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Interactions Between Nanosized Materials and the Brain

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Abstract: The current rapid development of nanotechnologies and engineered nanomaterials (ENM) will impact the society in a major fashion during the coming decades. This development also causes substantial safety concerns. Among the many promising applications of ENM, products that can be used for diagnosis and treatment of diseases, including conditions that affect the nervous system, are under development. ENM can pass the blood brain barrier (BBB) and accumulate within the brain. It seems that the nano-form rather than the bulk form of the chemicals pass the BBB, and that there is an inverse relationship between particle size and the ability to penetrate the BBB. Although translocation of ENM to the brain is possible during experimental conditions, the health relevance for real-life situations is far from clear. One major reason for this is that studies have been using nanoparticle concentrations that are far higher than the ones that can be expected during realistic exposures. However, very high exposure to the CNS can cause effects on neurotransmission, redox homeostasis and behavior. Available studies have been focusing on possible effects of the first generation of ENM. It will be necessary to study possible health effects also of expected novel sophisticated materials, independent of the outcome of present studies. The prospects for intended or targeted medical applications are promising since it has been shown that ENM can be made to pass the BBB and reach specific regions or cells within the brain.

Keywords: Axonal transport, blood-brain-barrier, brain, *in vivo*, *in vitro*, nanoparticle, translocation, unintended and intended exposure.

1. INTRODUCTION

The development of nanotechnologies is in a rapidly growing phase, characterized by the development of new methods, techniques and products that use or contain nanoscale materials. Experts claim that nanotechnology has the potential to provide technological solutions to large industrial challenges and they see nanotechnology as the next industrial revolution. In the coming years, this development will be a key driver of the economy in developed countries. It is relatively difficult to assess the direct impact on the real economy. However, recent studies pointed out that the value of goods of engineered nanomaterials (ENM) may reach US $ 2.6 trillion by 2014 at the global level. Other estimates of the volume of the world economy being associated with nanomaterials show that the global market value of nanotechnologies will be between U.S. $ 1 to 2.5 trillion by 2015 and 3 trillion U.S. $for the year 2020 [1]. Due to the current and future economic impact, the concerns regarding safety aspects of nanomaterials are increasing.

There is a multitude of ENM containing products on the market. This number is increasing every year, often without accompanying systematic toxicology tests [1], regulation or product information. Taylor *et al.* [2] listed the most common ENM: carbon, zinc, silica, titanium and gold. Another of the predominant nanomaterials is silver (Woodrow Wilson Database, www.nanotechproject.org), known as toxic for bacteria and often reported to be cytotoxic for mammalian cells [3].

Among promising applications of ENM, medical and biomedical research has shown that ENM can be used as a possible treatment for cancer and/or autoimmune diseases after being found to be selectively toxic towards potential disease-causing cells [4, 5]. The unique properties of the new nanomedicines also offer potential solutions for many of the current challenges in treating cancer, cardiovascular, and neuodegenerative diseases, as well as other diseases (for review see [6]). Many nanodiagnostics but also nanomedicines are already approved and in use. Of these, only a few are intended for treatment of neurological diseases (Table **1**). However, the ongoing research and the number of clinical trials in this area are growing rapidly.

The unique physicochemical properties of ENM are the drivers of the development of unique products. But exactly these unique physiochemical properties can induce unintended biological effects and cause cytotoxicity which in turn can lead to adverse health effects. Unintended exposure to ENM can occur via inhalation, which is the most likely pathway, by ingestion, or through the skin. However, the most common (intended) application route in nanomedicine is via the bloodstream. Potential cellular effects of ENM are mitochondrial damage, induction of free radicals, mutations, DNA damage, cytotoxicity, and related systemic effects which have been reviewed in many papers (e.g. [7, 8]).

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1875-533X/14 $58.00+.00

Table 1. Neurologically Related Nano-medicine Products on the Market

Indication	Composition	Product
Antiemetic	Nanocrystalline aprepitant, part of the group of neurokinin-1-receptor- antagonists	Emend
Atypical antipsychotic agent	Nanosized 9-hydroxyrisperidone, a dopamine antagonist	InvegaSustenna
Eating disorders	Nanocrystallinemegesterol acetate, synthetic derivative of progesterone	MegaceES
Multiple sclerosis	Copolymer of alanine, lysine, glutamic acid, tyrosine	Copaxone
Schizophrenia	Nanocrystalline paliperidone, 9-hydroxyrisperidone is a dopamine antagonist	InvegaSustenna

The main goal of the present review is to give a short overview of how ENMs can translocate from the respiratory tract to the circulation and possibly pass the blood-brain-barrier (BBB) and subsequently affect the brain. The review is built upon a previous paper of ours [9], and studies published thereafter. Here we differentiate between intended and unintended exposure to ENM and consider non-soluble nanomaterials only. Furthermore, the focus of this paper is on presenting illustrative data rather than a compilation of all available studies.

2. EXPOSURE

ENM are produced from numerous substances, in many different sizes and forms, and also with an assortment of surface coatings. Occupational exposure during the production of ENM, where handling large amounts of the material may occur, is the most probable condition for ENM exposure [10, 11]. In addition, occupational exposure to ENM may take place in medical applications, in research and development laboratories, as well as in manufacturing facilities where ENM are used for integration into other products. Handling of ENM containing waste is a further possible source for occupational exposure. Other possible targets for ENM exposure are consumers due to ENM containing products available on the market. Patients using EMN containing materials for diagnostics, therapy or theranostics are also exposed to ENM. A crucial difference is if the exposure is intended or unintended. This chapter gives a short example where exposure to ENM can occur.

2.1. Occupational Exposure –Unintended Exposure

At present it is very likely that occupational exposure (as in the production of nanomaterials, manufacturing of consumer products, research activities, the use of medical products, disposal etc.) far exceed the consumer / indoor exposure. As already mentioned, there are quite a few ENM-containing consumer products on the market, the expected large growth of the market, however, is still pending. It is reasonable to suppose that not only the number of ENM-containing products will increase dramatically in the near future, but also the number of occupationally exposed persons will experience a substantial increase.

Until now, only a few studies have been carried out on occupational exposure by nanomaterials, such as to titanium dioxide (TiO_2), carbon black, nickel powder, quartz dust or welding fumes, metal fumes, beryllium or diesel exhaust [12-19]. There is only limited knowledge available about in which companies and how much of ENMs are used for product manufacture. Thus, knowledge of occupational exposure outside the preparation of ENM is very limited.

Occupational exposure to nanomaterials may occur from three main sources: (1) by engineered nanomaterials, (2) process-generated nanoparticles (PG-NP), or (3) incidentally resulting nanomaterials by incineration or normally occurring as an ecological background. The ecological background concentration is derived from natural sources (volcanoes, weathering, etc.) and anthropogenic activities such as combustion [20, 21]. The identification of sources, and especially to distinguish between the process generated and the background nanomaterials is particularly difficult, but also very important [22] for the evaluation of the potential health risks of nanomaterials in occupational settings. There are already some exposure measurements performed in workplaces showing that the background concentration of particles varies greatly, often between 10,000 and 20,000 nanoparticles/cm^3 for industrial jobs, office, or moderately polluted districts [23-27].

