Microglia Determine Brain Region-Specific Neurotoxic Responses to Chemically Functionalized Carbon Nanotubes

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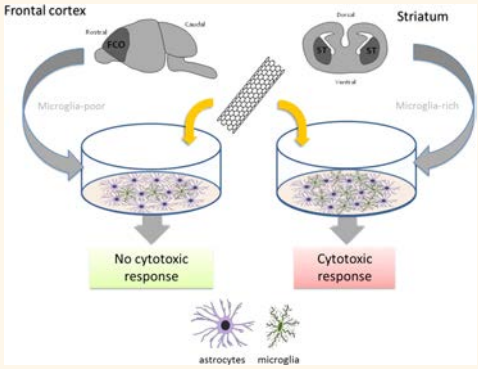
ABSTRACT Surface tunability and their ability to translocate plasma membranes make chemically functionalized carbon nanotubes (*f*-CNTs) promising intracellular delivery systems for therapeutic or diagnostic purposes in the central nervous system (CNS). The present study aimed to determine the biological impact of different types of multiwalled CNTs (MWNTs) on primary neuronal and glial cell populations isolated from fetal rat frontal cortex (FCO) and striatum (ST). Neurons from both brain regions were generally not affected by exposure to MWNTs as determined by a modified LDH assay. In contrast, the viability of mixed glia was reduced in ST-derived mixed glial cultures, but not in FCO-derived ones. Cytotoxicity was independent of MWNT type or dose, suggesting an inherent sensitivity to CNTs. Characterization of the cell populations in mixed glial cultures prior to nanotube exposure showed higher number of CD11b/c positive cells in the ST-derived mixed glial cultures. After exposure to MWNTs, CNT were uptaken more effectively by CD11b/c positive cells (microglia), compared to GFAP positive cells (astrocytes). When exposed to conditioned media from microglia enriched cultures exposed to MWNTs, ST-derived glial cultures secreted more NO than FCO-derived cells. These results suggested that the more significant cytotoxic response obtained from ST-derived mixed glia cultures was related to the higher number of microglial cells in this brain region. Our findings emphasize the role that resident macrophages of the CNS play in response to nanomaterials and the need to thoroughly investigate the brain region-specific effects toward designing implantable devices or delivery systems to the CNS.



KEYWORDS: brain · implant · nanomaterials · nanotoxicology · carbon nanotubes

The brain is a highly specialized organ composed of different regions with specific cell compositions and functions. Despite the numerous advances that have been made in the design of vector systems to bypass the highly restrictive blood–brain barrier, targeting specific brain regions still remains far from feasible.[1] Recent innovations using nanosized constructs have offered promising opportunities.[2] Polymeric nanoparticles, natural inorganic particles, cationic lipids, dendrimers, and carbon-based nanomaterials have been developed for imaging or therapeutic purposes, mainly against brain cancer but also other neurological conditions such as Alzheimer's, Parkinson's, and stroke.[3−5]

Among the different carbon-based nanomaterials, functionalized carbon nanotubes (*f*-CNTs) have gained particular interest thanks to their unique ability to cross biological membranes and translocate into different subcellular compartments.[6−8] Likewise, *f*-CNTs have established their capacity to transport drugs (*e.g.*, doxorubicin),[9]

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peptides,[10] plasmid DNA,[11] small interference RNA[12,13] or diagnostic molecules[14,15] intracellularly into many cell types,[16,17] including brain cells.[13] However, despite the unique opportunities that *f*-CNTs may offer, one of the concerns that may limit their further clinical translation is the uncertainty around their safety profile. Many studies have reported that CNTs can induce various *in vitro* and *in vivo* effects that could lead to toxic responses.[18] These include oxidative stress, free radical production, peroxidative products accumulation, DNA damage or inflammatory responses.[19−22] On the other hand, chemical functionalization of CNTs primarily developed to overcome their inherent hydrophobicity and lack of dispersibility in aqueous media[23−25] was found to significantly modulate the reported CNT toxicity or immunogenicity when compared to pristine (as produced) CNTs.[26−29]

Demonstration of the potential of CNT-based constructs for transport of therapeutic or diagnostic agents to the brain parenchyma has progressed; however, evaluation of the neurotoxicological profile of CNTs is still limited and certainly inconclusive.[30,31] In a study investigating the impact of CNTs on primary mixed neuro-glial cell cultures of the peripheral and central nervous system,[32] Belyanskaya *et al.* have demonstrated higher cytotoxicity on glial cells than on neurons, with peripheral neural cells being more affected than their central counterparts. The toxic effect reported was also dependent on the CNT dispersibility and concentration. Similarly, using human neuroblastoma cells (SHSY5Y) incubated with copolymer (Pluronic F127)-coated nanotubes, toxicity was shown to be dependent on concentration, incubation time (up to 2 weeks), material purity and the presence of carboxyl groups on the acid-treated CNT surface.[33] CNTs with high iron catalyst impurity content have also been found to decrease cell viability of pheochromocytoma cells and disrupt the cytoskeleton, hence reducing the ability to form mature neuritis.[34]

Some of us have previously shown that cortical stereotactic administration of different types of functionalized multiwalled carbon nanotubes (*f*-MWNT) can lead to a local and transient inflammation reported at early time points postadministration.[35] Presence of carboxyl groups on the *f*-MWNT constructs resulted in stronger inflammatory response compared to *f*-MWNT with only amino groups at the surface. Moreover, the stereotactic administration of oxidized MWCNTs induced sustained levels of activated microglia and astrocytes in the area around the injection site in comparison to nonoxidized MWCNTs. Conversely, F127-coated CNTs have been described as biocompatible when interacting with mouse primary cortical neurons or when injected directly into the mouse brain cortex.[36] Pluronic F108-coated CNTs were also reported to have no significant effect on cytokine release or proliferation of BV2 microglia or GL261 glioma cells, despite being internalized into microglia cells.[37] Taken together, these different studies demonstrate that the biological impact of CNTs on interaction with the neural environment and its different cellular subtypes remains largely unresolved. They also suggest that brain tissue/cell responses to CNT exposure are highly dependent on the physicochemical nature of the CNTs used.

The aim of the present study was to evaluate the toxicological impact of pristine and functionalized MWNTs on neural (neuronal and glial) cells isolated from two brain regions: the striatum (ST) and the frontal cortex (FCO). We hypothesized that once nanotubes gain accesses to the CNS they would either: (a) diffuse throughout the brain and interact nonspecifically with various cell types that comprise the CNS; or (b) interact selectively with specific cell populations more preferably than others. To test this hypothesis, the *in vitro* dose-dependent effects for a range of *f*-MWNTs were compared against pristine MWNT (*p*-MWNTs) and were studied in three different primary culture types: neuron-enriched, mixed glial and microglia-enriched cultures all isolated from either the ST or FCO of fetal rat brains. Our results suggested that microglial cells, acting as sentinel cells in mixed glial cultures, have a critical role in a brain region-selective sensitivity to such carbon nanomaterial exposures.

RESULTS AND DISCUSSION

Chemical Structure and Characterization of MWNTs. Four different types of chemically functionalized MWNTs were synthesized from the same batch of starting material to eliminate variations in the content of metal impurities. The chemical structure of the MWNTs studied is shown in Figure 1a. The five types of MWNTs include the following: (i) pristine MWNTs dispersed in the aqueous dispersion of the block copolymer Pluronic F127;[38] (ii) carboxylated MWNTs (ox-MWNTs) prepared by treatment in strong acid conditions;[39] (iii) amino-functionalized oxidized MWNTs (ox-MWNT-NH_3^+) prepared by 1,3 dipolar-cycloaddition reaction after their initial oxidation as described in literature;[40] (iv) amino-functionalized MWNTs (MWNT-NH_3^+) prepared following the 1,3 dipolar-cycloaddition reaction on pristine MWNTs as described previously;[41,42] and (v) amino-functionalized MWNTs (ox-MWNT-am-NH_3^+) prepared by amidation of the oxidized nanotubes.[43] Oxidation of the pristine MWNT led to introduction of carboxyl groups and shortening of the nanotubes (200−300 nm in length for ox-MWNT, ox-MWNT-NH_3^+ and ox-MWNT-am-NH_3^+), while pristine MWNTs or MWNTs functionalized without oxidation (*i.e.*, MWNT-NH_3^+) remained between 0.5 and 2 μm in length. The degree of amino group loading on the side walls and tips of the *f*-MWNTs as determined by the Kaiser test is summarized in Table 1. All MWNTs were prepared in either 5% dextrose (functionalized) or

