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Nanomaterials and neurodegeneration

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Abstract

The increasing application of nanotechnology in various industrial, environmental and human settings, poses the question of the potential adverse effects induced by nanosized materials to human health, including the possible neurotoxic and neuroinflammatory properties of those substances and their capability of inducing neurodegeneration. In this review we will focus on a panel of metal oxide nanoparticles (NPs), namely titanium dioxide, silicon dioxide, zinc oxide, copper oxide, iron NPs, ending with carbon nanotubes. An overview is provided of the *in vitro* and *in vivo* evidence of adverse effects to the central nervous system (CNS). Observations gathered have provided the evidence that these nanomaterials (NMs) can not only reach the brain but also cause a certain degree of brain tissue damage, including cytotoxicity, genotoxicity, induction of oxidative stress and inflammation, all potentially involved in the onset and progression of neurodegeneration. Surface chemistry of the nanomaterials may play an important role on their localization and subsequent effects on the brain of rodents. In addition, the shape difference of NMs may induce varying degrees of neurotoxicity. On the other hand one of the potential biomedical applications of NMs concerns nanodevices for early diagnostic and novel therapeutic approaches to counteract age related diseases. In this context, engineered NMs are promising vehicle molecules to carry diagnostic and therapeutic compounds across the BBB, thereby representing very timely and attractive theranostic tools in neurodegenerative diseases. Then a careful assessment of the risk-benefit ratio must be taken into consideration in using nanosized materials.

Keywords: Nanomaterials; Nanoparticles; Neurotoxicity; Oxidative Stress; Neurodegenerative diseases, Nanodevices.

Introduction

Nanomaterials are small molecules which behave with distinct biological activity and have been progressively and increasingly applied in various industrial and medical settings over the last 30 years [Robertson et al., 2010; Schröfel et al., 2014; Shannahan et al., 2012].

However, despite the great progress in nanotechnologies, comparatively little is known to date on the negative effects that exposure to NMs may have on the human brain, including the potential induction of pathways leading to neurodegeration [Cupaioli et al., 2014]. Although many NPs exhibit potential beneficial aspects for diagnostic and therapeutic purposes, some of these molecules can exert negative or harmful effects, suggesting that the beneficial and harmful effects should be compared prior to their application to human beings [Iqbal et al., 2013]. Indeed, NMs can enter the human body through several ways, including absorption through the skin or the digestive tract, airway inhalation, and blood injection, and they may cross the blood-brain barrier to reach the central nervous system, where they have been suspected to impair several molecular pathways and contribute to neurodegeration [Iqbal et al., 2013; Cupaioli et al., 2014].

Neurodegenerative diseases are a heterogeneous group of either hereditary or sporadic conditions all characterized by progressive nervous system dysfunction resulting from the degeneration of selected neurons in the CNS. Some of the most well known neurodegenerative diseases are Alzheimer's Disease (AD) and other dementias, Parkinson's Disease (PD), Amyotrophic Lateral Sclerosis (ALS), and Huntington's Disease (HD) [Migliore and Coppedè, 2009]. Despite the heterogeneous nature of neurodegenerative diseases, the application of recent genome-wide and omics approaches has provided novel insights into the critical molecular pathways of those disorders, revealing that aggregation and accumulation of misfolded proteins, mitochondrial dysfunction, oxidative stress, oxidative

DNA damage and impaired DNA repair, apoptosis, impaired autophagy-lysosomal activities, inflammation and microglia activation, perturbation of vesicle trafficking and synapse dysfunction, RNA processing and protein degradation pathways, as well as epigenetic deregulation of gene expression, are common pathways in neurodegenerative diseases [Coppedè and Migliore, 2010; Giordano et al., 2013; Golde et al., 2013; Ramanan and Saykin, 2013; Vanderweyde et al., 2013; Amor et al., 2014; Bäumer et al., 2014].

There is indication that NPs can impair dopaminergic and serotoninergic systems, the former relevant for PD and the latter for AD pathogenesis, respectively, and can lead to changes of neuronal morphology and cell death. In addition, NPs can also contribute to neurodegeneration by inducing mitochondrial dysfunction, redox imbalance and apoptosis, autophagy and impaired lysosomal activity, cytoskeletal damage and vesicle trafficking perturbations, neuroinflammation and microglia activation [Iqbal et al., 2013; Cupaioli et al., 2014]. Furthermore, *in vitro* evidence suggests that engineered NMs are able to induce changes in the expression of genes involved in DNA methylation pathways, as well as global changes in epigenetic marks such as DNA methylation and histone tail modifications, all potentially involved in human complex disorders, such as neurodegenerative ones [Stoccoro et al., 2013].

On the other hand, since biodegradable NMs can be engineered to load drugs, contrast agents, and cellular or intracellular component targeting moieties, they have emerged as potential alternatives for tracking and treating human diseases, including neurodegenerative disorders [Marrache et al., 2013]. Nanoparticulate drug carriers are able to cross the BBB by virtue of their size, surface potential or surface coatings, and are currently under investigation for effective delivery of pharmaceuticals or contrast agents active in the treatment and detection of AD and other neurodegenerative diseases [Garbayo et al., 2013; Oesterling et al., 2014]. Indeed, during the past decade, nanotechnology has been widely considered as a promising

tool for theranosis (diagnosis and therapy) of neurodegenerative diseases [Amiri et al., 2013]. Aim of this review is to critically discuss available *in vitro* and *in vivo* data on the potential neurotoxic effects of NPs in the context of neurodegeneration, with focus on the induction of cytotoxic and genotoxic effects, oxidative stress and inflammatory pathways. Moreover, we also provide some examples of the potential beneficial uses of NPs in the context of diagnosis and treatment of neurodegenerative diseases.

Cytotoxic, genotoxic, oxidative and inflammatory potential of NPs: implications for neurodegeneration

Thanks to their unique physico-chemical properties (i.e. small size, large surface area, composition and functionalization) several types of metallic NPs were shown to be able to cross the BBB and interact with the CNS components. However, despite the large number of both *in vitro* and *in vivo* investigations performed so far, the interactions between NPs and the CNS are still not completely understood and their toxic potential is still unclear. The majority of the data available in the literature report that metallic NPs induce toxic effects to the target cells or to the exposed animals, and the toxicity is mainly triggered via oxidative stress. The evidence that NPs induce cytotoxicity and genotoxicity, as well as oxidative stress and inflammation in various cell lines representative of body compartments such as the respiratory system, the intestine and the immune system, amplified the need of comprehensive studies on the neurotoxicity and the neurodegeneration induced by NPs engineered for the screening, diagnosis and therapy of CNS diseases. Moreover, the evidence that the CNS is a potential susceptible target for nanosized materials and that NPs can penetrate there through the olfactory bulb and deposit in the hippocampus [Oberdörster et al., 2004; Wang et al., 2008] enhanced the need of studies on the potential neuronal effects of NPs. Retention of the

particles into the CNS, neurotoxicity, apoptosis and oxidative stress, as well as changes in gene expression and neuropathological lesions were the most investigated parameters. The next sections discuss the evidence available on the most widely used NPs in industrial or biomedical applications, and a summary of *in vitro* (Table 1) and *in vivo* (Table 2) studies is provided.

Titanium dioxide nanoparticles

Titanium dioxide nanoparticles (TiO_2 NPs) represent one of the most frequently used NPs in industrial applications ranging from paints to ceramics and from food to cosmetics, and therefore of pivotal interest is the investigation of occupational exposure to TiO_2 NPs and the associated risk. Only in recent years studies have been devoted to test the neurotoxic potential of these NPs. Among the first to demonstrate the cytotoxic effects of TiO2 micro- and nanoparticles on human neural cells (U87 astrocytoma cells) as well as in human fibroblasts (HFF-1 cells), were Lai et al., [2008]. Either TiO2 microparticles (1–1.3 μ particle size) or nanoparticles (<25 nm), irrespective of their sizes, were found able to induce cell death on both human cell types, with mechanisms including apoptosis, necrosis, and possibly apoptosis-like and necrosis-like cell death types [Lai et al., 2008]

Afterwards Márquez-Ramírez and colleagues [2012] demonstrated that the *in vitro* proliferation of murine C6 and human U373 glial cells was linearly inhibited in the presence of 40-200 nm TiO_2 NPs; furthermore after 96 h exposure apoptosis was induced. Moreover, TiO_2 NPs were internalized in cytoplasmic vesicles and induced morphological changes, observable already after 24 h incubation [Márquez-Ramírez et al., 2012]. The toxicity of TiO_2 NPs on the same C6 and U373 cells was further confirmed by Huerta-García and collaborators [2014]. They observed by immunostaining, the morphological changes exerted by titania, including the impairment of the integrity of mitochondria. In addition, severe

changes in the redox-state of the cells and in lipid peroxidation, accompanied by increased levels of glutathione peroxidase, catalase and superoxide dismutase, demonstrated that TiO_2 NPs induced oxidative stress in rat and human microglial cells [Huerta-García et al., 2014]. The ability of TiO_2 NPs to induce oxidative stress had already been previously reported also in the murine microglial cell line BV-2, where the release of free radicals occurred in less than 5 min exposure to sub-toxic concentrations of Degussa P25 nanoparticles [Long at al., 2006]. In addition, the prolonged (2 h) release of reactive oxygen species (ROS) suggested that TiO_2 NPs interfered with the mitochondrial apparatus of BV-2 cells [Long at al., 2006]. Further investigation was performed to test the ability of TiO_2 NPs to cause inflammation and microglia-mediated neurotoxicity. Xue and co-authors [2012] demonstrated that the exposure of Sprague-Dawley freshly isolated microglia cells to TiO_2 NPs enhanced the release of nitric oxide (NO) via the upregulation of the expression of inducible nitric oxide synthase (iNOS), both at mRNA and at protein level. Moreover, the inflammation produced by TiO_2 NPs determined an increase in the expression of the monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein 1 alpha (MIP-1α), but also the secretion of TNF-α, IL-1ß and IL-6 was significantly enhanced upon exposure to titania. Finally, to test if TiO_2 NPs were able to initiate microglia-mediated neurodegeneration, the rat embryonic pheochromocytoma cell line PC12 was incubated with supernatants of the exposed microglial cells. The inflammatory cytokines TNF-α, IL-1ß and IL-6 contained in the supernatant from TiO_2 NP-treated microglia impaired the viability of PC12 and severely suppressed the expression of the tyrosine hydroxylase (*Th*) gene, which is involved in the dopamine secretion in the CNS [Xue et al., 2012].

