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RETROSPECTIVE PERFORMANCE ASSESSMENT OF THE TEST GUIDELINE 426 ON DEVELOPMENTAL NEUROTOXICITY

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FOREWORD

This document is the Retrospective Performance Assessment (RPA) of the Test Guideline (TG) 426 on Developmental Neurotoxicity (DNT). It has been developed to ensure that the Test Guideline 426 satisfies current OECD validation criteria. For this aim, the RPA reviews the history of the DNT test method, and demonstrates the extensive scientific efforts made for its development.

The work on the Test Guideline on Developmental Neurotoxicity started in1996 as part of the OECD Test Guideline Programme. In 2005, an expert group met in Tokyo to revise the draft TG 426 and respond to the comments received from the WNT. Based on the extensive supportive material for the performance of the method, the DNT study was considered relevant and reliable for its specific regulatory purpose and use. However, the meeting emphasized that the information available on the performance of the test should be brought forward to the WNT as a supportive retrospective performance assessment document to TG 426.

The RPA was developed by a TG 426 expert drafting group and sent to the WNT in October 2006. In April 2007, the WNT approved the draft TG 426. The draft RPA was also approved, pending the inclusion of additional information requested by Germany. In February 2008, the draft RPA was approved by the WNT by written procedure.

This document is published on the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology.

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PREAMBLE

1. The purpose and intent of this retrospective performance assessment document is to review the history of the developmental neurotoxicity (DNT) test method, and to demonstrate the extensive scientific efforts, including basic neurotoxicology research, inter-laboratory collaborative studies, expert workshops and validation studies, which form the foundation for this testing paradigm. The relevance, applicability and use of the DNT study in human health risk assessment are also reviewed. These considerations address the historical performance of the DNT study, in support of developing an OECD DNT Test Guideline (TG 426; OECD, 2007), that satisfies current OECD validation criteria.

2. In June 2005, the Joint Meeting declassified Guidance Document No. 34 (GD34) on the "Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment" (OECD 2005a). GD34 provides guidance on issues related to the validation of new or updated test methods. It was drafted by representatives of OECD member countries, based on advice from member countries and OECD stakeholders and comprises the OECD principles for validation and for regulatory acceptance of test methods.

3. The GD34 is based on the so called "Solna Principles" for validation and regulatory acceptance. During the development of GD34 it was recognized by the Experts involved and the Working Group of National Coordinators of the Test Guidelines Programme (WNT) that the rigorous principles developed at the Solna workshop may sometimes be too stringent to meet the regulatory needs in member countries. Therefore, a section was added (paragraph 13 of the GD34) that emphasizes the importance of flexibility and adaptability in the validation process without compromising the scientific rigour:

> *…"Given the continuing increase in the numbers and types of test methods being developed for varying purposes, the validation process should be flexible and adaptable. The extent to which these validation principles are addressed will vary with the purpose, nature, and proposed use of the test method. There are differences between in vivo assays and in vitro or ex vivo assays which should be considered in applying the validation principles. In general, the closer the linkage between the effect measured and the toxicological effect of interest, the easier it will be to establish the relevance of the assay. The more closely a test measures/observes (an) effect(s) identical to the effect(s) of concern, the greater the confidence that the test will accurately characterize or model the effect in the target species of concern."…[GD34, paragraph 13]*

4. The Developmental Neurotoxicity (DNT) study was developed by the US EPA and has been subjected to numerous validation studies, rigorous peer reviews and expert judgments over the years. The US EPA has deemed the method validated for its regulatory purposes and as described by this retrospective assessment document extensive supportive materials for the relevance, reliability and overall performance of the DNT study are available. Until the present, only the US EPA DNT study has been available for testing laboratories and the new TG 426 will fill a regulatory gap in OECD member countries.

5. The Expert Consultation Meeting in Tokyo, 24-26 May, 2005 (OECD 2005b) was faced with the task to make the final revisions to the draft Test Guideline 426 on Developmental Neurotoxicity, in response to the comments received after the 2nd round for comments in 2003. Based on the extensive supportive material for the performance of the method, the DNT was considered relevant and reliable for its specific regulatory purpose and use. However, the meeting emphasized that the information available on the performance of the test should be brought forward to the WNT as a supportive retrospective performance assessment document to the TG 426, and should subsequently be attached to the draft TG 426

when a 3rd revised version was circulated to the WNT for comments. This document encapsulates the enormous amount of work that has been performed in the development of the DNT study and provides the rationale for the regulatory acceptance of the DNT as a new OECD Test Guideline.

6. The US EPA DNT guideline (OPPTS 870.6300; US EPA, 1998), the prototype for TG 426, was founded upon an extensive scientific data base, including inter-laboratory validation studies, such as the Collaborative Behavioral Teratology Study, which was conducted in the mid-1980s. A separate group of experts at the Williamsburg Workshop (Kimmel *et al*., 1990) agreed that the methods in the DNT study are sensitive to known human developmental neurotoxicants. An Expert Consultation Meeting conducted in 2000 (OECD, 2003), discussed issues on validation, especially of individual test components versus the whole DNT test method. In doing so, they reviewed the extensive history of international validation, peer review and evaluation of DNT methods contained in the public record. Experts agreed that individual assays of the DNT test method have been shown to be relevant, reliable and sensitive, and there was agreement that there is extensive information available demonstrating the validity of individual components of the DNT test method.

INTRODUCTION

7. The field of developmental neurotoxicology evolved from the disciplines of neurotoxicology and developmental toxicology, through an extensive history of scientific research and regulatory consideration. Developmental toxicity has been defined in the draft OECD Guidance Document 43 on Mammalian Reproductive Toxicity Testing and Assessment (OECD, 2007b) as

> *"Any effect which interferes with normal development of the conceptus, either before or after birth, and resulting from exposure of either parents prior to conception, or exposure of the developing offspring during prenatal development, or post-natally, to the time of sexual maturation. These effects can be manifested at any point in the life-span of the organism. The major manifestations of developmental toxicity include; death of the developing organism; structural abnormality; altered growth, and; functional deficiency."*

8. The developmental neurotoxicity study is a specialized type of developmental toxicity study. Developmental neurotoxicity studies are unique among guideline toxicology studies in that they are designed to screen for adverse effects of pre- and post-natal exposure on the development and function of the nervous system and to provide dose-response characterizations of those outcomes.