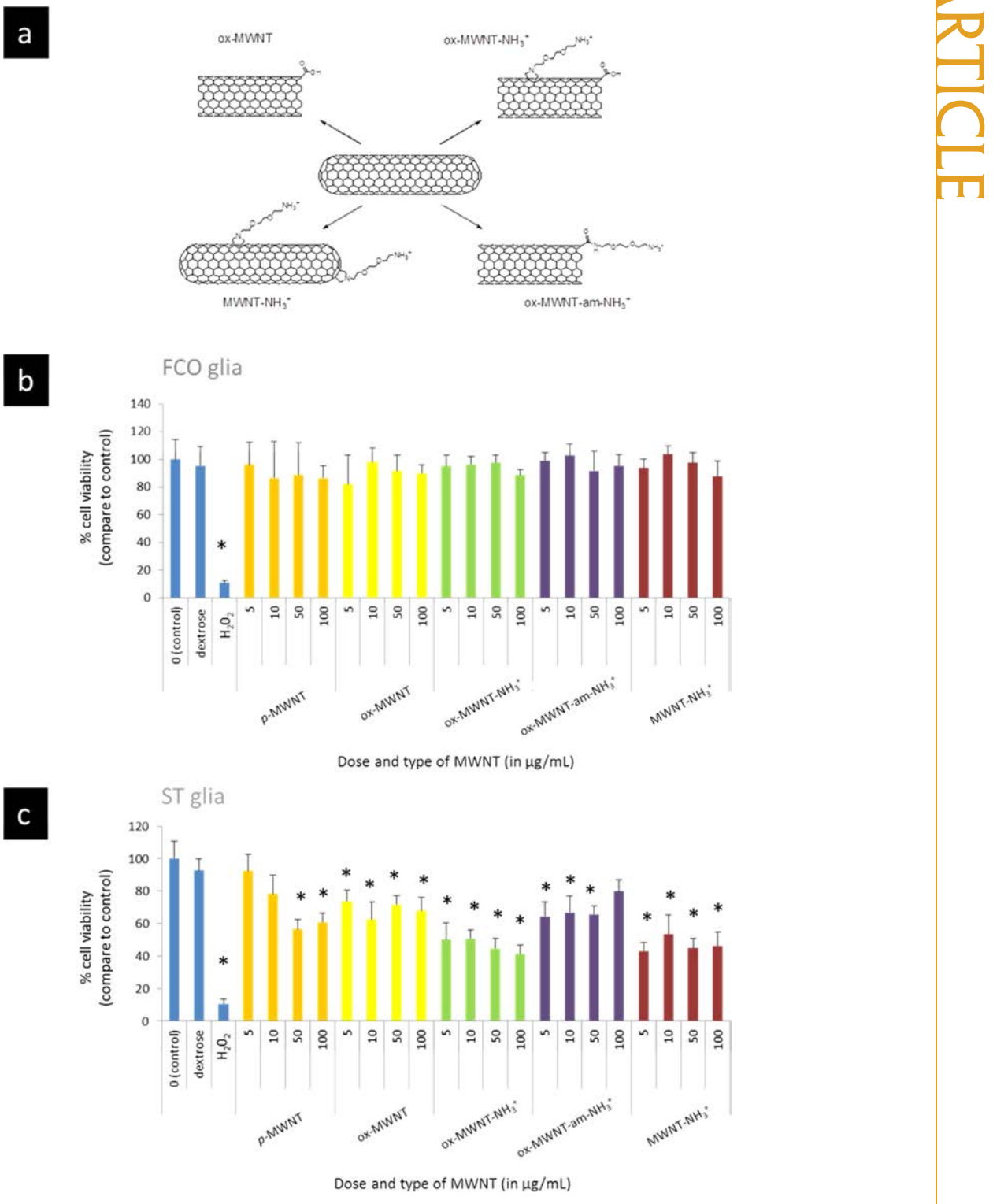

Figure 1. Cytotoxic effects of different MWNTs on mixed glial cell cultures by modified LDH assay. Mixed glial cultures were exposed during 24 h to an increasing range of different MWNTs (5−100 μg/mL). (a) Chemical structures of the different MWNT studied, pristine and chemically functionalized. (b) mLDH assay for mixed glia cells extracted from frontal cortex (FCO). No significant effect was observed compare to control in FCO cultures. (c) mLDH assay for mixed glia cells extracted from striatum (ST). The CNT exposure was toxic in ST cultures for all concentrations, except for 5 μg/mL of *p*-MWNTs. The values are presented as mean ± SD ;*$p < 0.05$ (Student's *t*-test).

1% Pluronic F127 (pristine), using bath sonication and they exhibited good dispersibility before incubation with cells.

Cytotoxic Effect on Primary Neuronal Cultures. We have previously reported the internalization of MWNT-NH_3^+ in primary neurons and described their ability to

TABLE 1. Description and Characteristics of Chemically Functionalized MWNTs Used in This Study[a]

short name	nature of MWNTs	amount of amino groups (Kaiser test)	length (TEM)
p-MWNT	pristine	-	0.5−2 μm
ox-MWNT	carboxylated	-	200−300 nm
ox-MWNT-NH_3^+	carboxylated and aminated (cycloaddition)	170 μmol/g	200−300 nm
ox-MWNT-am-NH_3^+	carboxylated and aminated (amidation)	320 μmol/g	200−300 nm
MWNT-NH_3^+	aminated (cycloaddition)	84 μmol/g	0.5−2 μm

[a] The table provides the length and amount of amino groups for the different *f*-MWNTs, as well as the abbreviations used throughout the manuscript.

transfer biologically functional siRNA both *in vitro* and *in vivo*.[13] In the present study, we aimed to interrogate the impact of exposure to the different MWNT types of primary neural (neuronal and glial) cultures isolated from the FCO and the ST. First, primary neuron-enriched cultures were incubated with increasing concentrations of MWNTs (5−100 μg/mL) for 24 h. Cell viability was then assessed by a modified LDH (*m*LDH) assay.[38] Results showed no significant reduction in cell viability induced by any MWNT type isolated from either the FCO or the ST (Supporting Information Figure.S1).

Cytotoxic Effect on Primary Mixed Glial Cultures. The response of non-neuronal (mixed glial) cell cultures isolated from either FCO or ST brain regions was then interrogated. The viability of the mixed glial cultures derived from FCO did not seem to be affected by any of the MWNT types, with no reduction in cell viability observed at all concentrations (5−100 μg/mL) (Figure 1b). Interestingly, mixed glial cultures derived from ST showed an overall reduction in cell viability by the *m*LDH assay. An exception was exposure to *p*-MWNT at the lowest concentration (5 μg/mL) with no toxicity observed after 24 h incubation (Figure 1c).

To confirm the results obtained by *m*LDH assay, cell viability was also determined using propidium iodide/4′,6-diamidino-2-phenylindole (PI/DAPI) staining. PI is an impermeable dye that can only stain dead cells (red channel) but not living cells. Cell nuclei of all cells (live and dead) were counterstained by the permeable dye DAPI (blue channel). Mixed glial cultures of both origins (FCO and ST) were incubated with MWNTs at 10 μg/mL for 24 h. Similar to *m*LDH results, only mixed glial cultures isolated from the ST region were clearly PI positive (Figure 2b,c), whereas FCO mixed glial cultures appeared mostly PI negative (Figure 2a,c) in comparison to both negative and positive controls. DMSO (10%) was used as positive control in this study and shown to be toxic to both ST and FCO mixed glial cultures. We therefore concluded from both *m*LDH assay and PI/DAPI staining results that ST-derived mixed glial cultures appeared to be more sensitive to MWNT exposure than FCO-derived mixed glial cultures.

Determination of Microglia and Astrocyte Content in Primary Mixed Glial Cultures. In an attempt to understand the enhanced cytotoxic response obtained in the ST-derived mixed glial cultures, we assessed the microglia and astrocyte content in naïve (not exposed to nanotubes) mixed glial cultures. Two immunocytochemistry markers were selected: the CD11b/c for microglia (Figure 3) and the glial fibrillary acidic protein (GFAP) for astrocytes (Supporting Information Figure S2).[44] All cell nuclei were counterstained with DAPI. To determine the distribution of microglia in the cultures in ST and FCO, the number of CD11b/c positive cells as a percentage of the whole cell population (DAPI stained) was determined. The number of CD11b/c positive cells (microglia) were significantly higher in mixed glial cultures isolated from fetal ST (30.6% ± 1.5) than from the fetal FCO (16.8% ± 1.8) (Figure 3b). These results suggested that the mixed glial cultures derived from ST and found to be more responsive to MWNT exposure contained a larger proportion of microglia.

Preferential Uptake of MWNTs by Microglia in Mixed Glial Cultures. Microglial cells are known for their capacity to engulf foreign particles as a mechanism of maintaining brain homeostasis and protection against injury.[45,46] In previous studies, we have reported preferential uptake of MWNT-NH_3^+ by microglia *in vivo* after intracranial administration in adult mouse brains.[13,35,47] The fact that mixed glial cultures from the ST exhibited a higher cytotoxic response and contained a larger proportion of microglia prompted us to question the critical role of microglia on the interaction with MWNTs using a mixed glia culture.

