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Review Article

Neurotoxicity of nanoscale materials☆

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ABSTRACT

Nanotechnology has been applied in consumer products and commercial applications, showing a significant impact on almost all industries and all areas of society. Significant evidence indicates that manufactured nanomaterials and combustion-derived nanomaterials elicit toxicity in humans exposed to these nanomaterials. The interaction of the engineered nanomaterials with the nervous system has received much attention in the nanotoxicology field. In this review, the biological effects of metal, metal oxide, and carbon-based nanomaterials on the nervous system are discussed from both *in vitro* and *in vivo* studies. The translocation of the nanoparticles through the blood–brain barrier or nose to brain via the olfactory bulb route, oxidative stress, and inflammatory mechanisms of nanomaterials are also reviewed.

1. Introduction

As a rapidly growing emerging science, nanotechnology has shown a significant impact on almost all industries and all areas of society. Nanomaterials, defined by the National Nanotechnology Initiative, have at least one dimension in the range of 1–100 nm. Due to their small size, the properties of nanomaterials may differ from those of their bulk materials, showing unique chemical, physical, optical, and electrical properties. Nanotechnology involves creating and applying engineered materials at the nanoscale to take advantage of these specific properties. Humans have been exposed to many nanoparticles (NPs) originating from various activities such as combustion, welding, and biomedical applications. People working in certain industries, for example, automobile, aerospace, electronics and communications, and chemical and paint industries are at high risk of being exposed to a large amount of NPs [1–10]. As NPs persist in the environment, people living in those environments are at higher risk of NP exposure. Copper, zinc, iron, cerium, silver, gold, iron, manganese, titanium, aluminum, silica, and other carbon-based nanomaterials are some of the NPs to which humans are exposed significantly and may cause several health-related problems including neurotoxicity.

☆ The views presented in this article are those of the authors and do not necessarily reflect the views or policies of the US Food and Drug Administration.

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In recent years, a significant number of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, or Huntington's disease have been diagnosed and treated. The increased amount of environmental pollutants, including NPs, may be responsible for increasing the number of these neurodegenerative diseases. The role of the blood–brain barrier (BBB) is crucial in understanding NP toxicity in the brain. BBB separates blood from cerebrospinal fluid in the central nervous system (CNS). The BBB is an extended plasma membrane that contains tight junctions between the adjacent endothelial cells of the cerebral capillaries. The permeability properties of the BBB are of interest [1,11]. Unlike noncerebral capillaries, the cerebral endothelium does not have vesicles for macromolecular transport. Astrocytic end feet cover most (85%) of the cerebral capillary endothelial cells and they also contain a thick basement membrane [12]. The presence of such complex combinations of astrocytes, cerebral capillaries and basement membrane strongly supports the BBB function [11,13], even though establishing the clear cut roles of the basal lamina and/or astrocytic end feet in maintaining BBB permeability needs further study. When NPs reach the circulation, they may interfere with the function of the endothelial cell membrane. The effect of NPs on the cell membrane may be due to their direct toxicity, or indirectly, they may induce some cascade mechanism that disrupts the tight junctions in the BBB or alters the permeability of the membrane. It has been shown that intravenous, intraperitoneal, or intracerebral administration of Ag, Cu, or Al NPs (50–60 nm) reportedly disrupts the BBB, as indicated by staining with albumin-bound Evans blue [14]. Vesicular transport may also be stimulated by NPs in order to gain access to the CNS microenvironment to exert toxic effects in the CNS. The unique size and surface modification of NPs could deliver drugs or therapeutic agents to the brain in the development of nanomedicine. Additional research is, however, necessary in order to understand fully how NPs are translocated from the blood to the brain across the BBB.

Nanomaterials could enter the human body by different routes including inhalation, dermal penetration, ingestion, and systemic administration, by which NPs may be accumulated in different tissues and organs including the brain [15,16]. It has been indicated that the olfactory nerve pathway may serve as a portal of entry for NPs into the CNS in humans who are environmentally or occupationally exposed to airborne NPs [17–19]. De Lorenzo [18] showed that when silver-coated colloidal gold particles (50 nm) were intranasally instilled in squirrel monkeys, the NPs anterogradely moved in the axons of the olfactory nerve to the olfactory bulbs. Olfactory epithelium that has been exposed to manganese, cadmium, nickel, and cobalt nanomaterials can translocate the nanomaterials to the brain via olfactory neurons [20–25]. Therefore, full understanding of the neurotoxicity of these nanomaterials may lead to the design of safer therapeutics and reduce the side effects of these nanomaterials in future.

Having a greater surface area than their bulk counterparts, metal oxide NPs are used in various fields such as water treatment, medicine, cosmetics, and engineering, and provide superior performance in their applications. Unfortunately, almost no federal or state laws have specifically established regulations for the manufacture, transportation, use, sale, or disposal of nanomaterials [26]. For metal oxide NPs, their widespread application, small size, and large specific surface area endow them with high chemical reactivity and intrinsic toxicity, and their health effects in living creatures, especially on the nervous system, have been of concern. Metal oxide NPs are capable of translocating along the olfactory nerve pathway to the brain after intranasal instillation, and accumulating in the olfactory bulb, cortex, and cerebellum. Moreover, NP deposition in the brain can stimulate oxidative stress, inflammatory responses, and pathological changes. These observations have provided evidence that metal oxide NPs can reach the brain and cause a certain degree of tissue damage.

Metal oxide toxicity can also be induced by dissolved metal ions from the oxides. Brunner et al [27] studied the toxicity of NPs in human and rodent cell lines. They divided the tested NPs into soluble and insoluble NPs, and showed that the toxicity of soluble NPs was from the soluble metal ions released from NP dissolution prior to or after the NPs entered the neural cells. Considering the unique physicochemical properties, including small size effect, large specific surface area, and high biological surface reactivity, NPs might induce the neurotoxicological behavior and effects in organisms.

