Introduction to OECD Case Studies for potential Testing Strategies and a Draft Framework for building a DNT Testing Battery

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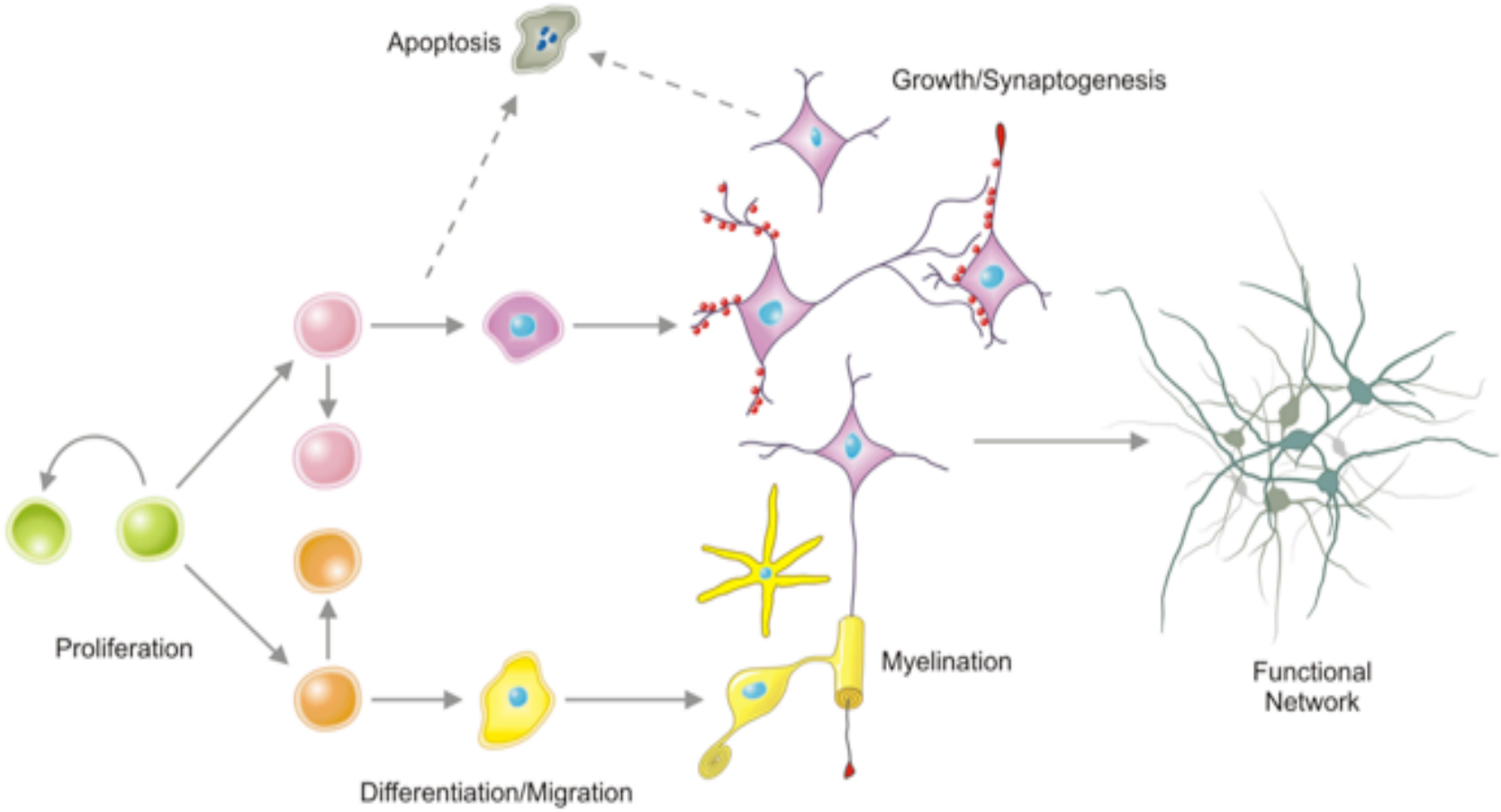
Ellen Fritsche

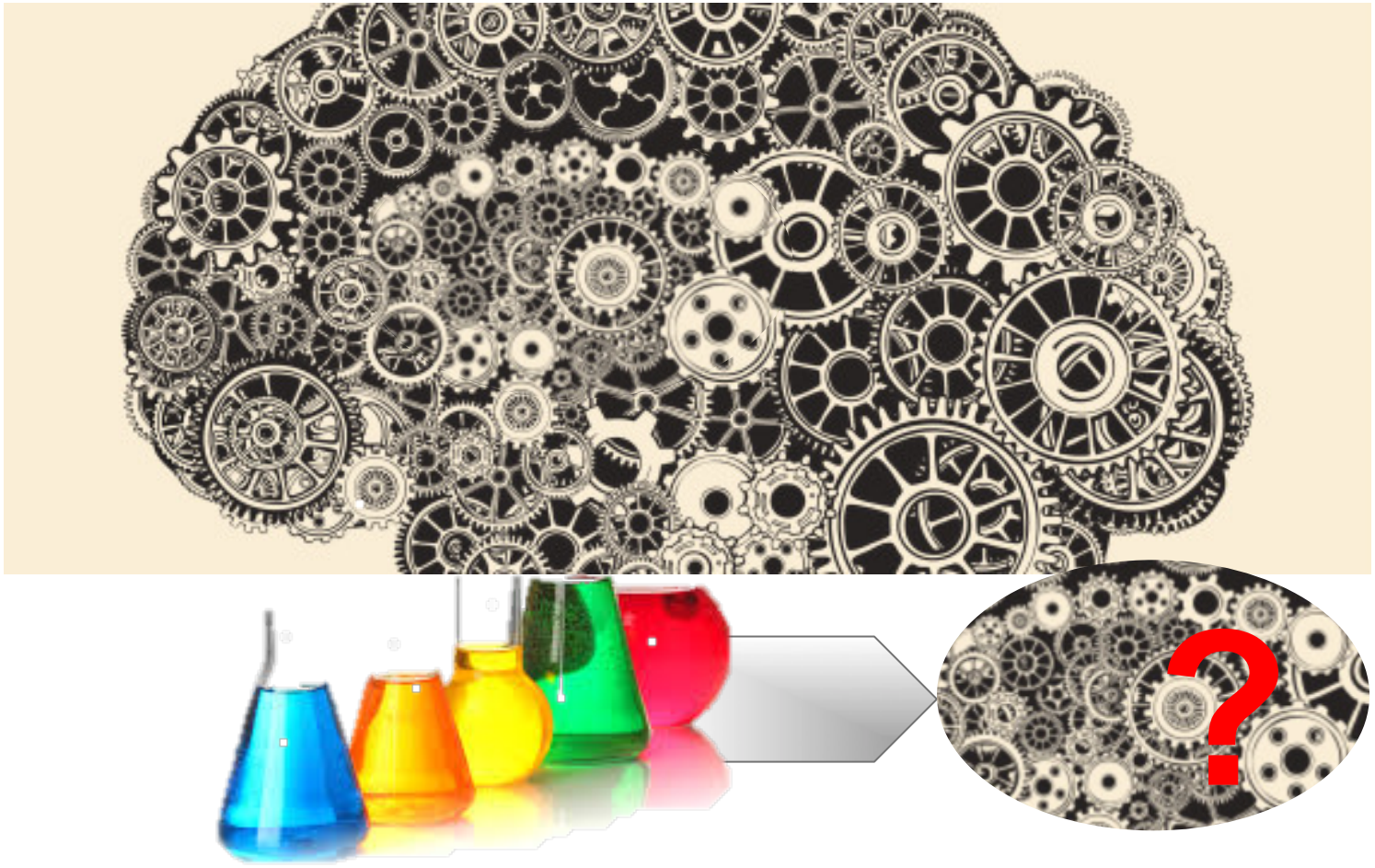
OECD/EFSA Workshop on DNT: the use of non-animal test methods for regulatory purposes
Brussels, 18-19 October 2016

Neurodevelopmental Processes serving as possible KE for DNT testing



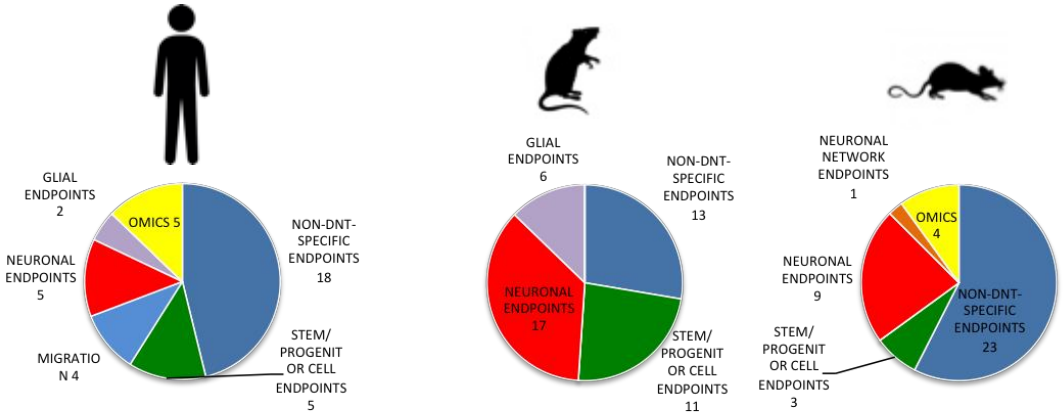
With courtesy from **William Mundy, U.S. Environmental Protection Agency** and **John Havel, SRA International, Inc.**

PROCESS-ORIENTED TESTING STRATEGY

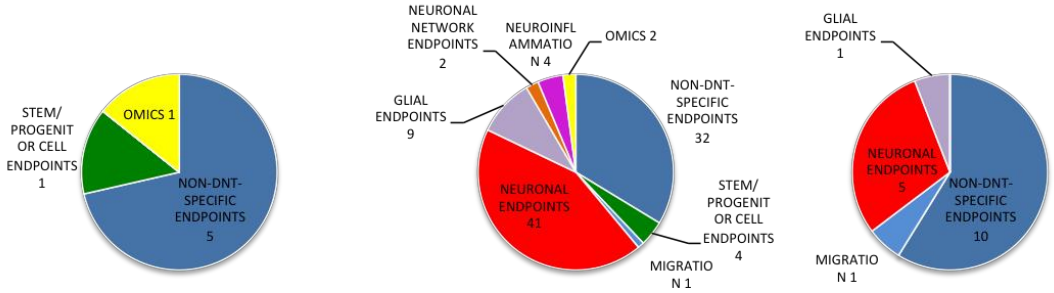


Correct Classification of Compounds: Endpoints by Cell Types & Species

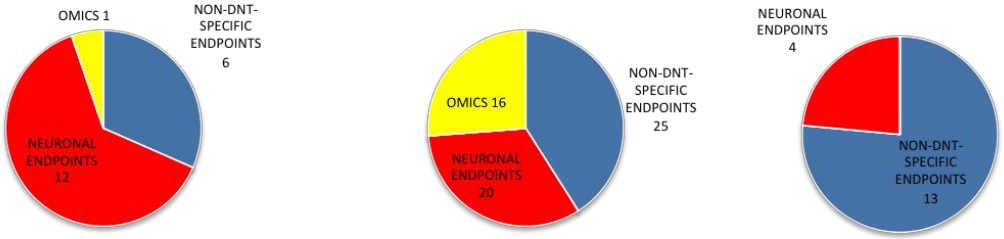
Stem-/ progenitor cells



Primary cells



Tumor/ immortalised cell lines



Fritsche et al. Literature review on in vitro and alternative Developmental Neurotoxicity (DNT) testing methods. EFSA supporting publication 2015:EN-778