Mixed glial cultures were exposed to 10 μg/mL MWNT for 24 h followed by CD11b/c marker staining in order to identify microglia cells (Figure 4a,b, Supporting Information Figures S3 and S4a) or by GFAP staining to identify astrocytes (Figure 4c,d and Supporting Information Figure S4b). We observed highly reproducible colocalization of the CD11b/c marker (yellow-green channel) with cells that contained large amounts of internalized MWNTs (black matter inside cells). CD11b/c positive cells appeared to engulf large amounts of MWNTs irrespective of MWNT type (Figure 4a,b, Supporting Information Figure S4a). In case of *p*-MWNT, much lower uptake was however observed, which correlated with low cytotoxicity obtained at low *p*-MWNT concentrations (Figure 1b,c). On the contrary, cells which were GFAP positive (CD11b/c negative) did not show a great correlation with the extent of MWNT internalization (Figure 4c,d and

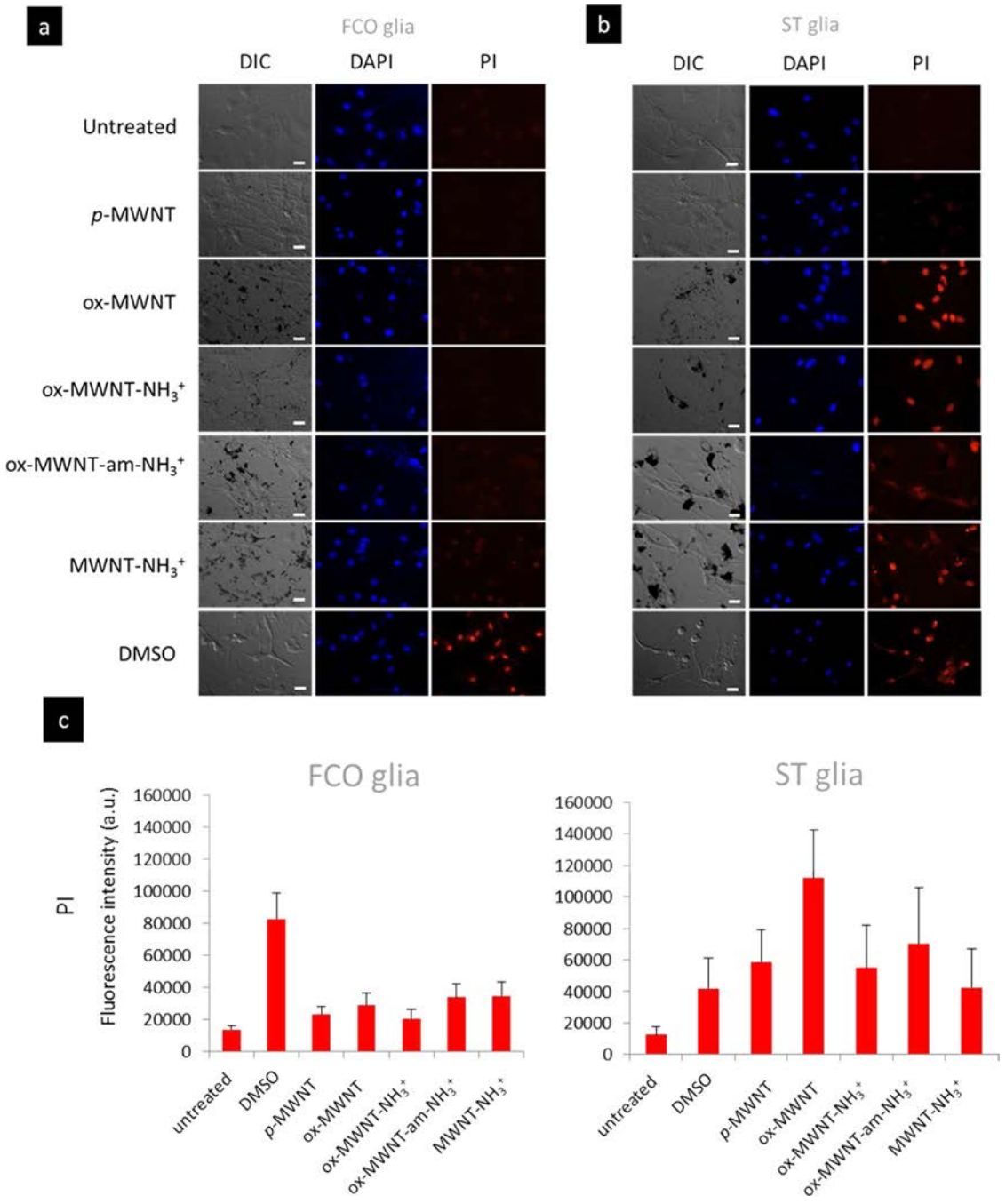

Figure 2. Cytotoxic effects of different MWNTs on mixed glial cell cultures by PI/DAPI assay. Mixed glial cultures were first exposed during 24 h to 10 μg/mL of the different MWNTs or to 10% DMSO as positive control, and then to 200 ng/mL of PI following by fixation and DAPI counterstaining. (a) Results of DAPI/PI assay for mixed glial cells extracted from frontal cortex (FCO). No uptake of PI was observed for any MWNT exposure in FCO cultures compared to DMSO that induced uptake of PI. (b)Uptake of PI by mixed glial cells extracted from striatum (ST) exposed to different MWNTs. In ST cultures, there was an uptake of PI for all functionalized MWNTs, while there was a lower uptake for *p*-MWNT (scale bars = 20 μm). (c) Fluorescence intensity of PI signal measured using ImageJ software.

Supporting Information Figure S4b). The pattern of intracellular spatial distribution of MWNTs inside glial cells was also different between microglia and astrocytes (Supporting Information Figure S5). Microglia cells (GFAP negative) contained significant amounts of MWNTs throughout their cytoplasm, positioning the nucleus of those cells peripherally (Supporting Information Figure S5; solid arrows). In contrast, astrocytes (GFAP positive) contained much less MWNTs, and maintained their nucleus in a central position (Supporting Information Figure S5, hollow arrows).

We concluded from this experiment that microglia were the primary cells responsible for MWNT uptake. This behavior was found to be independent of the brain region from which the primary cultures originated, since both ST- and FCO-derived mixed glial

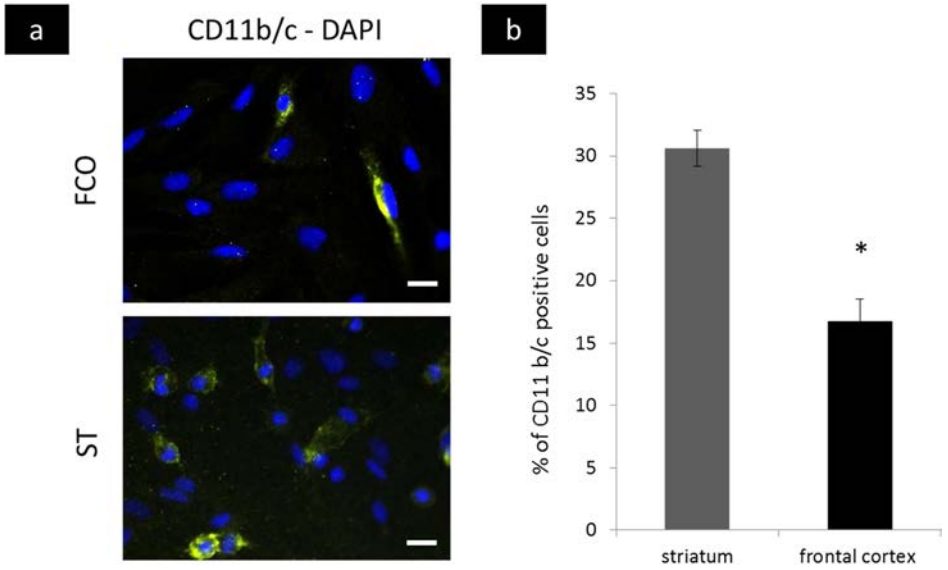

Figure 3. CD11b/c positive cells in nonexposed mixed glia cell cultures. Mixed glial cultures were fixed with precooled methanol, washed and immunostained with mouse anti-rat CD11b/c antibodies in combination with secondary anti-mouse Cy3 antibodies. (a) Merged images of DAPI stain with CD11b/c immunoreactivity of a mixed glia cell culture extracted from frontal cortex (FCO) and from striatum (ST). (b) Percentage of CD11b/c positive cells compared to all cells (DAPI positive) in FCO- and ST-derived mixed glial cultures. There were a higher number of CD11b/c positive cells in mixed glial culture extracted from ST compared to culture from FCO. The values are presented as mean ± SD; *$p < 0.05$ (Student's *t*-test). (Scale bars = 20 μm.)

cultures showed a similar trend (Figure 4 and Supporting Information Figure S5a). Differential interference contrast (DIC) images (Figure 4b,d) also indicated that ST-derived mixed cultures contained a larger proportion of high CNT content and CD11b/c positive cells, validating our previous quantitative assessment of the higher number of microglia cells in the ST-derived mixed cultures (Figure 3). Lastly, MWNTs with free carboxylic acid groups (ox-MWNT, ox-MWNT-NH_3^+, or ox-MWNT-am-NH_3^+) appeared to be internalized to a higher extent by microglia as qualitatively observed by the larger amounts of intracellularly localized nanotubes, in comparison to *p*-MWNT and MWNT-NH_3^+ (Supporting Information Figure S4); however, more work would be required to investigate this mechanistic correlation further.

