Review Article

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Toxicity of metallic nanoparticles in the central nervous system

https://doi.org/10.1515/ntrev-2019-0017 Received Nov 16, 2018; accepted Jul 1, 2019

Abstract: Metallic nanoparticles due to their small size and unique physico-chemical characteristics have found excellent applications in various branches of industry and medicine. Therefore, for many years a growing interest has been observed among the scientific community in the improvement of our understanding of the impact of nanoparticles on the living organisms, especially on humans. Considering the delicate structure of the central nervous system it is one of the organs most vulnerable to the adverse effects of metallic nanoparticles. For that reason, it is important to identify the modes of exposure and understand the mechanisms of the effect of nanoparticles on neuronal tissue. In this review, an attempt is undertaken to present current knowledge about metallic nanoparticles neurotoxicity based on the selected scientific publications. The route of entry of nanoparticles is described, as well as their distribution, penetration through the cell membrane and the blood-brain barrier. In addition, a study on the neurotoxicity in vitro and in vivo is presented, as well as some of the mechanisms that may be responsible for the negative effects of metallic nanoparticles on the central nervous system.

Keywords: nanoparticles, neurotoxicity, brain, environmental exposure, oxidative stress

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Graphical abstract: This review summarizes the current knowledge on the toxicity of metallic NPs in the brain and central nervous system of the higher vertebrates.

Abbreviations

AChE	Acetylcholinesterase
ATPase	ATP monophosphatase
BBB	Blood-brain barrier
BCEC	Brain capillary endothelial cells
CNS	Central nervous system
DA	Dopamine
GSH-Px	Glutathione peroxidase
IL-1β	Interleukin-1 beta

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LTP	long-term potentiation
NO	Nitric oxide
NPs	Nanoparticles
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SPION	Supermagnetic iron oxide nanoparticles
TJ	Tight junctions

1 Introduction

Nanotechnology is the design, production and application of structures, materials and devices sized at nanometre scale. It is assumed that nanoparticles (NPs) are structures having a critical dimension of less than 100 nm. Because of their size, greater surface area and volume to mass ratio, NPs present unique optical, mechanical, chemical, electrical and magnetic properties that makes them more reactive, compared to their bulk counterparts [1–3]. NPs can stimulate and affect certain cells, inducing and multiplying the desired physiological effects. On the other hand, NPs, due to their size comparable to biological molecules, can easily pass through cell membranes, penetrate cellular organelles and interfere with the normal cell physiology, and, as a result, cause damage at the cellular and sub-cellular level and/or triggering different cell/tissue responses [4-8].

The progress of nanotechnology has led to the development and commercialization of hundreds of products containing NPs. Nanotechnology offers many opportunities and benefits for medicine, energy production, environmental protection, the food industry, electronics, science, computer technology, cosmetics, textiles, agriculture and the defence industry [1, 3–5, 9–11]. Large scale production and use of NPs can lead, however, to unintentional human and the environmental exposure to this potentially hazardous substances [10, 12]. According to the Wilson Centre data, exposure to 45% among the 580 catalogued NPs have been classified as potentially dangerous [13]. The potential risk of exposure to toxic NPs becomes a burning issue for today's science, despite their wide use and many beneficial applications [10]. Although to-date no human disease has been officially attributed directly to NPs, certain studies that have been conducted suggest that some NPs may cause adverse biological reactions, leading to toxic effects [9].

Humans could be exposed to metallic oxide NPs from different environmental and occupational sources. NPs can come from natural phenomena, such as volcanic activity, or as a result of industrial processes with a lot of metal fumes, *e.g.* cutting, grinding, melting, casting and welding. Another potential source of human exposure to metallic NPs is its intentional use in commercial products, such as vectors of drugs, sunscreens, toothpaste, cosmetics, plastic products, textiles, paints, and gasoline components [9, 14–17]. Despite their origin, NPs can penetrate into the body by a number of different routes, including injection, inhalation or ingestion. Then, circling with blood, they can penetrate and accumulate in different organs and tissues including central nervous system (CNS) [9, 18, 19].

Some NPs seem to be very suitable for medical applications, such as drug delivery vectors or theranostics for therapeutic and diagnostic procedures [20]. Metal oxides are currently one of the most important tools used in the diagnosis of diseases (contrast to MRI, fluorescent dyes), drug delivery systems (photosensitizers), genes, antimicrobial substances, as well as scaffolding materials in tissue reconstruction [2, 3, 9, 18, 21]. Metallic NPs can resonate in the magnetic field, thus delivering energy directly to the target cancer cells [20]. Due to the increasing use of metallic NPs in medicine, more and more attention is paid to the safety of using NPs for CNS [22]. Numerous studies indicate inability of blood-brain barrier (BBB) to protect against NPs translocation to the brain [23]. Compared to other types of cells, nerve cells are more sensitive to toxins due to their limited ability to regenerate [24]. Thus, it is important to reliably analyze the neurotoxic impact of metallic NPs on the brain. Acquired knowledge can be used to develop safety guidelines for the potential use of NPs in industry and medicine, to minimize the negative health effects on the CNS [25].

The presented review is an attempt to summarize the current knowledge on the toxicity of metallic NPs in the brain and CNS of the higher vertebrates.

2 Modes of exposure *in vivo* and distribution of internalized NPs

Metallic NPs, due to their small size and specific physicochemical properties, can penetrate into living organisms through various modes of exposure, for example, inhalation or ingestion. In general, smaller NPs show a greater accumulation in the organs and induce a higher toxicity, compared to larger NPs [26]. Thus, it is important to fully understand the mechanisms responsible for their distribution and penetration into the target organs, and the effects that they might have there [27].

Several studies support the concept that the CNS may be a potential target for inhaled NPs [28, 29]. Inhalation is one of the main route of unintentional human exposure to metallic NPs. Due to the small size, NPs efficiently bind to nose mucosa [30] or reach into bronchi and alveoli in the lung [31, 32]. From the nasal cavity, NPs are further transported by olfactory epithelium and migrate along primary olfactory neurons to the glomeruli of the olfactory bulb, olfactory nerve or trigeminal, or the blood- cerebral spinal fluid to the choroid plexus [3, 31]. Transport via the olfactory nerve, is a direct way of penetration of NPs to the brain, bypassing the BBB [33]. This has been demonstrated for several metal NPs, e.g. ultrafine silver NPs (Ag NPs) [34], CdSe/ZnS quantum dots (CdSe/ZnS QDs) [35], copper oxide NPs (CuO NPs) [36], ultrafine manganese dioxide NPs (MnO₂ NPs) [37] or titanium dioxide NPs (TiO₂ NPs) [38, 39].

On the other hand, inhaled metal NPs can also get into the alveoli epithelial cells and further to blood and lymph circulation, finally accumulating in potentially sensitive target sites, such as the brain, bone marrow, lymph nodes, spleen or heart [18, 40, 41]. The process was observed for aluminum oxide NPs (Al₂O₃ NPs) [12, 40] or lead NPs (Pb NPs) [42]. Also, it is postulated that small metal NPs are uptaken inside the alveoli by macrophages and dissolved in the phagosomes. It was shown that ions were released even from the oxidized form of NPs, which are not normally soluble. Released ions can easily penetrate BBB [14].

A digestive rout is also important way of penetration of NPs into the body. It is postulated that NPs are absorbed by epithelial cells in the digestive system, from where they can further penetrate into bloodstream and secondary organs. NPs are mainly absorbed by M cells found in Peyer's patches through the transcytosis mechanism. It was also shown that extracellular transport through tight junctions (TJ) of epithelial cells might be involved; however, this applies only to very small NPs (d=0.5-6 nm) [43]. Loeschner et al. have shown that the digestive route constituted an important source of exposure to Ag NPs. They observed in rats after 28 days of dietary exposure to Ag NPs (14±4 nm) that Ag NPs were uptaken by the M cells and enterocytes [44]. The amount of NPs uptaken via digestive rout correlate with the size and charge of NPs. Twenty four hours after administration of different size gold NPs (Au NPs) (1.4-200 nm) with negative/positive charge, the highest accumulation in secondary organs (lung, spleen, heart) was for the smallest (1.4 nm), negatively charged NPs. In contrast, the highest accumulation in the brain was observed for NPs with a diameter of 18 nm [43].