2.2. Intended Exposure to ENM - Cosmetics

ENMs are used in the manufacture of cosmetics in two important areas: for encapsulation or as carrier systems forintended transport of agents to deeper skin layers, and as UV protective filter in sunscreens. According to the manufacturers information, nanoscale materials can be found in cosmetic products such as nanoparticulate gold and silver, ceramic nanoparticles, pigments, minerals, and also fullerenes. Normally, the skin is a very good barrier to the outer environment. Its histological structures and its fat and acid mantle prevent most foreign substances and pathogens from entering the skin. The cosmetics industry therefore uses nanodispersions and encapsulation or carrier systems, so that agents penetrate into deeper skin layers where they activate skin metabolism to improve the skin's appearance. The functions and benefits of the encapsulation and carrier systems are 1) the controlled release and optimization of the availability of cosmetic agents in certain skin layers, 2) the protection of sensitive agents, 3) longer availability and hence greater product effectiveness, and 4) the reduction in the amount of agents and additives used in the products. The ENMs or encapsulation systems, which are soluble and biodegradable, include liposomes, nanoemulsions, micelles and lipid-nanoparticles. In the present review these systems are not considered.

Titanium dioxide has been produced industrially for more than 100 years [28]. The increasing application of TiO_2 NPs in industry and daily life products explains why nano-TiO_2 is one the most investigated nanomaterial also in nanotoxicological studies. By using nanosized TiO_2 and zinc oxide, protection against UV radiation has been radically improved as a result of the smaller sized particles. The primary size of the nanoparticles used as UV filters is approximately 40 nm. Nano-TiO_2 has been used in sunscreens as a so-called physical UV-filter since about 1990. In contrast to chemical UV-protective substances, physical UV-filters do not absorb the UV-radiation but rather reflect or disperse them so that they cannot penetrate down to the skin surface. Both regular and nanosized TiO_2 in sunscreens are coated with organic compounds and metal oxides such as silica or aluminum in order to reduce the photocatalytic reactivity and hinder the formation of reactive radicals [29]. As sunscreens for allergy sufferers should not contain chemical UV-filters, thus TiO_2-NP-containing sunscreens are a good alternative. However, the question of health risks of TiO_2 both in its regular and nanosize form is highly relevant. A number of studies have investigated the potential toxicity of TiO_2 NPs but very few have focused on the CNS (for review see [30]).

2.3. Intended Exposure to ENM - Medical Applications

Nanotechnology and thus ENMs promises to dramatically change and improve the quality of life especially in the area of medicine. It is often stated that ENMs have the potential to reach and to affect target organs and tissues, even tumors in the brain and/or at the molecular and cellular levels. While medical and pharmacological research focuses on the applications of nanosized materials, induced side effects associated with the use of ENMs are generally not taken into consideration [31]. Regrettably, the knowledge about potential toxicity of ENMs is far from comprehensive. In fact - as it has been stated by Devadasu *et al.* [31]- it is essential to understand the drug and the disease properly before designing a medical delivery system. The main goal for drug delivery systems or nanocarriers is, to target the site of interest specifically, to overcome solubility or stability issues for the drug, to minimize the drug dose, and to reduce side effects. Unfortunately, ENMs themselves can also induce significant toxic effects via their specific physical and chemical properties. Size, electric, optical, magnetic properties, surface charge, agglomeration or chemical composition of the material can induce cytotoxicity e.g. via catalytic and oxidative reactions or by triggering of immune reactions etc. (for reviews see e.g. [32]). Although ENMs toxicity and directed or targeted delivery systems are better understood nowadays, concerns about safety aspects are still remaining.

Of course, the risk / benefit approach in medical application is quite different than for other situations. However, the fate and the behavior and the effectiveness of an applied ENM also for medical purposes have to be known.

Another issue regards the scientists or medical workers who prepare or administer drugs to patients. Exposure to ENM enabled drugs can occur through inhalation, skin contact, ingestion, and injection, when safe handling measures fail or when they are not followed, but also during (fresh) drug preparation, transport, or administration, during the disposal process, when handling patient excreta, and in the event of spills and other circumstances [33]. Inhalation and skin contact are the most common routes of exposure in health care settings [34]. There is no other monitoring of exposure to ENM than to follow traditional industrial hygiene and health care exposure assessment paradigms by using different techniques such as samplers placed at static locations (area sampling), samples collected in the breathing zone of the worker (personal sampling), or real-time measurements of exposure that can be personal or static. Therefore there is a need to continuously evaluate the adequacy of exposure-monitoring techniques for nano-enabled medical applications, as well as, to conduct further measurements of exposure levels to nano-enabled medical applications in R&D laboratories, manufacturing, and health care facilities [33].

3. TRANSLOCATION OF ENM FROM POINT OF ENTRY TO THE BRAIN

As previously stated, there are four principal routes by which ENM can enter the human body: i) by inhalation ii) by dermal exposure iii) via the digestive tract iv) systemic entrance via e.g. injections to blood vessels. In order to reach secondary organs, nanomaterials are transported via the circulatory system and thus reaching other organs. In addition, nanoparticles (NP) can under certain circumstances enter secondary organs such as the brain via axonal transport along the olfactory nerve. This is here considered to be a variant of inhalation exposure and does not constitute an own route.

3.1. The Blood-brain Barrier Structure and Function

The brain is probably the best protected of the organs in the human body. Besides the protection against mechanical damage exerted by the osseous structure of the cranium, the brain and also the peripheral nerves are shielded from possibly damaging compounds (circulating pathogens, toxins, and also endogenous signaling substances) in the blood by means of three structural barriers (BBB, the blood-cerebrospinal fluid barrier BCSFB, and the blood-neuron barrier BNB). The BBB is formed by brain capillary endothelial cells that in contrast to capillary endothelial cells in other tissues lack fenestrations but are tightly kept together by tight junctions. This barrier is further strengthened by processes from neighboring astrocytes, so that transfer of substances from the blood to the brain is restricted to small, low-molecular weight lipophilic compounds (cf. [35]).

The permeability of the BBB is affected by fluctuations of physiological conditions. Thus, normal life processes can trigger temporary fluctuations in permeability. Chronically altering the permeability of the BBB or of the capillary walls can enable the passage of substances and damage the surrounding nerve cells. For example, strong temperature increases promote BBB permeability. Medical therapies can take advantage of this phenomenon. At the same time, this barrier hinders or prevents many potential treatments of neurological diseases because many active substances cannot pass the BBB. Overcoming the BBB is therefore an important field of current research that seeks better treatments for diseases of the central nervous system.

The BBB – despite its function as a protective barrier – must also enable the transport of nutrients to the brain and the corresponding removal of metabolic products. Accordingly, water-soluble substances and peptides overcome this barrier via specific transporters or special channels in the cell membrane (diffusion, paracellular transport, specific transporter proteins, receptor-mediated transport, and adsorptive transport), while the other soluble compounds pass this barrier via passive diffusion.

Despite the efficiency of the BBB, even damaging compounds as well diagnostic or therapeutic substances can pass this barrier. This can be accomplished by the mechanism mentioned above, viz. simple diffusion, although that is in most cases unlikely. According to a review by Yang [36], the most likely pathways for BBB crossing are adsorptive transcytosis and receptor-mediated transcytosis. These are also the mechanisms that are most commonly used in developing e.g. nanomaterial-based drug delivery systems for the brain. A comprehensive overview of mechanisms for BBB crossing by ENM and axonal transport of ENM was given in a previous article [9].

