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Nano- and neurotoxicology: an emerging discipline

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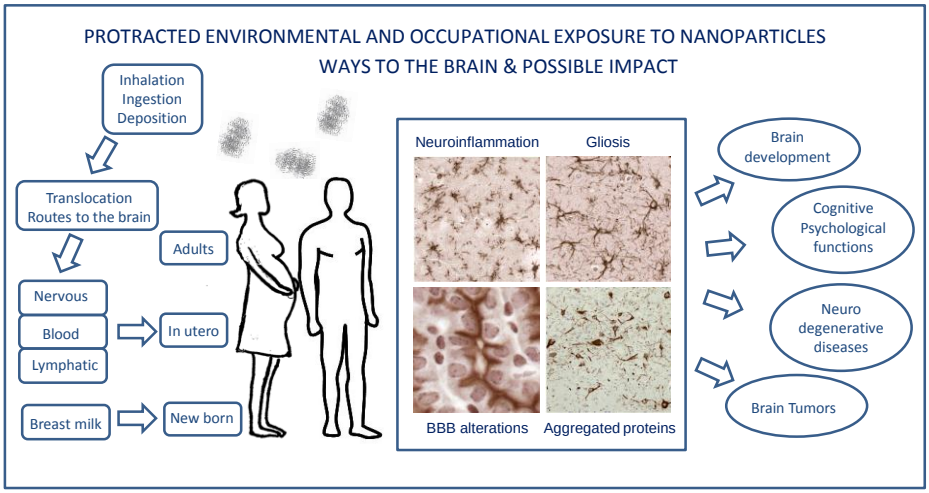
Graphical abstract



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Highlights

- Available evidence suggests incomplete effectiveness of protective barriers of the brain against nanoparticles translocation to the brain
- Nanoparticles from continuously growing industrial production and the use of nanoparticles may impact human brain health
- There is a need to specifically evaluate the interactions between nanomaterials and the nervous system: with original dedicated experimental models and tools for neurotoxicological research, with epidemiological studies of neurodegenerative diseases in manufactured nanoparticle-exposed populations
- This review warrants recognition of an emerging need to combine nanotoxicology and neurology and calls for novel specific tools and investigation methods for this discipline

Abstract

The present critical review analyzes the question of how nanoparticles from continuously growing industrial production and use of nanomaterials may impact human brain health. Available evidence suggests incomplete effectiveness of protective barriers of the brain against nanoparticles translocation to the brain. This raises concerns of potential effects of manufactured nanoparticles on brain functions, given that nanoparticle's potential to induce oxidative stress, inflammation, death by apoptosis, or changes in the level of expression of certain neurotransmitters. Most concerns have not been studied sufficiently and many questions are still open: Are the findings in animals transposable to humans? What happens when exposure is chronic or protracted? What happens to the developing brain when exposure occurs *in utero*? Are some nanoparticles more deleterious, given their ability to alter protein conformations and aggregation? Aside from developments in nanomedicine, the evidence already available fully justifies the need to specifically evaluate the interactions between nanoparticles and the nervous system. The available data clearly indicates the need for original dedicated experimental models and tools for neurotoxicological research on the one hand, and the need for epidemiological studies of neurodegenerative diseases in manufactured nanoparticle-exposed populations, on the other. A combination of nanotoxicology with neurology in a novel discipline, with its specific tools and methods of investigation, should enable answering still unresolved questions.

Abbreviation list

α, alpha; β, beta; δ, delta; ε, epsilon; APOE, apolipoprotein E ; Aβ, amyloid beta; BBB, blood–brain barrier; CERAD, The Consortium to Establish a Registry for Alzheimer's Disease; CNS, central nervous system; CRP, C reactive protein; CSF, cerebrospinal fluid; DRG, dorsal root ganglia; EBC, exhaled breath condensate; H_2O_2, hydrogen peroxide; HCECs, human cerebral endothelial cells; HR, hazard ratios ; IL, Interleukin; MDA, malondialdehyde; MIG, metal inert gas; MMSE, Mini-Mental State Examination; NPs, nanoparticles ; PEG, polyethylene glycol ; PM particulate matter; PNC, particle number concentration; PNS, peripheral nervous system; QD, quantum dots ; ROS, reactive oxygen species; SiO_2, silica dioxide; TIG, Tungsten Inert Gas; TiO_2, titanium dioxide ; TNF-α, tumor necrosis factor- alpha; UFPs, ultrafine particles ; ZnO, zinc oxide;

Key words

barriers; brain; chronic exposure; epidemiological studies; nanomaterials; neurodegenerative diseases; translocation; toxicity

Contents

1. Introduction

It is advisable today to distinguish between ultrafine particles characterized by various sizes, shapes and compositions, either of natural origin (erosion, volcanic eruptions) or anthropic activities (traffic, combustion and some industrial activities), and manufactured nanoparticles. The latter are produced intentionally in the framework of the flourishing nanotechnologies. Unlike ultrafine particles (UFPs), manufactured nanoparticles are produced for their particular properties shown only at the nanoscale, and have more controlled physicochemical characteristics. The properties exhibited at this scale (between 1 and 100 nm) can be of diverse type, i.e. electric, catalytic, mechanical, optical, or biological, enabling use of nanotechnology products in almost all the major sectors in today's world, including health, energy, electronics, the environment, and transport and information technology and telecommunications. This explains the enthusiasm of industry and the public authorities for these products. The use of manufactured nanoparticles in medical applications is particularly remarkable, including for diagnostic purposes, treatment of cancers, molecular imagery, surgery, and medical devices or tissue engineering (Bechet et al., 2008; Benachour et al., 2012; Chouikrat et al., 2012; Koffie et al., 2011; Wadghiri et al., 2013; Zhang et al., 2013). The development of optimized tools to diagnose, study, and treat central nervous system (CNS) diseases, including tumors or neurodegenerative diseases reputed incurable, is one of the expected applications in nanomedicine. Carbon-based (Riviere, 2009) and metallic nanoparticles (Yokel et al., 2013) are already widely used, especially for imaging. Nanoparticles may resolve key problems such as penetration and targeting of specific brain areas, difficulties resulting from the high level of protection of this particular organ. This is why some studies dealing with nanomedicine (*in vitro*, *in vivo* and

more rarely human studies) have focused mainly on the interactions between nanoparticles and the nervous system, and on drug delivery systems (like polymer-based, dendrimers, nanoliposomes ...) that target the CNS. Despite the different strategies that are currently used to overcome the remaining hurdles for nanoparticle transfer to the brain (increasing nanoparticle dose, use of polyethylene glycol (PEG) or other surface modifications), there is still little evidence that nanoparticles reach the brain *in vivo*, and there is a lack of studies about possible deleterious effects of these nanoparticles. Indeed, precautions must be taken in the use of these nano-designed treatments, as nanoparticles may also induce neurotoxic effects or accelerate existing brain damage (Figure 1).

Moreover, in addition to nanomedicine products that concern vulnerable populations, the rapid spread of nanotechnology raises serious questions about its impact on the general population's health and on the environment. Thousands of nanoproducts are already available on the market (JRC, 2014) (Tulve et al., 2015; Vance et al., 2015), raising concerns of massive consumer exposure by inhalation, ingestion, dermal contact, or a combination of these routes. While several studies have already pointed to possible respiratory and cardiovascular system damage following nanoparticle exposure (Cassee et al., 2011; Dockery et al., 1993; Ghio et al., 2012; Liao et al., 2014; McCreanor et al., 2007; Miller et al., 2013; Patel et al., 2013; Pieters et al., 2012; Pope et al., 2008; Pope et al., 2006; Robertson et al., 2012; Robertson et al., 2014; Seaton et al., 1995; Thurston et al., 2016; Xu et al., 2013b; Yamamoto et al., 2013), only few studies have looked at their impact on the highly vulnerable nervous system as underlined in several reviews (Cupaioli et al., 2014; Lung et al., 2014; Suh et al., 2009; van Berlo et al., 2014; Yokel, 2016a; Yokel and Macphail, 2011).

This review aims to 1) describe possible exposure routes and consequences of manufactured nanoparticles on the function and integrity of the nervous system in light of their specific structural and physiological properties, 2) assess the evidence in humans, with particular focus on neurodegenerative and neurodevelopmental outcomes, and 3) identify methodological and research gaps and possible solutions for a better understanding of how manufactured nanoparticles may affect the human nervous system.

2. The brain: a particularly vulnerable and highly protected organ

Compared to the other organs, the brain is unique in several ways (Figure 2): 1) high metabolic needs, for a cerebral mass accounting for approximately 2% of the whole body, the brain consumes nearly 20% of the total oxygen inhaled, making it one of the most active organs at the metabolic level; 2) complex organization with hundreds of neuroanatomic regions namely the “nuclei” having different sensitivities to physical or chemical insults; 3) specific cells with unique properties, the neurons, highly specialized to allow electric and chemical conduction; the glial cells (80% of CNS cells) responsible for myelination of the neuronal processes, metabolic support and regulation of neuronal synaptogenesis or chemical transmission within a synapse, as well as responses to brain damage; and 4) several levels of protection including the ventricular spaces filled with cerebrospinal fluid (CSF) that have a crucial function in preserving brain homeostasis by regulating the maintenance of the integrity of the CSF–brain barrier, but also the blood–brain barrier (BBB), a regulator of the flow of molecules between the blood circulation and cerebral parenchyma, also involved in the elimination of toxic metabolites and protection of the brain against xenobiotic agents (Abbott et al., 2006). Finally, the brain is highly vulnerable by nature, as it is extremely

sensitive to hypoxia, inflammation, and oxidative stress, and has very limited regenerative capacity of the neurons.

3. Routes for nanoparticles to reach the brain

The main paths of exposure to nanoparticles in humans are the respiratory route, which includes the nasal cavity, but also the digestive tract and potentially the cutaneous route (Buzea et al., 2007) (Figure 3). Once nanoparticles successfully cross these first barriers, the lungs, the gastrointestinal tract, or the skin, they may attain the blood stream, and next the brain, especially if they can cross the BBB. The possibility of nanoparticles reaching the brain via these various routes of exposure is supported by experimental evidence accumulated over the last fifteen years and Figure 3 provides a sum up of possible routes transposed to humans.