The neurodegenerative effect of TiO_2 NPs was additionally investigated taking into consideration the role played by the crystalline structure of the particles. Since TiO_2 NPs can mainly occur in the anatase and rutile forms, a comparison of the effects of these two

crystalline structures was performed on PC12 [Wu et al., 2010] and SHSY5Y [Valdiglesias et al., 2013b] neuronal cells. Anatase TiO_2 NPs were more efficient than rutile to exert a concentration-dependent decrease of cell viability in PC12 cells; in a similar way, membrane damage evaluated via lactate dehydrogenase (LDH) release assay was more effective in the presence of anatase TiO_2 NPs. At high doses (200 μg/ml) ROS production was significantly higher in PC12 exposed to anatase TiO_2 NPs than to rutile, and similarly for the cellular levels of glutathione (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA). Furthermore, annexin V-FITC and PI staining showed that apoptotic and necrotic PC12 cells increased significantly with anatase titania, but flow cytometry demonstrated that both crystalline forms were able to arrest the cell cycle in G2/M phase. Western blot analysis confirmed that anatase TiO_2 NPs were more potent than rutile in activating apoptosis and cell cycle checkpoint proteins: the expression of JNK, p53, p21, GADD45, as well as bax and bcl-2 was higher following exposure to anatase NPs than to the rutile ones [Wu et al., 2010].

In contrast, crystalline form-related cytotoxic effects were not observed in the human neuroblastoma SHSY5Y cell line [Valdiglesias et al., 2013b]. MTT test and neutral red uptake showed that up to 24 h exposure to 0-150 μg/ml pure anatase and P25 (80:20 anatase: rutile) TiO_2 NPs did not impair the viability of SHSY5Y. Moreover, no morphological alterations were observed and electron microscopy studies showed that TiO_2 NPs were internalized in a time- and concentration-dependent manner, although pure anatase TiO_2 NPs were slightly more efficiently taken up than P25. Additionally, as previously observed in PC12 cells [Wu et al., 2010], pure anatase TiO_2 NPs altered the SHSY5Y cell cycle and induced apoptotic and necrotic events, while no effects were observed in cells treated with P25. Interestingly, both types of TiO_2 NPs enhanced the formation of micronuclei and by means of the comet assay primary but not oxidative DNA damage was observed [Valdiglesias et al., 2013].

Since TiO_2 NPs induced *in vitro* neurodegeneration, *in vivo* studies were of fundamental importance to better investigate the toxic potential of these NPs. To this end, Zhang and co-authors [2011] focused their attention on the neurological lesions induced in the brain of female CD-1 mice by intranasally instilled TiO_2 NPs of various size and surface coating. The results indicated that surface properties play a role on the neurodegenerative mechanisms of TiO_2 NPs: after 30 days exposure hydrophobic TiO_2 NPs accumulated significantly in the cerebral cortex and in the striatum, while microsized and nano-hydrophilic (silica-coated) titania did not differ from the unexposed animals. Moreover, hydrophilic TiO_2 NPs caused morphological changes of neurons in the cerebral cortex and norepinephrine (NE) levels significantly decreased in the hippocampus, cerebral cortex, cerebellum and striatum after hydrophilic TiO_2 NPs instillation, whereas hydrophobic titania did not alter the monoamine neurotransmitter levels in the sub-brain regions [Zhang et al., 2011]. Shrivastava and collaborators [2014] exposed Swiss albino mice for 21 days to a single oral dose of TiO_2 NPs and observed enhancement of dopamine (DA) and norepinephrine (NE) levels, They also detected oxidative stress conditions with increased ROS and reduced SOD production, suggesting that TiO_2 NPs are neurotoxic. Noteworthy, mutations of superoxide dismutase 1 (*SOD1*) cause familial forms of amyotrophic lateral sclerosis [Rosen et al., 1993]. Since there were evidences demonstrating that TiO_2 NPs are able to induce oxidative stress *in vivo*, Ze and co-authors [2013] examined the activation of the P38-nuclear factor-E2-related factor-2 (P38-Nrf-2) signaling pathway in CD-1 mice. Ninety consecutive days of intranasal administration caused the overproliferation of spongiocytes and the development of brain hemorrhages, as well as significant increase of mRNA expression of the oxidative stress-related cytokines p38, Nrf-2, c-Jun N-terminal kinase and NF-κB, which were accompanied by significantly increased levels of superoxide radical, hydrogen peroxide and malondialdehyde (MDA) [Ze et al., 2013]. Moreover, the same group [Ze et al., 2014a]

demonstrated that, after prolonged exposure (90 days), increased titanium content in the brain of CD-1 mice, overproliferation of the glial cells, tissue necrosis and significant alterations in the expression of genes associated with oxidative stress occurred. Additionally, subchronic peroral exposure to TiO_2 NPs caused severe pathological changes and spatial recognition impairment in CD-1 mice [Ze et al., 2014b]. An interesting study was recently performed in pregnant Wistar rats, which received intragastric TiO_2 NPs (100 mg/kg body weight) daily, from gestational day 2 to 21. Exposure to TiO_2-NPs significantly reduced cell proliferation in the hippocampus and impaired learning and memory in offspring [Mohammadipour et al., 2014].

The TiO_2 NPs assessed in the reported experiments, in a variety of models both *in vitro* and *in vivo*, differed according to the cristalline form (anatase or rutile), or surface characteristics. or dose used. Analogously the endpoints taken into account (cell viability, inflammation, oxidative stress markers, cytogenetic effects) differed greatly making difficult any comparison among set of experiments. However both forms (anatase and rutile) have been demonstrated able to induce neurotoxicity at various levels, with the anatase form in general more active than the corrispective rutile form. Taken together the above findings indicate that single neurons, microglial cells and the whole central nervous system, including brain regions, critical for the onset of neurodegenerative dideases, are potentially susceptible targets for TiO_2 NPs.

Silicon dioxide nanoparticles

Another type of metal oxide used in industry and proposed for drug and gene delivery are silicon dioxide nanoparticles (SiO_2 NPs), whose mechanism of toxicity is linked to the overproduction of ROS and to the activation of pro-inflammatory responses [Liu and Sun, 2010; Park and Park, 2009]. SiO_2 NPs, in fact, stimulated the secretion of the pro-

inflammatory cytokines TNF-α, IL-1 ß and IL-6 in freshly isolated rat microglial cells, but they were not able to stimulate the secretion of NO, MIP-1α and MCP-1 as well as NF-κB, which is known to be involved in the induction of inflammation-related genes and in microglia activation [Xue et al., 2012]. Using primary microglial cells from Sprague-Dawley pups, Choi and collaborators reported that SiO_2 NPs were intracellularly stored in phagocytic membrane-bound vesicles and, although the cell viability was not affected, silica induced a significant release of ROS and nitric oxidative species (NOS) accompanied by an increased *COX-2* gene expression [Choi et al., 2010]. Moreover, the exposure of PC12 neuronal cells to SiO_2 NPs were in a concentration-dependent decrease of cell viability, depletion of GSH and enhanced ROS production; silica nanoparticles were internalized as agglomerates in the cytoplasm and induced significant morphological changes, with cells that appeared small and fragmented and that had a reduced ability to outgrow neuritis, impeding thus the development of intercellular contacts and the formation of mature cells [Wang et al., 2011]. Morphological alterations and concentration-dependent cytotoxicity were observed in human SK-N-SH and mouse Neuro2a (N2a), two common neuroblastoma cell lines, exposed to low doses (10 µg/ml) of 15 nm SiO_2 NPs. By electron microscopy SK-N-SH cells were shown able to internalize silica particles, throughout the cytoplasm, while in N2a cultures they were found stored in vesicles [Yang et al., 2014]. In addition, the treatment of SK-N-SH and N2a cells exerted ROS release and a significant and dose-dependent apoptosis, as shown by nuclear and TUNEL staining. Interestingly, Yang and collaborators [2014] reported that SiO_2 NPs (mean particles size 12.1nm) increased the deposit of intracellular ß-amyloid peptide ($Aß_{1-42}$) with upregulation of the ß-amyloid precursor protein (APP) and downregulation of the amyloid-ß-degrading enzyme neprylysin, suggesting thus a possible risk of SiO_2 NPs of developing Alzheimer's disease. Indeed, according to the amyloid cascade hypothesis of AD, changes in APP and/or $Aβ_{1-42}$ homeostasis foster the assembly of Aβ peptides into progressively higher

order structures, from dimers all the way up to the insoluble plaques which finally deposit in the brain; these events are sufficient to initiate the pathological and clinical changes of the disease [Hardy and Selkoe, 2002].

The use of SiO_2 NPs in many applications and their potential employment for drug and gene delivery makes it essential to conduct further studies on possible biological effects, especially because i*n vitro* studies have shown that SiO_2 NPs are cytotoxic [Eom and Choi, 2009; Akhtar et al., 2010], induce oxidative stress [Napierska et al., 2009; Zhang et al., 2011; Ahmad et al., 2012; Ahamed, 2013] and inflammatory responses [Panas et al., 2013; Kusaka et al., 2014; Mendoza et al., 2014] in many cell types, including cells representative of the CNS [Choi et al., 2010;Wang et al., 2011; Xue et al., 2012; Yang et al., 2014]. Furthermore, SiO_2 NPs have been reported to induce inflammation [Lee et al., 2011; Morishige et al., 2012; Brown et al., 2014], as well as pulmonary [Choi et al., 2008; Zhao et al., 2014] and hepatic [Nishimori et al., 2009; Liu et al., 2012] toxicity *in vivo*.

Wu and collaborators [2011] exposed SD rats to intranasal instillation of SiO_2 NPs and observed significant brain accumulation of nanoparticles, oxidative stress (increased H_2O_2 and MDA and significant decrease in GSH), augmented TNF-α and IL-1ß levels which indicated that inflammation took place. Interestingly, when a deeper analysis of the content of silica in the different sub-brain regions was performed, it was possible to establish a ranking of SiO_2 NPs accumulation that corresponded to olfactory bulb > striatum > hippocampus > brain stem > cerebellum > frontal cortex [Wu et al., 2011]. In Balb/c mice polyethylene glycol-coated silica nanoparticles (PEG-SiO_2 NPs) crossed the BBB, showing size-dependent transport efficiency. At short exposure times (15 min) 50 nm and 100 nm PEG-SiO_2 NPs were poorly able to migrate through the BBB but their uptake significantly increased after 60 minutes. Smaller (25 nm) PEG-SiO_2 NPs, in contrast, were significantly taken up already after 15 min incubation and their migration across the BBB was further enhanced after 1 h

[Liu et al., 2014]. Nevertheless, SiO_2 NPs were also reported to significantly increase behavioral impairment in rats, in addition to BBB disruption and neuronal damage [Sharma et al., 2013a]. Similarly, silica nanoparticles disturbed the neural behavior of zebrafish *Danio rerio* in a size-dependent manner, as 15 nm SiO_2 NPs significantly changed the color preference of the animals and, compared to 50 nm particles, caused Parkinson's disease-like behavior [Li et al., 2014].