3.2. Effects of ENM on the BBB

A relevant question is to what extent nanoparticles that reach the BBB are getting stuck there, and what the effects of ENM are on the BBB itself. The first question has been addressed by Ye *et al.* [37] who tested several ENM *in vitro*, with several models of the BBB. All types of ENM were internalized by the BBB cells, and TEM could also reveal that a few of the particles were passing through the BBB, apparently via transcytosis. The effects on the BBB physiology were investigated in a recent study by Raghnaill *et al.* [38]. The *in vitro* model consisted of human brain capillary endothelial cells (hCMEC/D3) cultured as a monolayer, with or without a co-culture of normal human primary astrocytes. A set of cytokines and their expression levels was investigated by means of an antibody array. After exposure (24 h) to carboxylated polystyrene nanoparticles (100 nm diameter), it was shown that the particles accumulated in the endothelial cells. When analyzing the cytokine data, it was seen that the co-culture of endothelial cells together with astrocytes released more pro-inflammatory cytokines than the culture consisting of only endothelial cells.

An effect on both permeability and inflammatory response was seen in two studies from Trickler and co-workers [39, 40]. They used silver NP [39] and gold NP [40] respectively in a model consisting of primary rat brain endothelial cells. In both studies, smaller particles (3-7 nm gold and 25 and 40 nm silver particles were studied) had more pronounced effects on pro-inflammatory cytokine release. The effect on BBB permeability was different for the two species of nanoparticles, where only the Ag particles caused increased permeability.

Brun *et al.* [41] employed a culture model of the BBB consisting of rat brain endothelial cells cultured together with glial cells. The authors performed two types of exposure to TiO_2NP (25 nm); either an apical exposure where the endothelial cells displayed the surface towards the particles (as in a systemic administration of ENM), or a basal exposure where the particles first encountered the glia components. Exposure was for 4 h, 24 h or 5 days at 0-500 μg/ml. None of the exposures had any effect on cell viability. Regarding permeability (sucrose flux), apical exposure caused increased permeability, whereas no significant effects on this parameter were seen after basal exposure. The basal exposure was also accompanied by a strong inflammatory response (increased transcript levels for inflammatory cytokines measured by quantitative RT-PCR) and down-regulation of the genes for tight junction proteins and certain membrane-based transporters (for insulin, glucose, transferrin).

In summary, several recent *in vitro* studies confirm that at least during specific conditions, the BBB allows ENM to be internalized into the endothelial cell component, and also that the presence of ENM influence the functional state of the BBB, e.g. by activating inflammatory or pro-inflammatory pathways.

3.3. ENM By-passing the BBB

A relevant question is also if unintended exposure of the brain to ENM can occur, and if so, if the local exposure can have damaging effects. This presupposes that the ENM can penetrate the BBB, or use a route which is not containing the BBB. First of all, the BBB is indeed a very powerful barrier in most cases. It has been estimated [42] that only fractions of a percent of inhaled or systemically injected NPs are found within the brain. Yokel *et al.* [43] also addressed this problem in a recent review and they presented data that indicated that very few of the NPs they included in their overview were actually found in the brain after systemic administration, and that the fraction of the applied dose which was found in the brain for those substances were generally below 0.5%.

Several recent animal studies have investigated if ENM exposure (not intended for medical purposes) causes accumulation of the particles in the brain or in specific parts of this structure. If that is seen, it is obviously a sign of incomplete protection by the BBB. Ma *et al.* [44] employed female ICR mice and exposed them to either anatase TiO_2 (5 nm) at various doses or to bulk TiO_2, for 14 days. The exposure was by daily injection into the abdominal cavity. The TiO_2 content in tissues was determined by ICP-MS which showed that the nano form of TiO_2 increased in accumulation with increased dose. When comparing the highest dose (150 mg/kg body weight) with the same dose for the bulk form of TiO_2, the nano form was at significantly higher levels (ca. 500 compared to 350 ng/g tissue).

A study by Lee *et al.* [45] used male and female 4-week-old Sprague-Dawley rats that were orally (gavage feeding) exposed for 28 days to silver nanoparticles (10 or 25 nm; vehicle control, 100 mg/kg/day, or 500 mg/kg/day). Tissue contents of silver were investigated by atomic absorption spectrometry immediately after exposure or after a recovery time (1, 2, or 4 months). Several tissues were investigated, and interestingly, clearance of the applied nano-silver was very high in all tissues except in testes and in the brain. Here, almost no tissue decrease of silver could be seen independent of particle size and recovery time. Such high silver biopersistence has also been seen in other studies such as in a 28 day oral administration study [46] and in a 5 day i.v. administration study [47].

Oral exposure was also employed by Singh *et al.* [48] who treated female Albino Wistar rats with a single oral dose of Fe_2O_3 (up to 2000 mg/kg body weight), either as bulk material or as 30 nm nanoparticles. Higher levels of Fe_2O_3 were detected (atomic absorption spectrometry) in the brains of animals treated with the nano-form of Fe_2O_3 than in those treated with the bulk form of the substance, suggesting that the nano-form preferentially pass the BBB.

A nose-only inhalation exposure for 1 h was used by Petitot *et al.* [49]. They exposed 16 weeks old Wistar rats to depleted uranium (UO_2) nanoparticles (38 nm). In this study, ICP-MS was used for detection of the tissue levels of the material. In contrast to other tissues, the UO_2 did not incorporate in brain or the olfactory portion of the brain at levels significantly higher than the background level.

Taken together, several but not all recent studies indicate that various types of administration allow ENM that are not modified for medical use to pass the BBB and be accumulating within the brain. Some studies further suggest that the nano-form rather than the bulk form of the chemicals pass the BBB, and that there is an inverse relationship between particle size and ability to penetrate the BBB.

In vitro models of the BBB are useful for mechanistic studies and can also to some extent complement and replace animal and human studies (see [50] for a recent review). Such models are typically consisting of a monolayer of (brain) endothelial cells, sometimes also with co-cultures of glia cells. Current developments even allow creating dynamic 3D-models of the BBB in culture. Significant interest is invested in producing and modifying ENM so that they can easily pass through the BBB for use in diagnosis and therapy. In addition, certain studies have also been published that show that, at least *in vitro* and during specific conditions, certain ENM not intended for medical use can pass the BBB.

Thus, using an *in vitro* model of the BBB consisting of murine brain endothelial cells, Hoff and co-workers [51] investigated the permeability of 11 different iron oxide NPs, both as ferrofluid formulations and as powders. The authors concluded that the ferrofluid samples were more prone to pass the BBB than the powders, and that the particle size showed the highest correlation to permeability, with smaller particles more permeable than large ones. Also silver nanoparticles were seen by Tang *et al.* [52] to penetrate in an *in vitro* model (rat brain vascular endothelial cells, BMVEC, together with astrocytes) of the BBB. The authors could document transcytosis as well as accumulation of the particles inside the endothelial cells after 4 h exposure.

3.4. Axonal Transport of ENM From the Nose to the Brain

Particles such as ENM can be taken up by sensory nerve endings embedded in airway epithelia. In the nasal region it is the olfactory and trigeminus nerve system, and in the tracheobronchial region it is the extensive sensory nerve network. Translocation to ganglia and the CNS can then be accomplished by axonal transport. Studies that were providing data supporting this translocation mechanism for nanoparticles to the brain were previously extensively discussed [9]. A recent review by Lucchini *et al.* [53] points to a number of studies (mainly *in vivo* experiments on rats) where a translocation by this pathway has been shown. It seems that the translocated materials can cross synapses and that translocation along both the olfactory and the trigeminal nerves have been experimentally substantiated. However, only a few new data are available where intranasal instillation was applied showing a possible axonal transport of NPs and also neurotoxic effects. It has to be pointed out that most of the experiments performed applied quite high doses of NPs.

