REVIEW

Developmental Neurotoxicity of Engineered Nanomaterials: Identifying Research Needs to Support Human Health Risk Assessment

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Increasing use of engineered nanomaterials (ENM) in consumer products and commercial applications has helped drive a rise in research related to the environmental health and safety (EHS) of these materials. Within the cacophony of information on ENM EHS to date are data indicating that these materials may be neurotoxic in adult animals. Evidence of elevated inflammatory responses, increased oxidative stress levels, alterations in neuronal function, and changes in cell morphology in adult animals suggests that ENM exposure during development could elicit developmental neurotoxicity (DNT), especially considering the greater vulnerability of the developing brain to some toxic insults. In this review, we examine current findings related to developmental neurotoxic effects of ENM in the context of identifying research gaps for future risk assessments. The basic risk assessment paradigm is presented, with an emphasis on problem formulation and assessments of exposure, hazard, and dose response for DNT. Limited evidence suggests that in utero and postpartum exposures are possible, while fewer than 10 animal studies have evaluated DNT, with results indicating changes in synaptic plasticity, gene expression, and neurobehavior. Based on the available information, we use current testing guidelines to highlight research gaps that may inform ENM research efforts to develop data for higher throughput methods and future risk assessments for DNT. Although the available evidence is not strong enough to reach conclusions about DNT risk from ENM exposure, the data indicate that consideration of ENM developmental neurotoxic potential is warranted.

Key Words: developmental neurotoxicity; engineered nanomaterials; risk assessment research.

Engineered nanomaterials (ENM) are increasingly used in consumer products and commercial processes, raising concern over the potential health impacts of increased human exposure (Hansen et al., 2008; Hendren et al., 2011). The definition of ENM continues to evolve as the nanotechnology field itself grows (Grieneisen and Zhang, 2011; Maynard, 2011). In this review, we use the National Nanotechnology Initiative definition: ENM are materials intentionally synthesized or manufactured with at least one external dimension of 1 to ~100 nm and that possess unique properties due to their size (NSTC, 2011). Products in which manufacturers claim to use ENM include consumer products such as cosmetics, fabrics and clothing, personal care items, cleaning solutions, sporting equipment and electronics, and goods specifically intended for use by children (e.g., toys) (Hansen et al., 2008). Within these various products is a diversity of ENM types, including nanoscale silver (nano-Ag), titanium dioxide (nano-TiO₂), and carbon (nano-C).

Based upon the variety of ENM products and applications, coupled with variability in ENM production methods and measurement techniques, it has been suggested that environmental health and safety testing of ENM could be both time consuming and expensive. In the United States, environmental health and safety testing could take 34–53 years and cost an estimated $0.25–$1.18 billion, depending on the extensiveness of testing required to determine safety (i.e., minimal testing required vs. long-term in vivo testing to establish safety; see Choi et al., 2009 for greater details). Notably, this estimate is based on a 2009 analysis of U.S. firms producing or using ENM and thus does not reflect resources that might be necessary to test new ENM coming into the market. Recognizing that the use and development of novel ENM applications will outpace environmental health and safety research, several
preliminary evaluations have used currently available data to highlight data gaps for assessing human health risks from ENM exposure (Aschberger et al., 2011; Savolainen et al., 2010; Wijnhoven et al., 2009). Such data gaps include exposure data, hazard identification and characterization information, quantitative dose-response data, standards related to metrics for ENM testing, and analytical approaches for reliable detection of ENM in biological and environmental milieux.

A variety of toxicity outcomes are associated with ENM exposure in animal models (Savolainen et al., 2010). Of these outcomes, of particular note for this article are indications that some of these materials translocate to the brain and evoke biological effects in the nervous system of animal models (Boyes et al., 2012; Hu and Gao, 2010; Oberdörster et al., 2009; Sharma and Sharma, 2010; Simkó and Mattsson, 2010; Yang et al., 2010). Biological changes in the brain of adult animals following ENM exposures include elevated inflammatory responses, increased oxidative stress levels, alterations in neuronal function, and changes in cell morphology (Simkó and Mattsson, 2010). These types of effects on the nervous system could result in functional neurotoxicity, and indeed, early evidence shows behavioral changes (e.g., decreased motility in open field tests) (Simkó and Mattsson, 2010). It remains unclear, however, whether the behavioral effects are ENM specific or not (e.g., conflicting findings on whether nanoscale vs. ionic Mg led to changes in open field motility as detailed in Oszlanczi et al., 2010a, b). However, neurotoxic effects of ENM in the adult brain suggest the potential for developmental neurotoxicity (DNT) from ENM exposure due to the greater vulnerability of the developing brain to toxic insults (reviewed by Bondy and Campbell, 2005 and Rice and Barone, 2000), as discussed further below.

Here, we highlight several characteristics of ENM and the developing brain that suggest a particular concern for DNT from ENM exposure. First, production volume estimates indicate that metal or metal-oxide ENM are widely used (Hendren et al., 2011), and some of these metals (e.g., silver, cadmium, and manganese) are associated with adverse neurological effects prior to being manufactured at the nanoscale (Burton and Guilarte, 2009; Lansdown, 2007; Méndez-Armenta and Rios, 2007). Secondly, even materials without known neurotoxic effects (e.g., carbon-based materials) are increasingly produced as ENM (Innovative Research and Products Incorporated, 2011) and may disrupt neurodevelopment when manufactured into ENM, as the higher surface area to volume ratio makes them more biologically reactive compared with larger size materials (i.e., those with dimensions greater than 100 nm) (Oberdörster et al., 2005). Thirdly, the blood-brain barrier (BBB) continues to mature up to about 6 months of age in humans, which when coupled with the small size of ENM, increases the likelihood of ENM reaching the developing brain (Watson et al., 2006). Finally, the immature status of several systems may lead to greater effects of ENM in the developing fetus. For instance, metabolic systems capable of protecting the body from toxicant exposures, such as cytochrome P450 enzymes, are still developing in the fetus (Bondy and Campbell, 2005). As a result, the ability to metabolize ENM or ENM components (e.g., surface coatings on ENM) will likely differ across life stages, and studies to determine such potential differences will need to account for the ontogeny of expression of metabolic enzymes (Wrighton and Stevens, 1992). Such studies might focus on determining whether there are particular types of ENM for which the fetus could serve as a “sink” for ENM in maternal circulation, suggesting a period of prolonged exposure to the developing brain should ENM cross the placental barrier. In fact, recent evidence indicates that certain sizes of ENM pass through the human placental barrier (Wick et al., 2010) and accumulate in fetal tissues, including the brain, of animal models (Gao et al., 2011; Yamashita et al., 2011).

In summary, the greater susceptibility of the developing brain, unique size and reactivity of ENM compared with larger sized materials, and differences in maturation of key systems between adults and young children, all suggest that attention to the potential for DNT is warranted. Here, we review current data through March 2013 related to the potential for ENM DNT and then use established testing guidelines (OECD, 2007; U.S. EPA, 1998) to highlight areas of research that could inform future ENM risk assessment efforts related specifically to DNT. We further note how current DNT guidelines could be used to help develop a tiered testing approach for the more rapid, inexpensive testing and characterization of ENM that will likely be necessary given the increase in ENM production (Hendren et al., 2011).

