Alternative Test Methods for Developmental Neurotoxicity: A History and Path Forward

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The views expressed in this presentation are those of the author and do not necessarily reflect the views or policies of the U.S. EPA.
Outline

- Brief History of DNT Guidelines and Efforts to Promote In Vitro
- The Problems
  - Evidence of Increasing developmental neuro ‘diseases’
  - Thousands and thousands of chemicals with no hazard info
- The Importance of Matching Data Type to the Decision Context – “fit for purpose”
- Demonstrating Progress
- Suggestions for Path Forward
A Brief History of DNT
Historical Contributions to DNT Guidelines

From Makris et al., EHP 2009

This work led to, and supported the development of EPA and OECD Guidelines

- OECD 2007
A Brief History of DNT Efforts to Encourage In Vitro

A long-series of workshops have been held specifically to promote the development and use of in vitro DNT for replacement of animal testing and regulatory use.

- 2005 - In Vitro Alternative Methods for DNT, Ispra, Italy (Coecke at al. EHP, 2007)
- 2006 - DNT TestSmart I (Lein et al. EHP, 2007)
- 2008 - DNT TestSmart DNT II (Crofton et al. ALTEX 2011)
- 2011 - DNT TestSmart III (Bal-Price et al. ALTEX 2012)
- 2014 - DNT TestSmart IV
- 2014 - ISTNET DNT (Bal-Price et al., Arch Toxicol 2015)
- 2016 – OECD/EFSA Workshop
Problem: Evidence for Increasing Incidence of Neurodevelopmental Disorders

- Prevalence of neurodevelopmental diseases in children increased (Atladottir et al. 2015; Landrigan et al 2012)
- Overall estimates that 10-15% in children (Grandjean & Landrigan, Lancet 2014)
- Genetic factors account for no more than 30–40% (NRC, 2000)
- Includes: autism spectrum, ADHD, dyslexia, OCD, Tourette’s

- McDonald and Paul (2010)
  - Identifies ‘break point” for increases in autism
  - Provides a time frame for before and after
Problem: The Chemical Universe

1974 US NRC report

- Major challenge is too many chemicals and not enough data
- Estimated number of chemicals = 65,725
- Number of chemical with no toxicity data of any kind = 46,000

<table>
<thead>
<tr>
<th>Category</th>
<th>Size of Category</th>
<th>Estimate Mean Percent In the Select Universe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pesticides and Inert Ingredients of Pesticides Formulations</td>
<td>3,350</td>
<td>10 24 2 26 38</td>
</tr>
<tr>
<td>Cosmetic Ingredients</td>
<td>3,410</td>
<td>2 14 10 18 56</td>
</tr>
<tr>
<td>Drugs and Excipients Used in Drug Formulations</td>
<td>1,815</td>
<td>18 18 3 36 25</td>
</tr>
<tr>
<td>Food Additives</td>
<td>8,627</td>
<td>5 14 1 34 46</td>
</tr>
<tr>
<td>Chemicals in Commerce: At Least 1 Million Pounds/Year</td>
<td>12,860</td>
<td>11 11 34 78</td>
</tr>
<tr>
<td>Chemicals in Commerce: Less than 1 Million Pounds/Year</td>
<td>13,911</td>
<td>12 12 76</td>
</tr>
<tr>
<td>Chemicals in Commerce: Production Unknown or Inaccessible</td>
<td>21,752</td>
<td>10 8 82</td>
</tr>
</tbody>
</table>

Complete Health Hazard Assessment Possible
Partial Health Hazard Assessment Possible
Minimal Toxicity Information Available
Some Toxicity Information Available (But Below Minimal)
No Toxicity Information Available

US National Research Council, 1984
Matching Data Type and Uncertainties to Decision Context

It is critical to understand the uncertainties in the data

and

Match them to the regulatory decision context
### Data Types & Chemical Risk Decisions