3.1. Neuronal uptake and transportation of nanoparticles

3.1.1. From nose to brain

A first and direct possibility of neuronal translocation is after inhalation, from the nasal cavity to the brain through the olfactory bulbs directly accessible from the nasal fossae. The demonstration of nanoparticle uptake from the nasal cavity to the brain can be found in a study using squirrel monkeys exposed intranasally to 50 nm colloidal gold (De Lorenzo, 1970). The gold nanoparticles detected by electron microscopy, were seen in the olfactory nerve within 30 min after exposure and in mitral cells of the olfactory bulb 1 hour after exposure (De Lorenzo, 1970). In 2004 Oberdörster's team showed that nanoparticles of ^{13}C of approximately 36 nm could be transferred straightaway from the olfactory epithelium to

the olfactory bulbs via olfactory nerve neural processes (Oberdorster et al., 2004). Since these studies, the observation has been repeated for other types of nanoparticles, such as those of manganese or iron oxide (Elder et al., 2006; Wang et al., 2007a). Thus a direct neuronal uptake of nanoparticles is possible and it is reasonable to assume that nanoparticles could be then transported to more distant brain areas, using the neuronal retrograde transport. Indeed the uptake of particulate matter, such as pigment particles or viruses (such as 30 nm poliovirus, 80 to 100 nm herpes virus …), followed by their neuronal retrograde transport along the olfactory tract is reported and admitted since a long time ago (Howe and Bodian, 1941; Rake, 1937). But even today, the exact mechanism of Nanoparticles uptake by sensory endings and their translocation to the brain are not understood.

Neuronal transport

It is well-known that neurons are able to internalize not only pathogens, toxins but other types of molecules such as trophic factors. Once inside the neurons, these molecules can be transported using either anterograde or retrograde axonal transport machinery (Gibbs et al., 2016; von Bartheld, 2004). The retrograde transport is mostly driven by cytoplasmic dynein, a protein that sustains the transport of various organelles such as vesicles, mitochondria, endosomes, lysosomes etc.…from the axon terminals to the neuronal cell bodies. It is usually accepted that axonal transport can be 'fast' (200–400 mm/day or 2 to 5 µm/s) or 'slow' (1–5 mm/day) (Shepherd, 1994). The fast transport with an average speed of 1 to 2 µm/s (Cui et al., 2007; Ori-McKenney et al., 2010), can be however less (0.4 µm/s) or more rapid (up to 5 µm/s) (Hill et al., 2004; Kural et al., 2005; Parton et al., 1992) depending on the model studied (Cui et al., 2007; Ori-McKenney et al., 2010) More recently, some studies have

brought evidence supporting the attractive speculation that Nanoparticles could be internalized by endocytosis and then transported using this retrograde machinery. For example, using a zinc binding dye, airborn ZnO particles (12-14 nm) were detected in the olfactory bulbs and brains of rats exposed 4h on 3 consecutive days using transmission electron microscopy (Kao et al., 2012). To test the olfactory uptake and axonal transport, quantum dots (QD) made of CdSe/ZnS nanocrystals were used in inhalation studies in mice (droplets size 84 nm at an average concentration of 250 $\mu g/m^3$ for 1 hour) (Hopkins et al., 2014). In this study, several techniques, including fluorescent and transmission electron microscopy on tissue samples that were previously washed and exsanguinated, allowed demonstrating that QDs were quickly transported from the nose to the brain mainly by olfactory uptake and via fast axonal transport. Axonal translocation was also reported for CeO_2 and SiO_2 Nanoparticles in frog sciatic nerve (*ex vivo* preparation) and the measured speed of translocation was compatible with the slow axonal transport (Kastrinaki et al., 2015). Computational studies may further clarify our understanding of the axonal transport of nanoparticle. Notably, Kuznetsov (Kuznetsov, 2012) provided a model of nanoparticle transport in neurons and described two modes of neuronal transport, depending on the intracellular location of Nanoparticles, inside or outside of endocytic vesicles. Assuming that in axons, Nanoparticles would be internalized only at axon terminals, and in dendrites, in which Nanoparticles could enter anywhere through the entire plasma membrane, this mathematical model integrated the differences between axons and dendrites compartments, resulting from these different uptake possibilities. The development of nanoparticle based tools for studying the function of dyneins on retrograde transport of endosomes in neurons (Chaudhry et al., 2008) also confirms the relevance of the nanoparticle translocation using the retrograde axonal machinery. Functionalization of

Nanoparticles enables them to be endocytosed and transported. It was shown by a study using nerve growth factor conjugated quantum dots that enter DRG neurons and are then transported axonally in a retrograde manner (Cui et al., 2007).

An additional main pathway to reach the CNS implicates the trigeminal nerve (Dhuria et al., 2010), a small portion of which terminates in the olfactory bulbs (Schaefer et al., 2002). The trigeminal and olfactory pathways of nanoparticles allow direct translocation to the brain (Elder et al., 2006; Lewis et al., 2005). These data suggest an innovative gateway of entrance into the CNS for Nanoparticles, avoiding the blood–brain barrier (Johnson et al., 2010).

Trans-synaptic transportation

The observation that several levels of brains areas connected to the nasal fossae through olfactory and trigeminal nerves were shown to contain inhaled Nanoparticles, suggests by itself that Nanoparticles could pass from a neuron to another through the synapses. Olfactory sensory neurons synapse on mitral cells, are themselves connected with tufted cells. From the olfactory bulbs, these neurons pass through to the olfactory tubercle and then a third-order neuronal projections (third synaptic contact) result in connections with several brain areas such as the anterior olfactory nucleus, prepyriform and the entorhinal cortex, the amygdala, as well as the hippocampus, hypothalamus and thalamus (Lledo et al., 2005). Most of the inhalation studies reported clearly nanoparticle deposition within the olfactory bulb (first order synapse) (De Lorenzo, 1970; Elder et al., 2006; Hopkins et al., 2014; Oberdorster et al., 2004; Wu et al., 2013). From there, Nanoparticles might travel to other areas of the brain (e.g., hippocampus, hypothalamus, etc.), following the olfactory and trigeminal pathways (Lledo et al., 2005). This property should be particularly taken into

account in the drug delivery strategies based on nasal instillation (Illum, 2000) (Mistry et al., 2009). The ability of Nanoparticles to pass the synapses between olfactory neurons in the olfactory bulb to the telencephalon and diencephalon structures or from trigeminal nerves to the projections from the spinal trigeminal nucleus are difficult to apprehend and might appear as less efficient (Oberdorster et al., 2004; Tjalve et al., 1996). Several parameters could explain this difficulty, first a question of time: nanoparticle axonal and trans-synaptic transport needed to reach other areas of the brain supposes more time. Secondly, a dilution effect during trans-neuronal dissemination and thirdly a potential clearance of some of the Nanoparticles during the journey.

Extracellular pathways excluding blood

In addition to the neuronal pathway, nanoparticles could gain access to the CNS through the extracellular pathways (perineuronal, perivascular and cerebrospinal fluid pathways). As an example, to comfort initial inhalation studies using uranium aerosol (Tournier et al., 2009) in rats, that suggested the existence of an olfactory transport, a second wave of experiments using instillation of soluble uranium in the nasal cavity was performed (Ibanez et al., 2014). Using SIMS microscopy, the analysis revealed that the uranium has been brought directly along the olfactory nerve to the brain through perineural and cerebrospinal fluid pathways.

3.1.2. From lungs to the brain

Translocation to the CNS after inhalation has been reported for Nanoparticles of very different chemical types and sizes: ferric oxide (Wang et al., 2016) silver (Patchin et al., 2016), gold (Yu et al., 2007; Zhang et al., 2012), copper (Zhang et al., 2012), titanium dioxide (TiO_2) (Wang et al., 2008a; Wang et al., 2008b), manganese dioxide (Elder et al., 2006),

iridium (Kreyling et al., 2009), and carbon (Kreyling et al., 2009), with dimensions reaching from 2 to 200 nm. It was also reported in the context of anthropic nanoparticles (Shimada et al., 2006). During inhalation, passage of nanoparticles from the lungs to the brain has also been suggested (Oberdorster et al., 2005; Oberdorster et al., 2004). In this case, it would appear that translocation to the brain results predominantly from secondary translocation, following preliminary nanoparticle crossing of the lung–blood barrier. It has been shown that metal nanoparticles, for instance uranium (38 nm) (Petitot et al., 2013), are translocated from the respiratory tract to extra-pulmonary organs, particularly the CNS, via blood capillary vessels (Simko and Mattsson, 2010). The presence of sensory nerve endings at the bronchiolar and alveolar level could also support the neuronal translocation of nanoparticles. As an example, C-nerve fibers present within the airways ending in the solitary nucleus and para-trigeminal nucleus within the medulla oblongata (McGovern et al., 2015) might contribute to the uptake and translocation to the brain. A review by Stapleton et al. points out several neurological links that may sustain the observed cardiovascular responses to xenobiotic pulmonary exposure (Stapleton et al., 2015). However there is a lack of data reporting nanoparticle neuronal uptake and retrograde transportation by this way.

3.1.3. From gut to the brain

The digestive tract appears to be another route of translocation after inhalation of nanoparticles; this well-known pathway can be studied specifically by intra-esophageal instillation. A very recent study by Kreyling et al. (Kreyling et al., 2017) investigated with a remarkable precision the quantitative biodistribution of TiO_2 NPs in rats after a single dose delivered by intra-esophageal instillation. They showed that despite very low absorption of TiO_2 nanoparticles across the gut (less than 0.6% of the dose applied), and despite the

physiological barriers of the CNS, TiO_2 nanoparticles were detectable weakly but clearly in the brain. This study does not allow determining precisely the cellular location of these nanoparticles (nervous cells, endothelial cells) neither the precise pathways (blood, neuronal) that contribute to this translocation in the brain. But, compared to other organs, these nanoparticles still detectable in the brain seven days after oral delivery suggest importantly retention of nanoparticles in this organ (Kreyling et al., 2017). Similar observation was reported for 80nm gold nanoparticle but with lower retention (Schleh et al., 2012). Furthermore, the possibility of nanoparticle passage from the gastrointestinal tract to the brain has been studied by direct exposure via drinking water or by gavage. The study of Hillyer and Albrecht investigated gold nanoparticle content in gastrointestinal tract and brain after 4, 10, 28 and 58 nm diameter colloidal gold nanoparticle oral delivery (Hillyer and Albrecht, 2001). The authors report nanoparticle accumulation in brain with the highest concentration recorded for 4nm nanoparticles and less strong but similar concentration for 10 and 58 nm. Other studies (Hu et al., 2010; Wang et al., 2007b; Wang et al., 2013) suggest nanoparticle accumulation in the brain after oral exposure, combined to dose-dependent brain damage, based on prior passage of the nanoparticles in the systemic circulation. However for all these studies it is not possible to distinguish the parenchymal from the blood nanoparticle content. A study on female rats exposed to a unique oral dose of iron oxide (Fe_2O_3 (30nm)) at different concentrations, has also showed, although with slight levels, a dose dependent content in Fe_2O_3 in the brain that was more effective for the nanoforms compared to the control bulk material (Singh et al., 2013). It is plausible that before being transported in a retrograde manner to the brain, nanoparticle could be captured by the numerous nerve endings along the digestive tract (Furness et al., 2013) Yet, this pathway has not been addressed in the nanotoxicology literature. It requires a thorough evaluation,

notably in a context of growing exposure by this route, to relatively high levels of engineered nanoparticles (Weir et al., 2012) and given the recent hypothesis in Parkinson disease (PD) etiology (Hawkes et al., 2007). According to Braak's theory, PD could be initiated outside the central nervous system, in peripheral areas, such as olfactory bulbs and enteric nervous system present in the gut, possibly triggered by xenobiotic agents that would be taken up locally and initiate neurotoxicity (Braak et al., 2006). This hypothesis was experimentally studied and sustained only recently (Pan-Montojo et al., 2012).