RISK ASSESSMENT OF ENM

A standard paradigm for the assessment of risks to human health resulting from environmental chemical exposures has been described by the National Research Council of the National Academy of Science as consisting of the processes of hazard identification, dose-response assessment, and exposure assessment (NRC, 1983). These components contribute to the development of a risk characterization that provides critical information for risk management decisions and actions within a broader social and regulatory context. The value and importance of approaching the risk assessment process with a clear understanding of the information that will be needed to characterize the risks have been recognized and formalized in a problem formulation and scoping step that precede the actual risk assessment (NRC, 1994, 2009) (Fig. 1).

In the context of human health risk assessment, DNT testing is a unique and important tool for hazard identification and dose-response assessment of potentially neurotoxic chemicals providing insight on functional, behavioral, and anatomical changes to the nervous system, which result from exposures during critical periods of neurological development and which are not evaluated in other testing procedures (Makris and Raffaele, 2009). The U.S. Environmental Protection Agency (EPA) DNT screening guideline (shown in Fig. 2) has been evaluated for
sensitivity in detecting changes in neurological development for a variety of neurotoxic chemicals (Makris et al., 2009; U.S. EPA, 1998). Included in this screening guideline, which is generally conducted in rats, are measures of (1) developmental landmarks (e.g., pup weight gain and sexual maturation), (2) neurobehavior (e.g., functional observations, auditory startle habituation, motor activity testing, and cognitive testing), (3) neuropathology, and (4) brain weight (U.S. EPA, 1998). The Organisation for Economic Co-operation and Development (OECD) guideline for DNT testing (GL 426) (OECD, 2007) is harmonized with the EPA DNT guideline but includes a number of refinements, such as a longer postnatal exposure period (see Table 2 in Makris and Raffaele, 2009). As discussed below, both of the EPA and OECD guidelines could help identify ENM DNT research that would support risk assessment work in this area; however, challenges specific to ENM necessitate that the study design will need to be carefully crafted. Thus, we note areas in which studies with ENM might need to expand upon current DNT testing guidelines. 

**AVAILABLE EVIDENCE FOR CONDUCTING ENM DNT PROBLEM FORMULATION**

As discussed above, problem formulation is critical to develop a clear understanding of what information to include in subsequent steps of the risk assessment process. This section thus steps through problem formulation for potential ENM exposure and hazard in preparation for subsequent sections that summarize available data on ENM DNT and related data gaps that could inform future assessments.

Regarding potential exposure scenarios for ENM, many ENM-containing products are consumer goods as outlined earlier (Hansen et al., 2008; Hendren et al., 2011). In turn, the production, use, and disposal of these products could result in a number of human exposure scenarios through either direct contact with ENM or ENM products, or environmental contact with ENM released during any of these stages of the product life cycle. Some of these scenarios could lead to ENM exposure to the developing nervous system, of which two primary potential exposure scenarios emerge: (1) *in utero* exposures following maternal inhalation, ingestion, or dermal contact of ENM, and (2) postpartum exposures from the same portals of entry in the young child (Fig. 3). Although biomedical applications (e.g., pharmaceuticals) could also result in iv exposures, the potential impacts on the developing nervous system are expected to be discussed with a medical professional. In turn, the focus here is on incidental exposures to ENM. Although exposure at conception is not expected to have significant latent effects on CNS development, the possibility of ENM persisting in the mother and affecting neural tube development or related processes several weeks following conception...
cannot be completely ruled out. In utero and postpartum ENM exposure could result in distinct neurological effects, owing to differences in the stage of nervous system developmental and the biological barriers that ENM need to cross to reach the developing nervous system.

The nervous system, and particularly the brain, undergoes a variety of spatiotemporally regulated developmental processes from around 2 weeks of gestation, when the neural plate forms in humans, to adolescence (Makris and Raffaele, 2009; Rice and Barone, 2000). Given the diversity of developmental changes occurring during this time period, the developing nervous system is particularly sensitive to toxicant exposures, and the outcome(s) of exposure is heavily influenced by the timing of exposure (Rice and Barone, 2000). For instance, although apoptosis and synaptogenesis continue from mid-gestation to the postnatal period, myelination occurs mostly in the late gestation and postpartum periods (see Fig. 1 in Rice and Barone, 2000). Thus, the dynamic maturation of the brain means that postpartum exposures could affect neurodevelopment in ways distinct from in utero exposures because different neurodevelopmental processes are operant at different times during gestation and postpartum development.

Throughout brain development, there are three categories of exposure sources to consider for ENM. They are (1) occupational exposures in which individuals manufacturing ENM come into contact with the material, either before or after incorporation into a product, (2) exposure from using consumer products containing ENM, and (3) environmental exposure to ENM released during product manufacturing, use, or disposal. Levels of actual ENM exposures in each of these categories are generally poorly understood, but some estimates from recent modeling efforts are summarized in Table 1. In general, occupational ENM exposures are expected to be higher than environmental ENM exposures, but as the application of ENM continues to increase, so does the likelihood of aggregate and cumulative exposures to multiple types of ENM via multiple routes in consumer products or other applications.

During in utero development, the large surface area of the placenta combined with the increased surface area to volume ratio of nanoparticles compared with larger sized materials increases the likelihood of fetal ENM exposure (Saunders, 2009). Further, maternal differences in absorption, distribution, metabolism, and excretion during pregnancy may influence prenatal ENM exposure levels. For instance, higher pulmonary

![Diagram of Developmental Neurotoxicity Test Design](https://example.com/dnt_diagram.png)
function during pregnancy results in an approximately 72% increase in air exchange volume over an 8-h period (Selevan et al., 2000). Although this increased air exchange could result in greater exposure levels, ENM exposure could itself impact respiratory function (e.g., decrease respiration rate due to inflammatory response; Mühlfeld et al., 2008), which would need to be taken into account as well when evaluating potential exposure levels during pregnancy.

Consideration of postpartum ENM exposure may be particularly relevant due to several factors unique to infants and young children (Selevan et al., 2000), including (1) greater surface area to body mass ratio (i.e., −2.7-fold greater ratio in infants than adults), (2) higher ventilation rates (i.e., 4 times higher in infants [44 breaths/min] compared with adults [12 breaths/min]; Besson et al., 2010; Sherwood, 2013), and (3) increased drinking water intake (i.e., 4 times greater intake in infants compared with adults per body weight; U.S. EPA, 2011). These characteristics of young children combined with the small size and increased reactivity of ENM compared with larger size materials suggest a greater potential for DNT as a result of ENM exposure throughout neurodevelopment. Notably, lactational exposure primarily occurs during the early postpartum developmental period with some rare exceptions (e.g., mothers choosing to nurse children past 1 year of age) and highlights the potential for direct exposure to the developing brain via particle uptake by afferent sensory fibers in the mouth, followed by axonal transport to the brain. Although such uptake has not been demonstrated for ENM to date, transport up afferent olfactory fibers has been demonstrated in adult rodents for nanosized gold and other types of ENM (Simkó and Mattsson, 2010). For lipophilic ENM (e.g., fullerenes, carbon nanotubes), lactational exposure might be a particularly important route of exposure to consider based on evidence of elevated levels of lipophilic compounds such as polychlorinated biphenyls in breast milk (Schwartz et al., 1983).