MWNT Internalization by Microglia Induces Higher Cytotoxic Responses. The different responses obtained from mixed glial cultures derived from the ST and the FCO could be due to differences in residing microglial cell content in the two brain regions. To test this, we determined cytotoxicity using the *m*LDH assay in microglia-enriched cell cultures derived from both FCO and ST. Microglia-enriched cultures were incubated for 24 h with increasing concentrations of ox-MWNT (5, 10, 20, or 50 g/mL). These nanotubes were chosen because they were shown to be uptaken by microglia to a larger extent than other types of chemically functionalized MWNTs. Figure 5 indicates a very similar dose-dependent reduction in microglia viability in cultures derived from either brain region for nanotube exposures of 20 μg/mL and above. This suggested that microglia-enriched cultures are sensitive to MWCNT exposure, regardless of their brain region of origin.

Role of Microglia Response to CNTs in Cytotoxic Effects on Mixed Glia. MWNTs were shown to induce cell death to microglia-enriched cultures at doses higher than 20 μg/mL irrespective of brain region (Figure 5), even though loss of cell viability was observed even at lower nanotube doses only in ST-derived mixed glia cultures but not in FCO-derived ones (Figure 1c). To further elucidate the reason behind this, a dual immunostaining study was performed with the aim to identify the nature of the dying cells in the mixed glia cultures. Both FCO- and ST-derived mixed glia cultures were exposed for 24 h to CNTs at 10 μg/mL, and were then stained with cell-specific markers (CD11b/c for microglia or GFAP for astrocytes) and Bax (BCL2 associated protein) to determine apoptosis (Figure 6 and Supporting Information Figure S6).

In the DMSO treated cultures (positive control), Bax immunoreactivity was very bright, with most cells staining positive. In untreated cells (negative control), Bax immunoreactivity in ST-derived cultures was generally higher than in FCO-derived cultures, suggesting that ST-derived mixed cells were more sensitive, prone to apoptosis. CNT-exposed mixed glia cultures showed moderately higher Bax signals in both FCO- and ST-derived mixed glia cultures (Figure 6). In the CNT-exposed mixed glia cultures, very limited colocalization between the Bax and the GFAP signals was observed, while more consistent Bax positive signals were colocalized with CD11b/c immunoreactivity, especially in

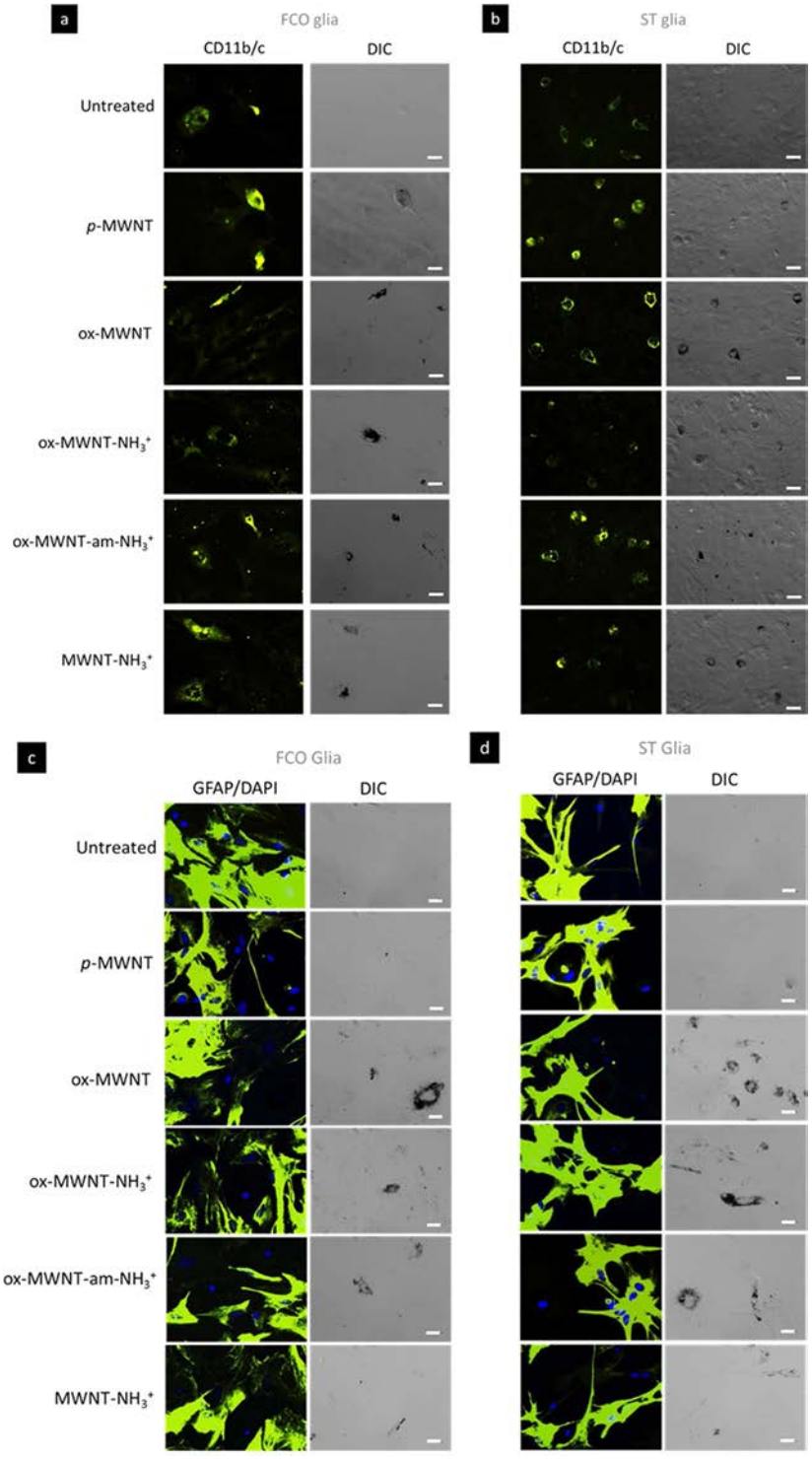

Figure 4. CNT uptake and CD11b/c or GFAP immunocytochemistry identification. Mixed glial cultures were exposed during 24 h to 10 μg/mL of the different MWNTs, fixed with precooled methanol, washed and immunostained with mouse anti-rat CD11b/c (a and b) or anti-GFAP (c and d) antibodies in combination with secondary anti-mouse Cy3 antibodies. DIC channel was used to assess the amount of CNTs present. (a) DIC images and CD11b/c immunoreactivity of mixed glia cells extracted from frontal cortex (FCO). (b) DIC images and CD11b/c immunoreactivity of mixed glia cells extracted from striatum (ST). (c) DIC images and CD11b/c immunoreactivity of mixed glia cells extracted from frontal cortex (FCO). (d) DIC images and CD11b/c immunoreactivity of mixed glia cells extracted from striatum (ST). For both FCO- and ST-derived cultures, there is a good correlation between CD11b/c immunoreactive cells and the highest CNTs concentration, as shown by black material in DIC channel. For both FCO and ST cultures, there was also a clear higher uptake of functionalized MWNTs by CD11b/c positive cells compared to *p*-MWNTs. In contrast, GFAP immunoreactive cells do not correlated with cells with the highest amount of CNTs indicated by the black signal in the DIC channel. (Scale bars =20 μm.)

