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Towards a regulatory use of alternative developmental neurotoxicity testing (DNT)

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ABSTRACT

There is a need for a more effective Developmental Neurotoxicity (DNT) screening which is scientifically driven by the fact that the developing nervous system might be more sensitive to exposures to some hazardous chemical. Additional concern comes from the recent societal concerns that toxic chemicals can contribute to the prevalence of neurodevelopment disabilities. Consequently, hazard identification and actions to reduce exposure to these chemicals is a priority in chemical risk assessment. To reach this goal a cost-efficient testing strategy based on a reliable in-vitro testing battery should be developed. Although this goal is representing a huge challenge in risk assessment, available data and methodologies are supporting the ultimate aim of developing a predictive model able to respond to different regulatory based problem formulations.

1. Introduction

There is concern among scientists and the public that exposure to chemicals is contributing in the development of certain neurological diseases and disorders. This concern is supported by a number of reported evidence that there is an increase in developmental disabilities including attention deficit hyperactivity disorder (ADHD), autism and learning disabilities. These complex disorders have multiple causes, such as genetic, social and environmental with a very high associated economic cost. (Grandjean and Landrigan, 2006; Grandjean and Landrigan, 2014; Smirnova et al., 2014; Gray and Billock, 2017; Boyle et al., 2011; Di Renzo et al., 2015; Gore et al., 2015; Lanphear, 2015; Chambers et al., 2004; Bellanger et al., 2015; Gould, 2009; Bennett et al., 2016).

Although it is accepted that increased awareness and better diagnostics are important factors determining the increased prevalence of reported diagnoses of childhood psychiatric disorders (Atladottir et al., 2015; YooHJ, 2013; Getahun et al., 2013), the contribution of environmental risk factors in adverse outcomes related to developmental neurotoxicity (DNT) remains a challenge for modern risk assessment. It is estimated that genetic factors account for no more than 30–40% of such disorders (NRC – National Research Council, 2007) and therefore there is concern that exposure to chemicals could have contributed to the observed increase in incidence. Indeed, for some chemicals a possible contribution to neurodevelopmental disorders is known, like for organophosphate pesticides, PBDE flame retardants, air pollutants, lead, mercury and PCBs, with evidence provided by epidemiological and toxicological studies (Bennett et al., 2016).

It is however a challenge to show causation in epidemiological studies (EFSA, 2017a, 2017b). In particular, evidence of causation between exposure to non-persistent compounds such as most modern pesticides and adverse outcomes has proven to be relatively weak (Ntzani et al., 2013) and multiple factors, such as, among others, the inconsistency of findings, high heterogeneity, inappropriate documentation of exposure and lack of a more precisely determined trend over time in both children and adults, are limiting the use of epidemiological studies in a regulatory setting and for the definition of the relevant contributory factors to the diseases (EFSA, 2017a, b).

Considering all these complexities, identifying and characterising hazards associated with the development of the nervous system is therefore key in chemical risk assessment. A number of scientific conferences and workshops on the need for more DNT testing have been held over the past decade. The consensus is that there is scientific evidence and health concerns that justify a regulatory need for more testing and that is therefore necessary to develop an effective strategy for a fit for purpose risk assessment of DNT (Bal-Price et al., 2015a).

The need for a more effective DNT screening is scientifically driven by the fact that the developing nervous system might be very sensitive to exposures to some classes of hazardous chemical substances (Fritsche, 2017). Moreover, there is in general a lack of understanding of DNT due to the paucity of tested chemicals; particularly tests performed in line with OECD-compliant in vivo guideline studies, which

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are the current standards for the identification and assessment of DNT hazardous chemicals.

The deficit in chemicals testing is due to a number of factors like: systematic testing for DNT is not a mandatory requirement, standard guideline studies are very resource intensive, in vivo regulatory studies are not intended to inform on mechanism of action.

For plant protection products (PPPs) – although the situation is similar for biocides – accepted guideline studies for DNT are not mandatory, but are triggered by observations in other studies or the mode of action of the pesticides. Often such observations become available from acute and repeated neurotoxicity studies where neurological effects, including structural, neuro-biochemical and behavioural effects, are expected to be captured and thus possibly trigger DNT data. Indications from developmental, multi-generation, acute and short-term neurotoxicity studies and, where performed, developmental neurotoxic effects will be relevant to conclude on the establishment of the regulatory reference doses – e.g. acute reference dose (ARfD) and acceptable daily intake(ADI).

In an analysis conducted by the JMPR (JMPR, 2002) of 14 pesticides evaluated by the US EPA, DNT-related toxicity endpoints were compared with effects retrieved from developmental, multi-generation, acute/short-term neurotoxicity studies. It was concluded that in general DNT studies were not used to identify lower points of departure (NOAELs and LOAELs) for the establishment of the regulatory reference doses.

Similarly, in a retrospective analysis of ARfDs for pesticides evaluated in the European Union (Solecki et al., 2010), ARfDs based on DNT studies in rats represented 1% of the overall ARfDs. However, when considering the impact of DNT studies on the setting of ARfDs and ADIs, it should be noted that only 35 *in- vivo* DNT studies were conducted for the 485 pesticide active substances currently approved in the EU. Of these 35 studies, 19 were considered positive, with 18 showing evidence of both DNT and neurotoxicity. There was evidence of DNT in only one case, but no evidence of neurotoxicity was observed in adult animals.