<table>
<thead>
<tr>
<th>EPA Office</th>
<th>Assessment “Workflows”</th>
<th>Historical Throughput</th>
<th>Data Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPPTS</td>
<td>Premanufacture Notice (PMN)</td>
<td>~1000/yr 90d/chem</td>
<td>III (II)</td>
</tr>
<tr>
<td></td>
<td>New chemicals</td>
<td>~84,000 total</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Significant New Use Rule (SNUR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Existing chemicals</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Current Chemical Risk (new program)</td>
<td>~10 total</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>DFE / Green Chemistry</td>
<td>~2500</td>
<td>I, II, III</td>
</tr>
<tr>
<td>OSCP</td>
<td>Endocrine Screening Program</td>
<td>~10-20/year</td>
<td></td>
</tr>
<tr>
<td>OPP</td>
<td>Pesticide registration (PR)</td>
<td>~10 new/yr ~50 old/yr</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pesticide re-registration</td>
<td>~1000/yr 24,576 total</td>
<td>I</td>
</tr>
<tr>
<td>OW</td>
<td>Chemical Contaminant List</td>
<td>~6yr ~6,000 total</td>
<td>I, II, III</td>
</tr>
<tr>
<td></td>
<td>Regulatory Actions on CCL</td>
<td>6yr 90 total</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Unregulated Contaminant Monitoring</td>
<td>30/5yr 90 total</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Drinking Water Health Advisories (MCLs)</td>
<td>~80 total</td>
<td>II, III</td>
</tr>
<tr>
<td>ORD NCEA</td>
<td>IRIS</td>
<td>~3/yr ~540 total</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>PPRTV</td>
<td>400-500</td>
<td>II, III</td>
</tr>
</tbody>
</table>

I. Data rich – Extensive guideline studies

II. Data partial – Some acute in vivo and in vitro data, SAR and exposure modeling

III. Data minimal to none – only chemical structure, SAR and exposure modeling

Courtesy of I. Shah
The aim is efficiency

- Develop only the data really needed for making regulatory decisions using the least amount of resources possible
- Provides a process for more efficient risk management
Progress To Date

In Vivo Guidelines

In Vitro Data
Progress to Date – In Vivo

How to visualize the problem of 60,000 Chemicals and not many DNT studies?

Black dot = no DNT study
Red dot = DNT study*

* ~0.2% DNT Guideline Studies
Progress to Date – In Vitro

Critical Science Challenges for DNT*

• Develop and evaluate in vitro assays for application to DNT
• Develop reference chemicals for demonstration of predictability
• Generate data for lots of chemicals
• Develop tiered testing and decision frameworks
• Build open databases to share and compare methods and results

Based on DNT I, DNT 2, and 2007 Talk at CAAT 25th Anniversary Meeting
Over the past 2 decades there has been development of in vitro assays for a variety of DNT processes:

- Differentiation into NPC
- NS/PC proliferation
- NPC apoptosis
- Radial glia proliferation
- NPC/NPC
- Neuronal/glial migration
- Astrocyte differentiation
- Oligodendrocyte differentiation
- Glia maturation
- Myelin formation
- Neuronal differentiation
- Dendritic spine formation
- Dendrite formation
- Neurite outgrowth
- Synaptogenesis

✓ = Ready-to-go Assay Available
✗ = No Ready-to-go Assay Available, yet cell system available

No reason not to start fit-for-purpose use

Fritsche, 2016
Progress to Date
Need for Reference Chemicals

• Over the past 5 years multiple reviews of in vivo and in vitro data to generate lists of reference chemicals
• Kadereit et al Front. Biosci 2012.
  – Criteria for selection and use of “gold standards”
  – List of XX chemicals
• Mundy et al 2015
  – GRADN list
  – 100 chemicals with evidence of development neurotoxicity
• Aschner et al ALTEX 2106
  – ~100 compounds (including negative controls) to address specificity, adversity and use of alternative test systems.
  – ~50 endpoint-specific controls and 33 “bona fide DNT toxicants”

Need consensus on lists
Progress to Date - Data Generation Examples

- There has been less progress on the generation of data for chemicals (see Fritsche EFSA/OECD Report)

- Data collections
  - Mundy & colleagues – synaptogenesis, proliferation, apoptosis, neurite outgrowth, viability
  - Leist & colleagues – neurite outgrowth, migration, viability
  - Shafer & colleagues – MEAs, viability
  - Biel et al (2015) - Proliferation, viability, neurite outgrowth, MEAs
  - NTP 80
    - Multiple labs and assays
    - EPA Organophosphates Project

* Based on my previous expectations and lack of patience!
Total of 70 chemicals

10 Endpoints
- human and rat cells
- viability
- neurite outgrowth
- synaptogenesis
- proliferation
- apoptosis

Values are - log(E30)
- Pink (0) = no effect
- darker red = more potent

- Ranking by clustering - combination of potency, neuro-endpoints and viability

Allows prioritization by:
1. Overall potency
2. Selectivity for neurodevelopment endpoints (not shown)

Note: High priority chemicals tend to be similar for both approaches.
Leist and colleagues – Neurite Outgrowth Comparison to Tox21 Assays

- Many compounds more sensitive in neurite outgrowth assays compared with current Tox21 assays