Concerning oral exposure during critical periods of life like in newborns, a very recent study suggests that infants can be exposed to silver and gold nanoparticles via breastmilk (Morishita et al., 2016). Silver nanoparticles administered intravenously or orally to lactating mice resulted in silver nanoparticle detection in breastmilk, and subsequently silver nanoparticles were found to accumulate in the brains of offspring. During the earliest months of life, the young mice did not show any neurologic abnormalities. However, the consequences of nanoparticle exposure on brain function may appear on a longer time scale and thus this should be evaluated.

3.2. Neuronal translocation of nanoparticles through the blood brain barrier

Regardless of the route of exposure, it would seem that nanoparticles could quickly reach the blood vessels, (Ragnaill et al., 2011; Sharma and Sharma, 2007; Zensi et al., 2009) via transcytosis through endothelial cells rather than between endothelial cells. Translocation from blood compartment through endothelial cells to reach the brain has been demonstrated *in vivo* in the mouse after intravenous injection of nanoparticles functionalized with Apo-E molecules (Kreuter et al., 2002; Zensi et al., 2009). These nanoparticles were identified in endothelial cells of the BBB 15 min after administration, and

in neurons 30 min after administration (Zensi et al., 2009). This was also shown *in vitro* for TiO_2 nanoparticles, which were detected within the endothelial compartment, then in astrocytes (Brun et al., 2012). The distribution of nanoparticles in the bloodstream raises a particular concern of placental nanoparticle transfer to the fetal CNS (Figure 3). Because the BBB develops gradually in the fetal brain (Ballabh et al., 2004), this type of direct exposure to nanoparticles *in utero* may have the most damaging consequences. Best studied in rodents, in which the BBB develops anatomically between embryonic day 11 and 17, the temporal development of an operational BBB has been found to vary with species (Ballabh et al., 2004). In addition, complete BBB functionality may need additional time, as shown in the rat brain by the increase of the expression of occludin, a tight junction protein expressed by BBB endothelial cells, between postnatal day 8 and 70 (Hirase et al., 1997).

3.3. The case of the peripheral nervous system

In case of dermal contact with nanoparticles, the peripheral nervous system (PNS) may be the first nervous compartment to be concerned by possible nanoparticle uptake and consecutive harmful effects. As an example, the presence in the superficial layers of the epidermis of free sensory nerve endings of C- and Aδ-fibers that innervate the skin represents a possible gateway of entrance of nanoparticles into the brain. In the event of nanoparticle uptake by these afferent sensory nerve endings, the substances may be transported through neuronal processes to their neuronal cell bodies grouped in the dorsal root ganglia (DRG), as discussed in the previous subsection. Secondarily from DRG, nanoparticles may pass through synapses in the spinal cord to reach neurons in the CNS. This pathway of nanoparticles in the PNS has been poorly investigated despite its importance, particularly considering that hundreds of consumer products containing nanoparticles are

designed for dermal application. Constitutively, neurons of the PNS are secured by a blood–neuron barrier thanks to the existence of tight junctions in the endothelial cells surrounded by pericytes. However, a noticeable difference with the CNS lies in the absence of foot processes from astrocytes at this barrier, and in the presence of Schwann cells instead of oligodendrocytes. These features may sustain PNS susceptibility to nanoparticle exposure that requires particular attention.

4. Cellular and subcellular locations of manufactured nanoparticles

Once in the brain, where can we find these nanoparticles? *In vitro* studies have helped to highlight the existence of nanoparticles in neurons, astrocytes and microglial cells, but potentially all cell types in the brain could be involved. Once in neurons or glial cells, the nanoparticles may be directed to the lysosomes or persist in the cytoplasm, offering the opportunity to interact with other organelles. Electron microscopy studies have shown the presence of nanoparticles within the glial and neuronal cells for instance silver nanoparticles of 20 nm were found mostly in the lysosomes of astrocytes (Haase et al., 2012; Locht et al., 2011), silica based nanoparticle engineered for nanomedicine tools, was detected in the endoplasmic reticulum (ER) and in the cytoplasm of microglial cells (Ducray et al., 2017), as wells TiO_2 nanoparticles were detected in the cytoplasm and in vacuoles of microglia (Long et al., 2006). This indicates that certain nanoparticles may be subject to classic cell degradation processes. More rarely, nanoparticles (like silver nanoparticles of 6-20 nm and cationic quantum dots (QD) of 2.2 nm) have been found in the nucleus (Asharani et al., 2009; Lovric et al., 2005), suggesting that the subcellular location can be highly reliant on the dimension of the nanoparticles. Surprisingly, the mitochondrial compartment of nerve cells

has not been identified as hosting nanoparticles, except in the study of De Lorenzo in which spherical 50nm gold nanoparticles were reported within the mitochondria of the mitral cells of the olfactory bulb (De Lorenzo, 1970). The lack of other evidence of mitochondrial implication results probably and predominantly from many research gaps in this area. Given that oxidative stress is regularly reported in association with exposure to nanoparticles, it is very likely that mitochondria host nanoparticles more frequently, as demonstrated for other cell types such as macrophages (Eidi et al., 2012).

5. Possible effects on the nervous system

Given the proven possibility of nanoparticles to reach the nervous system, together with the subcellular detection of these nanoparticles within nervous cells, several factors are to be taken into attention for the study and understanding of the possible effects on peripheral and central nervous system. In the case peripheral nervous system, according to Jaiswal *et al.,* SiO_2-nanoparticles exert differential cytotoxic effects on PNS neural cells and Schwann cells are more susceptible than DRG cells (Jaiswal et al., 2011). In more recent times, it has been proved that TiO_2-nanoparticles are taken on in DRG cells and generate apoptosis, produce reactive oxygen species (ROS) and changes in expression of pro-inflammatory cytokines (Erriquez et al., 2015). Further implication of the PNS has also been shown in rats exposed repeatedly to lead nanoparticles (20 nm) by intratracheal instillation (Oszlanczi et al., 2011). The action potential as well as conduction velocity of the tail nerve were altered in the exposed animals.

In the case of the CNS, it is important to consider the regional dimension of the brain affected by the presence of nanoparticles. In brain areas ensuring specific roles, a variety of

impacts are likely to be possible. It may be noted, for example, that the presence of nanoparticles in the BBB can induce changes in barrier properties. Similarly, interactions of nanoparticles with the hippocampus could be linked to memory impairment (Liu et al., 2012a). The nature of the possible consequences is also reliant on the cell type targeted. Schematically, the presence of nanoparticles in astrocytes may participate in the induction of reactive gliosis, while in the case of nanoparticle accumulation in neurons, there may be changes in neuronal metabolism, functions, or even viability. Finally, the sub-cellular scale also has its own importance, as suggested by a study reporting the loss of β-tubulin and actin filamentin cultured neurons exposed to 20 nm silver nanoparticles (Xu et al., 2013a); this observation was reproduced at sublethal concentrations of silver nanoparticles which disrupt also actin dynamics in SVZ-NSCs (Cooper and Spitzer, 2015). Among the possible expected effects, impacts on cell morphology, function, and viability are critical for the highly vulnerable nerve cells, when compared to other cell types.

Since neurons ensure the transfer of information, it is key to note that variations in electrical activities have been documented, including studies on neurons isolated from the hippocampus (Liu et al., 2012b; Xu et al., 2009; Zhao et al., 2009), or studies of primary murine cortical networks (Gramowski et al., 2010). These studies indicate that silver nanoparticles (50-100 nm, 10 µg/mL) inhibited postsynaptic currents in neurons from the CA1 region of the hippocampus, and that ZnO nanoparticles (20-80 nm, 10^{-4} g/mL) are able to increase the entry of sodium ions and the output of potassium ions from the neurons of the CA3 region, enhancing their excitability while CuO nanoparticles had small effects on transient outward potassium current. In primary cell cultures from the mouse frontal cortex, TiO_2 nanoparticles at 10 µg/mL induced severe inhibition of the electrical activity of the neural network.

The review by Simko and Mattsson (Simko and Mattsson, 2010) provides a summary of possible neurotoxic outcomes associated with exposure to nanoparticles; other reviews (Heusinkveld et al., 2016; Win-Shwe and Fujimaki, 2011; Yokel, 2016b) support the idea of a wide range of possible effects. Morphological changes such as number and length of neurites or astrocytes branches were reported for example *in vitro,* in neurons (rat DRG primary cells) exposed to Cu nanoparticles (40, 60 and 80 nm) (Prabhu et al., 2010), as well as in astrocytes exposed to ZnO nanoparticles (rod shaped 45 nm)(Wang et al., 2014). *In vivo*, morphological changes can also be seen, like in the hippocampus of mice repeatedly (3 times/week for 4 weeks) exposed to ZnO nanoparticles (20-80nm) by intraperitoneal (i.p.) injection at 5.6 mg/kg body weight (Tian et al., 2015). Observed in two vulnerable parts of the hippocampus, the CA1 and dentate gyrus (DG) regions, the morphological changes affecting pyramidal neurons consisted in a sparse arrangement, cellular distortions, dissolved and less numerous Nissl bodies. Neurotoxicity induced by nanoparticles exposure can be mediated by oxidative stress, characterized by overproduction of various reactive oxygen species (ROS). It is ascertained by the evaluation of the ROS production levels combined to the presence of antioxidant defenses (Hellack et al., 2017). This has been extensively studied *in vitro* and *in vivo* notably for metallic nanoparticles such as ZnO (Guo et al., 2013; Tian et al., 2015; Wang et al., 2014), iron oxides (Wu et al., 2013), TiO_2 (Huerta-Garcia et al., 2014; Long et al., 2006; Ma et al., 2010), silver (Haase et al., 2012) but also shown for non-metallic nanoparticles such as for fullerenes (C60) (Oberdorster, 2004). Neurotoxicity induced by nanoparticles can also be mediated by induction of an inflammatory state that can be assessed by different approaches like detection of an up-regulation of the transcription of various pro-inflammatory genes, of cytokines production, and in the brain an activation of microglial cells. As an example, *in vivo*, it has been shown