Overall data until now obtained either *in vitro* and *in vivo* indicate that SiO_2 NPs can pass through the BBB. The increased production of ROS and of pro-inflammatory response which seems a common feature of SiO_2 NPs can adversely affect different cell types. The major emerging finding is that transport efficiency of SiO_2 NPs across BBB was found to be size-dependent, with increased particle size resulting in decreased efficiency. A potential concern with small sized silica nanoparticles neurotoxicity in biomedical applications and occupational exposure in large-scale production seems thus quite justified.

Zinc oxide nanoparticles

Zinc oxide nanoparticles (ZnO NPs) are of industrial interest because of their exceptional optoelectronic, piezoelectric, ferromagnetic and optical properties. Moreover, ZnO NPs have been applied in sunscreens, biosensors, food additives and pigments [Ji and Ye, 2008]. Due to their antiseptic activity, they have also potential applications against bacteria-related infections and diseases. Although some data on the toxic potential of ZnO NPs are available in the current literature, yet little is known about their neurotoxic effects. Deng and co-authors [2009] showed that in neural stem cells (NSC) ZnO NPs, whose nominal mean size diameter ranged between 10 and 200 nm, impaired the cell viability in a dose-dependent manner; size, in contrast, did not play a role in inducing toxic effects as the comparison of the differently sized nanoparticles did not result in any significant difference in terms of cell vitality. By

electron microscopy analysis and nuclear staining ZnO NPs were observed to induce apoptosis in NSC cells [Deng et al., 2009]. Nevertheless, the authors speculated that the cytotoxicity and the apoptosis induced by ZnO NPs in NSC cells might result from the zinc ions dissolved either in solution or intracellularly, and this hypothesis was supported also by the fact that the internalized ZnO NPs were not detectable by electron microscopy [Deng et al., 2009]. ZnO NPs neurotoxicity was further evaluated in RSC96 rat Schwann cells comparing four different hierarchical structures: monodispersed spherical ZnO NPs of 35 nm size, hollow ZnO microspheres (2.7 μm), prism- (ca. 2.5 - 6.0 μm in diameter and ca. 18 - 60 μm in length) and flower-like (500 - 600 nm in diameter and several microns in length) structures [Yin et al., 2012]. Results demonstrated that prism- and flower-like ZnO NPs did not induce cytotoxic effects after 12 h exposure while significant impairment of the viability of RSC96 cells was observed at 48 h. Similarly, spherical monodispersed ZnO NPs and zinc microspheres exerted concentration- and time-dependent cytotoxicity. Moreover, they significantly enhanced apoptotic events and G2/M cell cycle arrest was observed when RSC96 cells were exposed for 12 h to 80 μg/mL ZnO nanoparticles and microspheres [Yin et al., 2012]. Interestingly, the analysis of the levels of zinc ions performed in culture media at increasing time points revealed that the observed time-related ion levels enhancement was the result of a leaching process occurring during the incubation period, which suggested that the cytotoxic effects observed in RSC96 rat Schwann cells were also due to the ionic fraction in the culture environment and not exclusively to the nanoparticulated fraction [Yin et al., 2012]. By confocal microscopy Kao and co-authors [2012] observed that ZnO NPs were internalized in membrane-bound vesicles in PC12 neuronal cells and caused the reduction of cell viability and mitochondrial impairment. In the human neuroblastoma SHSY5Y cell line an extensive study on Zn NPs was performed, testing several concentrations and exposure times, and employing a battery of cytotoxicity and genotoxicity assays. The internalization of the Zn

NPs was assessed by flow cytometry but it was not possible to demonstrate that ZnO NPs enter the neuronal cells. However a wide range of cytotoxic effects were induced , including apoptosis and cell cycle alterations, as well as genotoxic effects (micronuclei , H2AX phosphorylation and primary and oxidative DNA damage), in a dose- and time-dependent manner [Valdiglesias et al., 2013a].

Turning to the *in vivo* studies, Kao et al. [2012] showed that ZnO NPs intranasally administered to Sprague-Dawley rats (6 h exposure) translocated into the olfactory bulb and in the synaptosomes, as clearly shown by electron microscopy micrographs. The translocation of ZnO NPs across the BBB and into the CNS was then further confirmed by Cho and collaborators [2013]: after 13 weeks of repeated oral administration, enhanced ZnO NPs levels were measured in rats brain compared to the untreated group, although the uptake was not dose-related. Additionally, ZnO NPs were reported to disrupt the spatial memory and to significantly impair the synaptic responses of Swiss male mice with depressive-like behavior [Xie et al., 2012].

Even if the Zn NPs are able to induce neurotoxic effects in many experimental systems, often *in vitro* no cellular uptake was observed, differently sized nanoparticles did not induce significant difference in cytotoxicity, whilst a time-dependent increase of Zn^{2+} concentration in the culture media was sometimes found. This can be associated with the decomposition of ZnO hierarchical architecture and the subsequent release of ions, as already reported. However the question whether the increase of intracellular ions is due to the NPs being taken up by cells or to NPs dissolution in medium still remains not yet fully solved [Vandebriel and De Jong, 2012].

Copper oxide nanoparticles

Copper, an essential trace element vital for the life and the development of organisms, is known to be involved in neurodegenerative disorders such as Menkes [Kodama et al., 201], Wilson's [Lorincz, 2010], Alzheimer's, Huntington's and Parkinson's disease [Desai and Kaler, 2008; Rivera-Mancia et al., 2010; Greenough et al., 2013; Montes et al., 2014], acting through the induction of oxidative stress [Halliwell and Gutteridge, 1984] and the activation of microglial cells and inflammation [Zhang et al., 2011b]. Although it is important to understand how nanosized copper oxide (CuO NPs) can induce neurotoxicity, to date few investigations were performed. Wang and collaborators [2009] investigated the expression changes of genes associated with the dopaminergic system and their correlation with dopamine depletion in PC12 cells. Treatment with CuO NPs significantly reduced the content of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in PC12 cells, and induced a downregulation in the expression of the redox-status gene glutathione peroxidase 1 (*Gpx1*) and an upregulation of thioredoxin reductase 1 gene (*Txnrd1*). In addition, CuO NPs upregulated the expression of the monoamine oxidase A (*Maoa*), which is related to the dopamine metabolism, and of the alpha-synuclein gene (*Snca*) associated with the pathogenesis of neurodegeneration in Parkinson's disease [Wang et al., 2009]. Indeed, PD results from loss of neuromelanin containing dopaminergic neurons in the *substantia nigra* (SN) with the presence of eosinophilic, intracytoplasmic inclusions termed as Lewy bodies and containing aggregates of α-synuclein as well as other substances. Furthermore, human *SNCA* mutations cause autosomal dominant PD, and *SNCA* polymorphisms or epigenetic changes of *SNCA* gene expression are believed to contribute to the sporadic forms [Thomas and Beal, 2011]. An additional study on rat brain microvessel endothelial rBMECs cells showed that low concentrations of 40 and 60 nm CuO NPs increased the cellular proliferation while 50 μg/ml Cu-NPs were cytotoxic, and the extracellular concentration of the proinflammatory mediators Prostaglandin E2 (PGE2), TNF-

α and IL-1β were significantly increased [Trickler et al., 2012]. Moreover, Trickler and co-authors [2012] reported that the enhanced permeability of rBMEC upon exposure to CuO NPs suggests that the NPs can be neurotoxic and damage the blood-brain barrier even at low doses.

To better investigate their involvement in the etiology of neurodegenerative disorders, CuO NPs were studied *in vivo*. Cu NPs of approximately 50 - 60 nm mean diameter were able to induce brain dysfunction in rats, which, after 7 days exposure, exhibited mild cognitive impairment and cellular alterations in the brain [Sharma and Sharma, 2007]. Additionally, intraperitoneal, intravenous, intracarotid or intracerebroventricular administration of Cu-NPs significantly altered BBB function in several regions of the brain and spinal cord at 24 h after administration, and marked decrease in local cerebral blood flow (CBF) and severe brain edema were observed in brain areas associated with BBB leakage [Sharma et al., 2009]. Moreover, Sharma and co-authors [2009] observed that the injured brain areas exhibited neuronal cell damage, glial cell activation, heat shock protein upregulation and loss of myelinated fibers, and these changes were more evident in mice compared to rats. Furthermore, by means of Evans blue leakage, it was possible to show that brain edema formation took place in rats after intravenous, intraperitoneal and intracerebral administration of Cu NPs, and the mostly damaged areas were the ventral surface of brain and the proximal frontal cortex, whereas the dorsal surfaces of cerebellum showed mild to moderate damage [Sharma et al., 2010]. CuO NPs treatment led to toxic effects also on the cognitive functions of Wistar rats highlighted by poor performance of animals in behavioral tests. The occurrence of an imbalance of the oxidation–antioxidation homeostasis and of neuronal damages in the hippocampus, suggested the induction of oxidative damage and neuronal apoptosis [An et al., 2012]. Despite the scarcity of available studies, mainly carried out in a few experimental

centers, Cu NPs seem neurotoxic both *in vitro* and *in vivo*. Of concern the finding that nano-CuO can induce brain dysfunctions and affect the abilities of learning and memory in rodents.

Silver nanoparticles

Due to its bactericidal properties and as imaging contrast agent, silver nanoparticles (Ag NPs) are promising tools for biomedical applications. It is well known that the CNS is sensitive to silver [Carpenter, 2001], and that Ag can be retained in the CNS for long periods of time [Panyala et al., 2008] and induce neuronal degeneration and BBB malfunction.

The ability of Ag NPs to translocate into the brain by crossing the BBB was reported in 2010 by Tang and co-authors. Using an *in vitro* co-culture model composed of rat brain microvessel endothelial cells and astrocytes, Ag NPs were observed to pass the BBB by transcytosis and accumulate in endothelial cells, as shown by electron microscopy [Tang et al., 2010]. In freshly isolated rat brain microvessel endothelial rBMEC cells 25 - 40 - 80 nm Ag NPs accumulated in a dose- and size-dependent manner, and induced an impairment of the cell viability only at high concentrations (25 - 50 $\mu g/cm^3$); furthermore, size-related morphological changes and formation of perforations in the monolayer were observed in rBMECs [Trickler et al., 2010]. In a follow-up study using confluent porcine brain microvessel endothelial cells, Trickler et al. [2014] observed that 25 - 40 - 80 nm Ag NPs induced pro-inflammatory responses by enhancing the extracellular levels of PGE2, TNF-α and IL1ß, in addition to causing BBB leakage and significantly higher permeability.

