Screening for Developmental Neurotoxicity: An In Vitro Approach using High Content Analysis

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Allegheny-Erie Regional SOT
Testing Needs

- EPA is charged with protecting public health and the environment
- Public concerns/perceptions about neurodevelopmental disorders (e.g., ADHD, autism) has increased pressure to test environmental chemicals
- Large chemical inventories (1000’s) with little or no toxicity data
  - e.g., pesticide inerts, HPVs, CCLs, REACH

*Challenge: Provide data that characterizes hazard for use in risk decisions*

*No data does not equal no risk*
Regulatory Tools for DNT

Testing Guidelines
• EPA 870.6300 DNT Guideline
• OECD 426 DNT Guideline

• Basic Requirements – *in vivo – rat is primary species*
• Developmental exposure followed by assessments of:
  • Growth and developmental landmarks
  • Motor and sensory behaviors
  • Cognitive function
  • Neurohistopathology and morphometrics

  $0.7 – 1.0$ million per chemical
  1.5 years per study
Use of Current Regulatory Tools

Since 1991 we have tested only ~104 chemicals

<table>
<thead>
<tr>
<th>Chemical class</th>
<th>Number of studies</th>
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</thead>
<tbody>
<tr>
<td>Industrial Chemicals</td>
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<tr>
<td>Miscellaneous Agents*</td>
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<tr>
<td>Pharmaceuticals</td>
<td>3</td>
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<tr>
<td>Pesticides</td>
<td>73</td>
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<tr>
<td>Positive Control Chemicals</td>
<td>15</td>
</tr>
<tr>
<td>Solvents</td>
<td>7</td>
</tr>
</tbody>
</table>

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

Makris et al. EHP (2008)
Current Testing Approach versus Reality

- Assume that the list of untested compounds is 10,000
- Assume that a DNT take 6 months
- Assume that a DNT costs $750,000
- Assume that there are 10 contract/industry labs that can do a GLP DNT

- Total cost = $7,500,000,000
- Total time = 500 years
Current Testing Approach versus Reality

• We cannot test our way out of this problem using current methods!

_We need new approaches that are faster and more cost-efficient_
Alternative Methods for DNT (screening for prioritization)

Goal
• Develop a battery of *in vitro* tests that predict DNT

Result
• Use as 1st tier screen that is fast and efficient (*hazard identification*)

• Provide data for prioritization of chemicals for further testing (targeted based on MOA?)
Research Approach

- Battery of *in vitro* tests based on key events of brain development
  - proliferation, differentiation, migration, neurite growth, synaptogenesis, myelination
- Endpoints amenable to high throughput testing
  - cell-based endpoints, biomarkers, molecular signaling
- Show predictive ability based on “test set” including known developmental neurotoxicants
Development of a test battery based on assays for key events

1. Evaluate neural cell cultures as model of key event in neurodevelopment

2. Develop *in vitro* method using endpoint that is amenable to high throughput testing

3. Evaluate ability of assay to detect key event using a “training set” (chemicals with known effect *in vitro*)

4. Determine ability of test battery to correctly detect developmental neurotoxicants using a “test set”
Development of a test battery based on assays for key events

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Models of key events in neurodevelopment

- Does key event occur reliably in the cell culture model?
- Is cell type widely available?
- Are special culture conditions needed (e.g., expensive media, prolonged culture time)?

**Proliferation**
- cell lines
- neural stem cells

**Differentiation**
- cell lines
- neural stem cells

**Neurite growth**
- differentiated cell lines
- differentiated stem cells
- rodent primary neurons

**Cell Lines**

- **SH-SY5Y**
- **ReNcell Cx**
- **PC12**
Development of a test battery based on assays for key events

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In vitro high throughput screening assays

• Is the endpoint measured specific for the key event?

• Is the endpoint quantitative?

