Functional motor recovery from brain ischemic insult by carbon nanotube-mediated siRNA silencing

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Stroke is the second cause of death worldwide with ischemic stroke accounting for 80% of all stroke insults. Caspase-3 activation contributes to brain tissue loss and downstream biochemical events that lead to programmed cell death after traumatic brain injury. Alleviation of symptoms following ischemic neuronal injury can be potentially achieved by either genetic disruption or pharmacological inhibition of caspases. Here, we studied whether silencing of Caspase-3 using carbon nanotube-mediated in vivo RNA interference (RNAi) could offer a therapeutic opportunity against stroke. Effective delivery of siRNA directly to the CNS has been shown to normalize phenotypes in animal models of several neurological diseases. It is shown here that peri-lesional stereotactic administration of a Caspase-3 siRNA (siCas 3) delivered by functionalized carbon nanotubes (*f*-CNT) reduced neurodegeneration and promoted functional preservation before and after focal ischemic damage of the rodent motor cortex using an endothelin-1 induced stroke model. These observations illustrate the opportunity offered by carbon nanotube-mediated siRNA delivery and gene silencing of neuronal tissue applicable to a variety of different neuropathological conditions where intervention at well localized brain foci may offer therapeutic and functional benefits.

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Preventing injured neurons from undergoing apoptosis in the acute phase of cerebral ischemia (neuroprotection) is a widely studied strategy to counteract the effects of stroke. The central role of Caspase-3 in regulating cell death (1, 2) processes, constitutes this protease as a prime target for the development of neuroprotective treatments. Indeed, Caspase-3 activation contributes to brain tissue loss and downstream biochemical events leading to programmed cell death after traumatic brain injury (3, 4). Both genetic disruption and pharmacological inhibition of caspases after stroke can reduce ischemic neuronal injury inducing a neuroprotective effect (5–8). It has been suggested that silencing of apoptotic genes using in vivo RNA interference (RNAi) could be potentially an effective treatment for stroke (5, 9) . Moreover, nonviral delivery of siRNA directly to the Central Nervous System (CNS) has been reported to effectively normalize phenotypes in animal models of several neurological diseases (10–13). However, one of the major challenges is to achieve sufficient in vivo delivery of siRNA to target tissues, and into target cells, to induce effective gene silencing. Administration of naked siRNA by continuous pump infusion into the CNS (13) requires high siRNA doses, while the use of lipid transfection reagents or electroporation techniques may induce neuronal tissue damage and cell death (14–16).

Recently, a unique type of nonviral gene vector has emerged, based on functionalized carbon nanotubes (*f*-CNT). Various types of nanotube chemical functionalization approaches have led to improvements of their dispersibility in physiological media that broaden the spectrum of potential biological applications (17–19). *f*-CNT possess unique features that make them extremely attractive for the construction of unique gene delivery vectors. Most importantly, *f*-CNT have the ability to interact with biological components and be internalized efficiently in different cell types by different mechanisms (20–22). Several studies have shown promising results, exploiting *f*-CNT characteristics for pDNA and siRNA delivery in vitro and in vivo (17, 18, 20, 23–28). Chemically functionalized single-walled CNT have been able to deliver siRNA to knockdown the expression of telomerase reverse transcriptase in an Lewis Lung Carcinoma mouse tumor model (26). We have recently demonstrated that therapeutic silencing using ammonium functionalized multiwalled carbon nanotube-mediated siRNA delivery can lead to tumor growth arrest and prolonged survival of animals bearing a human lung tumor xenograft (Calu 6) (28). Some of us have also previously reported that Pluronic F127-coated CNT injected into the cerebral cortex did not induce any adverse toxicological effects (29).

Based on previous findings that biocompatible *f*-CNTs are effective intracellular transporters for siRNA and that down-regulation of the Caspase-3 gene can aid recovery after ischemic attack, we hypothesized in this work that *f*-CNT can act as nanoscale devices for the delivery of Caspase-3 siRNA (siCas 3) into neurons of the rodent motor cortex and lead to protection against ischemic insult. Herein we show that peri-lesional, stereotactic administration of siCas 3 delivered by *f*-CNT led to neuroprotection after focal ischemic damage induced by Endothelin-1 (ET-1) in the motor cortex of mice and rats, which promoted functional recovery as shown by the functional *"skilled reaching"* test in living rats.