ENM characteristics other than lipophilicity (e.g., production processes, size, surface, area, shape) will similarly influence potential exposures throughout in utero or postpartum development (see Yokel and Macphail, 2011 for discussion on how these characteristics might influence occupational exposures). The propensity of ENM to agglomerate or adhere to other materials means that individuals are likely exposed to ENM with different sizes, surface charge, or other properties than the materials as produced. In turn, these considerations need to be accounted for in efforts to understand the potential for exposure and effects in the developing nervous system. Importantly, the likelihood and level of any of the exposure scenarios discussed here remain to be determined. The references listed in Table 1 and others (Benn and Westerhoff, 2008; Dahm et al., 2011; Hendren et al., 2011; Wijnhoven et al., 2009) provide additional information.
Another consideration for problem formulation of ENM DNT is the vulnerability of the developing brain. As mentioned earlier, recent evaluations of ENM exposures in adult animal models show neurotoxic outcomes, which may have greater impact in the developing brain. Although the physiological relevance of many of these observations in adults is unknown due to the study design (i.e., relatively high-dose concentrations, lack of material characterization, methodological questions), the findings may have implications for the more vulnerable developing brain. For instance, exposure to 80 or 155 nm TiO₂ resulted in significant cell loss in the hippocampus of adult animals (Wang et al., 2008a). Given that cell death is programmed to occur in specific areas of the developing brain at certain times (for a review, see Costa et al., 2004b), alterations in apoptosis resulting from ENM exposure could interfere with neurological function later in life.

Similarly, neuronal cell migration is a highly orchestrated process that results in specific cells in particular places at selected times during brain development (Costa et al., 2004a). Data showing altered neuronal morphology in the adult brain after ENM exposure suggest that such migration events may be altered by ENM exposure during neurodevelopment (Ma et al., 2010; Wang et al., 2008a, b). Reports also show alterations in oxidative stress (Wang et al., 2008b) and indications of glial cell activation in the adult brain (Elder et al., 2006; Maysinger et al., 2007). Glial cells protect neurons from oxidative stress (Tanaka et al., 1999) and are a target of other known developmental neurotoxicants (e.g., nicotine, alcohol, methylmercury) (Abdel-Rahman et al., 2005; Guizzetti et al., 1997; Shanker et al., 2003). Importantly, the major period of glial cell proliferation does not begin until the third trimester of pregnancy and continues well into postnatal maturation (Costa et al., 2004b), leaving the developing brain vulnerable to elevations in oxidative stress that might result from ENM exposure. A number of factors, including higher availability of iron to catalyze free radical formation and lower levels of antioxidant, in the developing brain amplify vulnerability to oxidative stress from ENM (Blomgren and Hagberg, 2006). Data further indicate altered neurotransmitter levels in the adult brain after ENM exposure (Ma et al., 2010). Because neurotransmitters are critical to regulating cell proliferation, migration, and differentiation in the developing brain (Costa et al., 2004b), such effects in the developing brain could have long-term consequences on neurobehavior and neuronal function. Finally, observations in adult animal models show inflammation in the brain after ENM exposure (Elder et al., 2006; Maysinger et al., 2007; Tang et al., 2009a). Recent evidence suggests that systemic inflammation negatively affects neurodevelopment and may have long-term consequences on behavior and psychiatric health (Hagberg and Mallard, 2005), again suggesting that ENM exposure in the developing brain could lead to persistent changes later in life. As discussed in the Hazard Identification section below, initial data suggest that several observations noted in the brain of adult animal models also occur in the developing brain after in utero exposure or lactational exposure. Presumably, these exposures resulted in lower concentrations in the developing brain than the adult brain because the offspring were not directly dosed. The similarity in results thus supports the potential for greater vulnerability to ENM in the developing brain.

### AVAILABLE EVIDENCE FOR CONDUCTING ENM DNT EXPOSURE ASSESSMENT

As depicted in Figure 3, in utero exposures lead to the potential for indirect exposure to the developing nervous system as the material has to cross several barriers (e.g., maternal skin, digestive tract lining, and placenta). In contrast, ENM exposure during the postpartum period would need to cross fewer barriers (i.e., no placental barrier) to reach the developing nervous system; however, these barriers are more developed and thus likely less penetrable by ENM (Rice and Barone, 2000). Of these barriers, two are most relevant when discussing the potential for ENM to enter the developing fetal brain: the placental barrier and the BBB.

Early evidence in animal models shows that ENM with certain sizes and coatings can cross the placental barrier (Yamashita et al., 2011). Although differences between rodent...
and human placenta structures necessitate caution in interpreting such findings, a recent human ex vivo study demonstrated that ENM ≤ 240 nm can cross this barrier (Saunders, 2009; Wick et al., 2010). Animal models also indicate that metal and metal oxide ENM can cross the developing BBB (Takeda et al., 2009; Yamashita et al., 2011). This is in contrast to studies of the developed BBB in adult animals, which shows that similar types of ENM cross at low concentrations or not at all (Cho et al., 2009; De Jong et al., 2008; Hardas et al., 2010). Moreover, recent data show that ENM can disrupt the BBB integrity through elevating inflammatory responses or reducing expression of tight junction proteins (e.g., occludin-, claudin-5) in the BBB endothelium (Chen et al., 2008; Sharma and Sharma, 2007; Sharma et al., 2009; Tang et al., 2010; Trickler et al., 2010, 2011). Efforts to design therapeutics for the brain show that specific surface coatings and sizes can enhance ENM delivery to the brain (Kreuter, 2004). Importantly, the mechanism(s) by which ENM cross biological barriers such as the placenta and BBB is poorly understood and may involve passive diffusion, active transport, or more likely, a combination of the two depending on the ENM physical and chemical characteristics (Chithrani et al., 2006; Lockman et al., 2002).

Whether ENM cross these barriers will likely depend on the route of exposure (e.g., inhalation, oral, dermal). For example, inhalation exposure in animal models has demonstrated that ENM can bypass the BBB via anterograde axonal transport to the olfactory bulb (Oberdörster et al., 2009). Whether dermal exposure leads to systemic circulation of ENM is contested (Gulson et al., 2010; Popov et al., 2010), and no studies have specifically evaluated translocation to the developing brain after this type of exposure. Importantly, the extent to which ENM reaches the developing brain is expected to also be heavily dependent on the specific ENM physical and chemical characteristics because properties such as size, surface charge, and surface coating all influence biodistribution (Maynard et al., 2011).

AVAILABLE EVIDENCE FOR CONDUCTING ENM DNT HAZARD IDENTIFICATION

As discussed further in the subsections that follow, in vivo and in vitro studies have examined the DNT potential of ENM exposure. Perinatal exposure in animal models has resulted in electrophysiological, histopathological, and behavioral effects in offspring. In vitro studies have provided mechanistic data for the DNT effects observed in the in vivo studies. Together, these studies suggest that gestational exposure to materials not characterized as DNT agents may in fact lead to DNT effects when they are synthesized at the nanoscale; however, as discussed in greater detail below, there are a number of limitations to the currently available data.