Several studies, such as those on rats exposed to TiO_2 NPs [45–47] or porcine exposed to TiO_2 NPs or zinc oxide NPs (ZnO NPs) [48], demonstrated the inability of NPs to penetrate into the dermis or into the live layers of skin. This may be due to the ability of metallic NPs to form larger aggregates, as well as having a surface charge which prevents penetration to the stratum corneum (SC) and the deeper layers of the skin. Thus, it is believed that the inorganic NPs are not able to penetrate intact healthy skin. This is very important in the context of application of many metallic NPs, as a component of certain ointments and creams commonly used by people.

The CNS of vertebrates is isolated from the rest of body by BBB. Normal functioning of BBB is critical for homeostasis. BBB is responsible for the active exchange of nutrients and metabolites between the blood and brain, prevention of the xenobiotics penetration to the brain, and restricting immune cell infiltration [4]. Selective permeability is a result of the complex structure and biochemical properties of the brain capillary endothelial cells (BCEC), such as leakproof TJ connections and minimal endocytotic activity to prevent penetration of harmful substances from the blood directly to the brain [49, 50].

Nonetheless, Chen et al., showed that exposure of mice to aluminum NPs (Al NPs) caused disturbances in the normal functioning of TJ, which was associated with increased permeability of the BBB and further damage to brain tissue [4]. Moreover, high level of expression of transferrin receptor in BCEC cells may facilitate transcytosis across the BBB [51]. In line, Au NPs were capable of penetrating through the BBB and accumulating in the brain, after intravenous administration [52] or intraperitoneal administration in mice [20]. Further, the ability to penetrate the BBB and accumulation in the brain was demonstrated for many metallic NPs, such as Al NPs [53], Ag NPs [54], CuO NPs [55], manganese NPs (Mn NPs) [14] and TiO₂ NPs [56]. In the brain, NPs are able to enter neurons and move along axons or dendrites to other connected neurons [57]. Transneuronal transport may occur through the synaptic connections, endocytosis or passive diffusion [58].

3 Cellular uptake

Metallic NPs can enter to the cell *via* interaction with components of its membrane. The main mechanism of their cellular uptake is endocytosis. During endocytosis, the absorption of NPs occurs through membrane invaginations, then their budding and pinching off to form endocytic vesicles, transported to specialized intracellular compartments [9, 59–61]. Endocytosis is classified into sev-

eral types depending on molecules involved in the process. The two main classes of endocytosis are phagocytosis and pinocytosis. In addition pinocytosis can be further divided into four subclasses depending on the size of vesicles and proteins involed for their formation. On this basis, pinocytosis is differentiated into clathrin-mediated endocytosis. caveolae-dependent endocytosis, clathrin/caveolae independent endocytosis and macropinocytosis [62]. In contrast to phagocytosis, which takes place primarily in specialized phagocytes, pinocytosis are more prevalent and occurs in many kinds of cells. Interestingly, metallic NPs uptake in neurons and glial cells, also mainly based on the endocytosis process, includes all its types, even phagocytosis [61, 63, 64]. Research on astrocyte-rich primary cultures indicated endocytosis as the uptake mechanism of Ag NPs [65]. Comparable, the ZnO NPs were absorbed by neuronal cells line PC12 through endocytosis, which was required for interneuron translocation of these NPs [66]. The iron NPs (Fe NPs) coated with dimercaptosuccinic acid were also efficiently taken into astrocytes. Transmission electron microscopy showed their congregations in intracellular vesicles. Due to the negative charge, as indicated by their zeta potential, the mechanism of passive diffusion was excluded [67].

Clathrin-mediated endocytosis leads to the formation of clathrin-coated vesicles and it is the main mechanism for nutrients and membrane components cellular uptake. In contrast, another mechanism, the caveolae-dependent endocytosis, is equally important and participates in many biological processes, among others transcytosis, signaling and nutrients regulation and depends on the integral membrane protein - caveolin. Clathrin/caveolae independent endocytosis goes through other pathways, without the involvement of these proteins. The unique pinocytosis process of forming membrane extension or ruffles as a result of cytoskeleton reorganization is macropinocytosis. As a consequence of these membrane changes the large amount of extracellular fluid with dissolved molecules is collected, regardless of the presence of any specific receptors [68, 69]. Luther et al. showed that Fe NPs were successfully taken up by microglial cells chiefly through macropinocytosis and clathrin-mediated endocytosis, in the course of which absorbed particles were directed into the lysosomal compartment [70]. A predominance of endocytosis was also observed in another study on neural stem cells incubated with Ag NPs [71]. Likewise, the uptake of TiO₂ NPs by glial cells was based on the mechanism of endocytosis. However, chemical immobilization of the cytoskeleton significantly reduced the entry of TiO₂ NPs into the cells, suggesting macropinocytosis as the main process of their uptake [72].

In addition to various types of pinocytosis, metallic NPs may enter to the interior of the cell through the phagocytosis mechanism. Typically, the opsonization of NPs by immunoglobulins or other blood proteins precedes this process and enables recognition by the appropriate cells. This initiates a signaling cascade that allows engulfing and internalization of NPs to form so-called "phagosomes" [61]. Valentini *et al.* observed a characteristic phenotype of activated microglia cells caused by the presence of TiO₂ NPs. Their morphological changes, such as the larger size and formation of membrane protrusions typical of phagocytosis, indicated the uptake of TiO₂ NPs through this process [73].

Some studies indicated that various types of endocytosis might act simultaneously for the metallic NPs uptake by neuron- and glia-like cells. TiO₂ NPs were mainly taken by astrocyte-like ALT cells and BV-2 microglia *via* disparate types of endocytosis: clathrin-mediated endocytosis and caveolae-dependent endocytosis in ALT cells, phagocytosis and clathrin-mediated endocytosis in BV-2 cells [74]. An analogous research of the absorption of Ag NPs showed less uptake by neuroblastoma N2a cells, as compared to ALT and BV-2 cells. In ALT cells prevailed phagocytosis and clathrin/caveolae independent endocytosis, while in BV-2 Ag NPs were taken mainly by micropinocytosis and clathrin-mediated endocytosis [75].

4 Toxicity of NPs

4.1 Toxicity in vitro

Numerous reports indicate detrimental effects of metallic NPs on neural cells in culture. In a triple coculture BBB model consisting of microvascular endothelial cells, astrocytes and pericytes exposed to Ag NPs, Xu et al. observed a severe shrinkage of mitochondria, endoplasmic reticulum expansion and vacuolations in astrocytes. An analysis of gene expression showed changes in 23 genes associated with metabolic and biosynthetic processes, cell death and response to stimuli. Another research on primary rat cortical cells showed that Ag NPs disrupted development and functioning of the nervous system. Ag NPs were toxic to nerve cells by causing abnormalities in formation of cytoskeleton, pre- and post-synaptic proteins, and functioning of the mitochondria, leading to cells death [76]. In line, Coccini et al. observed toxic effects after shortterm (4-48 h, 1-100 µg/ml) or long-term exposure (up to 10 days, 0.5-50 μ g/ml), even at low doses (0.5 μ g/ml) [77]. Size dependent toxicity of Ag NPs was also observed for

organotypic mouse midbrain cells [78]. The mechanisms involved in Ag NPs-induced toxicity of primary cultures of rat cerebellar granule cells (CGCs) include activation of N-methyl-D-aspartate receptor, destabilization of mitochondrial function, production of free radicals [79], oxidative stress leading to caspase activation and apoptosis [80]. Apoptosis was also reported as a main cause to Ag NPs-induced death of N2A cells growing in co-culture with ALT and BV-2 cells. However, this was not a direct effect, because the NPs were effectively uptaken only by ALT and BV-2 cells. N2A cells death followed the release of toxic compounds, nitric oxide (NO) from BV-2 and hvdrogen peroxide (H_2O_2) from ALT. These results suggested that the key factor in Ag NPs neurotoxicity may be the induction of reactive oxygen and nitrogen species by astrocytes and microglia [75]. The assessment of the effects of Ag NPs on human and rat embryonic neural stem cells (NSCs) led to similar conclusions. Apoptosis and necrosis of NSCs occurred as a result of increased mitochondrial production of reactive oxygen species (ROS) [81]. Huang et al. examined the effect of Ag NPs on the expression of genes involved in inflammation and neurodegenerative disorder on brain mouse neural cells. The study showed a significant increase in interleukin-1 beta (IL-1 β) secretion, and increased expression of C-X-C motif chemokine 13, macrophage receptor with collagenous structure and glutathione synthetase (GSS). Furthermore, exposure to Ag NPs, formation of amyloid- β (A β) plaques responsible for Alzheimer's disease [82].