9. Developmental neurotoxicity studies as described in the OECD TG 426 recommend administration of the test substance during gestation and lactation. Cohorts of offspring are randomly selected from control and treated litters for evaluations of gross neurological and behavioral abnormalities during postnatal development and adulthood. These include assessments of physical development, behavioral ontogeny, motor activity, motor and sensory function, learning and memory, and post-mortem evaluation of brain weights and neuropathology.

HISTORY OF DNT TEST GUIDELINE DEVELOPMENT

10. The evolution of developmental neurotoxicity studies has its roots in scientific publications that began to appear in the early 1960s; the science has continued to develop over the past four decades. An extensive scientific literature exists of studies evaluating the potential for physical, pharmaceutical, and environmental agents to affect the development and function of the nervous system after prenatal and early postnatal exposure. This body of information, which provides a strong foundation for guideline development, implementation, and validation, is summarized in Table 1.

Table 1. Historical contributions to the Developmental Neurotoxicity Guideline

Date	Event	Summary	References
1960's-1980's	Published research on developmental neurotoxicity and behavioural testing	Evidence that developmental exposure to chemicals and drugs can alter behavioural functioning in young and adult animals.	Butcher, 1985
1978-84	Collaborative Behavioural Teratology Study (CBTS)	Study to examine intra- and inter-laboratory reliability and sensitivity of behavioural test methods.	Buelke-Sam *et al*., 1985*
1982-85	Collaborative studies of the Japanese Teratology Society	Inter-laboratory methods evaluations and assessment of 6 reference chemicals.	Tanimura, 1986*
1985-88	European Inter-laboratory Collaborative study	Inter-laboratory study to assess sensitivity of behavioural test procedures to detect neurotoxicity of methyl mercury.	Elsner *et al.,* 1986, 1988; Suter and Schon, 1986
1989	Williamsburg Workshop	Workshop to evaluate the qualitative and quantitative comparability of animal and human data for developmental neurotoxicity.	Kimmel *et al.*, 1990; Francis *et al.*, 1990*
1993-97	Collaborative studies of the Japanese Teratology Society	Three inter-laboratory studies using behavioral teratogens to evaluate a core battery of tests.	Fukunishi *et al.*, 1998; Tachibana *et al.*, 1998; Nishimura *et al*, 2001
1995	International Programme on Chemical Safety (IPCS) Collaborative Study	Inter-laboratory study using neurotoxic chemicals to evaluate test validity, reliability and measurement variability.	MacPhail *et al.,* 1997; Catalano *et al*., 1997; Tilson *et al.,* 1997
2000	International Life Sciences Institute workshop on developmental neurotoxicity testing	Workshop to review EPA DNT behavioural test methods, pharmacokinetics and neuropathology.	Mileson and Ferenc, 2001; Cory-Slechta *et al.,* 2001; Dorman *et al.,* 2001; Garman *et al.,* 2001
2003	Japanese Inter-laboratory Study	Inter-laboratory study using neurotoxic chemicals to determine sensitivity of behavioural measures.	Okazaki *et al.,* 2003
2003 (Sept)	Behavioral Test Methods Workshop	Expert Workshop to address design, conduct and analysis of behavioural tests for neurotoxicity evaluation.	Slikker *et al*., 2005

* Additional citations are detailed below.

11. Table 2 provides a brief historical summary of EPA and OECD DNT guideline development. While prenatal developmental toxicity test guidelines have existed for some time (*e.g.*, OECD, 1983), the first regulatory protocol specifically designed to evaluate developmental neurotoxicity was developed and implemented by the US Environmental Protection Agency (EPA) in support of hazard evaluation for specific solvents (US EPA, 1986). The EPA toxicology testing guidelines were developed by the Office of Toxic Substances (since renamed the Office of Pollution Prevention and Toxics [OPPT]) and the Office of Pesticide Programs (OPP), and first proposed and published for public review and comment in the US in 1986. The DNT guideline was finalized in 1991 (§83-6; US EPA, 1991). In 1998, it was revised (OPPTS 870.6300; US EPA, 1998) as part of a broader US effort to harmonize testing guidelines across OPPT and OPP, and with OECD.

12. As illustrated in Table 2, OECD initiated the development of TG 426 following the recommendations of the OECD Working Group on Reproduction and Developmental Toxicity in Copenhagen in 1995. The first draft of TG 426 was prepared following a 1996 Expert Consultation Meeting. While using the US EPA developmental neurotoxicity guideline as the basis for design of developmental neurotoxicity studies, TG 426 addressed a number of important issues and incorporated improvements recommended at the expert consultation meeting in 1996. The draft TG 426 was distributed to National Coordinators for comment in 1998, and significant technical issues that were identified by this review were further discussed at an Expert Consultation Meeting in Washington in 2000 (OECD, 2003). A revised draft was then circulated to National Experts for review and comment. Comments from member countries were addressed at a 2005 Expert Consultation meeting in Tokyo (OECD, 2005b).