that TiO_2 nanoparticles induce cerebral inflammation (Elder et al., 2006) via activation of microglial cells in the brain (Balvay et al., 2013). These aspects are very important given the high sensitivity of the CNS to oxidative stress and inflammation (Appel et al., 2010; Brochard et al., 2009; Glass et al., 2010; Perry et al., 2007; Pott Godoy et al., 2008; Tansey and Goldberg, 2010; Wu et al., 2002). More radically, the possibility of neuronal cell death by apoptosis has been described *in vitro* and *in vivo,* specifically in the context of exposure during development (Shimizu et al., 2009). This possibility is particularly worrying for the brain, which has very limited regenerative capacity. Importantly for brain function, changes in neurotransmitter expression have been identified *in vitro* and *in vivo* (Hu et al., 2010; Hussain et al., 2006; Wang et al., 2009). As an example, TiO_2 nanoparticles administered by ingestion are able to deregulate the monoaminergic and serotonergic systems in mice (Hu et al., 2010). These changes can further result in behavioral abnormalities, and altered spatial memory or motricity, as shown by a significant decrease in locomotor performance (Balvay et al., 2013). In a very significant manner, it has been shown that some nanoparticles have the ability to accelerate aggregation of certain proteins such as β-amyloid protein, and α-synuclein protein, demonstrated more recently (Alvarez et al., 2013; Linse et al., 2007). Of note, accumulation of these aggregated fibrillar proteins is typical of human neurodegenerative diseases such as Alzheimer's disease or Parkinson's disease. In this context, a plausible impact of nanoparticles on brain function could be an induction or acceleration of these human proteinopathies. The effect induced could be reliant on the dimension and concentration of the nanoparticles, as indicated by Alvarez *et al*. (Alvarez et al., 2013). They showed that 10 nm gold nanoparticles can lead to a 3-fold increased speed of α-synuclein aggregation at concentrations as low as 20 nM (Alvarez et al., 2013).

The list of possible nanoparticle effects is still lacunar, since there are very few relevant studies on the subject. The list should clearly be completed with the indirect effects, unrelated to nanoparticle accumulation in the brain. When exposure to nanoparticles is not sufficient to induce severe changes in the affected target organs or systems (liver, lungs, cardiovascular system, and circulating monocytes), it may however induce peripheral changes and result in indirect neurotoxicity, mediated by circulating cytokines synthesized by the affected organs. Cytokines are known to be able to promote the onset or the acceleration of brain disorders (Perry et al., 2007; Pott Godoy et al., 2008), whether they alter the integrity of the BBB or not (Murta et al., 2015).

6. Evidence from epidemiological and human exposure studies

Although no epidemiological data have specifically investigated the neurotoxic effects of manufactured nanoparticles, studies of populations exposed to anthropic nanoparticles provide an interesting perspective on the concerns related to the possible effects of nanoparticles described above in humans. Studies of workers exposed to occupational pollutants released at the nanoscale (welding fumes and other non-intentional combustion-related, mineral or metallic nanoparticles) present the greatest interest for assessing this evidence.

6.1. Studies of nanoparticulate components of welding fumes and their effects on human central nervous system

Exposure characterization studies have shown that welding entails release of metal, but their elemental composition and size vary depending on the welding process, the electrode,

the material being welded, and many other parameters (Buonanno et al., 2011). For example, fumes from metal inert gas (MIG) soldering consisted of 60% zinc, 17% copper, 1% iron, and 0.3% manganese, whereas MIG welding of aluminum generated fumes with 51% aluminum, 5% magnesium, and 0.1 manganese (Hartmann et al., 2014). In 2014, Andujar *et al*. found predominantly iron of around 20-25 nm, but also chromium and/or manganese, titanium, aluminum, silica and nickel in the lung tissue sections of welders (Andujar et al., 2014). Regarding tungsten inert gas welding, Graczyk *et al.* (2016a) (Graczyk et al., 2016a) reported that iron was a minority among elements measured at the welders' breathing zone, mostly composed of aluminum and tungsten nanoparticles of 45 nm in average. However, Miettinen *et al.* (2016) reported that percentages of Fe, Mn, Cr, Ni, and Mo of the total particle mass collected were 8.7, 2.7, 2.6, 1.4, and 0.3%, respectively, with multimodal particle size distribution in the range of 10–30 nm (Miettinen et al., 2016). Among the most common types of welding, only shielded metal arc welding generated the highest manganese release (up to 10% of PM2.5 mass concentration), although this was 4-fold less than iron concentrations (Oprya et al., 2012). Actually, many epidemiological studies of neurobehavioral impairments in welders have focused on manganese exposure. In 2013, Park performed a systematic review of 28 such studies (Park, 2013). He concluded that welders and other workers with known or likely sustained exposures to respirable Mn are at risk for developing neurological effects, such as performance decrements on standardized neuropsychological tests and adverse symptoms, and that these effects are consistent with signs of early manganism. Some data suggest that Mn exposures increase the risk of Parkinson's disease and may disrupt dopamine metabolism (Park, 2013). However, the hypothesis of accelerated onset or early detection of Parkinson's disease in these workers remains unconfirmed, even when considering more recent studies. Tescke *et al.* (Teschke et

al., 2014) found a three-fold increased risk of Parkinson's disease in welders, but this finding was not statistically significant. In contrast, Van der Mark et al. observed reduced Parkinson's disease risk associated with exposure to welding fumes (van der Mark et al., 2015). The effects of other nanoparticulate components of welding fumes have attracted scientific interest only recently. Nevertheless, despite potential indirect neurotoxicity of nanoparticles, all identified studies were performed but regardless of their potential neurobehavioral consequences (Brand et al., 2014; Jarvela et al., 2013; Kauppi et al., 2015; Kim et al., 2005; Scharrer et al., 2007). These studies investigated the association between nanoparticle exposure and inflammation and oxidative stress at both, pulmonary and systemic levels in voluntary welders.

Kauppi *et al.* (Kauppi et al., 2015) analyzed platelet counts, leucocytes and their differential counts, hemoglobin, sensitive C reactive protein (CRP), lipids, glucose and fibrinogen, interleukins IL-1β, IL-6, IL-8, TNF-α, endothelin-1, and E-selectin in plasma samples collected from 16 welders with suspected occupational asthma. Based on the observed increased level of blood leukocytes, neutrophils, and platelets, and the decreased level of hemoglobin and erythrocytes, they concluded that a mild systemic inflammatory response takes place during welding exposure, in line with the results of earlier studies (Jarvela et al., 2013; Kim et al., 2005; Scharrer et al., 2007). However, they observed no statistical difference whether in CRP or in acute-phase mediators IL-6, IL-8, and TNF-α, while pro-inflammatory cytokine IL-1β and E-selectin levels decreased significantly. Although no study allows comparison with the latter finding, existing studies on CRP are still contradictory: Kim *et al.* (2005) (Kim et al., 2005) support significant CRP changes related to welding fume exposure, while Brand *et al.* (2014), Järvelä *et al.* (2013), and Scharrer *et al.* (2007), (Brand et al., 2014; Jarvela et al., 2013; Scharrer et al., 2007) do not. Very recently, Grazcyk *et al.* (Graczyk et al., 2016b) collected

exhaled breath condensate, blood and urine from 20 non-smoking male welding apprentices at different time points: 1)-before exposure, 2)-immediately after exposure, 3)-one hour after exposure, and 4)-three hours after exposure to assess oxidative stress biomarker concentrations (8-hydroxy-2'-deoxyguanosine, malondialdehyde, hydrogen peroxide, and total reducing capacity) at each time point. Significant increases in the measured biomarkers were observed at 3 h after exposure (a 24%-increase in concentrations of plasma hydrogen peroxide, a 91%-increase in urinary hydrogen peroxide, a 14%-increase in plasma 8-hydroxy-2'-deoxyguanosine, and a 45%-increase in urinary 8-hydroxy-2'-deoxyguanosine). After doubling the particle number concentration, a significant 22%-increase in plasma 8-hydroxy-2'-deoxyguanosine level was observed at 3 h post-exposure. The authors concluded that one hour exposure to Tungsten Inert Gas (TIG) welding fumes in an experimental, well-ventilated setting during one hour resulted in acute oxidative stress three hours after exposure in healthy, non-smoking apprentice welders with no previous chronic exposure to welding fumes. Since inflammation and oxidative stress (Song et al., 2016) are currently considered the two main mechanisms of nanoparticle-related neurotoxicity, these findings suggest that nanoparticulate components of welding fumes might be harmful for the human CNS.

6.2. Studies of occupational combustion-resulting nanoparticles and their effects on human central nervous system

Since manufactured nanoparticles may also induce direct and indirect genotoxicity on the basis of experimental studies, epidemiological case-control studies of CNS cancers were recently reanalyzed in order to assess the relationship with incidental occupational nanoparticle exposure. In 2016, Lacourt *et al.* (Lacourt et al., 2016) reanalyzed data from the

CERENAT study (Coureau et al., 2014) including 596 cases of CNS cancer diagnosed between 2004 and 2006, and 1,192 controls. Exposure to nanoparticles was assessed via the job exposure matrix "MatPUF", linked to the individual occupational histories of study participants. A significant association between occupational exposure to nanoparticles and CNS tumors was seen among men, who had a 50% increased risk of CNS tumors compared to controls (OR=1.5; 95% CI: 1.1, 2.2). The increased risk was particularly pronounced at high exposure levels and for exposure durations longer than 30 years (OR=1.9; 95% CI: 1.2, 2.8), as well as after exposure to carbonaceous nanoparticles (OR=1.5; 95% CI: 1.1, 2.3) and polycyclic aromatic hydrocarbons (OR =1.6; 95% CI:1.1, 2.4) (Lacourt et al., 2016). The results of analyses by histological subtype of CNS tumor (neuroepithelial tumor/meningioma) were not statistically significant, although the risk observed for meningioma was increased two-fold in the nanoparticle-exposed subgroup (OR=2.0; 95% CI: 0.8, 5.3). In the INTEROCC case-control study no association was found between occupational exposure to combustion resulting particulate material that could be emitted at nanoscale, namely benzo(a)pyrene, gasoline and diesel exhaust emissions and glioma tumors (Lacourt et al., 2013). These three combustion products presented a very similar pattern of results. There was neither an indication of increasing risk when comparing subgroups according to their exposure status (ever/never), nor clear dose–response relationship. Nevertheless, a borderline significant odds ratio of 1.3 (95% CI: 1.0, 1.8) was found in the highest category of diesel exhaust exposure duration (Lacourt et al., 2013). Although an increased brain cancer risk has been reported among motor vehicle operators (Carozza et al., 2000; Cocco et al., 1998; Krishnan et al., 2003), no other studies support the association between diesel or gasoline exhaust emissions and CNS cancer.

