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Review

Nanoparticles and potential neurotoxicity: focus on molecular mechanisms

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Abstract: The last decades have seen an explosive increase in the development of nanoparticles and in their use in consumer, industrial and medical applications. Their fast diffusion has also raised widespread concern about the potential toxic effects on living organisms, including humans: at the nanoscale, they can interact with subcellular components such as membranes, proteins, lipids, nucleic acids, thus inducing unpredicted functional perturbations in cells and tissues. The nervous tissue is a particular sensitive target, because its cellular components (mainly neurons and glial cells) are tightly regulated and metabolically exigent biological entities. While the literature on the potential toxicity of nanoparticles has grown in parallel with their utilization, the available data on neurotoxicity are less abundant. In particular, information on the neuronal molecular targets of nanoparticles is still largely incomplete. A better understanding of this issue is highly relevant for the rational and controlled design of nanoparticles, both for their general utilization and more specifically for their use in the promising field of nanoneuromedicine. In this review, we will discuss the available information on the mechanisms involved in the interaction between nanoobjects and cells of the nervous system, focusing on the known molecular actors, both at the plasma membrane and in intracellular compartments.

Keywords: nanoparticles; neurons; toxicity; molecular targets; oxidative stress; membrane proteins

1. Introduction

Nanoparticles (NPs) are defined by having at least one dimension < 100 nm. While some of them have always been present in the environment in the form of small size components (ultrafine particles, UFPs) of dusts and smokes, their presence has dramatically increased in the last decades due to the industrial production of engineered NPs for technological implementations, consumer goods and, of specific relevance for the human exposure, for biomedical applications [1]. Due to their size, comparable to the dimensions of typical subcellular and molecular structures in the biological material, they can interact with living tissues and cells and induce unknown and/or presently not well understood alterations of their functions. This new scenario has raised widespread concern about their potential toxicity in animals and humans.

In this review, we will make some reference to the interaction of UFPs, the most widespread nanoobjects to which animals and humans are exposed, with the nervous system (NS), but will focus specifically on the effects of engineered NPs.

The literature on the subject has significantly expanded in recent years, and a relevant number of excellent and exhaustive reviews is available; only some of the most recent and comprehensive ones will be cited here [2–7]. There is a general agreement on the use of the term "potential toxicity", since, while almost all kinds of environmental and engineered NPs have been reported to induce harmful effects in living beings, and specifically in the NS, for most of them lack of toxicity has also been reported; this criticity depends on several parameters, mainly chemical structure, size, surface properties (e.g. charge) and concentration [8–10].

For nanobiomedical applications, in particular, the task is, therefore, to carefully evaluate the range of the above cited parameters that can allow administration to animal and humans for the desired use (imaging, drug delivery) without exerting unwanted noxious effects. The range depends on the specific particle type and, critically, on the cell/tissue to be targeted. From this point of view, the nervous tissue is a highly sensitive and delicate target, due to its strict metabolic requirements and its basically non-renewable number of neuronal cells. Considering these critical requirements, a present limitation is that the amount of data on potential neurotoxicity of NPs is still less abundant than on other tissues. One of the reasons is probably due to the initial uncertainty about the accessibility of NPs to the central NS (CNS). Now a consistent set of data, both *in vivo* and *in vitro*, confirms that many types of NPs can reach the CNS. The two main pathways are by crossing the blood-brain barrier (BBB), when administered orally or injected, or through the nasal epithelium to the olfactory bulb and the brain, when inhaled [3,5,11,12]. The number of papers on NPs and neurotoxicity is steadily growing, even if most of them are based on *in vitro* experiments, many with high NP concentrations [13]. *In vivo* animal studies have reported morphological and functional alterations in several brain regions [14–16], and epidemiological data are mostly based on the correlation between exposure to PM (often with incomplete characterization of particle size) and neurological damages and behavioral impairments [17].

In this perspective, the understanding of the mechanisms activated by the interaction between the NPs and the biological target, specifically neuronal and glial cells, will be crucial for a more reliable evaluation of the safety of specific NPs and nanoobjects. Most of the data available are related to effects at the cellular level, the most common being oxidative stress (OS), inflammation, apoptosis, changes in gene expression and also epigenetic modifications and genotoxic effects [18–22]. While the amount of data on molecular mechanisms activated by NPs and neuronal targets is more limited, it is

useful to review the existing knowledge, not only when toxic effects have been reported but also at subtoxic doses, based on the finding that the border between toxic and subtoxic effects is not sharp in many instances, and on the rationale that the mechanisms activated at different doses may share common properties.