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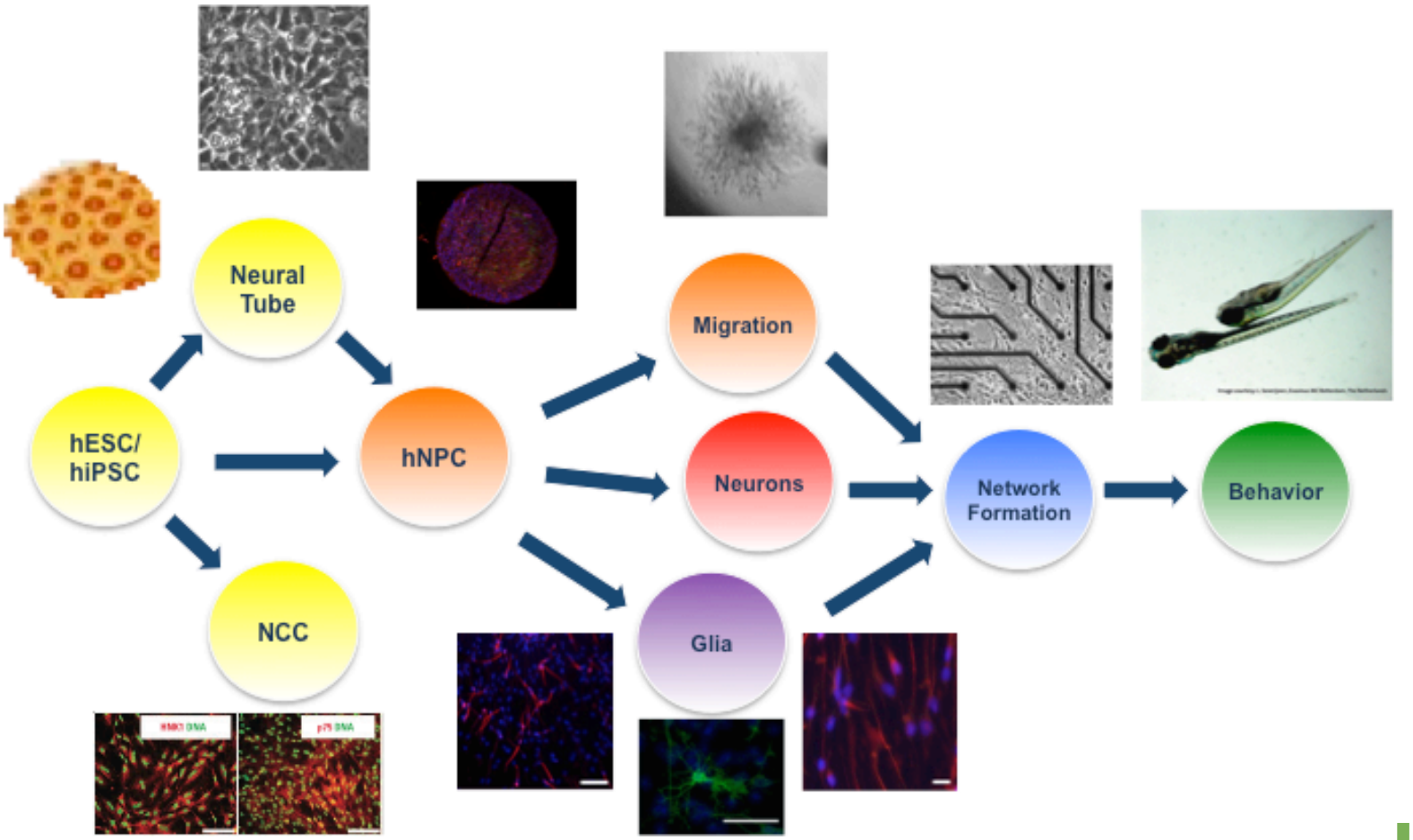
OVERVIEW OVER POTENTIAL DNT TESTING STRATEGY



Fritsche et al. EFSA supporting publication 2015

OECD REPORT ON IN VITRO METHODS FOR DNT TESTING

Strategy:

1. Identification of DNT AOPs: 6 (numbers 8, 13, 42, 54, 134, 152; https://aopwiki.org/wiki/index.php/Main_Page)

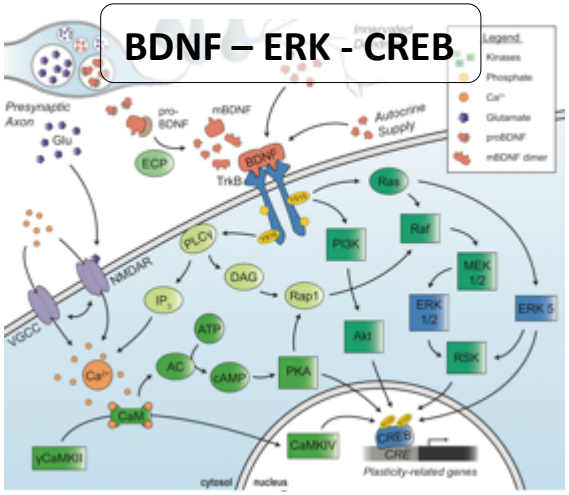
2. Compound-based MoA evaluation with regards to signaling pathways and neurodevelopmental functions

3. Signaling pathways contributing to human brain development by guiding neurodevelopmental processes

⇨ Extraction of neurodevelopmental processes necessary for brain development: **readiness analyses**

EXAMPLES OF SIGNALING PATHWAYS DRIVING NEURODEVELOPMENTAL PROCESSES

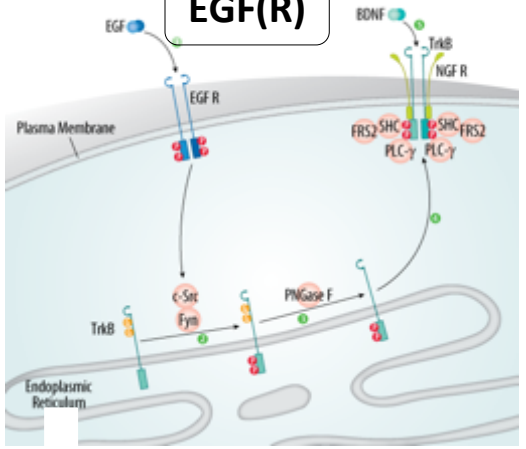
BDNF – ERK - CREB



- Proliferation/ Survival
- Dendritogenesis
- GABA circuit maturation

(Ehrlich and Josselyn, 2016)

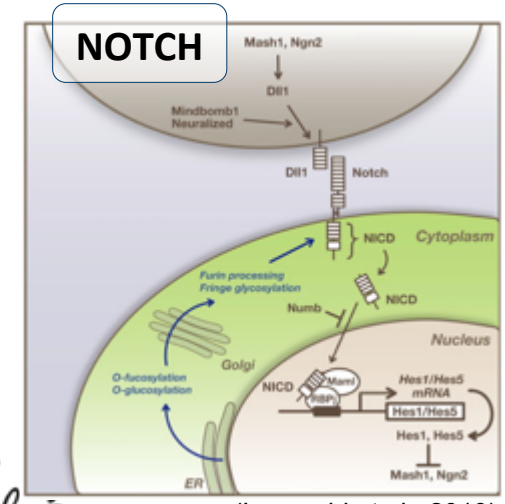
EGF(R)



- Migration ↑

(Puehringer et al. 2013)

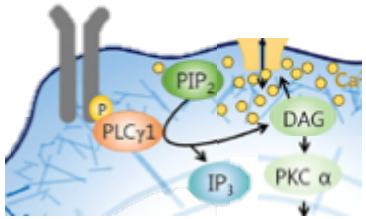
NOTCH



- Neural Stem Cell maintenance
- Neurogenesis ↓
- Gliogenesis ↑

(Imayoshi et al., 2013)

PLCγ1

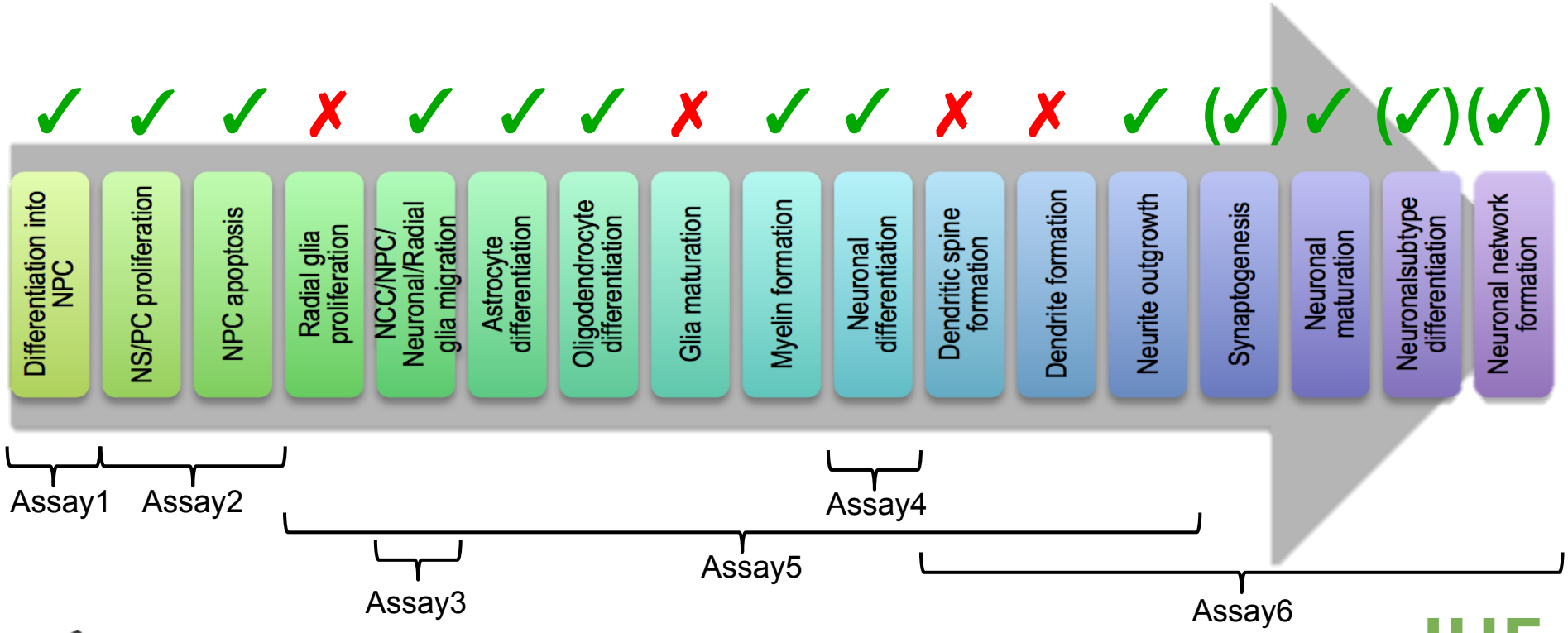


(Kang et al., 2016)

- Neurite outgrowth
- Neuronal migration
- Axon guidance
- synapse formation/ maturation

NEURODEVELOPMENTAL PROCESSES INVOLVED IN BRAIN DEVELOPMENT

Availability of HUMAN in vitro method(s) for compound testing on neurodevelopmental endpoints:



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DNT ASSAYS FOR HAZARD IDENTIFICATION ON NEURODEVELOPMENTAL ENDPOINTS

Proposed In vitro testing battery:

Assay 1: NPC differentiation

Assay 2: NPC proliferation & apoptosis

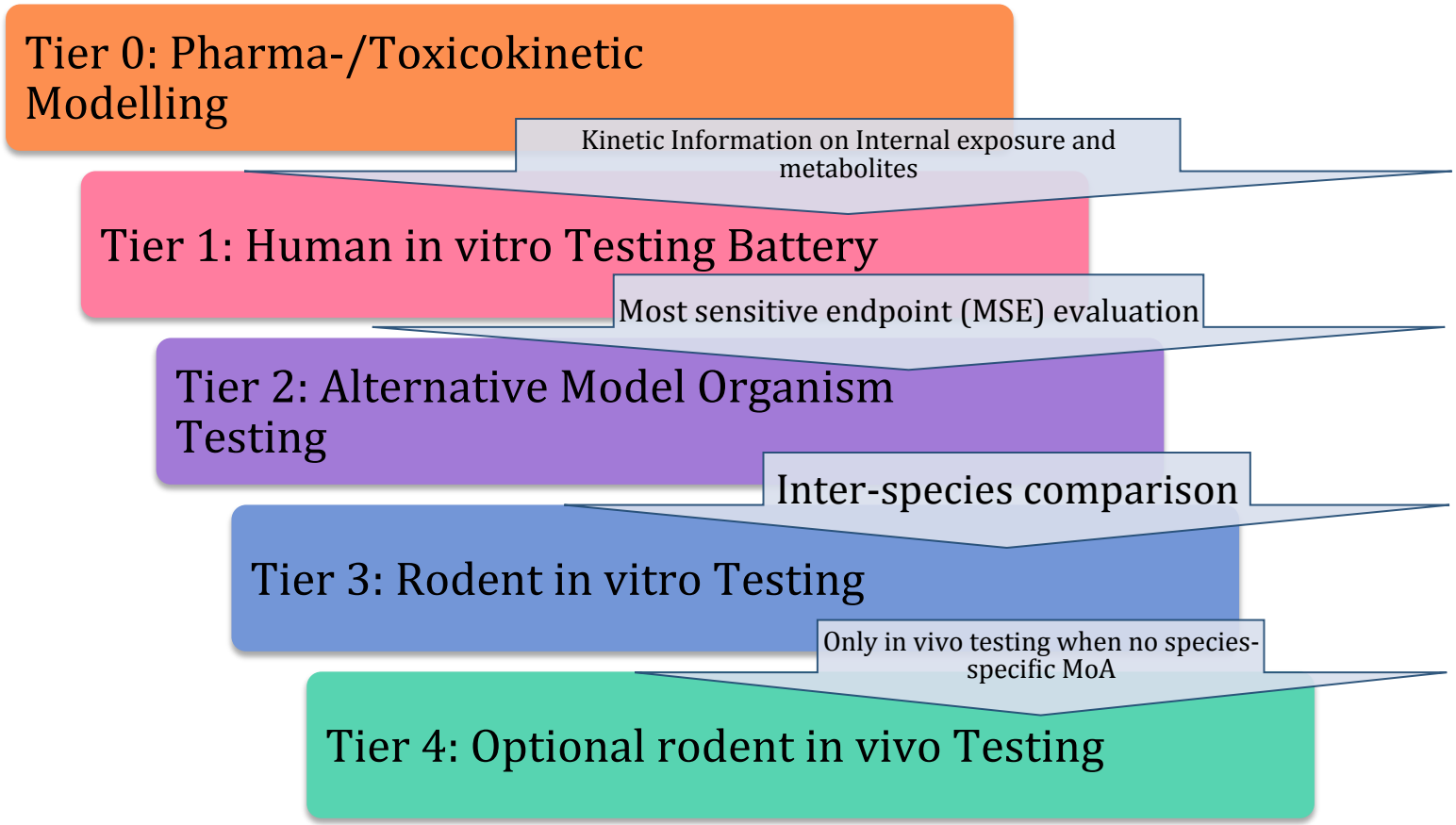
Assay 3: NCC migration

Assay 4: Embryonic phase: neuronal differentiation (hESC/hiPSC)

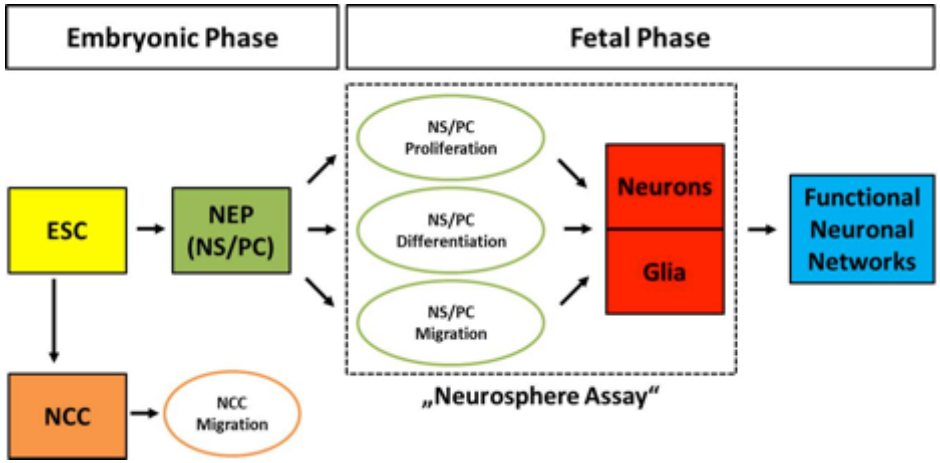
Assay 5: Fetal phase: radial glia/hNPC migration, neuron/astrocyte/ oligodendrocyte differentiation (hNPC)

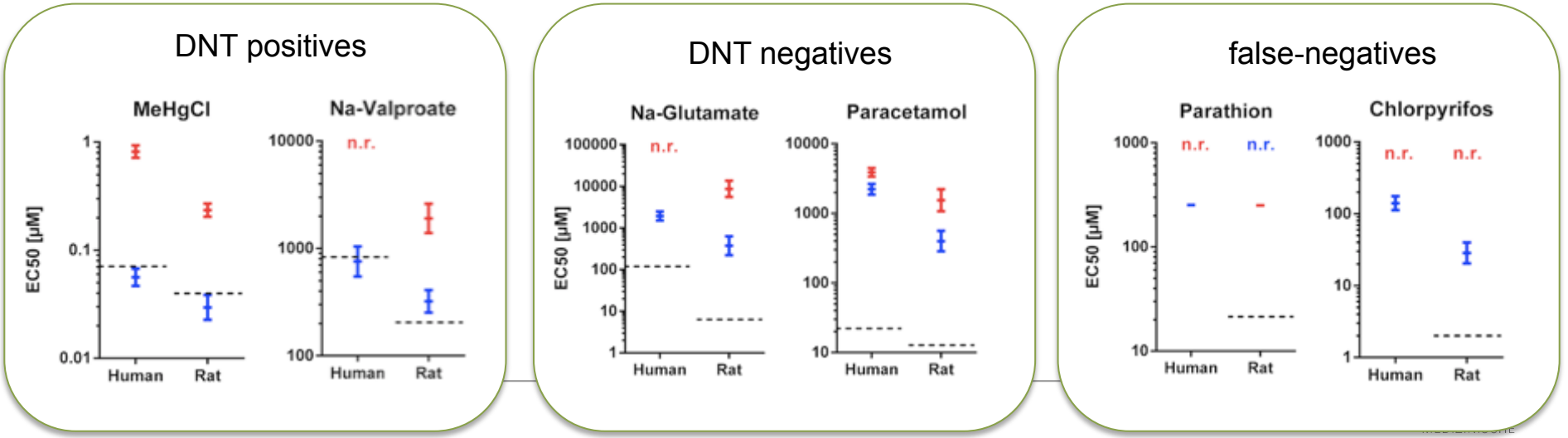
Assay 6: Neuronal maturation (i.e. neurite outgrowth)/synaptogenesis/neuronal network formation (hNS/PC-based method, preferably with neurons and glia present)

ITS FOR DNT HAZARD IDENTIFICATION: TIERED TESTING STRATEGY



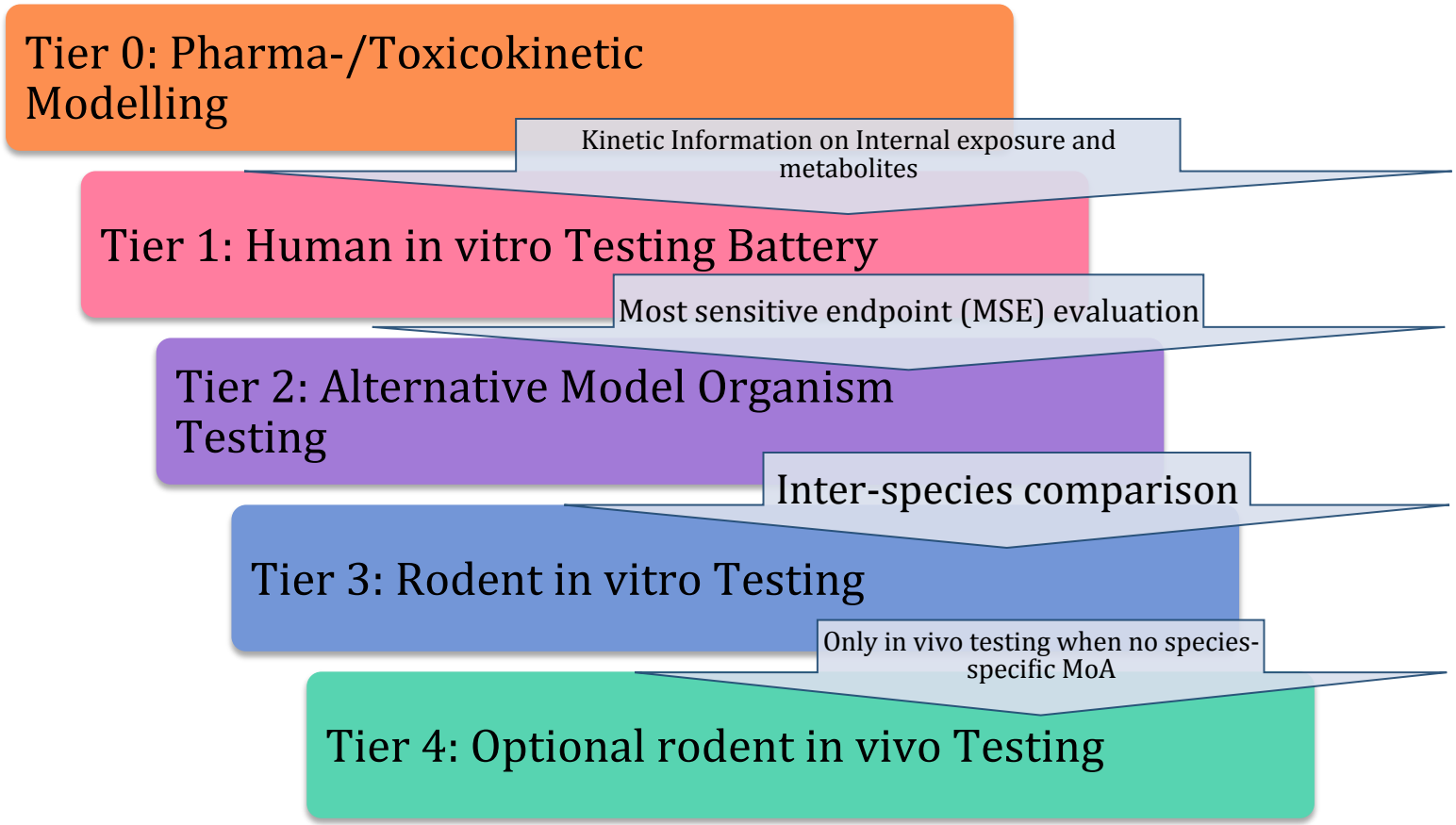
Identification of Most Sensitive Endpoint (MSE) across Testing Battery