Table 2. History of the Developmental Neurotoxicity Guideline

Date	Event	Reference
1986	US EPA OPPT published first draft DNT protocol for peer review and public comment	US EPA, 1986
1991	US EPA OPPTS published final DNT guideline (§83-6)	US EPA, 1991
1995	OECD Working Group on Reproduction and Developmental Toxicity (Copenhagen) recommended development of OECD Developmental Neurotoxicity Test Guideline	OECD, 1995a
1996	OECD Expert Consultation Meeting (Copenhagen) provided recommendations for development of Draft OECD 426	OECD, 1996
1998	US EPA OPPTS issued minor revisions and harmonization of DNT guideline (OPPTS 870.6300)	US EPA, 1998
1998	Draft TG 426 submitted to National Coordinators for expert review and comment	OECD, 1998
2000	OECD Expert Consultation meeting (Washington) held to review technical issues	OECD, 2003
2003	Draft TG 426 submitted to National Coordinators for expert review and comment	
2005	OECD Expert Consultation Meeting (Tokyo) convened to respond to remaining comments on Draft TG 426	OECD, 2005b

13. In the context of toxicological screening and testing to support human health risk assessment and chemical regulatory activities, the DNT study fills an information requirement that is not satisfied by other OECD Test Guidelines. Notably, it is the only Test Guideline that includes functional, behavioral, and anatomical evaluations of the nervous system at multiple time points, in test subjects that were exposed to test substance during critical pre- and early postnatal periods of nervous system development. This test method has been used extensively in the past two decades on a wide variety of chemicals (Table 3).

Table 3. Number of chemicals studied using the EPA DNT Guideline or draft OECD 426 Guideline

Chemical class	Number of studies
Industrial Chemicals	8
Miscellaneous Agents*	4
Pharmaceuticals	3
Pesticides	73
Positive Control Chemicals	15
Solvents	7

* Food additives, cigarette smoke, dietary restriction, and maternal separation

SCIENTIFIC BASIS OF DNT GUIDELINE

14. DNT study methodology has been extensively reviewed and evaluated over the last 25 years. This has included the conduct of a number of meetings and collaborative studies involving experts from academic, industry, regulatory and public interest groups. Pivotal influences and key events in the history of the development of the DNT test guideline (Table 2) include both research on test methods development and efforts to characterize and document the sensitivity, reliability, and performance of the test methods. The development of test methods in neurotoxicology includes a long history of intra-laboratory efforts to determine the sensitivity and reliability of the test methods now used in the DNT study design. In the 1970's Butcher and colleagues began publishing a series of papers in which rats were developmentally exposed to a variety of xenobiotics and subsequently tested during postnatal development using a battery of neurobehavioral tests (e.g., Butcher and Vorhees, 1979; Vorhees *et al*., 1979). At this same time Tilson and colleagues began efforts using behavioral and histological batteries, focused on sensory and motor function, in adult rodents exposed to a wide variety of neurotoxicants (e.g., Tilson *et al.*, 1979; Pryor *et al.*, 1983). A large body of research has provided an immense database on the ability of the functional observational battery to detect and characterize the effects of drugs and environmental chemicals (c.f., Irwin, 1968; Gad, 1982; Moser *et al*., 1988). This work was important in determining which methods would be most suitable for screening for neurotoxicity and developmental neurotoxicity. This early work was followed by a wide-ranging effort to understand the specificity of these test methods and the impact of both organismal and experimental factors (e.g., noise, species, strain, gender, test history) (cf., Gerber and O'Shaughnessy, 1983; Spencer *et al.*, 1993; MacPhail *et al*., 1989; Levine and Butcher, 1990). Clearly, the literature is too large to properly review herein. However, the result of over 30 years of work in this area is a consensus opinion of neurotoxicologists that proper use and interpretation of the data derived from these test methods provides unique insight into the impact of xenobiotics on the developing and adult nervous system.

15. The development of test methods in neurotoxicology also includes a long history of endeavors to characterize the inter-laboratory reliability and sensitivity of the test methods now used in the DNT study design. In 1979, Butcher and colleagues published a seminal paper comparing a learning and retention

method among three laboratories (Butcher et al., 1979). This was followed by the Collaborative Behavioral Teratology Study (CBTS) (Buelke-Sam *et al.,* 1985; Kimmel and Buelke-Sam, 1985), and the "Williamsburg Workshop" on Qualitative and Quantitative Comparability of Human and Animal Developmental Neurotoxicity (Kimmel *et al.,* 1990). These efforts addressed various aspects of DNT study design and conduct, providing a sound scientific basis for the test method and its use in hazard evaluation. Since the 1991 publication of the US EPA DNT guideline (US EPA, 1991), there has been a continued scientific effort to review and update methodologies, for neurotoxicology in general and for developmental neurotoxicology in particular. Examples of such reviews include the IPCS collaborative study on neurobehavioral screening methodologies (MacPhail *et al.,* 1997), an International Life Sciences Institute (ILSI) Risk Sciences Institute (RSI) workshop on Developmental Neurotoxicity and Risk Assessment held in 2000 (Mileson and Ferenc, 2001), a collaborative study on neurobehavioural screening in eleven Japanese laboratories (Okazaki *et al.,* 2003), and the 2003 Behavioral Test Methods Workshop (Slikker *et al.,* 2005). Descriptions of each of these efforts and their contributions to the scientific basis for DNT testing follow.

The Collaborative Behavioral Teratology Study (CBTS) - Several of the test procedures developed in early behavioral teratology studies underwent validation in a large inter-laboratory effort, the CBTS (Buelke-Sam *et al.,* 1985), which compared performance of a standardized behavioral test methodology in six different laboratories after *in utero* and lactational exposure to two known neurotoxicants, methyl mercury and amphetamine. Conducted from 1978 to 1984, it was, at the time, the largest study ever undertaken to examine the intra- and inter-laboratory reliability and sensitivity of several behavioral test methods. The study also examined the effects of a number of other variables, including which tests had been administered to the animals, whether pups from the same litter responded more or less like their litter mates than pups from other litters, and the effects of pup gender on response to testing. The results of the CBTS were published in the peer-reviewed literature, and included descriptions of the background and overview (Kimmel and Buelke-Sam, 1985), protocol and test procedures (Adams *et al.,* 1985b), data entry and test systems (Adams et al., 1985c; Voorhees, 1985), preliminary research (Adams *et al.,* 1985a), statistical approach (Nelson *et al.,* 1985), results (Buelke-Sam *et al.,* 1985), and implications, current applications, and future directions (Kimmel *et al.,* 1985). Additionally, the results of a workshop held to review the CBTS data were published (Butcher and Nelson, 1985; Geyer and Reiter, 1985; Kutscher and Nelson, 1985; Sobotka and Voorhees, 1985; Tilson and Wright, 1985). The study showed that replicability of data among laboratories using a standardized protocol was excellent, and that both positive effects (*e.g.,* with methyl mercury exposure), and the lack of effects (*e.g.*, after low-level amphetamine exposure) were replicable. The CBTS also demonstrated that the DNT test procedures were sufficiently sensitive; no more than a 5-20% change from control values was required to detect an effect.