In the present review, we will deal with the effects of NPs on neuronal and glial cells, and will not discuss the impact on the endothelial cells that compose the BBB (for an example of these effects, see [23]).

2. NPs and oxidative stress

As stated above, the most common perturbation elicited by NPs in neurons and glial cells is a more or less massive production of reactive oxygen species (ROS) and other reactive molecules, leading to OS. The outcome may be the onset of inflammatory responses, with the release of cytokines, finally leading to neuronal death, mainly through apoptotic mechanisms [5,24–26]. The production of ROS and reactive nitrogen species (RNS) is a process basically active in all cells, including those of the CNS. However, its dysregulation can cause damage when production is not counterbalanced by scavenging mechanisms. This reflects the delicate border between toxicity and lack of it: when a NP induces ROS production, this may not necessarily lead to neuronal damage and neurotoxicity. While the agreement on this point is almost universal, less clear are the mechanisms by which interaction of NPs with neuronal (or glial) cells induces production of ROS. It is from this issue that our review of the NP-activated molecular mechanisms will start.

2.1. Molecular mechanisms involved in the generation of ROS

Several reviews list many pathways activated following ROS production, such as activation of enzymes and kinases [6,27], oxidation of proteins that leads to their aggregation, oxidation of mitochondrial DNA, and also perturbation of calcium homeostasis (see below), but these are all downstream targets: where are ROS generated?

It has been proposed that ROS can be generated by NPs in the extracellular environment [28], even if no specific data relative to neuronal or glial cells are presently available; on the other hand NPs can interact directly with the plasma membrane, thus activating surface receptors and downstream pathways, potentially leading to ROS production [22]; however, also in this case no evidence specific for neuronal cells can be found in the literature.

Most data refer to intracellular targets, activated following NP internalization: enzymes responsible for the oxidative balance (such as NADPH oxidase); interaction with mitochondrial membranes, leading to their damage and perturbation of the mitochondrial membrane potential, MMP; direct production of ROS via Fenton or Fenton-like reactions [6].

Before entering into details on the subject of NP-cell interactions and ensuing generation of ROS, it should be mentioned that it is generally considered that NPs tend to adsorb on their surface proteins and other molecules present in the dispersion medium, forming the so-called protein corona: this dynamic structure is involved in the interaction with the plasma membrane, in NPs uptake and, at least potentially, in the interaction with surface receptors and activation of signalling pathways [6,29–31].

Cytosolic enzymes involved in ROS production and degradation are potential targets of internalized NPs. Cytosolic (and nuclear) localization of glucose-coated Au nanoparticles has been reported for neuronal and glial cells [32]. However, data on activation of these pathways by NPs in neurons is quite scarce; it has been reported that 25 nm SiO_2 NPs (25–200 μg mL^{-1}) induce oxidative stress and alfa-synuclein aggregation in PC12 cells by inhibiting the ubiquitin-proteasome complex [33].

Localization in cellular subcompartments, such as lysosomes, has been characterized in more detail [34–36], and in the case of 30 nm TiO_2 NPs (5 μM), it can be associated with ROS production and changes in expression of proinflammatory cytokine IL-1β [35]. Interestingly, it has been reported [37] that the high toxicity of 45 nm iron oxide NPs (3 mM) in microglial cells as compared to neurons and astrocytes is a consequence of the strong accumulation of the NPs in the lysosomal compartment of the former.

Data on mitochondrial localization of NPs in cells of the CNS are more abundant [6,38]; most of them refer to glial cells and particularly to microglia. TiO_2 NPs were shown to generate ROS and disturb the mitochondrial machinery in brain microglia (P25; 2.5 ppm) and to induce morphology changes, mitochondrial damage, and increase of mitochondrial membrane potential in glial cell lines (50 nm; 20 μg cm^{-2}) [39,40]. Conversely, decreased mitochondrial membrane potential and ROS production was reported in PC12 cells in the presence of 1–2 nm single-walled carbon nanotubes (SWCNTs) at concentrations above 5 μg mL^{-1} [41,42], in hippocampal neurons of rats exposed to 5–30 μg mL^{-1} of 5 nm TiO_2 NPs and in rat cortical astrocytes exposed to 25–100 ppm of 20–50 nm TiO_2 NPs [43,44]. Similar results were obtained with 75 μg mL^{-1} of 5–30 nm Ag NPs on cultured rat cerebellar granule cells [45], but in this case the effects were shown to be dependent on activation of surface receptors and perturbation of intracellular calcium homeostasis (see below). Impairment of mitochondrial function by Ag NPs has also been reported for brain cortical neurons (20 nm; 1–10 μg mL^{-1}), SHSY5Y neuroblastoma and D384 astrocytoma cells (70 nm; 15–250 μg mL^{-1}) [11,46]. 10–50 ppm of 5–45 nm Ag NPs have been shown to cause inhibition of mitochondrial respiratory chain complexes I and III in rat brain tissue [47].