Studies performed on astrocytes exposed to CuO NPs showed a significant decrease in viability, reduced lactate dehydrogenase activity and increased permeability of the cell membrane. Generation of ROS was point as a main cause of the CuO NPs toxicity [83]. ROS generation was also proposed as a main cause of time- and concentration-depended increase of apoptosis in CuO NPs treated HT-22 mouse hippocampal neuronal cells [84]. Impaired viability was also observed for cultured primary rat astrocytes exposed to CuO NPs. Moreover, Cu²⁺ ions released from CuO NPs induced nerve cells glycolytic flux, and synthesis of glutathione and metallothioneins [85]. CuO NPs and Ag NPs significantly increased release of prostaglandin E2, tumor necrosis factor (TNF), IL-1 β in cells, while the Au NPs did not cause such an effect [86].

Rivet *et al.* studied the effect of the coating substance on the toxicity of encapsulated supermagnetic iron oxide NPs (SPION, Fe_3O_4 NPs). *In vitro* studies on cortex neuronal cells indicated that the toxicity of SPION depended on the coating substance. The polydimethyloamine coating resulted in cell death at all tested concentrations due the rapid and complete disruption of the cell membrane,

whereas aminosilane coating affected cell metabolic activity at higher concentrations, leaving the cell membrane intact. Coating with dextran, even at high concentrations. did not affect the viability of the cells [87]. Toxicity of Fe₃O₄ NPs on brain cells was also studied by De Simone et al. They developed two types of CNS spheroids from SH-SY5Y neuron-like cells and human D384 astrocytes. After shortterm (24 or 48 h, 1-100 µg/ml) and long-term (30 days,0.1- $25\mu g/ml$) exposure of 3D-spheroids to Fe₃O₄ NPs a cytotoxic effect was observed, more pronounced for neurons compared to astrocytes [23]. Other studies have shown that Fe₃O₄ NPs induced oxidative stress and activation of c-Alb tyrosine kinase, which was associated with neurotoxicity. In addition, Fe₃O₄ NPs caused alterations in the α synuclein expression associated with neuronal damage, occurring among others in Parkinson's and Alzheimer's diseases [88].

In a study conducted by Wang et al., it was shown that Mn NPs, Ag NPs, and Cu NPs caused dopamine (DA) depletion by alteration of the expression of dopaminergic system-related genes, associated with induction of oxidative stress in PC12 cells treated for 24 h [89]. In line, a concentration-dependent decrease in the synthesis of DA by PC12 cells exposed to Mn NPs [57]. Unlike most metallic NPs, cerium dioxide NPs (CeO₂ NPs) were not cytotoxic in neuronal cells and had antioxidant activity, inhibiting ROS production. Despite this results, disruption of redox balance inhibited neural stem cell differentiation [90]. Decrease in viability and increase of apoptosis were observed in primary cortical neurons after incubation with gallium trioxide NPs modified with chromium ions and hyaluronic acid (HA/Ga₂O₃:Cr³⁺ NPs). The toxicity was explained by activation of calpain and disruption autophagy signaling [91]. Also no toxicity was observed for 145 nm tungsten carbide particles (WC) in any of the cell lines tested including oligodendrocytes, rat primary neurons, rat primary astroglial cells, however, doping WC NPs with cobalt (WC -Co) resulted in an increase in the toxicity. The plausible explanation of the enhanced toxicity was increased oxidative stress and DNA damage [92].

Valdiglesias *et al.* compared the effects of two types of TiO_2 NPs differing in crystal structure on the viability of nerve cell SH-SY5Y. The results indicate that a different structure of the NPs did not significantly impact on cells, as no decrease in cell viability was observed in neither case. Besides the NPs were efficiently taken-up by the cells, resulting in dose-dependent changes in cell cycle and genotoxicity, however not associated with formation of double-stranded breaks [93]. In contrast, low doses of TiO_2 NPs caused cytotoxic effects in SH-SY5Y and glial cells

References	[75]	[76]	[77]	[78]	[62]	[80]	[81]	[82]	[06]
Outcome	Ag NPs induced of ROS and NO production in astrocytes and microglia, which trigger apoptosis of neurons	Ag NPs induced toxicity in neurons, expressed as degradation of cytoskeleton components, perturbations of pre- and postsynaptic proteins expression, and mitochondrial dysfunction.	Ag NPs induced cytotoxic effects after short-term and prolonged exposure	Greater cell susceptibility to the cytotoxic effects of smaller Ao NDs	Excitotoxicity via activation of NMDA receptor, followed by calcium imbalance, destabilization of mitochondrial function and ROS production	Ag NPs caused apoptosis based on the caspase activation-mediated signaling and oxidative stress	Ag NPs increased mitochondrial production of ROS leading to cell apoptosis and necrosis	Ag NPs could alter gene and protein expressions of $A\beta$ deposition potentially to induce AD progress in neural cells	CeO ₂ NPs exhibited antioxidant activity and disturbed the redox balance, thus inhibiting cell
Exposure duration	24 h or 48 h	2, 3 days	4-48 h (short-term) or up to 10 days (prolonged exposure)	24 h	24 h	24 h	24 h	24 h	24 and 28 h
Concentration	1, 3, 6 µg/ml	1, 5, 10, 50 μg/ml	1100 µg/ml (short-term) or 0.5-50 µg/ml (prolonged exposure)	6.25, 12.5, 25, 50 μg/ml	2.5-100 µg/ml	0.01, 0.05, 0.1, 0.25, 0.5, 1, 2.5 μg/ml	1, 5, 10, 20 μg/ml	5, 10, 12.5 μg/ml	5-100 µg/ml
Model	Murine astrocyte-like ALT, murine microglial BV-2 cells and murine neuroblastoma N2a cells	Primary rat cortical cell cultures	Human neuronal SH-SY5Y cells and human glial D384 cells	Primary organotypic mouse midhrain cells	Primary rat cerebellar granule cells (CGCs)	Primary rat cerebellar granule cells (CGCs)	Human and rat embryonic neural stem cells (NSCs)	Murine brain astrocyte-like ALT, murine microglial BV-2 cells and murine neuroblastoma N2a cells	Neuronal progenitor cells (C17.2)
Surface coating	None	None	None	Citrate; nolvvinvlnvrrolidone	None	None	None	None	None
Size (nm)	10	20	20	20 and 110	<100	22.1-26.2	23	35	10
NPs	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	CeO ₂

Table 1: In vitro models for metallic nanoparticles neurotoxicity assessment.

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References	[91]	[57]	[89]	[93]	[46]	[95]	[96]	[3]
Outcome	HA/Ga ₂ O ₃ :Cr ³⁺ NPs induced calpain activation and disturbance of autophagy signaling	Mn NPs depleted DA, DOPAC, and HVA in a dose-dependent manner	Enzymatic alterations were involved in dopaminergic neurotoxicity induced by Mn NPs and Cu NPs	TiO ₂ NP did not reduce the viability of neuronal cells but were effectively internalized by the cells and were found to induce dose-dependent cell cycle alterations, apoptosis by intrinsic pathway, and genotoxicity not related with DSB production	TiO ₂ NPs caused cytotoxic effects with alterations of the mitochondrial function and cell membrane damages	Hippocampal neuron apoptosis caused by TiO ₂ NPs may be associated with mitochondria-mediated signal pathway and endoplasmic reticulum-mediated signal pathway	TiO ₂ -NPs induced damage of neurons via ROS and JNK/p53 mediated-apoptosis and caused G2/M arrest by activation of p53/p21 pathway	TiO ₂ NPs induced microglial activation and subsequently caused release of proinflammatory factors that contributed to dysfunction and cytotoxicity in PC12 cells
Exposure duration	12 h	24 h	24 h	3, 6, 24 h	4, 24, 48 h (acute) or 7-10 days (prolonged exposure)	24 h	6 and 24 h	24 h or48 h
Concentration	1, 5, 25, 50, 100 μg/ml	1-100 µg/ml	10 µg/ml	20-150 µg/ml	1.5-250 µg/ml (acute) and 0.05-31 µg/ml (prolonged exposure)	5, 15, 30 μg/ml	25, 50, 100, 200 μg/ml	0.25, 0.5 mg/ml
Model	Primary cortical and SH-SY5Y neuronal cells	Rat PC-12 cells	Rat PC-12 cells	Human SH-SY5Y neuronal cells	Human neuronal SH-SY5Y cells and human glial D384 cells	Primary cultured hippocampal neurons	Rat PC-12 cells	Primary microglia and rat PC-12 cells
Surface coating	Cysteine modified hyaluronic acid (HA)	None	None	None	None	None	None	None
Size (nm)	125.7, 200.6 and 313.9	40	40 90	25	68.9-69.7	5-6	20	20
NPs	HA/Ga ₂ O ₃ :Cr ³⁺	Mn	Mn Cu	TiO ₂	Ti0 ₂	Ti0 ₂	Ti0 ₂	TiO ₂