The European Inter-Laboratory Collaborative Study - In the 1980's, the European Inter-laboratory Study group on Behavioural Teratology conducted an inter-laboratory study of behavioral test methods (Elsner, 1986; Elsner *et al.*, 1986; Schreiner *et al.*, 1986; Suter and Schon, 1986). Three laboratories, one each from industry, academia and government, collaborated to examine the sensitivity and applicability of behavioral methods for routine toxicological testing. Results from animals perinatally exposed to methyl mercury indicated that behavioral tests were sensitive and that automated procedures and measures aimed at specific functional capacities were more sensitive than non-specific behavioural measures (Elsner *et al.,* 1986, 1988).

The Williamsburg Workshop - In 1989, the US EPA held a workshop on the Qualitative and Quantitative Comparability of Human and Animal Developmental Neurotoxicity (also known as the "Williamsburg Workshop") to provide scientific input into DNT protocol design and to evaluate its appropriateness for use in risk assessment (Kimmel *et al.,* 1990). Expert scientists from government, industry, public interest groups, and academia reviewed a range of representative chemicals and environmental exposures including: drugs (cannabis, cocaine, methadone, and phencyclidine) (Hutchings, 1990), ethanol (Driscoll

et al., 1990), the anticonvulsant phenytoin (Adams *et al.,* 1990), as well as environmental contaminants such as methyl mercury (Burbacher *et al.,* 1990), lead (Davis *et al.,* 1990), polychlorinated biphenyls (Tilson *et al.,* 1990), and ionizing radiation (Schull *et al.,* 1990). Based on data available for these known human developmental neurotoxicants, the workshop participants concluded that DNT methodologies were adequate for detecting developmental neurotoxicity. A number of specific issues directly relevant to design and usefulness of DNT studies were extensively evaluated by participants: 1) the comparability of measures of developmental neurotoxicity in humans and laboratory animals (Stanton and Spear, 1990), 2) testing methods in developmental neurotoxicity for use in human risk assessment (Buelke-Sam and Mactutus, 1990), 3) weight of evidence and quantitative evaluation of developmental neurotoxicity data (Tyl and Sette, 1990), and 4) triggers for developmental neurotoxicity testing (Levine and Butcher, 1990). In addition, the participants were asked to address the relationship between biological endpoints specified by DNT guidelines and adverse findings observed in humans following exposure to the developmental neurotoxic agents under consideration. A major conclusion of the workshop was that the DNT protocol would have identified each of the agents presented at the workshop as a potential developmental neurotoxicant (Francis *et al.,* 1990), although the critical effects, and the dose at which the effects were observed, could vary across species. The predictive power of DNT guideline studies was attributed largely to the scope of neurobehavioral and neuropathological tests used that can evaluate neurological functions across multiple domains (*i.e.,* sensory, motivational/arousal, cognitive, and motor). The laboratory animal serves as an adequate surrogate for humans because many of the biological and behavioral mechanisms underlying these neurological functions are shared between humans and laboratory animals.

Collaborative Studies of the Japanese Teratology Society - The Japanese Teratology Society established the Behavioral Teratology Meeting (BTM) as a satellite meeting in 1982. This group sponsored a number of collaborative studies conducted primarily by pharmaceutical, industry, and contract laboratories (Tanimura, 1985). The first effort involved 21 institutions that investigated the effects of parametric variables (water temperature, number of trials) on performance in a water T-maze and two-way shuttle box (Mizutani, 1984). This was followed by a larger study involving 46 laboratories that investigated the effects of six chemicals (chlorpromazine, ethanol, hydroxyurea, methylazoxymethanol, phenylalanine, and vitamin A (Mizutani, 1985). The results of these studies are summarized by Tanimura (1986) who concluded that the T-maze test was reliable, but possibly not as sensitive as needed, and suggested the use of more complicated learning paradigms for this method. Workshops were held between 1988 and 1990, with three subgroups: reflexes and sensory function, activity and emotionality, and learning (Tanimura, 1992). Subsequently, a core battery test draft for behavioral developmental toxicity was proposed, and its utility was examined with three positive behavioral teratogens in 1993-97. They were phenytoin (Fukunishi *et al.*, 1998), retinoic acid (Nishimura *et al.*, 2001) and nicotine (Tachibana *et al.*, 1998). The numbers of participating laboratories were 32, 28 and 18, respectively. It was concluded that the proposed core battery of tests is useful as a screening method to detect postnatal developmental disorders, including behavioral dysfunction, in rats. Activities of the BTM of the Japanese Teratology Society have continued to the present as the Behavioral Teratology Committee.