Results and Discussion

Chemical Functionalization of CNTs. Chemical functionalization of nanotubes was achieved by introducing an ammonium group onto the multiwalled carbon nanotube (MWNT) backbone using the 1,3-dipolar cycloaddition reaction as described previously (30, 31). The chemical structure of the *f*-CNT studied is shown in Fig. 1*A*. Chemical functionalization has shown to significantly improve the aqueous dispersibility and individualization of the

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MWNTs manifested in the quality of their aqueous dispersion in 5% dextrose, in comparison to pristine CNT which required the addition of a dispersing agent or surfactant. The improved dispersibility of the *f*-MWNTs was confirmed by transmission electron microscopy (TEM) (Fig. 1*A*, Fig. S1*A*). Pristine carbon nanotubes used in this work had a length between 0.5 and 2 μm and average diameter of 20–30 nm. Following the 1,3-dipolar cycloaddition reaction, the morphology and the length of the nanotubes were not markedly affected. Most of the nanotubes (>80%) were below 1 μm long. The length distribution of *f*-MWNT used in this study based on TEM analysis is shown in Fig. S1*B*. Ammonium functional groups corresponded to 0.147 mmol/g material, as determined by Kaiser Test and thermogravimetric analysis (TGA) (Fig. S1*C*).

Cytoplasmic Delivery of siRNA in Primary Neuronal Cultures In Vitro. We have recently reported the ability of this type of chemically functionalized CNT to complex and transfer siRNA into mammalian cells (27, 28, 32). To confirm such results in neurons, noncoding siRNA fluorescently labeled with AlexaFluor 546 (siNEG-AF546) was complexed with *f*-CNT at 1:8 mass ratio and incubated for 24 h with primary neurons isolated from the mouse motor cortex. Epifluorescence imaging (Fig. 1*B*) indicated that siNEG-AF546 signals were higher into neurons treated with the complexes in comparison to neurons treated with siNEG-AF546 alone. Confocal laser scanning microscopy (CLSM) confirmed that the fluorescence signal was localized within the cells and that siRNA was not only bound to the plasma membrane. This data indicated that *f*-CNT could effectively transport siRNA into primary neurons in agreement with previous studies using other cell types.

Caspase-3 Silencing in Murine Neuro2a (N2a) Cells In Vitro. In order to validate the siCas 3 sequence in vitro, the mouse neuroblastoma N2a cells were incubated with *f*-CNT:siCas 3 complexes (8:1 mass ratio) for 24 h or 48 h. The level of full length caspase-3 (35 kDa) expression in N2a cells was assessed by rtPCR and Western blotting. Fig. 2*A* shows that preincubation of N2a cells with *f*-CNT:siCas 3 complexes decreased the number of mRNA copies of the full length Caspase-3 to 62% ± 7% at 24 h, and by 69% ± 8% at 48 h posttreatment by rtPCR as a percentage of naive cells. The same results were not seen in cells treated with the vehicle alone (5% dextrose), siCas 3 alone or noncoding siRNA (siNEG) complexed with *f*-CNT at the same mass ratio. Similarly, Western blot analysis at 48 h posttreatment showed that full length Caspase-3 expression in the *f*-CNT:siCas 3 treated group was significantly ($p < 0.05$) decreased compared to all other treated groups (Fig. 2 *B* and *C*), with a reduction of 56.0% ± 16.0% in Caspase-3 level as a percentage of naive cells. GAPDH was used as housekeeping gene and no change in its expression was observed in all test groups. This data validated the siCas 3 sequence used in this experiment and indicated that siCas delivered into cells is biologically active.

Internalization of *f*-CNT by Neurons In Vivo. Several studies have been performed showing internalization of *f*-CNTs into variety

