in glial activation or cellular morphology were seen, indicating that *a*-SWNT itself is inert on the contralateral side after MCAO (Supplementary Fig. S5). Nanotubes were also seen in the dorsal third ventricle and dentate gyrus on the ipsilateral side of *a*-SWNT-injected animals.

The levels of proinflammatory cytokines such as IL-1β and TNF-α in the ipsilateral cortex were lower in *a*-SWNT-treated animals than in PBS-injected animals (Fig. 3o,p), indicating that *a*-SWNT treatment protects against ischaemia and inhibits glial activation and post-ischaemic inflammation.

Cellular defences against brain ischaemic injury normally involve neurorestorative processes, including the antiapoptotic, neurogenic and angiogenic functions that are stimulated in response to ischaemia[14,15]. MAPK/extracellular signal-regulated kinase (ERK) and PI3K/Akt pathways are important intracellular signalling pathways that promote cell survival and regulate apoptosis via antiapoptotic proteins, such as Bcl-2 or Bcl-xL[16–18]. The potential involvement of ERK/Akt signalling pathways was examined by western blot analysis of the ipsilateral cortex tissue. p-ERK levels in PBS-treated rats were ~60% higher than levels in *a*-SWNT-treated rats (Fig. 4a,b). Increased ERK activation in the infarcted region of PBS-treated rats was likely related to the increased extent of infarction.

In contrast to ERK, p-Akt levels normalized to total Akt levels (both p-Akt and non-phosphorylated Akt) were similar in PBS- and *a*-SWNT-treated animals. Because pathological conditions such as stroke would induce survival mechanisms by elevating p-Akt levels, for a-SWNT-treated rats that showed little brain damage to have p-Akt levels similar to PBS-treated animals suggests that the enhanced p-Akt levels in nanotube-treated animals cannot explain the differences in injury. However, this does not mean that the Akt pathway was not important in limiting the extent of injury. To further investigate the involvement of Akt activation in the protective role of *a*-SWNTs in the MCAO stroke model, the Akt inhibitor LY294002 was injected into the ipsilateral ventricles of rats 20 min before MCAO surgery. All PBS-treated rats injected with 2.5 μg kg^{21} LY294002 died after MCAO-induced ischaemia (Supplementary Fig. S6a–c). In contrast, no deaths occurred among *a*-SWNT-pre-treated animals, despite findings of severe infarction and impaired behavioural function. Treatment with 0.25 μg kg^{21} LY294002 (a 10-fold lower dose) was associated with anatomical and functional damage in all PBS-treated animals after MCAO-induced ischaemia (Table 1). *a*-SWNT-pretreated rats subjected to ischaemia did not exhibit anatomical damage, although they showed mild changes including shorter latency time in the Rota-Rod treadmill test (Table 1; Supplementary Fig. S7). Therefore, the Akt pathway may play a critical role in limiting MCAO-mediated brain damage in both PBS- and *a*-SWNT-treated animals. However, Akt phosphorylation is not the only mechanism of protection afforded by *a*-SWNTs, as *a*-SWNT treatment can overcome the inhibition of the Akt pathway.

N-Cadherin (N-Cad), which is important for cell-to-cell interactions and adhesion in the central nervous system, promotes neural survival and synaptogenesis, including axonal guidance, synaptic remodelling and plasticity[19,20]. N-Cad modulates cell survival, tissue repair, synaptic function and transcriptional activation by interacting with other synaptic adhesion molecules after cerebral ischaemic injury[21]. To determine whether the *a*-SWNT-induced protective effects against ischaemia are related to N-Cad protein levels, N-Cad expression was measured in PBS- and *a*-SWNT-treated rats. As shown in Fig. 4a,b, N-Cad expression was ~1.8-fold greater in *a*-SWNT-treated animals than in PBS-treated animals. Maintenance of elevated N-Cad expression by *a*-SWNTs may be important for maintaining cellular adhesion.