Baumann et al. 2015 Arch Toxicol

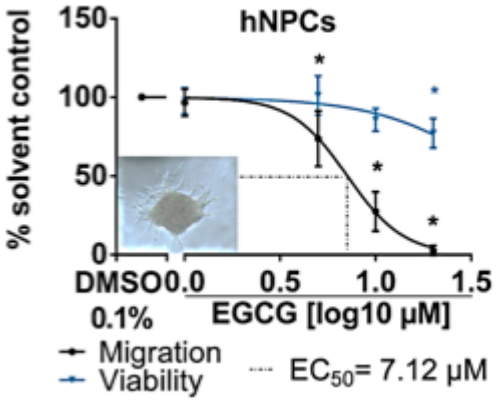
ITS FOR DNT HAZARD IDENTIFICATION: TIERED TESTING STRATEGY



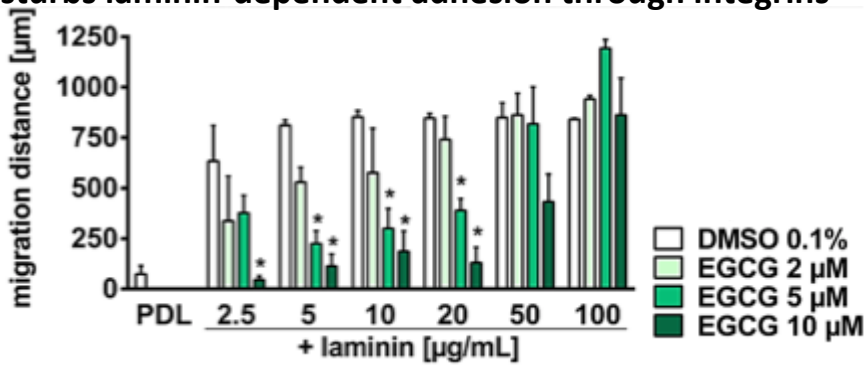
Example: Food Supplement EGCG as an unknown Compound

From **Pharmacokinetics** in Humans and Rats it is estimated that a 3g EGCG/d intake (two tablespoons of commercially available food supplement) of a pregnant woman might lead to a **1 to 3 µM fetal brain concentration**

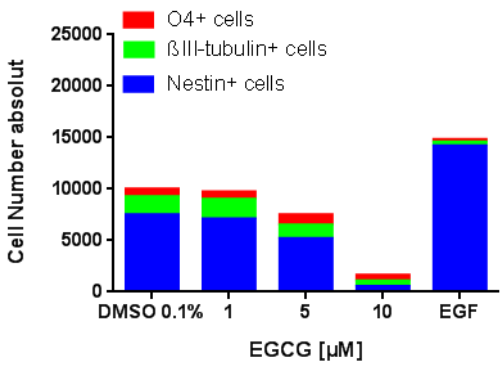
EGCG disturbs hNPC migration



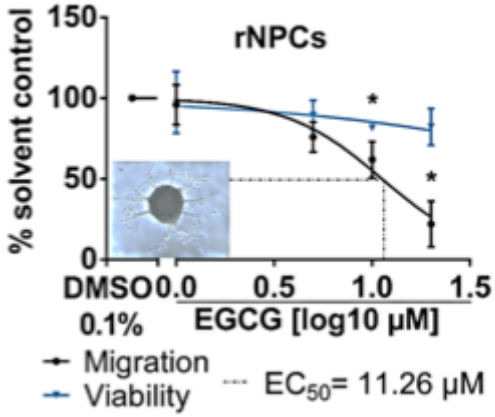
EGCG disturbs laminin-dependent adhesion through integrins



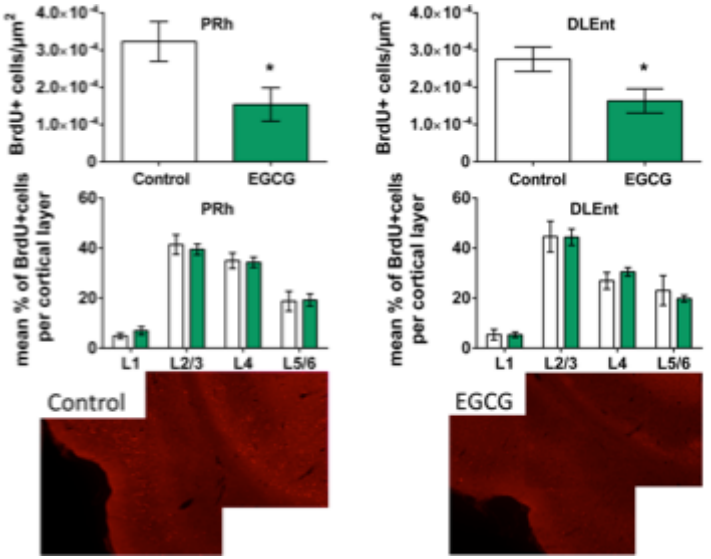
....causing reduction of radial



EGCG disturbs rNPC migration



....and a reduction of BrdU+ cells in P28 cortical layers



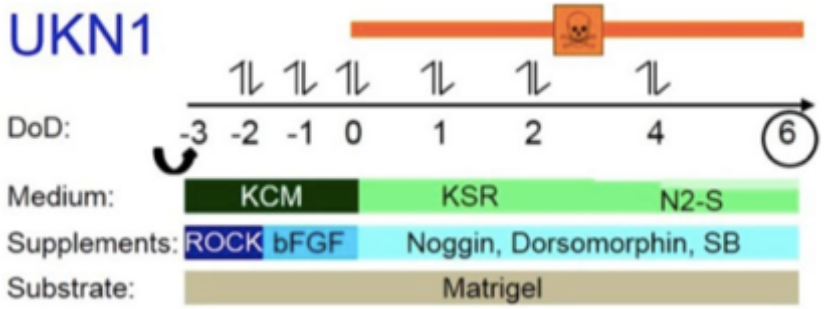
there is a concern of high dose food supplement intake during pregnancy. More studies are needed.

CASE STUDIY: MEHGCL

- Methylmercury causes adverse neurodevelopmental outcomes in children
- It is one of the most data-rich DNT compound
- Data for MeHgCl produced with the proposed assays of the in vitro testing strategy will be displayed

MeHgCl – hESC differentiation into NEP (Assay1)

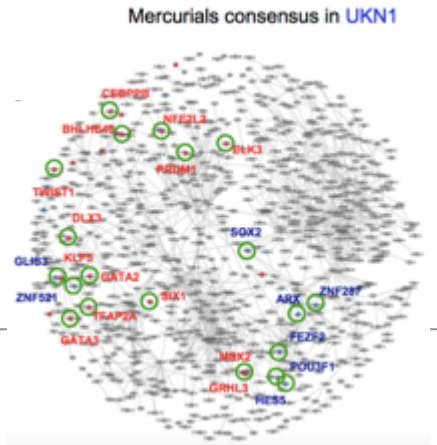
hNEP differentiation, MeHgCl treatment for 12 days



- Cytotoxicity: NOAEC 25 nM
- Functional readout: -
- Gene expression changes: suppressed NCAM1, NEUROD1 and MAP2 expression.

Stummann et al. 2009 Toxicology

- Cytotoxicity: Benchmark concentration (10): 1,5 µM
- Functional readout: -
- Gene expression changes:



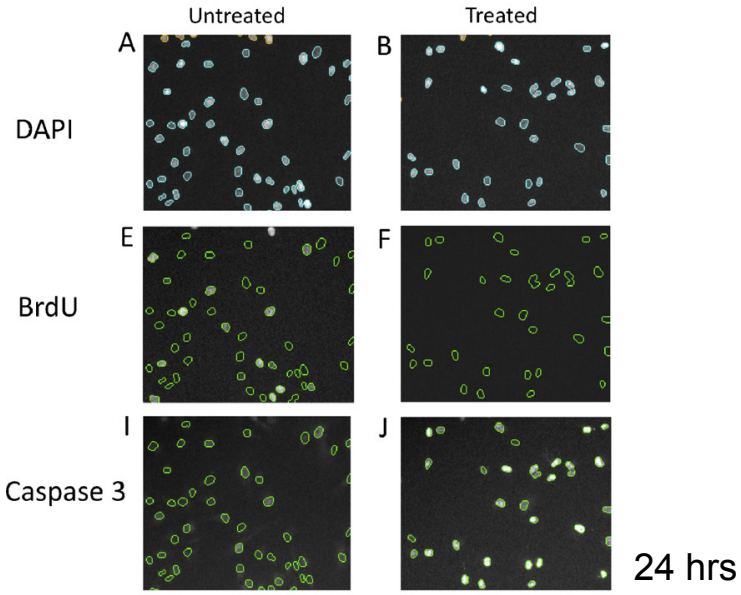
General toxicity factors:
Affected by HDACis and mercurials in UKN1

TWIST1	CEBPPB	NFE2L2
PRDM1	ELK3	SIX1
TFAP2A	GATA3	GLIS3
KLF5	GATA2	DLX3
SOX2	ARX	FEZF2
MSX2	GRHL3	POU3F1
HES5	ZNF521	BHLHE40
ZNF287		

Krug et al. 2013,
Shinde et al. 2016 Arch Toxicol

MeHgCl – NPC Proliferation & Apoptosis (Assay2)

ReN CX cells (Millipore)

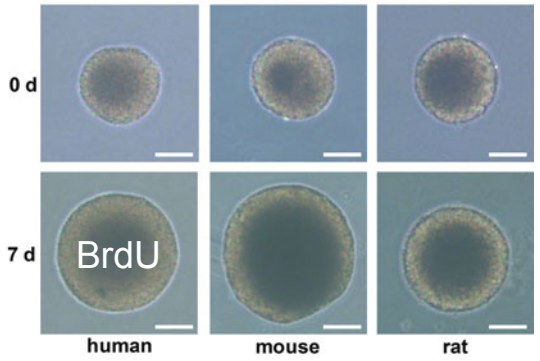


24 hrs

- Cytotoxicity >50%: >10 µM
- BrdU incorp. <50%: 30 µM
- Caspase-3 act. >2-fold: -

Culbreth et al. 2012 NeuroToxicol

hNPC (Lonza)



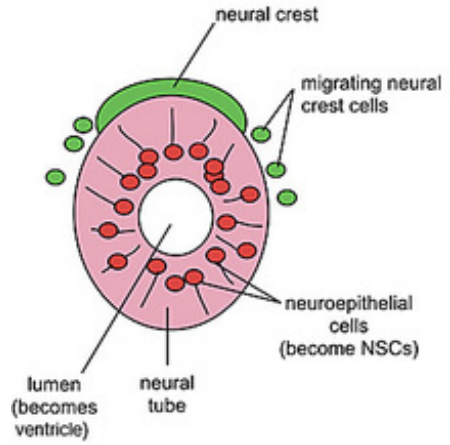
BrdU
72 hrs

- Cytotoxicity >50%: 1,100 nM
- BrdU incorp. <50%: 700 nM
- Caspase-3 act. >2-fold: n. d.