  - This suggests value of adding these models to expand current biological space of Tox21

- May want to consider testing some of these compounds in vivo for further hazard characterization

Courtesy of M. Leist
Shafer and colleagues
Screening ToxCast Chemicals with MEAs

“Acute” Assay
• 1080 ToxCast Phase 1 & 2 single concentration
• 384 ‘hits’ were then run in concentration
• Good separation between cell viability and reduced firing rates
• Provides functional measure of neural activity

Public release of data release via ToxCastDB in 2017

Courtesy of T. Shafer
Shafer and colleagues
Results for “Developmental” MEA

- Total of 170 chemicals (so far)
- Mundy List (70), NTP80 (50), ToxCast (50)
- 15 measures of neural activity
- Exposure throughout network development

- Allows prioritization by overall potency
- Provides functional measure of neural activity in a “developmental” context
- Can a signature pattern be developed that predicts targets?

- Purpose: Compare BFRs to replacement OP-FRs via bioactivity
- Use battery approach – in vitro devtox and DNT assays
  - proliferation, viability, neurite outgrowth, MEAs, cytotoxicity, devtox assays
- 11 organophosphate and brominated flame retardants
- Compare in vitro PODs

Conclusions:
- no one endpoint was always best
- similarity of bioactivity for replacements suggests need for follow-up testing
Leist and colleagues

NTP80 and Neural crest assay

- Measures both migration and viability with good separation for hits
- Great example of how larger datasets allows for examination of relationships between chemical properties and bioactivity

(Nyffeler et al., unpublished)
EPA OPP-NHEERL Organophosphate Project

- The effects of OPs on neurodevelopment are likely multi-target based. Some caused by AChE inhibition and others due to unknown mechanism(s).
  - Epi studies in children show DNT outcomes at doses that cause AChE inhibition.
- In 2015 OPP - ORD started a project to develop data for 27 OPs using in vitro DNT assays as well as zebrafish in order to:
  - determine whether such data may be useful in reading across from data rich to data poor (no in vivo DNT data) chemicals
- Work is ongoing

Preliminary Data
- In vitro only
- Suggest not all are the same
- Does not contain MEAs or zebrafish endpoints

Courtesy of W. Mundy
Proposed tiered DNT testing:

Tier 0: Pharma-/Toxicokinetic Modelling
- Kinetic Information on Internal exposure and metabolites

Tier 1: Human in vitro Testing Battery
- Most sensitive endpoint (MSE) evaluation

Tier 2: Alternative Model Organism Testing
- Inter-species comparison

Tier 3: Rodent in vitro Testing
- Only in vivo testing when no species-specific MoA

Tier 4: Optional rodent in vivo Testing

Major Discussion Topic At Meeting
Progress to Date – Build Open Databases

Multiple databases available to deposit datasets
No use yet for DNT data
Critical Science Challenges for DNT*

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*Based on DNT I, DNT 2, and 2007 Talk at CAAT 25th Anniversary Meeting
Ideas for Focusing Research Efforts Going Forward

• Must develop data for MORE CHEMICALS – testing of large chemical libraries inform:
  – Potential assay confounds - auto fluorescence, protein denaturation etc
  – Allows for better predictive models – read across,
  – Will foster development of DNT ‘chemotypes”
  – Patterns across multiple assays at relevant concentrations will increase confidence in use for more than prioritization “risk” decisions

• Better relationships between risk managers and scientists
  – Don’t just develop a new assay – develop assays and data that provide the information needed to make risk decisions
  – Scientists - talk to the risk managers here at the meeting – If you don’t understand their problems how do you solve them?

• Build data sharing opportunities
  – Start combining work to compare across multiple labs and multiple types of assays
A Couple of Cautionary Issues

• On the issue of “validation”
  – Remember that the idea is “fit-for-purpose’
  – Amount of effort to validate for replacement of animal guidelines must be very different than use for prioritization or support for read across

• Time is against us – technology is evolving at a very rapid pace
  – New biotechnologies promise better biological coverage
  – Currently testing new ‘global’ genomics technologies that promise ability to tests entire genome for low prices
    • e.g., Biospyder – whole human genome on cell lysates
      http://biospyder.com/technology/
  – Don’t wait for perfection
  – Always be willing to adapt to new and better technologies
    (remember - the DNT guidelines are based on technologies from the 70’s and 80’s)
“Do not let the perfect be the enemy of the good”  

Voltaire

“Do not let the perfect get in the way of developing and using in vitro data for use in risk assessments”  

Crofton