Wu *et al.* [54] for example used Fe_3O_4-NPs which were intranasally instilled in rat nostrils for 7 days (20 μg total). The authors showed that Fe_3O_4-NPs were deposited from highest to lowest concentration in the olfactory bulb, striatum, hippocampus, brain stem, cerebellum, and frontal cortex, respectively, and there were no significant deposition in any of the sub-brain regions except in the olfactory bulb. It was shown that the clearance rate of Fe_3O_4-NPs from the rat brain regions was slow, since more than 50% of the Fe_3O_4-NPs remained in the striatum and hippocampus 14 days post-instillation. The authors investigated the H_2O_2, GSH-PX, SOD, and MDA levels in striata and hippocampi where the NPs may have induced oxidative damage. However, no injuries were observed.

Zhang *et al.* [55] tested four different types of TiO_2 particles (rutile phase) for their toxic effects on the brain of mice after intranasal instillation (two hydrophobic particles types [1 μm, 10x40 nm] without coating and two types of water-soluble 10x50 nm hydrophilic particles with silica surface coating) every second day for 30 days (500 μg TiO_2 per mouse/per instillation). A significant increase of TiO_2 (hydrophilic NPs) contents in the cerebral cortex and striatum was detected as well as morphological changes of neurons in the cerebral cortex and significant disturbance of the monoamine neurotransmitter levels in the sub-brain regions. The hydrophilic TiO_2 nanoparticles were more toxic than the hydrophobic ones with similar size, and also the different shapes of NPs induced different neurotoxicity. The same group [56] investigated the potential influence on the neurotransmitter secretion of intranasally instilled Cu-NP (23.5 nm) in mice at three different doses (1, 10 and 40 mg/kg) every second day for 15 or 21 days. The levels of various neurotransmitters (norepinephrine, dopamine and its metabolites, acetylcholine, and glutamic acid) changed in some brain regions, but especially in the olfactory bulb after exposure to a high level of Cu-NP. The authors even detected changes of neurotransmitter levels in parts of the brain where Cu-NP were not accumulating. However, it still seems to be unclear if these effects were mainly caused by the NP themselves, or by the dissolved ions from the particles.

In a recent study by Hopkins *et al.* [57] PEG2-PE-encapsulated CdSe/ZnS quantum dots (QD) were instilled in transnasally to mice (average particle size 84 nm, 250 μg/ml, 1 h) to test the olfactory uptake via axonal transport. Already 3 h after the acute exposure, QD were detected in the olfactory bulb, indicating the olfactory uptake and axonal transport. Moreover this effect was accompanied by the activation of microglial cells suggesting a pro-inflammatory response.

4. EFFECTS ON THE NERVOUS SYSTEM

This section includes references to a large number of studies. Technical details regarding these studies are given in (Table **2**) (for *in vivo* studies) and (Table **3**) (*in vitro* studies), and not necessarily repeated in the main body of the text.

4.1. *In Vivo* Studies

TiO_2 is one of the most and best investigated ENP ENMs. However, there are still contradicting results about its toxicity and especially about its neurotoxicity. Scuri *et al.* [58] therefore investigated the effects of TiO_2-NPs *in vivo* inhalation exposure on neuroimmune responses in rat airways. The authors detected up-regulation of the expression of lung neurotrophins in an age-dependent fashion (in weanling (2 week old) and newborn (2 d old) rats but not in adult (12 week old) animals. In another study Cui *et al.* [59] investigated the prenatal exposure of rats to TiO_2 NPs and found signs of the induction of oxidative damage in the offspring brain. Furthermore the results suggested an effect on the emotional behaviors in adult rats, since the force swimming test and the sucrose preference test showed an enhanced depressive-like behavior. In order to study the mode of action of the effects of TiO_2 NPs on the brain, Ma *et al.* [44] injected mice with anatase TiO_2 (5 nm) of various doses into the abdominal cavity daily for 14 days. The daily injection of high-doses of TiO_2 induced morphological changes in some neurons. The authors detected a statistically significant increased level of enzymes related to oxidative stress and injury of the brain after daily injections of high doses of TiO_2 NP. To investigate whether intravenous injection of TiO_2 NP carriers directly into the bloodstream can cause toxicity, an intravenous single injection of TiO_2 NPs at high doses was administered to mice. Only the highest dose caused acute toxicity effects in the brain, lung, spleen, liver, and kidney [60]. In a recent study Umezawa *et al.* [61] investigated the gene expression changes in brain tissues from male mice fetuses on embryonic day 16 and from postnatal days 2, 7, 14 and 21, after subcutaneous injection of TiO_2 into pregnant mice on gestational days 6-15. The prenatal TiO_2 exposure resulted in alteration of genes expression in the cerebral cortex, olfactory bulb and some regions intimately related to the dopaminergic system [61]. To explore the mechanisms by which TiO_2-NPs induce oxidative injury in the brain Ze *et al.* [62] studied the activation of the P38-Nrf-2 signaling pathway which is associated with oxidative stress in the mouse brain. TiO_2 NPs (5-6 nm) were intranasally administered for 90 days. A significant activation of the expression of different proteins associated with the p38-Nrf-2 signaling pathway was detected in the brain, namely p38, c-Jun N-terminal kinase, nuclear factor kappa B, Nrf-2 and heme oxygenase-1, which in turn led to increased production of reactive oxygen species, and lipid, protein and DNA peroxidation. Also the TiO_2-NPs caused increased proliferation of "spongiocytes" and hemorrhage in the mouse brain.

Ag-NP applications currently have quite a high degree of commercialization and is already present in a wide range of consumer products ranging from disinfecting medical devices and home appliances to water treatment (for review see [63]). In an interesting study different neurotoxic effects of 14 nm Ag-NPs and ionic silver were compared *in vivo*. In rats, Ag-NPs and ionic silver increased the dopamine concentration in the brain following 28 days of oral administration. The authors detected that the concentration of 5-hydroxytryptamine (5-HT, serotonin) in the brain was increased only after exposure to Ag-NP. Ionic silver however, increased the noradrenaline concentration in the brain. On the other hand, shorter exposure time (14 days) and lower Ag-NPs concentrations decreased the dopamine concentration, suggesting different, exposure-time dependent effects of silver on dopamine [64]. Liu *et al.* [65] found no cognitive changes or hippocampal neurogenesis in adult mice after intraperitoneal administration of Ag-NPs daily for 7 days. Neither reference memory nor working memory was affected by Ag-NPs and also no differences were observed in hippocampal progenitor proliferation, in newborn cell survival or differentiation.

Copper oxide (CuO), a semi-conducting compound, shows a range of potentially useful chemical and physical properties. Since it is cheaper than silver, easily mixable with polymers, and has relatively stable chemical and physical properties [66], CuO NPs are currently more and more present in different applications. Unfortunately, little is known about the neurotoxicity of the CuO nanoparticles *in vivo*. Therefore the cognitive impairment in rats induced by CuO-NPs and its possible mechanisms was studied by An *et al.* [67]. Wistar rats were treated with CuO-NPs via intraperitoneal injection for 2 weeks, and the Morris water maze (MWM) test and electrophysiological examinations were performed to examine the possible effects. After the exposure period the copper accumulation in hippocampus was significantly increased compared to the control group. CuO-NP also had toxic effects on the cognitive functions of rats. The authors suggest that the mechanism of neurotoxicity was due to in the impairments of synaptic plasticity, and in the induction of the imbalance of redox homeostasis in the hippocampus.

Autophagy is known as a cellular stress response, degrading damaged cellular components, which can be activated by many types of nanoparticles. CdSe/ZnS QDs can impair synaptic transmission and synaptic plasticity in the dentate gyrus (DG) area of the brain. Chen *et al.* [68] showed that elevated autophagy is partly responsible for the synaptic dysfunction induced by QDs in rats (intrahippocampal infusion). Autophagy inhibitors (wortmannin, 3-MA, or chloroquine) counteracted the induced autophagy, partly blocked LTP impairment, coincident with down-regulation of synapsin-I and synapse deficits by QDs in the hippocampal CA1 area [68].