In Vivo Evaluations of Hazard

A limited number of in vivo studies evaluating potential hazardous effects of ENM in the developing nervous system were identified (See Fig. 4 and Table 2), and none of these studies utilized a standard guideline DNT testing approach. In addition, several identified studies used sc exposures to determine potential ENM effects, which is not expected to be a relevant exposure route for ENM products; however, due to the limited number of studies available, these studies are included here to help guide future research.

DNT Observations Following Oral ENM Exposure

Only one study examined DNT effects of oral exposure to ENM. Pregnant or lactating rats were treated orally with a nano-TiO2 suspension in distilled water (final dosage 100 mg/kg body weight) from gestational day (GD) 21 to day 2 or postnatal day (PND) 2–21, respectively. Results indicated that the exposure during pregnancy or lactation impaired offspring short-term synaptic plasticity in the hippocampal dentate gyrus (DG) area (Gao et al., 2011). In addition, after lactational exposure, both long-term synaptic plasticity of the hippocampal DG area and basic synaptic transmission were weakened, effects which were not seen in offspring exposed during pregnancy. These results occurred in the presence of significant increases in nano-TiO2 in the hippocampus and indicate that developmental nano-TiO2 exposure could impair synaptic plasticity in rats’ hippocampal DG area, especially during lactational exposure.

DNT Observations Following sc ENM Exposure

Three studies examined the effects of sc exposure to nano-TiO2 (100 µl of 1 mg/ml TiO2) in pregnant mice during gestation (Shimizu et al., 2009; Takahashi et al., 2010; Takeda et al., 2009). In a gene expression study, Shimizu et al. (2009) exposed dams on GD 6, GD 9, GD 12, and GD 15 and then collected brain tissue from male offspring on GD 16 and on PND 2, PND 7, PND 14, and PND 21. Based on microarray data, the authors reported significant changes in gene expression at each time point examined, with the largest number of significant changes observed at PND 21. Authors categorized the gene expression changes using gene ontology (GO) and medical subject headings (MeSH) related to neurodevelopment and determined statistical significance using an enrichment factor (ratio of differently expressed genes in a category to ratio of differentially expressed genes in the microarray) for each GO or MeSH category. In general, data showed altered gene expression associated with apoptosis, brain development, oxidative stress, inflammation, and neurotransmitters (epinephrine, norepinephrine, serotonin, and glutamic acid) in what could be an age-dependent manner. Results suggest that maternal nano-TiO2 exposure impacted prenatal gene expression and that alterations may persist after cessation of maternal exposure although data are difficult to interpret without accompanying functional measurements or accounting for basal expression differences in different gene categories.

A similar exposure paradigm elevated apoptotic cells in the olfactory bulb of 6-week-old male offspring and increased...
levels of TiO$_2$ in the olfactory bulb and cerebral cortex compared with controls (Takeda et al., 2009). Microscopy combined with spectroscopy data demonstrates translocation of nanomaterials to the developing nervous system following maternal exposure although the authors did not indicate whether TiO$_2$ nanomaterials were observed at an earlier time point on PND 4 and did not examine other areas of the brain for apoptotic markers. The observation of nanomaterials and increased apoptosis in the olfactory bulb at 6 weeks of age suggests that both nanomaterials and the neurotoxic effects of nanomaterials may persist in the developing brain even after cessation of maternal exposure; however, the authors did not provide a clear rationale regarding the mechanism through which nano-TiO$_2$ might have resulted in the observed effects after sc exposure.

Takahashi et al. (2010) exposed pregnant mice to 100 µl of 1 mg/ml nano-TiO$_2$ sc at similar time points in gestation. Of the 10 offspring brain regions examined, statistically significant changes in dopamine and dopamine metabolites were only observed in the prefrontal cortex and the neostriatum. In the prefrontal cortex, maternal nano-TiO$_2$ exposure significantly decreased the monoamine levels. Conversely, monoamine levels in the neostriatum were significantly increased following maternal exposure. Interestingly, the authors did not observe any monoamine level changes in the olfactory bulb, despite previous reports from this lab, which identified the olfactory bulb as a target of prenatal nanomaterial exposure (Takeda et al., 2009). This study demonstrated that maternal nano-TiO$_2$ exposure impacted monoamine levels after cessation of maternal dosing. However, the lack of an exposure assessment or characterization of the functional impact of monoamine level changes limits the ability of this study to inform the potential neurodevelopmental impact of gestational exposure to nanomaterials.

**DNT Observations Following Inhalation ENM Exposure**

One study evaluated DNT after inhalation exposure to nano-TiO$_2$ in pregnant mice (Hougaard et al., 2010). After maternal exposure for 1 h/day to 42 mg/m$^3$ aerosolized powder from GD 8–18, a number of endpoints were examined including maternal lung inflammation, gestational and litter parameters, offspring neurofunction, and fertility. In addition, particle physicochemical properties and exposure were characterized. Gestationally exposed offspring displayed moderate neurobehavioral alterations as young adults, including altered open field behavior (males and females) and enhanced prepulse inhibition (females). No effect was observed on cognitive function.
### TABLE 2
Summary of Hazard Identification Studies With In Vivo ENM Exposures

<table>
<thead>
<tr>
<th>ENM</th>
<th>Size (nm)</th>
<th>[ENM]</th>
<th>Exposure</th>
<th>Main findings</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO₂ anatase</td>
<td>&lt; 25</td>
<td>100 mg/kg</td>
<td>SD rats, Oral, GD 21–PND 2; PND 2–21 C57BL/6JomTac mice, Inhalation, GD 8–18, 1 h/day</td>
<td>10 offspring/group: ENM in hippocampus ↓ hippocampal DG I/O (PND) ↓ paired-pulse reaction (PND)</td>
<td>(Gao et al., 2011)</td>
</tr>
<tr>
<td>TiO₂ rutile</td>
<td>10–100</td>
<td>40 mg/m³</td>
<td>Inhalation, GD 8–18, 1 h/day</td>
<td>10–12 offspring/group: ↓ visits (M), time (F) ↑ prepulse inhibition (F) Maternal lung inflammation</td>
<td>(Hougaard et al., 2010)</td>
</tr>
<tr>
<td>TiO₂ anatase</td>
<td>27, 2,429</td>
<td>100 μl of 1 mg/ml in saline + 0.05% Tween80</td>
<td>SD rats, sc, GD 6, 9, 12, 15, and 18</td>
<td>8 males/group: 6 weeks: ↑ DA and metabolite (DOPAC, HVA, 3-MT) levels: Neostriatum and prefrontal cortex</td>
<td>(Takahashi et al., 2010)</td>
</tr>
<tr>
<td>TiO₂ anatase</td>
<td>25–70</td>
<td>100 μl of 1 mg/ml in saline + 0.05% Tween80</td>
<td>ICR mice, sc, GD 3, 7, 10, and 14</td>
<td>8 males/group: 4 and 6 weeks: 22% ↓ bw @ 6 weeks: ENM in olfactory bulb and cerebral cortex ↑ apoptotic markers, olfactory bulb</td>
<td>(Takeda et al., 2009)</td>
</tr>
<tr>
<td>TiO₂ anatase</td>
<td>2570</td>
<td>100 μl of 1 μg/μl in saline + 0.05% Tween80</td>
<td>ICR mice, sc, GD 6, 9, 12, and 15</td>
<td>8–10 males/group: Gene expression change, e.g.— Cell death (PND 2, 7, 14, and 21) Brain development (PND 2, 7, and 14) Oxidative stress (GD 16, PND 2, 7, 14, and 21) Neurotransmitter activity (PND 14 and 21) Motor activity (PND 2, 7, and 14)</td>
<td>(Shimizu et al., 2009)</td>
</tr>
<tr>
<td>Carbon black</td>
<td>140</td>
<td>11, 54, and 268 μg/animal</td>
<td>Mice, Intratracheal, GD 7, 10, 15, and 18</td>
<td>Females (high dose only) habitation change (Activity ↑, ↓) Maternal inflammation</td>
<td>(Jackson et al., 2011)</td>
</tr>
<tr>
<td>TiO₂ rutile</td>
<td>35</td>
<td>100 μl of 0.8 mg</td>
<td>BALB/c mice, iv, GD 16 and 17</td>
<td>TiO₂ in placenta, fetal liver and brain ↓ maternal weight</td>
<td>(Yamashita et al., 2011)</td>
</tr>
<tr>
<td>C₆₀</td>
<td>143</td>
<td>100 μl of 0.8 mg</td>
<td>BALB/c mice, iv, GD 16 and 17</td>
<td>Maternal weight ↓ No effect</td>
<td>(Yamashita et al., 2011)</td>
</tr>
</tbody>
</table>