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References	[92]	[66]
Outcome	Doping of WC NPs with Co noticeably increases their cytotoxic effect	Newport-Green DCF-2 K ⁺⁻ -conjugated ZnO NPs along with the membrane probe FM1-43 demonstrated endocytosis of ZnO NPs by PC12 cells;
Exposure duration	3 days	24 h
Concentration	7.5, 15, 30 μg/ml for WC NPs and 8.25, 16.5, 33 μg/ml for WC-Co NPs	10, 100, 1000, 10000 µМ
Model	Oligodendrocyte precursor cell line OLN-93 and primary neuronal and astroglial cell cultures from cortices of Wistar rat fetuses	Rat PC-12 and human SH-SY5Y neuronal cells
Surface coating	None	None
Size (nm)	145	<50
NPs	WC (tungsten carbide), WC – Co	ZnO

fluozin-3 measurement showed elevation of cytosolic Zn²⁺ concentration in cells

D384, evidenced both after acute and prolonged exposure, and manifested as alterations of mitochondrial function and cell membrane damage [94]. In line, apoptosis associated with mitochondria-mediated and endoplasmic reticulum-mediated signaling was reported in primary cultured hippocampal neurons exposed to TiO₂ NPs for 24 hours [95]. While Wu et al. observed that TiO₂ NPs may directly interfere with rat PC12 neuronal function and cause damage mediated by activation of p53 and/or JNK pathway [96]. An alternative or supplementary mechanism of neurotoxicity was proposed by Xue et al. They observed a significant cytotoxicity of PC12 cells when the cells were incubated with supernatant from microglia culture exposed to TiO₂ NPs and proposed that NPs-induced neurotoxicity corresponded to release of NO and pro-inflammatory factors, such as tumor necrosis factor alfa (TNF α) by the activated microglia cells [3].

Significant neurotoxicity was also observed in the rat and human neuronal cells in culture treated with ZnO NPs, however in this case toxicity was attributed rather to the action of Zn^{2+} than to NPs *per se.* ZnO NPs were uptaken *via* endocytosis, but low pH 5.5 in endosomes facilitated release of Zn^{2+} ions that penetrated to cytosol and disturbed divalent ions homeostasis, resulting in cell death [66].

The presented *in vitro* studies on the neurotoxicity of various metallic NPs confirmed induction of diverse adverse effects in CNS-derived cells. The vast majority of reports suggested that increased mitochondrial production of ROS and increased oxidative stress is a main mechanism of cytotoxicity of metallic NPs. This resulted in disruption of structure and function of other cell organelles, changes in gens expression, ionic imbalance and activation of apoptotic pathways. The effects of *in vitro* exposure to metallic NPs are summarized in Table 1.

4.2 In vivo toxicity in mammals

Neurotoxicity of metallic NPs has been confirmed in many mammalian studies. Chen *et al.* observed that Al NPs accumulated in the mouse brain endothelial cells, causing damage to the neurovascular system. Systemic administration of Al NPs resulted in elevated autophagy-related genes expression and autophagic activity in the brain, decreased tight-junction protein expression and increased BBB permeability [4].

The ability to cross BBB was further confirmed for Au NPs that were found in the brain of intraperitoneally administered mice, however, the concentration of gold in the brain was the lowest of all examined organs. Interestingly, no obvious toxicity to the CNS nor changes in behaviour of mice were observed [20]. In contrast, acute exposure of male Wistar rats to Au NPs resulted in a reduction of thiobarbituric acid reactive substances and carbonyl protein levels in the rats brain. In addition, suppression of catalase activity and inhibition of energy metabolism in hippocampus, striatum and cerebral cortex was observed. Interestingly, long-term exposure to Au NPs resulted only in inhibition of catalase in the brain and suppressed energy metabolism in cerebral cortex [97].

Also prolonged exposure of rats to citrate-stabilized Ag NPs caused a severe synaptic degeneration, mainly in the hippocampal region of brain, which may consequently lead to an impairment of normal nerve function and cognitive processes [98]. In line, Ag NPs given intragastrically to adult female rats induced a slight shrinking of the hippocampus, neuron shrinkage and swelling of astrocytes after 7-day exposure. The study also showed a significant increase of interleukin-4 (IL-4) in blood. Researchers suggested that neurodegeneration after exposure to Ag NPs occurred through inflammatory effects [99]. Neurotoxicity and impaired BBB functions were also observed after intraperitoneal administration attributed to the change of level of trace element in serum and brain, reduction of antioxidant enzyme activity, apoptosis and induction of inflammatory processes and down regulated tight junction proteins expression [100]. Similarly, chronic, intragastrically exposure of rats to low doses of Ag NPs resulted in the presence of silver in various parts of the brain, including the hippocampus and impairment of hippocampal dependent memory and cognitive coordination processes [101]. In contrast Dąbrowska-Bouta et al. showed in their studies on male Wistar rats that Ag NPs administered in low concentration did not cause visible neurotoxicity in behavioral tests; however, more accurate studies showed abnormalities in the myelin sheaths and an altered expression of myelin proteins [102].

Study conducted by An *et al.* have shown that CuO NPs accumulated in the brain when administered *via* intraperitoneal injection to adult male Wistar rats, and had a toxic effect on the hippocampus, inducing learning and memory deficits. The results suggested neuronal damage, induced by an imbalance of redox homeostasis that led to the impairment of hippocampal long-term potentiation (LTP) and poor performance of animals in behaviour tests [55]. Similarly, Liu *et al.* examined the effect of CuO NPs after nasal instillation into mice. They observed that NPs were taken directly to the brain by the olfactory bulb, and that exposure to CuO NPs resulted in severer lesions in the mouse brain, which could be due to the induction of oxidative stress in nerve cells [36]. In line, Bai *et al.* observed nerve damage to astrocyte cells and abnormal neurotrans-

mitter levels in murine after intranasally instillation of Cu NPs [103]. It was also shown that in male Wistar rats CuO NPs impaired glutamate transmission presynaptically and postsynaptically that might result in diminished LTP and other cognitive deficits [25].

In yet other studies, rats were exposed orally to cadium oxide NPs (CdO NPs) by intratracheal instillation alone, or in sequential combination with CdCl₂ solution. A 3-weeks oral administration, plus 1 week delivery to the trachea, resulted in significant loss in weight of the rats and decreased open field motility. In addition, the lengthening of latency evoked potentials of sensors, and the conduction velocity of the tail nerve was decreased [16]. Similar electrophysiological effects were observed after 4-12 weeks of oral or intratracheal exposure to cadmium [17, 104]. Translocation from the lungs to the secondary organs, such as the brain, was also reported for lead oxide NP (PbO NPs) after acute or subchronic PbO NPs inhalation by female mice. Inhaled PbO NPs caused mild pathological alterations in the hippocampus area [105].

In line, intranasal administration of Fe₂O₃ NPs caused damage to the olfactory bulb, hippocampus and striatum, likely due to elevated levels of ROS and NO causing neurons degeneration that occurred primarily in the CA3 area of hippocampus. In addition, excessive microglial cell recruitment, proliferation and activation, especially in the olfactory bulb, was observed and proposed as an additional cause of brain injury [32]. Interestingly, no induction of DNA damage was also observed in female Wistar rats exposed to Fe₂O₃ NPs or Fe₂O₃ bulk material, suggesting that Fe₂O₃ NPs did not have genotoxic potential [9]. Neurotoxic were also Fe₃O₄ NPs when administered directly into the dorsal striatum or hippocampus of mice. Fe₃O₄ NPs administration reduced TH⁺ fiber in both dorsal striatum and hippocampus and caused motor memory deficits, attributed to activation of MAPK and JNK signalling pathways [22].