Trpkovic *et al.* [69] recently reviewed the toxicity of pristine versus functionalized fullerenes showing that the limited number of studies, especially for the CNS, is not allowing conclusions about the toxicological behavior of any of the fullerene formulations. However, most of the fullerene preparations are not very toxic with exception of photoexcitation and/or at very high concentrations.

Taken together, many of the studies cited here show possible translocations of ENMs to the CNS, independent of the kind of application (Table **2**). As already pointed out, one of the main remaining questions is about the biological and the possible health relevance since dose-response relationships

Table 2. Investigations on the Nervous System After ENM Exposure *In Vivo*

ENM	Animal	Administration	Parameters / Dose	Effect	Refs.
TiO_2	CD-1 (ICR) mice	intranasal instillation	hydrophobic 1 μm or 10x40 nm; hydrophilic 50 or 10x50 nm with silica surface coating, 500 μg /per instillation, every second day for 30 days	accumulation in cerebral cortex and striatum of hydrophilic TiO_2, morphological changes of neurons in cerebral cortex, significant disturbance of the monoamine neurotransmitter levels in the sub-brain regions	[55]
	80 CD (ICR) mice	intranasal administration	5-6 nm, 2.5, 5 and 10 mg/kg for 90 days	oxidative damage in the brain via p38-Nrf-2 signaling pathway	[62]
	Rats	inhalation	21 nm, 12 mg/m^3, 5.6 h/d for 3 d	age-dependent up-regulation of neurotrophin expression	[58]
	Sprague-Dawley rats	subcutaneous injection of pregnant rats	25-70 nm; surface area 20-25 m^2/g; anatase	oxidative damage in the offspring hippocampus , depressive-like behavior in adulthood	[59]
	ICR mice	subcutaneous injection of pregnant mice	25-70 nm, total 0.4 mg	changes in gene expression of offspring's brain	[61]
	CD-1 (ICR) mice	injection into abdominal cavity	5 nm, various doses from 5 to 150 mg/kg, daily for 14 days, anatase	at high doses some neurons changed morphology, increased oxidative stress	[44]
	ICR mice	intravenous injection	42 nm, 0, 140, 300, 645, or 1387 mg/kg, anatase	high dose caused acute toxicity effects in the brain, lung, spleen, liver, and kidney	[60]
Ag	Wistar rats	oral administration	14 nm, 4.5 and 9 mg Ag-NP/kg/day, 9 mg kg/day Ag-ions	AgNP affected 5-HT, Ag-ions noradrenaline, both affected dopamine	[64]
	Sprague-Dawley rats	oral administration	10 or 25 nm, 100, 500 mg/kg/day for 28 days	silver accumulation in the brain did not clear after 4 months	[45]
	Sprague-Dawley rats	oral exposure	<20 nm noncoated, or <15 nm PVP-coated, 90 mg/kg , or $AgNO_3$ 9 mg/kg, for 28 days	AgNP and Ag ions showed similar effects, long retention of silver in brain and testis	[46]
	ICR mice	intraperitoneal injection	25 nm, 10, 25, and 50 mg/kg for 7 days	no effects on memory or on hippocampal neuro-genesis	[65]
	Wistar rats	intravenous administration	20, 80 and 110 nm, 23-28 mg/ml, once daily for 5 consecutive days	small part of total silver accumulated in the brain	[47]
CuO	CD-1 (ICR) mice	intranasal instillation	23.5 nm, 1, 10 and 40 mg/kg, every second day for 15 or 21 days	high level induced changes in neurotransmitter secretion	[56]
	Wistar rats	intraperitoneal injection	60.6 nm, 0.5 mg/kg/day for 2 weeks	increased Cu accumulation in hippocampus, cognition deficits	[67]
CdSe/ZnS quantum dots	C57BL/6 mice	intranasal instillation	PEG2-PE-encapsulated micelle with a total diameter of 15–20 nm with a QD core of 1.9 nm, average size 84 nm, 250 μg/ml, 1 h	3 h post exposure within the olfactory tract and olfactory bulb via axonal transport, activation of microglial cells	[57]
	Wistar rats	intrahippocampal infusion	20 nm, (5 μl) for 2 h	induced autophagy	[68]

(Table 2) contd….

ENM	Animal	Administration	Parameters / Dose	Effect	Refs.
Fe_3O_4	SD rats	intranasal instillation	30 nm, 20 µg total for 7 days	deposited from highest to lowest concentration in olfactory bulb, striatum, hippocampus, brain stem, cerebellum, and frontal cortex, and no other significant changes, 50% of Fe_3O_4-NPs remained in striatum and hippocampus 14 days post-instillation	[54]
	Albino Wistar rats	oral application	30 nm or bulk, single dose of 500, 1000, 2000 mg/kg	small part of the total applied nano-Fe accumulated in the brain	[48]
depleted UO_2	Wistar rats	nose-only inhalation	38 nm, 10^7 particles per cm^3, 1 h	no incorporation in brain or olfactory portion of the brain	[49]

Table 3. Investigations on the Nervous System After ENM Exposure *In Vitro*

ENM	Cell Type	Parameters / Dose	Main Findings	Refs.
TiO_2	PC12 cells	20 nm anatase, rutile; 24 h; 25-200 µg/ml	Anatase more toxic, concentration dependent; increased oxidative stress, apoptosis induction, G2/M arrest	[70]
ZnO	Retinal ganglion cells	60 nm; 24, 48, 72 h; 0.63-10.0 µg/ml	Increased ROS; apoptosis induction	[71]
	SHSY5Y neuroblastoma cells	60 nm; 3, 6, 24, 48 h; 0-80 µg/ml	Viability decrease; S-phase accumulation; apoptosis induction, genotoxicity	[72]
Ag	Primary rat cortex cells; mixed, neuron-enriched, astrocytes	20 and 40 nm; Au as control; 7, 14, 21 days; 5-100 µg/ml	Uptake in astrocytes; increased cell death, Ca^{2+} influx, oxidative stress; stronger effects from 20 nm NP	[73]
	Primary rat cortex cells	20 nm; 3 days; 1, 5, 10, 50 µg/ml	Decreased cell viability; neurite and synapse degeneration	[74]
	astrocytes	review	Up to 7 days treatment, no acute effects; instracellular accumulation of NP; increased HO-1 and MT levels	[75]
	PC12 cells	14 nm PVP-coated; 0.5, 5, 10 µg/ml; sub-nano fraction and AgAc in corresponding Ag-concentrations; 4-48 h	No uptake of Ag NP but Ag-presence detected intracellularly; apoptosis induction in all conditions on a comparable level	[64]
FeO	astrocytes	review	Endocytotic uptake of NP; ferritin-binding of NP	[75]
	Primary cultures from rat cerebellum and spinal cord; PC12 cells	24 h; 100 µg/ml	Uptake in microglia more pronounced than in other primary cells; PC12 uptake even stronger; microglia proliferation	[76]
	PC12 cells	11 nm Fe_3O_4 PEG-shell; 24 h; 5-40 µg/ml	Lysosomal uptake; NGF-potentiation	[77]
SiO_2	PC12 cells	20 and 50 nm; 12-48 h; 25 and 30 µg/ml	Decrease in survival; increased oxidative stress; inhibited NGF-induced differentiation	[78]
	U87 astrocytoma cells	12 nm; 48 h; 0.1-100 µg/ml	Decreased survival $\geq$25 µg/ml; mitochondrial effects	[79]

(Table 3) contd….