Note. 3-MT, 3-methoxyxramine hydrochloride; bw, body weight; DOPAC, 3,4-dihydroxyphenylacetic acid; F, female; HVA, homovanillic acid; ICR, imprinting control regions; I/O, input/output; M, male; SD rats, Sprague Dawley rats.

*Major mode as determined by hydrodynamic number size distribution was noted by the study authors as 50–60 nm.*
(in a Morris water maze test). Results also showed persistent maternal lung inflammation (26–27 days postexposure) after exposure termination, which could increase offspring vulnerability to neurodevelopmental effects (e.g., impaired learning and memory, similar to observations in offspring after maternal lipopolysaccharide exposure; Golan et al., 2005). The authors of the study concluded that these results suggest that inhalation of nano-TiO₂ can induce long-term (i.e., greater than 20 days postexposure) lung inflammation in pregnant mice and resulting neurobehavioral alterations in gestationally exposed offspring. The authors further noted that the effects in offspring likely derived from a combination of indirect and direct pathways and were related to the observed maternal inflammation.

Another study examined effects of pulmonary exposure in pregnant mice on offspring neurodevelopment. Jackson et al. (2011) intratracheally instilled animals with Carbon Black (Printex 90) dispersed in Millipore water on GD 7, GD 10, GD 15, and GD 18, with total doses of 11, 54, and 268 µg Printex 90 per animal. Similar to findings described above from the same lab with nano-TiO₂ (Hougaard et al., 2010), maternal lung inflammation was observed after prenatal exposure to Printex 90. In offspring, no treatment effects were observed for acoustic startle or prepulse inhibition. However, the authors did observe an altered habituation pattern in open field testing for female offspring exposed to 268 µg of Printex 90. Similarly, the authors of the study suggested that the change in neurobehavior of exposed offspring was likely an indirect effect resulting from maternal pulmonary inflammation. The mechanism(s) by which such indirect effect occurs is unknown.

**In Vitro Evaluations of Mechanisms Relevant to Neurotoxicity and DNT**

A larger body of evidence exists using in vitro models to evaluate potential ENM effects, and some of those that might inform DNT evaluations are discussed here (Table 3). Questions about the relationship between in vitro and in vivo dosimetry and alterations in ENM behavior in the in vivo environment may limit the utility of in vitro studies in risk assessment efforts; however, a few findings are briefly reviewed here to highlight the mechanistic findings that may support the observed in vivo DNT effects, namely, cell death, oxidative stress, alternations in neurotransmitters, and cell type–specific effects.

**Mechanisms**

In vitro studies have shown elevations in oxidative stress and decreases in cell viability after ENM exposure, supporting the observed increase in apoptotic cells in vivo by Takeda et al. (2009). Powers et al. (2011) found that silver nanomaterials (different sizes and coatings) elicited oxidative stress and decreased cell viability in undifferentiated and neuronal PC12 cells, but at a lower potency than Ag ions. Nano-Ag and Ag ions were prepared in stock solutions equivalent to a nominal concentration of 1mM Ag and diluted to achieve nominal concentrations of Ag between 0–100µM (See Powers et al., 2011 for additional study details). Treatment with ascorbate, an agent that blocks ionic Ag-mediated oxidative stress, was ineffective in blocking oxidative stress from 10µM nano-Ag. These results suggest that observed effects may be due to a combination of the ENM form of the material and the ENM core composition (silver) because negative controls (silica ENM and particle-coating material alone) had no effect. A more recent adult neurotoxicity study (Zhang et al., 2013) extended the findings from Powers et al. (2011) by showing that neurotoxicity is associated with Ag ENM exposure in adult male rats.

Similarly, Phenrat et al. (2009) evaluated oxidative stress and cell viability with several types of iron ENM (1–20 µg/ml) in different cell lines. In immortalized mouse microglia (BV2 cells), fresh and aged (> 11 months) nanoscale zerovalent iron (nZVI) elevated intracellular H₂O₂ (a possible indicator of oxidative stress and/or microglial activation), but magnetite (end oxidative product of nZVI) and polyaspartate surface modified nZVI (SM-nZVI) elicited no effect at the concentrations tested. In immortalized rat dopaminergic neuron (N27) cells, levels of intracellular adenosine triphosphate (ATP) were reduced by all nZVI species (fresh [≥ 5 µg/ml, 1 h], SM-nZVI [20 µg/ml, 1 h], aged and magnetite [20 µg/ml, 6 h]). In general, findings show that nZVIs may exhibit differential toxicity to neural cell subtypes (BV2 vs. N27), with the fresh nZVI representing the most toxic species tested. Differences in particle chemistry and aging of the particle were characteristics that influenced toxicity (e.g., fresh nZVI was more toxic than aged nZVI). Fresh nZVI may have been more toxic because of the higher content of nanosized iron particles and thus a potential higher “redox” activity than aged nZVI. This would align with previous findings that nanosized particles exhibit higher reduction potential compared with their larger, non-nanoscale size counterparts (Buza et al., 2007; Stone et al., 2007). Even though SM-nZVI had more iron than aged nZVI, the surface coating might have prevented reactive oxygen species generation in BV2 cells. These results suggest that there is neurotoxicity associated with exposure to nZVI particles with a potential for developmental neurotoxicity. These in vitro evaluations of ENM suggest that ENM characteristics such as surface coating, charge, and particle size influence the effects of ENM exposure. This highlights the need for future in vitro studies to directly compare different types of ENM and subsequently translate these findings to in vivo animal models.