From all these data, it can be concluded that NPs can interfere with mitochondrial functions and generate OS either by causing damage of mitochondrial membranes or by perturbing some component of the respiratory chain.

3. Other mechanisms

3.1. Cytoskeletal proteins

The cytoskeleton is the backbone of the eukaryotic cell; in neurons and glial cells, its dynamic modifications have key roles in development, survival, formation and stabilization of synaptic structures and establishment of the correct connections of neuritic processes to form neuronal networks. Several reports have addressed the role of several kinds on NPs in perturbing the cytoskeletal architecture in cells of the CNS, thus potentially inducing toxicity and cell death. It has been found that 1–10 μg mL^{-1} of 20 nm Ag NPs induce degradation of the key cytoskeletal proteins β-tubulin and F-actin in rat cortical neurons in culture [11], resulting in reduced survival of both neurons and glial cells, inhibition of neurite growth in developing neurons and degeneration of neuronal connections in mature neurons; moreover, they dramatically disrupted synaptic structures,

affecting key proteins both at the presynaptic (synaptophysin) and postsynaptic (PSD-95) level. Similar effects on microtubule and microfilament proteins, leading to morphological changes and reduced cell viability were observed in PC12 cells presented with 0.15–15 mM of 5–12 nm Fe_2O_3 NPs [48]. Also, exposure of rat dorsal root ganglia (DRG) neurons to Cu NPs (55–70 nm, 40–100 μM) caused disruption of neurite outgrowth [49]. In rat hippocampal neurons, suppression of neurite outgrowth by TiO_2 NPs (5 nm, 5–30 μg mL^{-1}) was ascribed to a reduced activity of ion channels and transporters (see below) and to impairment of glutamate NMDA receptors [50]. Finally, it was found that SiO_2 NPs (20–50 nm, 25–100 μg mL^{-1}) induced OS and reduction of neurite growth in PC12 cells [51], thus suggesting a link between OS and cytoskeletal modifications.

A comprehensive review of the impact of NPs on cytoskeletal neuronal proteins and of the underlying mechanisms can be found in [52]

3.2. Interactions at the plasma membrane: Ion transporters and channels

The plasma membrane is the first site of interaction between nanoobjects and the cell; before any internalization process is initiated, the highly organized lipids and proteins of which it is composed are likely targets. In neurons (and glial cells) receptors for neurotransmitters and neurohormones, transporters and channels are the main modulators of neuronal activity and of its changes in responses to external stimuli. It is therefore to be expected that in these tightly regulated and highly responsive cells, NPs can induce significant and potentially damaging effects by interfering with these proteins. Some NPs have been shown to regulate the expression of neuronal channels and other proteins involved in excitability and neurotransmission [50,52,53]; here, we will discuss in detail only direct modulation of the activity of membrane transporters and channels, even if non-specific interactions between NPs and phospholipids influencing in turn the activity of membrane proteins cannot be ruled out. Moreover, we will not deal with the activation of channels by NPs engineered to be sensitive to chemical or physical (such as thermal or magnetic) stimuli (for a recent review on this expanding field, see e.g. [54]).

Data on ion transporters, the main regulators of cytosolic ion homeostasis, are quite scarce. Inhibition of Na^+-K^+, Mg^{2+} and Ca^{2+} ATPases in rat brains following administration of Fe_2O_3 NPs have been reported [55]; TiO_2 NPs (5 nm, 5–30 μg mL^{-1}) caused a reduction of the activities of Ca^{2+} ATPase and Na^+-K^+ ATPase in cultured rat hippocampal neurons [50]. Similar effects on the plasma membrane of rat retinal ganglion cells Ca^{2+} ATPase by ZnO NPs (30 nm, 2.5–10 μg mL^{-1}) has been reported [56].