6.3. Studies of environmental air pollution nanoparticles and their effects on human central nervous system

6.3.1. Studies in adult populations

Given the similarity of air pollution components, namely fine (PM2.5) and especially ultrafine (PM0.1) particles, with carbonaceous and metal manufactured nanoparticles, studies of healthy or vulnerable adults and children heavily exposed to outdoor air pollution also shed interesting light on nanoparticle-related neurobehavioral effects. Tzivian *et al.* (2015) identified ten studies published up to November 2013 focused on the effects of environmental exposure on mental health in adult population, including mood disorders, neurocognitive function, and neurodegenerative diseases (Tzivian et al., 2015). This review was supplemented by adding four studies published post 2013 (Bakian et al., 2015a; Bakian et al., 2015b; Kioumourtzoglou et al., 2016; Schikowski et al., 2015; Wu et al., 2015) as summarized in Table 1. Although the results were presented in a heterogeneous way, hampering quantitative comparison between studies, this review supports, in overall, a possible role of airborne environmental particulate pollutants on neurocognitive function in the adults. No study analyzed the relationship between the PM0.1 fraction and neurocognitive outcomes. PM2.5 was significantly associated with global cognitive decline, assessed by the Mini-Mental State Examination (MMSE), in two Chinese (Sun et al., 2012; Zeng et al., 2010) and two American studies (Loop et al., 2013; Weuve et al., 2012), but not in a German study (Schikowski et al., 2015). The latter study was conducted in elderly women and used a cross-sectional design. The cognitive performance of 789 participants was assessed by the neuropsychological test battery of 'The Consortium to Establish a

Registry for Alzheimer's Disease (CERAD)-Neuropsychological Assessment Battery and CERAD-Plus subtests for four specific cognitive domains: executive function, constructional praxis, semantic memory, and episodic memory. Figure copying, a subtest of constructional praxis, was negatively associated with all particulate components of air pollution. The strongest association was observed for NO_2, NOx and PM10. For PM2.5, the association was of similar magnitude but not statistically significant (Schikowski et al., 2015). The association between PM2.5 and traffic load was evident only among carriers of the APOE ε4 risk allele. The *APOE* gene regulates cholesterol/lipid metabolism and the ε4 haplotype is a well-described risk factor for impaired cognitive function and Alzheimer's disease (Corder et al., 1993; Strittmatter et al., 1993). In contrast, Gatto *et al.* (Gatto et al., 2014) reported an association between PM2.5 and poorer verbal learning in a cross-sectional study of two distinct populations, while no association was found between PM10 and gaseous pollutants. Four studies where traffic-related air pollution effects were investigated using two exposure variables: black carbon concentrations and proximity to the road (Power et al., 2011; Power et al., 2013; Wellenius et al., 2012), and a study performed by Ranft *et al.* (Ranft et al., 2009), supports an association between the increase in black carbon levels and worse MMSE scores and poorer results on additional specific cognitive tests. The association between particulate air pollution and neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease and dementia has been investigated in five studies. Two studies performed by Calderón-Garciduenas *et al.* (Calderon-Garciduenas et al., 2004; Calderon-Garciduenas et al., 2010) pointed out more extensive changes related to Alzheimer's disease in individuals exposed to higher air pollution. This changes included accumulation of Aβ42, increase in expression of COX2, and reduction in olfactory functions, respectively. Finkelstein and Jerrett (Finkelstein and Jerrett, 2007) reported that Parkinson's disease prevalence was associated

only with increasing ambient manganese concentration, but not with NO_2 concentrations. Wu *et al.* (Wu et al., 2015) found that the highest tertile of PM10 (49.23 mg/m^3) and ozone (21.56 ppb) exposure was associated with increased risk of vascular dementia and Alzheimer's disease. PM2.5 exposure data were unavailable, but given that PM10 and PM2.5 had a high correlation (0.81) in this study, the authors considered that PM10 may serve as a surrogate of PM2.5 (Wu et al., 2015).

Most recently, Kioumourtzoglou *et al.* (Kioumourtzoglou et al., 2016) studied the effects of PM2.5 on first hospital admission for dementia, Alzheimer's disease, and Parkinson's disease among around 9.8 million Medicare beneficiaries ≥ 65 years in 50 northeastern U.S. cities. The cohort was followed up from 1999 towards 2010 and allowed analyzing relationships between long-term city-wide exposure to PM2.5 and the three health outcomes. The hazard ratios (HR) were reported per 1μg/m^3 increase in annual PM2.5 concentrations: 1.08 (95% CI: 1.05, 1.11) for dementia, 1.15 (95% CI: 1.11, 1.19) for Alzheimer's disease, and 1.08 (95% CI: 1.04, 1.12) for Parkinson's disease admissions. The authors interpreted these findings as an indication that PM2.5 environmental exposure might accelerate the progression of neurodegeneration, especially after the disease onset, and emphasized the role of inflammation as an intermediate outcome between PM2.5 exposure and the neurodegeneration (Block and Calderon-Garciduenas, 2009). Three studies assessed associations between air pollution index and anxiety, mood disorders, and activities of daily living, and reported that increase in air pollution was associated with poor self-reported health (Persson et al., 2007; Sun and Gu, 2008; Zeng et al., 2010). Lim et al. examined a relationship between air pollution and depression in a longitudinal study of 357 residents in Korea (Lim et al., 2012). Increased levels of PM10, O_3 and NO_2 were associated with increase

in the emotional symptoms of depression, while PM10 exposure additionally increased the frequency of somatic and affective symptoms (Lim et al., 2012).

6.3.2. Studies in pediatric populations

Calderón-Garcidueñas *et al.* (Calderon-Garciduenas et al., 2015) reviewed studies on the impact of air pollution on the CNS in pediatric populations, mostly considering studies in children from the highly polluted Mexico City Metropolitan Area. The authors concluded that the emerging picture for children in the Mexico City Metropolitan Area consists of systemic inflammation oxidative stress and immuno-dysregulation at both the systemic and brain levels, neuroinflammation, disorders in small blood vessel, intrathecal inflammatory process, as well as the early neuropathological hallmarks of Alzheimer's and Parkinson's diseases. They considered that the developing brain in exposed children responds to the harmful environment by structural and volumetric changes, leading to cognitive, olfactory, auditory and vestibular deficits, and long-term neurodegenerative consequences (Calderon-Garciduenas et al., 2015). When considering this alarming picture, it is important to keep in mind the high level of uncertainty and methodological limitations in most studies, which may undermine credibility. These limitations include small study samples, selection and exposure misclassification bias, and use of poor-quality aggregated exposure data, hampering control even for well-established confounders. For instance, Tzivian *et al.* (Tzivian et al., 2015) criticized the lack of joint consideration of noise exposure when studying the effects of traffic-related air pollution, due to possible synergistic relations or interactions between these two exposures. Kristiansson *et al.* (Kristiansson et al., 2015) emphasized the need for prospective longitudinal epidemiological investigations of associations between high concentrations of air pollutants, poverty, violence and health. To date, only one study

has met these requirements (Sunyer et al., 2015), confirming reduced cognitive development in children highly exposed to traffic-related air pollutants in Spanish schools. In this study, outdoor ultrafine particle (PM0.1) concentrations were associated with inattentiveness and reduction of superior working memory in the 12-months change model, after adjustment for sex, age, maternal education, air pollution exposure at home, and residential neighborhood socioeconomic status. In this model, the individual and school were treated as nested random effects and residual confounding by noise. Moreover, any residual confounding by noise was ruled out after checking the correlation between noise and different pollutants in the same classrooms, and the robustness of the coefficients for the pollutants by additional adjustment for noise and for the interaction between age and noise (Sunyer et al., 2015).

Overall, the available evidence points to vulnerability of the developing brain to particulate components of air pollution. This is compatible with most currently considered neurotoxic mechanisms of ultrafine particles, such as inflammation, altered innate immune response, and chronic microglial stimulation. Given that microglial inflammation may result from both local deposition of ultrafine particle in the brain and systemic inflammation originating in more distant ultrafine particles-exposed organs (Block et al., 2012; Viana et al., 2014), the risk of neurobehavioral impairments in adults occupationally exposed to anthropic and manufactured nanoparticles cannot be excluded.

7. Gaps in research and methodological challenges

Currently available studies are insufficient to elucidate the complex issue of how the brain manages nanoparticles. Are they modified, removed or instead retained in the CNS? The fact that certain manufactured nanoparticles, like TiO_2, could be detected in the vesicles of

microglial cells suggests that the brain is able to activate classic elimination processes. However on a longer time scale in particular, there is no evidence on the actual ability of nerve cells to eliminate nanoparticles. Instead, recent findings are in favor of nanoparticle accumulation in the brain over time. As an example of intracerebral persistence of manufactured nanoparticles, multi-wall carbon nanotubes have been observed nearly one year after single aerosol exposure in mice (at 5 mg/m^3 for 5 h) (Mercer et al., 2013). Another example from nanomedicine area could be found with biostability studies of gallium phosphide (GaP) nanowires (Gallentoft et al., 2016). Characterized with a diameter in the nanometer range and a length on the micrometer length scale, these nanowires have been shown to promote neurite outgrowth and reduce glial cell spreading (Piret et al., 2013; Piret et al., 2015), thus offering useful advantages in medical applications such as nanostructured electrode surfaces, by improving recording properties and reducing tissue responses (Piret and Prinz, 2016). The biostability in the brain of female rats was compared between degradable nanowires (coated with a 20nm layer of SiOx) and biostable nanowires (coated with a 20 nm layer of HfOx) known to persist in the brain for long periods of time (Gallentoft et al., 2015). In both cases, residues from nanowires remained detected in brain tissue 1 year post injection, trapped in the microglia/macrophages, indicating a very slow clearance (dissolution and removal) of nanoparticles from the brain (Gallentoft et al., 2016). These rare long term studies stress the possible limits of degradation pathways in the brain and raises the question of the consequences that would have been observed on chronic exposure.

Furthermore, it is highly likely that the nature of the nanoparticles may also be a key element in the ability of the brain to eliminate or in contrast to accumulate nanoparticles. Despite scant available evidence, based on often imperfect studies, it seems reasonable to point out that metallic nanoparticles are the most dangerous to the brain due to their

natural tendency to accumulate over time. Interestingly, metals such as iron, manganese, copper or zinc, are in fact retained in the brain during normal development with regional specificity (Tarohda et al., 2004), a phenomenon that worsens over time. This point is noteworthy when studying long-term impacts of nanoparticles on the brain, since abnormal levels of metals are also associated with neurodegenerative diseases, and more specifically the neuronal populations affected by the disease (Davies et al., 2014).