2. Neurotoxicity and mechanism of nanomaterials

2.1. *Titanium dioxide NPs*

Among several metal-based NPs, those originating from titanium have been used widely and in large quantities. Titanium dioxide (TiO_2) is the most common compound of titanium that has found a variety of uses in our lives. TiO_2 is a white, odorless, water-insoluble material that was believed to have low toxicity [28–31]. TiO_2 is a relatively stable, nonflammable material that is found naturally in the form of various ores such as rutile, anatase, and brookite. TiO_2 can also be extracted from an iron-containing mineral ($FeTiO_3$) known as ilmenite [32–36]. TiO_2 possesses certain physiochemical properties that make it useful for multiple applications. Corrosion resistance, biocompatibility, mechanical strength, whitening property, opacity, and photocatalytic, optical, and electrical activity are some of the attractive properties that have paved the way for large-scale applications of TiO_2 [37]. The National Nanotechnology Initiative of America classifies nanoparticulate TiO_2 particles as one of most widely manufactured NPs globally [38].

Industrially, 80% of TiO_2, including its nanoparticulate form (globally), is used to produce paints, varnishes, plastic, and papers. Besides these applications, nanoparticulate TiO_2 has major uses in developing various products such as cosmetics, foodstuffs, toothpaste, sun blocks, printing ink, car materials, rubber, cleaning products, materials for industrial photocatalytic applications including solar cells, and catalysts for remediation of organic matter in wastewater [39]. Toxicity of nanosized TiO_2 has yet to be completely understood despite its widespread uses. Recent toxicological studies have indicated harmful effects of TiO_2 NPs in biological systems, which is of major concern [40]. It has been recently recognized that TiO_2 may be carcinogenic to humans if inhaled [31]. As a

result, it is of great importance to understand the risks and hazards including neurotoxicity associated with nanoparticulate TiO_2 exposure and its dose-dependent response [41]. Irrespective of the different forms of TiO_2, exposure route and particle size, it has been found that TiO_2 NPs translocate to different parts of the brain [39,42–46]. The NPs accumulate in this organ and induce structural changes in the neuronal architecture [39,43,45]. As mentioned previously, when NPs are inhaled, they can translocate to the CNS using the olfactory nerve as a means of entry. Several studies in mice have indicated that rutile NPs can translocate to the brain and accumulate throughout the organ, primarily in the hippocampus regions [39,43,45]. Such a neuronal translocation pathway of TiO_2 NPs may be responsible for neurotoxicity. TiO_2 NPs when instilled intratracheally in mice accumulate in the brain via the blood circulation and penetration of the BBB. This type of accumulation is responsible for inducing tissue damage [42]. Accumulation of nanoparticulate TiO_2 in the brain induces release and metabolism of neurotransmitters such as norepinephrine and 5-hydroxytryptamine [39,43,45,46]. After intranasal exposure of TiO_2 NPs, enhanced levels of the above-mentioned compounds were detected [43]. However, a decrease in response was detected when anatase TiO_2 NPs were administered intragastrically [45]. Reduced levels of homovanillic acid, dopamine, 5-hydroxyindole acetic acid, and 3,4-dihydroxyphenylacetic acid were detected when TiO_2 NPs were administered intranasally or intragastrically [43,46]. Enhanced catalase and acetylcholinesterase activity was detected during intranasal instillation of rutile [39] and intragastric administration of anatase TiO_2 NPs [46]. Acetylcholine, glutamic acid, soluble protein carbonyl, and nitric oxide content were also increased by such NP treatments. When anatase TiO_2 NPs were intraperitoneally injected, increased nitric oxide but decreased acetylcholine and glutamic acid were detected [44]. Hu and colleagues [46] showed that the levels of sodium, potassium, magnesium, calcium, iron, and zinc in the brain were changed after nanoparticulate TiO_2 exposure. In that study, the treated mice had impaired spatial recognition memory, which could be linked to the disturbed homeostasis of neurotransmitters, trace elements, and enzymes in the brain [46]. Proteomic analysis showed differentially expressed proteins in the brain in response to TiO_2 NP exposure, even though no NPs were detected in the tissue [47]. Oxidative-stress-related damage with a consequent change in the balance between oxidative and antioxidative activities was observed both *in vitro* [48–50] and *in vivo* [39,42,44,45,47]. Levels of malondialdehyde, an oxidative marker, increased after intranasal instillation [39,44] of TiO_2 NPs. A similar effect was also found with intraabdominal injection and intratracheal instillation of TiO_2 NPs in mice [42]. Reactive oxygen species (ROS) such as superoxide [42], hydrogen peroxide [42,45], and hydroxyl radical [42] were also found to be increased in animals treated with TiO_2 NPs. Increased cytokine levels, which are indicative of inflammatory effects in the brain, were detected in animals treated with TiO_2 NPs [44,51]. TiO_2 NPs (P25 Degussa TiO_2 and rutile forms) when injected intraperitoneally in mice induce an increase in lipopolysaccharides, and alter the mRNA levels of interleukin IL-1β and tumor necrosis factor (TNF)-α, as well as IL-1β protein. Lipopolysaccharide induction was necessary to cause this phenomenon, which suggests the importance of a trigger element or a possible synergistic role in tissue responses to nanoparticulate TiO_2. The embryotoxic role of TiO_2 was also studied by maternal intravenous injection of TiO_2 NPs, which yielded no characterized TiO_2 NPs [52], and by subcutaneous injection of TiO_2 NPs in the anatase form [53–55].

In the case of subcutaneous injections, TiO_2 accumulation was found in the offspring cerebral cortex and olfactory bulb. A large number of olfactory bulb cells were found to be positive for markers of apoptosis [53]. Altered gene expression was detected for prenatal TiO_2 NP exposure, which was involved in cell death, brain development, and the response to oxidative stress in newborn pups [54]. Finally, the influence of prenatal TiO_2 NP exposure on the dopaminergic system was established as increased levels of homovanillic acid, dopamine, 3,4-dihydroxyphenylacetic acid, and 3-methoxytyramina hydrochloride in the prefrontal cortex and neostriatum of exposed mice [55]. These findings indicate that TiO_2 NPs can be carried from the mother to the fetal brain, which ultimately has a toxic effect on fetal brain development, leading to several nervous system disorders. More in-depth studies are necessary in order to understand fully the toxic effect of TiO_2 NPs on neurons in various stages of life, including during pregnancy and early stages of development.

2.2. *Zinc oxide NPs*

Like TiO_2, another metal-based NP is zinc oxide (ZnO), which has broad uses and applications. ZnO is also white, thermally stable, and a naturally occurring material. It can be used to develop sunscreens, biosensors, food additives, cement, rubber, ceramics, pigments, plastic, catalysts, and electronic materials. ZnO shows antibacterial activities and in recent years studies have also focused on the effect of nanoparticulate ZnO on various microorganisms [56,57].

