Comparative study of neurologic effects of nano-
TiO$_2$ versus SiO$_2$ after direct intracerebral
exposure in mice

To cite this article: A Balvay et al 2013 J. Phys.: Conf. Ser. 429 012027

View the article online for updates and enhancements.

Related content
- Experimental evaluation of the resistance of nitrile rubber protective gloves against TiO$_2$ nanoparticles in water under conditions simulating occupational use
  L Vinches, P Dolez, K J Wilkinson et al.
- Predictive tests to evaluate oxidative potential of engineered nanomaterials
  Mara Ghiazza, Emanuele Carella, Simonetta Oliaro-Bosso et al.
- Application of in vitro BBB model to measure permeability of nanoparticles
  S Hanada, K Fujoka, Y Inoue et al.
Abstract. Titanium and silicon dioxide nanoparticles (TiO$_2$ and SiO$_2$ NPs) are now in daily use in many commercial products of which food, sunscreens, toothpastes or cosmetics. However, their effects on human body, especially on the central nervous system, are still unclear. The aim of this study was to determine whether direct exposition of the brain to TiO$_2$ and SiO$_2$ NPs results in alternations in nervous system function. C57Bl6 mice were exposed to 5 and 10 µg doses of TiO$_2$ and SiO$_2$ NPs through intracerebroventricular administration using a stereotaxic approach. Then the neurologic effects were investigated using motor performance parameters, measured on a rotarod at 20 rpm or at an accelerating rod (from 4 to 40 rpm). Before and after injection, motor activity is registered individually for each mouse exposed, once a week, for 8 weeks. Besides, a group of 3 mice is culled at 1, 2, 3, 4 and 8 weeks after exposure in order to study the timedependant effect on the histopathology of the brain (gliosis, inflammatory process...). Both rotarod tests (accelerating and at 20 rpm) showed that TiO$_2$ and SiO$_2$ NPs exposure could significantly impair the motor performances, even several weeks after initial acute exposure. The first examination of the brain histopathology revealed microglial activation. As it appeared to grow throughout the brain in a timedependant manner this suggests the induction of a long lasting neuroinflammation. These primary findings indicated that exposure to TiO$_2$ and SiO$_2$ NPs could possibly impair the locomotor ability and this deficit may be possibly attributed at least to an inflammatory process maintained till 8 weeks after exposure in the mouse brain. To fully investigate the neurotoxicological consequences of TiO$_2$ and SiO$_2$ NPs exposure, brain contents in these NPs will be also investigated as well as other alterations like neurotransmitter levels. These preliminary data already underline the necessity of more in vivo studies to better characterize TiO$_2$ and SiO$_2$ NPs exposure effects especially on human brain for long-term and low-dose treatment.

1. Introduction

The expanding development and production of engineered nanomaterials (ENMs) cover varied and extensive potential benefits in consumer products, food, drugs… and thus increase consumer’s exposure to ENMs. The unique properties of ENMs have also raised concerns about the potential non intended consequences on human health and the environment. A potential risk for neurotoxicity arises since ENMs are able to reach the brain and to accumulate there. NPs inhaled through the nose can translocate to the brain via olfactory neurons [1, 2], similarly NPs from the lung are able to reach the brain after lung-blood barrier crossing [3]. Recently NPs were demonstrated as being able to cross the brain blood barrier [4] thus whatever the way of exposure, if NPs enter the systemic circulation they
can reach the brain. Considering the susceptibility of the brain towards different kind of injuries and because of the limited regenerative capability of the brain, NPs presence in the brain should be considered cautiously. After a certain time of exposure, NPs might as well participate to the appearance of neurodegenerative diseases like Parkinson’s or Alzheimer’s diseases. However, neurotoxicity remains poorly documented \textit{in vivo}. Thus, to consider whether any brain exposure may trigger a neurotoxic effect, a first approach based on direct injection of nanoparticles (NP) into the brain of mice using a stereotaxic apparatus is proposed. Even if this is not reflecting any natural way of exposure, this way, as other classical experimental routes of administration like intra peritoneal, allows assessing quickly neurotoxicity. Ventricles were chosen as the site of NPs delivery, because through the cerebro-spinal-fluid, it brings the advantage to ease the dissemination of NPs to the entire brain in a short time. In this preliminary study the doses of NP tested were 5 to 100% lower than those detected in the brain of mice exposed by intragastric route for consecutive 60 days [5], in an attempt to avoid acute toxic effect. Two different ENMs were selected, titanium dioxide (TiO$_2$) a metal oxide and silicon dioxide (SiO$_2$) a non-metal oxide as they are already highly used in several manufactured products as diverse as additives in cosmetics, food, inks, textile…In addition they offer to study different properties of these NPs such as their metal or non-metal nature, their opposite charges in biological media (pH 7-8). The objective of this paper was to set up these studies on \textit{in vivo} evaluation of potential neurotoxicity of ENMs in C57B16 mouse line through the analysis of neurological tests. Neurologic effects of NP brain exposure were studied quantifying motor performance using a Rotarod system. Before and after injection motor activity is registered individually for each mouse exposed, once a week, for 8 weeks. Besides, a group of 3 mice is culled at 1, 2, 3, 4 and 8 weeks after exposure in order to study the time dependant effect on the histopathology of the brain like on gliosis, inflammatory process.