In line, MnO_2 NPs instilled into the trachea of adult male rats penetrated to the brain causing damage to nerve tissue. In addition, in the open field activity, the percentage of ambulation and rearing decreased, while local activity increased. The latency of the evoked potentials was lengthened, while the conduction velocity of the tail nerve decreased [41]. Also intratracheal exposure of mice to MnO NPs caused an increase in evoked potential latency and change in cortical electrical activity to the higher frequencies. Co-exposure with Fe₃O₄ NPs also resulted in increase in evoked potential latency [106]. Detrimental health effects were also observed for nickel oxide NPs (NiO NPs) and Mn_3O_4 NPs administered intraperitoneally to rats. Mn_3O_4 NPs were more harmful in the most nonspecific toxicity symptoms, and caused more nerve damage in the caudate nucleus and hippocampus, as compared to NiO NPs [107].

Despite the neurotoxicity manifested in exposed adult animals, several experiments showed that exposure to metallic NPs might be detrimental also for the future generations. Intragastrically exposure to TiO₂ NPs of pregnant rats resulted in significant inhibition of cell proliferation in the hippocampus of the offspring and significantly impaired their learning skills and memory [108]. A similar study by Hong et al. demonstrated that exposure to TiO₂ NPs of mice during pregnancy or lactation had detrimental effects on developing CNS in offspring. TiO₂ NPs were shown to negatively affect the learning and memory processes. Researchers suggested that this might be the result of down regulation of Rac 1 and Cdc42 protein expression, and upregulation of Rho A protein expression, and increased ratio of RhoA/Rac 1 proteins [24]. This was further confirmed for intraperitoneally administered TiO₂ NPs. The brains of foetuses from treated mice had changes in anatomical structure linked with perivascular edema [109].

On the other end, Amara et al. showed that acute intravenous injection of ZnO NPs to adult rats caused no changes in neurotransmitter contents (norepinephrine, epinephrine, DA and serotonin), nor deterioration in locomotor activity and spatial working memory [110]. No toxicity was also observed when ZnO NP were administered intraperitoneally [111]. Similarly, Shim et al. studied the effects of 28-day oral exposure of ZnO NPs on BBB tightness in rats. In line, no damage to BBB or the brain, nor behavioural changes were observed after 28-day oral exposure of ZnO NPs [112]. In turn, de Souza et al. demonstrated that environmentally relevant concentration of ZnO NPs cause behavioral changes of male Swiss mice [113]. Also Aijie et al. reported injury in cerebral cortex and hippocampus and impaired learning and memory of rats administered with NPs ZnO and TiO₂. Interestingly, in this experimental set up the NPs were administered to tongue of male Wistar rats. The experiment confirmed that NPs can enter the CNS through the taste nerve pathway [114].

As demonstrated by many *in vivo* studies, metallic NPs are harmful to the rodents' CNS. NPs easily translocate and accumulate in different regions of the brain, in particular in hippocampus striatum and cerebral cortex. Damage caused by metallic NPs may result in motor deficits, and impairment of learning and memory. The effects of *in vivo* exposure to metallic NPs are summarized in Table 2.

References	[98]	[66]	[100]	[101]	[102]	[122]	[124]	[125]	[126]
Outcome	Ag NPs caused severe synaptic degeneration, mainly in the hippocampal region of brain	Ag NPs induced neuronal degeneration, due to inflammatory effects	Ag NPs induced neurotoxicity and disrupt BBB because of perturbation of serum and brain trace elements contents, reduction of antioxidant defense, and induction of inflammation and apoptosis, along with inhibition of expression of tight junction proteins	Ag NPs induced detrimental effect on memory and cognitive coordination processes	No differences between treated and control animals were observed in behavioral tests, however, transmission electron microphotographs showed abnormalities in myelin sheaths and altered expression of myelin proteins	Ag NPs (2 mg/l) stimulated the brain antioxidant system, but inhibited it at high concentration	Ag NPs shifted into the yolk and gallbladder during embryonic development; NPs were capable of penetrating BBB and reach the brain; toxicity was salinity-dependent	PVP-Ag NPs and AgNO ₃ affected pathways involved in Na ⁺ , K ⁺ , and H ⁺ homeostasis and oxidative stress	Ag NPs induced neurotoxicity
Exposure duration	14 days, oral exposure	14 days, intragastrically	Intraperitoneally, 3 times a week, for 30 days	Orally <i>via</i> gavage, for 28 days	14 days, administration <i>via</i> gastric tube	24, 48 and 96 h, aqueous exposure	a,b) 3 days, aqueous exposure c) 7 days, aqueous exposure	96 h, aqueous exposure	96 h or 40 days
Concentration	0.2 mg/kg b.w.	1, 10 mg/kg b.w./day	50 mg/kg b.w.	30 mg/kg b.w. or 1 mg/kg b.w.	0.02 mg /ml	2 and 4 mg/l	a) 1mg/1 b) 30 mg/1 c) 10 mg/1	4.8 μg/l of AgNO ₃ or 61.4 μg/l of PVP-Ag NPs	0.2, 2 and 20 mg/ml
Model	Male Wistar rats	Six-week-old female Sprague-Dawley rats	Male Wistar Albino rats	Male Wistar rats	Male Wistar rats	Nile tilapia (Oreochromis niloticus) and Redbelly tilapia (Tilapia zillii)	a, b) See-through medaka (<i>Oryzias</i> <i>latipes</i> , ST II strain) eggs c) adults see-through medaka (<i>Oryzias</i> <i>latipes</i> , ST II strain)	Fathead minnow (Pimephales promelas)	Neotropical fish (Hoplias intermedius)
Surface coating	None	None	None	Bovine serum albumin	None	None	Fluorescent polystyrene	Polyvinylpyrrolidone (PVP)	Polyvinylpyrrolidone
Size (nm)	10±4	3-30	44.13	20±5	10±4	<100	39.4	30±3	20
NPs	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag

Table 2: In vivo models for metallic nanoparticles neurotoxicity assessment.

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References	[127]	[4]	[134]	[20]	[77]	[16]
Outcome	Ag NPs caused hyperexcitability in developing embryos without mortality, significant hatching impairment or morphological abnormalities	Al NPs caused elevation of autophagy-related genes and autophagic activity in the brain, decreased tight-junction protein expression, and elevated blood-brain barrier (BBB) permeability, increased brain infarct volume in mice subjected to a focal ischemic stroke model	Al ₂ O ₃ NPs caused extensive histological changes in various organs, including severe necrosis of brain cells	Au NPs crossed BBB and accumulated in the neural tissue; importantly, no evidence of toxicity	Acute resulted in reduction of thiobarbituric acid reactive substances and carbonyl protein levels in rats brain; inhibition of catalase activity and energy metabolism was observed in the hippocampus, striatum and cerebral correx	NPs caused significant reduction of open field motility; lengthening of latency of sensory evoked potentials; conduction velocity of the tail nerve was decreased in all treated groups
Exposure duration	4-120 h, aqueous exposure	1 week, carotid artery surgery to deliver Al NPs directly into the cerebral circulation	96 h, aqueous exposure	Every day for 8 days, intraperitoneal administration	Single intraperitoneal injection or repeated injections once daily for 28 days	3-4 weeks, intratracheal instillation
Concentration	0.03, 0.1, 0.3, 1 and 3 ppm	1.25 mg/kg	120, 150 and 180 ppm	40, 200, and 400 μg/kg/day	70 μg/kg b.w.	0.04 mg/ kg b.w.
Model	Zebrafish (<i>Danio rerio</i>) embryos and larvae	C57BL/6 mice	Mozambique tilapia (<i>Oreochromis</i> mossambicus)	Male C57/BL6 mice	Adult male Wistar rats	Adult male Wistar rats
Surface coating	None	None	None	None	None	None
Size (nm)	20-40	8-12	40	12.5	10 and 30	65
NPs	Ag	AI	Al ₂ O ₃	Au	Ац	CdO ₂