In recent years, ZnO toxicity has been demonstrated both *in vitro* and *in vivo* in various mammalian cells. Dissolved Zn^{2+} from the NPs is responsible for the toxicity. ROS were detected in these studies and may have been responsible for the inflammatory effects associated with ZnO toxicity. The neurotoxic effect of ZnO has not been studied much. In one of the early works, neurotoxicity of different-sized ZnO NPs (10–200 nm) in mouse neural stem cells (NSCs) was investigated. As determined by cell viability studies, ZnO NPs showed dose-dependent toxic effects towards NSCs. However no size-dependent toxic effects on NSCs were found in this study [58]. Using confocal microscopy, transmission electron microscopy, and flow cytometry, apoptotic cells were detected and analyzed in this toxicity study. Like previous studies, the results indicate that ZnO NP toxicity originates from the dissolved Zn^{2+} in the culture medium or inside the cells [58]. The effects of ZnO NPs on voltage-gated sodium and potassium pumps and action potential generation have been studied by Zhao et al [59]. The study on isolated rat hippocampal CA3 pyramidal neurons demonstrated that ZnO NP solution was able to generate neuronal injury by inducing depolarization through activation of voltage-gated sodium channels, and led to higher Na^+ influx and intracellular accumulation of Na^+ and Ca^{2+}, release of glutamate, and neuron excitability. ZnO

NPs are also able to induce neuronal apoptosis by depleting intracellular K^+ level due to increased ion efflux [59]. An *in vivo* toxicity study involving rats showed that intraperitoneal ZnO altered synaptic plasticity, which changed spatial learning and memory ability [60]. In that study, 20–80-nm ZnO NPs (4 mg/kg body weight) twice weekly for 8 weeks were administered to rats. ZnO NPs synthesized using the sol–gel method and starch as a template have been tested for *in vitro* cytotoxicity in neuro2A cells. A dose-dependent toxicity profile was obtained, whereas nontoxic effects were seen at a concentration < 6 μg/mL [61].

More studies have shown that the antibacterial activity or adverse effects of ZnO NPs are partly due to the generation of ROS [62–69], or causing membrane damage through the direct NP–cell membrane interaction or generation of ROS [56,65], or release of Zn^{2+} ions in the ZnO NP suspensions [27,67]. Studies in mammals have suggested that oral exposure of ZnO NPs causes an increase in blood viscosity and pathological lesions in the stomach, liver, kidney, pancrea, and spleen [70]. However, the potential hazards of high concentrations of manufactured nanoscale ZnO on the CNS need further investigation.

2.3. *Manganese oxide NPs*

Manganese is an important metal. It is a trace element and necessary for survival. In plants in photosystem II, a manganese-containing metal cluster is responsible for oxygen generation from water activity and there are several enzymes that use manganese for their activity [71]. Manganese has found several other uses in our lives. Manganese is a major component of making different types of steel and cast iron [72]. Manganese chloride is used in batteries, disinfectants, dyes, paint driers, and dietary supplements. Oxides of manganese, such as manganese oxide (MnO), are used in colored glass, ceramics, paints, textile printing, fertilizers, and in food supplements and additives. Manganese dioxide (MnO_2) is used in batteries and may also be generated from the welding of manganese alloys. Use of manganese-containing welding rods is a major source of occupational exposure to welders. Manganese tetroxide (Mn_3O_4) may be generated in situations where other oxides of manganese are heated in air [73]. Methylcyclopentadienyl manganese tricarbonyl is used as an antiknocking agent in some unleaded gasolines. The compound is released to the environment during fuel combustion in the form of manganese sulfate, phosphate, and oxides. Farm workers who work with Maneb (manganese ethylene-bis-dithiocarbamate) may also be exposed to a significant amount of manganese [74].

As manganese is known for its neurotoxicity, toxicity studies associated with manganese-containing nanomaterials provide a useful test case in the evaluation of nanomaterial toxicity [75]. The occupational disease associated with manganese exposure and toxicity is known as manganism. The disease in later stages resembles Parkinson's disease [76]. It has been found that if manganese is inhaled in water-soluble and water-insoluble forms, it is translocated to the brain, crossing the BBB via the olfactory nerve pathway [77]. It has been found that, among many metals, manganese is preferentially taken up via the olfactory nerve route [21,78]. After nasal exposure to manganese oxide NPs (MnO, MnO_2, Mn_2O_3, and Mn_3O_4), the concentration of manganese in the olfactory bulb, striatum, frontal, and other brain regions is increased. Macrophage inflammatory protein-2, glial fibrillary acidic protein, and neuronal cell adhesion molecule mRNA is also increased in the olfactory bulb. The results indicate that the olfactory neuronal pathway is efficient for translocating inhaled manganese oxide as solid ultrafine particles to the CNS and can result in inflammatory changes [24]. Although absorption of manganese in the lungs is dependent on particle size and solubility [24,79], for neuronal manganese uptake and further translocation into the CNS, dissolution of manganese is not necessary. As mentioned earlier, major sources of ultrafine manganese oxide particles include the iron and steel industries, battery production, ferroalloy production, and power plant and coke oven combustion emissions [80]. Use of glass, paints, and ceramics may also provide major sources of manganese oxide. Methylcyclopentadienyl manganese tricarbonyl is presently used in gasoline, mainly in Canada and Australia [81,82], and decomposition and oxidation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) during combustion may release manganese oxide of nanoparticulate size into the environment. In all of these cases, the most likely route of human exposure is through inhalation. Toxicity of various manganese oxide nanomaterials has been investigated in a neuronal precursor cell model. The Promega CellTiter Aqueous One Solution Cell Proliferation (MTS) assay was used to evaluate mitochondrial function in living cells and the lactate dehydrogenase (LDH) assay was used to quantify the release of the enzyme as a result of damage to the cell membrane. Both assays indicated that manganese toxicity was dependent on the type of manganese oxides and their concentration. State of cell differentiation also contributed to varying NP toxicity. Manganese oxide NPs are responsible for the generation of ROS and cell death due to apoptosis, as revealed by flow cytometry. During cell division, exposure to manganese oxide NPs results in elevated levels of the transcription factor nuclear factor NF-κB. Such enhanced levels of NF-κB mediate the cellular inflammatory response [83]. In another study, Hussain et al [84] investigated the effect of manganese oxide NPs (40 nm) on dopamine production in PC12, neuronal phenotype cells. Manganese oxide NPs induced depletion of dopamine and its metabolites dihydroxyphenylacetic acid and homovanillic acid in PC12 cells, with a similar mechanism as Mn^{2+} [84]. In an *in vivo* study, adult male Wistar rats were exposed to MnO_2 NPs of ~23 nm diameter. The experiment was a model study to understand the inhalational risks associated with MnO_2 NPs. MnO_2 NPs were instilled into the trachea for several weeks in daily doses of 2.63 mg/kg and 5.26 mg/kg. The endpoints of functional neurotoxicity (open field behavior and electrophysiology) and general toxicity (body and organ weights) were investigated. Animals treated with MnO_2 did not gain weight after 6 weeks exposure. High levels of manganese were detected in brain and blood samples of the treated animals after 9 weeks exposure. The open field behavior of treated rats showed decreased ambulation and rearing, and increased local activity and immobility were observed. Electrophysiological studies of animals treated for 9 weeks indicated a shift in spontaneous cortical activity to higher frequencies,