The International Programe on Chemical Safety (IPCS) Study - The IPCS collaborative study, was an inter-laboratory evaluation of neurobehavioral screening methodologies used adult and developmental neurotoxicity studies (MacPhail *et al.*, 1997; Moser *et al.*, 1997e). A total of 8 laboratories participated in the proficiency studies (Moser *et al.*, 1997a) and the full study, using 7 neurotoxic positive control chemicals (triethyl tin, acrylamide, parathion, *p,p'*-DDT, toluene, N,N'-methylene bis-acrylamide, and lead acetate) in adult male rats (Moser *et al.*, 1997b,c). A principal focus of the study was to examine the amount of variability that was likely to occur with the test methods, and to explore the reasons that differences occurred. The overall conclusion of this extensive study was general "agreement across laboratories in terms of their ability to detect dose-related changes in behavioral endpoints with prototypic neurotoxic agents" (Catalano *et al.*, 1997). The study results were also reviewed at a workshop held in

1995 in Capri, Italy (Tilson *et al.*, 1997), and were presented at the 1996 meeting of the Society of Toxicology (Moser *et al.*, 1997d).

The ILSI workshop on DNT testing - Enhancements to the published US EPA DNT guideline method are included in the OECD TG 426, and some were implemented by the EPA Office of Pesticide Programs (OPP) when it issued Data Call-In (DCI) notices for organophosphate pesticides with tolerances in 1999 (US EPA, 1999b). Specifically, these enhancements included extension of the offspring dosing period through to the age of weaning, demonstrating that the pups are receiving the test substance when only the mother is treated (or adjusting the study protocol to ensure that this occurs), increasing the number of offspring evaluated neuropathologically, and collecting chemical class specific biomarker data. The extension of the dosing period during the lactation period raised several issues, specifically in the areas of pharmacokinetic or toxicokinetic data needs, behavioral testing, and neuropathological evaluation. To address these topics, the International Life Sciences Institute (ILSI), under a cooperative agreement with EPA, established a working group of scientists from government, industry, and academia, to assemble and evaluate the available science. The conclusions of the working group (OECD, 2003) were presented in a joint OECD-ILSI public workshop held in Washington, DC on October 24-25, 2000, and were published in the peer reviewed literature in 2001 (Mileson and Ferenc, 2001: Cory-Slechta *et al.,* 2001; Dorman *et al.,* 2001; Garman *et al.,* 2001). Overall, the working group agreed that the current DNT test protocol was based upon solid scientific principles and experience, that there were opportunities to revise and improve some aspects of the US EPA guideline study, and that further research will be valuable in providing the scientific basis for future revisions of TG 426. Further consideration of methodological issues related to the conduct of the DNT study include an ILSI workshop on the direct dosing of preweaning mammals. This workshop culminated in a monograph on direct dosing that has broad application to study design for many areas of research, *e.g.,* pharmaceuticals, environmental pollutants, academic research, etc. (Zoetis and Walls, 2003; Moser *et al*., 2005).

The Japanese Inter-laboratory Study - An inter-laboratory evaluation of neurobehavioral screening methodologies (used in DNT studies as well as adult neurotoxicity studies) was carried out by laboratories in Japan (Okazaki *et al.,* 2003). The study focused on examining technical problems in evaluating neurotoxic potential of chemicals using a common testing protocol. A total of eleven laboratories conducted a variety of neurobehavioural tests on rats after either acute or repeated (28-day) exposure to acrylamide or 3,3'-iminodiproprionitrile. The overall conclusion of this study was that there was general agreement that all laboratories detected neurotoxicity of both chemicals. The reports pointed out inter-laboratory differences and concluded that it is most important to standardize the methods and criteria, and improve observers' skills (Okazaki *et al.*, 2003).

The Behavioral Test Methods Workshop - In 2003, a workshop on behavioral testing was conducted in order to discuss experimental procedures and practices that could help enhance the utility of behavioral data as a reliable index of neurotoxicity and in the safety evaluation of chemical substances (Slikker *et al.,* 2005). Workshop participants included individuals from all sectors of the neuroscience community, include academia, government, testing laboratories, industry, and non-profit non-government organizations (NGOs). The overall conclusions from the workshop were that consensus can be reached on the fundamentals of behavioral assessment, and that aspects of behavioral assessment, including experimental design, test method selection, training of technical staff, validation, control of confounding factors, data variability, data analysis and data interpretation should be carefully considered in the planning and conduct of behavioral safety assessment (Slikker *et al.,* 2005).

16. In summary, the scientific basis of the DNT test method has been subjected to an extensive history of international validation, peer review and evaluation which is contained in the public record. Through the various collaborative efforts and workshops that have been conducted, a number of important conclusions have been drawn. The individual test methods utilized in the DNT study have been found to be highly

relevant and based upon solid scientific principles and experience. Utilizing exposures to known human developmental neurotoxicants, the DNT study has been shown to adequately identify the potential for adverse effects of chemical exposure on neurological development. The intra- and inter-laboratory reproducibility, reliability and sensitivity of the DNT test method has been demonstrated, utilizing a spectrum of test substances.

VALUE AND USE OF THE DNT IN RISK ASSESSMENT

24. There is a clear regulatory need for DNT testing to support risk assessments in OECD member countries. Many pesticides and other chemicals are known to affect the nervous system, and there are concerns regarding the potential for developmental neurotoxicity following early life exposures to these substances (NAS, 1993, 2000). This is particularly important since the unique behaviors and activities of children place them at greater risk for increased exposure to xenobiotics by multiple routes (Brent et al., 2004; Weiss *et al.,* 2004). The call for a more rigorous assessment of the potential for developmental neurotoxicity has been issued by scientists from multiple and diverse sectors with an interest in public health protection, *e.g.,* academia, government, NGOs, and public interest groups.

25. An examination of the historical and potential uses of the DNT study in risk assessment is critical to an overall evaluation of its value in protecting human health. At this point in time, the largest collection of DNT guideline studies resides with the US EPA OPP, since this regulatory body has conducted a concerted effort to obtain information on developmental neurotoxicity for specific chemicals to satisfy the mandates of the US Food Quality Protection Act (FQPA) that was promulgated in 1996. US EPA has furthermore engaged in a continuous, on-going scientific analysis and discourse regarding the conduct of DNT studies, the interpretation of the data from these studies, and their regulatory impact.