The involvement of angiogenesis-related factors such as HIF-1α and VEGF in *a*-SWNT-mediated neuroprotective effects was also determined after MCAO. HIF-1α and VEGF expression levels in *a*-SWNT-treated animals were 40 and 80%, respectively, of those in

PBS-treated animals (Fig. 4c,d). Expression of doublecortin (DCX), a marker of neurogenesis, was much lower in a-SWNT-treated rats than in PBS-treated animals. These data are consistent with the increased tendency of brain tissues injured by stroke to activate angiogenesis and neurogenesis to promote functional recovery[14]. Reduced HIF-1α, VEGF and DCX expression levels in *a*-SWNT-treated animals reflect a lesser need for restorative processes due to effective inhibition of brain damage by *a*-SWNTs.

To date, the role of carbon nanotubes in protecting against ischaemic brain damage has not been well characterized *in vivo*. Our results show that carbon nanotubes can protect the brain from ischaemic damage by reducing apoptosis, inflammation and glial cell activation. Furthermore, the plasma amination, positive charge and high surface energy of *a*-SWNTs provide a favourable environment for neurons[11,22,23]. Our results also demonstrate that activation of restorative mechanisms against ischaemic damage such as angiogenesis and neurogenesis, which were observed in PBS-treated animals, did not occur in *a*-SWNT-treated rats, reflecting the effective blockage of ischaemic damage by *a*-SWNTs.

Our results suggest that the Akt pathway is necessary for the full protective role of *a*-SWNTs against ischaemic brain damage. Another possible mechanism mediating the effect of *a*-SWNTs is the maintenance of cell-to-cell interactions, as implied by higher levels of N-Cad after *a*-SWNT treatment.

a-SWNT injection resulted in increased tolerance of neural tissue to ischaemic injury and decreased infarction area caused by transient MCAO. Human application will require further study of *a*-SWNTs pharmacokinetics, including the persistence of their effects in the brain and improved delivery methods. Nevertheless, our work lays the foundation for further studies to elucidate the protective effects of SWNTs against ischaemic brain injury.

Methods

Synthesis of *a*-SWNTs

Commercially available raw SWNTs (ASP-100F, Hanwha Nanotech) and carboxyl-functionalized SWNTs (*c*-SWNTs) (90% pure; diameter, 4–10 nm; length, 500–1,500 nm, Sigma-Aldrich) were purchased. Fourier transform infrared spectroscopy measurements confirmed the presence of carboxyl groups in the *c*-SWNTs (not shown). *c*-SWNTs were ultrasonicated in a distilled water bath for 30 min and dried on silicon wafers. *a*-SWNTs were obtained by treating *c*-SWNTs with N_2/H_2 plasma in an inductively coupled plasma reactor with no substrate biasing; treatment was performed at a top electrode power of 600 W and a bottom bias power of 170 W (self-bias voltage of 150 V) with N_2/H_2 flow rates of 50/50 s.c.c.m. and a plasma treatment time of 35 s.

Focal cerebral ischaemia–reperfusion injury model

Under a surgical microscope, a middle incision was made in the neck, and the right common carotid and external carotid arteries were carefully exposed and dissected from the surrounding tissue. A 5–0 monofilament ethilon suture (Ethicon) was inserted at the bifurcation of the external and internal carotid arteries. The suture tip was rounded by heating and then coated with a 0.1% poly-L-lysine (PLL) solution in water before insertion.

PLL coating facilitates adhesion of the suture to the endothelial lining of the vessel, resulting in improved, more consistent occlusion of the blood vessel. After 90 min of ischaemia, the filament was withdrawn.

In the sham-operated group (n = 50), the middle cerebral artery was not occluded. In the *a*-SWNT treatment group (n = 136), 19 animals died during MCAO surgery, and 14 rats were not analysed further due to poor induction of stroke. Raw SWNT-treated (n = 15) and *c*-SWNT-treated (n = 25) animals were included as controls and underwent surgery with the same procedure. LY294002 treatment with the indicated concentration (n = 65, Sigma-Aldrich) was administered by injection into the lateral ventricle 20 min before MCAO. As positive controls, 30 min before MCAO, the lateral ventricles of the rats were injected with humanin (n = 10, Peptron) and BF-7 (n = 10, Rural Development Administration, Korea) neuroprotective peptides. In the PBS treatment group (n = 145), 41 rats died following MCAO, and 12 rats were not analysed further due to poor induction of stroke (see Supplementary Information for more details).