lengthened cortical evoked potential latency, and slowed nerve conduction. Many of these neurofunctional and general parameters were significantly correlated with the tissue manganese levels. It can be concluded that the instilled manganese in the NP form was absorbed and the NPs were responsible for the neurotoxic effects [85].

The acute oral toxicity of MnO_2 NPs and MnO_2 bulk particles in female albino Wistar rats was investigated [86]. MnO_2 NPs (45 nm) exhibited higher absorption and tissue distribution compared with MnO_2 bulk particles. The histopathological analysis revealed that MnO_2 NPs caused alterations in the liver, spleen, and brain. The neurotoxicity of 45-nm MnO_2 NPs in the brain and red blood cells, as determined through acetylcholinesterase activity, was significantly inhibited at doses of 1000 mg/kg and 500 mg/kg. MnO_2 NPs (45 nm) disrupted the physicochemical state and neurological system of the animals through alterations in ATPases via the total Na^+–K^+, Mg^{2+}, and Ca^{2+} levels in the brain. Toxicity of Mn_3O_4 NPs was investigated in ST-14 rat striated neuroblasts, a neuronal precursor cell model, using the MTS assay to evaluate mitochondrial function in living cells and the LDH assay to quantify the release of the enzyme as a result of damage to the cell membrane [87]. Both assays showed that the toxicity of Mn was dependent on the type of manganese oxide NPs and their concentration, as well as the state of cell differentiation. Following exposure to manganese oxide NPs, ROS were generated, and flow cytometry experiments suggested that cell death occurred through apoptosis. During exposure to manganese oxide nanomaterials, increased levels of the transcription factor NF-κB (which mediates the cellular inflammatory response) were observed.

2.4. *Silver NPs*

Silver is a bright, silvery white, soft metal that has been used for thousands of years. Silver ornaments, utensils, and art work have been around for a long time. Silver has monetary value and silver coins and jewelry are considered as valuables. Silver is used in large quantities as catalysts, mainly in the production of ethylene oxide. It is also used industrially for conductors, mirrors, and photographic applications. One of the interesting properties of silver is its antibacterial and antifungal activity. As a result, the use of nanoparticulate silver is one of the fastest growing areas of commercial NP applications [88]. Due to their excellent antibacterial properties, silver NPs have been used in food services, building materials, textile industry, medical instruments, personal care products, and washing machines [89]. Silver NPs (Ag NPs) are used as room sprays, deodorants, wall paints, and laundry detergents, and are also used for indoor air purification and water detoxification [90,91]. As a result of these widespread uses and exposure of silver NPs to humans, it is likely that Ag NPs enter the body and accumulate in various tissues and organs [92]. Previous research has indicated that Ag NPs can accumulate in several organs, which includes the kidney, liver, testis, lung, and brain [93].

In vitro studies have shown that Ag NPs are capable of inducing toxicity in cells derived from a variety of tissues, including liver, skin, vascular system, lungs, and reproductive organs. Previous studies have shown that Ag NPs induce cell death and oxidative stress in human skin carcinoma and fibrosarcoma cells [94]. The same group have also reported that Ag NPs can enter cells, causing DNA damage and apoptosis in liver cells and fibroblasts [95]. Cell viability is decreased when alveolar macrophages and lung epithelial cells are treated with Ag NPs [96]. *In vitro* studies have shown Ag NP toxicity in neural-like cell lines, such as PC12 cells, which is a rat cell line with a neuronal-like phenotype [97].

It has been shown that Ag NPs could come across through and be accumulated in brain microvessel vascular endothelial cells. An *in vitro* BBB model composed of primary rat brain microvessel vascular endothelial cells, it has been shown crossing and accumulation capability of silver nanoparticles [98]. Ag NPs can induce inflammation and affecte the integrity of this BBB model, and be readily translocated to the brain [99]. Ag NPs can also induce BBB damage, astrocyte swelling, and neuronal degeneration [100]. Ag NPs can translocate to the brain using the nasopharyngeal system as a gateway during inhalation exposure [17]. *In vivo* studies by Liu and coworkers have shown the effects of Ag NPs on hippocampal synaptic plasticity and spatial cognition in rats. Their studies have revealed that intranasally administered Ag NPs induce impairment of hippocampal function [101]. These results suggest that Ag NPs cause neurotoxicity in humans and other animals. More recently, a significant finding indicated that 7-nm Ag NPs decreased motor activity and body weight in a time- and dose-dependent manner after intravenous injection, suggesting that the nervous system may be targeted by Ag NPs [102]. Yin and coworkers tried to establish the mechanism of Ag NP neurotoxicity both *in vitro* and *in vivo* using rat cerebellar granule cells. Their studies indicated that Ag NPs, depending on the caspase-activation-mediated signaling, drastically decreased the survival of primary neuronal cells through apoptosis coupled to oxidative stress [103].