26. A review of twelve developmental neurotoxicity studies evaluated by the EPA Office of Pollution Pesticides and Toxic Substance (OPPTS) in support of the registration and/or use of 9 pesticides and 3 solvents (Makris *et al.,* 1998) was presented to a FIFRA (Federal Insecticide, Fungicide and Rodenticide Act) Scientific Advisory Panel (SAP) in 1998 (US EPA, 1999a). For the 9 pesticides examined, the EPA analyses concluded that the No-Observed-Effect-Level (NOEL) for DNT was lower than that of the fetal NOEL from the prenatal developmental toxicity study (TG 414; OECD 2001) for eight of the nine pesticides tested, and demonstrated an equivalent dose for one. The offspring NOEL for the DNT study was lower than the offspring NOEL for the reproduction study (TG 415; OECD 1983) for six of the nine pesticides, and equivalent for one. Overall, in two of nine cases, the NOEL for DNT was lower than or equal to that for any adult or offspring endpoint from the prenatal developmental, reproduction, or neurotoxicity (TG 424; OECD 1997) studies. Even though limited by the paucity of DNT studies available at that time, this review indicated that the DNT study includes unique endpoints which are not examined in any other Test Guideline, thereby enabling detection of neurobehavioral and neuropathological effects in offspring following exposure during sensitive periods of neurological development. Therefore, the DNT study, when present in a chemical data base, is often identified as a sensitive study and an important source of quantitative and qualitative information for risk assessment.

27. At the same SAP meeting in 1998, the Panel reviewed the use of the DNT study in risk assessment and agreed that DNT results are appropriate for use to support acute and chronic dietary risk assessments and short- and intermediate-term occupational and residential risk assessments for pesticides (US EPA, 1999a). DNT endpoints have been shown to be the determining factor in the selection of endpoints and doses for risk assessment for some chemicals for which these data have been required by the US EPA (Makris *et al.*, 1998). As might be expected of a study that utilizes short term exposures (approximately 25

to 40 days) during development, where a single exposure during a critical period may result in developmental insult (Rice and Barone, 2000; Rodier, 1980, 1986, 1994), the predominant use of the DNT study in pesticide risk assessment has been for acute (single dose) reference doses (RfD), and for short-term (1-30 days) and intermediate-term (1-6 months) non-occupational exposures, which are especially applicable to risk assessments for children. While there is potentially a more limited use of the DNT study for chronic risk assessment (*i.e.,* in calculating a chronic RfD for lifetime exposure to a toxicant), the DNT study has also been used for this exposure scenario when it has been shown to be the most sensitive study in the toxicology data base.

28. Since 1998, the data base of available DNT studies has increased substantially. By early 2006, approximately 114 DNT studies had been completed using either the EPA guideline or the draft OECD guidelines (Table 4). This list of agents is included here for the purpose of demonstrating the extensive history and experience that already exists regarding the conduct and interpretation of DNT studies; however, it is not suggested that the outcomes of these efforts comprise a focused attempt to validate the study protocol or specific endpoints. In fact, it is noted that a few of these studies did not include all the endpoints recommended by EPA or OECD guideline. For example, 1,1,1,-trichloroethane was tested under a consent agreement which allowed for the addition of extensive neurophysiologial testing *in lieu* of motor activity (USEPA, 1989). Others were conducted prior to the adoption of the early guidelines, and therefore contained limited assessments (*e.g.*, DEET), or tested with a slightly modified protocol used for some pharmaceuticals (*c.f.*, atorvastatin and CI-943; Henck *et al.*, 1995; 1998). As of August 2006, 75 DNT studies had been submitted to the EPA Office of Pesticide Programs (OPP) in support of pesticide registration. Official statistics regarding the uses of these studies in chemical risk assessments have not yet been released, pending finalization of the relevant chemical risk assessments. Nevertheless, a preliminary survey of the use of DNT studies in risk assessment in OPP was conducted in March 2007 (Rowland *et al.*, 2007). In this analysis, the impact of the DNT study was examined by identifying its specific use in the selection of endpoints and doses for the risk assessment (as compared to the 1998 retrospective analysis which compared the NOELs of DNT studies and other studies in the chemical database).

29. It was noted that for 58 pesticide chemicals where a DNT study had been considered in the weight-of-evidence review of the toxicology data base, the DNT study was utilized to select endpoints and doses for risk assessments for eight of those chemicals (Rowland *et al*., 2007). Four studies were used to establish an acute RfD, four were used for a chronic RfD and six were used for short- or intermediate-term non-dietary assessment. In this analysis, a single study may have been used for multiple risk assessment scenarios. An important finding of this review was that for four of the eight DNT studies the critical effects either included or were solely based upon offspring behavioral and neuropathological parameters that are not evaluated in other guideline studies (*i.e.,* motor activity, auditory startle habituation, learning and memory, and morphometric analysis). In addition, based on an evaluation of the doses and effects identified in the DNT study review, in comparison to those used as points of departure in the most recent risk assessment, an additional 17 cases were identified where an endpoint from a DNT study could potentially be selected for use in one or more risk assessment scenarios. The outcome of this evaluation is consistent with the conclusion of the earlier retrospective analysis (Makris *et al.,* 1998). It provides further evidence of the sensitivity of the DNT study in identifying adverse effects in the young, and the importance of the role of DNT studies in human health risk assessments.

30. In addition to using DNT data for regulatory decisions, some regulatory agencies have also, on a case-by-case basis, incorporated an additional database uncertainty factor into their regulatory decisions because of the absence of DNT data. The use of these uncertainty factors in risk calculations reflect regulator views that DNT data are valuable in refining permissible exposure levels, and the absence of these data can increase the uncertainty about the toxicity of the chemicals (US EPA, 2002a, b).

Table 4. Examples of Chemicals Tested Using the US EPA DNT Guideline or OECD Draft 426.