Neurological assessment

The Rota-Rod treadmill test (ROTA-ROD Jones & Roberts for Rat 7750, UGO BASILE) was used to evaluate the sensorimotor coordination of each rodent at different times after surgery. Animals were habituated to the Rota-Rod and trained to remain on a rotating drum (constant speed of 6 r.p.m.) for a minimum of 90 s to provide a preoperative baseline; those that did not achieve baseline criteria were excluded from further study. In the testing sessions, animals were placed on the Rota-Rod and the rotational speed accelerated from 6 to 19 r.p.m. over 180 s. The time at which the animal first fell off the drum (latency time to fall) was recorded.

Analysis of protein extracts from brain tissues

Animals were killed 7 days after MCAO. Brains were harvested and the right hemispheres containing the ischaemic sites were excised and homogenized in lysis buffer (137 mM NaCl, 20 mM Tris, pH 8.0, 1% NP40, 10% glycerol, 1 mM PMSF, 10 mg ml^{21} aprotinin, 1 mg ml^{21} leupeptin and 0.5 mM sodium vanadate). Protein extracts were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Blots were probed with antibodies specific for the following proteins: p53, Bax, p-Akt, Akt1, N-Cad, HIF-1a, VEGF and DCX (1:500, Santa Cruz Biotechnology); b-actin (1:5,000, Assay Designs); and p-ERK and total ERK (1:1,000, Cell Signaling Technology). Blots were further probed with secondary antibodies, and immunoreactive proteins were detected using the ECL detection system (Amersham Pharmacia Biotech). Western blot analysis was performed on samples from three separate experiments.

GFAP and Iba-1 staining was carried out using standard immunohistochemistry, and rat IL-1β and TNF-α were measured using standard enzyme-linked immunosorbent assay (ELISA) kits (see Supplementary Information for details).

Statistical analysis

Data were expressed as means±standard error of the mean (s.e.m.) values. The statistical significance between groups was determined by oneand two-way analysis of variance (ANOVA). *P*-values of <0.001 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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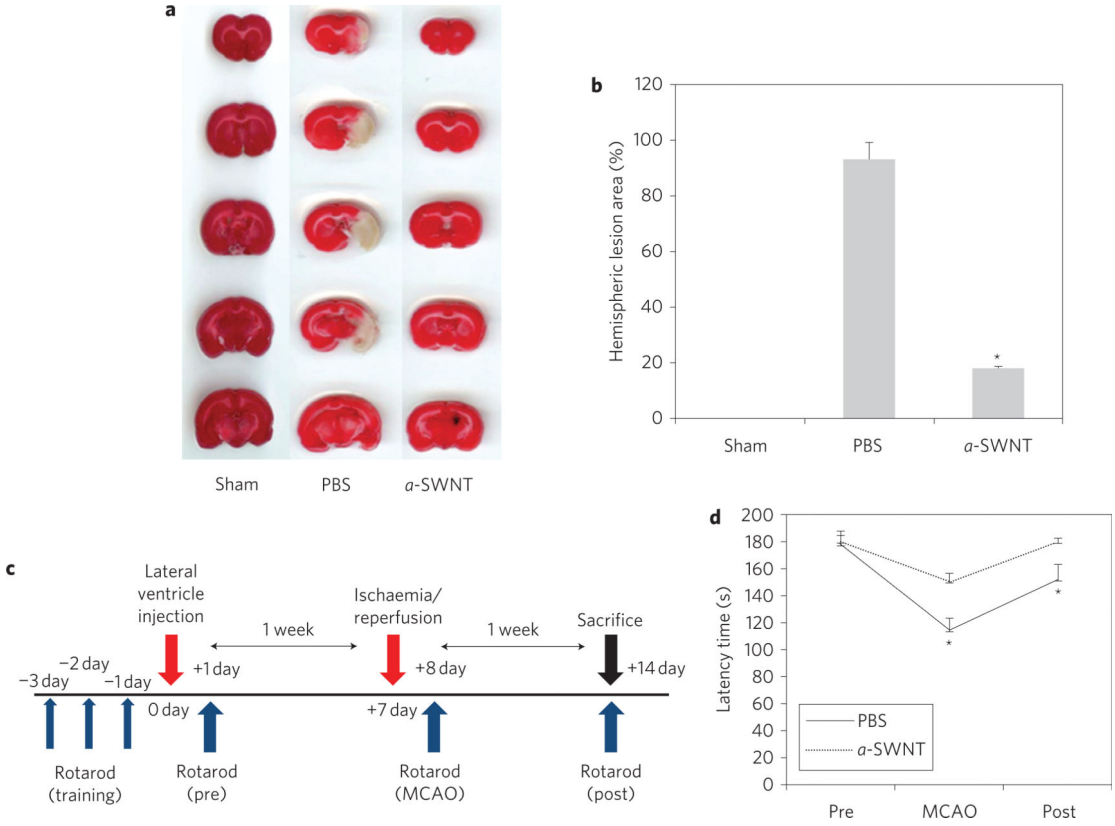