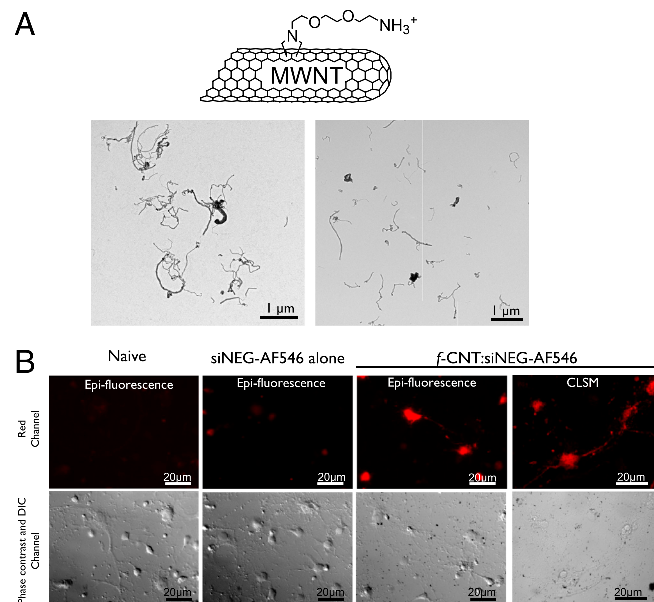

Fig. 1. Neuronal uptake of *f*-CNT: Alexa Fluor 546-labeled siNEG (siNEG- AF 549) complex in vitro. (*A*) Chemical structure of *f*-CNT and TEM images of *f*-CNT dispersed in 5% dextrose at 250 μg/mL final concentration. (*B*) Epifluorescence and confocal laser scanning microscopy images of primary neuronal culture isolated from mouse brain motor cortex and incubated with media containing free uncomplexed siNEG-AF 549 (100 nM) or *f*-CNT:siNEG-AF 549 complex (8:1 mass ratio) for 24 h. siRNA uptake is shown in red.

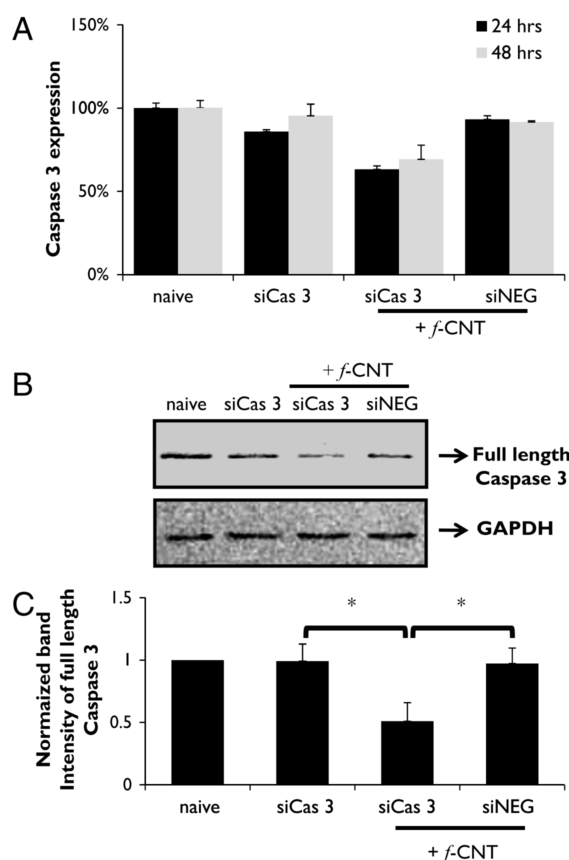

Fig. 2. Silencing of Caspase-3 in murine Neuro2A (N2a) neuroblastoma cell line by *f*-CNT:siCaspase-3 (siCas 3) complexes. (*A*) mRNA expression levels of full length caspase-3 (35 kDa) in N2a cells was analyzed by real time PCR ($n = 3$) after treatment with siCas 3 alone, *f*-CNT:siCas 3 or *f*-CNT:siNEG complexes. Cells were incubated with the complexes for 4 h at 37 °C, and analyzed for mRNA expression after 24 or 48 h posttransfection. Both β-actin and total RNA were used as internal controls. Full length Caspase-3 mRNA level was reduced only in cells treated with *f*-CNT:siCas 3 complexes at both time points. Error bars indicate means + SEM (*B*) Western blot of N2a cells treated as in (*A*) and assessed 48 h posttransfection. Full length Caspase-3 was detected by a Caspase-3 antibody. Caspase-3 silencing was seen when N2a cells were pretreated with *f*-CNT:siCas 3 but not with uncomplexed siCas 3 or *f*-CNT:siNEG. No difference in band intensity was seen for GAPDH housekeeping gene in all treated groups. (*C*) Quantification of full length Caspase-3 silencing, the *ordinate* shows the normalized band intensity of full length Caspase-3 which was significantly reduced compared to its level in siCas 3 alone or *f*-CNT:siNEG treated groups (*, $p \leq 0.05$, $n = 3$).