Given the limited literature available, an attempt to classify the deleterious effects induced by nanoparticles is still impossible. However, based on *in vitro* studies dealing with the integrity of the BBB, it can be suggested that silver nanoparticles may be more deleterious than copper nanoparticles, themselves more harmful than aluminum nanoparticles. While the combination of several metallic nanoparticles is often more realistic in occupational and environmental settings, there is no study dealing with mixed nanoparticle exposure. A recent review article proposes an in-depth examination of the physicochemical properties of engineered nanomaterials that may impact their ability to interact with the nervous system, reporting how they contribute to nanoparticle distribution in the brain as well as how they may induce effects on it, bringing further evidence of possible nano-brain interactions (Yokel, 2016b).

Given the specificities of brain composition and function, it is clear that very careful attention must be paid to the diverse experimental designs used when studying the effects of nanoparticle exposure on brain health. Nano-brain studies must indeed follow specific strategies and require a minimum of knowledge in neuropathology. Unfortunately, several published studies do not meet such requirements. Despite an attempt to alert the scientific community on the limitations of these studies (Bencsik and Lestaevel, 2015; Bencsik et al.,

2013; Jonaitis et al., 2010), some of the results are still quoted as proven observations, needlessly fueling controversy that may lead to incorrect scientific conclusions and inappropriate regulatory responses. Any over-interpretation of data collected on one type of cell, on stressed or anesthetized animals, using unrealistic amounts of nanoparticles, or unrealistic routes of exposure, should be carefully avoided. With respect to *in vivo* access of nanoparticle within nervous cells of the brain, attention must be paid on how the data were collected notably to differentiate between nanoparticles within the brain proper (brain cells and brain extracellular space) *versus* nanoparticles in brain that also contains the vascular compartment. This is important to avoid a false conclusion, often drawn, that a nanoparticle entered the brain, when it might be on the blood side of the BBB or within the BBB components, but not the brain. The *in vivo* studies are more illustrative of the situation in living beings. However, experiments in animals are challenging to control and could be affected by several random issues. Additionally, other parameters, such as the bio-distribution of nanoparticles, could possibly lead to inaccurate results.

The *in vitro* models could help to assess the neurotoxicity of nanoparticles. These models reflect our current mechanistic understanding of different cerebral effects. Protocols and methods are broadly well-known. *In vitro* models involve low costs, and provide high numbers of replicates. They raise few ethical difficulties, with the prominent exceptions of human tissue gift and embryonic stem cells. Cell models for practically all neuro-cellular types are available. For TiO_2 nanoparticles for example, PC12, primary microglia, primary hippocampal neurons, human SH-SY5Y neuronal cells, human cerebral endothelial cells (HCECs), and human stem cell lines have previously been used (Coccini et al., 2015; Huerta-Garcia et al., 2014; Rihane et al., 2016). Although the above-mentioned models were all dedicated on the harmful effects of TiO_2 nanoparticles on the central nervous system,

different conclusions were found. Several factors, such as crystal type or size of nanoparticles, might influence the neurotoxicity of TiO_2 nanoparticles examined in these *in vitro* models. Moreover, the wide relations among cells and/or tissues cannot be totally reproduced in these models.

Current *in vitro* approaches for assessment of nano-neurotoxicity have important limits. *In vitro* tests are poorly interpretable *in vivo*, since the genotoxicity of nanoparticles in cultures are not found in animal tests (Hartung, 2010). This divergence may be produced by limited nanoparticle bio-distribution within a cell compared with an entire organism, resulting in fast nanoparticle overload. A possible explanation of these limitations may result directly from the biological coating of the nanoparticles. Although the phenomenon is far from being understood, it is recognized that the protein corona probably plays a crucial role in nanoparticle behavior in living beings once in contact with the complex biological milieu. The nature of the proteins that would bind to the surface of nanoparticles will affect the properties of the nanoparticles, guiding their interactions with cells (allowing endocytosis or not), and participating in the translocation process from one biological compartment to the other. Thus, protein coronas on nanoparticles may act not only on bio-distribution, but may also play an important role for the functional properties of coated nanoparticles, which can be beneficial as well as toxic for the body. As an example, the composition of the protein corona was studied for four different nanoparticles of SiO_2 (combining 20 and 100 nm, with positive and negative charges), taking into account the composition in proteins specific to blood compared to brain homogenates. In plasma, the corona is mainly made of albumin, lipoprotein and proteins related to coagulation, while in the brain, it is mainly composed of tubulin, suggesting completely different interactions and functions of these nanoparticles in

brain tissue (Shim et al., 2014). These major obstacles to assessing the neurotoxicity of nanoparticles are summarized in Figure 4.

Besides, the knowledge in nanotoxicity and CNS in the biomedical field is deeply lacunar. Even if by definition brain nanomedicines will be optimized, today biocompatibility and biodegradability of nano-drugs are far to be understood. To our knowledge, there is no specific method to identify targeted drug release or the toxicity level in the brain. It appears that to improve safe nanotechnology–based drug for CNS disorders, specific guidelines should be also advanced and followed.

8. Research avenues to gain insight in the raised questions

Over the past few years, there has been a continual debate on the most appropriate strategies to use for evaluating the human health risks of nanoparticles on the brain. One of the utmost defies facing the neurotoxicology community is the prioritization of nanoparticles to estimate and the complexity of toxicological evaluation that should be directed. However, to date, the understanding of nanoparticle neurotoxicology has been particularly incomplete. The conclusions from classic *in vitro* and *in vivo* studies are not always comparable and are sometimes contradictory. Hence, it is crucial to normalize experiments that assess the neurotoxicity of nanoparticles. This appears to be a key aspect in the biomedical context and probably some important advanced should come from nanomedicine studies. As an example, Kaushik *et al*. developed a promising biocompatible nanocarrier that could be helpful to follow brain delivery of nanoparticle and to evaluate their toxicity (Kaushik et al., 2016). Relaying on the first step, *in vitro* cytotoxicity tests applied on primary human astrocytes and SKNMC neuroblastoma cell line, the authors completed their study by *in vivo* cytotoxicity tests in a mouse model (intra venous delivery at

a dose transposable to humans). Toxicity was assessed by histopathology, blood toxicity profile completed with neurobehavioral evaluation (grip test, horizontal bar test, rotarod test). The transmigration of the nanocarrier within the brain tissue, the particles size distribution as well as uptake in brains were analyzed using *in situ* transmission electron microscope (TEM) experiments. Brain tissues were also subjected to various analyses to evaluate elemental and structural analysis of the nanocarrier (scanning transmission electron microscopy (STEM), convergent beam electron diffraction (CBED), energy dispersive spectroscopy (EDS). A quantitative estimation of the nanocarrier concentration within the brain was performed using inductively coupled plasma mass spectroscopy (ICP-MS), validated by the previous establishment of calibration curves and appropriate control (of which control injected mice). Still this study does not evaluate the efficacy of the drug itself, neither the biodegradability of the nano-delivered medicine that would need long-term studies, in animal models but also in humans. For example, there is no long-term human safety information available for *Nanotherm* a magnetofluid consisting of superparamagnetic iron oxide nanoparticles (SPIONs), currently used for treating brain tumors (Gobbo et al., 2015). The main hypothesis is based on the dissolution of the nanoparticle into ions that would be then recycled into the iron pool of the body in a homeostatic fashion.

Moreover, because nanoparticles could disturb brain homeostasis, the possible relationship between exposure to manufactured nanoparticle and neurodegenerative disorders needs further investigation. To do this, there are animal models for the common of nanoparticle-induced neurodegenerative diseases, showing that the animal models have the same molecular targets or paths as humans. These animal models can be used for both sexes and at different life stages. Concerning animal studies, the importance of the life stage should be examined. First, fetal life and early childhood are critical stages and specific attention should

be focused on determining the effect of exposure to nanoparticle exposure at these developmental periods. Aging may also act as a significant feature in susceptibility to nanoparticle-induced neurotoxicity. The exposure scenarios should be realistic (exposure route, dose rate, exposure regimen, etc.), but conventional *in vivo* drug neurotoxicity testing methodologies are unfeasible, costly and time-consuming, even for nanoparticles that have already been developed.

Alternative neurotoxicity testing methods could help us to carefully study nanoparticle-brain interactions, such as innovative technologies that are rapidly developing and that contain imaging technologies as well as the different ‘‘omic’’ technologies. Because neurotoxicity investigations require extensive testing, the development of an intermediate *in vivo* screening platform would be most appropriate. Ideally this neuro-screening platform would rely on the mechanistic knowledge acquired *in vitro* and would provide relevant *in vivo* nano-neuro-interfaces, perfectly controlled in a spatiotemporal point of view. This platform must be in the same place and performs significant *in vitro* and *in vivo* studies on nanoparticles in the same time. It is also central to acquire information on how and where nanoparticles can accumulate in the brain, as well as nanoparticle elimination paths. It is possible that the cerebrospinal fluid could be an excretory path used by the central nervous system, and this subject should be studied as soon as possible.

Most neurotoxic data on nanoparticles gathered from experimental studies are based on rodents or their cells, and might be inappropriate to determine neurotoxicity in humans. Thus, studies on cells derived from humans would enhance our understanding of nanoparticle effects on the human nervous system, along with epidemiological and human controlled exposure studies. In conclusion, while the effect of nanoparticles on the brain has recently received substantial attention, the data obtained from *in vivo* and *in vitro* studies

are nonetheless incomplete. In light of the most recent evidence of the presence of magnetite pollutant nanoparticles detected in the brains of people living in contaminated cities, such as Mexico City or Manchester (Maher et al., 2016), better evaluation systems are urgently needed.

9. Conclusion

Scientific articles dealing with neuro-nanotoxicology are received with particular interest, because in the field of nanotechnology, they will most likely have a significant impact not only on our perception of hazards potentially associated with nanomaterials, but also on regulatory decisions regarding their use in consumer products. Since the nervous system has many specificities in terms of vulnerability and protection systems, it needs particular attention and specific experimental and epidemiological studies relying on suitable approaches. Unfortunately, among the articles dealing with the specific question of brain–nanoparticle interactions, only a few follow a suitable design and allow accurate conclusions that might be transposed to humans. In this context, particular attention must be paid to these studies.

Thus, at a time when the use of the nanoparticles is becoming increasingly widespread across different application areas, workers and consumers are exposed more and more, and by multiple pathways. This context of chronic exposure favors a potential impact on the vulnerable brain, in particular in susceptible periods of life (at the fetal age, and in young and elderly populations), as suggested by the most recent epidemiological studies. All the evidence already available fully justifies the need to evaluate the interactions between

nanoparticles and the nervous system specifically, and must lead to heightened awareness of the possible impact of nanoparticles on brain function.