Baumann et al. 2016, Arch Toxicol

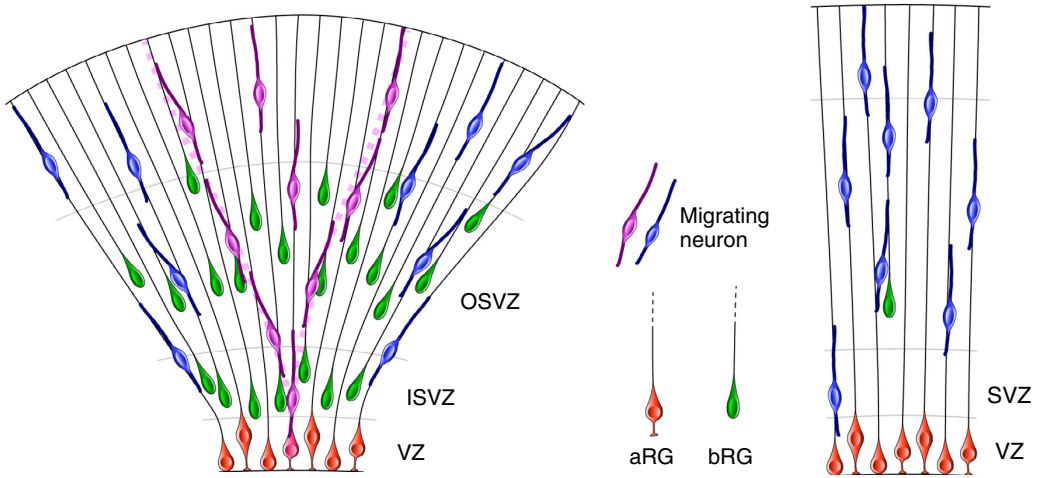
MIGRATION DURING BRAIN DEVELOPMENT

NCC/NEP migration



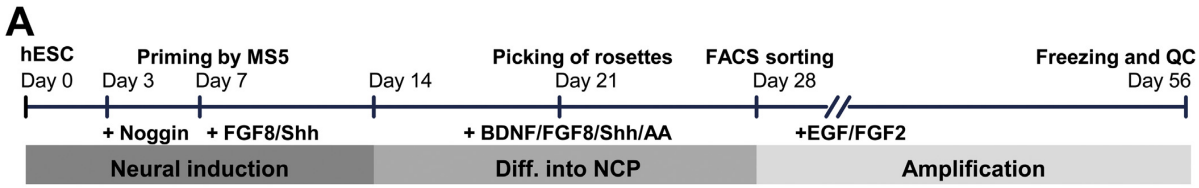
http://www.glycoforum.gr.jp/science/hyaluronan/HA30/HA30E.html

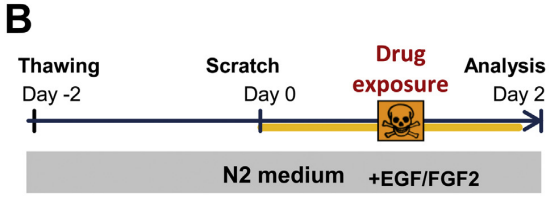
Radial glia cell/Neuronal migration



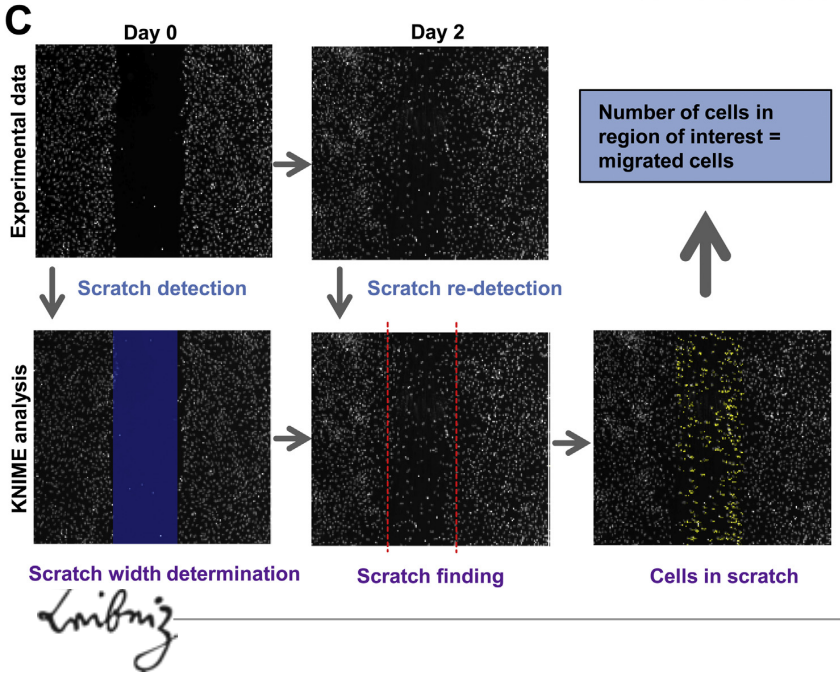
Borrell & Götz 2014 Curr Opin Neurobiol

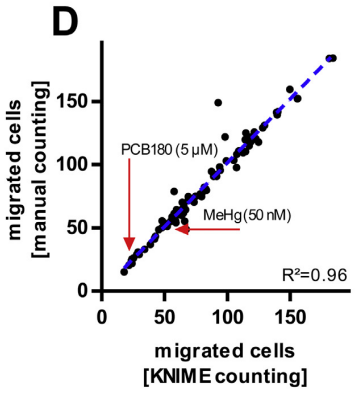
MeHgCl – Neural crest cell migration (MINC) assay (hESC-based, Assay 3)

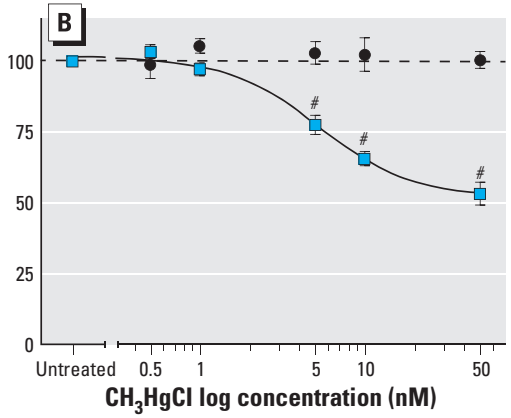




FGF - fibroblast growth factor
Shh - sonic hedgehog
BDNF - brain derived neurotrophic factor
EGF - epidermal growth factor
AA - ascorbic acid
MS5 - feeder cells
MeHg - methylmercury
QC - quality control







- Cytotoxicity >50%: n.r.
- Migration <50%: n.r., significant red.: 5 nM

Zimmer et al., 2012 Environ Health Perspect

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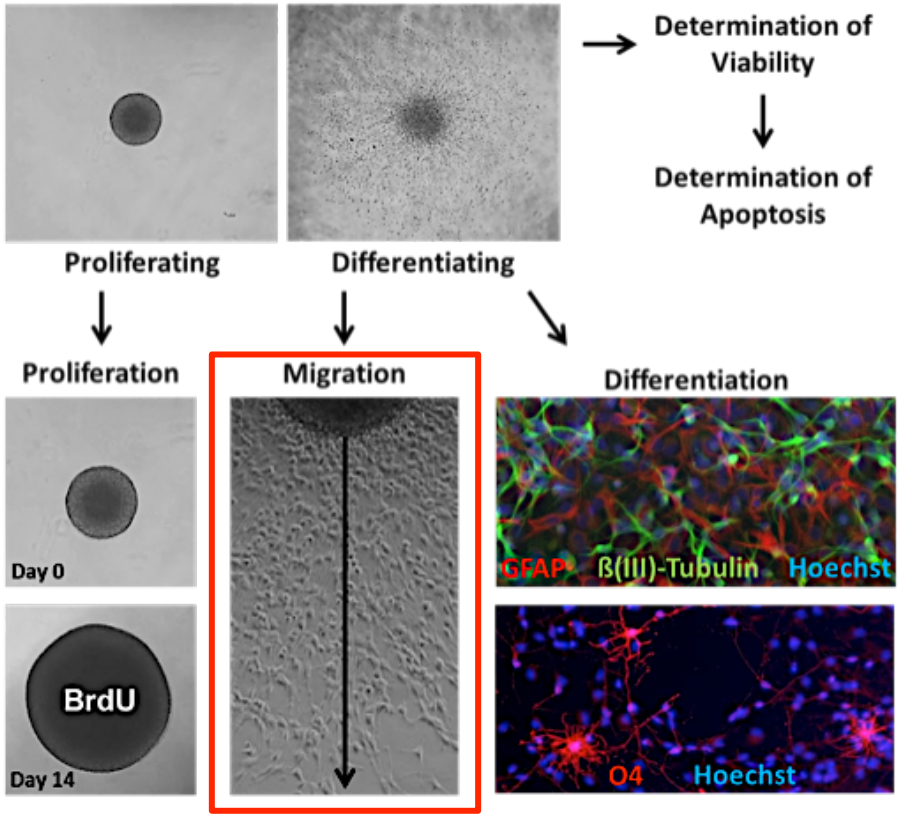
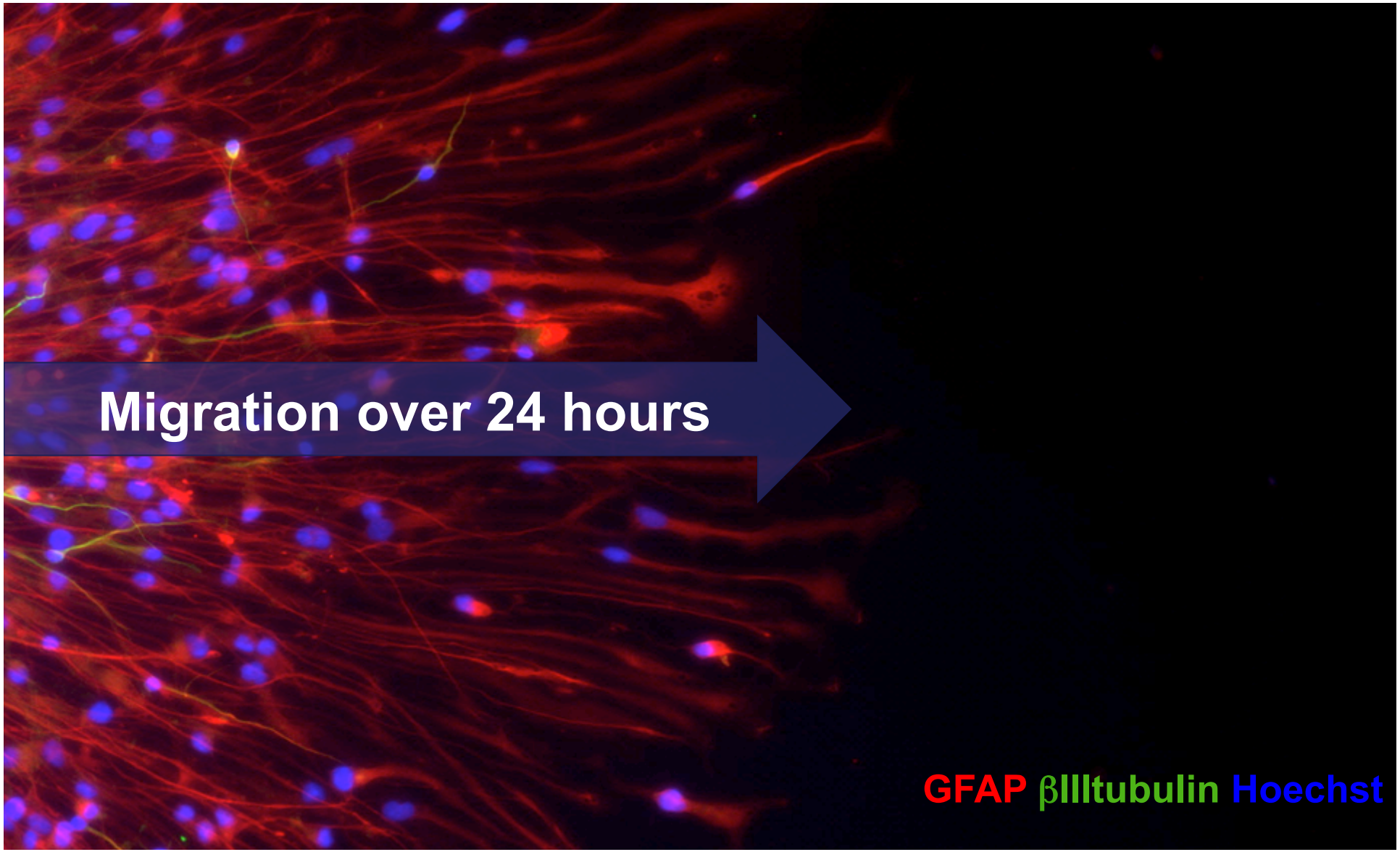
MIGRATION ASSAYS (II) – NPC MIGRATION ASSAY (PART OF ASSAY 5)