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of cells in vitro and in vivo (17, 33–36). In this study, we directly administered stereotactically *f*-CNT (without siRNA) into the brain cortex to confirm their ability to be internalized by neuronal cells in vivo. Intraparenchymal injection of *f*-CNT into the motor cortex resulted in *f*-CNT internalization by a variety of brain cells within 48 h as shown by TEM of brain sections (Fig. 3*A*). Neurons were able to internalize *f*-CNTs (Fig. 3 *B* and *C*) and TEM images showed that *f*-CNTs located within the cytoplasm of neurons were individualized (Fig. 3*D*). Cell types were identified based on their morphological characteristics. Neurons were identified by the large round nucleus, and by the large number of organelles, including mitochondria (M) and Golgi apparatus (G). *f*-CNT were also found to internalize into other types of neuronal cells in vivo (Fig. S2). Furthermore, to confirm the capacity of primary neurons to internalize *f*-CNT, an isolated primary neuronal culture was incubated with *f*-CNT for 24 h, and cells were imaged either intact by cryo-SEM or sectioned and imaged by TEM. These microscopic techniques confirmed that nanotubes were able to bind and translocate the plasma membrane of neurons (cryo-SEM) and localize intracellularly (TEM) (Fig. S3).

f-CNT:siCas 3 Complex Treatment Before and After Endothelin-1 (ET-1) Exposure Decreases Expression of TUNEL in Murine Brain Cortex In Vivo. To determine the silencing efficiency of *f*-CNT:siCas 3 in vivo, the ET-1 model of focal stroke was employed. ET-1 is a potent vasoconstrictor that has been used to induce ischemic injury resembling a thrombo-embolic stroke event (37) when injected directly into rodent brain. In the treatment protocol before ET-1 exposure, mice were pretreated (1 μL) with the vehicle alone (5% dextrose), siCas 3 (4.7 pmol), or the *f*-CNT:siRNA complexes (8∶1 mass ratio), and after 24 h reinjected at the same site with ET-1 (+ET-1 group) to induce ischemic injury (Fig. 4*A*). The group of 5% dextrose-treated animals was reinjected with vehicle alone (-ET-1 group). All injections were aimed at the cortical area containing the representation of the forelimb and neuroprotection was studied by TUNEL staining (apoptotic cells only) (Fig. 4*B*). Previous studies have indicated that injury-induced apoptosis can be detected from hours to days following induced injury and may contribute to neurological dysfunction. Therefore, TUNEL staining was performed on brain sections 24 h after ET-1 injection (48 h posttreatment). As expected, TUNEL-positive cells were observed in the +ET-1 group preinjected with 5% dextrose (156.0 ± 15.3 cells/mm^2) mainly in the penumbra area compared to only 80.8 ± 16.7 cells/mm^2 in -ET-1 group (Fig. 4). Only the group preinjected with *f*-CNT:siCas 3 (+ET-1) led to statistically significant reduction ($p \leq 0.05$) in the number of apoptotic cells (83.0 ± 18.0 cells/mm^2) compared to +ET-1 group preinjected with 5% dextrose (Fig. 4). No significant neuroprotection was detected in brains preinjected with siCas 3 alone or noncoding siRNA (siNEG) complexed with *f*-CNT at the same mass ratio, indicating the specific biological activity of siCas delivered by *f*-CNT.

It was considered prudent and clinically relevant to also perform this experiment after the ET-1 induced ischemic damage had been initiated to assess the siRNA treatment. *f*-CNT:siCas 3 complexes were stereotactically injected 1 h postischemic damage. We observed that the number of apoptotic cells in the penumbra area decreased mostly in the *f*-CNT:siCas 3 treated group in comparison to 5% dextrose or free siCas 3 treated groups (Fig. 5), however this trend was not statistically significant as seen in the case of the treatment protocol before ischemic damage was induced. Similar observations of moderate therapeutic effects obtained postischemic damage induction (compared to pretreatment) have previously been reported for other therapeutic modalities (6). We therefore concluded that a neuroprotective effect could be reproducibly obtained from a single *f*-CNT:siCas 3 stereotactic administration before and after ET-1 induced ischemic damage, however more significant effects were obtained when the treatment was carried out prior to the ischemic insult.