• Can you assess multiple endpoints at the same time? (e.g. key event and viability)
Cell-based Assays using High Content Screening

Provides data at the level of the individual cell

High throughput due to automated data acquisition and analysis in multi-well plates

*High content* based on amount of data in a single image

• Microscope and digital camera *in a box*
• Automated stage movement, exposure, and focusing capabilities
• Computer algorithms analyze the images to provide cell-based data (*e.g.* size, shape, location, fluorescence intensity)
Neurite Outgrowth – process by which a relatively undifferentiated/immature cell elaborates specialized processes (neurites) to achieve a mature neuronal phenotype
Neurite Outgrowth – high content assessment

Automated image acquisition and analysis of PC12 cells using Cellomics ArrayScan

- culture cells in 96-well plate
- at desired time fix cells
- immunostain for markers of nucleus, cell body, neurites

channel 1
nuclei (DAPI)

channel 2
cell body and neurites (tubulin)

image analysis software
Neurite Outgrowth – high content assessment

Data report from automated image analysis of NGF-induced neurite outgrowth in PC12 cells using Cellomics ArrayScan

- n = 250 cells from one well (x 8 endpoints = 2000 data points = high content)
- image acquisition and analysis time = 15 sec per well
Control studies in PC12 cells

NGF stimulates neurite growth (96 hours)

Neurite growth increases over time (100 ng/ml NGF)

Pharmacologic inhibition (internal control)

Cells were treated with NGF and Bis-1 (a PKC inhibitor) at time 0 and neurite length assessed at 96 hours
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Evaluation for chemical screening

- Develop a training set (positive and negative controls) for the key event.
- Determine concentration range for testing.
- Evaluate selectivity for endpoint (compared to measures of cell viability).
- Is assay capable of high(er) throughput (100’s – 1000’s of chemicals per week)?
## Training Set for Neurite Outgrowth

### Positive

<table>
<thead>
<tr>
<th>Chemical</th>
<th>DNT in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>K252a</td>
<td>nd</td>
</tr>
<tr>
<td>U0126</td>
<td>nd</td>
</tr>
<tr>
<td>Okadaic Acid</td>
<td>nd</td>
</tr>
<tr>
<td>Vincristine</td>
<td>+</td>
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<tr>
<td>Lead Acetate</td>
<td>+</td>
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<tr>
<td>Valproic Acid</td>
<td>+</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>+</td>
</tr>
<tr>
<td>Methylmercury</td>
<td>+</td>
</tr>
<tr>
<td><em>trans</em>-Retinoic Acid</td>
<td>+</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>+</td>
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</tbody>
</table>

*nd = no data*

### Negative

<table>
<thead>
<tr>
<th>Chemical</th>
<th>in vitro/in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl phthalate</td>
<td>-</td>
</tr>
<tr>
<td>d-Sorbitol</td>
<td>-</td>
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<tr>
<td>Acetaminophen</td>
<td>-</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>-</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>-</td>
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<tr>
<td>Diphenhydramine</td>
<td>-</td>
</tr>
<tr>
<td>Saccharin</td>
<td>-</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>-</td>
</tr>
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*nd = no data*
Patterns of Effects - Neurite Outgrowth and Cytotoxicity (96hr exposure)

1) No effect

2) Outgrowth inhibition at cytotoxic concentrations

3) Outgrowth inhibition at concentrations that are not cytotoxic

Diphenhydramine

Dexamethasone

trans-Retinoic Acid

Graphs show the effects of different compounds on neurite outgrowth and cytotoxicity.

- Diphenhydramine: No significant effect on neurite outgrowth.
- Dexamethasone: Outgrowth inhibition at cytotoxic concentrations.
- trans-Retinoic Acid: Outgrowth inhibition at concentrations that are not cytotoxic.

Graph details:
- X-axis: Concentration in Molar (M)
- Y-axis: % Control
- Green line: Total Neurite Length
- Red line: Cell Titer Glo Viability

RESEARCH & DEVELOPMENT
Building a scientific foundation for sound environmental decisions
4) Outgrowth facilitation at concentrations that are not cytotoxic

Omeprazole

- Neurite Outgrowth and Cytotoxicity (96hr exposure)

Patterns of Effects - Neurite Outgrowth and Cytotoxicity (96hr exposure)

- Neurite Outgrowth and Cytotoxicity (96hr exposure)
## Training Set Results

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8/10

### Negative

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<th>Neurite Growth</th>
</tr>
</thead>
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<tr>
<td><em>Dimethyl phthalate</em></td>
<td>+*</td>
</tr>
<tr>
<td>d-Sorbitol</td>
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<td>Saccharin</td>
<td>-</td>
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<tr>
<td><em>Glyphosate</em></td>
<td>+*</td>
</tr>
</tbody>
</table>

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* Increase at highest concentration tested
High content screening for Proliferation