References	[17]	[36]	[103]	[115]	[132]	[133]	[25]	[55]
Outcome	Spontaneous cortical electrical activity was shifted to higher frequencies, the latency of sensory-evoked potentials was lengthened, and the frequency following ability of the somatosensory evoked potential was impaired, despite detectable Cd deposition in the brain	Cu NPs induced severe lesions of the brain with no connection to oxidative stress	Cu NPs entered the brain after nasal inhalation and induced damages to the CNS	Significant decrease in Na ⁺ /K ⁺ -ATPase activity in the brain; depletion of plasma and carcass ion concentrations; Cu NPs have similar types of toxic effects to CuSO ₄	Toxicity of CuSO ₄ and Cu NPs was similar, but Cu NPs caused more damage in the intestine, liver and brain	Cu NPs caused a significant increase in the ratio of oxidized to reduced glutathione in brains	CuO NPs impaired glutamate transmission presynaptically and postsynaptically, diminished LTP and caused other cognitive deficits	CuO NPs impaired oxidation–antioxidation homeostasis, and hippocampal LTP, which was associated with poor performance of animals in behavior tests
Exposure duration	3 and 6 weeks, intratracheal instillation	1 week, nasal instillation	21 days, intranasal instillation	4, 10 days, semi-static aqueous exposure	4, 10 days, semi-static aqueous exposure	12 h, aqueous exposure	Intraperitoneal, for 14 days	Once a day for 14 consecutive days, intraperitoneal injection
Concentration	0.4 mg/kg	40 mg /kg b.w.	1, 10, 40 mg/kg b.w.	0, 20 and 100 μg/l	20 or 100 μg/l	50 µg/1	0.5 mg/kg b.w.	0.5 mg/ kg
Model	Male Wistar rats	Mice	CD-1 (ICR) female mice	Juvenile rainbow trout (Oncorhynchus mykiss)	Juvenile rainbow trout (Oncorhynchus mykiss)	Juvenile rainbow trout (Oncorhynchus mykiss)	Male Wistar rats	Adult male, specific-pathogen free (SPF) Wistar rats
Surface coating	None	None	None	None	None	None	None	None
Size (nm)	~20	23.5	23.5	87±27	87±27	20.3±0.9	15-90	10-70
NPs	Cd	Cu	Cu	Cu	Си	Cu	CuO	CuO

Table 2: ...continued

	Outcome	Cholinesterase activity was inhibited by CuO NPs exposure, attributed to	the free Cu ²⁺ ions dissolved inside the carp body	Cu NPs exposure leaded to abnormal phenotypes in zebrafish embryonic	development, induced an inflammatory response and delay in	retinal neurodifferentiation accompanied by reduced locomotion	Fe ₂ O ₃ NPs caused no significant	DNA damage	Fe ₃ O4 NPS reduced 1.H ⁻ fiber in both dorsal striatum and hippocampus:	and caused motor memory deficits in	mice due to the activation of the	MAPK and JNK pathways	Ratio of Fe (III)/Fe (II) increased in	olfactory bulb and brain stem; Fe ₂ O ₃ NPs were nossibly transported <i>via</i>	untake hv sensory nerve endings of	the olfactory nerve and trigeminus	MnO ₂ NPs translocated from airways	to the brain, lengthened evoked	potential latency and decreased	conduction velocity of the tail nerve	MnO ₂ NPs caused a shift of	spontaneous and evoked cortical	electrical activity to higher	frequencies, and lengthened evoked	poternual fatericy, writch were also	heavily reduced by co-exposure with	$Fe_{3}O_{4} NPs$	Both NPs negatively affected rats	health; Mn ₃ O ₄ NPs were more
	Exposure duration	a) 96 h b) 30 days		48, 72, 96 h			72 h, oral	exposure	into the dorsal	striatum and	hippocampus		Single dose,	intranasal instillation			3, 6, and 9 weeks,	instilled into the	trachea		Intratracheal	instillation once a	day, for 4 weeks				:	Intraperitoneally 3	times a week (up +^ 18 injections)
	Concentration	a) 0, 10, 50, 100, 200, 300, 500, 1000 mg/l	b) 100 mg/ml	1, 6.25, 12.5, 25 or 50 mg/L	5		500, 1000 and 2000	mg/kg	ر الم/10 Jul 2 الم 2				40 mg/kg b.w.				2.63, 5.26 mg				2 mg/kg b.w., MnO ₂	NPs, also in 4 mg/kg	b.w.					0.25, 0.50 mg	
	Model	Juvenile carp (Cyprinus carpio)		Zebrafish (<i>Danio</i> <i>rerio</i>) embrvos and	larvae		Adult female Wistar	rats	even to the test of test o				CD-ICR male mice				Adult male Wistar rats				Male Wistar rats							White female rats	
	Surface coating	None		None			None	;	None				None				None				None						;	None	
מפת	Size (nm)	20-40		20-130			30		29./8-68.09				280±80				23				3.6,	8.8,	13.5					16.7±8.2	18.4±5.4
	NPs	CuO		CuO			Fe_2O_3	¢	Fe3 U4				Fe_2O_3				MnO_2				MnO_2 ,	Fe ₃ 04,	$Cr(OH)_3$					NiO	Mn_3O_4

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nerve damage of the caudate nucleus and the hippocampus as compared to NiO NPs

harmful in most nonspecific toxicity symptoms; Mn₃O₄ NPs caused more

References	[105]	[108]	[24]	[109]	[116]	[118]	[119]	[120]
Outcome	Inhalted PbO NPs translocated from the lungs to secondary organs such as brain and caused mild pathological alterations in hippocampus area	TiO ₂ NPs significantly reduced cell proliferation in the hippocampus, impaired learning and memory in offspring	Pregnancy/lactation exposure to TiO ₂ NPs inhibited development of brain and decreased learning and memory in weaning mice, which was associated with down regulation of Rac 1 and Cdc42 protein expression, upregulation of Rho A protein expression and increased ratio of RhoA/Rac1	TiO ₂ NPs accumulated in heart and brain fetus. Fetus and placenta sizes were lower than in control group	TiO ₂ NPs (100 and 200 mg/l) caused decrease in SOD, CAT and POD activities and significant increase in LPO levels in tissues, suggesting oxidative stress	TiO ₂ NPs caused brain injury and reductions of spatial recognition memory	TiO ₂ NPs induced Parkinson's disease-like symptoms and faster embryos hatching	TiO ₂ NPs with difficulty penetrated into the brain
Exposure duration	Whole body inhalation chambers for 4–72 h in acute experiment, for 1–11 weeks in subchronic experiment	From prenatal day 2 to day 21, orally expose (gavage)	Orally <i>via</i> gavage, from prenatal day 0 to postnatal day 21	Single dose, intraperitoneal iniection	8 days, aqueous exposure	45 days, aqueous exposure	For 96 h post fertilization, aqueous exposure	24 h and 14 days, aqueous exposure
Concentration	Acute experiment 4.05×10 ⁶ NPs/cm ³ , subchronic experiment 3.83×10 ⁵ NPs/cm ³ or 1.93×10 ⁶ NPs/cm ³	100 mg/kg b.w.	1, 2, 3 mg/kg b.w.	2 mg/ml	10, 50, 100, 200 mg/l	5, 10, 20 and 40 $\mu g/L$	0,1, 1, 10 µg/ml	500 and 5000 µg/l
Model	Adult female mice (ICR line)	Pregnant Wistar rats	Pregnant CD–1 (ICR) mice	Pregnant NMRI mice	Carp (Cyprinus carpio)	Zebrafish (Danio rerio)	Zebrafish (Danio rerio) larvae	Zebrafish (Danio rerio) and rainbow trout (Oncorhynchus mykiss)
Surface coating	None	None	None	None	None	None	None	None
Size (nm)	Fraction of 99.9% PbO NPs 7.64–120, fraction of 90% of NPs 7.64–44.5	10	6.5	20	50	5-6	3,4±1,9	32.47-35.93
NPS	PbO	TiO ₂	TiO2	TiO ₂	TiO ₂	TiO ₂	TiO ₂	TiO_2