The 'Neurosphere Assay' (hNPC, Lonza)

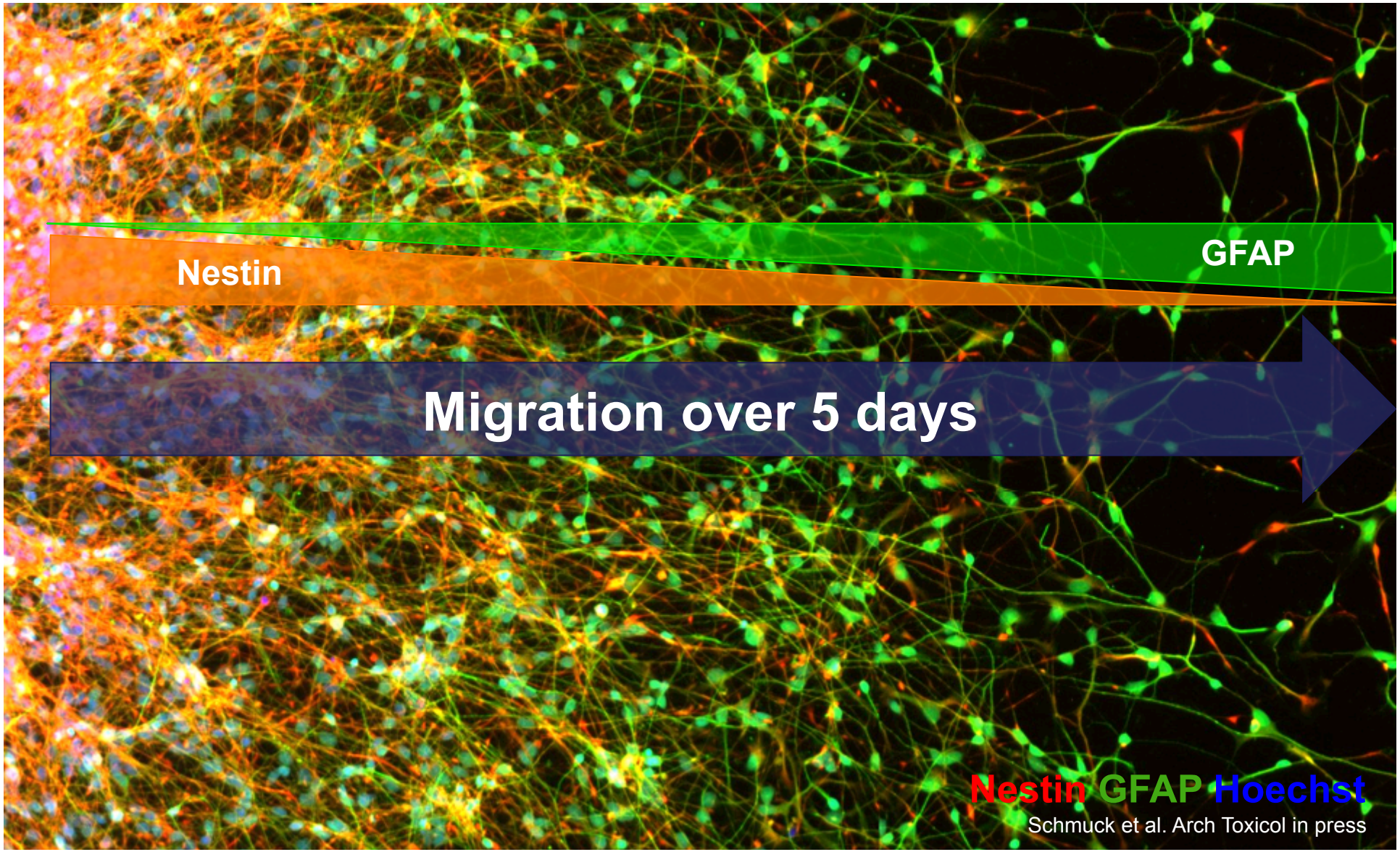


modified from: Breier et al. 2009

NPC Migration assay – Radial Glia Migration

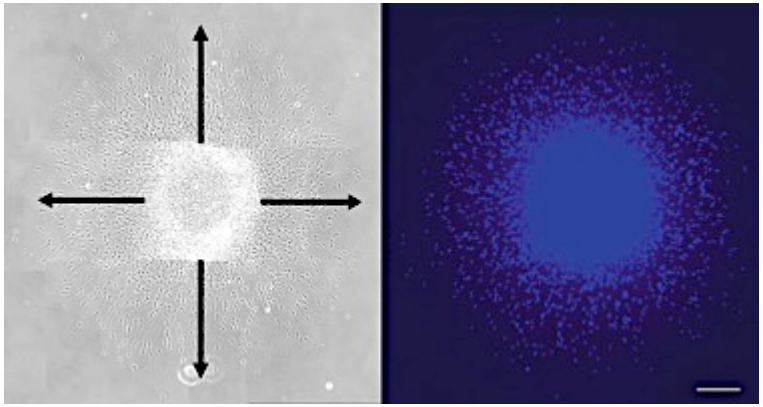


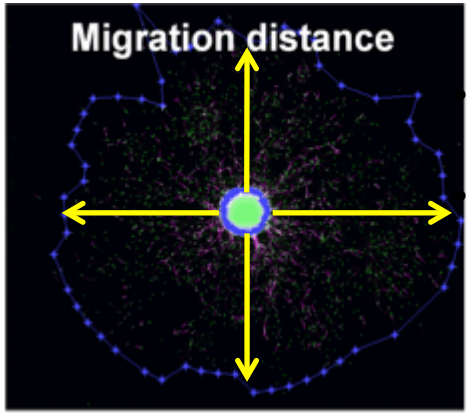
Migration Assays (II) – NPC Migration assay – Radial Glia Migration



Migration Assays (II) – NPC Migration assay (Part of Assay 5)

Radial Glia Migration, 24 hrs



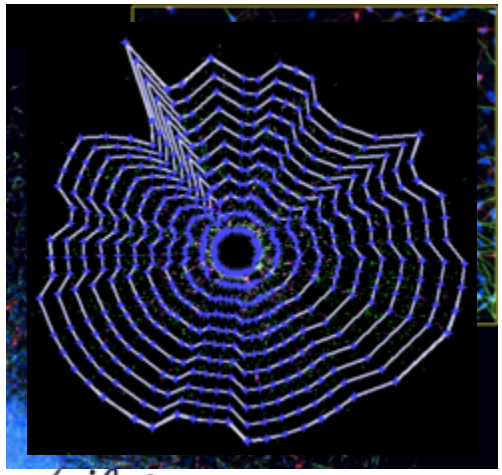


Cytotoxicity >50%: >3 µM

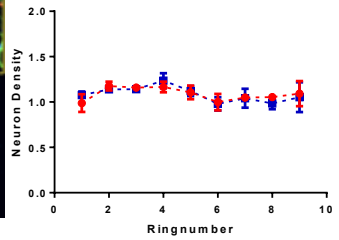
Migration <50%: 650 nM, significant red.: 30 nM

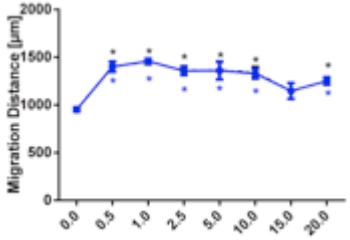
Moors et al. 2007, Baumann et al. 2015,

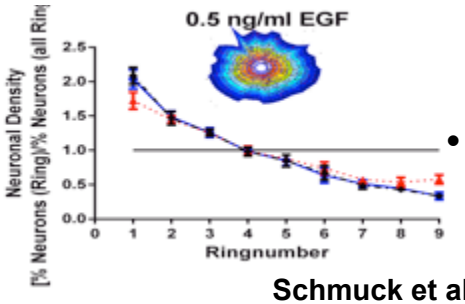
Neuronal migration (5 days)

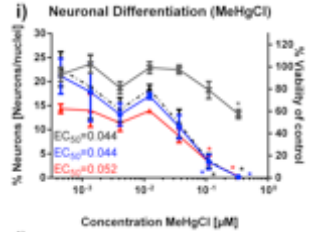


$$\sigma_n = \frac{\frac{n_n(Neurons)}{n_n(Nuclei)}}{\sum_1^n \frac{n_n(Neurons)}{n_n(Nuclei)}}$$









- Cytotoxicity >50%: n.r., significant red.: 330 nM (40%)
- Neuronal migration <50%: -

Schmuck et al. Arch Toxicol in press

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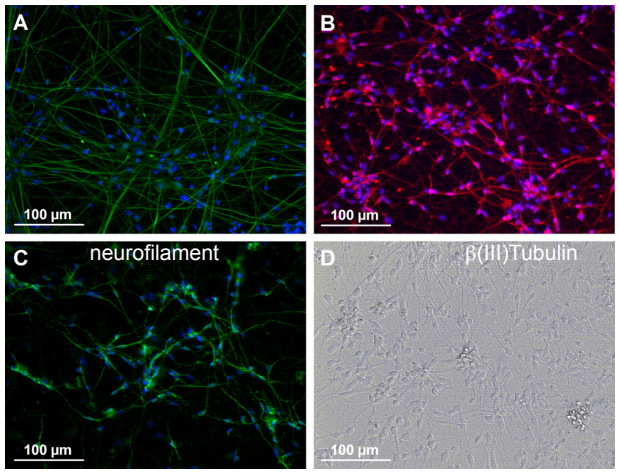
NEURONAL DIFFERENTIATION

hESC-based (Assay 4)



Stummann et al. 2009 Toxicology

After 12 days:

- Cytotoxicity >50%: 39 nM
- Neuronal Differentiation (Map2/Ncam/ NeuroD gene expression) at NOAEC: 25 nM

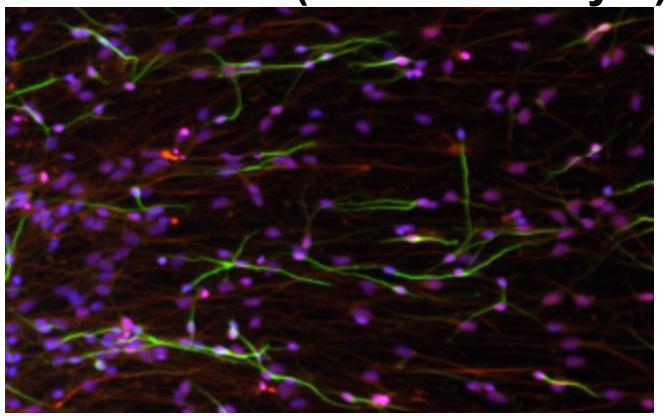
Stummann et al. 2009, Toxicology

After 13 days:

- Cytotoxicity >50%: 100 nM
- Neuronal Differentiation (nº Map2+ cells) <50%: ≥ 1 nM

He et al. 2012, Tox Lett

hNPC-based (Part of Assay 5)



After 72 hrs:

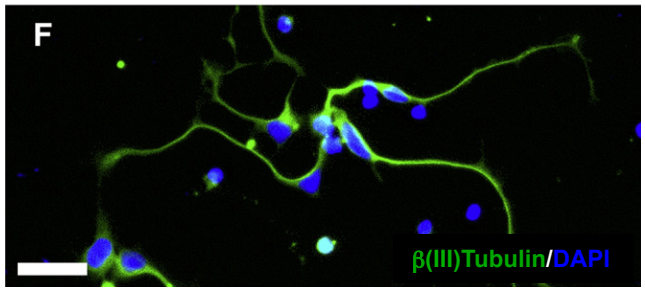
- Cytotoxicity >50%: 800 nM
- Neuronal Differentiation <50%: 60 nM
- significant red.: 12 nM