Doubling time $\approx 35$ hr

Doubling time $\approx 40$ hr
High content screening for Proliferation

Detection of cells in S phase
- grow cells in 96-well plate
- treat with chemical for 20 hr
- add BrdU for 4 hr (incorporation into replicating DNA)
- fix in presence of DAPI dye and perform ICC for BrdU
- detect and quantify BrdU+ cells using ArrayScan

Channel 1
DAPI Dye
(detect all nuclei)

Channel 2
BrdU
(staining in mask)

Channel 2
DAPI Dye
(create mask around nuclei)
Control studies in PC12 cells
- inhibit proliferation with aphidicolin
- induce differentiation with NGF

Control studies in SH-SY5Y cells
- inhibit proliferation with aphidicolin
- induce differentiation with *trans*-retinoic acid

BrdU positive cells (% total)
## Training set chemicals for Proliferation

### Positive *(in vitro)*
- 5-FU
- Cytosine Arabinoside
- Aphidicolin
- Ochratoxin A
- Dexamethasone*
- t-Retinoic acid*
- Cadmium*
- Methylmercury*

### Negative
- Amoxicillin
- Sorbitol
- Saccharin
- Acetaminophen
- Dimethyl phthalate
- Diphenhydramine
- Omeprazole
- Glyphosate

* *developmental neurotoxicant*
Example Responses - Positives

PC12

Cytosine Arabinoside

% Control ± S.E.

log Chemical (M)

BrdU Positive

Viability

SH-SY5Y

Cadmium

% Control ± S.E.

log Chemical (M)
## Summary - Positives

<table>
<thead>
<tr>
<th>Chemical</th>
<th>PC12</th>
<th>SH-SY5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU</td>
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<td>+</td>
</tr>
<tr>
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<td>Methylmercury</td>
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<td>5/8</td>
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</table>

+  selective effect on proliferation
-  effect on proliferation and viability
-  no effect
### DNT In Vitro Status

**In Vitro Models** *(blue = human cells)*

<table>
<thead>
<tr>
<th>Key Event</th>
<th>PC12 (NS-1)</th>
<th>N1E-115</th>
<th>SH-SY5Y</th>
<th>1° Cortex</th>
<th>1° CGC</th>
<th>ReNcell VM</th>
<th>ReNcell Cx</th>
<th>EnStem A</th>
<th>ArunA hN2</th>
<th>Glial</th>
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<tbody>
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</table>

**D** = methods development (high throughput)  
**V** = validation (10-20 chems)  
**S** = Screening (NCCT_320)
# Alternative Methods - *In Vitro*

## Major Research Efforts

<table>
<thead>
<tr>
<th>Lab</th>
<th>Research Projects</th>
<th>Model systems</th>
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<tbody>
<tr>
<td>EPA - US</td>
<td>Neurite outgrowth</td>
<td>Rodent cell lines</td>
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<tr>
<td></td>
<td>Proliferation</td>
<td>Human progenitor cells</td>
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<tr>
<td></td>
<td>Differentiation</td>
<td>Primary rodent cells</td>
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<tr>
<td>ECVAM - Italy</td>
<td>Cell line development</td>
<td>Human stem cells</td>
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<td>Evoked potentials</td>
<td>Mixed re-aggregated cultures</td>
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<td></td>
<td>Membrane potential</td>
<td>Rodent cell lines</td>
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<td>ROS production</td>
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<td>TNO  Netherlands</td>
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<td>Heinrich-Heine Universität Germany</td>
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<td>apoptosis</td>
<td>Human aggregated cultures</td>
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<td>MAP Kinase signaling</td>
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How Do Go From Where We Are Now to Where We Need to Be

There need to be transitional stages

- Use existing methods/technology to
  - Evaluate methods
  - Generate data
- Initially build tiered testing frameworks
  - Screening for prioritization
- Shift to predictive ‘pathways’ as they become available
Transitioning To A New Paradigm

Current Practice

- Expensive, low throughput In Vivo Based Testing
  - Limited In Vivo Triggered Follow-up Studies
    - Specialized Mechanistic Studies
      - “Supporting” In Vitro Studies
Transitioning To A New Paradigm

“Near Future” – Screening for Prioritization

HTP Screening Batteries

Second Tier (alt species?)

Limited Mammalian Testing

Specialized Testing
Acknowledgements

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Theresa Freudenrich
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