EFSA conference: New approach methods (NAM) in toxicology for mechanism-based hazard assessment

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Chair inaugurated by the Doerenkamp-Zbinden Foundation,
University of Konstanz, Germany
Need of human cell-based models in toxicology

Predictivity

Species barriers

Mechanisms

Ressources, costs, throughput

https://joshmitteldorf.scienceblog.com/category/uncategorized/

https://www.innovativetesting.nl/news?page=3

https://thedailyblog.co.nz/2015/07/10/money-as-a-social-technology/

https://www.innovativetesting.nl/news?page=3
Why not the good old way…?

fashion?
Why not the good old way…?
Why not the good old way…?

Reach: > 8000 chemicals (high tonnage),
TSCA: > 8000 high production vol. chemicals,
Hundreds of pesticides,
Thousands of food additives,
etc.…

- About 200 regulatory DNT studies
- About 10 industrial chemicals
- EFSA 'claims' 34 pesticides tested*
- Number of positives unclear (no survey)
- About 14 substances with human evidence

* (unpublished)
Two reasons to consider mechanisms

I. Making sense of data

II. Generation of data
Two reasons to consider mechanisms

I. Making sense of data → Examples

A. Animal studies
   Aa: eye opening delayed by 0.5 days; altered gender balance; etc.
      (implication; relevance?)
   Ab: hyperactivity (species extrapolation; implication?)

B. Epidemiological studies
   Ba: Parkinsonism & childhood leukemia in areas of high pesticide use
      (plausibility, causality?)
   Bb: Methylmercury from fish intake and cognitive performance
      (modulation by nutrients; causality; confounding?)

C. In vitro studies
   Ca: Positive outcome in the embryonic stem cell test (EST)
      (relevance; association with adverse outcome?)
   Cb: Zebra fish altered movement in the dark
      (relevance; association with adverse outcome?)
Two reasons to consider mechanisms

**II. Generation of data**

**Principle:** ‘process control’ instead of ‘end stage control’

**Assumption I:** there are **key neurodevelopmental processes** required to form a fully functional and intact nervous system.

**Assumption II:** if **key neurodevelopmental processes** are disturbed, functional or structural deficits may arise.

**Procedure:** define and establish test methods for **key neurodevelopmental processes** and evaluate interference by test chemicals
Key neurodevelopmental processes

**Proliferation**
- Erythromyeloid Progenitor
- Neuroepithelium
- Pluripotent Stem Cell (PSC)

**Migration**
- Radial Glia
- Glial Progenitors
- Apoptosis

**Differentiation**
- Microglia (CD45, CD11b etc)
- Astrocytes (GFAP)
- Oligodendrocytes (O4, GalC, CNPase)
- Neurons (diverse neuronal markers)

**Network formation and function**
- Network formation and function
- Synaptogenesis
- Myelination

**Apoptosis**
Eventually, any DNT finding (man or animal) must be due to a combination of disturbed neurodevelopmental processes.

<table>
<thead>
<tr>
<th>In vivo Finding</th>
<th>Disturbed neurodevelopmental processes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain weight up/down</td>
<td><strong>Proliferation, Apoptosis</strong></td>
</tr>
<tr>
<td>Holoprosencephaly</td>
<td><strong>Apoptosis, Neurodifferentiation</strong></td>
</tr>
<tr>
<td>Lissencephaly</td>
<td><strong>Apoptosis, Neurodifferentiation, Migration</strong></td>
</tr>
<tr>
<td>Neuroinflammation</td>
<td><strong>Astrocyte activation, Gliosis, Neurodegeneration</strong></td>
</tr>
<tr>
<td>Cortical layer thickness</td>
<td><strong>Proliferation, Migration, Myelination</strong></td>
</tr>
<tr>
<td>Disturbed reflexes</td>
<td><strong>Neurodifferentiation, Myelination, Synaptic transmission</strong></td>
</tr>
<tr>
<td>Anxiety behaviour</td>
<td><strong>Neurodifferentiation, Synaptic transmission, Synapse formation</strong></td>
</tr>
</tbody>
</table>

*If a compound does not disturb at least one process, it cannot be associated with a DNT hazard.*
Two reasons to consider mechanisms