Loss of cytoskeleton structure with degradation of beta-tubulin and F-actin was observed in primary rat cortical cells exposed to Ag NPs (20 nm mean size diameter), and phase contrast images showed that Ag NPs inhibited neuronal extension, neuritic overlap, and impaired the viability of the rat cortical cells [Xu et al., 2013]. Size- and time-dependent TNF-α and IL-1ß secretion were detected, while PGE2 was not released in the presence of 40 and 80 nm Ag

NPs. In addition, Ag NPs selectively affected the permeability of rBMECs: small Ag NPs (25 nm) induced an increased permeability of fluorescein across rBMECs, whilst 40 nm Ag NPs only slightly damaged the integrity of the barrier and 80 nm particles did not exert any effect [Trickler et al., 2010]. In PC12 cells the expression changes in genes associated with the dopaminergic system were analyzed following exposure to 15 nm Ag NPs: *Gpx1* was the only upregulated gene whereas genes related to dopamine metabolism (*Th*, *Maoa*, and *Comt*) and the genes *Gpr37*, *Snca* and *Park2*, which are associated with the pathogenesis of neurodegeneration in Parkinson's disease, did not show any significant variation [Wang et al., 2009]. In human-derived SHSY5Y neuroblastoma and D384 astrocytoma cells the exposure to 20 nm Ag NPs revealed that at short exposure times (4 - 48 h) Ag NPs induced dose- and time-dependent impairment of the mitochondrial metabolism and cell membrane damage, and similarly at longer exposures (10 days) SHSY5Y and D384 cells treated with increasing concentrations of Ag NPs showed dose-dependent reduction in colony forming efficiency. Since Ag NPs are known to release silver ions in solution, a comparison with $AgNO_3$ was performed: the cytotoxicity, both at short (4-48 h) and at long (10 days) time points, was more severe when SHSY5Y and D384 cells were incubated in the presence of $AgNO_3$ compared to Ag NPs [Coccini et al., 2014]. Ziemínska and co-authors [2014] investigated the role of Ag NPs in the induction of excitotoxicity, a pathological process by which nerve cells are damaged and killed by excessive stimulation of neurotransmitters such as glutamate, which is linked to alterations of intracellular calcium levels and deregulation of intracellular calcium signaling pathways, and determines ROS production, mitochondrial dysfunction and, ultimately, cell death. To this end, primary cultures of rat cerebellar granule cells exposed to Ag NPs activated the glutamatergic N-methyl-d-aspartate receptors (NMDAR) and induced calcium imbalance, changes in mitochondrial membrane potential and significant ROS production, thus suggesting that Ag NPs have neurotoxic potential [Ziemínska et al., 2014].

Interestingly, Ziemínska and co-authors [2014] showed that the toxic effects exerted by Ag NPs were attenuated in the presence of MK-801, a non-competitive inhibitor of NMDAR.

In vivo studies have demonstrated that Ag NPs accumulate in liver [Kim et al., 2008; Kim et al., 2010] and lungs [Sung et al., 2009; Song et al., 2013], but Ag NPs are able to translocate also into the CNS. In fact, 25 nm Ag NPs were detected by autometallography in the olfactory bulb and in the lateral brain ventricles of C57BL/6J mice [Genter et al., 2012], and mass spectrometry showed size-related internalization of Ag NPs in young ICR mice, with 22 - 71 nm particles distributed into the brain whereas 300 nm Ag NPs were not detected in the tissue after 14 days oral administration [Park et al., 2010].

50 - 60 nm Ag NPs administered into rats and mice systemic circulation or brain ventricular spaces showed severe BBB leakage, formation of brain edema and decrease in local cerebral blood flow, as well as glial activation and loss of myelinated fibers [Sharma et al., 2009]. Size-dependent BBB breakdown, NOS upregulation, neuronal damage and glial fibrillary acidic protein upregulation were observed in inbred male Sprague-Dawley rats: small Ag NPs (20-30 nm) induced more severe damages in young (9 - 10 weeks old) and old (30 - 35 weeks old) rats compared to mid-age (18 - 20 weeks) animals, and the effect significantly reduced in the presence of 50 - 60 nm and 130 - 150 nm Ag NPs [Sharma et al., 2013b]. The evidence that very young and old rats showed the most severe neurodegeneration induced Sharma et al. [2013b] to suggest that children and elderly might be more susceptible to Ag NPs-induced brain damage.

An altered expression of mouse oxidative stress and antioxidant genes was observed in different regions of C57BL/6N mice exposed by injection to Ag NPs, and suggested thus that 25 nm Ag NPs were able to induce oxidative stress and oxidative DNA damage and could be involved in the development of neurotoxicity and in the pathogenesis of neurodegenerative disorders [Rahman et al., 2009].

Silver NPs in solution are known to release Ag-ions which induce significant toxicity as already reported *in vitro* [Singh and Ramarao, 2010; Hamilton et al., 2014] and *in vivo* [Radiecki et al., 2011; Yang et al., 2012; Visnapuu et al., 2013]. Is therefore pivotal to understand if the neurotoxic potential of Ag NPs is due to the nanosized fraction or to the silver ions, which leached in solution. When the neurotoxicity induced by Ag NPs and Ag-ions was compared interesting results were reported. Hadrup and co-authors [2012] observed that in female Wistar rats 28 days of 14 nm Ag NPs and silver ions oral administration induced an increase in dopamine levels; in contrast, 5-hydroxytryptamine (5-HT) was enhanced exclusively following exposure to Ag NPs whereas noradrenaline was upregulated only following exposure to silver ions. Similar effects were also reported in Wistar Hannover Galas rats: animals were exposed by repeated oral administration for 28 days and the analysis of homogenates revealed that both nanosized and ionic silver accumulated in the brain with comparable distribution [Loeschner et al., 2011]. Moreover, silver was detected in brain of 28 days exposed Sprague-Dawley rats and, while it was eliminated from liver and spleen, a biopersistance of silver was observed in the brain [van der Zande, 2012]. Interestingly, Ag NPs were detected also in $AgNO_3$ exposed animals, supporting the evidence that nanoparticles can originate from Ag-ions *in vivo* and explaining thus the fact that Ag NPs and Ag salts exhibited similar distribution and clearance [van der Zande, 2012]. Additionally, Dziendzikowska and co-authors showed that at short and mid-term exposures (24 h and 7 days) the brain was the organ with the lowest concentration of silver, while a significant increase was measured after 28 days Ag NPs intravenous administration in Wistar rats, hence demonstrating that Ag NPs displayed time-dependent deposition in the brain [Dziendzikowska et al., 2012].

Therefore, based on findings in animals, Ag NPs seem able to distribute and accumulate during the time in many organs, including the brain. Increasing evidence suggests that Ag

NPs-induced neurotoxic effects may occur via silver ions that are released from the particle surface, as happens for other metal oxide NPs. A size-dependent effect was found *in vitro* and *in vivo* (small-sized AgNPs were more active). Moreover a higher susceptibility in the age groups most vulnerable (in the younger or older animals) has been highlighted *in vivo*.

Magnetic nanoparticles

The use of magnetic nanoparticles (MNPs) has become an area of increasing interest in biomedicine. MNPs have unique features such as their reaction to a magnetic force which can be utilized in drug targeting and cell sorting. Moreover, MNPs have gained interest because of their potential use as contrast agents for magnetic resonance imaging (MRI) and as heating mediators for hyperthermia and cancer therapy [Ito et al., 2005]. However, their potential neurotoxicity has been poorly investigated. Au and colleagues [2007] exposed astrocytes from the cerebral cortices of newborn Sprague-Dawley rats to 10 μg/ml iron oxide superparamagnetic particles (Fe_3O_4 or γ-Fe_2O_3) and reported that, although the cell membrane integrity was not affected, viability and cell adhesion were significantly impaired. Anionic magnetic nanoparticles (AMNPs) were shown to severely affect the viability of PC12 neuronal cells that underwent morphological alterations such as reduced microtubules protrusion, reduced formation of actin microfilaments within the soma and loss of organized actin in the cellular body, inducing thus PC12 cells to assume a spheroidal shape [Pisanic et al., 2007]. In rat primary microglia cells, while NO and MCP-1 production and NF-κB binding activity were comparable to the untreated control cells, Fe_3O_4 NPs were found to exert a mild increase in the expression of the pro-inflammatory cytokines TNF-α, IL-1ß and IL-6, indicating that other inflammatory signaling pathways may act independently of NF-κB activation [Xue et al., 2012]. However, since following incubation with the supernatant from Fe_3O_4 NPs-treated microglia significant cytotoxicity in PC12 cells was not observed, Xue and

collaborators [2012] concluded that the pro-inflammatory activity exerted by iron NPs was not sufficient to cause neurotoxicity and neurodegeneration.

The interaction of iron oxide nanoparticles (IONPs) with astrocytes has been extensively investigated during the past years, and Hohnholt and co-authors [2013] summarized in a review the main results. Astrocytes play an important role in the CNS because they regulate the metal homeostasis in the brain [Tiffany-Castiglioni and Qian, 2001; Dringen et al., 2007; Jones, 2012] and protect the brain from metal toxicity and oxidative stress [Hirrlinger and Dringen, 2010; Macco et al., 2013]. Time- [Geppert et al., 2011], concentration- [Geppert et al., 2011; Hohnholt et al., 2012; Lamkowsky ey al., 2012] and temperature-dependent [Geppert et al., 2009; Lamkowsky ey al., 2012] accumulation of IONPs was shown in cultured murine astrocytes, and IONPs were observed to stably remain in the cells without inducing cytotoxicity [Lamkowsky et al., 2012; Yiu et al., 2012]. Furthermore, the resistance of astrocytes to IONPs cytotoxic effects was suggested to depend on the fact that particles are stored in intracellular vesicles and are not freely dispersed in the cytosol [Hohnholt et al., 2010; Geppert et al., 2011; Geppert et al., 2012], but also the sequestration of IONPs-leached ions by proteins (such as ferritin) has a protective effect to the cells [Geppert et al., 2012].

The *in vivo* uptake and the potential adverse effects of IONPs in brain have been reviewed by Petters et al. [2014]. They highlighted that although IONPs are able to cross the BBB [Kim et al., 2006; Kwon et al., 2008; Wang et al., 2010; Wang et al., 2011] and to induce the activation and the proliferation of microglial cells in the olfactory bulb, still unclear is under which conditions IONPs migration occurs and which regions of the brain are targeted by the particles.

Wu and co-authors [2013] demonstrated that after 7 days intranasal instillation 30 nm Fe_3O_4 NPs differentially deposited in the brain of SD rats: olfactory bulb, striatum and hippocampus were the regions were IONPs mostly accumulated compared to brain stem, cerebellum, and

frontal cortex, and the clearance of Fe_3O_4 NPs from the brain was slow, as striatum and hippocampus still retained more than half of IONPs up to 14 days post-instillation. In addition, Fe_3O_4 NPs upregulated the oxidative damage markers GSH, H_2O_2, SOD and MDA in the striatum, emphasizing thus the neurotoxic potential of magnetic NPs [Wu et al., 2013]. Intraneural injection of maghemite (Fe_2O_3) and magnetite (Fe_3O_4) NPs coated with dimercaptosuccinic acid (DMSA) and PEG into the sciatic nerve of Sprague-Dawley rats resulted in an accumulation of macrophages, monocytes and lymphocytes at the injection sites, together with increased levels of ERK, caspase-3, IL1ß, matrix metallopeptidase 9 (MMP-9) and heme oxygenase 1 (HO-1), confirming thus that IONPs are able to induce oxidative stress, inflammation and apoptotic events [Kim et al., 2013]. The accumulation of IONPs and the induction of apoptosis was demonstrated also in the brain of zebrafish, where increased levels of ferric iron and enhanced mRNA levels of caspase-8 (*casp8*), caspase-9 (*casp9*) and transcriptional factor AP-1 *jun* were detected [de Oliveira et al., 2014].