"Skilled Reach" Assay in Rats. In order to study if the neuroprotective effect observed in the brain cortex was sufficient to warrant a significant improvement in the behavior of treated animals, the functional *skilled reaching* test in rats was used. Damage of the forelimb representation area of the motor cortex results in impaired reaching and grasping movements of the contralateral forelimb that can be evaluated using the *skilled reaching* test. This

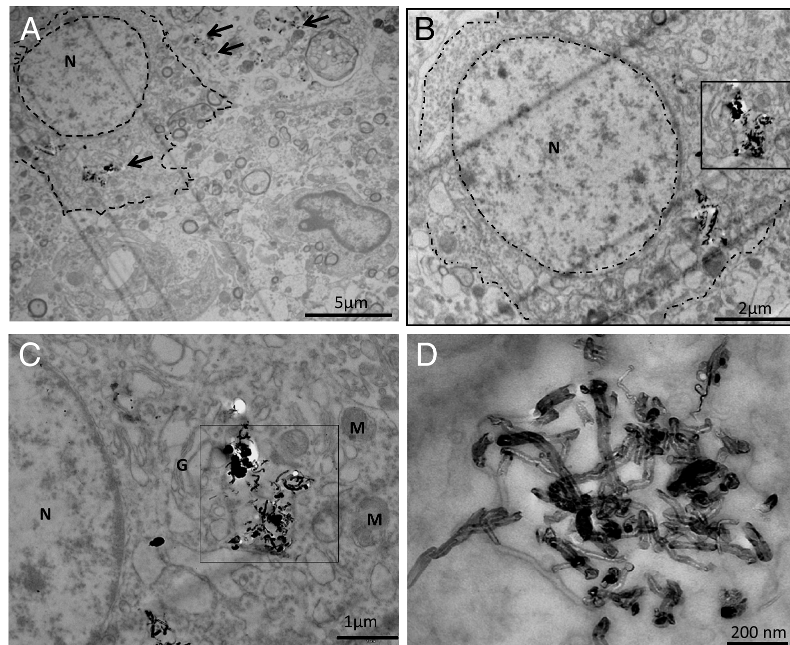

Fig. 3. Internalization of *f*-CNT by neuronal tissue in vivo. TEM of brain cortical parenchyma at 48 h after stereotactic administration of *f*-CNT showing nanotubes internalization into brain cells (*A*). Cells are identified by their morphological characteristics; neurons are marked by a dotted line and zoomed in with internalized *f*-CNT marked in a sqaure. G, M, and N stands for Golgi apparatus, mitochondria, and nucleus respectively (*B–D*).

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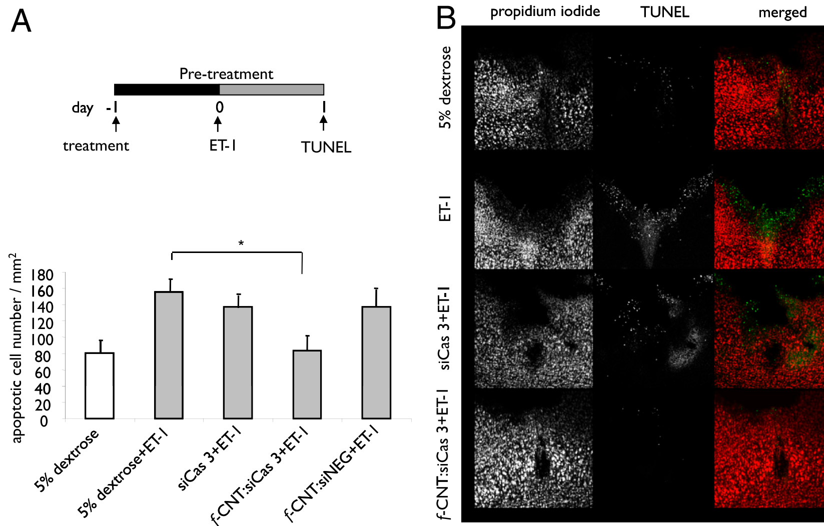

Fig. 4. Caspase-3 silencing in vivo by *f*-CNT:siCas 3 complexes. (*A*) Dosage regimen used in the preischemia treatment protocol and Endothelin-1 (ET-1) administration in mice. C57Bl/6 mice were pretreated with 5% dextrose, siCas 3 alone (4.7 pmol), *f*-CNT:siCas 3, or *f*-CNT:siNEG complexes (8 : 1 mass ratio) at 24 h before exposure to ET-1 (30 pmol) injury. Mice were killed 24 h postlesion (ischemia) induction, and brains were sectioned and stained for TUNEL. Quantification of TUNEL-positive cells/mm^2 from CLSM shown in (*B*), showed that *f*-CNT:siCas 3 treatment group showed significantly lower number of apoptotic cells compared to 5% dextrose treatment group after lesion induction. (*B*) TUNEL staining of brain sections, apoptotic cells are stained in green (TUNEL) and all nuclei are counterstained in red (propidium iodide). Data were collected from mice killed in two to three independent experiments (*, $p \leq 0.05$, $n = 7$–12).