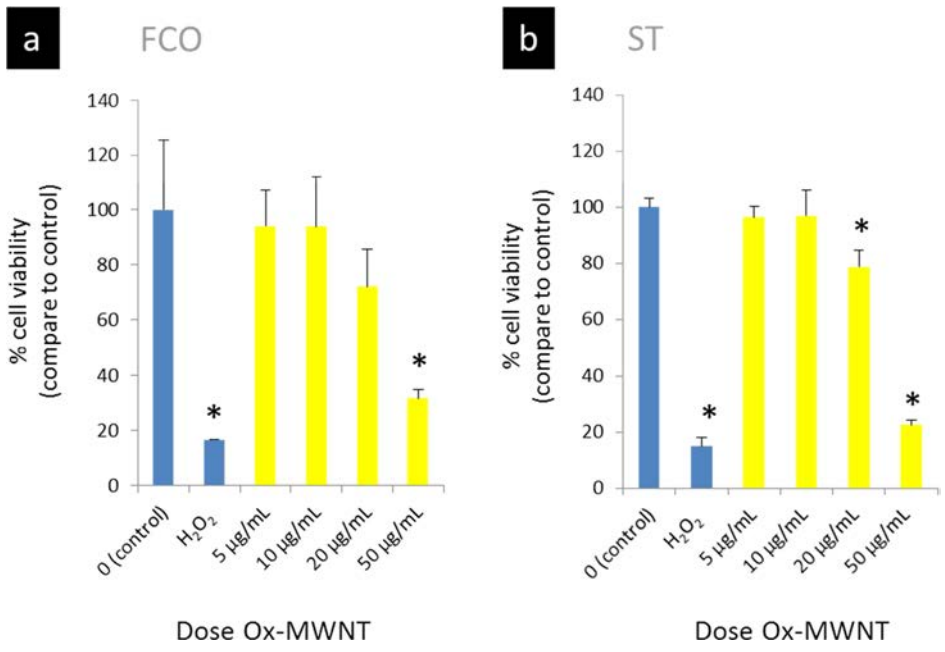

Figure 5. Cytotoxic effects of ox-MWNT on microglia-enriched cell cultures by modified LDH assay. Twenty-four hours after isolation by mild trypsination, microglia enriched cultures were exposed to an increasing range of ox-MWNTs (5−50 μg/mL). mLDH assay for microglia enriched cultures from frontal cortex (FCO, a) or striatum (ST, b). In both cases, a dose−response relationship was observed with 5 and 10 μg/mL as nontoxic concentrations and 20 and 50 μg/mL as toxic ones. There was no significant difference between the cultures derived from FCO or from ST. The values are presented as mean ± SD; *$p < 0.05$ (Student's *t*-test). (Scale bars = 20 μm)

ST-derived cultures. This again suggested that microglia were primarily associated with apoptotic signaling in response to CNT exposure, mainly in ST mixed glia cultures.

To further investigate whether microglia could impact other surrounding cells and potentially cause downstream cytotoxicity, NO release—a marker of nitrosative stress—was evaluated in the supernatant of mixed glia cultures exposed to different CNTs (Figure 7a,b), and in the supernatant of mixed glia treated with conditioned media from microglia-enriched cultures exposed to CNTs (Figure 7c,d). The NO content in the conditioned media from enriched microglia cultures exposed to CNTs was also determined (Supporting Information Figure S7). Similar CNT dose-dependent NO release was found in mixed glia cultures from both brain regions and all CNT types, compared to untreated cells (Figure 7a,b). However, in mixed glia cultures treated with conditioned media from microglia-enriched cultures exposed to CNTs, evident brain region-specific differences were found. ST-derived cultures showed higher levels of NO release, for all CNT exposures, despite using the same conditioned media to induce NO release from FCO-derived mixed glia (Figure 7c,d). CNT dose-dependent responses were also observed here for both brain regions. Interestingly, the values of NO obtained in mixed glia derived from the ST (Figure 7d) and exposed to conditioned medium were higher than in isolated microglia (Supporting Information Figure S7) or in mixed glia derived from FCO (Figure 7c). This suggested that mixed glia cultures have a sensitivity to CNTs that is different from isolated microglia and that this sensitivity to CNTs is higher for ST-derived mixed glia than for FCO, which could explain the toxicity observed in ST-derived mixed glia cultures at 10 μg/mL (Figures 1 and 2), whereas the same concentration was found not toxic in microglia enriched cultures (Figure 5).

The brain is a complex structure that consists of various types of cells that constitute each distinctive brain region. Therefore, introduction of nanomaterials to a specific brain region could have an impact that would be different, determined by the neural cell types and their proportional content in each region. Since no previous study was available regarding selective cytotoxic responses from brain region-specific cell types, we included three types of primary brain cell cultures, including a neuron-enriched culture, a mixed glia culture (consisting mainly of astrocytes and microglia), and a microglia-enriched culture, all extracted from two brain regions, the FCO and the ST. Both brain structures have been associated with dopaminergic pathways and are implicated in key neurological processes.[48,49]

In the present work, pristine (nonfunctionalized) nanotubes (*p*-MWNT) were studied as a case of clinically irrelevant material that has been associated with cytotoxic responses in previous studies not related to the brain tissue.[19−21,50] Chemically functionalized nanotubes (*f*-MWNT) with different surface functionalities were also used as cases of more clinically relevant candidate nanoscale vectors in neurological applications.[13,35,47]

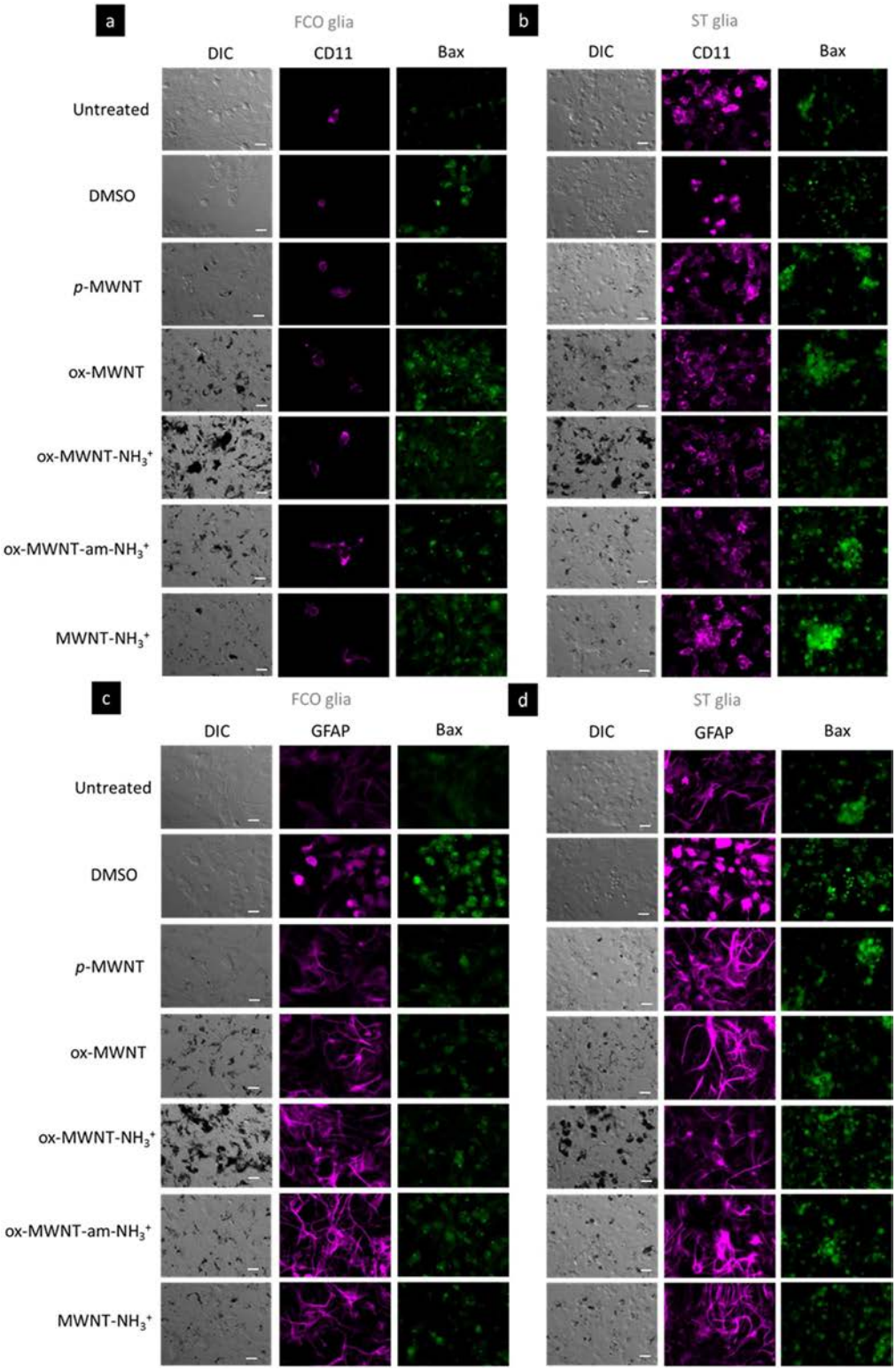

Figure 6. Bax positive cells in mixed glia cell cultures. Mixed glial cultures were first exposed during 24 h to 10 μg/mL of the different MWNTs. Then, cells were fixed with 4% paraformaldehyde, washed and immunostained first with mouse anti-rat CD11b/c antibodies or mouse anti-rat GFAP in combination with secondary anti-mouse Cy3 antibodies, and second with rabbit anti-rat Bax antibodies in combination with secondary anti-rabbit biotinylated antibodies revealed with fluorescein avidin. (a) DIC images, CD11b/c and Bax immunoreactivities of mixed glia cells extracted from frontal cortex (FCO). (b) DIC images, CD11b/c and Bax immunoreactivities of mixed glia cells extracted from striatum (ST). (c) DIC images, GFAP and Bax immunoreactivities of mixed glia cells extracted from frontal cortex (FCO). (d) DIC images, GFAP and Bax immunoreactivities of mixed glia cells extracted from striatum (ST). Bax signal was generally higher for ST derived cells compared to FCO derived cells; there was also a better correlation between CD11b/c immunoreactive cells and the highest Bax immunoreactivity, in comparison to GFAP positive cells. (Scale bars = 20 μm.)

