AN INTERSPECIES COMPARISON OF TOXICITY PATHWAYS MEDIATING NEURODEVELOPMENTAL TOXICITY IN NEUROSPHERES - AND NOVEL COMPUTATIONAL APPROACHES FOR HIGH CONTENT IMAGE ANALYSES (HCA)

Ellen Fritsche
EPA’s Computational Toxicology Communities of Practice
September 25th 2014
Cell Biological Processes performed by NPC

With courtesy from William Mundy, U.S. Environmental Protection Agency and John Havel, SRA International, Inc.
Epi-genetics

Hormones

ROS

OxPhos

Transmitter

Signaling molecules
The Neurosphere Method

Fetal Brain → Neural Stem/Progenitor Cells (NPCs, Lonza) → Culture as Neurospheres → Neural Stem/Progenitor Cells self-prepared → Fetal Brain

NPC Proliferation:
- Increase in size/time
- BrdU incorporation

NPC Migration:
- Total migration distance
- Number of migrated cells
- Neuronal migration

NPC Differentiation:
- Neurons
- Astrocytes
- Oligodendrocytes

Neural Stem/Progenitor Cells (Lonza)
Neural Stem/Progenitor Cells self-prepared
Fetal Brain

IUF
LEIBNIZ-INSTITUT FÜR UMWELT MEDIZINISCHE FORSCHUNG
The ‘Neurosphere-Assay’

Neurosphere Culture

Proliferating

Differentiating

Determination of Viability

Apoptosis

Proliferation

Migration

Differentiation

3 days

5 days

BrdU

Day 0

Day 14

GFAP β(III)tubulin DAPI

O4 DAPI

From Breier...Fritsche.. et al. Neurotoxicol Teratol 2009

Fritsche et al. Environ Health Perspect 2005
Moors et al. Toxicol Appl Pharmacol 2007
Moors et al. Environ Health Perspect 2009
Moors et al. Genes & Immunity 2010
Schreiber et al. Environ Health Perspect 2010
Gassmann et al. Environ Health Perspect 2010
Verner et al. Toxicol in Vitro 2011
Fritsche et al. Methods Mol Biol 2011
Gassmann et al. Toxicol in Vitro 2012
Bal-Price et al. ALTEX 2012
Fritsche Methods Pharmacol. Toxicol. 2014
Alépée et al. ALTEX 2014
Outline

• Thyroid Hormone (TH)
• Arylhydrocarbon Receptor (AhR)
• Valproic Acid
  – Histone Deacetylase (HDAC) inhibition
  – Apoptosis
• HCA (Cellomics Arrayscan & self-written algorithms ‘Omnisphero’)

Growth/Synaptogenesis
Apoptosis
Proliferation
Differentiation/Migration
Myelination
Functional Network
Thyroid Hormone

E11-E14 = GW 6-9

P1-7 = GW 18-30

P8-14 = GW 32-40

Ontogeny of T3 receptors in rat

Perez-Castillo et al, Endocrinology 117:2457-2461, 1985
Thyroid Hormone

Hairless expression

<table>
<thead>
<tr>
<th></th>
<th>hNPC</th>
<th>mNPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>3 nM T3</td>
<td>250</td>
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Oligodendrogenesis

<table>
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<tr>
<th></th>
<th>hNPC</th>
<th>mNPC</th>
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<tbody>
<tr>
<td>C</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>T3</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>T4</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>T3</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>T4</td>
<td>300</td>
<td>300</td>
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</table>

Oligodendrogenesis of THR α/- mNPCs

<table>
<thead>
<tr>
<th></th>
<th>Wildtype (n=4)</th>
<th>THR α/- (n=2)</th>
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</thead>
<tbody>
<tr>
<td>O4* cells [% control]</td>
<td>150 ± 10</td>
<td>50 ± 5</td>
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</table>

Oligodendrogenesis of THR β/- mNPCs

<table>
<thead>
<tr>
<th></th>
<th>Wildtype (n=4)</th>
<th>THR β/- (n=1)</th>
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<tbody>
<tr>
<td>O4* cells [% control]</td>
<td>100 ± 10</td>
<td>50 ± 5</td>
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</tbody>
</table>

Human oligodendrogenesis

Transgenic animals kindly provided by Heike Heuer, IUF
Thyroid Hormone

- Thyroid Hormone
- BDE-99
- T3
- OL formation
- OL maturation
- THRα
- IC₅₀ = 14 µM

IC₅₀ = 14 µM
Thyroid Hormone

MBP expression - hNPC

MOG expression - mNPC

BDE-99

OL maturation

T3

THRα

OL formation

IC\textsubscript{50} = 2 \mu M
AhR - migration

Gassmann et al. Environ Health Perspect 2010
AhR - gene/protein expression

Human NPC are protected against AhR-dependent toxicity of PAH due to lack of AhR expression.
Molecular mechanisms:

Proliferation:
wnt – β-catenin (Foti et al. 2013)

Apoptosis:
Bcl-2 expression (Go et al. 2011)

Differentiation:
suggested indirectly by increasing the NPC pool?
HDAC inhibition suggested (Montgomery et al. 2004)