3. Iron oxide (FeO, Fe_2O_3, Fe_3O_4) NPs

Iron oxide or superparamagnetic iron oxide nanoparticles (SPIONs) have become one of the most favorable and exciting choices in both the industrial and biomedical fields, due to their superparamagnetic property and other physicochemical characteristics unique to nanomaterials. SPIONs (Feridex) are small NPs composed of a Fe_3O_4 (magnetite) or Fe_2O_3 (maghemite) core. Although maghemite is naturally ferromagnetic, with the decreasing size (< 30 nm), it becomes superparamagnetic. Their potential application ranges from biomedical imaging (magnetic resonance imaging, positron emission tomography, or ultrasound as contrast agent), gene and drug delivery, tissue regeneration, hyperthermia in cancer treatment, catalysis, and magnetic storage [104]. They are extensively used specifically for brain imaging or brain-targeted drug and gene delivery, due to their ability to move across the BBB [105]. SPIONs are metal oxide NPs that have been clinically approved, although recently they have been taken off the market [106,107].

In spite of their desirable traits, there is a critical need to investigate their toxicity both *in vivo* and *in vitro*. SPIONs have already been shown to have potential toxicity that can lead to altered gene expression, actin modulation, interference with

cell cycle regulation and signaling pathways, excessive ROS generation, and disruption of iron homeostasis [108]. According to the recent findings, environmental factors are a major contributor to the development of neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease [109]. Peters et al [110] have emphasized the significance of oxidative stress generated by NPs in the brain, along with the evaluation of the possible connection between long-term NP exposure and neurodegenerative disease. With increased use of Fe_3O_4 NPs in industry and biomedical sciences, the risk related to occupational exposure has escalated considerably. Involvement of ultrafine particulate materials in polluted air leads to protein fibrillation. Fibrillation of specific proteins, for example, Aβ42 and α-synuclein, may play a role in the development of Alzheimer's disease and Parkinson's disease [111]. SPIONs have further been shown to form a corona with plasma proteins. This corona can lead to several toxic side effects because the initial cellular interaction of magnetic nanoparticle (MNP) changes lead to downstream modification of cellular and tissue interaction [112,113]. In 2007, Pisanic et al [114] used PC12 cells as a quantifiable *in vitro* model system to study the toxic effect of anionic Fe_3O_4 MNPs in a dose-dependent manner. In that study, it has been established that when PC12 cells were exposed to the anionic MNPs at an increasing concentration ranging from 0.15mM to 15mM iron, they lost their viability and were unable to generate normal neurite growth in the presence of nerve growth factor. They have concluded that the anionic MNPs were possibly interfering with transcriptional regulation and protein synthesis, for example, Growth associated protein (GAP)-43 leading to cellular death and phenotypic changes.

In 2009, Wang et al [115] discussed the ability of submicron level Fe_3O_4 NPs to be transported to the brain via the olfactory nerve pathway, leading to oxidative-stress-related damage in the brain. They also discussed changes in the ultrastructure of the olfactory bulb nerve cells. Recently, Wu et al [116] have focused on the neurotoxicity of iron oxide NPs in the rat brain (*in vivo*). The study investigated the effect of uptake and retention of Fe_3O_4 NPs in rat brain hippocampus and striatum, including oxidative injuries. The olfactory bulb, striatum, and hippocampus seemed to be the main sites for Fe_3O_4 NP deposition after intranasal instillation [117]. Approximately 80% of NPs were still found in the striatum at 7 days after instillation and about 50% were found in both the striatum and hippocampus after 14 days. The striatum in the instillation groups exhibited comparatively more susceptibility to oxidative stress, as indicated by increased levels of H_2O_2 and decreased Glutathione peroxidase (GHS-PX) activity in the control group at 7 days after exposure. The group also investigated the effect of Fe_3O_4 NPs in PC12 cells *in vitro*. The PC12 cells showed dose-dependent cytotoxicity, as measured by LDH release and MTT assay, demonstrating membrane disruption and mitochondrial enzyme activity, respectively. The oxidative stress was also evident by the reduced GSH-PX and superoxide dismutase activity, and increased ROS level and lipid peroxidation. Fe_3O_4 NPs also had a substantial cytotoxic effect on PC12 cells by modulating the cell cycle and inducing apoptosis. JNK is usually activated by oxidative stress and modulates apoptosis, neurodegeneration, cell cycle control, and cellular proliferation [118]. The cells also exhibited phosphorylation of p53 protein at ser15 position and elevated levels of bax and bcl-2 proteins upon exposure to NPs.

It has been demonstrated that intranasally instilled Fe_2O_3 NPs are transported into the brain via the olfactory route [119], and additional investigation has been made of the size-related effect. After a single intranasal exposure of 21-nm Fe_2O_3 NPs, there was a significant increase in iron content in almost all the brain regions, from the olfactory bulb, hippocampus, cerebral cortex, and cerebellum to the brainstem [120]. However, a single intranasal exposure of 280-nm Fe_2O_3 NPs led to a significant increase in iron content only in the olfactory bulb and hippocampus, with no significant alteration of iron content in other brain regions. At 30 days after instillation of 280-nm Fe_2O_3 NPs in mice, the iron content in the olfactory bulb and hippocampus also increased but was lower than that in mice treated with 21-nm Fe_2O_3 NPs.

It is widely known that brain iron accumulation is associated with the oxidative stress induced by the formation of the highly reactive •OH via the Fenton reaction [121–123]. The excess iron in the brain suggests an association with the oxidative stress response. The generation of ROS is a well-established paradigm to explain the toxic effects of NPs [40]. It has been demonstrated that intranasal exposure of iron oxide NPs causes a certain degree of oxidative stress response in mouse brain [119]. Significant oxidative stress responses in the brain of mice have also been observed after intranasal exposure of 21-nm and 280-nm Fe_2O_3 NPs [124]. Alterations of iron and zinc levels in the brain are more evident in mice exposed to nano-sized than submicron-sized Fe_2O_3. Furthermore, the strong positive correlation between the iron and zinc levels in the sub-brain regions may contribute to the understanding of zinc homeostasis in the brain after Fe_2O_3 particle inhalation.