1,1,1-Trichloroethane	Dichlorvos (DDVP) (2)	Methyl parathion
Abamectin	Dicrotophos	Methylazoxymethanol (2)
Acephate	Dietary restriction	Methyl mercury
Acetamiprid	Dimethoate	Molinate
Acibenzolar-s-methyl	Disulfoton	Naled
Acrylamide	Emamectin	Nelfinavir
AE-0172747	Epidermal growth factor	Nitrous oxide
Aldicarb	s-Ethyl dipropylthiocarbamate	n-Methylneodecanamide
Alitame	Ethoprophos	Perchlorate
Amicarbazone	Ethylbenzene	Phorate (2)
Atorvastatin	Etofenprox	p-Methane-3,8-diol
Azinphos methyl	Fenamidone	Prochloraz
BAS 510F	Fenamiphos	Profenofos
BAS 670H	Fipronil	Propylthiouracil (2)
Bifenthrin	Fluazinam	Pymetrozine
Carbaryl	Flubendiamide	Pyrasulfotole
Carbofuran	Flufenacet	Spirodiclofen
Chlorfenapyr	Glufosinate ammonium	Prothioconazole
Chlorite, sodium	Glyphosate trimesium	Styrene
Chlorpyrifos	GN1180 (MN rgp120/HIV-1)	TBBPA
CI-943	Hydrogen sulfide	Tebuconazole
Cigarette smoke	Imidacloprid	Terbufos
Clodinafop propargyl	Iminodiproprionitrile	Tetrachlorvinphos
Clothianidin	Indoxacarb	Thiamethoxam
Coumaphos	Isopropanol	Thiocloprid
Cyclohexanemethanol	Isoxaflutole	Thiram
Cyfluthrin, beta-cyfluthrin	Lambda-cyhalothrin	Fentin hydroxide (TPTH)
Cymoxanil	Lasofoxifene	Triallate
Octamethylcyclotetrasiloxane	Lead nitrate	Tribufos
Decamethylcyclopentasiloxane)	Lindane	Trichlorfon
DDT	Malathion	Trichloroethylene
DEET	Maternal separation	Triethylene glycol monomethyl ether
Deltamethrin	Methamidaphos	Trimethyltin
Diazepam	Methimazole (6)	Zeta-cypermethrin
Diazinon	Methyl bromide	Ziram

The number in parentheses represents the number of studies

31. Cross-laboratory comparisons of methodologies and results from DNT studies have been conducted by US EPA scientists, in an on-going evaluation of OPP study submissions (Crofton *et al.,* 2001; Crofton *et al.,* 2004; Raffaele *et al.,* 2003, 2004, 2005, 2006; Sette *et al.,* 2004; Makris *et al.,* 2005, 2006). Other efforts have retrospectively examined specific endpoints across multiple DNT studies conducted under EPA and/or OECD guidelines (e.g., neuropathological assessments of offspring, Kaufmann and Gröters, 2006), for the purposes of demonstrating the value of current methods in hazard characterization and exploring further opportunities for methodological refinement. In an ILSI Risk Science Institute (RSI) workshop entitled "An Evaluation and Interpretation of Neurodevelopmental Endpoints for Human Health Risk Assessment" a working group consisting of scientists from government, industry, academic, and public health sectors is examining the interpretation of DNT study data and addressing a number of critical issues (*i.e.,* public health considerations, overall data interpretation, data variability, positive control data, and statistical analysis) (Fenner-Crisp *et al*., 2005; Crofton *et al*., 2008 in press; Tyl *et al*., 2008 in press;

Raffaele *et al*., 2008 in press; Holson *et al*., 2008 in press) with the expectation that the final results of this effort will soon be published in its entirety in the peer reviewed literature. Overall, these various review efforts and resulting publications have provided and will continue to provide transparent decision criteria for the analysis and interpretation of DNT test results, in accordance with the principles described in GD34 (OECD, 2005a). Additionally, they have demonstrated test method reliability, reproducibility, and relevance, which is attributable in part to the high level of standardization of the test methods that are recommended in the test guideline.

FUTURE ACTIVITIES

32. The US EPA DNT guideline was developed and promulgated over fifteen years ago in response to the need for regulatory-based screening methods to assess developmental neurotoxicity (US EPA, 1986, 1991). The discussion above reviewed the overall performance of the DNT test method, as well as the ability to detect effects of concern from a regulatory perspective (Buelke-Sam *et al.,* 1985; Elsner *et al.,* 1986; Francis *et al.,* 1990; MacPhail *et al.,* 1997; Makris *et al.,* 1998; Mileson and Ferenc, 2001; Slikker *et al.,* 2005; Tilson *et al.,* 1997). The recent increase in the number of regulatory DNT studies being conducted (US EPA, 2002c), has refocused attention on this test method. While some have argued that some tests are insensitive (*e.g.,* assessment of cognitive and sensory dysfunction are inadequate), others have suggested that the tests are overly sensitive and have a high rate of false positives (AIHC, 1995; Claudio *et al.,* 1999; Claudio *et al.,* 2000; Cory-Slechta *et al.,* 2001; US EPA, 2006). A number of diverse groups have advocated increased testing for developmental neurotoxicity (Andersen *et al.,* 2000; Nelson, 1986; NRC, 1992, 1993; OTA, 1990; Stein *et al.,* 2002; Vorhees, 1986). There have also been calls to include evaluations of endpoints not currently assessed, such as social behavior (Cory-Slechta *et al.,* 2001), pharmacokinetics (including the use of exposure and kinetic data to determine the need for direct dosing of preweaning pups) and neurochemistry (Andersen *et al.,* 2000; Dorman *et al.,* 2001), or changes during senescence (Cory-Slechta *et al.,* 2001). In addition, there have been extensive criticisms of the complexity of the study, accompanied by calls for deleting some test components from the protocol (Li, 2005) or utilizing screening approaches that incorporate DNT testing into other testing protocols (Ladics *et al*., 2005; Cooper *et al*., 2006). Critics also claim that variability of some endpoints (*e.g.,* motor activity, morphometrics) is too great to be useful (CMA 1987; Nolen 1985; York *et al.,* 2004), and that this *in vivo* test is not necessary to detect developmental neurotoxicity (Balls and Combes, 2005). These controversial opinions do not invalidate the DNT study as an important protocol for use in hazard identification and risk assessment, but rather they highlight the need for ongoing scientifically-based evaluation of this test method and the incorporation of appropriate revisions as scientific knowledge advances and as experience with the DNT studies warrants.