Figure 1. Pretreatment with *a*-SWNT-protected animals following ischaemia–reperfusion

One week before MCAO, animals received either PBS or *a*-SWNTs. Animals were killed 1 day after reperfusion. **a**, Coronal brain sections (stained with triphenyltetrazolium chloride (TTC) solution) of sham-operated control and PBS- or *a*-SWNT-treated rats. Brain slices were arranged in sequence. White areas represent the infarcted region after MCAO. **b**, Quantification of the ischaemic lesion of brain sections in **a**. Data are means±s.e.m. **P* <, 0.001 versus the PBS group. **c**, Schematic of injection and training schedule. **d**, Graph showing that pretreatment with *a*-SWNTs reduced neurological damage after ischaemia. Neurological score was evaluated by the Rota-Rod treadmill test. Data are means±s.e.m. **P* <, 0.001 versus pre-MCAO.

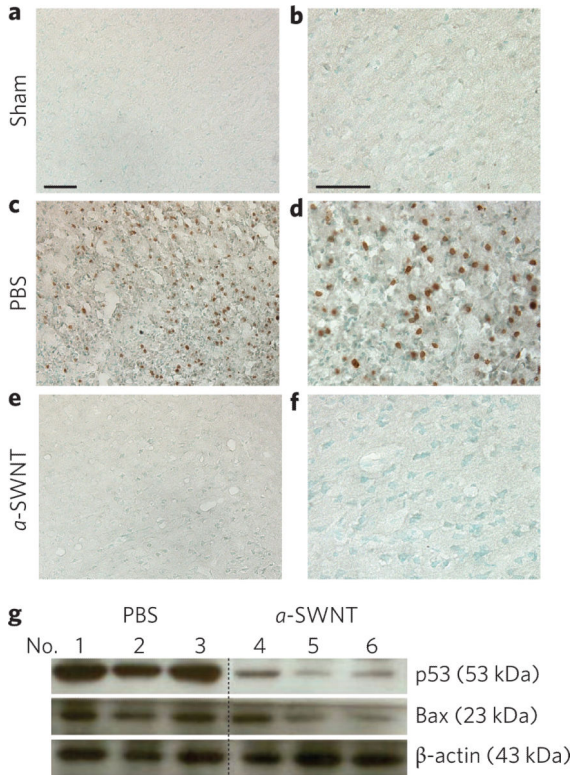

Figure 2. Reduction of apoptosis in *a*-SWNT-treated animals

a–f, Representative photomicrographs showing TUNEL staining in the penumbral region of brain sections from the sham-operated group (**a,b**), PBS group (**c,d**) and *a*-SWNTs group (**e,f**) 7 days after MCAO ($n = 5$). Images in **b**,**d**,**f**, are magnified areas from **a**,**c**,**e**, respectively. Scale bars, 200 μm. **g**, Western blot analysis of cerebral cortex tissue 7 days after MCAO, showing lowered expression of pro-apoptotic proteins (p53 and Bax) in *a*-SWNT-treated rats. β-actin is the loading control. Data represent the results of three independent experiments.