The biomarkers of oxidative stress, activity of nitric oxide synthases, and release of monoamine neurotransmitters in the brain have been studied as well [115]. It was shown that significant oxidative stress was induced by the two sizes of Fe_2O_3 NPs. The activities of GSH-PX, copper, zinc superoxide dismutase, and constitutive nitric oxide synthase were significantly elevated and the total glutathione and glutathione/glutathione disulfide ratio were significantly decreased in the olfactory bulb and hippocampus after treatment with nano- and submicron-sized Fe_2O_3 particles. The nano-sized Fe_2O_3 generally induced greater alteration and a more significant dose–effect response than the submicron particles did. Transmission electron microscopy showed that nano-sized Fe_2O_3 treatment induced some ultrastructural alterations in nerve cells, including neurodendron degeneration, membranous structural disruption, and increased lysosomes in the olfactory bulb, dilation in the rough endoplasmic reticulum, and increased lysosomes in the hippocampus. The results indicated that intranasal exposure of Fe_2O_3 NPs could induce more severe oxidative stress and nerve cell damage in the brain than the larger particles did.

Fe_3O_4 NPs also exert cytotoxic effects by influencing the cell cycle and apoptosis [116]. For example, cells are arrested at the G_2/M phase after 24 hours exposure to NPs. Arrest at the G_2/M phase provides time for these cells to instigate DNA repair and delay cell death. However, cells with impaired DNA

repair processes enter apoptosis. The study indicates that Fe_3O_4 NPs are deposited and retained in the striatum after intranasal instillation, and the NPs may then cause oxidative damage in the striatum. The results of *in vitro* studies on dopaminergic neurons have demonstrated that Fe_3O_4 NP exposure decreases cell viability and induces marked oxidative stress. Furthermore, Fe_3O_4 NPs mediated apoptosis signaling pathway included JNK and c-Jun phosphorylation, p53 phosphorylation, Bax upregulation, Bcl-2 downregulation, and apoptosis.

3.1. *Copper and copper oxide NPs*

Copper is one of the essential trace elements for energy production in biological systems. Copper is a requirement for the synthesis of different enzymes, including cytochrome c oxidase, superoxide dismutase, tyrosinase, lisyl oxidase, and cupro-protein [125,126]. Copper is also responsible for stimulating the production of neurotransmitters such as epinephrine and norepinephrine in the brain and can be found there at a high concentration [127]. However, at higher than normal levels, unbound copper become toxic to the human body because it disrupts homeostasis. Its redox activity can give rise to ROS, leading to oxidative stress and modification of protein, lipid, and nucleic acid [128,129]. Compounds of copper such as copper oxide (CuO) NPs have found a broad use in various areas. CuO NPs are used in inks, lubricants, coatings, semiconductors, heat transfer fluids, antimicrobial preparations, and intrauterine contraceptive devices [130]. Copper-based NPs are used as lubricant additives because they reduce friction and wear, and worn surfaces can be repaired by an addition of copper NPs in lubricants. As more copper NPs are currently in use, it is likely that human exposures to copper NPs will increase gradually.

Due to their nanolevel size, CuO NPs are capable of crossing the BBB and pose a threat to the CNS. Studies have shown that copper NPs can cause BBB dysfunction, swelling of astrocytes, and neuronal degeneration once introduced into the bloodstream [1,131]. Li et al [132] showed neurotoxicity of CuO NPs in a dose-dependent manner in H4 neurogiloma cells using an automated image analysis technique.

Primary cultures of dorsal root ganglion of neonatal rat pups were investigated to measure neurotoxicity of copper NPs of varying size and concentration by Prabhu et al [133]. After exposured to 10–100μM copper NPs (40 nm, 60 nm, and 80 nm) for 24 hours, the neurons started to develop vacuoles and became detached from the substratum. They also exhibited disruptive neurite growth. LDH and MTT assays have also shown the significant toxicity of copper NPs, and the smaller size is associated with higher toxicity.

The whole-cell patch-clamp technique was used to study the influence of CuO NPs on voltage-dependent potassium current in acutely isolated rat CA1 pyramidal neurons of the hippocampus [134]. Although the CuO NPs did not have a significant effect on the outgoing potassium current, they did inhibit the delayed rectifier potassium current at a relatively high concentration. CuO NPs shifted the inactivated curve of rectifier potassium current negatively but did not show any significant effect on transient outgoing potassium current. These blockades of the potassium current might inhibit the normal functional activity of nerve cells. In another study, Trickler et al [135] has determined the effect of copper NPs on induction of proinflammatory mediators, followed by their influence on rat brain microvessel endothelial cells. At a low dosage, the copper NPs enhanced cellular proliferation, whereas at a high concentration, they started to express toxicity. NP exposure increased prostaglandin E2 release. Extracellular levels of TNF-α and IL-1β were considerably higher in the exposed cells. This resulted in the disruption of cerebral microvasculature by increasing its permeability.

According to Karlsson [136], nano-CuO is highly toxic when compared with other metal oxide NPs. However, few studies have investigated the direct effects of nano-CuO on neurotoxicity and the potential mechanisms involved in these effects.

A study explored the potential neurotoxicity of nano-CuO on ion channels of neuron, voltage-dependent sodium current (INa) in rat hippocampal slices with whole cell patch-clamp technique [137]. The results showed that nano-CuO inhibited the peak amplitude of INa, which might have decreased intracellular Na^+ concentration due to decreased Na^+ influx. This could inhibit the exchange of Na^+ for Ca^{2+} by Na^+–Ca^{2+} exchangers [138]. The exchanger was shown to generate inward current during the repolarization phase of the action potential [139], thus, the effect on INa could contribute to the change in action potential shape by nano-CuO.

It is well established that voltage-gated sodium current (VGSC) plays a role in neurotransmitter release [140]. Thus, the effects of nano-CuO on INa also mean that modulation may produce functional effects on neurotransmission in the CNS. It has been shown that nano-CuO produces a hyperpolarizing shift in the activation curve. The S4 segment in a subunit of VGSCs contains 4–8 positively charged residues at three residue intervals. They serve as voltage sensors and initiate the voltage-dependent activation of VGSCs by moving outward under the influence of the electric field [141,142]. The results suggest an effect on the S4 segment of the activation gating, resulting in conformational changes of the channel. The findings also confirm that the effects of nano-CuO on hippocampal neurons are mediated through activation of ROS–INa–action potential signaling cascades and are independent from the G-protein pathway. These results show the primary mechanisms underlying nano-CuO-induced INa amplitude inhibition and improve our understanding of nano-CuO neurotoxicology.