ENM	Cell Type	Parameters / Dose	Main Findings	Refs.
CeO	HT22 hippocampal cells	6 nm and 100 nm CeO, Al_2O_3 300 nm; 8, 16, 24 h; 0.04 M	Specific gene expression pattern for 6 nm CeO including induction of genes for neurological disease	[80]
	PC12 cells	5-80 nm; 24 or 72 h; 10, 20, 50, 100 μg/ml	Stimulation of NGF-induced differentiation; increased dopamine production; decreased ROS levels	[81]
Mn	Mesencephalic dopaminergic N27 cells	20 nm or 100-900 nm aggregates; 3, 6, 9 h; 25-400 μg/ml	Internalization; cytotoxicity; autophagy; apoptosis. Cell death independent of oxidative stress	[82]
	Murine primary mesencephalic nerve cells	20 nm or 100-900 nm aggregates; 3, 6, 9 h; 25-400 μg/ml	Increased cell death; decreased neurite length	[82]
Cu	Primary dorsal root ganglion cells	40, 60, 80 nm; 24 h; 10-100 μM	Smaller particles and higher concentrations more toxic (morphological effects, increased LDH-release)	[83]
TiO_2, HAP, Fe_3O_4	Primary microglia	TiO_2 20 nm, HAP 60 and 12 nm (short/long diameter), Fe_3O_4 45 nm; 2 h; 0.25 and 0.50 mg/ml	Cytokine production and NO production; strongest effects of TiO_2	[84]
MWCNT	Primary dorsal root ganglion cells	25 nm diameter, 10-20μm length; 24 h; 0.1, 1, 5, 10 μg/ml	Decreased axonal regeneration in crush-lesioned ganglia from 1 μg/ml	[85]
Fullerene	Primary hippocampal neurons	24, 48, 72 h; 1, 5, 25, 100 μM	Increased viability at 1 and 5 μM; increased DNA damage and apoptosis at 25 and 100 μM	[86]
CdTe QD	PC12 cells	2.4-4.7 nm with/without thioglycolic coating; up to 17 days; 1 nM	Decreased cell viability and apoptosis induction, more prominent for smaller particles and without coating	[87]
WC (tungsten carbide)	Hippocampal slices	5-20 nm primary particles, 115-704 nm aggregates; 10^{-7} – 10^{-4} g/ml;	Interference with both rectifying K^+-channels and voltage-gated Na+-channels, affecting action potentials	[88, 89]
CB, Fe_2O_3, TiO_2	Primary frontal cortex cells	CB 575 nm, Fe_2O_3 50 nm; TiO_2 91 nm; 0.0001-300 μg/cm^2	Concentration-dependent effects (strongest by TiO_2) on electrophysiological parameters. ROS increase by TiO_2	[90]

have not yet been carried out. It is still challenging to extrapolate the majority of these experiments to realistic chronic low-dose exposures since very high doses of ENMs have been applied. Regarding the physiological effects of these exposures, it is unclear to what extent, and at what exposure levels, nervous system functions can be affected by ENMs. However, taking together all available data, it seems that after very high exposure to the CNS, effects on neurotransmission, redox homeostasis and possibly on behavior can be expected.

4.2. *In Vitro* Studies

Wu *et al.* [70] investigated the effects of TiO_2 macro and nanoparticles) at various doses on several end-points in differentiated (NGF-induction) PC12 cells (exhibiting a phenotype resembling dopaminergic neurons) that were exposed for 24 h. For all investigated end-points, the anatase form of the particles exerted much stronger toxicological effects on the cells than rutile particles and the nanosized particles were much more potent than the microsized particles of the same substance. Studied end-points included cell survival and death, cell cycle regulation, and oxidative stress. In summary, the anatase particles were causing significant toxic responses in PC12 cells in a concentration dependent manner. The authors suggested that exposure caused increased oxidative stress that in turn triggered induction of apoptotic pathways via the mitochondria, also arresting the cells in the G2/M phase of the cell cycle.

Guo *et al.* [71] used rat retinal ganglion cells that they exposed to ZnO particles (60 nm) for 24, 48, or 72 h. The authors did not study uptake but suggested that the NPs are taken up and causing ROS (H_2O_2, and OH^-) increases which in turn cause increased expression of apoptosis markers. The apoptosis was induced via the intrinsic pathway from the mitochondria, which displayed a decreased membrane potential in the exposed cells. However, the authors did not investigate uptake of particles, so the possibility exists that the noted effects were emanating from the cell´s exterior. Valdiglesias *et al.* [72] used cultures of SHSY5Y human neuroblastoma cells that they exposed to 60 nm ZnO NP for different exposure times. The authors found both cytotoxic and genotoxic effects due to exposure. Since no uptake of

particles into the cells or any membrane leakage could be documented, the authors concluded that toxic effects were not due to internal accumulation and direct effects on mitochondria, but that indirect effects mediated via the cell membrane and possibly for some end-points also dissolved Zn^{2+} ions in the external medium. The findings included decreased viability, and increased levels of apoptosis markers as well as cell cycle disturbances (cells accumulating in the S-phase). Genotoxicity was seen as increased micronucleus production, H2AXγ phosphorylation, and DNA damage.

Both Haase *et al.* [73] and Xu *et al.* [74] used mixed cortical primary cultures from rats for their studies on effects of silver NP on primarily neurons. Haase *et al.* employed 20 and 40 nm Ag-particles and Au-NPs as controls. They exposed the mixed cultures for 7, 14, or 21 days. In addition, the study also employed neuron-enriched and astrocyte cultures for comparisons. Uptake of Ag-NPs was documented by TEM only in astrocytes, not in neurons. Both cell types exhibited increased rates of cell death (LDH-leakage) when exposed to the NPs, more so for astrocytes than for neurons, and more so when exposed to 20 than 40 nm particles. In addition to the cytotoxicity, the exposure caused an acute Ca^{2+}-response (within minutes) that was seen in both cell types. A later response was induction of oxidative stress, which was studied by measuring ROS in real-time in single live cells with fluorescence microscopy and also in cell populations with flow cytometry. In both cases, the response was stronger to 20 than 40 nm particles, and even stronger than the response to Ag^{+} ions ($AgNO_3$). Another sign of induced oxidative stress was the presence of protein carbonyls and induction of the oxidative stress-protecting protein Hemoxygenase-1 (HO-1). The authors concluded that their findings suggest a sequence of events starting with an immediate Ca^{2+} response preceding oxidative stress, which after longer time spans negatively affect cell viability. Another observation in the study is that the used doses (5-20 μg/ml for the mechanistic studies) are very high compared to the doses used in *in vivo* studies. The study by Xu *et al.* [74] used primary rat cortical cultures (newborn Sprague-Dawley pups) that were subjected to Ag-NP for three days. Unfortunately, the study does not provide any physical-chemical characterization of the used nanomaterials, and also lacks proper controls, decreasing the usefulness of the study for evaluation of NP toxicity. The authors reported effects by the exposure on all investigated parameters: cell viability; degeneration of neurites, and decreased levels of β-tubulin and F-actin; destruction of synaptic contacts and down-regulation of presynaptic (synaptophysin) and post-synaptic (PSD-95) proteins. Since the cultures contained both neurons and glial cells, it is likely that the reduced viability due to exposure is affecting both cell types. However, although this study seems to partly confirm findings from the study by Haase *et al.* [73], it is not to exclude that the observed effects are due to silver ions, and not from Ag-NPs.