II. Generation of data: expectations and challenges

De novo (no prior knowledge) evaluation of a new unknown compound for classification and labelling

Screening of libraries of compounds to check for 'alerts‘ and to prioritize for further more comprehensive (resource-consuming) testing

alert: preliminary indication that there is a hazard potential; needs verification by other methods
Two reasons to consider mechanisms

De novo (no prior knowledge) evaluation of a new unknown compound for classification and labelling

II. Generation of data: expectations and challenges

Read-across (RAX):
1. anchoring toxicity of unknown compound by comparison to similar* known compound (s);
2. comparison within a category of related* compounds

Screening of libraries of compounds to check for ‘alerts‘ and to prioritize for further more comprehensive (resource-consuming) testing

* similarity extended from structure to mechanisms (and metabolism)
Two reasons to consider mechanisms

I. Making sense of data

- Plausibility, relevance
- Species extrapolation
- Causality

II. Generation of data

- Key neurodevelopmental processes
- Gap-filling / screening / prioritization
- Read-across (RAX)
- De novo evaluation
What is wrong with descriptive data

(Often outdated technology)

Data always describe a **model** – **not the reality**!

→ always an extrapolation required (**uncertainty**)
→ poor explanation of uncertainty
→ implicit mechanistic assumptions (not rationalized and validated)

Example: mouse cancer bioassay
Perfect description, but wrong model (< 60% concordance)
Is a mechanistic approach less direct?

<table>
<thead>
<tr>
<th>Level</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct observation</td>
<td>Altered light-dark behaviour</td>
</tr>
</tbody>
</table>

Processes disturbed (during development)

Migration/Differentiation

Mechanistic correlate/endpoint

Hit in Migration/Differentiation assay
Is a mechanistic approach less direct?

<table>
<thead>
<tr>
<th>Level</th>
<th>Parameter</th>
<th>Human situation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct observation</td>
<td>Altered light-dark behaviour</td>
<td>Meaningless</td>
</tr>
<tr>
<td>Interpretation (theoretical construct)</td>
<td>Anxiety</td>
<td></td>
</tr>
<tr>
<td><strong>Endophenotype</strong> (measurable change in structure or connectivity)</td>
<td>Altered function/structure of amygdala (limbic system)</td>
<td></td>
</tr>
<tr>
<td>Processes disturbed (during development)</td>
<td>Migration/Differentiation</td>
<td></td>
</tr>
<tr>
<td>Mechanistic correlate / endpoint</td>
<td>Hit in Migration/Differentiation assay</td>
<td></td>
</tr>
</tbody>
</table>
Example:
Test of early brain/spinal cord development
Valproic acid (VPA) (anti-epileptic)

→ Failure of neural tube closure

forms spinal cord
Cellular model: Neural differentiation from hiPSC

iPSC (Oct4, Nanog)  
day -3  
human pluripotent stem cells

NEP (Pax6, Otx2)  
day 6  
neuroectodermal progenitors

Rosettes  
day 14

-3 -2 -1 ROCK, bFGF, Noggin, dorsomorphin, SB 431542  
11 13 14 FGF2, AA  
DMEM-F12 KSR
Cellular model: Neural differentiation from iPSC

**hiPSC**
- day -3

**lineage specification**
- ectoderm / neuroectoderm

**NEP**
- day 6

**differentiation**
- functional anchoring

**Rosettes**
- day 15

**Images:**
- Oct4
- Pax6 / Nestin
- PAX6/Nestin
Relevant concentration range – concentration response of valproic acid (VPA)

Gene expression changes start with 350 µM VPA
no cytotoxicity observed in this range
Functional anchoring after VPA treatment