2. Material and Methods

2.1. Nanoparticles
TiO$_2$ (99% purity, aeroxide TiO$_2$ P-25, spherical 22 nm, 85% anatase, 15% rutile) and SiO$_2$ (96.5% purity, primary particle of 5-35 nm, specific surface 220 m$^2$/g), are both provided by the EC-JRC in the framework of the OECD sponsorship program as representative nanomaterial for research purposes. The dispersion protocol developed by Nanogenotox Joint Action was used in the present study. This standard operation procedure was determined in order to harmonize and standardize the dispersion of ENMs. This generic Nanogenotox dispersion protocol produces ENM exposure media suitable for \textit{in vitro} and \textit{in vivo} toxicity testing (www.nanogenotox.eu). Briefly, NPs were suspended and highly dispersed in sterile-filtered 0.05% w/v BSA-water at a fixed concentration using a high energy probe sonication at 4°C. The protocol ensures stable dispersion for 0.5 to 1 hour. The pH value was 7 for each suspended NPs solution.

2.2. Mice exposure
All procedures were carried out in complete compliance with the guidelines laid down by the French (Decree 87-848) Ethical Committee and European Community Directive 86/609/EEC. The intracerebral exposure protocol was approved (11-0042) by the ComEth (National Committee on the Ethics of Animal Experiments). Animal experiments were performed in the ANSES animal facilities (PFEA) which have the relevant approval to carry out animal work (A 69 387 0801) by licensed people working in the animal experiment unit (license numbers AB: 69 387 531, LL: 69 387 191).

Groups of 15 female C57B16 mice, 6-week-old (average weight 19 g), were deeply anesthetized before unique acute administration of NPs. A volume of 2 µL of 2.5 or 5 µg/µL NPs solution freshly sonicated was stereotaxically injected into the right side of lateral ventricle over a period of 10 min under the control of a motorized microinjection pump. A sham experiment was performed with the same volume 2 µL of solvent (sterile-filtered 0.05% w/v BSA-water) into the right side of lateral ventricle of a control group of C57Bl6 mice. None of the mice used in this study presented an overt
neurological impairment at the time of NPs injection. At 1, 2, 3, 4, and 8 weeks after initial exposure, 3 mice were euthanized by lethal injection of pentobarbital. Brains were removed, fixed in 10% buffered formalin solution and either processed for histochemical analysis, or kept for programmed study of elemental content analysis (Ti brain mapping using µ-X-ray fluorescence).

2.3. Histopathology
Fixed brains were routinely embedded in paraffin blocks. Brain slices of 5 µm thickness were placed onto glass slides. Once dewaxed, brain slices were rehydrated, stained using hematoxylin-eosin (HE) or immunostained using markers for glial or microglial cells following procedures described elsewhere [6]. Slides were observed under a light microscope coupled to an image analysis station.

2.4. Nanoparticles impact on motor performances
To assess the effect of NPs direct exposure of the brain on motor function, mice were trained to remain on a rotarod (Bioseb, in vivo Research Instruments, Spain), through a 3-day training program on a 3-cm diameter rotarod (mice-sized). During the training period, each mouse was placed on a horizontal rod rotating at a gradually increasing speed from 4 to 40 rotations per minute (rpm) for a maximum of 10 min by which time a steady baseline level of performance was attained. This is the acceleration test. A second test consisting in measurement of endurance at a fixed speed of 20 rpm was similarly taught to mice. The day after training, the motor performances (coordination and endurance) were recorded for each animal during three trials. The latency to fall off the rotarod was recorded and the time limit was fixed at 3 min. One day prior surgery, baseline values were registered. After NPs or sham exposure, motor performances were registered individually for each mouse, once a week, for 8 weeks following the same procedure.