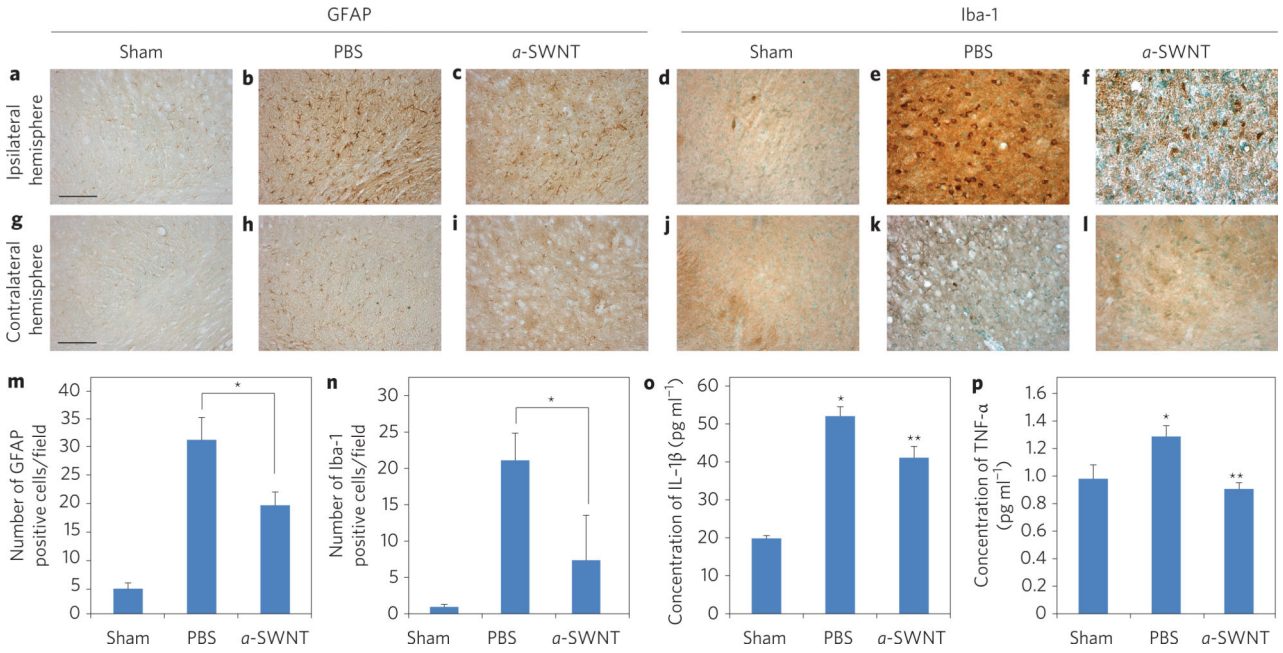

Figure 3. Immunohistochemical analysis of GFAP and Iba-1 expression after MCAO

a–l, Representative photomicrographs of GFAP and Iba-1 expression in ipsilateral (**a–f**) and contralateral (**g–l**) hemispheres of sham-operated, PBS-treated and *a*-SWNT-treated rats following MCAO (n = 5). Scale bars, 200 μm. **m**,**n**, Bar graphs showing quantification of GFAP- (**m**) and Iba-1- (**n**) positive cells on the ipsilateral side. Data are the mean number of cells from five random fields. Data are presented as means±s.e.m. *P <, 0.001 versus the PBS group. **o**,**p**, Effects of *a*-SWNTs on the production of proinflammatory cytokine IL-1b (**o**) and TNF-α (**p**). Cytokines, determined by ELISA, were normalized against the cortex of the contralateral hemisphere. Immunohistochemical analysis and ELISA were performed 7 days after MCAO. Data are expressed as means±s.e.m. *P < 0.001 versus the sham-operated group; **P < 0.001 versus the PBS group; n = 6.