Baumann et al. 2016, Arch Toxicol

NEURITE OUTGROWTH ASSAYS

hESC/hNPC-based methods:

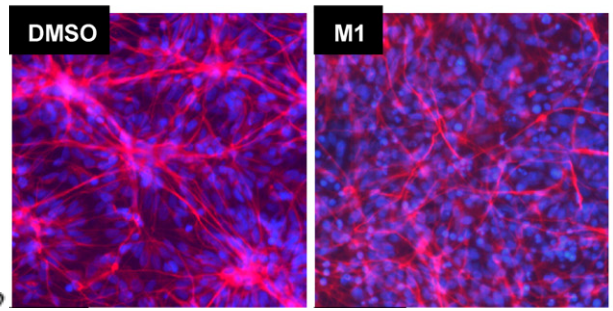
- hN2^{TM} (Aruna, Assay 6): single cell type (neuronal) cultures, HCA



After 24 hrs:
- Cytotoxicity (neurons/field) <75%: 90 nM
- Neurite Count <75%: 70 nM
- Neurite Length <75%: 200 nM

(Harrill et al. 2011 TAAP)

- He et al. (2012) uses hESC-derived neural mixed-cultures, HCA (Assay 4)



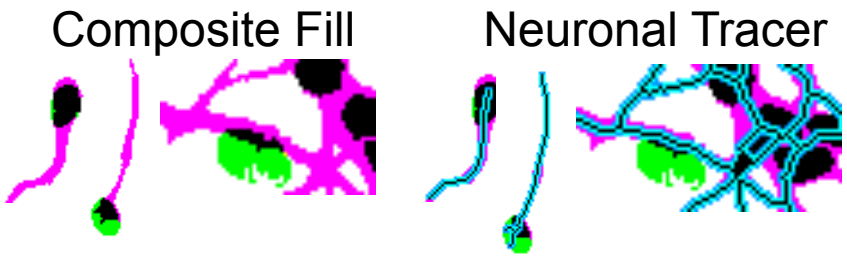
After 13 days:
- Cytotoxicity (MTT assay) <75%: 100 nM (stat.)
  IC_{50} ca. 400 nM
- Neurite Length <60%: 1 nM (stat.)
- Branching points <60%: 100 nM

(He et al. 2012 Tox Lett)

NEURITE OUTGROWTH ASSAY (CTD.)

hESC/hNPC-based methods:

- hNPC-derived young neurons in mixed and mixed-density cultures (Assay 5)

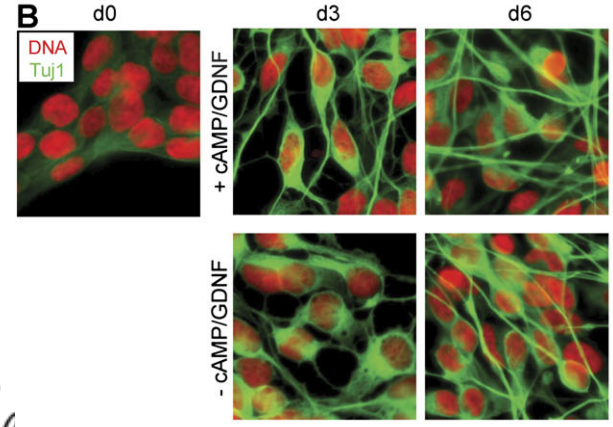


After 5 days:

- Cytotoxicity >50%: n.r., significant red.: 330 nM (40%), neur. diff. <50%: 45 nM
- Neurite Length/neuron <50%: ≈200 nM
- Branching points/neuron <50%: ≈200 nM
- Neurites/neuron <50%: ≈200 nM

(Schmuck et al. in press)

- LUHMES cells (Assay 6) for neurite outgrowth assessment (Stiegler et al. 2011, Krug et al. 201



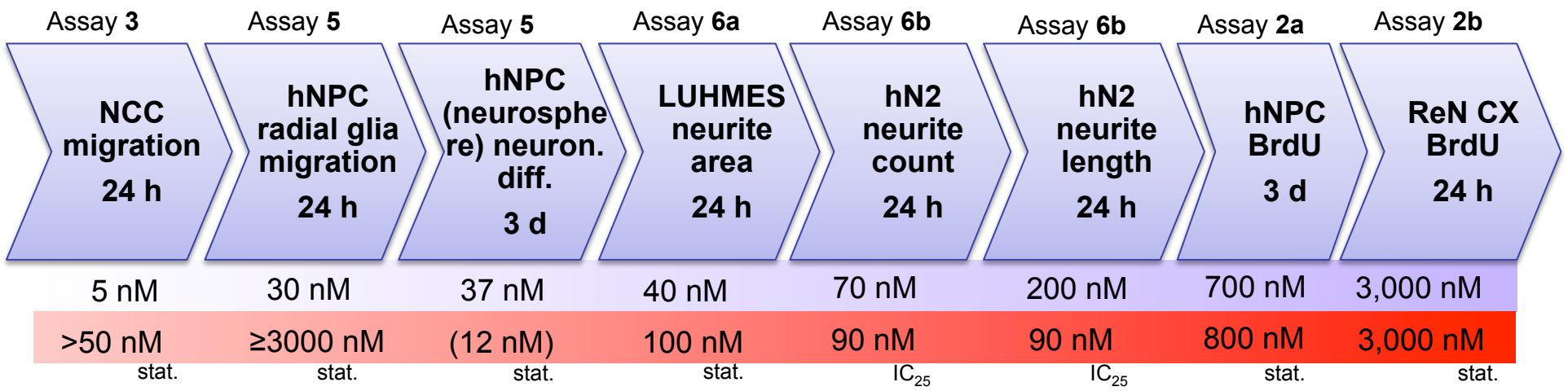
After 24 hrs:

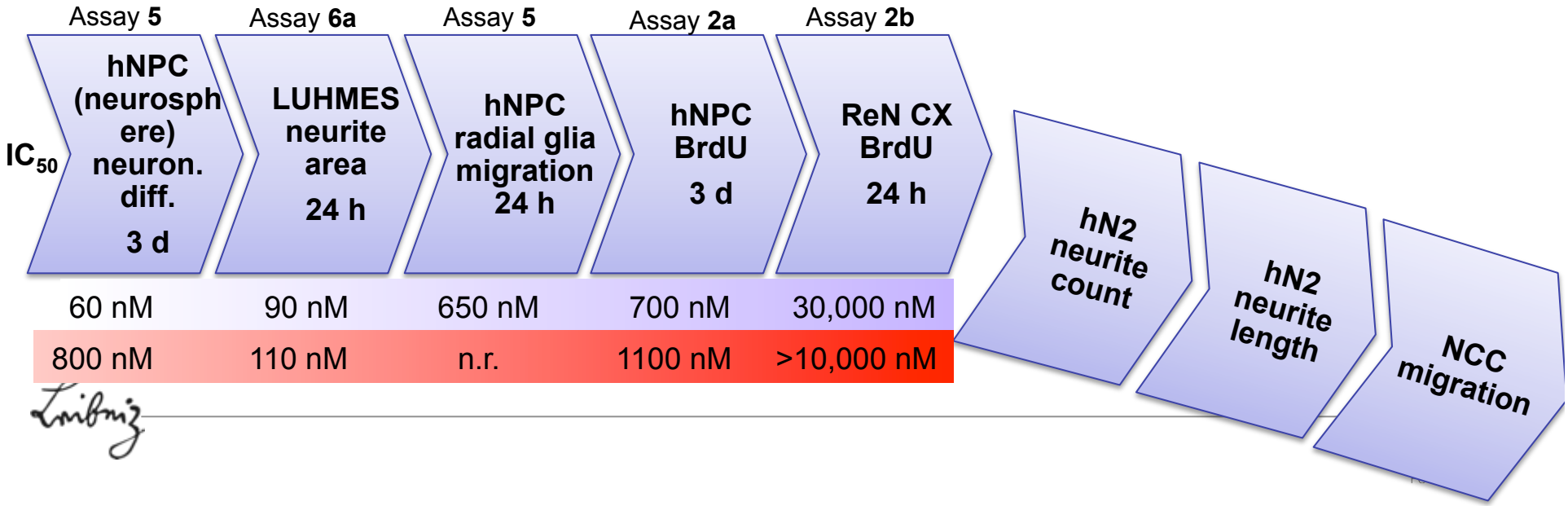
- Cytotoxicity (neurons/field) <50%: 110 nM
- Neurite Area <50%: 90 nM

(Stiegler et al. 2011 Tox Sci)