It is established that magnetic NPs are able to pass through the BBB and enter the CNS, and ROS production is one of the main mechanisms by which they induce toxicity.

A very recent review taking into account a wide range of toxic effects induced by IONPs, including neurotoxicity, indicate that surface coatings and particle size seem to be crucial for the observed IONPs-induced effects [Valdiglesias et al., 2014].

Carbon nanotubes

Carbon nanotubes (CNT) are a class of nanomaterials whose structure is exclusively composed of carbon atoms. CNT, which display high electronic and thermal conductivity, can occur in two main types: single-walled carbon nanotubes (SWCNT), consisting of a single sheet of carbon benzene rings rolled up into a tubular structure; and multi-walled carbon nanotubes (MWCNT) consisting of multiple concentric layers of carbon sheets. The use of

CNT in biomedicine has grown favored by their improved aqueous dispersibility, as some functionalized forms are also water dispersible (e.g. carboxylated MWCNT [Ntim et al., 2012]).

Nevertheless, the comprehension of the interactions between CNT and the CNS, both *in vitro* and *in vivo*, is still limited and their potential short and long-term neurotoxicity is still unclear. SWCNT were reported to induce time- and dose-dependent impairment of the cell viability and membrane damage in PC12 neuronal cells, as well as decrease in mitochondrial membrane potential; moreover, SWCNT induced the formation of ROS, enhanced the levels of lipid peroxide and decreased SOD, glutathione peroxidase, catalase and GSH in a time- and dose-dependent manner [Wang et al., 2011b; Wang et al., 2012]. Additionally, condensed chromatin, fragmented nuclei and a block of the cell cycle in G2/M phase characterized PC12 cells, indicating that apoptotic events were enhanced by the exposure to SWCNT [Wang et al., 2011b], but prevented by a pre-incubation with vitamin E [Wang et al., 2012]. CNT have been proposed as substrates for neuron growth and in some experiments have shown cell culture toxicity. In order to reduce their toxicity there is the possibility to modify SWCNT surface to make the contact between cells and nanotubes less close. This can be achieved by enveloping the CNT molecule with surfactants or polymers, such as polypyrrole (PPy). While the viability of co-cultures of primary embryonic rat hippocampal neurons and glial cells was impaired in SWCNT substrates, for the PPy-SWCNT-substrates, the toxicity was lower, [Hernández-Ferrer et al., 2014]. Even the different degrees of agglomeration of SWCNT can influence neurotoxicity. In chicken embryos primary mixed neuronal and glial cells from spinal cord or dorsal root ganglia agglomerated SWCNT significantly decreased the DNA content and reduced the amount of glial cells, whereas bundle SWCNT had only mild effects [Belyanskaya et al., 2009].

CNT can retain metal impurities and to test the role of these impurities in inducing neurotoxicity, MWCNT with increasing concentration of iron (Fe-MWCNT) were investigated in PC12 cells. The results showed that highly impure Fe-MWCNT impaired the cell viability, increased cytoskeleton disruption, diminished the ability to form mature neurites and influenced the neuronal dopaminergic phenotype in NGF-treated rat pheochromocytoma cell line PC12 cells [Meng et al., 2013].

Upon injection, in C57/Bl6 mice MWCNT functionalized with amino groups were internalized in microglia, astrocytes and neurons, and stimulated a transient induction of the pro-inflammatory cytokines TNF-α, IL-1ß, IL-6 and IL-10 at early time points (< 16 h) [Bardi et al., 2013]. Moreover, the oxidation of nanotubes induced significant levels of glial fibrillary acidic protein (GFAP) and CD11b in the areas of injection, indicating that astrocytes and microglia were locally activated by MWCNT [Bardi et al., 2013]. In Wistar rats, gadolinium (Gd-SWCNT) and iron (Fe-SWCNT) single-walled carbon nanotubes were found to accumulate as aggregates in the cerebral cortex of the brain without altering the tissue architecture nor inducing inflammation [Avti et al., 2013]. The ability of MWCNT to cross the BBB was further demonstrated using C57BL/6J mice, where after 12 days inhalation monodispersed MWCNT accumulated in the brain in a time-related manner [Mercer et al., 2013]. Moreover, 50 nm MWCNT were reported to induce brain deformity via an indirect mechanism: MWCNT, in fact, crossed the blood-placental barrier of $p53^{+/-}$ pregnant C57BL/6J mice and induced crown-shaped tissue malformations of the brain, but they did not migrate through the BBB as demonstrated by the fact that CNT did not accumulate in fetal brains [Huang et al., 2014]. Although CNT have shown much promise in many applicative fields, including biomedicine and neurobiology, a limited number of studies is available on their neurotoxicity both *in vitro* and *in vivo*. Toxicological studies performed *in vivo* have often evaluated the specificity of many tissues and organs, but the nervous system was almost

never included. Of current interest is the research of safer groups for CNT functionalization, which however should be tested *in vivo* as possible, bearing in mind also their possible accumulation in the medium-long term.

Use of NPs in diagnosis and treatment of neurodegenerative diseases

Although many evidences have proven that many NPs can induce toxic mechanisms and cause cytotoxicity, genotoxicity, inflammation and oxidative stress *in vitro* and *in vivo*, the design, the development and the synthesis of engineered NPs for biomedical applications is a very dynamic field. Screening, diagnosis and treatment of diseases are the expected applications of engineered NPs, but the use of NPs in the diagnosis and in the treatment of neurodegenerative diseases implies that NPs migrate through the BBB, which is known to be tightly regulated and presents a very low rate of transcytotic vesicles and acts as a restrictive paracellular diffusion barrier, protecting thus the neural tissue from toxins and toxicants [Wolburg and Lippoldt, 2002]. The ability of NPs specifically engineered for the diagnosis and the treatment of neurodegenerative diseases to cross the BBB and enter the CNS depends on the physico-chemical properties of NPs, on their composition and on their functionalization [Kreuter, 2004]. The use of lipidic (liposomes, nanoemulsions and nanocapsules), polymeric (micelles, dendrimers, nanogels and polymeric particles) and inorganic (quantum dots and iron oxide) NPs for CNS targeting, diagnostic and therapeutic purposes was recently reviewed [Modi et al., 2009; Modi et al., 2010; Garbayo et al., 2013; Rocha, 2013; Cupaioli et al., 2014] and a number of suitable and promising nanocarriers has been identified (Figure 1). Therefore, we only mention some of several recent examples highlighting the potential application of nanotechnology in diagnosis and treatment of neurodegenerative diseases.

Due to their lipophilic nature which allows them to cross the BBB by passive diffusion [Brasnjevic et al., 2009; Redzic et al., 2011], in the recent past nanolipidic structures have been coupled to drugs and used for the treatment of AD and PD. For instance, the encapsulation of rivastigmine, an inhibitor of acetylcholinesterase (AChE) and butyrylcholinesterase, into liposomes showed potential therapeutic effects in an aluminium chloride-induced Alzheimer's rat model. The administration of rivastigmine-loaded liposomes to $AlCl_3$-treated rats normalized *BACE1* (the gene coding for the β-secretase which cleaves APP producing Aβ peptides), *AChE* (coding for the enzyme acetylcholinesterase which inactivates the neurotransmitter acetylcholine by catalyzing its hydrolysis to choline and acetic acid), and *IL1B* gene (coding for a member of the interleukin 1 cytokine family, mediator of the inflammatory response) expression. In contrast the co-treatment with rivastigmine solution caused a significant down-regulation of these genes [Ismail et al., 2013]. To overcome the poor bioavailability and solubility of curcumin, a pleiotropic molecule with anti-inflammatory and anti-oxidant activity, nanoliposomes loaded or functionalized with curcumin have been designed. *In vitro*, curcumin liposomes showed very high affinity [Mourtas et al., 2011] for $Aß_{1-42}$ and inhibited its aggregation [Taylor et al., 2011]. Moreover, Lazar and co-authors [2013] demonstrated that mono-dispersed curcumin-conjugated nanoliposomes are biocompatible and bind selectively to $Aß_{1-42}$ deposits. *In vitro* these nanolipidic structures were not toxic to HEK human embryonic kidney and human neuroblastoma SHSY5Y cells and down-regulated the secretion of the amyloid peptide. E*x vivo* they were reported to strongly bind to $Aß_{1-42}$ deposits in post-mortem brain tissue of AD patients, and *in vivo* they specifically stained $Aß_{1-42}$ in APPxPS1 mice, a transgenic animal model of AD expressing mutant APP and presenilin 1, both involved in $Aß_{1-42}$ production. Furthermore, anti-apoptotic and neurotrophic effects were demonstrated in a rat model of PD by using liposomal-formulated curcumin targeting histone deacetylase [Chiu et al., 2013].

Polymeric nanoparticles are stable NPs characterized by high drug loading capacity, because they protect against degradation the loaded drug which can specifically be delivered to the CNS [Behan et al., 2001]. Poly(n-butylcyanoacrylate) nanoparticles coated with 1% polysorbate 80 were shown to be more efficient in delivering rivastigmine into the brain of male Wistar rats than the free drug [Wilson et al., 2008],. Orally administered Tween80-coated polylactide-co-glycolide (PLGA) NPs containing estradiol resulted in significantly higher brain estradiol levels after 24h as compared to uncoated ones in an ovariectomized rat model of AD [Mittal et al., 2011]. Moreover, the conjugation of polyethylene glycol-polylactide-polyglycolide nanoparticles (PEG-PLGA NPs) with lactoferrin was shown to facilitate NPs internalization in brain endothelial cells *in vitro* and to enhance NPs accumulation in an *in vivo* mice model of PD [Hu et al., 2011]. Also *in vivo*, in a PD rat model, repeated injections of lactoferrin-modified PEG-PLGA NPs loading the human glial cell line-derived neurotrophic factor gene *hGDNF* improved locomotor activity, reduced dopaminergic neuronal loss and enhanced monoamine neurotransmitter levels [Huang et al., 2009].