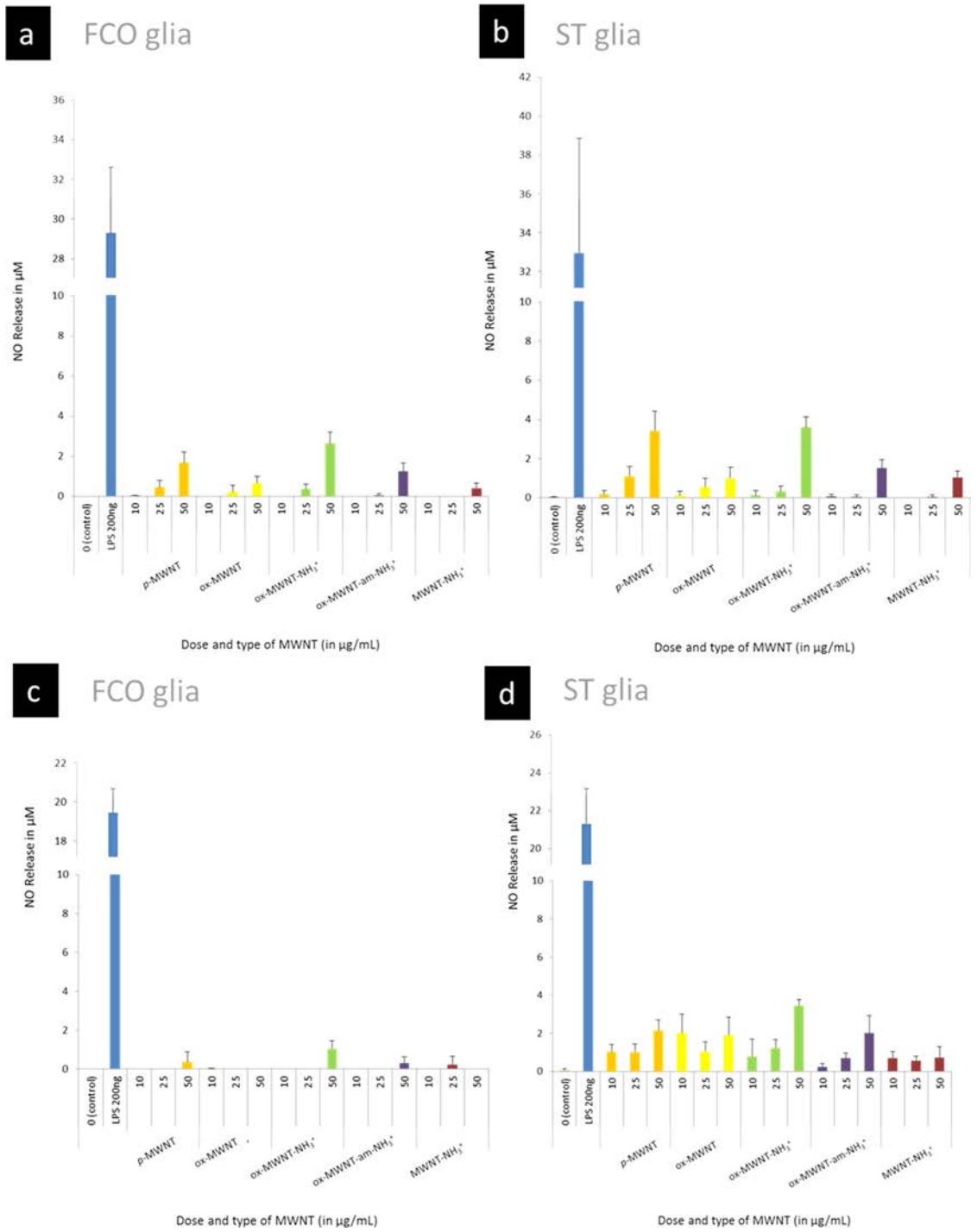

Figure 7. NO release from mixed glia exposed to CNTs or mixed glia treated with conditioned media from CNT-exposed microglia enriched cultures. Mixed glial cultures were either exposed for 24 h to 10, 25, or 50 μg/mL of different MWNTs, or to the conditioned media of microglia that were exposed to CNTs for 24 h at the same dose range (10, 25, or 50 μg/mL). (a) NO release in mixed glia from frontal cortex (FCO) exposed to CNTs. (b) NO release in mixed glia from striatum (ST) exposed to CNTs. (c) NO release in mixed glia from FCO treated with conditioned media from CNT-exposed microglia. (d) NO release in mixed glia from ST treated with conditioned media from CNT-exposed microglia. Release of NO was overall higher in mixed glia directly exposed to CNTs in comparison to mixed glia treated with conditioned media. Brain region differences were observed in mixed glia treated with conditioned media, showing higher NO release in ST-derived cultures than in FCO-derived cultures. The values are presented as mean ± SD.

The absence of cytotoxic responses for any type of MWNTs studied on neurons isolated from either FCO or ST was in line with observations by others. Bardi *et al.* have reported no loss in neuron viability when treated with Pluronic F127-coated MWCNTs after local administration into mouse brains.[36] Similarly, Matsumoto *et al.* have shown that incubation of dorsal root ganglion neurons and a rat pheochromocytoma cell line

(PC12) with 1,4-diaminobutane or 1,8-diaminooctane functionalized MWNTs at low concentrations (0.11−1.7 μg/mL) increased the neurite outgrowth of neuronal cells.[51] Such observations of beneficial effects of CNTs on neurons[52] agreed with the results reported here regarding a slight increase of viability of FCO derived neurons when exposed to the highest concentration of CNTs in comparison to untreated neurons isolated from the same brain region (Supporting Information Figure S1, for *p*-MWNT, ox-MWNT and ox-MWNT-am-NH_3^+). For neurons isolated from ST, we did not observe such trends. We attributed this difference between FCO and ST to the specific population of neurons present in these two regions, with dopaminergic, serotoninergic and pyramidal neurons in the frontal cortex[53,54] and a more heterogeneous population of neurons in the striatum[55] (GABAergic, cholinergic, dopaminergic projections, or even neuroblasts). Differences in neuron density are also considered hallmarks of primary neuron cultures from these two brain regions, as can be optically observed between the two neuron cultures (Supporting Information Figure S1a). The response to CNTs of neurons derived from specific brain regions will need further investigation to understand the reasons behind the possible neuroprotective effects of CNTs.

When mixed glial cultures rather than isolated neurons were used, two independent cytotoxicity assays (*m*LDH assay and PI/DAPI staining) indicated that mixed glial cultures were consistently more sensitive to MWNT exposure than neurons, irrespective of the nanotube considered. Belyanskaya *et al.* compared peripheral and central nervous system sensitivity to CNTs to also demonstrate a higher cytotoxic effect on glial cells compared to neurons.[32] Such selective vulnerability of specific cell types in the CNS (consisting of different proportions of mainly neurons, astrocytes and microglia) has been previously reported for other neurotoxins, such as bilirubin[56] or diesel exhaust particles.[57]

In our experiments, we have additionally observed a brain region-selective cytotoxicity, with ST-derived glial cultures significantly more sensitive to CNT exposure than those derived from the FCO. Various other agents (*e.g.*, organophosphate pesticide, cadmium, or methamphetamine) that reach the CNS are known to cause region-specific injuries throughout the brain.[58−60] With relation to nanomaterial exposure, Sharma *et al.* have reported a brain region-dependent impact of metallic nanoparticles (Al, Ag and Cu) that was attributed to selective alteration of the blood brain barrier permeability in these regions.[61] In another study investigating the neural impact of diesel engine exhaust particles after inhalation, a brain region-specific toxic effect was also reported and attributed to the function and unique cellular composition of brain regions considered.[62]

Analysis of the cell population in the two brain regions (FCO and ST), from where the mixed glial cell cultures were isolated, found that ST-derived cultures contained more microglia than the FCO-derived ones. In the central nervous system, microglia are thought to constitute at least 20% of the non-neuronal cell population and 5−10% of all glial cells found in the normal adult brain.[63] In addition, their distribution in the normal brain is not homogeneous.[64] Lawson *et al.* have reported that in the adult mouse brain there is a higher density of microglia in the neostriatum (*caudae putamen*) compared to the cortex.[64] In adult rat brains, while microglial cell population was found to be about 123 ± 10 LC1-positive cells (marker for microglia) per mm^2 in frontal cortex, between 123 and 138 LC1-positive cells were found in the different subareas of the striatum, the highest proportion compared to the whole brain (103 ± 3 cells per mm^2).[65] In the developing mouse brain, at embryonic day 16 (E16), hot spots of premicroglia have been found in the white matter of the developing cortex and corpus callosum, increasing in these regions and in the subventricular zone (which was included in our ST extraction) from stage E16 to E19.[66] However, no specific cell numbers were reported for the two embryonic regions used in this study, namely FCO and ST. Overall, the reported differences between the FCO and ST regions in adult brains are in line with the differences in microglia populations between ST- and FCO-derived mixed glial cultures observed in the present study (Figure 3). It is noteworthy that the proportion of microglial cells found in our derived mixed glial culture was found higher (15−30%) than what has been reported[64] for the whole adult brain (5−10%). This difference could be justified by the fact that our cultures were maintained under conditions that favored glial cell proliferation from the pool of progenitor cells extracted from the fetal brain.[66] The difference in microglia population between the two regions suggested that the brain region-specific sensitivity to CNT exposure could be related to microglia, both in terms of microglial cell number and microglia response to CNTs following internalization.