Additionally, both Wang et al. (2009) and Powers et al. (2011) reported dopaminergic alterations in undifferentiated and differentiated PC12 cells, respectively, after ENM exposure, supporting the in vivo changes in this neurotransmitter discussed above (Takahashi et al., 2010). Wang et al. (2009) showed that 10 µg/ml of copper (Cu-90), silver (Ag-15), or manganese (Mn-40) nanoparticles significantly decreased the content of all DA metabolites measured (DA, 3,4-dihydroxyphenylacetic acid, and homovanillic acid). They subsequently evaluated gene expression changes at this concentration and...
### TABLE 3
Summary of Hazard Identification Studies With *In Vitro* ENM Exposures

<table>
<thead>
<tr>
<th>ENM</th>
<th>Size (nm)</th>
<th>[ENM]</th>
<th>Exposure</th>
<th>Main findings</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag citrate coated</td>
<td>10</td>
<td>1, 3, 10, 30, and</td>
<td>Pheochromocytoma (PC12) cells,</td>
<td>Undifferentiated cells: Impaired DNA and protein synthesis</td>
<td>(Powers et al., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000µM</td>
<td>undifferentiated and differentiated</td>
<td>Differentiated cells: Oxidative stress, impaired differentiation into the Ach phenotype</td>
<td></td>
</tr>
<tr>
<td>Ag polyvinylpyrrolidone coated</td>
<td>10 and 50</td>
<td>10, 30, and 50µM</td>
<td>PC12 cells, undifferentiated and</td>
<td>Undifferentiated cells: Impaired DNA synthesis</td>
<td>(Powers et al., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>differentiated</td>
<td>Differentiated cells: Oxidative stress, impaired differentiation into the Ach phenotype, augmented differentiation into the DA phenotype (10nm only)</td>
<td></td>
</tr>
<tr>
<td>Zero-valent Fe bare, oxidized, two-surface modified and magnetite</td>
<td>&lt; 1000 to &gt; 2000</td>
<td>1–20 µg/ml</td>
<td>Cultured rodent microglia (BV2) and neurons (N27)</td>
<td>BV2 cells: Oxidative stress, morphological evidence of mitochondrial swelling and apoptosis</td>
<td>(Phenrat et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N27 cells: Reduced ATP levels, morphological evidence of perinuclear floccular material, cytoplasmic granularity, and particle deposition.</td>
<td></td>
</tr>
<tr>
<td>Mn, Ag, and Cu</td>
<td>Mn-40, Ag-15, Cu-90</td>
<td>10 µg /ml</td>
<td>PC12 cells</td>
<td>Dopamine depletion (Cu and Mn), altered gene expression associated with the dopaminergic system</td>
<td>(Wang et al., 2009)</td>
</tr>
<tr>
<td>Polystyrene unmodified, carboxylated, and amino-modified polystyrene particles.</td>
<td>60</td>
<td>6, 25, or 100 µg/ml depending on cell type and assay</td>
<td>Macrophage (RAW 264.7) and epithelial (BEAS-2B) cells, human microvascular endothelial (HMEC), hepatoma (HEPA-1), and PC12 cells</td>
<td>ENM uptake, intracellular localization, and toxicity</td>
<td>(Xia et al., 2008)</td>
</tr>
</tbody>
</table>

*Note.* Ag, silver; Ach, Acetylcholine; Cu, copper; Mn, Manganese; ppm, parts per million.
found that nano-Mn and nano-Cu may contribute to pathological processes related to altered DA metabolism, impaired proteasomal activity, and, in the case of nano-Cu, disrupted redox balance, which leads to dopaminergic neurotoxicity. Powers et al. (2011) observed that 10 nm polyvinylpyrrolidone (PVP)–coated nano-Ag augmented differentiation of cells into the dopaminergic phenotype. In contrast, all three types of Ag ENM tested (10 and 50 nm PVP-Ag ENM and 10 nm citrate-Ag ENM) impaired differentiation into the acetylcholine phenotype. Together, these studies suggest that ENM of different compositions can perturb dopamine production and/or metabolism and alter neurotransmitter phenotypes in this cell line.

As suggested by the findings of Phenrat et al. (2009), differences in cell type may further influence ENM mechanisms in the developing nervous system, in addition to the influence of ENM characteristics. Xia et al. (2008) demonstrated that ENM uptake, intracellular localization, and toxicity depended on cell type in a study comparing effects of cationic polystyrene (PS) ENM (6, 25, or 100 μg/ml depending on cell type and assay) in cell lines representing five different cell types. In this study, PS ENM did not affect PC12 cell viability, unlike nano-Ag (Powers et al., 2011), but PS ENM did significantly increase cytotoxicity in both macrophage and epithelial cell lines (Xia et al., 2008). Effects in the later two cell lines were tied to mitochondrial damage and ATP depletion, similar to results by Phenrat et al. (2009) that showed decreased ATP levels in immortalized rat dopaminergic cells after exposure to Fe ENM. Together, data point to nuanced effects in different cell types that will be informed by pairing mechanistic, in vitro studies with in vivo studies evaluating effects in the multicellular matrix of the developing brain.

In vitro studies of ENM toxicity in other culture systems could provide additional mechanistic information that may have particular relevance to in vivo human exposures. For example, induced pluripotent stem cells (iPSCs) could be used as a potential platform to study the effects of ENM on either undifferentiated or differentiated neural cells, and because iPSCs can be derived from human tissues, these cultures may provide relevant information on the potential for early or late DNT effects in humans (reviewed in Kumar et al., 2012). Similarly, zebrafish cultures (reviewed in de Esch et al., 2012) could be used as a platform to evaluate neural cell responses to ENM within intact organisms in a more “high throughput” fashion.

DOSE-RESPONSE INFORMATION

Although the early evidence discussed above suggests that the developing brain may be exposed to ENM and that ENM exposures can cause DNT effects, there is little information on the dose-response relationship between ENM and neurodevelopmental effects. Of the identified in vivo studies, only Jackson et al. (2011) used multiple doses to evaluate effects of maternal exposure to ENM; however, no dose-response relationship was observed because offspring neurobehavioral function was only tested at the highest concentration (268 μg Printex 90/animal). Notably, this concentration also caused maternal inflammation, which confounds findings related to neurodevelopmental effects because maternal inflammation has also been shown to influence neurodevelopment (Golan et al., 2005 and reviewed in Hagberg and Mallard, 2005). As discussed above, differences in ENM coating, size, and core composition can result in differences in effects; thus, these parameters need to be accounted for in future dose-response studies. As discussed further below, additional dose-response information will help to fill important gaps in current understanding of the potential for DNT from ENM exposures.

DISCUSSION

As the use of ENM in a variety of applications, including cosmetics, clothing, and children’s toys, continues to rise, the likelihood of human exposures may increase as well. In turn, an understanding of potential human health risks would inform decisions about possible trade-offs between the economic and societal value of using ENM applications and impacts on human health. Evidence suggests that ENM evoke biological changes in the brain of adult animal models (Hu and Gao, 2010; Oberdörster et al., 2009; Sharma and Sharma, 2010; Simkó and Mattsson, 2010; Yang et al., 2010). As discussed above, the greater vulnerability of the developing brain to toxic insult combined with unique characteristics of ENM suggests that DNT may be a potential outcome of ENM exposure. We discussed the potential for ENM exposure in the developing brain, highlighted recent findings on ENM DNT endpoints, and discussed the need for dose-response information.