Among the inorganic NPs, IONPs are widely used in therapeutic and diagnostic applications. IONPs, which depending on their size can be classified in superparamagnetic iron oxide (SPIONs, 60 - 150 nm in diameter) and ultrasmall superparamagnetic iron oxide (USPIONs, 10 - 40 nm), have a Fe-core and can be coupled to organic materials and drugs. USPIONs chemically coupled with $A\beta_{1-42}$ were successfully proposed as poorly invasive diagnostic tools for the *in vivo* detection of amyloid plaques by magnetic resonance microimaging [Yang et al., 2011]. Due to their increased relaxivity during MRI and *in vitro* binding to ß-amiloid aggregates SPIONs were proposed as ultra-sensitive nanoprobes for AD imaging [Zhou et al., 2014]. Several examples of nanovehicles to carry monoclonal antibodies agains $A\beta_{1-42}$ into the brain have been recently developed as theranostic tools, some of them also being able to

carry conjugated drugs to the Aß deposits [Poduslo et al., 2011; Agyare et al., 2014; Jaruszewski et al., 2014]. Similarly, quantum dots proved to be highly efficient in a microarray to detect the potential AD biomarker apolipoprotein-E [Morales-Narváez et al., 2012], and SWCNT were reported to be able to deliver acethylcholine in the brain of Kunming mice [Yang et al., 2010].

The treatment of neurodegenerative diseases is a major challenge, both because for most of them suitable drugs have not yet been identified, and because of the limited access of bulky molecules, such as peptides and proteins, through the BBB. To overcome the latter problem, it is proposed a growing number of nanotechnology-based delivery systems that are likely to become a innovative modality for either the diagnosis and treatment of neurodegenerative disorders. Many approaches are being tested with promising results, which go beyond the limited number of examples shown here. However, studies are still at the beginning. Among the important issues to be taken into consideration there are certainly the affinity between the drug and the nanobiocarrier (whereas there are drugs still to be identified) and the subsequent removal of the nanodevices from the brain.

Concluding Remarks

The ever-growing use of nanomaterials in several human settings, including their medical applications, raises the question of safety of humans employed in the manufacturing of those materials, as well as that of consumers of NPs-containing products. In this regard, several authors have suggested that NPs can be toxic to various human organs and systems, including the CNS, thereby potentially contributing to the onset of human complex pathologies such as neurodegenerative diseases. On the other hand, the global aging of the population in both developed and developing countries, coupled with the fact that there is actually no available

treatment to halt the progression of most neurodegenerative conditions, lead to projections that those disorders will soon represent a serious health and socio-economic concern, reinforcing the demand for early diagnostic tools and novel therapeutic approaches. Nanotechnology has therefore the possibility to impact the two sides of the same coin as it could contribute to the onset and progression of several human pathologies, due to the toxic properties of many nanosized particles, but, taking advantage of the physico-chemical properties of NPs, can also be of extreme importance for the delivery of either diagnostic or therapeutic compounds to the site of disease lesion that might be difficult to reach with other methodologies. In this review we presented an overview on studies to assess the impact on the nervous system by some the most widespread nanoparticles. For *in vitro* approaches various cell models, representing the main cell types composing the brain: neurons and neuroglial cells (oligodendrocytes, astrocytes, microglia) or Schwann cells, responsible for the myelination of axons, or endothelial cells, which compose the BBB, have been used for the assessment of neurotoxicity and of other related effects. The main cell lines employed were non-neuronal tumor cell lines such as pheochromocytoma (PC12) cells and neuronal tumor cell lines represented for instance by the human neuroblastoma SH-SY5Y. In other cases primary cells obtained from mouse brain were used (mainly glial). In *in vivo* studies many of the most known mammalian models (rat and mouse) have been employed, as well as the invertebrate zebrafish (*Danio rerio*), including a transgenerational model. Even in the *in vivo* experiments different routes and times of administration have been comprised. Quite all the reported studies clearly demonstrate the potential for several NPs to reach the CNS and induce toxic effects and pathways, such as oxidative stress, genotoxicity, apoptosis, inflammation and microglia activation, which are common to most of the human neurodegenerative disorders, suggesting that many of them are potentially able to contribute to neurodegeneration. It is however hard to compare the studies, both for the various cell

models used, both for the different NPs employed, which can differ for the same element, in relation to chemical and physical properties. Sometimes the dimension can influence the behaviour of the particle, more often microsized NPs are less active than nanosized ones. For instance micron-sized TiO2 did not exhibit any toxic response in PC12 cells, in contrast with nanosized TiO2 [Wu et al., 2010]. Also smaller Ag NPs produced stronger inflammatory responses correlated with increased cerebral microvascular permeability, in primary rat brain microvessel endothelial cells, whereas the effects produced by the larger Ag-NPs were much less intense [Trickler et al., 2010]. Likewise, *in vivo* the influence of the size was shown: in different animal models (mouse and rat) nano-sized AgNPs internalize better if smaller and may cause BBB damage, organ toxicity and inflammatory response, in a size-dependent manner [Park et al., 2010; Dziendzikowska et al., 2012; Sharma et al., 2013b]. Conversely in some cases the size does not influence the toxic effects, such as TiO_2NPs when tested in human astrocytoman cells [Lai et al., 2008] or ZnO NPs, when tested in NSC mouse neural stem cells [Deng et al., 2009]. Even the shape can exert an influence on neurotoxicity: ZnO nanoparticles and microspheres displayed significant cytotoxic effects on RSC96 rat Schwann cells in dose- and time-dependent manners, while no or low cytotoxic effect was observed when the cells were treated with the prism-like and flower-like ZnO [Yin et al., 2012]. The surface modification of the NPs seems to play as well a role on their effects on the brain as shown in the *in vivo* study of Zhang and coworkers [2011] where mice were intranasally instilled with four different types of TiO_2 NPs varying in size and coating. Hydrophobic particles without coating resulted less neurotoxic than hydrophilic particles with silica surface coating. Particular concern should be devoted to metallic substances for which may be foreseen airborne exposure: they can enter the brain directly via retrograde transport through the olfactory nerve. In the brain, NPs may induce inflammation, apoptosis and oxidative stress as accumulating evidence strongly suggested that ROS generation and the

induction of oxidative stress is a major toxicological paradigm for engineered metal oxide nanoparticles. For all the NPs considered in this review it has been demonstrated that NPs deposited in the nasal epithelium of animals may enter the brain via olfactory bulb. Another portal of entry of NPs to brain is from systemic circulation. Also in case of other routes of administration (e.g. oral, intraperitoneal) employed in the *in vivo* studies, neurotoxic effects have been demonstrated.

In an overall assessment of the studies conducted so far, what emerges is a deficiency in the use of models and standardized methods, which is ever more desirable in case of nanomaterials, so that the data could be used more appropriately in a perspective of risk assessment strategy. This can be improved by introducing a concern-driven strategy for NMs potentially at risk or employed for specific purposes in the area of CNS [Oomen et al., 2013]. Moreover it shoud be necessary to take into account as much as possible biopersistence and accumulation of NMs, as well as their fate within critical tissues. Moreover the solubility should be taken into account when metal NPs are investigated, highlighting the importance of including proper controls to the experimental design, in order to discriminate between the toxicity triggered by the ionic part and the effects induced by the particles themselves. It is desirable the use of new models, in line with the 3Rs principle (by using fewer animals, but obtaining more informations at the same time), as well as the exploitation of the potential of emerging technology that employs iPSCs (induced pluripotent stem cells, increasingly used as cell model in vitro for neurodegenerative diseases), and the inclusion of new endpoints (such as epigenetic marks).

Collectively those data indicate an obvious need for a better assessment of the human risk following exposure to NPs, including a clear comprehension of the destiny of those compounds inside the human body and their potential aggregation, accumulation and target molecules, particularly for those compounds designed for clinical applications or to be in

direct contact with human tissues, for which a careful assessment of the risk-benefit ratio is compulsory.

Conflict of interest statement

The authors declare that there is no conflicts of interests

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Legend to Figure

Figure 1. A variety of materials of nanometric size through the different strategies summarized here may be useful for the diagnosis and treatment of neurodegenerative diseases

Abbreviations

5-HT	5-hydroxytryptamine
AChE	acetilcholinesterase
AD	Alzheimer's disease
Ag NPs	silver nanoparticles
$AgNO_3$	silver nitrate
ALS	Amyotrophic Lateral Sclerosis

1	AMNPs	amorphous silica nanoparticles
2	APP	ß-amyloid precursor protein
3	$Aß_{1-42}$	amyloid-$ß_{1-42}$ protein
4	*BACE1*	beta-secretase 1 gene
5	bax	BCL2-associated X protein
6	BBB	blood-brain barrier
7	bcl-2	B-cell lymphoma 2 protein
8	*casp8*	caspase-8 gene
9	*casp9*	caspase-9 gene
10	CBF	cerebral-blood flow
11	CNS	central nervous system
12	CNT	carbon nanotubes
13	*Comt*	catechol-O-methyltransferase gene
14	*COX-2*	Cyclooxygenase-2 gene
15	CuO NPs	copper oxide nanoparticles
16	DA	dopamine
17	DAPI	4',6-Diamidino-2-Phenylindole
18	DMSA	dimercaptosuccinic acid
19	DNA	Deoxyribonucleic acid
20	DOPAC	3,4-dihydroxyphenylacetic acid
21	ERK	extracellular-signal-regulated kinase
22	Fe_2O_3 NPs	maghemite nanoparticles
23	Fe_3O_4 NPs	magnetite nanoparticles
24	FITC	Fluorescein isothiocyanate
25	GADD45	growth Arrest and DNA Damage-inducible 45

1	GFAP	glial fibrillary acidic protein
2	*Gpr37*	G protein-coupled receptor 37 gene
3	*Gpx1*	glutathione peroxidase 1 gene
4	GSH	glutathione
5	GSH-PX	glutathione peroxidase
6	H_2O_2	hydrogen peroxide
7	HD	Huntington's disease
8	*hGDNF*	human glial cell-derived neurotrophic factor gene
9	HO-1	heme oxygenase 1
10	HVA	homovanillic acid
11	IL-10	interleukin-10
12	IL-1ß	interleukin-1 beta
13	IL-6	interleukin-6
14	iNOS	inducible nitric oxide synthase
15	IONPs	iron oxide nanoparticles
16	LDH	lactate dehydrogenase
17	JNK	c-Jun N-terminal kinases
18	J*un*	transcriptional factor AP-1
19	*Maoa*	monoamine oxidase A gene
20	MCP-1	monocyte chemoattractant protein-1
21	MDA	malondialdehyde
22	MIP-1α	macrophage inflammatory protein 1 alpha
23	MMP-9	matrix metallopeptidase 9
24	MNPs	magnetic nanoparticles
25	MRI	magnetic resonance imaging