Table 2: ...continued

References	[110]	[111]	[112]	[113]	[117]	[121]	[123]	[114]
Outcome	ZnO NPs caused no changes in neurotransmitter contents; no deterioration in locomotor activity and spatial working memory was observed	ZnO NPs did not affect neurotransmitter contents, locomotor activity nor spatial working memory in adult rats	BBB was not compromised and was able to block penetration of ZnO NPs	Environmentally relevant concentration of ZnO NPs, even for a short period of time, induced behavioral changes related to anxiety in mice	ZnO NPs (50 mg/l) caused significant decrease of several enzymatic activities; gill, liver and brain were most sensitive organs	ZnO NPs (500µg/l) stimulated the brain antioxidant system, but inhibited it at high concentration	ZnO NPs were retarded development of nervous and vascular system	NPs were deposited in the nerves and brain. The learning and memory of rats were impaired
Exposure duration	Every day for 14 days, intravenous injections	Single dose, intravenous injection	a) 28 days, oral exposure, b) 90 days, intravenous administration	Aqueous exposure	14 days, aqueous exposure	15 days, aqueous exposure	48, 72, 96 h aqueous exposure	30 days, NPs were tongue-instilled
Concentration	25 mg/kg b.w.	25 mg/kg b.w.	a) 500 mg/kg, b) 0.1, 10 mg/animal	5.625×10 ⁵ mg/ kg b.w., 300 mg/kg b.w.	0.5, 5, 50 mg/l	500 and 2000 μg/l	1, 5, 10, 20, 50 and 100 ppm	50 mg/kg b.w.
Model	Male Wistar rats	Male Wistar rat	Rats	Adult male Swiss mice	Carp (Cyprinus carpio)	Nile tilapia (Oreochromis niloticus) and Redbelly tilapia (Tilapia zillii)	Zebrafish (Danio rerio) embryos	Male Wistar rats
Surface coating	None	None	Citrate (for negative charge), and L-serine (for positive charge)	None	None	None	None	None
Size (nm)	30-40	30-40	20 and 100	68.96±33.71	30	35	10-30	50 25
NPs	ZnO	ZnO	ZnO	ZnO	ZnO	ZnO	ZnO	Zn TiO ₂

Table 2: ...continued

4.3 Toxicity in non-mammalian vertebrates

In water reservoirs metallic NPs are mainly present in a dispersed form or in the form of emulsion. Thus, it is believed that they may be more toxic in water, compared to the bulk counterparts, as they easier dissolved and release the metal ions [115]. Although dissolution of metallic oxide NPs is more difficult, as it must be accompanied by disruption of covalent bonding, it could be facilitated by humic acids and other chemical substances present in the water.

Studies on carp (*Cyprinus carpio*) showed that exposure to ZnO NPs or TiO₂ NPs induced a significant increase in lipid peroxidation, oxidative stress and a decrease in the activity of antioxidant enzymes in the brain [116, 117]. Similar study conducted on zebrafish (*Danio rerio*) revealed that subchronic exposure to low doses of TiO₂ NPs resulted in brain injury, reduction of spatial recognition memory, thus impairing behavioural response [118]. Hu *et al.* reported that exposure to 1 and 10 μ g/ml of TiO₂ NPs produced a loss of dopaminergic neurons on the level 50-70%, and induced Parkinson's disease-like symptoms zebra fish larvae. This was confirmed in *in vitro* studies on PC12 cells [119]. On the contrary, Johnston *et al.* observed that TiO₂ NPs hardly penetrated into the brain of zebrafish [120].

Effective uptake of metallic NPs from water was also noted for ZnO NPs and Ag NPs in two different fish models: Nile tilapia (Oreochromis niloticus) and redbelly tilapia (Tilapia zillii). Exposure to high concentration of ZnO NPs $(2000 \ \mu g/l)$ and Ag NPs $(4 \ mg/l)$ resulted in a destructive effect on the brain antioxidant system, while the low concentration (500 μ g/l) of ZnO NPs and (2 mg/l) of Ag NPs produced completely opposite effect, supporting the antioxidant activity [121, 122]. In the study on zebrafish, ZnO NPs impaired development of the nervous and vascular system [123]. Waterborn exposure to Ag NPs resulted in the presence of NPs in the brain of medaka (Orvzias latipes) [124]. Whereas studies conducted on fathead minnow females (*Pimephales promelas*), revealed that Ag NPs interacts directly in the form of intact NPs and indirectly, by releasing ions from the surface of NPs, each with different pathways of neurotoxicity [125]. Also Klingelfus et al. described neurotoxic effects of Ag NPs on neotropical fish (Hoplias intermedius) [126]. A Ag NPs caused hyperexcitability in developing zebrafish was reported far below concentrations found in some aquatic environment [127].

Similarly to Ag NPs, Cu NPs suspended in water, dissolve slowly and release copper ions that might accumulate initially in the gills reflecting entry gate, and then in the other internal organs [128]. CuO NPs and ions released from the NPs penetrated into the brain and thus DE GRUYTER

may be toxic to the CNS [129]. A dramatic decrease in cholinesterase activity was observed in the brains of juvenile carp exposed to CuO NPs, corresponding to a significant increase of copper concentration in the major organs of the fish, including the brain. Interestingly, cholinesterase activity returned to the control level after 10 days, most likely due to the adaptative capabilities of the fish. However, this was attributed rather to the action of the copper ions released from NPs in the fish body than to the NPs effects per se, as no CuO NPs were found in the brain tissue. A study by Zhao et al. included the effect of CuO NPs exposure on juvenile carp. Copper has a number of important characteristics in the brain of fish, including antioxidant functions in melatonin, by the modulation of the excitability of nerve cells and biological rhythms [115, 130]. Further, the NPs inhibited enzyme cholinesterase activity was important for the proper functioning of the neurotransmitter acetylcholine. The research Zhao et al. suggest that this is a consequence of the release of Cu^{2+} ions from CuO NPs inside the body of the carp. Thus it can be concluded that the CuO NPs have neurotoxic properties for carp. The study also showed that CuO NPs were more toxic than its copper molecular counterparts [130]. Sun et al. observed a disturbance of embryonic zebrafish development as a result of exposure to CuO NPs manifested by the delay of retinal neurodifferentiation [131]. Similarly, the study conducted by Al-Bairuty et al., where they compared the toxicity of CuO NPs and CuSO₄ NPs on rainbow trout (Oncorhynchus mykiss), showed in the brain, mild changes in neuronal cell bodies in telencephalon, and some changes in the thickness of the midbrain and vasodilation in a the ventral surface of the cerebellum. Furthermore, similar toxicity was observed for Cu NPs and their molecular equivalent. However, the Cu NPs caused more damage to the brain, compared to equivalent concentrations of CuSO₄. Al-Bairuty postulated that brain damage occurred indirectly as a result of gill damage and systemic hypoxia in the body of rainbow trout. The etiology of CuO NPs in the brain pathology requires further study, but it can be assumed that oxidative stress and disruption of osmotic and metal homeostasis may contribute to the pathology of the CNS [132]. Moreover, recent studies have shown that exposure of juvenile rainbow trout on Cu NPs resulted in a significant increase in the ratio of oxidized to reduced glutathione in brains of fish, which may indicate the induction of oxidative stress [133]. Also, studies on freshwater fish Mozambique tilapia (Oreochromis mossambicus) exposed to Al₂O₃ NPs revealed extensive histological changes in various organs, including severe necrosis of nerve tissue [134].

Although quoted studies were done only on fishes, the toxic effect of metallic NPs on non-mammalian vertebrates is clearly noticeable. Water favors a more efficient penetration of NPs into organisms enhancing their adverse effects. In larvae, metallic NPs interfere with development of embryonic brain leading to decreased vitality. Their destructive effects on nervous system of adult animals are manifested by oxidative stress, acetylcholinesterase activity disturbances, and changes in cellular signalling. The effects of *in vivo* exposure to metallic NPs are summarized in Table 2.

4.4 Other responses

The NPs also affect biochemical properties of the brain and iono-regulatory processes. Kumari et al. studied female albino Wistar rats after a 28-days repeated oral dose of 30 nm Fe_2O_3 NPs. They observed inhibition of Na⁺-K⁺, Mg²⁺ and Ca²⁺-ATP monophosphatase (ATPase) in rat brain; it was also shown that smaller Fe₂O₃NPs were more toxic [135]. In line, acute oral exposure of female Wistar rats to 30 nm Fe₂O₃ NPs resulted in more than 50% inhibition of total Na⁺-K⁺, Mg²⁺ and Ca²⁺-ATPases activity in the brain [136]. Similarly, intragastric administration of TiO₂ NPs for 60 days resulted in disturbance of brain trace elements homeostasis and inhibited the activity of Na⁺/K⁺- ATPase, Ca²⁺ -ATPase, Ca²⁺/Mg²⁺ - ATPase [137]. Also, a 14 days exposure of rainbow trout to TiO₂ NPs resulted in dose-dependent changes in the brain concentration of Cu and Zn ions and decreased in the activity of Na⁺/K⁺ - ATPase [138]. Lowering of Na^+/K^+ - ATPase activity in the brain was also observed in juvenile rainbow trout after waterborne exposure to Cu NPs [115]. NPs-dependent decrease in activity of AChE and increased protein oxidative damage in brain was also observed in juvenile fish Prochilodus lineatus exposured to TiO₂ NPs and ZnO NPs [139]. In line Xia et al. showed that 1 mg/l of TiO₂ NPs in water increased the level of AChE activity in gills (after day 5) and in digestive gland (after day 12) in the scallop (Chlamys farreri) [140]. In turn, de Oliveira et al. observed inhibition of AChE activity and a reduction in the exploratory performance in adult zebrafish exposed to SPION coated with cross-linked aminated dextran [141]. On contrary, Boyle et al. reported no accumulation of NPs neither decrease in the activity of Na^+/K^+ - ATPase and AChE in brain of trout exposed to TiO₂ NPs for 14 days, despite a significant increase in the total glutathione pool [142]. Similarly, in a study conducted by Ramsden et al., after 14 days of aqueous exposure of zebrafish to TiO₂ NPs, no changes were observed in Na^+/K^+ -ATPase activity in the brain [143].