Although the identified hazard identification studies provide initial evidence of effects on the developing brain after early life exposures, there are several limitations to their ability to inform future evaluations of ENM DNT. In general, authors did not provide detailed ENM characterization data from their own analyses but rather relied on manufacturer supplied specifications. Such independent evaluations are critical for understanding the relationship between specific types of ENM and observed effects (Oberdörster et al., 2005). In some studies, maternal effects were observed that may obscure any direct effects of ENM on neurodevelopment. Without exposure assessments, it is challenging to determine whether neurobehavioral or other endpoints reported are due to direct ENM effects or result from indirect effects (e.g., maternal inflammation). We would recommend minimally invasive techniques such as measurement of maternal and infant blood or urine samples in humans. With regards to animal studies, it would be ideal to measure maternal and fetal blood and brain ENM levels. Additionally, most studies excluded exposure during lactation, and this early postnatal period includes a number of developmental milestones for the brain (Rice and Barone, 2000). The lack of functional characterization of the developing nervous system in most studies limits the ability to tie changes in gene expression or cell viability to DNT endpoints.
Almost all of the studies exclusively evaluated males on the premise that a number of neurobehavioral disorders are seen to a greater extent in males; however, evaluating both males and females, as recommended by current guidelines (OECD, 2007; U.S. EPA, 1998), would be informative for understanding any sex differences in susceptibility. Further, the majority of studies investigated effects of nano-TiO₂, which is only one of many types of available ENM that would be informative to evaluate. Finally, most available studies did not include comparisons to materials that do not have nanoscale dimensions, which would provide insight on whether effects are due to specific characteristics of the nanosized material or to the material itself (Powers et al., 2011). Table 4 summarizes the research needs for ENM with respect to developmental neurotoxicity. In the following section we provide a strategy for addressing these data gaps for ENM exposure and developmental neurotoxicity.

**Strategy for Planning ENM Research Protocols for DNT Testing**

**Exposure Research Needs**

Although measurements of actual child exposure via multiple routes are necessary to inform future risk assessment efforts, the time and resources required to gather such data, coupled with the rapid rise in ENM use, demand another, parallel approach to study potential ENM DNT. As indicated above, the EPA and OECD DNT Study Guidelines present a robust battery of tests to query potential effects during the most rapid period of nervous system development (Makris et al., 2009; OECD, 2007; U.S. EPA, 1998). Such an approach lends itself to identifying the most sensitive window(s) during which to evaluate dose-response relationships and exposure, which are critical aspects of risk assessment (Selevan et al., 2000). In these guidelines, exposure extends from GD 6 to PND 11 or PND 21 to ensure that offspring are exposed to maternal circulation in utero and maternal milk in the postnatal period (Makris and Raffaele, 2009). Although the temporal rate of development of individual nervous system structures generally differs substantially between humans and rodents, the overall patterns of progression are largely the same, and the tests in this screening battery have been previously evaluated for interspecies reliability (Makris and Raffaele, 2009; Makris et al., 2009; Rice and Barone, 2000). In turn, such a well-established, screening-level approach should provide important evidence for moving forward with future risk assessment efforts focused on ENM and neurodevelopment.

For instance, data from DNT screening efforts that include toxicokinetic data could inform the development of physiologically based pharmacokinetic models that account for changes in adsorption, metabolism, and excretion that occur during pregnancy and early development (Selevan et al., 2000). Pairing such models with measurements of exposure in occupational, residential, and other settings relevant to early life could then support an understanding of dosimetry in the developing nervous system. Importantly, future measurements and modeling efforts should include the variety of parameters that are likely to influence exposure levels, such as product formulation (e.g., liquid vs. cream), use environment (e.g., indoors vs. outdoors), and use duration (e.g., one application vs. multiple applications per day), in addition to ENM characteristics (e.g., particle size, surface area, and charge) (Hansen et al., 2008). Further, based on the potential for ENM exposure to continue well beyond the early postnatal period, the longer exposure period included in the OECD guidelines (i.e., to PND 21) or

<table>
<thead>
<tr>
<th>Research area</th>
<th>Research gap</th>
</tr>
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<tbody>
<tr>
<td><strong>Exposure</strong></td>
<td>1. Identification of sensitive exposure windows in offspring using DNT guideline studies with extended exposure periods for offspring (i.e., PND 21 or beyond). Comparisons of sensitive exposure windows between different types of ENM could support the prioritization of in vitro and/or in vivo follow-up studies.</td>
</tr>
<tr>
<td></td>
<td>2. Development of PBPK models using toxicokinetic measurements of ENM in offspring after maternal dosing or direct dosing of ENM in offspring. Models would be developed with consideration of appropriate material characteristics (e.g., size and charge) and exposure parameters (e.g., route, frequency, and direct vs. indirect contact).</td>
</tr>
<tr>
<td></td>
<td>3. Measurements of exposure in occupational, residential, and other settings relevant to early life. The utility of measurements increases with the inclusion of parameters that are likely to influence exposure levels, such as product formulation (e.g., liquid vs. cream), use environment (e.g., indoors vs. outdoors), and use duration (e.g., one application vs. multiple applications per day), in addition to ENM characteristics (e.g., particle size, surface area, and charge). The development of tools and approaches to accurately and quickly evaluate ENM exposure in the environment parallels this research gap.</td>
</tr>
<tr>
<td><strong>Hazard</strong></td>
<td>1. Evaluation of neuropathology and behavior (see text and DNT guideline for discussion of endpoints) at multiple time points in developing offspring (i.e., PND 4, 11, 21, 35, 45, and 60) following maternal ENM exposures.</td>
</tr>
<tr>
<td></td>
<td>2. Evaluations of glia cell number and function in offspring during the postnatal period following maternal ENM exposure during prenatal and lactational periods.</td>
</tr>
<tr>
<td></td>
<td>3. Evaluation of visual function in offspring following maternal ENM exposure or direct dosing of offspring during the early postnatal period.</td>
</tr>
<tr>
<td><strong>Dose response</strong></td>
<td>1. Evaluation of dose response using dose levels suggested in DNT guidelines with well characterized ENM (e.g., data on size, surface charge, and shape).</td>
</tr>
<tr>
<td></td>
<td>2. Development of analytical methods capable of characterizing multiple ENM characteristics (e.g., size, surface charge, and composition).</td>
</tr>
</tbody>
</table>
perhaps even longer (to the age of puberty or adulthood) may be useful. Additionally, although direct dosing of preweanling offspring is not stipulated in either EPA or OECD guidelines, given observations of maternal inflammation by Hougaard et al. (2010) and Jackson et al. (2011), it may be informative in ENM studies to distinguish between direct and indirect ENM effects. Alternatively, toxicokinetic measurements of ENM in offspring could be used in lieu of direct dosing to establish whether the material reaches offspring and distinguish direct from indirect effects. Both direct dosing of preweanling offspring and maternal dosing studies would be informative for developing a better understanding of whether, and the extent to which, different types of ENM cross the blood-brain and placental barriers.