1	mRNA	messenger RNA
2	MWCNT	multi-walled carbon nanotubes
3	MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
4	NE	norepinephrine
5	NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
6	NMDAR	N-methyl-d-aspartate receptors
7	NO	nitric oxide
8	NOS	nitric oxide species
9	NPs	nanoparticles
10	Nrf-2	NF-E2-related factor 2
11	NSC	neural stem cells
12	p21	cyclin-dependent kinase inhibitor 1
13	p53	Tumor protein p53
14	*Park2*	Parkinson's disease (autosomal recessive, juvenile) 2 gene
15	PD	Parkinson's disease
16	PEG	Polyethylene glycol
17	PGE2	prostaglandin E2
18	PI	propidium iodide
19	PLGA	polylactic-co-glycolic acid
20	RNA	ribonucleic acid
21	ROS	reactive oxidative species
22	SiO_2 NPs	silicon dioxide nanoparticles
23	SN	substantia nigra
24	*Snca*	synuclein gene
25	*SOD1*	superoxide dismutase-1 gene

SOD	superoxide dismutase
SPIONs	superparamagnetic iron oxide nanoparticles
SWCNT	single-walled carbon nanotubes
Th	tyrosine hydroxylase gene
TiO_2 NPs	titanium dioxide nanoparticles
TNF-α	tumor necrosis factor-alpha
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
Txnrd1	thioredoxin reductase 1 gene
USPIONs	ultrasmall superparamagnetic iron oxide nanoparticles
ZnO NPs	zinc oxide nanoparticles

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Table 1. Neurotoxic effects of engineered nanoparticles *in vitro*

Nanoparticle	Cell system	NP characteristics	Effects	References
Titanium dioxide	BV-2 murine microglial cells	Degussa P25 (a mixture of the anatase (70%) and rutile (30%) forms of TiO_2, <100 nm particle size)	TiO_2 NPs induced rapid (< 5 min) and prolonged (2 h) release of ROS Interference with the mitochondrial machinery	Long et al., 2006
	U87 astrocytoma cells HFF-1 human fibroblasts	Anatase TiO_2 microparticles (1–1.3 µ particle size) or nanoparticles (<25 nm),	Dose-related cytotoxicity (apoptosis, necrosis, apoptosis and necrosis-like cell death) . Both TiO_2 microparticles and nanoparticles,are equally effective in human neural cells and fibroblasts	Lai et al., 2008
	PC12 rat embryonic pheochromocytoma cells	Anatase (20 nm in size) and rutile (20 nm in size) TiO_2-NPs and micrometer-TiO_2 particles (1µm)	Concentration-dependent cytotoxicity and membrane damage ROS, GSH, SOD and MDA increased production Apoptosis and necrosis; cell cycle arrested in G2/M phase Anatase TiO_2 NPs more toxic than rutile. Micron-sized TiO2 did not exhibit any toxic response	Wu et al., 2010
	C6 murine glial cells and U373 human glial cells	Anatase (96%) and rutile (4%) forms of TiO2,, 4-200 nm particle size.	Dose-related cytotoxicity Induction of apoptotic events Internalization on TiO_2 NPs in membrane-bound vesicles Morphological changes	Márquez-Ramírez et al., 2012
	Primary rat microglial and PC12 rat embryonic pheochromocytoma	TiO_2,20 nm particle size	Oxidative stress via increased NO release Increased secretion of TNF-α, IL-1ß and IL-6 Increased expression of MCP-1 and MIP-1α	Xue et al., 2012

	cells			
	SHSY5Y, human neuroblastoma cells	Anatase (100%) and a mixute of anatase (80%) and rutile (20%) forms (2% nm in size) TiO_2-NPs	No cytotoxicity nor morphological alterations Time- and dose-dependent internalization Pure anatase TiO_2 NPs altered cell cycle and induced apoptosis/necrosis Micronuclei formation and primary oxidative DNA damage	Valdiglesias et al., 2013
	C6 murine glial and U373 human glial cells	Anatase (96%) and rutile (4%) forms of TiO2, ≤ 50 nm particle size.	Cytotoxicity Morphological changes Increased ROS, lipid peroxidase, GSH-PX, CAT and SOD	Huerta-García et al., 2014
Silicon dioxide	Primary rat microglial cells	Amorphous SiNPs embedded with a fluorescent dansylamide dye, spherical in shape and about 150–200 nm in diameter	No cytotoxicity SiO_2 NPs internalization in membrane-bound vesicles Oxidative stress	Choi et al., 2010
	PC12 rat embryonic pheochromocytoma cells	SiO_2 NPs of different sizes (20 and 50 nm)	Concentration-dependent cytotoxicity Dose-related GSH depletion and enhanced ROS production Cytoplasmic accumulation of SiO_2 NPs agglomerates Morphological changes	Wang et al., 2011a
	Primary rat microglial cells	SiO_2 NPs (20 nm in diameter)	Enhanced secretion of TNF-α, IL-1ß and IL-6 pro-inflammatory cytokines	Xue et al., 2012
	SK-N-SH human neuroblastoma and N2a mouse neuroblastoma cells	SiO_2 NPs, 12.1 nm mean particles size	Concentration-dependent cytotoxicity, apoptosis and ROS release Morphological changes SiO_2 NPs dispersed in the cytoplasm of SK-N-SH cells SiO_2 NPs stored intracellularly in vesicles in N2a cells	Yang et al., 2014
Zinc oxide	NSC mouse Neural Stem Cells	ZnO NPs with zincite crystal structure, of different size (10, 30, 60, and 200	Concentration-dependent, but no size-dependent toxic effects Altered morphology and induction of apoptosis	Deng et al., 2009

		nm)	Significant release of Zn-ions	
	PC12 rat embryonic pheochromocytoma cells	Zn NPs, <50 nm particle size	Mitochondrial impairment and reduced cell viability Internalization of ZnO NPs in membrane-bound vesicles	Kao et al., 2012
	RCS96 rat Schwann cells	ZnO NPs with different architecture: spherical (35 nm in diameter); microsphere (45 nm in diameter); hexahedral, prism-like (~2.5 to 6.0 µm in diameter and ~18.0 to 60.0 µm in length.); flowers-like (~500 to 600 nm in diameter and several microns in length.)	Shape- and time-dependent neurotoxicity Apoptosis induction and G2/M phase cell cycle arrest Significant release of Zn-ions	Yin et al., 2012
	SHSY5Y human neuroblastoma	ZnO NPs, 100 nm mean particles size	Cytotoxicity: apoptosis and cell cycle alterations and genotoxicity (micronuclei , H2AX phosphorylation and primary and oxidative DNA damage), in a concentration- and time-dependent manner	Valdiglesias et al., 2013a
Copper oxide	PC12 rat embryonic pheochromocytoma cells	CuNPs, 90 nm particle size	Reduced levels of DA, DOPAC and HVA Downregulation of *Gpx1* gene Upregulation of *Txnrd1*, *Snca* and *Maoa* genes	Wang et al., 2009
	rBMEC rat brain microvessel endothelial cells	Cu-NPs , 40 and 60 nm particle size	Size-related cytotoxicity Increased levels of PGE2, TNF-α and IL-1ß Enhanced barrier permeability	Trickler et al., 2012
Silver	PC12 rat	Ag NPs, 15 nm particle size	Upregulation of *Gpx1* gene	Wang et al., 2009

	embryonic pheochromocytoma cells		No variations of the genes associated to DA metabolism and Parkinson's pathogenesis	
	Rat brain microvessel endothelial cells co-cultured with astrocytes	Ag NPs	Ag NPs crossed the BBB and accumulated in endothelial cells	Tange t al., 2010
	Primary rat brain microvessel endothelial cells	Ag-NPs, 25, 40, or 80 nm particle size	Dose- and size-dependent Ag NPs internalization Cytotoxicity and morphological changes with monolayer perforations Size- and time-dependent increase of TNF-α and IL-1ß levels Size-selective impairment of the barrier permeability	Trickler et al., 2010
	Rat cortical cells	Ag NPs, 20 nm particle size	Loss of cytoskeleton structure and F-actin and ß-tubulin degradation Inhibition of neuronal extension and cytotoxicity	Xu et al., 2013
	SHSY5Y human neuroblastoma and D384 human astrocytoma cells	Ag NPs, 20 nm particle size	Dose-related impaired mitochondrial metabolism and membrane damage Dose-related inhibition of colony forming efficiency Significant release of Ag-ions	Coccini et al., 2014
	Primary porcine brain microvessel endothelial cells	Ag-NPs, 25, 40, and 80 nm particle size	Increased PGE2, TNF-α and IL-1ß levels BBB leakage Enhanced barrier permeability	Trickler et al., 2014
	CGC primary rat cerebellar granule cells	0.2%polyvinylpyrrolidone (PVP)-coated AgNPs < 100 nm.	Calcium imbalance Activation of NMDAR Oxidative stress by ROS production	Ziemínska et al., 2014

			Impaired mitochondrial membrane potential	
Iron oxide	Primary rat cerebellar cortex astrocytes	Nanosized iron oxide superparamagnetic particles (Fe_3O_4 or γ-Fe_2O_3).	Severe reduction of cell viability and cell adhesion No damage to the cell membrane integrity	Au et al., 2007
	PC12 rat embryonic pheochromocytoma cells	Anionic Fe_2O_3 nanoparticles with surface coating (DMSA); nanoparticle diameters between 5 and 12 nm.	Impaired cell viability Morphological changes	Pisanic et al., 2007
	Primary rat astrocyte-rich cultures	DMSA-coated Fe-NPs, 60 nm average diameter	Temperature-dependent IONPs uptake	Geppert et al., 2009
	OLN-93 spontaneously transformed primary rat brain oligodendroglial cells	Citrated-coated Fe NPs	Cellular Fe-content significantly increased in a concentration dependent manner IONPs stored in membrane-bound perinuclear vesicles	Hohnholt et al., 2010
	Primary rat astrocyte-rich cultures	DMSA-coated Fe-NP, 60 nm average diameter	Time- and dose-dependent IONPs internalization	Geppert et al., 2011
	Primary rat astrocyte-rich cultures	DMSA-coated Fe-NP, 60 nm average diameter	IONPs stored in membrane-bound perinuclear vesicles Fe-ions sequestered by proteins to protect cells from cytotoxic effects	Geppert et al., 2012
	Primary rat	DMSA-coated Fe-NP, 60 nm average	Time-, concentration- and temperature-dependent uptake of	Hohnholt et al.,