In a comprehensive review made by Raffaele (Raffaele et al., 2010), in 15 out of 72 pesticide active substances for which in vivo DNT testing was performed, the study was used to determine the point of departure for at least one risk assessment scenario. This indicates that DNT testing is relevant and sensitive though all the substances included in the analysis were considered neurotoxic; therefore, an understanding of DNT by other toxicity pathways is overall lacking. A conclusive summary suggests that although the majority of risk assessments can be considered protective in regard to in vivo DNT effects, in general DNT testing cannot be considered sufficient given the relatively low number of substances tested. Furthermore, if a similar evaluation is made globally in regard to health-hazard assessment of chemicals in general, the conclusion is even more substantiated (Crofton et al., 2012).

This observed deficit in testing for DNT has been frequently associated with the methodological and scientific uncertainties of the in vivo studies. In vivo testing for DNT is currently based on studies (OECD TG 426 and OECD TG 443) intended to evaluate all developing life stages i.e. prenatal and postnatal periods up to puberty, by means of investigating gross neurological and behavioural abnormalities (including physical development), neuropathology and toxicokinetic (TK) endpoints. The protocols were, however, developed to be applicable to testing of any chemical and for this reason chemicals belonging to different regulatory frameworks could have undergone different testing. It should be noted that the methodologies for assessment of learning and memory are flexible, making it difficult to compare studies and findings. DNT guidelines studies are complex and very resource demanding (time and cost intensive) with raised concerns in terms of animal welfare. Interpretation of the results is frequently difficult, possibly due to lack of knowledge regarding brain development (Beronius et al., 2013). Additional issues have been comprehensively described in a Scientific Opinion of the European Food Safety Authority (EFSA), Plant Protection Products and their Residues (PPR) Panel (EFSA, 2013) and they include excessive variability in results, difficulties to interpret minor statistically significant changes and to correctly interpret the findings in a complementary way. The Scientific Opinion is also quoting that a high level of expertise is necessary to assess the study results and evaluate their reliability and relevance for risk assessment. As most of the regulatory guideline studies, they are intended to explore and possibly capture hazards and are consequently conducted at high doses with the highest dose expected to be selected close to the maximum tolerated dose. However, the relevance for human exposure scenarios which is likely to be represented by exposure to low doses over prolonged time periods is questionable (Smirnova et al., 2014) and there is a limited understanding of impact of maternal toxicity (Kaufmann, 2003). The predictivity for protection of the human brain is also questionable when considering differences in developmental timing and toxicodynamics between humans and rodents (Dorman et al., 2001; Kaufmann, 2003; Tsuji and Crofton, 2012).

Although the current guideline studies are considered to represent the best available science for assessing the potential DNT in human risk assessment (Makris et al., 2009), the sensitivity and reproducibility of in vivo testing is also questionable (Crofton et al., 2004; Smirnova et al., 2014; Bal-Price et al., 2015a) and, in some cases, negative or non- reproducible results have been observed with substances or mechanisms known to be of human concern, including methylmercury (Radonjic et al., 2013) or for substances acting on the hypothalamic–pituitary–thyroid (HPT) axis (European Commission, 2017). Also importantly, extrapolation between the test species (rodents) and humans carries in this case substantial uncertainties due to: i) differences in TK and metabolism among species when taking sensitivity and reproducibility into account; ii) timing differences in brain development between rodents and humans; iii) use of non-homologous functional test, particularly in regard to cognitive performances; iv) and the fact that rodents do not capture or represent human relevant diseases such as for example autism, ADHD and Tourette's syndrome.

All these factors need to be carefully considered and weighted in order to develop a DNT testing strategy dependent on a regulatory context, based on the specific problem formulation and an acceptable level of uncertainties, to ultimately inform risk management decisions. It is therefore acceptable a different level of uncertainty when the problem formulation is intended to address testing for chemical screening and/or prioritisation or single substance hazard identification and characterization or mechanistic investigations.

For all these reasons discussed herein, consequently, DNT is regarded as an area where alternative methods should be developed to provide a scientifically validated, time- and cost-efficient testing strategy. In addition, understanding of the uncertainties associated with in vivo data is important when proposing testing strategies using in vitro assays or alternative animal models (e.g. zebra fish) and considering which validation approach to use. Indeed, a number of exemplary DNT test methods have been reported though some of them were not developed for regulatory use. A literature review was committed by EFSA with the goal of building a systematic and comprehensive literature search and data collection from past 20 years until mid-April 2014 on the state of the art of in vivo DNT testing methods including novel and alternative non-mammalian models, in vitro test methods, in silico methods, read across and combination of testing methods in test batteries (Fritsche et al., 2015). The systematic review identified a variety of methods covering early and later stage of neurodevelopment with considerations on their ability to predict DNT of chemicals, definition of biological application domain, validation and protocol standardization needs.

Over the last decade a number of scientific initiatives have concluded that development of in vitro assays and other alternative methods could provide the basis for a non-in-vivo-based testing strategy to assess the impact of chemicals on DNT, and that such new test methods should be matched with the different regulatory needs (Bal-