- After 6 days of treatment, rosettes formation is disturbed.
- The gene expression changes have functional consequences on differentiation.
<table>
<thead>
<tr>
<th>VPA analogues</th>
<th>CAS-Nrs</th>
<th>structure</th>
<th>In vivo response</th>
</tr>
</thead>
<tbody>
<tr>
<td>VPA</td>
<td>99-66-1</td>
<td><img src="image" alt="VPA structure" /></td>
<td>+++</td>
</tr>
<tr>
<td>2-ethyl-hexanoic acid</td>
<td>149-57-5</td>
<td><img src="image" alt="2-ethyl-hexanoic acid structure" /></td>
<td>+++</td>
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<tr>
<td>4-ene-VPA</td>
<td>1575-72-0</td>
<td><img src="image" alt="4-ene-VPA structure" /></td>
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<tr>
<td>2-n-propyl-heptanoic acid</td>
<td>31080-39-4</td>
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<tr>
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<td>4536-23-6</td>
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<tr>
<td>2-ethyl-butyric acid</td>
<td>88-09-5</td>
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<tr>
<td>2,2-dimethyl-pentanoic acid</td>
<td>1185-39-3</td>
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<tr>
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<td>Hexanoic acid</td>
<td>142-62-1</td>
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</tr>
</tbody>
</table>

Valproate (VPA) analogues and their *in vivo* response
Testing Strategy

Concentration – Response
C1 = 5 mM
Endpoint: Resazurin reduction

Curve fitting (Graph Pad, 4 parameter fit)
Determination of EC10

Concentrations around EC10
Endpoint day 6: RT-qPCR, gene expression

EC10 > 2.5 mM?

Concentrations around EC10
Endpoint day 15: rosettes formation

PAX6, OTX2 & AP2 changed?

Rosettes reduced > 75% of control?

No hit

Hit
Example for a hit: 4-ene-VPA

Viability

Gene expression: 1.2 & 0.625 mM

Testing in non-cytotoxic range
Expected gene expression changes
Inhibition of rosettes formation
## Summary Table

**3 clear hits:**
- Valproic acid: in vivo positive ✓
- 2-Ethylhexanoic acid: in vivo positive ✓
- 4 ene VPA: in vivo positive ✓

**3 are unclear:**
- Hexanoic acid: in vivo unknown
- 2-Methylhexanoic acid: in vivo negative
- 2-methyl-pentanoic acid: in vivo unknown

**2 clear Negatives:**
- 2 Ethylbutyric acid: in vivo negative ✓
- 2,2-Dimethylvaleric acid: in vivo negative ✓
Results from a test battery

<table>
<thead>
<tr>
<th>Analogues</th>
<th>In vivo NTD</th>
<th>ZET EC10</th>
<th>ZET reporter</th>
<th>EST (c) IC50</th>
<th>UKN IC10</th>
<th>CALUX</th>
</tr>
</thead>
<tbody>
<tr>
<td>VPA</td>
<td>+++</td>
<td>10</td>
<td>+++</td>
<td>378</td>
<td>600*</td>
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</tr>
<tr>
<td>4-ene-VPA</td>
<td>++</td>
<td></td>
<td>++</td>
<td>518</td>
<td>534*</td>
<td></td>
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<tr>
<td>2-ethyl hexanoic acid</td>
<td>+++</td>
<td></td>
<td>++</td>
<td>1115</td>
<td>943*</td>
<td></td>
</tr>
<tr>
<td>2-propyl heptanoic acid</td>
<td>+++</td>
<td>10</td>
<td>+++</td>
<td>365</td>
<td>208*</td>
<td></td>
</tr>
<tr>
<td>2-ethyl butyric acid</td>
<td>-</td>
<td></td>
<td>-</td>
<td>&gt;3000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,2-dimethyl pentanoic acid</td>
<td>-</td>
<td></td>
<td>+</td>
<td>&gt;3000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-methyl pentanoic acid</td>
<td>?</td>
<td>250</td>
<td>+</td>
<td>&gt;3000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(note: data without PBPK correction)

**negative** (in vitro / in vivo)

**unclear/ intermediate** (in vitro / in vivo)

**positive** (in vitro / in vivo)
Summary

1. Mechanistic risk assessment adds value to data

2. Mechanistic risk assessment allows for new NAM-based approaches

3. A battery of tests for key neurodevelopmental processes is available and has been successfully used in case studies

4. There is an educational need on all sides to understand strengths and weaknesses of the new approaches; discussions of case studies can provide a platform
Acknowledgement

Bob van de Water
Hennicke Kamp and many others