The most abundant data that have accumulated in recent years refer to ionic channels involved in neuronal excitability and synaptic transmission. A list of some of the channels modulated by several kinds of NPs can be found in [3].

3.3. Voltage operated channels (VOCs)

Ionic channels whose open or closed state is dependent on the electric potential across the plasma membrane are present in many different types of cells, but in neurons they are the key players in the generation of self-sustained transient changes in membrane potential (action potentials), the main mechanism for coding information in the NS and for its transmission and transfer to other cells. A functional perturbation of this class of membrane proteins may potentially disrupt the complex

neuronal networks that compose the NS. Below we review the known effects of some type of NPs on Na^+ and K^+ VOCs, reserving a separate paragraph for calcium permeable channels.

3.3.1. Na^+ VOCs

Channels selectively permeable to Na^+ are the prime generators of action potentials (APs) in neurons and other electrically excitable cells. Changes in their biophysical properties may influence the action potential shape as well as their rate of firing, thus either enhancing or reducing excitability. The most direct and reliable way of studying these changes is by means of patch clamp electrophysiological recordings [57]. A few papers have reported an increase in the amplitude of the current carried by Na^+ channels (I_{Na}) following *in vitro* administration of several kinds of NPs: 5–30 μg mL^{-1} of 5 nm TiO_2 in rat hippocampal neurons [50], 100 μg mL^{-1} of 20–80 nm ZnO in the same preparation [58]. In these two cases, the Authors also reported an increase in amplitude of K^+ currents: these currents are responsible for the recovery phase of the action potential. For ZnO NPs, the combined effects caused an increase in peak amplitude of the AP, a reduction of its duration, and an increase in the frequency of AP firing in response to electrical stimulation. On the contrary, inhibitory effects on I_{Na} have been reported by several groups. In hippocampal rat neurons 10–20 nM of 2.4 nm CdSe quantum dots (QDs) induced a shift of the channels to the inactivated state and a reduction of available channels [59]. Based on these data, the authors hypothesized that QDs could bind to a receptor site at one extracellular end of the voltage-sensing S4 residue of the α subunit, the ion-carrying subunit of the channel, thus altering its biophysical properties. Inhibition of I_{Na} and reduction of AP amplitude was observed in cultured CA1 hippocampal neurons following administration of Ag (10 μg mL^{-1}, hydrodinamic diameter 244 nm) and CuO (50 μg mL^{-1}, hydrodynamic diameter 246 nm) NPs [60,61]. In the latter case, again, involvement of the S4 residue was suggested. Ag NPs (130 μM, 5 nm) caused inhibition of I_{Na} and decrease of AP amplitude also in mouse chromaffin cells [62].

3.3.2. K^+ VOCs

In contrast with the two reports cited above [50,58], other groups have described inhibitory effects of NPs on K^+ currents. In hippocampal neurons, several types of K^+-selective channels were inhibited by Ag (10 μg mL^{-1}, 224 nm hydrodynamic diameter) [63], tungsten carbide (0.1 μg mL^{-1}, 5–20 nm) [64], CuO (50 μg mL^{-1}, 60 nm) NPs [65] and by Au nanostars (3 nM, 180 nm) [66]. In the last case, an increase in AP firing rate was observed. A suppression of K^+ channel activity by multi-walled carbon nanotubes (MWCNTs) was observed in PC12 cells [67]. Modification of the biophysical properties of calcium-activated K^+ channels (BK channels) by MCNTs (30–263 μg mL^{-1}, 50–100 nm x > 10 μm) has been also reported in chromaffin neurosecretory cells [68].

3.4. Calcium permeable channels

Calcium-permeable channels are a sensitive target, since influx of these ions from the extracellular medium is a key regulator of physiological and pathological responses in cells from the NS, and disregulation of calcium homeostasis may have a role in neurotoxicity [69]. Even if several

papers have reported activation of calcium influx in neuronal and glial cells by NPs (for a review see [70]) only a few provide information on the specific type of channel involved.