E11-E14 = GW 6-9
P1-7 = GW 18-30
P8-14 = GW 32-40

Mech.: HDAC inhibition

Foti et al. 2013

Yochum et al. 2011
Valproic Acid - NPC proliferation

**RAT**

IC50 = 380 µM

**HUMAN**

IC50 = 756 µM

Baumann et al. in preparation
Valproic Acid- NPC differentiation

RAT

IC50 = 321 µM

HUMAN

IC50 = 3177 µM

Baumann et al. in preparation
Valproic Acid

- NPC proliferation is inhibited by VPA-dependent HDAC inhibition (Baumann et al. in prep)
- VPA does not inhibit neuronal differentiation of rat NPC, but induces neuronal apoptosis due to formation of ROS.
- Human neurons are protected against VPA-induced apoptosis.
Long-Term Culture (25d) of hNPCs in Mal-PVA Hydrogels (Cellendis)
(Hellwig et al. in preparation)
Summary (I)

- Primary Neurospheres seem to conserve NPC molecular signatures and functions *ex vivo* into *in vitro*.

- Due to the multiple neurodevelopmental processes neurospheres are apt to mimic *in vitro*, they are well suited for investigations ‘from pathway to function’.

- A variety of interspecies differences in NPC signaling seem to exist between human and rodent NPCs.

- Studying the molecular similarities/differences of neurospheres will contribute to human risk assessment for DNT, especially in the context of the ‘Adverse Outcome Pathway’ concept.
HCA in differentiating NPC

- Information on the whole migration area, not just random areas of a well.

- Software needs to distinguish between different cell types.

- Issue of a high density culture needs to be overcome and neurons correctly identified.

- Varying densities in different areas around sphere core.
High Content Image Analyses (HCA) for DNT testing

Neurite Outgrowth

Proliferation/
Viability

Synaptogenesis

From: Breier et al., Toxicological Science, 2008
Harrill et al., Neuro Toxicology, 2010
Harrill et al., Toxicology in vitro, 2011
HCA in differentiating NPC

Omnisphero

Neuron/Nuclei Identification

Neuronal Quantification

Neuronal Morphology

Migration

Neuronal Positioning

Schmuck et al. in preparation
Omnisphero: Image Pre-processing

Raw images

Thresholding (Isodata)
Watershade

Thresholding (Isodata)

Binary images

Schmuck et al. in preparation
Omnisphero: Neuron Identification

Composite Fill  Neuron Tracer  Optional: Manual annotation

Schmuck et al. in preparation
Omnisphero: Automated Analyses

Schmuck et al. in preparation
Neurogenesis: comparison of methods (IC$_{50}$ values)

<table>
<thead>
<tr>
<th>Method</th>
<th>EC$_{50}$-Values</th>
<th>EGF [ng/ml]</th>
<th>Acrylamide [mM]</th>
<th>MeHgCl [µM]</th>
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<tbody>
<tr>
<td>Manual</td>
<td>0.96</td>
<td>0.30</td>
<td></td>
<td>0.045</td>
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<tr>
<td>Neuronal Profiling</td>
<td>1.442</td>
<td>0.34</td>
<td></td>
<td>0.049</td>
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<tr>
<td>Composite Fill</td>
<td>0.67</td>
<td>0.31</td>
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<td>0.104</td>
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<tr>
<td>Neuron Tracer</td>
<td>1.022</td>
<td>0.41</td>
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<td>0.051</td>
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</tbody>
</table>

Schmuck et al. in preparation
Neurogenesis: Accuracy & Precision

Detection Power (DP)

- Acrylamide
- EGF
- Methylmercury

False-Positives (FP)

- Acrylamide
- EGF
- Methylmercury

Schmuck et al. in preparation
Neurite outgrowth: Accuracy & Precision

a) Neurite Length

b) Neurite Area

c) Number of Branching Points

Schmuck et al. in preparation
Sphere-specific Endpoints

Floodfill-Filter
Sphere-specific Endpoints: Migration distance

a) EGF

b) Acrylamide

c) MeHgCl
Neuronal Density Distribution

\[ \sigma_n = \frac{n_n(\text{Neurons})}{n_n(\text{Nuclei})} \]

\[ \sum_i^n \frac{n_n(\text{Neurons})}{n_n(\text{Nuclei})} \]
EGF transactivation of Trk receptors regulates the migration of newborn cortical neurons

Dirk Puehringer¹, Nadiya Orel¹, Patrick Lüningschrör¹, Narayan Subramanian¹, Thomas Herrmann¹, Moses V Chao² & Michael Sendtner¹
Neuronal Density Distribution

- Control (EGF)
- 0.5ng/ml EGF
- 5ng/ml EGF

- Neuron Tracer
- Neuronal Profiling
- Manual

Average slope (EGF)

Concentration EGF [ng/ml]
Summary (II)

- The Neurosphere assay needs specific algorithms for HCA: development of ‘Omnisphere’.
- Composite fill and Neuron tracer significantly improve true neuronal detection rate as a ‘conventional’ HCA endpoint.
- Sphere-specific endpoints like migration and neuronal positioning can be assessed by ‘Ominsphere’.
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LRI Innovative Science Award 2006

Thank you for your attention!
DNT – Translating Time

Translated Time

Processes:
Brain Growth & Neurogenesis, whole brain

www.translatingtime.net