test consists of training the animals to retrieve a food pellet from a well and it is particularly effective in the detection of cortical motor deficits in rats. The animals were trained and monitored for the first week exhibiting improved ability to retrieve as evidenced by the increase in the number of positive trials from day 1 to day 6 during training (Fig. 6). We followed the protocol that was described above that offered maximum neuroprotective effects, consisting of treatment prior to induction of an ischemic lesion. According to the preischemia protocol,, all treatments were carried out on day 7 and an ET-1 ischemic lesion was induced on day 8. Assessment of functional effects showed that the ability to retrieve food pellets in the *f*-CNT:siCas 3 treated group was significantly retained after induction of ischemic damage (Fig. 6; black squares). Indeed, only the group treated

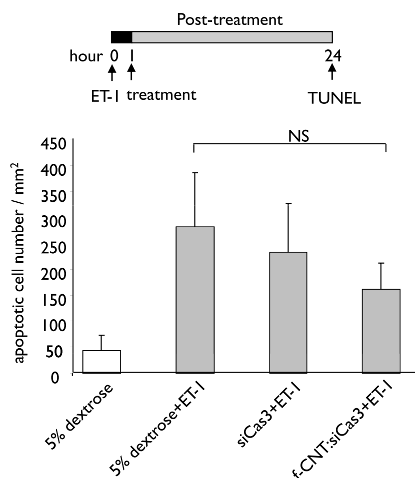

Fig. 5. Neuroprotective effect of *f*-CNT:siCas 3 complex injected 1 h after ET-1 focal stroke induction in mouse motor cortex. The number of apoptotic cells per mm^2 were detected 24 h after treatment delivery, by TUNEL staining of 50 microns fixed tissue slices (number of slices per case = 4, number of animals per group $n > 5$). The number of apoptotic cells in the group treated with *f*-CNT:siCas 3 complex is reduced if compared with the values measured in animals treated with 5% dextrose+ ET-1 or with siCas 3 alone+ET-1, however this apparent trend did not quite meet statistical significance (NS: not significant).

with *f*-CNT:siCas 3 maintained the same level of positive trials attained before the lesion was induced (day 6), and at a level significantly higher ($p \leq 0.05$) than the 5% dextrose-treated group. In the vehicle treated rats, positive trials after lesion induction (day 16) decreased to 38.6 ± 25.2% (Fig. 6; inverted triangles) of the average preischemic performance ($p < 0.05$). Conversely, positive trials on day 16 remained at 101.4 ± 7.8% of the preischemic levels only in *f*-CNT:siCas 3 treated group. These results further confirmed the neuroprotective effect achieved by carbon nanotube-mediated siCas 3 treatments and further illustrated that this could translate to functional preservation of motor skills after local ischemic damage in the rat motor cortex.

The current work was motivated by previous findings that *f*-CNTs are able to translocate into the cell cytoplasm (17) and act as transporters of nucleic acids, including siRNA, in vitro (27, 32) and in vivo (28). The delivered siRNA has proven to be functional with the ability to silence specific genes. Furthermore, we have previously reported that intratumoral delivery of an siRNA sequence mediating apoptotic responses by ammonium functionalized multiwalled carbon nanotubes into human lung (Calu 6) xenografts resulted in biological effects and a therapeutic outcome, evidenced by significant tumor growth suppression and improved animal survival (28). In the present study, ammonium functionalized CNTs were also shown to be capable of effectively transporting siRNA into primary neurons in the absence of any cell morphology alterations (e.g., dendrite contraction) or unwanted cytotoxic responses at therapeutically relevant doses, confirming previous in vitro studies showing axonal growth in primary neuronal cultures treated with positively charged CNTs (38). In addition, siCas 3 was biologically active leading to a reduction in the levels of full length Caspase-3 expression in N2a cells treated with the *f*-CNT:siCas 3 complex, while free siCas 3 was neither able to internalize in neurons nor exhibit any appreciable biological activity.