Presented studies indicate that different types of metallic NPs have the ability to change the biochemical processes taking place in individual nerve cells, thus disturbing the proper functioning of the entire nervous system.

5 The role of oxidative stress in NPs-induced toxicity and DNA damage

Although molecular mechanism underlying the neurotoxicity of NPs are still obscured, generation of intracellular ROS seems to be a main cause of the toxicity. Oxidative stress is caused by an imbalance between oxidants and antioxidants in the cell. Also is considered a major cause of damage to DNA, lipids and protein.

Induction of oxidative stress by various NPs, both in vitro and in vivo, is well documented, thus is was also expected in the context of their effects on the brain. Indeed, Ag NPs and TiO₂ NPs stimulated the activation of glial cells to release proinflammatory cytokines and to generate ROS and the production of NO, causing neurological inflammation [144, 145]. A decrease in antioxidant enzymes activity in neurons was also observed after exposure of rats to Ag NPs thus the level of ROS in brain tissue has increased [146]. Also exposure to Fe NPs reduced superoxide dismutase activity and an increased lipid oxidation in medaka (Oryzias latipes) embryos, however in adult animals oxidative stress was observed only at the beginning of treatment. After a certain time of exposure oxidantsantioxidants balance returned to the normal level [147]. A dose dependent, intrasynaptosomal formation of ROS was also reported for 7 nm iron particles coated ferritin [21]. Toxicity of ZnO NPs was also associated with their ability to produce ROS. Melatonin treatment, which has usually a protective effect against oxidative stress caused by external factors, was ineffective for these NPs [148].

In line, CuO NPs induced damage to hippocampal neurons and increased malonaldehyde level in Wistar rats was ascribed to increased ROS production and reduced activity of SOD and glutathione peroxidase (GSH-Px) [55]. Oxidative stress and decreased activity of antioxidant enzymes in the brain was pointed as a main cause of Au NPs-induces increase in 8-hydroxydeoxyguanosine and heat shock protein 70 level and caspase-3 activity, which might lead to cell death. In addition, the study showed a significant increase in the cerebral levels of IFN- γ in the treated animals [27]. Oxidative stress was also attributed to adverse effects in

brain tissue, such as increased inflammatory cytokines, DNA fragmentation and stimulation of apoptosis, in rats exposed orally for 7 days to ZnO NPs [149]. Chen et al. observed in human brain microvascular endothelial cells that oxidative stress, manifested as decreased mitochondrial potential and decreased the expression of TI proteins. is also induced by Al NPs [18]. Also various forms of Al₂O₃ NPs induced ROS formation, protein oxidation, lipid peroxidation, glutathione reduction and mitochondrial dysfunction [150]. In line, oxidative stress was point as a main cause of genotoxicity observed for tungsten oxide NPs (WO₃ NPs) [151], chromium oxide NPs (Cr₂O₃ NPs) [152] and MnO₂ NPs [153]. Oxidative stress arising after treating neural stem cells with Fe₃O₄ NPs caused by an imbalance in the ROS formation and antioxidant cell-defence system, resulted in depleted intracellular glutathione levels, hyperpolarization of mitochondrial membrane, disturbed cellmembrane potential and DNA damages [154].

Hardas et al. studied pro-oxidant effect of 5 nm CeO₂ NPs administered peripherally to the Sprague Dawley rats. After 30 days of exposure to the CeO₂ NPs, they observed elevated levels of protein carbonyls and Hsp70 protein in the hippocampus and cerebellum, while nitrotyrosine and inducible nitric oxide synthase (NOS) levels were elevated in the cortex. Whereas GSH-Px and catalase activity were decreased in the hippocampus, glutathione reductase decreased levels occurred in the cortex, and GSH-Px and catalase levels were decreased in the cerebellum. The GSH: glutathione disulfide ratio, an index of cellular redox status, was decreased in the hippocampus and cerebellum. This suggests that the CeO₂ NPs have a pro-oxidant potential in the rat brain [5]. On the other hand, Hardas et al. showed previously that 5 nm CeO₂ NPs administered intravenously to rats did not cause a significant increase in ROS, and oxidative stress was observed [31]. In turn, Hussain et al. observed an up to 10-fold increase of ROS in PC12 cells exposed to 40 nm Mn NPs that might be responsible for depleted level of DA and its metabolites, dihydroxyphenylacetic acid and homovanillic acid [57].

Significant increase in the ROS formation, oxidative stress and decreased activity of antioxidant enzymes was observed in the brain of mice treated with TiO_2 NPs, ZnO NPs or Al_2O_3 NPs [155].

Many reports suggest that neurotoxicity of metallic NPs is associated with induction of oxidative stress in the brain by disturbing the delicate redox balance in both neurons and glial cells. This mechanism is connected with genotoxicity and activation of the apoptotic pathway.



Figure 1: Mechanism of action of metallic nanoparticles in the central nervous system.

6 Summary

During the past decade, a dynamic development of nanotechnology has been observed. Products containing metallic NPs in their composition can be found everywhere in a wide range of commercial products commonly available to virtually everyone. In addition, NPs have excellent applications in medicine, in medicaments and diagnostics. NPs, due to their small sizes, have remarkable properties which determine their widespread use and immense bioavailability in the environment.

However, the metallic NPs, in addition to their many advantages, unfortunately also have drawbacks. Figure 1 summarizes the present stage of knowledge about toxicity of metallic NPs in CNS reviewed in this work. Studies have shown a greater toxicity and reactivity of metallic NPs compared to their bulk counterparts. There are several main routes of exposure to metallic NPs, of which perhaps the most dangerous in the context of neurotoxicity NPs are those suspended in the air. It was found that NPs easily penetrate the human body, as well as rodents and aquatic organisms. In addition, researchers have observed that some of them rapidly spread throughout the body and accumulate in various organs. Numerous studies confirm that NPs can penetrate into the brain by means of a fixed route (olfactory bulb - olfactory nerve-brain), thus bypassing the BBB. On the other hand, some NPs which enter the body can pass through the BBB, and circulate in the blood vessels in the form of ions released from the surface of the NPs, as well as through damage to the integrity of the BBB which increases its permeability. Of course, it is a serious consequences to bring on the influx of unauthorized substances into the brain, and violation of the delicate homeostasis of the microenvironment. The brain, due to its sensitive structure, is particularly vulnerable to the adverse properties of metallic NPs. Numerous studies performed both in vitro and in vivo show that NPs are toxic to neuronal cells. It has also been demonstrated that NPs can penetrate into the cells, mainly through the mechanism of endocytosis, although there are other possibilities, such as transport facilitated by some membrane receptors. The primary mechanism to induce neurotoxicity can be attributed to the generation of free radicals and induction of oxidative stress, which is known to damage the cell elements (proteins, lipids, nucleic acids). In particular, oxidative damage to DNA is dangerous due to its nature and mediation of mutation in cancer formation. Although metallic NPs have not yet been directly linked to the etiology of any neurodegenerative diseases, more and more studies are being conducted which provide new evidence in support of this thesis.

It is therefore important both for public health and for the environment to continue monitoring of metallic NPs and their effects in the context of the safety use of metallic NPs in medicine, especially in the CNS.

Acknowledgement: This study was financed by the National Science Centre Fund Projects No. DEC-2013/09/B/NZ7/03934 and No. DEC-2014/15/B/NZ7/01036.

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