A recent review from Hohnholt *et al.* [75] somewhat modifies this picture. The work points to that cultured mammalian astrocytes have been used in a number of studies on NP toxicity, including Ag. The authors summarize that in studies for up to at least seven days of exposure, astrocytes are not acutely damaged by the NPs, even at high concentrations. Astrocytes can accumulate significant amounts of NPs, which are stored preferentially in lysosomes, where the acid environment causes release of ions to the cytosol. These ions can cause the above mentioned immediate Ca^{2+} response and also up-regulation of the protective proteins HO-1 and metallothioneins, thus counteracting possible effects of the released ions that otherwise would be toxic. In conclusion, the authors argue that astrocytes can protect other brain components by taking up and safely storing NPs that can release ions, and thus be toxic.

Hadrup *et al.* [64] employed PC12 cells that were exposed to 14 nm AgNP (PVP-coated), or to a sub-nano filtered fraction of the AgNP which correspond to the same concentrations of silver ions that are found in the AgNP preparation, or to AgAc at 0.05, 0.5, 5, or 10 μg/ml. Exposure lasted for 4-48 h. No AgNP could be found inside the cells but the exposed cells still were found to contain Ag (as detected with ICP-MS), allowing the authors to conclude that either Ag-NP were internalized but rapidly degraded, or that silver ions released from the NPs could enter the cells. Furthermore, the AgNP induced apoptosis (TUNEL-staining) to the same extent as the other experimental conditions.

Hohnholt and co-workers [75] have also overviewed the literature pertaining to astrocytes and uptake and effects of magnetic iron oxide NPs. Their conclusion is that astrocytes by means primarily of endocytosis can accumulate significant amounts of the metal, increasing the intracellular concentration with a factor of 100-1000. The main storage of the ingested NPs occurs in the lysosomes, where the acid conditions also here may cause release of Fe^{2+}/Fe^{3+} ions, which in turn when reaching the cytosol can be sequestered by the protein ferritin. In this way, the authors argue that at least short-term detrimental effects of NP uptake can be avoided.

Also Pinkernelle *et al.* [76] found significant FeO uptake by glial populations, most notably microglia. The cells were primary cultures from neonatal rats and contained cells from the cerebellum and also organotypic spinal cord cultures cultured together with peripheral nerve grafts incubated with magnetic (iron) NPs (24 h) with a shell consisting of a green fluorescent dye and a polysaccharide mixture of glucuronic acid. Although all cell types internalized the particles, the microglia cells were by far the most active in that respect. In addition, incubation with the NPs also stimulated microglia to proliferate, which suggests that the NPs activated the cells. When comparing the uptake in primary cells with that in PC12 cells, it was shown that PC12 cells exhibited a much stronger uptake. This cell line was also the tool for a study by Kim *et al.* [77] who employed 11 nm Fe_3O_4 NPs with a PEG-phospholipid shell. After a 24 h incubation with various concentrations of NPs, TEM analysis documented substantial uptake into vesicular structures, presumably lysosomes. Interestingly, the presence of NPs potentiated the effect of NGF on differentiation of the PC12 cells. This was seen as increased neurite outgrowth, increased number of neurite-expressing cells, and increased levels of β3-tubulin compared to cells treated only with NGF. Furthermore, the cell adhesion molecule integrin β1 and phosphorylation of the signal transduction enzyme ERK1/2 was increased in NP-exposed cells. No cytotoxic effects were seen at any concentration at 1 day of exposure, whereas a modest cytotoxic response was noted at the highest (40 μg/ml) concentration at days 3 and 5.

Both Wang *et al.* [78] and Lai *et al.* [79] investigated effects of SiO_2 NPs on cell survival and other end-points in two human cell lines with origin in the nervous system. The work by Wang *et al.* employed NGF-treated PC12 cells that were subjected to 20 and 50 nm SiO_2 NPs for 12-48 h. The treatment caused a concentration and time-dependent decrease in cell survival which seemed to be mediated by increased oxidative stress. In addition, the treatment had negative effects on the NGF-induced differentiation where number of neurites/cell, neurite length, and intercellular contacts/cell all decreased as a function of dose. A similar effect on survival was seen in the work by Lai *et al.* [79] where U87 astrocytoma cells were exposed for 48 h to 12 nm SiO_2 NP. A dose-dependent decrease in cell survival was seen at $\geq$25μg/ml, accompanied by an increased activity of two mitochondrial enzymes and decreased levels of certain mitochondrial proteins and decreased ERK signaling.

Studies on naked CeO NP were performed by Lee *et al.* [80] who compared the effects of 6 nm CeO particles with 100 nm CeO particles and Al_2O_3 particles (300 nm) on murine HT22 hippocampal cells in culture. Incubation was for 8, 16, or 24 h (0.04 M) and the main end-point was gene expression. Each treatment gave rise to a distinct expression pattern, where the 6 nm CeO particles displayed the highest number of unique gene responses (230 genes that were not affected in any of the other conditions). The genes belonged to a wide range of disease groups including "neurological disease" (13 genes). Remarkably, these genes included Htt (huntingtin) that was strongly down-regulated in the array analysis, also confirmed by separate studies on the RNA (quantitative RT-PCR) and protein (Western blot) levels. Also Ciofani and co-workers [81] focused on CeO NP in their study on PC12 cells. Size distribution analysis revealed that the particles covered a broad range in size (5-80 nm). Treatment of NGF-treated cells was for 24 or 72 h, at concentrations up to 100 μg/ml. During those conditions, no effect on cell viability was documented, whereas differentiation (measured as neurite length and expression levels of β3-tubulin and neurofilament-66 proteins) was stimulated. Also dopamine production was stimulated by the presence of nano-ceria. Interestingly, the authors suggest that these beneficial effects were due to the decreased ROS levels that also were seen.

The cytotoxic effects of nano-sized Mn were shown by AfesehNgwa *et al.* [82] in a study where they employed rat mesencephalic dopaminergic cells (N27 cell line). According to the authors, the NPs were either single NP (20 nm) or agglomerates (100-900 nm sizes) during the experimental conditions. TEM and DIC microscopy both showed that the NPs were internalized by the cells. Furthermore, the particles were shown to be cytotoxic in a dose and time-dependent manner. Furthermore, the NPs induced oxidative stress, transferrin upregulation, autophagy and apoptosis. The cell death induction was independent of oxidative stress as seen by the use of an antioxidant cocktail. Also in murine primary mesencephalic nerve cell cultures, Mn NPs induced cell death and decreased neurite length. The study shows that the detrimental effects of Mn on dopaminergic neurons seen in chemical toxicology studies also are caused by Mn in the form of ENM.

In a study employing rat dorsal root ganglion (DRG) primary cells, Prabhu *et al.* [83] could show that Cu NP display cytotoxicity in a size and dose-dependent manner. The cells were treated with 40, 60, 80 nm NPs for 24 at concentrations between 10 and 100 μM. As seen by TEM, the particles tended to aggregate in the culture medium. Staining with rubeanic acid showed intracellular presence of Cu. The NP exposure exerted morphological (vacuoles, detachment from substrate, disrupted neurites) and toxic (increased LDH release) effects. The most pronounced effects were seen for smaller sized NPs at the higher concentrations.