Chen et al. (9) have reported the early inhibition of the proapoptotic factor HIF-1alpha with siRNA delivered intracerebrally by a commercially available transfection agent that resulted in reduced ischemic-reperfused brain injury in rats. The study suggested that HIF-1alpha and its downstream VEGF and other apoptotic-related proteins such as p53 and Caspase-3 may present an opportunity for the early treatment of ischemic cerebral

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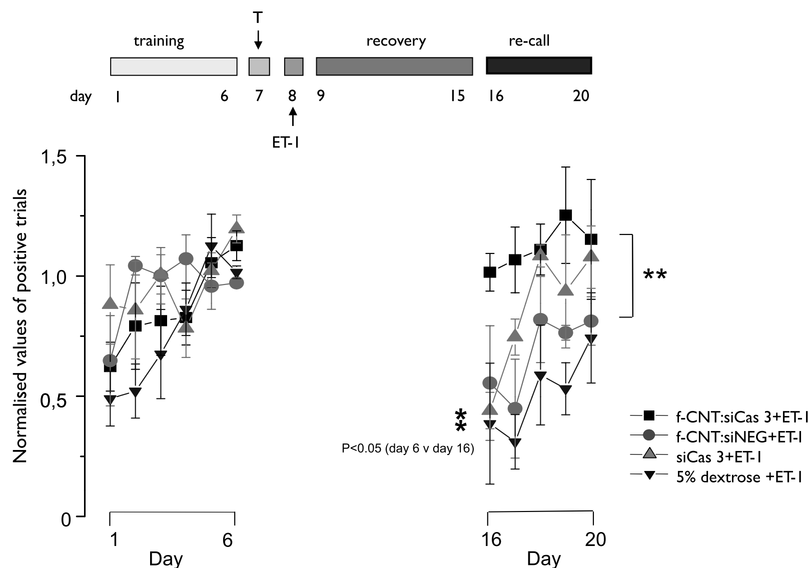

Fig. 6. Behavioral analysis using "skilled reaching" test in rats. Functional improvement in ET-1 ischemic rat forelimb function with or without pretreatment was measured. Rats were pretreated with 5% dextrose, siCas 3 alone (4.7 pmol), *f*-CNT:siCas 3 or *f*-CNT:siNEG complexes (8∶1 mass ratio) 24 h before exposure to ET-1 (30 pmol) leading to unilateral lesions made contralateral to the preferred (for reaching) forelimb. During recall, rats treated with *f*-CNT:siCas 3 performed a significant higher number of positive trials in food pellet reaching compared to all other treatment groups. (*, $p \leq 0.05$; **, $p \leq 0.01$, $n = 4$) and reaching prelesion levels

stroke. In another study by Karatas et al. (6), it was reported that the systemic delivery of chitosan nanospheres targeting the transferrin receptor and encapsulating a peptide caspase-3 inhibitor provided neuroprotection in mice subjected to 2 h of middle cerebral artery occlusion and 24 h of reperfusion. Both studies have directly or indirectly validated Caspase-3 as a target, however neither established Caspase-3 silencing by RNA interference as a possible therapeutic approach to be explored. Moreover, functional recovery and rehabilitation was not studied in order to assess the efficacy of either treatment strategies. In agreement with the results obtained in the present study, Karatas et al. (6) showed that treatment before the onset of ischemia was more effective in reducing the infarct volume than treatment after the ischemic insult.

Intriguingly, a very recent study showed that preinfarct intracerebroventricular treatment with amine-functionalized single-walled carbon nanotubes could improve tolerance of neurons to ischemic injury in the middle cerebral artery occlusion stroke model (39). In our experiments, control intraparenchymal injections of a complex between amino-functionalized MWNT and scrambled siRNA did not affect apoptosis or functional recovery after endothelin-induced focal stroke, suggesting little neuroprotective activity of CNTs alone. However, important methodological aspects differ between our experiments and those reported by Lee et al. (e.g., injection of CNT-siRNA complex vs. CNT alone; CNT type, dimensions, manufacturer, dosage, location of injection, and stroke model). Furthermore, in our work a specific biological hypothesis was explored based on the gene silencing of Caspase-3 mediated by effectively delivered siRNA. Further work is indeed required to understand and explore further any potentially beneficial effects of amino-functionalized CNTs in stroke.