Statistical analyses were done using Software R. Rotarod results were compared with reference to baseline data before injection using ANOVA, and subsequently Bartlett test, and for the last data registered 8 weeks post surgery, the number of animals being <5, a non parametric Kruskal-Wallis test was applied. Bonferroni method was also used when needed (test the means two by two). A p-value<0.05 was considered as significant.

3. Preliminary results and discussion
Figure 1 shows the results on motor performances registered in the control experiment. Performances on accelerated rotarod (ACC) were slightly progressing reflecting a possible positive effect of the regular week training of the mice. On the rotarod test fixed at 20 rpm there was no effect at all on the motor performance before and after surgery. This shows that the surgery by itself does not induce loss of motor performances. Compared with sham controls, NPs exposed mice had neurological deficits as revealed by the results of rotarod tests.

![Figure 1](image)

**Figure 1.** Direct brain exposure: control experiment using water + 0.05% BSA. Motor activity
3.1. Impact of SiO$_2$-NPs on motor performances

Performances recorded on an accelerated rotarod (ACC) were early significantly reduced at 1, 2, 3 and 4 weeks after surgery compared to control mice (before injection) (*$p<0.05$) (Figure 2). Performances were even more reduced until 8 weeks after initial exposure whatever the dose injected (5 and 10 µg). On the rotarod fixed at 20 rpm, whereas control mice run at least 129 s +/- 105 (means +/- SEM), nano-sized SiO$_2$ exposition induced a deficit in motor performance as soon as 1 week after surgery (29 s +/- 52 *$p<0.05$). Then the significant effect is observed solely for the 10 µg dose that lead to progressive and regular loss of motor performances until week 4 after surgery, which became null thereafter.

**Figure 2.** SiO$_2$ direct brain exposure reduced motor performances measured using a rotarod equipment. Motor activity histograms represent the time that mouse stays on the bar of rotarod one week before surgery and then every week till 8 weeks after surgery. (A) ACC test reveals no reduction on motor performances, on the contrary a slight positive progression was observable especially from week 4 post surgery. (B) 20 rpm test induces no changes on motor performances. Values are the mean ± SEM. Data from exposed mice ($n=9$) were compared with control baseline (before surgery) using ANOVA, Barlett test, and a non parametric Kruskal-Wallis test. * $p<0.05$

3.2. Impact of TiO$_2$-NPs on motor performances
Nano-TiO$_2$ induced a more progressive deterioration of each motor performance (*p=0.0001) (Figure 3). At 20 rpm the initial performance of 104 s +/-86 decline slowly to 84 +/- 100 at 1 week and became null after 4 weeks. The only test that reveals significant difference depending on the dose injected is the endurance test at 20 rpm as shown in figure 3.

Figure 3. TiO$_2$ direct brain exposure reduced motor performances measured using a rotarod equipment. Motor activity histograms represent the time that mouse stays on the bar of rotarod one week before surgery and then every week till 8 weeks after surgery. (A) 5 µg and (B) 10 µg of freshly suspended SiO$_2$ NPs. Values are the mean±SEM. Data from NP exposed mice (n=15) were compared with control baseline (before surgery) using ANOVA, Barlett test, and for the last data registered 8 weeks post surgery, the number of animals being <5, a non parametric Kruskal-Wallis test. * P<0.05, ** P<0.01, *** P<0.001

These first results indicate that both NPs studied were able to induce neurological effects after a direct intracerebral exposure, for several weeks after exposure. Both appear to induce a decline in motor performance but the effects appears to be different in the time-dependant response. Compared to SiO$_2$, TiO$_2$ NPs seem to induce a more progressive motor deterioration. Nano-TiO$_2$ and nano-SiO$_2$ were selected as a large body of literature already exists in terms of toxicity. However, neurotoxicity remains poorly documented and in particular if some neurotoxic effects were reported for TiO$_2$ [7, 8], less is known about SiO$_2$ potential neurotoxicity. In addition, nano-TiO$_2$ and SiO$_2$ were also selected because they are probe particles with positive (TiO$_2$) and negative charges (SiO$_2$) in biological media (pH 7-8). Surface charges are important to probe since they will be strongly related to the transfer of NPs [9]. With both nano-TiO$_2$ and SiO$_2$, two opposite possible trends were tested; the motor abilities were affected in both cases but in a different manner that may be linked to one or several of these specificities that will be studied later.