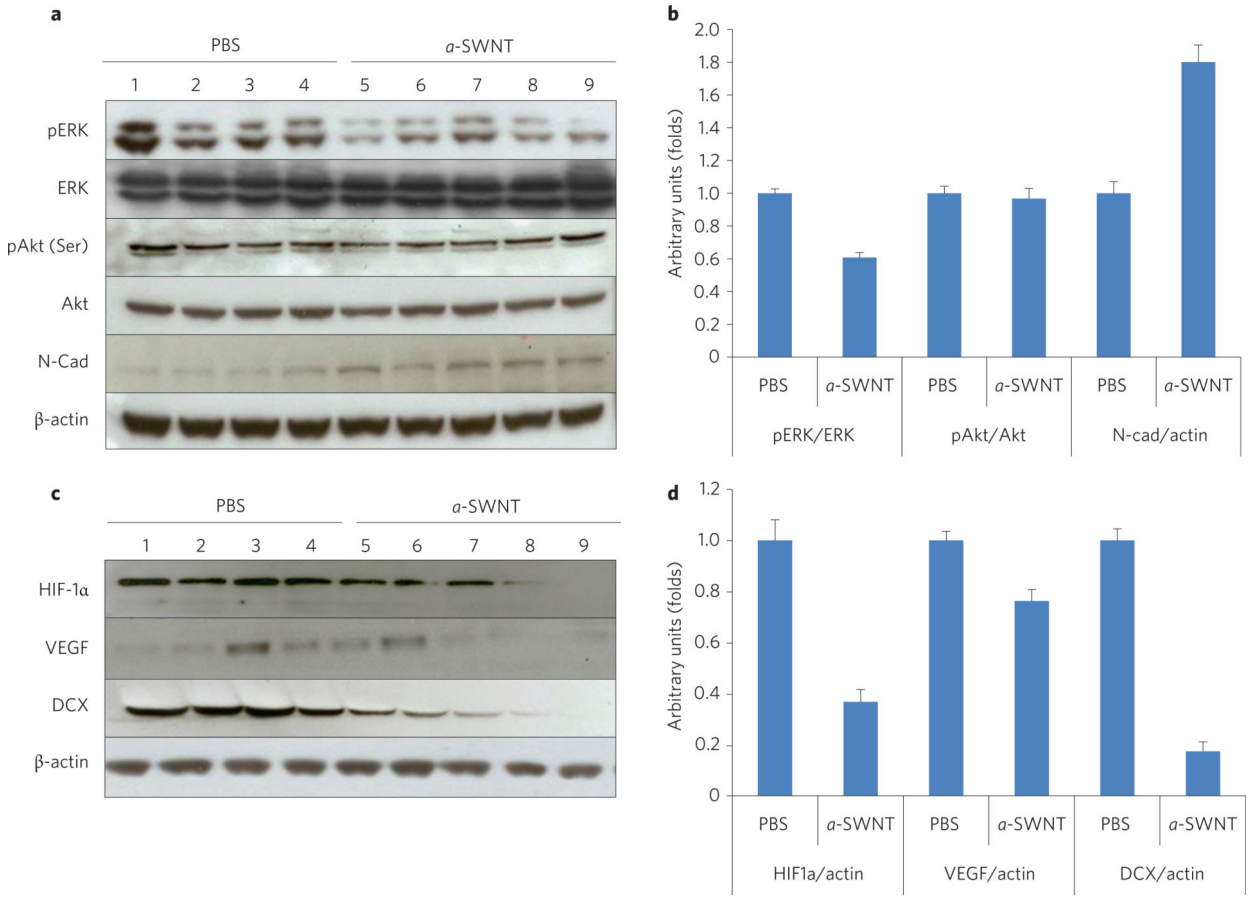

Figure 4. Western blot analysis of cerebral cortex lysates after MCAO in PBS- or *a*-SWNT-treated rats

a,**c**, Lanes 1–4 show data from individual rats of the PBS group; lanes 5–9 show data from individual rats of the *a*-SWNTs group. **b**,**d**, Bar graphs showing quantification of the expression level of each protein compared with the expression of β-actin or the non-phosphorylated isoform. Data are expressed as means±s.e.m. Western blots are representative of four independent experiments.

Table 1

Dose-dependent effect of LY294002 on anatomical damage and functional damage after ischaemia/reperfusion.

	Mock	LY294002 (25 μg kg^{-1})		LY294002 (2.5 μg kg^{-1})		LY294002 (0.25 μg kg^{-1})	
		Mock	*a*-SWNT	Mock	*a*-SWNT	Mock	*a*-SWNT
Anatomical damage	7(10)	ND	ND	ND	8(10)	9(10)	1(10)
Functional damage	9(10)	ND	ND	ND	10(10)	10(10)	6(10)

Anatomical damage was assessed by TTC staining, and functional damage was assessed by Rota-Rod treadmill tests. Mock: vehicle- or PBS-injected rats.Each group (n = 10) was assessed 1 day after MCAO surgery. Values represent the number of rats with impaired anatomical or neurological features. Numbers in parentheses indicate the total number of rats that underwent the operation. ND indicates that all rats died before analysis.