To determine the potential neurotoxicity of CuO NPs, human SH-SY5Y neuroblastoma and H4 neuroglioma cells were exposed at a concentration range of 0.01–100 μM for 48 hours [132,143]. The data indicated that exposure of CuO NPs induced differential toxic effects in both SH-SY5Y and H4 cells, and the cells had dose-dependent toxic responses to the CuO NPs. The toxic effects of CuO NPs were also investigated in a semiadherent, fast-growing, mouse neuroblastoma cell line (N2A cells), to provide a better understanding of the toxicological risks of CuO NPs in future nanotechnology developments [144]. N2A cells were less sensitive to CuO NP effects than other cultured cells were. The lower sensitivity may have been due to the agglomeration of CuO NPs in the culture medium, which resulted in an average particle size $>$ 300 nm. Agglomeration of CuO NPs reduced surface-specific effects

specific to nanoscale materials, and increased the contribution of particle solubilization in the toxic response induced in N2A cells. Agglomerated CuO NPs induced both cytotoxic and genotoxic effects in N2A cells.

3.2. *Aluminum oxide (alumina, Al_2O_3) NPs*

In recent years, the areas of nanotechnology and nanomedicine have expanded rapidly, aluminum oxide (alumina) NPs, having good electric and abrasive properties, are widely used as abrasive agents or insulators in motor vehicles, electronics, energetics, exterior coatings, personal care products, scratch-resistant coatings, alloys, and sensors [145]. This has led to increased human exposure to aluminum oxide NPs (nano-alumina).

An *in vivo* study in ICR mice aimed to investigate the effects of nano-alumina, with a focus on the effects on neurobehavioral defects and possible mechanisms of action. It showed that nano-alumina induced apoptosis via increased caspase-3 gene expression and impaired spatial learning behavior, which suggests that mitochondrial impairment plays a key role in the neurotoxicity of nano-alumina [146]. The research could help to understand the underlying mechanisms of toxicity of nano-alumina, particularly with respect to neurobehavioral function. The authors declared that impairment of the mitochondria played an important role in the neurotoxicological effects of nano-alumina and might be a direct cause of neurobehavioral defects.

The possible neurotoxic effects of nano-alumina and bulk alumina have been compared in nematodes [147]. The relatively large surface area of nano-alumina compared with bulk alumina might also explain the differences in toxicity between nano-alumina and bulk alumina. The decrease in locomotive behavior in nematodes chronically exposed to nano-alumina was associated with both an increase in ROS generation and disruption of ROS defense mechanisms. *Drosophila* was used as another model organism to explore the effects of nano-alumina on the CNS [148]. The rhythmic and electrophysiological activities in the antennal lobe of *Drosophila* were recorded using patch clamps. Fifteen minutes after application of nano-alumina, the average frequency of spontaneous activity was significantly decreased compared with that of the control groups. The results indicate that nano-alumina might have adverse effects on the CNS in *Drosophila*.

The hypothesis that nano-alumina can affect the BBB and induce endothelial toxicity has been proposed [149]. In the first series of experiments, human brain microvascular endothelial cells were exposed to nano-alumina and control NPs in dose- and time-responsive manners. Treatment with nano-alumina markedly reduced human brain microvascular endothelial cell viability, altered mitochondrial potential, increased cellular oxidation, and decreased tight junction protein expression as compared to treatment with control NPs. Alterations of tight junction protein levels were prevented by cellular enrichment with glutathione. In the second series of experiments, rats were infused with nano-alumina at a dose of 29 mg/kg and brains were stained for expression of tight junction proteins. Treatment with nano-alumina resulted in marked fragmentation and disruption of integrity of claudin-5 and occludin. The results indicate that the cerebrovasculature could be affected by nano-alumina. In addition, the data indicate that alterations of mitochondrial function might be the underlying mechanism of nano-alumina toxicity.

As far as the assessment of toxicological properties of nanoparticles is concerned, it is important to know whether cultured neural cells take up NPs, and if so, what mechanisms are involved [150]. Ultrastructural examination has shown that nano-alumina penetrates the cell membrane and that some particles are engulfed by the cells and mainly accumulated in the cytoplasm. It has been demonstrated that the NPs entering the cells are likely to have an effect on cellular function. Bulk-alumina-treated cells show apoptotic characteristics, whereas nano-alumina-treated cells demonstrate both apoptotic and necrotic morphological changes. Photomicrographs show that the vesicles with individual particles and aggregates remain in the cytoplasm and the nucleus. According to transmission electron micrographs, NPs form aggregates inside the lysosomal vesicles and their internalization in lysosomal bodies is arranged in a perinuclear fashion. The presence of an elevated amount of lysosomes might reflect enhanced phagocytosis of exogenous particles.

Microglia and astrocytes are dominant glial and major immune cells in the CNS. They are sensitive to changes in the microenvironment of the CNS and are rapidly activated in almost all conditions that affect normal neuronal functions. Activation of microglia and astrocytes in the cortex and hippocampus following peripheral administration of nano-alumina have been analyzed in Sprague–Dawley rats [151]. There was significant glial activation induced in rat brain after nano-alumina administration.

3.3. *Silicon dioxide (silica) NPs*

Silica (SiO_2) NPs have been developed for mechanical polishing, additives to food and cosmetics, and have various applications in biomedical fields, including diagnosis, optical imaging, targeted drug delivery for the CNS, cancer therapy, and controlled drug release for genes and proteins. In particular, being considered more biocompatible than other imaging NPs, silica NPs are emerging as ideal materials for medical applications. For applications of potential drug delivery, imaging, and diagnostics in the CNS, silica NPs are also being modified or used for coating or stabilization of other optical materials. However, to date, little is known concerning the potential adverse effects on the brain associated with exposure to silica NPs.

Research has indicated that silica NPs via intranasal instillation enter the brain and show a distinct pattern of biodistribution, and are especially deposited in the striatum, except for the olfactory bulb [152]. Such an accumulation could result in oxidative stress, inflammatory changes, and functional damage of the striatum. In addition, silica NPs appeared to induce depleted dopamine in the striatum, and the main contribution was downregulation of tyrosine hydroxylase protein.

In vitro studies on dopaminergic neurons have demonstrated that silica NPs have marked cytotoxic effects and oxidative stress activity against PC12 cells [152]. Furthermore, activation of the p53 pathway is involved in the mechanism of the silica-NP-

induced G_2/M arrest and apoptosis. Additionally, the decrease in dopamine levels is most likely attributable to the reduction of dopamine synthesis. The authors have claimed that although extrapolation of the animal effects to humans remains a challenge, their results for the neurotoxic effect on rat brains could be suggestive of human exposure, because different species may respond differently to the same substance.