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Figure Legends

Figure 1: In nanomedicine, nano-carriers help drug delivery to the brain by enabling passage across the BBB. Because nanoparticles (NPs) may induce neurotoxic effects, their use in the brain should be considered cautiously. *Adapted from Nature Reviews, Drug discovery and from Khanna et al Nanomaterials 2015.*

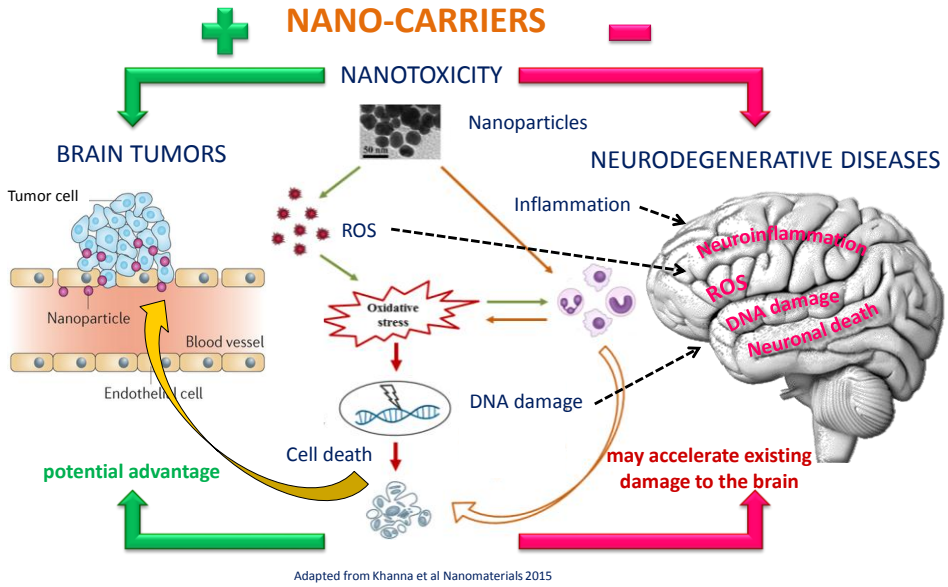

Figure 1 Bencsik et al. *Progress in Neurobiology*

Figure 2: Schematic illustration of the nervous system's main characteristics in terms of organization, composition, and protection.

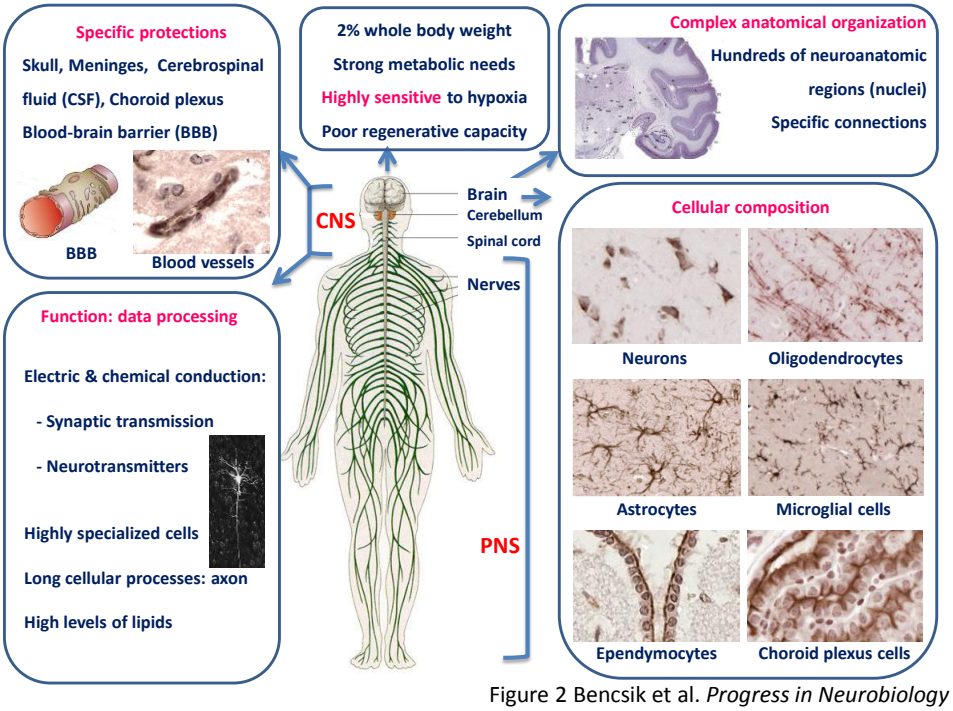

Figure 2 Bencsik et al. *Progress in Neurobiology*

Figure 3: Nanoparticle (NP) translocation to the nervous system: a summary of the main routes to the brain at the fetal, juvenile and adult stages of life, considering various routes of exposure, sites of uptake, and translocation pathways.

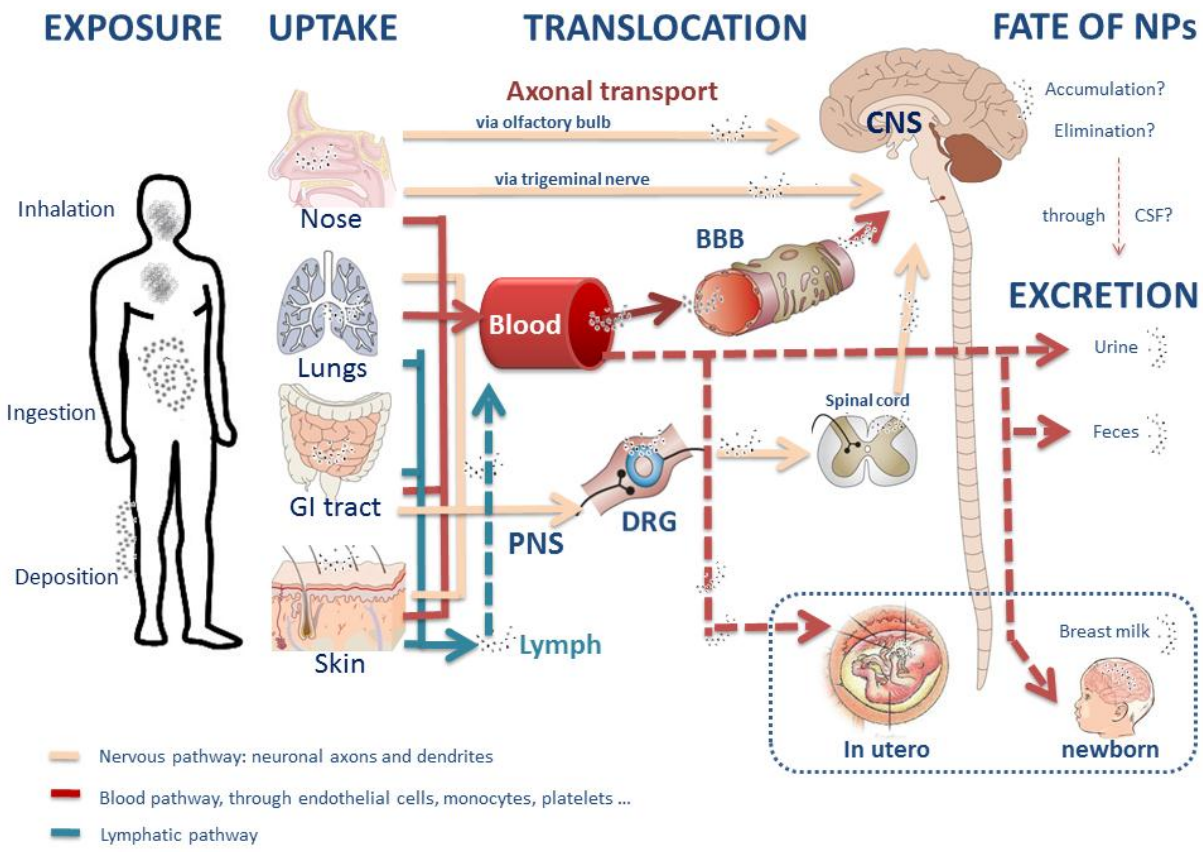

Figure 3 Bencsik et al. *Progress in Neurobiology*

Figure 4: A schematic summary of the major obstacles to assessing the neurotoxicity of nanoparticles (NPs).

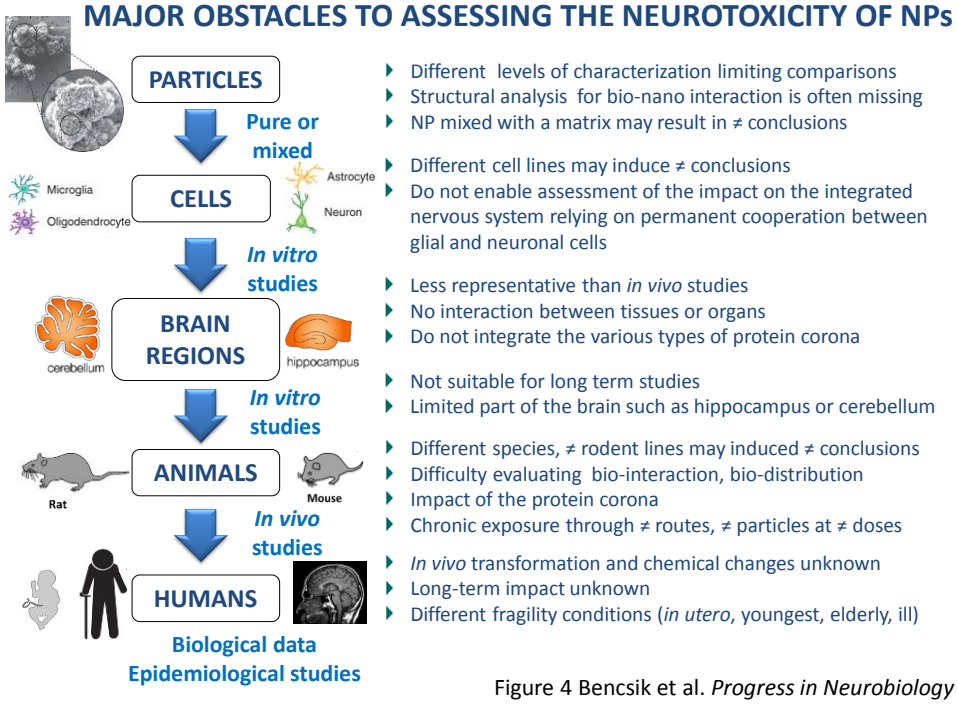

Figure 4 Bencsik et al. *Progress in Neurobiology*

Tables

Table 1: Results of studies on the effects of long-term air pollution on cognitive and psychological functions in adults.