Xue *et al.* [84] performed a comparative study where they exposed primary cultures of microglia cells from postnatal Sprague-Dawley rats to several NPs. The NPs were SiO_2 (20 nm), TiO_2 (20 nm), hydroxyapatite (HAP; 60 and 12 nm respectively for the long and short diameter) and Fe_3O_4 (45 nm) at different concentrations. The study also included a positive control, the bacterial lipopolysaccharide LPS (1 μg/ml; 24 h). Treatment of the microglia with TiO_2 and HAP caused NO production via iNOS activation and also chemokine (MCP-1, MIP-1α) production. All four investigated NPs caused cytokine (TNF-α, IL-1β, IL-6) production (at a level lower than LPS treatment). For all investigated end-points, the TiO_2 treatment gave the strongest responses in the microglia cultures. The authors furthermore took cell-free culture supernatant from cells treated with the NPs and exposed PC12 cells to these supernatants. The supernatants from TiO_2 and HAP-treated cells caused decreased levels of tyrosine hydroxylase and also modest induction of cell death in the PC12 cultures.

Wu *et al.* [85] recently published a study where crush lesions were induced in DRG sensory neuron axons on 8-week-old CD-1 mice. Subsequently, the DRG neurons were cultured *in vitro* and there subjected to treatment with MWCNT (25 nm diameter; 10-20 μm length). The axonal regeneration (length of axonal elongation after 24 *in vitro* treatments with 10 μg/ml MWCNT) was evaluated with light microscopy and apoptosis was investigated with TUNEL-staining. The DRG neurons incubated with MWCNT exhibited stunted neurite outgrowth (69 μm vs 330 μm in the controls), and fewer processes and branches than the vehicle-treated control cell cultures. No effects on apoptosis were seen. Also lower concentrations were causing inhibition of axonal regeneration, albeit at lower levels. No effects were seen only in cultures treated with the lowest concentration.

Zha *et al.* [86] investigated viability in primary hippocampal neurons from newborn Wistar rats that were treated with polyhydroxyfullerene (fullerenol) for 24, 48 or 72 h. Viability was affected by treatment in concentration-dependent manner. Viability was increased at 1 and 5 μM compared to the controls, whereas DNA damage (Comet assay) and apoptosis (Caspase-activation) was seen at 25 and 100 μM fullerenol.

Possible cytotoxic effects of CdTe QDs were published by Prasad *et al.* [87] who used PC12 cells cultured in the presence of particles for up to 17 days in their study. The cells were subjected to two types of QDs ("red" and "orange" respectively) that in turn were with or without a coating (thioglycolic acid). Cell viability, cytotoxicity and early apoptosis markers (caspase activation) were investigated. In

general, the smaller particles were more toxic than the larger particles, and particles with the gel coating were less toxic to the PC12 cells than the un-coated forms.

Functional studies of neurons *in vitro* include various types of electrophysiological investigations, where the whole-cell patch-clamp technique is one of the most informative approaches. Shan and co-workers [88, 89] employed this technique to hippocampal slices from male Wistar rats (at post-natal days 10-14). The slices were incubated with tungsten carbide (WC) NPs (primary particle size 5-20 nm; aggregate sizes 115-704 nm). In the first study, [88] it was seen that the WC NPs (at 10^{-7} g/ml) significantly decreased K^+-currents and thus prolonged action potential duration and lowered the firing rate. Effects were also noted on voltage-gated Na^+-channels [89] where the particles (at concentrations of 10^{-6}–10^{-4} g/ml) inhibited the opening of the channels so that peak amplitudes, overshoots and V-thresholds of the action potentials were reduced. These studies suggest that WC NPs have the potential to act in a neurotoxic fashion by directly interfering with ion channels responsible for the basic functions of neurons.

Another form of electrophysiological approach was used by Gramowski *et al.* [90] in their study on primary cultures from the frontal cortex of embryonic mice (embryonic day 15; crl:NMRI mice). The cultures contained both glia cells and neurons and were placed on multielectrode array glass chips that allowed simultaneous exposure to NPs and measurement of a battery of electrophysiological parameters. The cultures were treated with carbon black (575 nm), hematite (Fe_2O_3) (50 nm) and TiO_2 (91 nm). TEM could confirm intracellular presence of all particles. All three substances affected various electrophysiological parameters (e.g. spike rate, burst rate, burst duration, number of spikes within a burst) in a concentration-dependent manner, with the strongest effects exerted by TiO_2. ROS was also measured and were up-regulated only by TiO_2 at 5 and 10 $\mu g/cm^2$ (24 h exposure).

To summarize, a significant number of *in vitro* studies using either primary cultures from the mammalian nervous system (including experiments on both neurons and glial cells) or established cell lines with nervous system origin have been performed. Many types of NPs and many endpoints have been studied. Although the studies are incoherent regarding many aspects, and lacking many critical considerations, it is clear that detrimental effects to cells can be caused by NPs. These effects are, however, not necessarily unique to NPs but can appear as consequences of exposure to the bulk material as well. What seems to be consistent though, is that there are classical linear dose-response patterns (with chemical concentrations providing the "dose"), and that smaller particles of a certain species are more reactive than larger ones. Furthermore, almost all studies reporting effects also see NPs exerting oxidative stress, if investigated. Beyond that, observations are often too singular to allow more general conclusions.

5. CONCLUSION AND SUMMARY

Certain conditions would allow ENM to enter and subsequently pass over the BBB and thus reach the brain. Furthermore, present knowledge suggests that the nano-form rather than the bulk form of the chemicals pass the BBB, and that there is an inverse relationship between particle size and ability to penetrate the BBB. The possibility that intranasal deposition of ENM can lead to entrance to the brain without passing the BBB and instead via axonal transport has been shown in several studies. The question remains if this is a realistic pathway to the brain, or just occurring under very specific experimental conditions.

Both *in vivo* and *in vitro* studies provide evidence for effects of ENM on several aspects of nervous system function, including detrimental consequences. However, a weakness in the majority of the studies is the lack of positive controls, reference materials, and also that the bulk material was not included in the studies. In summary, exposures at very high levels to the CNS seem to be able to affect neurotransmission, redox homeostasis and possibly behavior.

Most of the studies that are referred to here have been using NP concentrations that are far higher than ones that can be expected for any consumer or even in a situation where an unintended occupational exposure takes place. There is almost no specific ENM regulation for occupational exposure. One exception though, is a recommended exposure limit issued by NIOSH [91] for airborneTiO_2NP. The suggested limit is based on available toxicity studies regarding tumor formation and exposure. The limit is set to 0.3 mg/m^3 as a time-weighted average for up to 10 hours per day during a 40h (work) week. Another NIOSH based suggested exposure limit concerned carbon nanotubes (CNT) and carbon nanofibers (CNF) which is also based on available data on toxicity [92]. This suggested limit would be 0.2 and $2g/m^3$ CNT in the air. Since the employed exposures include conditions that in most cases are not to be found, and sensitive *in vivo* or *in vitro* conditions are used, a conclusion must be that real-life situations would in very few, if any, cases allow acute effects on the nervous system.

The prospects for intended or targeted medical applications, based on existing toxicological knowledge should be very promising since it has been shown that ENM can pass the BBB and reach specific regions or cells within the brain.

Importantly, studies up to now have been focusing on possible detrimental effects of the first generation of ENM. Development of more "sophisticated" materials is under way, and will be parts of consumer products in the near future. It will continue to be necessary to study possible health effects of these materials, independent of the outcome of present studies.

CONFLICT OF INTEREST

The preparation of this article was financed by intramural grants from the AIT, Austrian Institute of Technology. The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

Both authors conceived of the article and participated in data collection and screening, data analysis, drawing of conclusions, drafted the manuscript, read and approved the final manuscript.

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Received: May 26, 2014 **Revised: July 04, 2014** **Accepted: July 11, 2014**