This work is a proof-of-principle study demonstrating that in vivo gene silencing of Caspase-3 in neuronal tissue mediated by *f*-CNT:siRNA vectors administered stereotactically can lead to neuroprotection and significant functional improvement before and after ischemic insult. Such an approach can offer more flexibility than the administration of a caspase-3 inhibitor intracerebrally (3) or systemically (6), because siRNA-mediated gene silencing can target any potential mRNA that may be involved in stroke-induced neurodegeneration. In contrast, pharmacological inhibitors can only target certain types of cellular receptors and enzymes. Such an approach could offer genetic-based combinatory modalities to various currently used neurosurgical interventions in a variety of CNS diseases. In particular, neuropathologies for which well localized brain foci are identified and known to be implicated in the phenotypic expression of disease (such as stroke and Parkinson's disease). Further work is needed in order to determine any implications and residence patterns of carbon nanotubes within cerebral tissue, the viability of stereotactic administration, and the therapeutic validity for the best target gene. Moreover, further studies are needed to improve the observed neuroprotective effect following ischemic insults in order to determine the clinical relevance of such approaches for post cerebral stroke treated patients. Overall, effective stereotactic administration of nanotube-mediated gene silencing with functional improvements, even though clinically more challenging compared to systemic treatment strategies (40, 41), can offer significant benefits for a variety of currently untreatable debilitating neuropathologies.

Experimental Methods

Carbon Nanotubes and Other Chemicals. All details are described in *SI Text*.

Chemical Functionalization and Characterization of Carbon Nanotubes. *f*-CNT were prepared following the 1,3 dipolar cycloaddition reaction as previously described (30, 31). Details of chemical functionalization procedures are described in *SI Text*.

Transmission Electron Microscopy (TEM) of *f*-CNT Dispersions and Brain Sections. TEM processing of brain tissues is described in *SI Text*.

Cell Cultures. N2A cell culture was performed as described in *SI Text*.

Evaluation of Fluorescent siRNA Complex Uptake in Primary Neurons. All details are described in *SI Text*.

In Vitro Silencing of Caspase-3 in N2a Cells. All details are described in *SI Text*.

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Western Blot and Real Time PCR (rtPCR). The experimental procedures of Western blotting and rtPCR are described in *SI Text*.

Animal Handling Procedures. All animal experiments were performed in compliance with the United Kingdom Home Office (1989) and Code of Practice for the housing and care of Animals used in Scientific Procedures and in agreement with protocols approved by the Italian Ministry for Scientific Research. More details are described in *SI Text*.

***f*-CNT:siRNA Complexation for In Vivo Studies.** siRNA complex preparation for in vivo experiments was prepared by adding an equal volume of *f*-CNT dispersion (1 mg/mL in 10% dextrose) to siRNA (siCas 3 or siNEG) solution (10 μM or 125 μg/mL in water), mixed by rapid pipetting, to achieve *f*-CNT:siRNA mass ratio of ∼8∶1. One microlitre containing the complex of 500 ng *f*-CNT and 62.5 ng (4.7 pmol) siRNA was injected into the cerebral cortex of each animal. For comparison, groups of mice were injected with 1 μL containing the vehicle alone (5% dextrose), *f*-CNT (500 ng) complexed with siNEG or uncomplexed siCas 3 (62.5 ng) in 5% dextrose.

Stereotactic Administration of *f*-CNT or *f*-CNT:siRNA Complexes. Mice or rats were anesthetized with Avertin (0.5 mL/100 g) and secured on a stereotactic apparatus. Injections were made at stereotactic locations (at one site in mice:+0.5 mmAP, +1.5 mmML from bregma; at two sites in rats: 0 mm, +2.5 mmML, +2.5 mmML, +2.3 mmAP from bregma) in the primary motor cortex by means of a glass pipette (30-μm tip diameter) mounted on a motorized (0.1-μm step) three-axis micromanipulator (Sutter Instruments) connected to a manual injector. In mice, a total of 1 μL of *f*-CNT (500 ng) dispersion was released at 700 μm below the cortical surface. In rats, a total of 500 nL was released at 700 μm below the cortical surface and another 500 nL was released 400 μm below the cortical surface to allow homogeneous dispersion of *f*-CNTs along the cortical depth at the two sites of injection. More details are described in *SI Text*.

Endothelin (ET-1) Stroke Model. All details are described in *SI Text*.

TUNEL Staining for Apoptosis Detection. The TUNEL staining procedure is described in more details in *SI Text*.

The Skilled Reaching Test. The reaching test was performed in adult rats. All experimental details are described in *SI Text*.

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