Another study demonstrated that exposure to 300 ppm silica NPs in differentiating cells showed less cytotoxicity than in undifferentiated cells [153]. Silica NPs at 100 ppm had no significant effect on the viability of either undifferentiated or differentiating neuroblastoma (SH-SY5Y) cells. Neurite outgrowth in differentiating cells after 48 hours exposure to 100 ppm silica NPs was not significantly changed. Thus, silica NPs appeared to have no effects in the early initiation of neurites. Although the production of ROS was not induced, neurotoxicity induced by silica NPs may be the result of increased DNA damage, apoptosis, and cell cycle arrest in undifferentiated and differentiating cells, which is independent of neuronal differentiation of SH-SY5Y cells.

4. Carbon-based nanomaterials

Owing to their unique chemical and physical properties, carbon-based nanomaterials have a potential use in a variety of biomedical applications, including early diagnosis of cancer, imaging, targeted photothermal therapy, drug delivery, and tissue engineering. Based on the shape, organic carbon-based nanomaterials are categorized as carbon nanotube, fullerene, graphene, or carbon NPs. Carbon nanotubes are one-dimensional forms of graphitic material and are present in many forms, depending on the number of graphene sheets used: single-walled carbon nanotubes, double-walled carbon nanotubes, and multi-walled carbon nanotubes with diameters of 1–2 nm and lengths of 0.05–1 μm. Graphene has similar chemical composition and crystalline structure with a flat sheet with a single layer or multilayer graphene with several layers. The fullerenes (C60) are named after Richard Buckminster Fuller as buckminsterfullerene, or the “bucky ball”. This allotrope of carbon consists of 60 carbon atoms joined together to form a cage-like structure. C60 is soluble in aromatic solvents (e.g., toluene or benzene), but insoluble in water and alcohol. However, C60 can be functionalized (e.g., with −OH, −COOH, or $-NH_2$) to increase its hydrophilicity. By contrast, aqueous fullerene aggregates can be generated by mixing pure C60 in water or through solvent extraction. Some fullerenes have been shown to inhibit human immunodeficiency virus (HIV) activity through inhibiting an HIV-associated protease, an essential enzyme for viral survival. It has been reported that some fullerenes can interact with biological membranes to elicit antimicrobial action, antitumor activity, enzyme inhibition, DNA photo cleavage, and neuroprotective activity via antioxidant actions. At present, fullerenes are commercially used in products including fuel cells, semiconductors, and product coatings, for example, bowling ball surfaces.

Studies of carbon nanomaterials have indicated the potential neurotoxic effects after inhalation or systemic exposure. Oberdörster and co-workers [17] showed that inhalation of elemental ^{13}C NPs of 36 nm by rats, which were exposed for 6 hours whole-body exposure, led to a significant and persistent increase in the accumulation of ^{13}C NPs in the olfactory bulb, and the NP concentration gradually increased. A recent study has shown that different shapes of carbon nanomaterials elicit different toxicity in neuronal culture models. Specifically, pure graphene is less toxic than highly purified single-walled carbon nanotubes in a concentration-dependent manner after 24 hours exposure of PC12 cells, involving the apoptosis pathway [154]. Subsequently, the impact of surface functionalization on the toxicity of carbon nanotube has been demonstrated using the same culture model. Carbon nanotubes with surface-coating polyethylene glycol are less toxic than uncoated carbon nanotubes, by measuring mitochondrial function and membrane integrity. A mechanistic study has shown that oxidative stress is involved in this toxic pathway, with surface coating playing an important role [155]. It has been reported that 14-nm carbon black particles might translocate to the olfactory bulb through olfactory neurons, resulting in the activation of microglial cells, which induces proinflammatory cytokines and chemokines, suggesting an inflammatory response [156]. Additional systematic evaluations and mechanistic *in vivo* studies are needed to understand the effect of surface coating on the biocompatibility of these carbon-based nanomaterials prior to use in humans.

5. Future perspectives

Physical and chemical characterization is considered to be the key element in assessing the neurotoxicity of nanomaterials. The nanomaterials used in the study require a comprehensive physicochemical characterization before during, and after the biological testing models are exposed to nanomaterials. As mentioned previously, the size, size distribution, purity, shape, crystal structure, composition, surface coating, surface charge, and surface reactivity may result in a different distribution, accumulation, and transport of the nanomaterials to the target organs, as well as across the BBB. Research findings are meaningless for hazard identification in the absence of adequate evaluation of the physical and chemical properties of nanomaterials. For example, impurities that contaminate the nanomaterials being tested may contribute most to neurotoxicological responses. The dissolution of metal ions from metal oxide nanomaterials may play an important role in neurotoxicity. The size or surface charge of nanomaterials might change the biokinetics of the nanomaterials, resulting in different pharmacological or toxicological actions in biological systems. However, batch-to-batch inconsistency is a major challenge when nanomaterials are produced by different manufactures/laboratories.

The exposure dose level should be carefully considered when laboratory animals or *in vitro* models are exposed to nanomaterials. The practically exposure level to human should be used as a reference when calculateing the relevant dose exposed to the animals or *in vitro* models. This will support studies for understanding the dosimetry in the nervous system. The characteristics of the nanomaterials should also be considered in physiologically based pharmacokinetic modeling to better predict the environmental hazard of the

nanomaterials. To date, the data gap of well-designed neurotoxicity assessment of nanomaterials still exists, and further *in vivo* studies will be considered as an urgent demand in the future.

Appropriate dose–response research should be considered in neurotoxicological studies. Recent inhalation studies have shown that the surface area or particle number, instead of the nanomaterials mass, is considered as the major dosimetry unit in term of the dose-response relationship. Cellular or target organ dose will provide a better understanding of the neurotoxicological responses, because the physical properties might change quickly in the biological system under the experimental conditions. Sensitive and specific methods need to be developed to quantify the nanomaterials, including metal NPs or carbon-based nanomaterials. The nanomaterials may interfere with the enzymatic assay during the measurement of neurotransmitters (such as acetylcholine or dopamine) using traditional methods. Therefore, the traditional approaches using chemicals should be carefully validated because they are used in nanoneurotoxicological studies.

Conflicts of interest

All authors declare no conflicts of interest.

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