Notably, as the development of ENM and ENM applications continues to place new products on the market, efforts to develop the tools and techniques to evaluate human exposures to ENM in the environment will likely continue to be a critical research area.

**Hazard Identification Research Needs**

The observations included in the DNT screening guidelines provide a starting point for building upon current knowledge about neurological endpoints sensitive to ENM exposure. For instance, existing work evaluated animals for gross neurological and behavioral changes at a limited number of time points after developmental ENM exposure (Gao et al., 2011; Hougaard et al., 2010; Takeda et al., 2009); thus, follow-up studies that include multiple time points throughout development (i.e., PND 4, 11, 21, 35, 45, and 60) could be informative (U.S. EPA, 1998). Only one study noted changes in body weight after ENM exposure (Takeda et al., 2009), but recording such developmental landmarks will be informative in future work (U.S. EPA, 1998). Similarly, only one of the identified studies evaluated motor activity changes, auditory startle responses, and cognitive (learning and memory) effects (Hougaard et al., 2010). As such, follow-up work could provide additional measurements of motor activity at multiple time points for time periods of at least 10 min, coupled with measurements of auditory startle, prepulse inhibition, and learning and memory at the time of weaning and around PND 60 (U.S. EPA, 1998). Notably, observations of negative effects on synaptic transmission and plasticity in the hippocampus after exposure to TiO$_2$ ENM by Gao et al. (2011) are similar to those noted by Tang et al. (2009b) in adult animals after ENM (cadmium selenium [Cd/Se] and CdSe/ZnS) were applied directly to the hippocampus, suggesting that this endpoint may be informative to include in future studies. Finally, neuropathological evaluations in several areas of the brain on PND 11 or PND 21 and at the end of study would supplement data from the study by Takeda et al. (2009), which indicated elevations in apoptotic markers in the olfactory bulb and cortex of 6-week-old offspring. Specific observations to include in investigations of neuropathology can be found in the DNT guidelines (OECD, 2007; U.S. EPA, 1998). In addition to the endpoints outlined in available guidelines, evaluations related to glial cell function may be informative for ENM because proliferation of this cell type continues well into the postnatal period (Rice and Barone, 2000), and current data indicate that ENM are taken up by microglia and astrocytes (Hutter et al., 2010; Luther et al., 2011; Tang et al., 2010). Evidence further suggests that ENM can alter glial cell differentiation (Shimizu et al., 2009) and activation (Sharma et al., 2009). Finally, recent in vitro data show phototoxicity of certain ENM to ocular tissues (Roberts et al., 2008; Sanders et al., 2012; Wielgus et al., 2010). Photoactive compounds, such as nano-TiO$_2$, are excited by ultraviolet A radiation, which could potentially reach the retina of infants or young children to a greater extent than in adults because the lens is still developing (Boyes et al., 2012). In turn, evaluating effects on visual function might be particularly relevant for future hazard identification efforts for ENM DNT testing.

**Dose-Response Research Needs**

As noted above, only one of the identified in vivo studies included multiple dose levels. Future work should build upon current findings by using at least three dose levels in addition to control groups. Specifics on selecting dose levels can be found in the DNT guidelines, but generally, the highest dose should elicit observable maternal effects, whereas the lowest dose should not result in any observable neurological changes in dams or offspring (OECD, 2007; U.S. EPA, 1998). Intermediate doses should be equally spaced between the highest and lowest selected doses. Notably, quantitative dose-response data for ENM will need to take into account not only the concentration of the material but also specific ENM characteristics (e.g., surface charge, shape, size, and composition) that may cause different effects. Because a single ENM batch generally contains a mixture of individual particles with different characteristics (e.g., TiO$_2$ ENM containing 10, 50, and 100 nm particles), it is necessary to explicitly evaluate these parameters. Measuring and characterizing ENM in biological media are challenging and require a multitude of techniques and instruments (Johnston et al., 2010; Lehman et al., 2011). Thus, the development of analytical methods capable of characterizing multiple ENM characteristics such as those noted above would support the development of detailed dose-response studies, which would inform future assessment efforts.

In addition to applying the DNT guidelines in evaluating DNT effects associated with ENM, information on dose response could also be used from adult neurotoxicological evaluations. For example, Zhang et al. (2013) treated adult male rats with 5, 10, and 45 mg/kg-day of Ag ENM for 3 days. Significant decreases in rearing behavior were only observed at the highest dose (45 mg/kg-day). Therefore, these dose levels could also be used as a guide for setting dosing regimens in a DNT study for Ag ENM.

**Key points for ENM DNT evaluations.** It is unlikely that the research outlined above using DNT screening guidelines can be carried out for every available ENM type. However,
initial work to compare ENM types using established guidelines could guide the design of higher throughput studies to show the relationship(s) between in vivo findings and in vitro or alternative model studies. Although high-throughput screening approaches are beginning to be applied to predict ENM toxicity in general (i.e., for endpoints other than DNT) (Nel et al., 2012), the complexity of the developing nervous system combined with current limitations in understanding related to ENM-specific issues in vitro (e.g., dosimetry [Teegarden et al., 2006], assay compatibility [Monteiro-Riviere et al., 2009], standards and methods for material characterization [Bouwmeester et al., 2011]) suggests that high-throughput methods for ENM DNT will not likely be available in the near term. Given the rapid rate of new ENM applications, producing data to inform decisions in the near term on potential ENM DNT may be best achieved through the use of established methods, while efforts to refine high-throughput approaches to inform risk assessments continue. The use of traditional DNT guidelines to plan and conduct research in the interim could not only support more near-term decision making but also help inform the development of a tiered higher throughput screening method(s) for longer term decisions about ENM DNT. This approach has been discussed in a recent report by the National Research Council: A Research Strategy for Environmental, Health, and Safety Aspects of Engineered Nanomaterial (NRC, 2012). Importantly, data from ENM DNT guideline studies should be reported in a standardized format to increase the utility of the information for both near-term decision making and the development of higher throughput screening method(s) (see Thomas et al., 2013 for an example data-sharing approach).

Ultimately, decisions regarding the health and safety of ENM-enabled products and applications will require an examination of the trade-offs between economic and societal benefits of using the product versus potential health risks. Such trade-offs will differ between individual types of ENM and each product or application they are proposed to be used in. For some, the use of ENM may decrease the use of materials with relatively well-understood health and environmental effects (e.g., carbon nanotubes replacing polybrominated flame retardants). Yet, other types of ENM may be proposed to replace chemicals for which health effects are less well understood (e.g., nano-Ag vs. conventional disinfectant products). In each instance, decisions will need to be made as to whether the ENM-enabled product is in fact favorable. The objective here is not to argue for or against the use of ENM but rather to encourage efforts to gather sufficient data to support knowledgeable decisions about these products. In doing so, we have the opportunity to avoid past experiences with overlooking the potential for DNT from chemicals in mass production, which has cost billions of dollars in economic terms alone (Grandjean and Landrigan, 2006). The evidence for ENM DNT to date is not strong enough to reach any conclusions about potential risk; however, it indicates that a closer look is warranted.

REFERENCES


DNT ENM RISK ASSESSMENT RESEARCH


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