Microglial cells assume the function of representative cells of the immune system and are known for their capacity to engulf foreign particles as a mechanism of maintaining brain homeostasis and protection against external injuries.[45,46] In mixed glial cell cultures, CD11b/c (microglia marker) positive cells were consistently found to have a larger intracellular CNT content compared to GFAP (astrocyte marker) stained cells, observed by fluorescence and optical microscopy. Phagocytic microglial cells have been previously reported to efficiently internalize carbon nanotubes[37,67,68] along with other nanomaterials, such as magnetic nanoparticles.[69] With regards to the role of chemical functionalization onto MWNTs, we found that

surface character was a determinant factor in microglia uptake. Higher degree of internalization was achieved for chemically functionalized MWNTs compared to *p*-MWNTs dispersed in F127 block copolymer; this agreed with our previous findings using human monocyte derived macrophages.[70] For various phagocytic cells, surface functionalization appears therefore as a common physicochemical feature that improves material engulfment. However, an accurate determination of the uptake efficiency and kinetics correlated to nanomaterial features that determine the overall internalization process was not the objective of the present study and will require specific investigations.

Apart from their phagocytic capability, microglia also constitutes a cell population that can produce different substances (cytokines, chemokines, ROS, RNS) potentially deleterious to surrounding cells in response to tissue assaults.[71] Among the different *f*-MWNTs studied, nanotubes bearing free available carboxylate groups on their surface resulted in the highest internalization by microglia. Interestingly, a higher uptake of nanomaterials bearing functional groups, known as more biologically reactive,[72,73] could lead to higher biological responses *in vitro*, but also *in vivo* as recently reported.[35] In the latter study, oxidized MWNTs compared to nonoxidized MWNTs induced a stronger and longer inflammatory response in brain regions injected with the oxidized nanomaterials, in correlation with a higher number of microglia in areas close to the site of injection.[35] Here, despite a higher interaction of carboxylated MWNTs with mixed glia in comparison to pristine or amino-functionalized MWNTs (Figures 4 and 6, DIC channel), we did not obtain clear evidence of higher cytotoxicity (Figures 1 and 2), Bax signaling upregulation (Figure 6) or NO release (Figure 7) for these nanotubes compared to other functionalized derivatives studied.

While the effects of MWCNTs on mixed glia cells was not surface chemistry dependent, we observed brain region-specific cytotoxic sensitivity (ST > FCO) in those mixed glial cultures. Both the concentration-dependent cytotoxic effects of MWNTs on microglia-enriched cultures (Figure 5) and the higher microglia number in ST compared to FCO (Figure 3) suggested that the brain region-specific sensitivity was due to microglia responses to CNT exposure. In addition, microglia exposed to MWNTs could secrete various substances,[71] such as NO, to trigger an adverse effect on surrounding cells, especially in a mixed culture. The number of dead cells in mixed glial cultures derived from ST could be correlated not only to the number of dead microglial cells, but also to the number of cells dying from the substances released by microglia in response to CNT exposure. Indeed, no cytotoxicity was observed in microglia-enriched cultures exposed to 10 μg/mL of MWNTs (Figure 5), whereas this dose was found to be toxic to mixed glial cells isolated from the ST (Figures 1c and 2b). This suggested involvement of substances released by microglia in response to MWNT exposure, as demonstrated by the higher NO release (and Bax expression) in ST-derived mixed glia for all MWNTs. The number of microglia present in the mixed glia cultures was still the determinant factor in terms of overall reactivity. Additional studies using neuronal-glial mixed cultures should also explore further the potential risks from microglia-mediated cytotoxicity toward neurons that the present study did not attempt to address. Long-term exposure studies to determine whether such events are indicative of a transient biological response to exogenous agents or a persistent pathophysiological effect are also essential toward determination of the safety limitations for potential CNT-based tools on interaction with the neuronal milieu.

CONCLUSION

We demonstrate here that exposure to different types of *f*-MWNTs did not induce any deleterious effects to neurons at doses up to 100 μg/mL in isolated neuronal cultures from the FCO and the ST within 24 h. In contrast, exposure to different types of functionalized MWNTs induced cytotoxic responses to microglia-containing cultures. The number of microglial cells in mixed cultures and different brain regions and the microglial response to MWNT exposure were revealed as key factors to be ascribed for the observed brain region-specific sensitivity to MWNT exposure.

EXPERIMENTAL SECTION

Materials. Pristine MWNTs (*p*-MWNTs) were purchased from Nanostructured and Amorphous Materials, Inc. (Houston, TX; Lot #1240XH, 95%). Outer average diameter was 20−30 nm, and length between 0.5 and 2 μm. Dulbecco's modified eagle medium with nutrient mixture F12 (DMEM:F12), fetal bovine serum, neurobasal medium, B27 supplement, glutamine, penicillin/streptomycin, HBSS (Ca^{2+}/Mg^{2+} free), and PBS (Ca^{2+}/Mg^{2+} free) were purchased from Gibco, Invitrogen, Life Technologies (U.K.). Poly-D-lysine and propidium iodide were purchased from Sigma-Aldrich (U.K.). Promega Cytotox 96 Nonradioactive cytotoxicity assay and Griess Reagent Assay were purchased from Promega (U.K.). Mouse monoclonal primary Anti-Glial Fibrillary Acidic Protein Cocktail (GFAP) were purchased from Calbiochem, Merck-Millipore (U.K.) and mouse monoclonal anti-CD11b/c [OX42] and anti-Bax [E63] antibodies were purchased from Abcam (U.K.). Goat anti-mouse Cy3 conjugated antibodies were purchased from Jackson Immuno Research Lab, Inc., Stratech Scientific Ltd. (U.K.). Vectashield (H1200) mounting medium containing antifading agent and DAPI was purchased from Vector Laboratories Ltd. (U.K.).

Chemical Functionalization of Multiwalled Carbon Nanotubes. Oxidized MWNTs (ox-MWNTs) were prepared as reported previously.[39] Oxidized and 1,3-dipolar cycloaddition functionalized MWNTs (ox-MWNT-NH_3^+) were prepared as reported in literature.[27,40] The amount of amino groups was determined with the Kaiser test, corresponding to 170 μmol/g. Transmission electronic microscopy characterization showed that the

morphology of the nanotubes was not modified by the 1,3-dipolar cycloaddition. Amidation reaction on oxidized MWNTs (ox-MWNT-am-NH_3^+) was performed as reported previously.[43] The amount of amino groups was determined with the Kaiser test, corresponding to 320 μmol/g. 1,3-Dipolar cycloaddition reaction on pristine MWNTs (MWNT-NH_3^+) was performed as previously described.[41,42] The amount of amino groups was determined with the Kaiser test, corresponding to 84 μmol/g. Transmission electronic microscopy characterization showed that the morphology of the nanotubes was not modified by the 1,3-dipolar cycloaddition.

Preparation of MWNTs for Cell Exposure. One milligram per milliliter stock suspension of pristine MWNTs was prepared using 1% Pluronic F127 in sterile distilled water, sonicated for 45 min and stored at −20 °C before further use. Before each experiment and after thawing, the suspension was sonicated 10 min. Functionalized MWNTs were prepared following the same protocol apart from dispersion that was made with 5% dextrose in sterile distilled water. Consequently, solutions of 1% Pluronic F127 or 5% dextrose in sterile distilled water were used as negative controls.

Primary Cell Cultures. *Primary Neuron Enriched Cell Cultures.* Neuron enriched cell cultures were prepared from frontal cortex (FCO) and striatum (ST) extracted from E16−E18 Wistar fetal rat brains (standard Witschi stages 33−34). Following isolation under stereomicroscope, FCO and ST brain pieces were dissociated to single cell suspensions by trypsin treatment followed by mechanical trituration in Ca^{2+}/ Mg^{2+} free HBSS solution. After determination of the number of live cells, cells were plated on 50 μg/mL poly-D-lysine-coated 96 wells plate at a density of 40 000 cells per well in serum-free neurobasal medium containing B27 supplement, glutamine (0.5 mM), penicillin (100 U), and streptomycin (100 μg). B27 supplement was used as a means to reduce glial cells growth and to obtain a nearly pure neuronal cell culture. Cultures were incubated in a humidified 37 °C/5% CO_2 incubator for 10 days before experimentation. Medium was changed by half every 4 days.