(Chen and Schwartz, 2009)

Table 1. Results of studies on long-term air pollution effect on cognitive and psychological functions in adults.

Authors, year of publication	Study design	Study population N (age)	Exposure	Covariates	Outcome	Obtained results
Sun and Gu, 2008	Cross-sectional	N = 7358 (83.6 ± 11.4)	API	P, L, E	Cognitive functions	1 unit air pollution index (API): For high gross domestic product (GDP): MMSE score β=−2.67, p < 0.001. For medium GDP: MMSE score β=−1.84, p<0.001
Chen and Schwartz, 2009	Cross-sectional	N = 1764 (37.4 ± 10.9)	PM, O_3	P, L, H	Cognitive functions	Increased PM10: SDLT β =0.48, 95% CI=0.27–0.68; SDST β = 0.10, 95% CI=0.05–0.15. After adjustment for race and SES—non-significant effect. Increased O3: SDST β=0.11, 95% CI=0.01–0.22; SDLT β=0.52, 95% CI 0.03–1.01
Ranft et al., 2009	Cross-sectional	N = 399 (74.1 ± 2.6)	PM_{10}	P, L, H, E	Cognitive functions	Traffic exposure: CERAD-plus battery β=−3.8, p < 0.1; Stroop test β= −5.1, p < 0.01; Sniffing test β=−1.3, p < 0.05 (age ≤ 74). No independent effect of PM10
Zeng et al., 2010	Cohort follow-up 7 years	N = 15,873 (86.3)	API	P, L	Cognitive functions	1 unit API: cognitive impairment OR = 1.009, p < 0.05
Power et al., 2011	Cross-sectional	N = 680 (71.0 ± 7.0)	BC	P, L, H	Cognitive functions	BC (doubling concentration, µg/m3): MMSE OR=1.3, 95% CI 1.1–1.6 Wellenius et al., 2012 100 m from major road: MMSE < 26 for at least college education OR= .54, 95% CI 1.10–2.17; for ≤77 years OR=1.34, 95% CI 1.01–1.76. Not associated with HVLT-R recognition, TMT Part A ad CIB. Interquartile increase in BC (0.11 _g/m3): MMSE < 26, OR=1.15, p = 0.06; worse performance of HVLT = R immediate recall, p=0.046
Wellenius et al., 2012	Cohort follow-up (median), 16.8 years	N = 765 (78.1 ± 5.4)	Proximity to nearest road, BC	P, L, H	Cognitive functions	BC (doubling concentration, µg/m3): MMSE OR=1.3, 95% CI=1.1–1.6; 100 m from major road: MMSE < 26 for at least college education OR=1.54, 95% CI=1.10–2.17; for ≤77 years OR=1.34, 95% CI=1.01–1.76. Not associated with HVLT-R recognition, TMT Part A ad CIB. Interquartile increase in BC (0.11 µg/m3): MMSE < 26, OR=1.15, p=0.06; worse performance of HVLT= R immediate recall, p = 0.046

Weuve et al., 2012	Cohort follow-up 4.3 years	N = 10,409 (74.0 ± 2.2)	PM_{10}; $PM_{2.5}$, Coarse PM	P, L	Cognitive functions	PM2.5–10: worse global cognitive score (p for trend 0.01); worse for highest vs. lowest level (p = 0.003). Highest vs. lowest quintile of PM2.5: changes in global cognitive score for women (p = 0.03). Global cognitive score (SD/2 years) per 10 μg/m3 increment: PM2.5–10 −0.020 (95% CI 0.32 to −0.008); PM2.5: −0.018 (95% CI −0.035 to 0.002)
Gatto et al., 2014	Cross-sectional	N = 1496 (60.5 ± 8.1)	O_3, NO_2, $PM_{2.5}$	P, L	Cognitive functions	None association with global cognition. Per 10 μg/m3 PM2.5: lower verbal learning (β= −0.32, p = 0.05). NO2 > 20 ppb: lower logical memory (β=−0.62, p = 0.095). O3 > 49 ppb: lower executive function (β= −0.66, p = 0.059). O3 range 34–49 ppb: higher logical memory—women (β= 0.46, 95% CI 0.09–0.83), adults ≥ 60 y. o. (β= 0.51, 95% CI 0.11–0.91)
Loop et al., 2013	Cross-sectional	N = 20,150 (64.0 ± 9.2)	$PM_{2.5}$	P, L, H, E	Cognitive functions	Per 10 μg/m3 PM2.5: for urban area—incident cognitive impairment OR=1.40 (95% CI 1.06–1.85); for mixed areas—incident cognitive impairment (OR = 0.32, 95% CI 0.11–0.98). No associations for rural area and total population
Power et al., 2013	Cross-sectional	N = 629 (70.0 ± 7.1)	BC	P, L, H	Cognitive functions	BC (doubling concentration, μg/m3): for lacked an HFE C282Y low MMSE (OR=1.37, 95% CI=1.08–1.73); for at least one HFE H63D variant (OR=1.74, 95% CI=1.06, 2.87). HFE C282 modifies the association between BC and global cognitive function
Schikowski et al. 2015	Cross-sectional	N=789 (73.4 ± 3.05)	NO_2, NOx, $PM_{2.5}$, PM_{10}	P, L, H, APOE ε4 allele	Cognitive functions	Negative association with cognitive function and cognitive performance in the subtests for semantic memory and visuo-construction. Significant associations could be observed for figure copying with an interquartile range increase of NO2 (β=–0.28 (95%CI:–0.44;–0.12)), NOx (β=–0.25 (95%CI:–0.40;–0.09)), PM10 (β=–0.14 (95%CI:–0.26;–0.02)) and PM2.5 (β=–0.19 (95%CI:–0.36;–0.02)).The association with traffic load was significant in carriers of one or two ApoE ε4 risk alleles
Calderón-Garciduenas et al., 2004	Cross-sectional	N = 19 (51.2 ± 4.9)	PM, O_3	none	Alzheimer's disease	Frontal cortex tissue: Elevation of COX2 mRNA in high-exposure group (p=0.009); elevation of COX2 immunoreactivity (p=0.01). Hippocampus tissue: Elevation in COX2 mRNA in high-exposure group (p=0.045); no differences in COX2 immunoreactivity between high and low-exposure groups (p=0.37)
Calderón-Garciduenas et al., 2010	Cross-sectional	N = 87 (21.0 ± 2.6)	$PM_{2.5–10}$, $PM_{2.5}$	none	Alzheimer's disease	Mean UPSIT scores lower for high-exposure group (p=0.03). No differences in UPSIT scores in different APOE statuses (p=0.52)
Wu et al., 2015	Case–control	249 AD patients, 125 VaD cases, and 497 controls	PM_{10}, O_3	P, L, H, APOE ε4 allele	Alzheimer's disease (AD) & vascular dementia (VaD)	The highest tertile of PM10 (49.23 mg/m3) or ozone (21.56 ppb) exposure was associated with increased AD risk (highest vs. lowest tertile of PM10: AOR 5 4.17; highest vs. lowest tertile of ozone: AOR 5 2.00). Similar finding was observed for VaD. The association with AD and VaD risk remained for the highest tertile PM10 exposure after stratification by APOE 34 status and gender.

Finkelstein and Jerrett, 2007	Case–control	N = 1764 (37.4 ± 10.9)	PM_{10}, O_3	P	Parkinson disease (PD)	10 µg/m3 increases in Mn: PD or Dopa prescription for men OR=1.041, 95% CI 0.997–1.09; for female: OR=1.035, 95% CI 0.97–1.10. With type of clinic as confounder OR=1.044 (95% CI=1.00–1.09)
Kioumourtzoglou eta l., 2016	Cohort, follow-up 10 years	N=9817806 (75.6± 7.6)	Annual 50 city-average $PM_{2.5}$ mass concentrations for the period of 1999–2010	P, L, H,	Dementia, Alzheimer's & Parkinson's diseases	Significant associations of long-term PM2.5 city-wide exposure with all three outcomes: HR=1.08 (95% CI: 1.05, 1.11) for dementia, an HR= 1.15 (95% CI: 1.11, 1.19) for AD, and HR=1.08 (95% CI: 1.04, 1.12) for PD admissions per 1µg/m3 increase in annual PM2.5 concentrations.
Persson et al., 2007	Case–control	N = 22,693 (43.0 ± 13.0)	NOx, Wood burning; industrial smells	P, H	Anxiety	Exhaust form traffic: anxiety OR=1.66, p=0.001
Sun and Gu, 2008	Cross-sectional	N = 7358 (83.6 ± 11.4)	API	P, K, E	Activity of daily living (ADL)	1 unit API: For high GDP: difficulties in ADL (β=−1.41, $p < 0.01$); instrumental ADL (β=−0.98, $p < 0.001$), self-related health (OR = 2.20, $p < 0.001$). For medium GDP instrumental ADL β=−0.6, $p < 0.001$, self-related health, (OR = 1.87, $p < 0.001$), no associations with ADL
Zeng et al., 2010	Cohort follow-up 7 years	N = 15,873 (86.3)	API	P, L	Activity of daily living (ADL)	1 unit API: increased ADL disability (25%, $p < 0.001$); increased health deficits (8%, $p < 0.05$)
Lim et al., 2012	Longitudinal follow-up 3 years	N = 357 (71.0 ± 5.0)	PM_{10}, CO, SO_2, NO_2, O_3	P, L, H	Depression	Interquartile increase of PM10: Increase in composite score of emotional symptoms: 38.2%, $p < 0.01$, NO2 118.2%, $p < 0.05$. Increase in somatic symptoms score 38.9%, $p < 0.05$. Increase in affective symptoms score 11.5%, $p < 0.01$. O3: Increase in composite score of emotional symptoms 132.5%, $p < 0.05$

API-Air pollution index that includes SO2, NO2, PM10, CO, O3; BC-Black carbon; P—personal factors, including age, sex, socio-economic status (SES), occupation, marital status, ethnicity, childhood SES, education level, marital status, number of surviving children, country of origin number of living children. L—lifestyle factors, including smoking, drinking, exercise, leisure activities, dark fish consumption, computer experience, BMI. H—health-related factors, including number of consultations with general physician, asthma, diabetes, incident stroke, presence of depressive symptoms, dyslipidemia, hypertension, blood pressure, triglycerides, HDL. E—environmental factors, including indoor air pollution, temperature, season. OR-odds ratio; 95% CI, 95% confidence interval; HR-Hazard ratio;* modal age class; the relationship with suicide risk was analyzed based on the air pollutant concentration on the day of the suicide (lag day 0) and on each of the 3 days preceding the suicide (lag day 1, lag day 2, and lag day 3) for single and cumulative air pollutants.