33. A number of efforts are underway, reviewing data from existing DNT studies, to identify ways to refine the DNT test and, if possible, reduce the number of animals used. It has been proposed that by applying certain statistical approaches to the behavioral analysis, a reduction in animal use can be achieved (Chiarotti and Puopolo 2000; Puopolo and Chiarotti 2000; Puopolo *et al.,* 2004). Reviews of historical and positive control data have pointed out the need for more standardized reporting requirements (Crofton *et al.,* 2004). Further, reviews of historical control data have identified differences among laboratories in data quality, including some laboratories with excessive variability for some parts of the test method, and suggested methods to decrease this variability (Crofton *et al.,* 1991*,* 2004; Raffaele *et al.*, 2003, 2004, 2006; Sette *et al.*, 2004). Another review project has evaluated various neuropathology assessments (*e.g.,* brain weight, standard histopathology, and morphometric assessments), concluding that variability of these measures are low (Crofton *et al.,* 2001), and that no one postmortem measure is more sensitive, with each providing important data (Raffaele *et al.,* 2005). The outcome of this continuing effort will allow better

data interpretation, help refine requirements for future testing, and also guide new methods development. Efforts are also underway to update existing reproductive toxicity testing guidelines; the inclusion of DNT endpoints, as one potential component of these updates, would be an appropriate way to reduce the number of animals required for the conduct of a stand-alone DNT study.

34. In addition to the goal of refinement of the current approach to DNT testing, there is another and more pressing driver for change in the science arena of developmental neurotoxicity. There are currently thousands of chemicals that lack even simple, basic toxicological data (*e.g.,* High Production Volume chemicals, pesticide inert ingredients, anti-microbial pesticides), but have a high potential for human exposure (NRC, 1984). Assessing potential neurotoxicological effects for these chemicals is a challenge confronting the chemical industry, international and national regulatory agencies, and associated stakeholders. New tools and methods are required to move towards a more sustainable risk assessment paradigm for these types of chemicals. While the current DNT guideline generates useful data for risk assessment purposes, this *in vivo* test is costly, time consuming, and uses a relatively large number of animals when conducted as a stand-alone study (as compared to incorporating the DNT testing into other on-going studies, such as a reproductive toxicity study). A pressing goal of future research is to develop a validated true first tier screening paradigm (*e.g.,* a high-throughput *in vitro* screening battery) that can rapidly screen large numbers of chemicals for their potential to cause developmental neurotoxicity (Lein *et al.,* 2005; Coecke *et al.,* 2006; US EPA, 2006). Coupled with development of decision frameworks (e.g., Combes *et al.,* 2003), data from these high-throughput screens will foster prioritization of any further testing *in vivo*. Data generated by the current DNT test method will be vital in the validation of these high-throughput *in vitro* methods, providing information on the utility and limits of these methods, as well as guidance on the potential use of data from these alternative methods in a risk assessment context.

CONCLUSIONS

35. OECD Test Guidelines are periodically reviewed in the light of scientific progress, changing assessment practices and animal welfare considerations, and the TG 426 should be no exception. Currently, a number of activities are underway for development of alternative methods to TG 426, or for replacement of certain parts of TG 426. An adopted TG 426 used by OECD member countries will generate new data for risk assessment and further the development of new approaches to DNT testing. The OECD supports the 3R's (*i.e.*, refinement, reduction, replacement), works on alternatives to TG 426 (or parts of it) and would welcome any revision of TG 426 that would better meet the animal welfare considerations. However, any suggestion for replacements of components of the TG 426 needs to be in compliance with the OECD submission and adoption process of new or updated Test Guidelines (OECD, 2006) and subjected to approval by the WNT. In addition, the performance of a revised TG will have to be demonstrated before being adopted as a new TG 426, as described by the Guidance Document No. 34 in the section on test batteries:

> *"…Component test methods of test batteries are treated as individual test methods for validation purposes and it is necessary to demonstrate that the combination of test methods produces reliable and relevant results and is more effective than the individual tests. In general, substitution of any component of the battery should improve its performance." [GD34, paragraph 28]*

36. The DNT TG 426 represents the best available science for assessing the potential for developmental neurotoxicity in human health risk assessment, and data generated by DNT testing are relevant and reliable for the assessment of these endpoints. The test methods used in the DNT have been subjected to an

extensive history of international validation, peer review and evaluation which is contained in the public record. The reproducibility, reliability and sensitivity of these methods have been demonstrated, utilizing a wide variety of test substances. Multiple, independent, expert scientific peer reviews affirm these conclusions, as described in this document. The DNT TG provides an outline of behavioral domains and morphological endpoints that are relevant to human neurodevelopment that should be examined to assess potential developmental neurotoxicity of a test compound. The results from DNT studies are used for hazard/risk assessment purposes and in cases where data from a DNT study are not presented, safety factors may be employed by regulators to address the need for DNT data from a regulatory standpoint. This document shows that a variety of chemicals have been tested for DNT constituting a sampled spectrum of the chemical universe that the test is proposed to investigate. Several published reports outlined herein show that the DNT study is robust and can be conducted in multiple laboratories with consistent performance.

37. The TG 426 is considered to meet the regulatory needs and regulatory requirements of OECD member countries as partly outlined in Guidance Document No. 34, and as is described by the extensive documentation of the performance of the DNT study in this and other documents. The DNT study has received extensive validation and is considered valid for its intended purpose and regulatory use.

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