Primary Mixed Glial Cell Cultures. Mixed glial cultures were prepared from FCO and ST of E16-E18 Wistar fetal rat brains. After trypsination and mechanical trituration in Ca^{2+}/Mg^{2+} free HBSS solution, cells were plated onto 75 cm^2 flask with DMEM:F12 medium completed with 10% heat inactivated fetal bovine serum and incubated at 37 °C in a humidified 5% CO_2 incubator for 1 week. Cells were used for CNT exposure after 2 passages as a means to reduce neuronal cell population and to increase the number of glial cells.

Primary Microglia Enriched Cell Cultures. Microglia enriched cell cultures were prepared from FCO and ST mixed glial cultures at passage 2 or 3, according to previously described method, based on mild trypsination.[74] Briefly, mixed glial cultures were seeded on poly-D-lysine coated 12 wells plate and incubated at 37 °C in a humidified 5% CO_2 incubator as described above. At confluence, the cell monolayer was treated with 0.05% trypsin in serum-free DMEM:F12 medium until all cells detached, except microglial cells which remained attached to the plate. After washing with serum-free DMEM:F12 medium, cells were cultured in DMEM:F12 medium completed with 10% heat inactivated fetal bovine serum. After 24 h, microglia enriched cell cultures were treated with CNTs.

Cell Viability Assessed by Modified LDH Assay. Neurons were incubated with the different CNTs in serum-free Neurobasal-B27 medium for 24 h. Mixed glial cell cultures or microglia enriched cell cultures were incubated with the different CNTs in serum-free DMEM:F12 medium for 4 h, after which heat inactivated fetal bovine serum was added to reach a final concentration of 10%, and cells were allowed to interact with CNTs for 24 h. Cell viability was assessed by a modified LDH assay as previously described[38] using the Promega Cytotox 96 Nonradioactive cytotoxicity assay. The assay was modified to avoid interference between CNTs and the LDH in the survived cells, and was determined after chemically lysing all remaining cells (0.9% triton X100 in distilled water) instead of detecting the LDH released in supernatant from the cells exposed to CNTs as this would contain MWCNTs. Cells were treated with *f*-CNTs at 5, 10, 50, and 100 μg/mL concentrations for neuron enriched and mixed glia cell cultures, and with 5, 10, 20, and 50 μg/mL for microglia enriched cell cultures. In addition, 10% DMSO or 1 mM H_2O_2 were used as positive controls to induce cytotoxicity. After 24 h of exposure, cells were lysed with 10 μL of lysis buffer per 100 μL serum-free media and left for 45 min at 37 °C. After centrifuging (13 000 rpm, 5 min), 50 μL of cell lysate supernatant was mixed with 50 μL of substrate mixture in a new microtiter plate and the sample was incubated for 15 min at room temperature. Absorbance at 490 nm was read in a multiwell plate reader (Fluostar Omega, BMG Labtech Ltd.). The amount of LDH detected after cell lysis was an indication of the number of cells which survived treatment. Hence, the percentage cell survival is expressed as [LDH released from tested cells − Blank (media alone)/LDH released control cells − Blank (media alone)] × 100.

Cell Viability Assessed by PI/DAPI Stain. Mixed glial cells seeded on poly-D-lysine coated glass coverslips (60 000 cells) were incubated with 10 μg/mL CNTs as described above (initially 4 h serum free, followed by 24 h complete medium). Ten percent DMSO or 1 mM H_2O_2 were used as positive controls to induce cytotoxicity (*i.e.*, propidium iodide uptake). After 24 h of exposure, the cell monolayer was washed with cold PBS once and then stained with 200 ng/mL of propidium iodide for 10 min, protected from light. The cell layer was then gently washed twice with cold PBS, fixed with 4% formaldehyde in PBS for 20 min at room temperature, and washed twice with PBS. The glass coverslips were finally mounted with appropriate medium containing antifading agent and DAPI. Epifluorescence images were obtained on a Carl Zeiss Axio Observer A1 inverted microscope.

Immunocytochemistry. Mixed glial cells were seeded on poly-D-lysine coated glass coverslips (13 mm diameter; 60 000 cells) or on poly-D-lysine coated plastic tissue-culture treated dishes (35 mm diameter; 200 000 cells). After reaching confluence, cells were exposed to CNT for 24 h. At the end of exposure, cells were fixed with precooled methanol (−20 °C) for 10 min, air-dried, and then washed twice with PBS containing 0.1% Triton (TPBS, pH 7.4) before staining. Staining with primary antibody was performed after 1 h incubation (*i.e.*, blocking) with 5% of normal goat serum in TPBS. Primary antibodies used were as follows: mouse monoclonal anti-CD11b/c (1:200) and mouse monoclonal anti-GFAP (1:800), both diluted in 2% BSA in TPBS and incubated for 2 h at room temperature (CD11b/c) or overnight at 4 °C (GFAP). The secondary antibody (goat anti-mouse Cy3 labeled) was used at the dilution of 1:250 in 2% BSA in TPBS for 1 h at room temperature. After a wash step, cell preparations were mounted in appropriate medium containing antifading agent and DAPI. Immunoreactivity was assessed on an inverted epifluorescence microscope (Carl Zeiss Axio Observer A1).

Dual Immunostaining. Mixed glial cell cultures were prepared as described above. At the end of a 24 h exposure to CNTs, cells were fixed with 4% paraformaldehyde for 20 min at room temperature. After fixation, cell monolayers were washed once with PBS and then air-dried. The first staining with primary antibodies (mouse monoclonal anti-CD11b/c (1:200) or mouse monoclonal anti-GFAP (1:800)) was performed after 1 h incubation (*i.e.*, blocking) with 5% of normal goat serum in PBS containing 0.1% Triton (TPBS, pH 7.4). Incubation was performed overnight at 4 °C for both primary antibodies. The secondary antibody (goat anti-mouse Cy3 labeled) was used at the dilution of 1:250 in 1% BSA in TPBS for 1 h at room temperature. A second blocking with 2% BSA was performed at room temperature for 1 h, followed by 2 h reaction at room temperature with the primary antibody anti-Bax (rabbit monoclonal, 1:250). The secondary antibody (biotinylated goat anti-rabbit, Calbiochem, U.K.) was used at the dilution of 1:200 in 1% BSA in TPBS for 1 h at room temperature. Fluorescein avidin revealed the secondary antibodies (8 μg/mL, 30 min room temperature).

Distribution of Microglia Population, Counting Method for CD11b/c Immunoreactivity. CD11b/c positively labeled cells were counted in 10 fields randomly chosen but covering the whole sample (35 mm diameter Petri dishes) in 3 independent samples. The percentage of CD11b/c positive cells was calculated from the ratio of CD11b/c positive cells compared to DAPI labeled cells.

NO Release. The concentration of NO present in the supernatant was quantified following the supplier's instructions. Briefly, 50 μL of supernatant to test is mixed with 50 μL of the sulfanilamide solution; after 8 min of reaction, 50 μL of the *N*-1-naphthylethylenediamine dihydrochloride solution is added and left to react for another 8 min. Absorbance is measured at 540 nm on a plate reader (Fluostar Omega, BMG Labtech Ltd.). Supernatant from cell cultures were centrifuged for 10 min at 4000 rpm before proceeding to measurement in order to spin down the CNTs present in suspension and prevent interference/inhibition with the NO detection system. The content of NO in the supernatant was measured for mixed glia cultures from FCO and ST exposed directly to different CNTs (10, 25, and 50 μg/mL), for mixed glia cultures from FCO and ST treated with conditioned media coming from isolated microglia exposed to different CNTs (10, 25, and 50 μg/mL), and for microglia enriched cell cultures used to prepare conditioned media. Conditioned media from microglia enriched cell cultures was centrifuged for 10 min at 4000 rpm to remove CNTs.

Statistics. Results are expressed as mean $\pm$ SD. Statistical differences were analyzed using the Student's *t* test, and $p < 0.05$ (*) were considered as significantly different.

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: Figure S1, primary neuron enriched cell cultures and effects of *f*-MWNTs; Figure S2, GFAP immunostaining of mixed glial cell cultures; Figure S3, intracellular localization of MWNTs in mixed glial cell cultures; Figure S4, fluorescence intensity measurement of the CD11 signal for both mixed glia cultures; Figure S5, intracellular localization of MWNTs in mixed glial cell cultures; Figure S6, fluorescence intensity measurement of the Bax signal for both mixed glia cultures; Figure S7, NO release from microglia-enriched cell culture exposed to MWNTs. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.5b02358.

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