	astrocyte-rich cultures	diameter	IONPs by endocytotic processes	2012
	Primary rat astrocyte-rich cultures	DMSA-coated Fe-NP, 60 nm average diameter	Time-, concentration- and temperature-dependent uptake of IONPs IONPs accumulation enhanced by the presence of a magnetic field	Lamkowsky et al., 2012
	Primary rat cerebellar cortex astrocytes	Fe_3O_4 NPs coated with polyethyleneimine and tagged with rhodamine B isothiocyanate (Fe_3O_4-PEI-RITC), mean core size 24.3±5.7 nm	No cytotoxic effects Rapid and extensive IONPs uptake	Yiu et al., 2012
	Primary rat microglial cells	Fe_3O_4 ,45 nm average	Increased expression levels of TNF-α, IL-1ß and IL-6 No changes in NO, MCP-1 and NF-κB production	Xue et al., 2012
Carbon nanotubes	Primary chicken embryos mixed neuronal and glial cells	SWCNT-agglomerates (SWCNT-a), with a diameter of approximately 100 nm SWCNT-bundles (SWCNT-b), with a diameter of approximately 20 nm.	Agglomerated SWCNT induced cytotoxicity	Belyanskaya et al., 2009
	PC12 rat embryonic pheochromocytoma cells	Long single-walled carbon nanotubes (LSWCNT: Outer Diameter 1–2 nm, Length 20 μm), short single-walled carbon nanotubes (SSWCNT: OD 1–2 nm, Length:0.5–2 μm).	SWCNT induced time- and concentration-related cytotoxicity Decreased mitochondrial membrane potential Oxidative stress via ROS and lipid peroxidation increase Time- and dose-dependent decreased SOD, GSH-Px, CAT and GSH Cell cycle arrested in G2/M phase Dose-dependent apoptotic rate	Wang et al., 2011b
	PC12 rat embryonic	SWCNTs, OD 1–2 nm, length 20 μm	Time- and dose-dependent apoptotic cell death Formation of ROS	Wang et al., 2012

	pheochromocytoma cells		Decreased levels of lipid peroxide Increased levels of GSH, SOD, GSH-Px and CAT Reduced mitochondrial membrane potential Activation of caspase-3 Vitamin E protects cells from the toxicity induced by SWCNT	
	PC12 rat embryonic pheochromocytoma cells	Fe-low and Fe-high MWCNTs based on their metal impurities, outer diameter 2–50 nm, length 50 μm	Cytotoxicity Cytoskeleton disruption Effects amplified by the presence of metal impurities such as iron	Meng et al., 2013
	Co-cultures of primary embryonic rat hippocampal neurons and glial cells	Substrates containing polypyrrole and/or SWCNTs	Impaired cell viability Neuroprotective effects in the presence of polypirrole	Hernández-Ferrer et al., 2014

Table 2. Neurotoxic effects of engineered nanoparticles *in vivo*

Nanoparticle	Animal model	Administration	NP characteristics	Effects	References
Titanium dioxide	CD-1 mice, female	Intranasal	4 types rutile-phase TiO2 NPs (<5μm;<100 nm; hydrophilic and with silica-coated surface)	Hydrophobic rutile TiO_2 NPs accumulated in the cerebral cortex and striatum and cause morphological changes to the neurons Hydrophilic rutile TiO_2 NPs were not internalized in the brain but induced reduced NE levels in hippocampus, cerebral cortex, cerebellum and striatum	Zhang et al., 2011
	Swiss albino mice, male	Oral	Mixture of rutile and anatase TiO_2 NPs (<75nm)	Enhanced DA and NE levels Oxidative stress with increased ROS	Shrivastava et al., 2014
	CD-1 mice, male	Intranasal	Anatase (5 to 6 nm)	Spongiocytes overproliferation Brain hemorrhages mRNA increased expression of p38, Nrf-2 and NF-κB	Ze et al., 2013
	CD-1 mice, male	Intranasal	Anatase (5 to 6 nm)	Glial cells overproliferation Tissue necrosis Altered expression of oxidative-stress associated genes Internalization of TiO_2 NPs in the brain	Ze et al., 2014a
	CD-1 mice, male	Oral	Anatase (5 to 6 nm)	Severe pathological changes Impaired spatial recognition	Ze et al., 2014b
	Wistar rats,	Intragastric	Anatase (10 nm)	Reduction of cell proliferation in the hippocampus and	Mohammadipour

	pregnant			impaired learning and memory in offspring	et al., 2014
Silicon dioxide	SD rats	Intranasal	SiO2 NPs (15 nm)	Internalization of SiO_2 NPs in the brain Oxidative stress Inflammation	Wu et al., 2011
	SD rats, male	Intraperioneal injection	SiO2 NPs (50 to 60 nm)	BBB disruption Neuronal damage Behavioral impairment	Sharma et al., 2013a
	Zebrafish (*Danio rerio*)	Exposure in water	SiO2-NPs (15-nm and 50-nm)	Size-dependent behavioral changes Parkinson's like behavior	Li et al., 2014
	Balb/c mice	Intraperitoneal injection	Mesoporous hollow silica nanoparticles (MHSNs) (110 nm)	Size-dependent migration through the BBB of PEGylated silica Time-related NPs accumulation in the brain	Liu et al., 2014
Zinc oxide	SD rats, male	Intranasal	ZnO NPs (<50 nm)	ZnO NPs translocation in the olfactory bulb and in the brain	Kao et al., 2012
	Swiss mice, male	Intraperitoneal injection	ZnO NPs (20-80 nm)	Impairment of synaptic responses Disrupted spatial memory	Xie et al., 2012
	SD rats, male	Oral	ZnO NPs (40nm)	ZnO NPs translocation in the brain	Cho et al., 2013
Copper	SD rats, male	Intraperitoneal injection	Cu NPs (50-60 nm)	Neuronal alterations Brain dysfunction Cognitive impairment	Sharma and Sharma, 2007
	SD rats, male	Intraperitoneal injection	Cu NPs (50-60 nm)	BBB damage Brain edema formation	Sharma et al., 2009
	SD rats, male	Intraperitoneal	Cu NPs (50-60 nm)	Neuronal cell damage, glial cell activation, loss of	Sharma et al., 2010

		injection		myelinated fibres Brain edema formation	
	Wistar rats	Intraperitoneal injection	CuO NPs (10 to 70 nm)	Oxidative damage in hippocampus Altered cognitive functions (poor performance of animals in behavioral tests)	An et al., 2012
Silver	C57BL/6N mice, male	Intraperitoneal injection	Ag NPs (25 nm)	Altered expression of oxidative stress and antioxidant genes ROS enhancement DNA damage	Rahman et al., 2009
	SD rats, male	Intraperitoneal injection	Ag NPs (50-60 nm)	BBB leakage Brain edema formation Glial activation Reduced cerebral blood flow Loss of myelinated fibers	Sharma et al., 2009
	ICR mice, male and female	Oral	Small-sized AgNPs (22nm, 42nm, and 71nm) and large-sized AgNPs (323nm)	Size-dependent Ag NPs internalization (small-sized NPs were distributed to the organs including brain, lung, liver, kidney, and testis). Increase of TGF-ß levels in serum by small-sized Ag NPs	Park et al., 2010
	Wistar Hannover Galas rats, female	Oral	Ag NPs (∼14nm)	Ag NPs and Ag-ions uptake in the brain Significant release of Ag-ions	Loeschner et al., 2011
	Wistar rats, male	Intravenous injection	AgNPs (20 and 200 nm)	Time-related Ag NPs uptake 20 nm Ag NPs better internalized than 200 nm Ag NPs	Dziendzikowska et al., 2012

	C57BL/6J mice, male	Inhalation	Ag NPs (25 nm)	Translocation of Ag NPs into the olfactory bulb and lateral brain ventricles	Gentner et al., 2012
	Wistar rats, female	Oral	Ag NPs (14 nm)	Increased DA and 5-HT levels Significant release of Ag-ions	Hadrup et al., 2010
	SD rats, male	Oral	Ag NPs <20 nm, noncoated) and <15 nm PVP-coated	Uptake and biopersistence of Ag NPs into the brain PVP-coated particles show limited ion dissolution	van der Zande et al., 2012
	SD rats, male	Intraperitoneal injection	Ag NPs (50-60 nm)	Size- related BBB disruption Age-related BBB damage Neuronal NOS upregulation Neuronal impairment	Sharma et al., 2013b
Magnetic	ICR mice, male and females	Intraperitoneal injection	PVP-stabilized cobalt ferrite silica-overcoated. [MNPs@SiO_2(RITC)]	IONPs passed through the BBB	Kim et al., 2006
	ICR mice, male and females	Inhalation	Fluorescent magnetic nanoparticles (50 nm)	IONPs passed through the BBB	Kwon et al., 2008
	ICR mice, male and females	Oral	Fe3O4 MNPs (∼20nm)	IONPs passed through the BBB	Wang et al., 2010
	SD rats, male	Intraneural injection	4 IONPs of different surface and core chemistries: DMSA-Fe_2O_3, DMSA-Fe_3O_4, PEG-Fe_3O_4 and PEG-Au-Fe_3O_4, roughly spherical at 8–10 nm	Macrophages, monocytes and lymphocytes accumulation at sites of injection Increased levels of ERK, caspase-3, MMP-9, HO-1 and IL-1ß	Kim et al., 2013
	SD rats, male	Inhalation	Fe_3O_4-NPs (30 nm)	IONPs deposited in olfactory bulb, striatum and hippocampus Oxidative stress via upregulation of GSH, H_2O_2, SOD	Wu et al., 2013

				and MDA	
	ICR mice, male	Inhalation	α-Fe_2O_3 and γ-Fe2O3 NPs (~22 nm and ~31 nm respectively)	Brain pathological alteration, microglial proliferation, activation and recruitment in hippocampus and striatum, especially in olfactory bulb	Wang et al., 2011
	Zebrafish (*Danio rerio*)	Oral	Superparamagnetic iron oxide nanoparticles (dextran-coated)	Induction of apoptosis Brain accumulation of IONPs Enhanced mRNA levels of *casp-8*, *casp-9* and *jun*	de Oliveira et al., 2014
Carbon nanotubes	Wistar rats, male	Intravenous injection	Gadolinium-catalyzed SWNTs (Gd-SWCNTs) . Average diameter 2.05 nm, length 500 nm to 1.5 µm	Accumulation of SWCNT into the cerebral cortex No inflammation No altered tissue morphology	Avti et al., 2013
	C57BL/6J, female	Injections at specific stereotactic locations in the motor cortex	MWNT shortened (by oxidization) and amino-functionalized (oxMWNT-NH3) and only amino-functionalized (MWNTNH3)	Presence of both functionalized MWCNT in astrocytes, microglial and neuronal cells Induction by both types of MWNT of a transient enhancement of TNF-α, IL-1ß, IL-6 and IL-10 Microglia and astrocytes activation with increased levels of GFAP and CD11b	Bardi et al., 2013
	C57BL/6J, male	Inhalation	MWCNT	Time-dependent accumulation of MWCNT in brain	Mercer et al., 2013
	C57BL/6J $p53^{+/-}$ mice, male and female	Intravenous injection	CNT	MWCNT passed the blood-placental barrier Brain deformity and malformations via an indirect neurotoxic mechanism No evidence of MWCNT into the brain of the pups	Huang et al., 2014

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Figure 1