Hematoxylin-eosin (H&E) staining was performed to examine brain histological changes at 1, 2, 3, 4 and 8 weeks after exposure. At the moment, only one/three mouse per each time point was analyzed,
thus no quantitative and definitive data are yet available. Still, several preliminary histopathological data could be collected. As an example, it was possible to visualize the site of injection, to follow the cicatrisation of the mechanical lesion induced by the needle during injection. At 1 week after surgery, the needle scar was already replaced by a fibroglial tissue as shown in figure 4. Immunohistochemical studies showed the activation of astrocytes and microglial cells thus demonstrating a local gliosis with an inflammation at the initial point of injury.

This was not linked to the NPs themselves as this feature was also observed in brain of sham injected mice. Obviously the early loss of motor performance in NPs exposed mice, cannot be attributed to this local histopathological change as sham operated mice did not present this reduction of motor function. Interestingly, in the weeks after, whereas gliosis progressively vanished, microglial activation seems to grow throughout the brain suggesting an induction of a long lasting neuroinflammation (figure 5).

**Figure 4.** A. Site of injection, cerebral cortex one week after surgery (H&E). B. & C. Local glial scar: astrocytes (in brown) increase their synthesis of glial fibrillary acidic protein (GFAP) and extend their processes. D. & E. Activated microglial cells are the second most prominent cell type present within the glial scar.

**Figure 5.** A.-C. Time-dependant loss of glial activation at the injection site at 1, 3 and 8 weeks post-injection. D.-G. microglial activation at 1, 2, 3 and 4 weeks after SiO$_2$ injection, in other brain areas than the injection site.
This may most probably be linked to the NP, but this needs to be checked on all the other brains available. As well, the results of NPs brain distribution to be studied by μ-X-ray fluorescence will allow easing the possible link between NP presence and induction of neuroinflammation. To take into consideration the loss of motor performances observed in the present study, which could be related to a neuroinflammatory process it will be also relevant to study disturbances in other different neurotransmitter systems.

4. Conclusion and future work
This preliminary study indicates already that nano-sized SiO$_2$ and TiO$_2$ are both able to induce some neurological effects after direct exposure into the brain as shown by loss of motor performance of exposed mice. Comparison with sham experiment indicates that NP exposure is most probably at the origin of these and not the initial needle injury itself. Microglial activation may contribute to these behavioral deficits. These results indicate that rotarod tests are useful in assessing brain response to NP presence within cerebral tissue and therefore could be commonly used in rodent model of nanotoxicology. Still, to fully investigate the neurotoxicological consequences of TiO$_2$ and SiO$_2$ NPs exposure, brain contents in these NPs will be also investigated. Other alterations like neurotransmitter levels will be studied as they may be more directly involved in motor deficits. The necessity of more in vivo studies to better characterize TiO$_2$ and SiO$_2$ NPs exposure effects especially on human brain for long-term and low-dose treatment are required and other experiments using peripheral exposure are already going on.

5. Acknowledgements
Aurélie Balvay was supported by a grant from ANSES. We thank Émilie Antier, Coralie Pulido, Rabah Belkheir, Damien Gaillard, members of the plateforme expérimentation animale (PFEA) of l’ANSES - Lyon, for their respective contributions to this work. We thank Jean-Michel Bridon for technical assistance in preparation of NP solutions as well as Marion Gay for histotechnical assistance.

References
[1] Elder A, Gelein R, Silva V, Feikert T, Opanashuk L, Carter J, Potter R, Maynard A, Ito Y, Finkelstein J and Oberdorster G 2006 Environ Health Perspect 114 1172
[2] Oberdorster G, Sharp Z, Atudorei V, Elder A, Gelein R, Kreyling W and Cox C 2004 Inhal Toxicol 16 437
[3] Shimada A, Kawamura N, Okajima M, Kaewamatawong T, Inoue H and Morita T 2006 Toxicol Pathol 34 949
[4] Brun E, Carriere M and Mabondzo A 2012 Biomaterials 33 886
[5] Hu R, Gong X, Duan Y, Li N, Che Y, Cui Y, Zhou M, Liu C, Wang H and Hong F 2010 Biomaterials 31 8043
[6] Relano-Gines A, Lehmann S, Bencsik A, Herva M E, Torres J M and Crozet C A 2011 J Infect Dis 204 1038
[7] Wang J, Chen C, Liu Y, Jiao F, Li W, Lao F, Li Y, Li B, Ge C, Zhou G, Gao Y, Zhao Y and Chai Z 2008 Toxicol Let 183 72
[8] Zhang L, Bai R, Li B, Ge C, Du J, Liu Y, Le Guyader L, Zhao Y, Wu Y, He S, Ma Y and Chen C 2011 Toxicol Let 207 73
[9] Lockman P R, Koziara J M, Mumper R J and Allen D D 2004 J Drug Target 12 635