3.4.1. Ca^{2+} VOCs

Calcium-selective voltage operated channels have a crucial role in coupling electrical activity to influx of calcium into the cytosol and in the control of synaptic transmission, gene expression, and other key neuronal processes. Despite their importance, very few data are available about the effects of their interaction with NPs. In rat hippocampal neurons CdSe QDs (10–20 μg mL^{-1}, 2.4 nm) induced an elevation of intracellular calcium concentration, $[Ca^{2+}]_i$, dependent on influx through N-type Ca^{2+} VOCs but also through Na^+ channels that in some cases may become permeable to Ca^{2+} ions, indicating a peculiar change in their biophysical properties [71]. Even if not related to a neuronal model, it is worth citing that carbon nanotubes (CNTs) have been reported to inhibit neuronal N-type channels heterogously expressed; the effect was shown to be dependent on traces of yttrium released from the CNTs [72].

3.4.2. Other Ca^{2+} channels

Calcium-permeable cationic channels activated by neurotransmitters, hormones and a vast set of chemical and physical stimuli are another ubiquitous and functionally relevant pathway for calcium influx. In rat cerebellar granule cells, Ag NPs neurotoxicity is dependent on activation of NMDA receptors, a subtype of glutamate receptors forming ion channels permeable to Ca^{2+} (75 μg mL^{-1}, 5–30 nm) [45]. An excitatory effect of Ag NPs (10–100 μg mL^{-1}, hydrodynamic diameter 234 nm) was also reported in rat CA1 pyramidal neurons at the postsynaptic level [73]; also in this case the data suggest an involvement of calcium permeable glutamate receptors. Conversely, TiO_2 NPs (5–30 μg mL^{-1}, 5 nm) impaired NMDA receptor function in rat hippocampal neurons [50]. A few data are available on non-neurotransmitter activated calcium permeable channels: the increase of $[Ca^{2+}]_i$ induced by SiO_2 NPs (20 μg mL^{-1}, 50 nm) was partially blocked by antagonists of TRPV4, a channel activated by different mechanical and chemical stimuli [74]; in glial cells (rat cortical astrocytes) ultrafine carbon black (10–40 μg cm^{-2}, 124 nm) activated both connexin 43 (Cx43) and pannexin1 (Panx1) hemichannels [75], large conductance non selective channels involved in the release of ATP and other messengers from neurons and glial cells.

3.4.3. Intracellular calcium channels

In addition to influx from the extracellular medium, also release from intracellular stores can contribute to increases in $[Ca^{2+}]_i$ [76]. CdSe QDs (10–20 μg mL^{-1}, 2.4 nm), in addition to the calcium influx mechanisms described above, can induce release of Ca^{2+} ions from stores of the endoplasmic reticulum (ER) [71]. The proposed mechanism is as follows: Na^+ influx through Na^+ VOCs; activation of the Na^+-Ca^{2+} exchanger in the mitochondrial membrane, with release of Ca^{2+} into the cytosol and increase of $[Ca^{2+}]_i$; activation of calcium-sensitive ryanodine receptors of the ER, leading to further increase in $[Ca^{2+}]_i$ via a positive feedback loop.

Several other reports are available regarding increase of $[Ca^{2+}]_i$ induced by NPs, but many of them do not describe the molecular players of these responses. Here we will cite two of these papers,

because they point to a relevant controversial issue: the relationship between calcium signals and onset of OS. Even if OS has been shown to be upstream of activation of several types of neuronal channels, including Ca^{2+} channels [77], the calcium signals activated by Ag NPs (20 μg mL^{-1}, 20–40 nm) in mixed neural cell cultures (neurons and astrocytes) were independent on oxidative stress [78]. In this context, it must be recalled that contrasting findings are available for the dependence of inhibition of other channel types from OS: evidence for a role of ROS in inhibition of I_{Na} by Ag NPs (50 μg mL^{-1}, hydrodynamic diameter 246 nm) has been provided [61]; on the contrary, in PC12 cells, suppression of K^+ channel activity by MWCNTs was independent from ROS generation [67].

To conclude this section on NPs and ion channel modulation, several other papers have reported that NPs can induce perturbations of neuronal electrical activity, excitability and synaptic transmission [10,79–82], even if these papers do not provide information on the specific channels responsible for the observed effects.

Conclusions

Even if the literature on molecular targets activated in cells of the NS by nanoobjects and their potential involvement in neurotoxicity is rapidly expanding, the picture is far from being complete. More data on the dependence of the described effects on cell type, nanoparticle physicochemical characterization (chemical nature, size, surface properties), and concentration, as well as on the potential side effects due to the presence of contaminants, are needed. The progress in this field will allow a better and rational design of nanoparticles for safe and controlled use, particularly for biomedical applications.

Conflict of interest

All authors declare no conflicts of interest in this paper.

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