Sensitivity of in vitro Assays towards MeHgCl: Short-term Assays up to 72 hrs

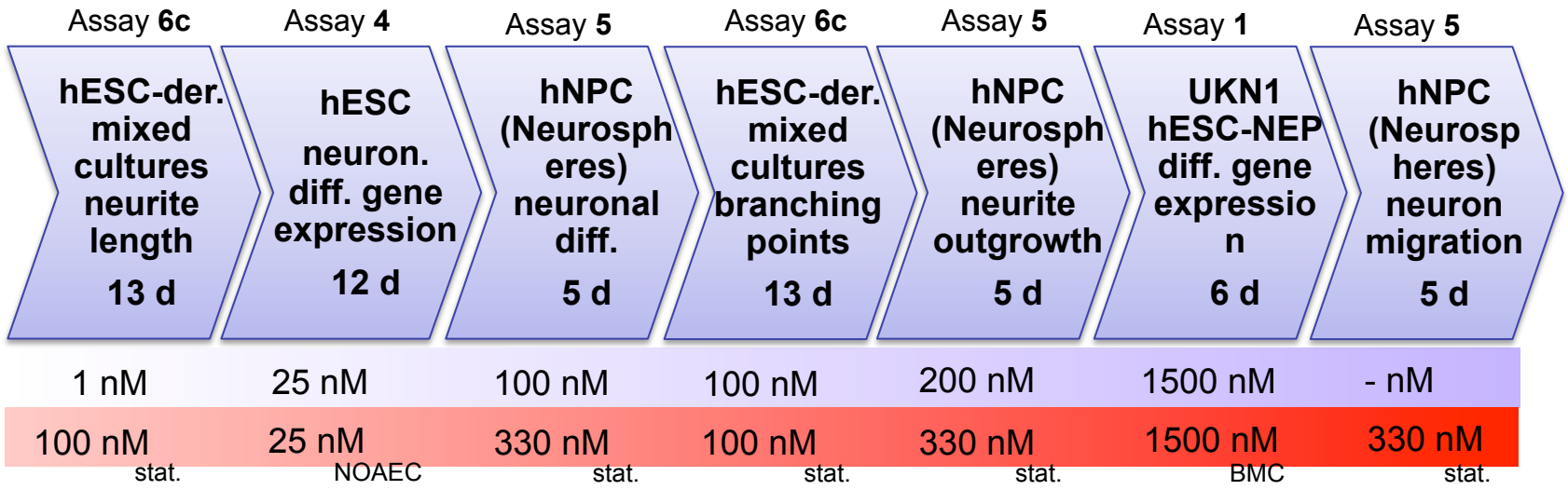
IC_{25} & statistical significance



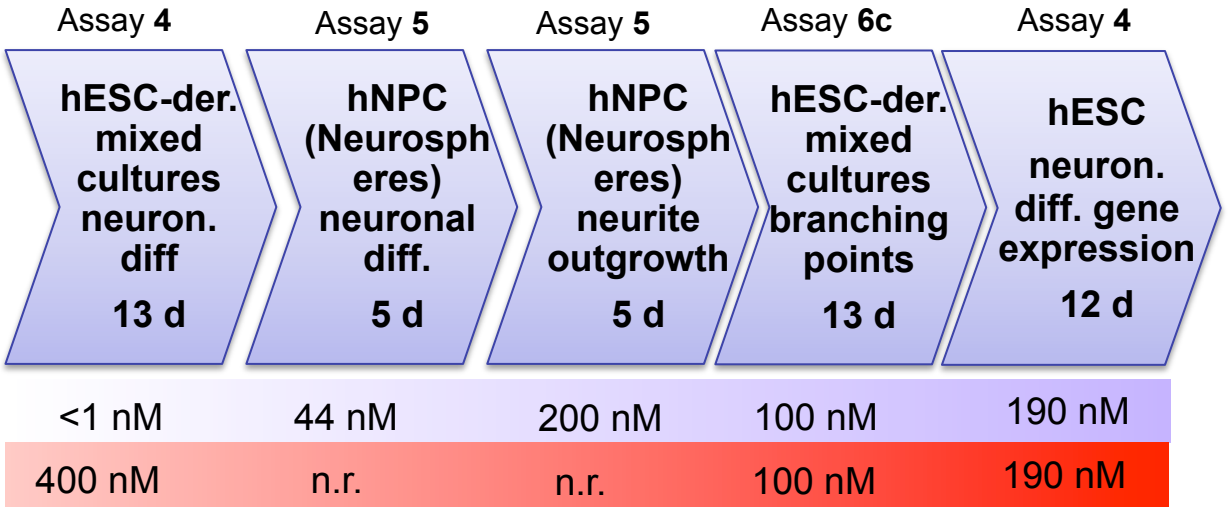


SENSITIVITY OF IN VITRO ASSAYS TOWARDS MEHGCL: LONG-TERM ASSAYS 5 TO 13 DAYS

IC_{25}, NOAEC, BMC & statistical significance



IC_{50}



MeHgCl: in vivo – in vitro extrapolation approach

Maternal hair concentration of 4.5 ppm

(LOAEL)

Neuropsychological deficits in children (Castoldi et al. 2001)

(Burbacher et al. 1990; Lewandowski et al. 2003)

approx. 72 nM

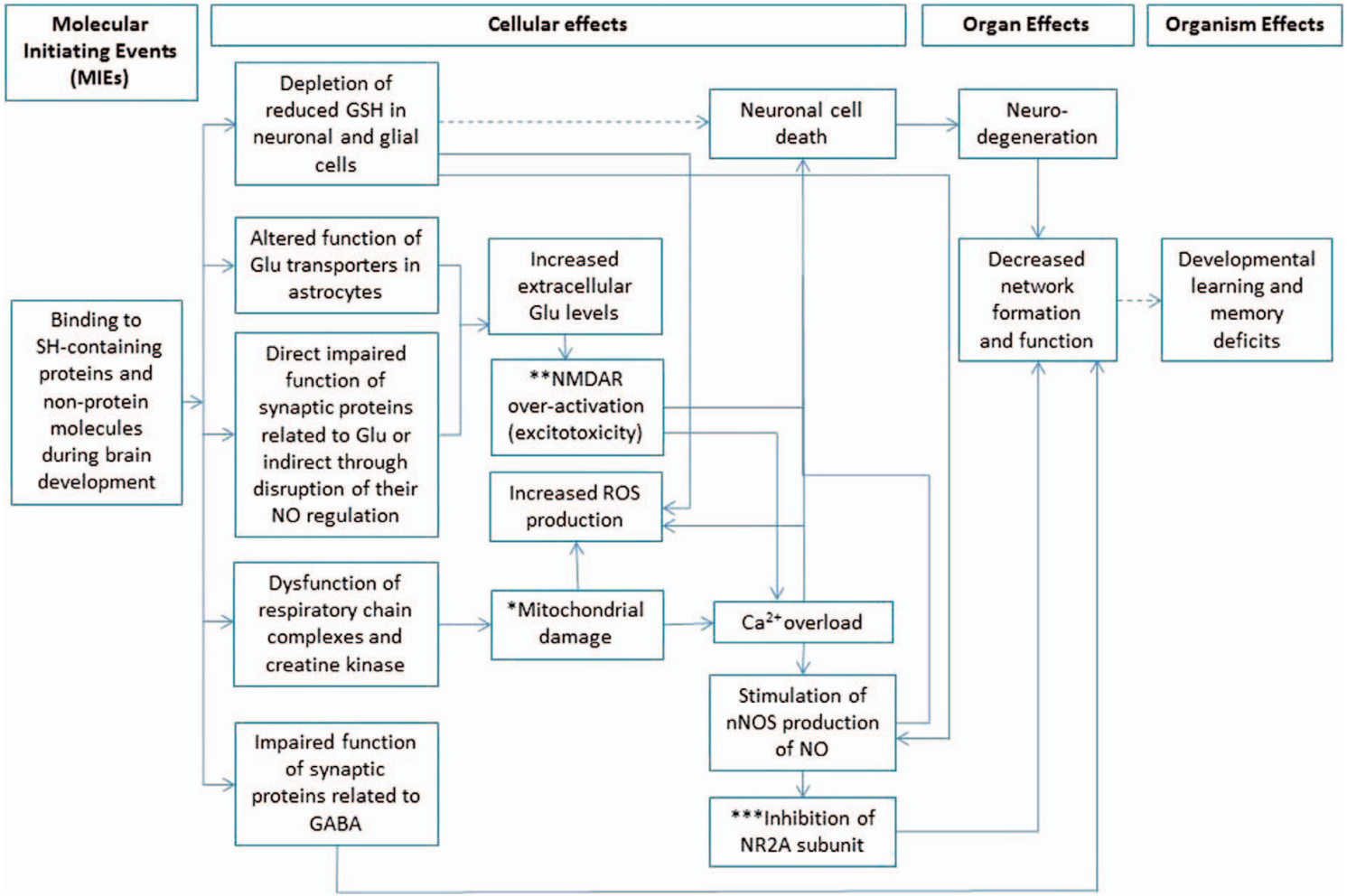
- Migration
- Neuronal Differentiation
- Neuronal Maturation (Neurite Outgrowth)

Burbacher TM, Rodier PM, Weiss B (1990) Methylmercury developmental neurotoxicity: a comparison of effects in humans and animals. Neurotoxicol Teratol 12(3):191–202

Castoldi AF, Coccini T, Ceccatelli S, Manzo L (2001) Neurotoxic- ity and molecular effects of methylmercury. Brain Res Bull 55(2):197–203

Lewandowski T, Ponce R, Charleston J, Hong S, Faustman E (2003) Effect of methylmercury on midbrain cell proliferation during organogenesis: potential cross-species differences and implica- tions for risk assessment. Toxicol Sci 75(1):124–133

MeHgCl: putative AOP on Chemicals binding to SH-groups

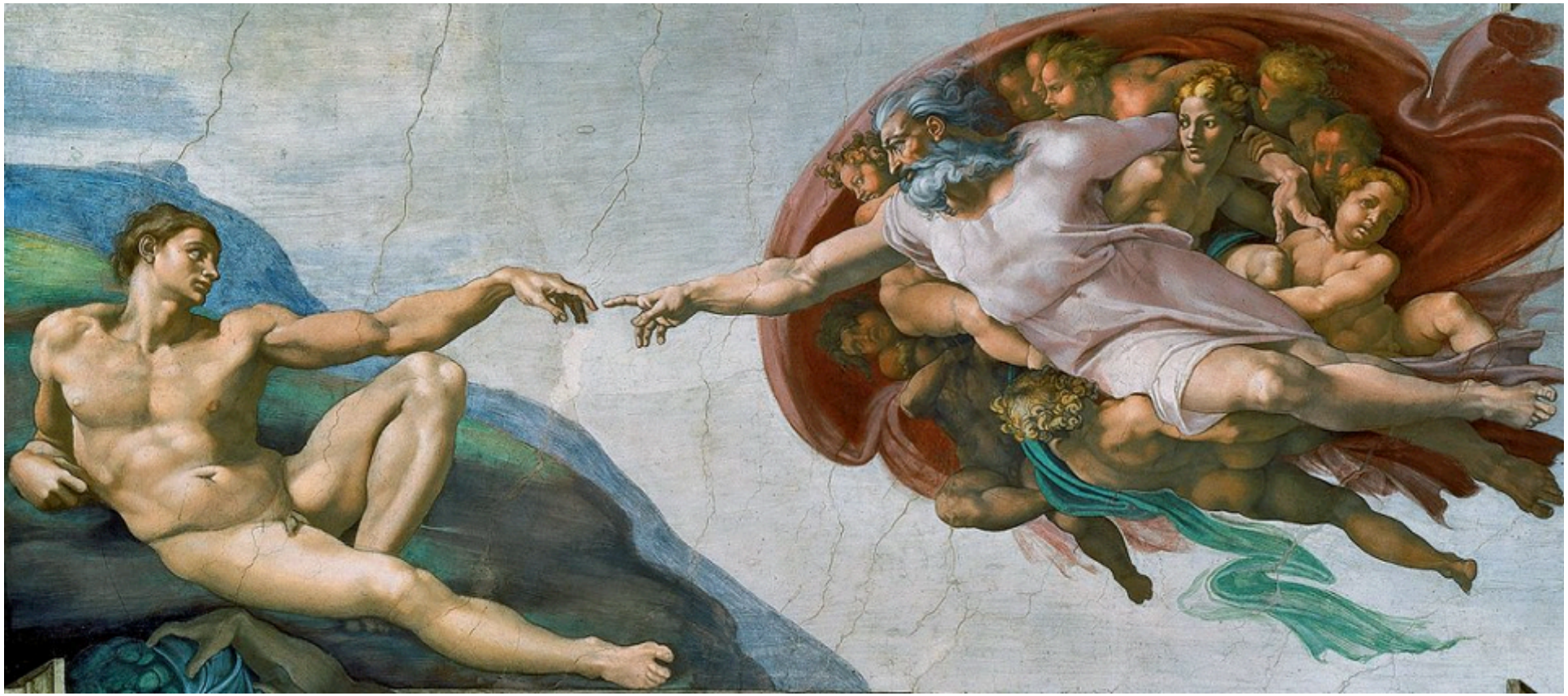


Bal-Price et al. 2015 Crit Rev Toxicol; AOP VI

SUMMARY & CONCLUSION

- From the current state of science, DNT in vitro testing can be based on neurodevelopmentally-relevant processes that can serve as KE in an AOP-based framework
- 'Signaling pathway' to 'process function' analyses improve confidence in assays, which is necessary for regulatory acceptance
- A large variety of neural stem/progenitor cell-based DNT in vitro assays is available NOW
- Compound testing across a battery of in vitro tests covering timing and processes of brain development is the next step forward

SUMMARY & CONCLUSION



ACKNOWLEDGEMENTS

- OECD funded Report on ‘Report on Integrated Testing Strategies for the identification and evaluation of chemical hazards associated with the developmental neurotoxicity (DNT), to facilitate discussions at the Joint EFSA/ OECD Workshop on DNT’, October 18th/19th, Brussels

- EFSA funded ‘Literature review on in vitro and alternative Developmental Neurotoxicity (DNT) testing methods’ (2015)

CASE STUDIES: MEHGCL SUMMARY OF IN VITRO HAZARD

Endpoint	In vitro system	Assay number	Time of Exposure	Measure	Effective concentration Endpoint	Measure	Effective concentration Viability
NEP differentiation (MAP2 gene expression)	hESC	**1**	12 Days	Endpoint determination	25 nM	NOAEC	25 nM
NEP differentiation (global gene expression)	hESC	**1**	6 days	Endpoint determination	1000 nM	Benchmark concentration (10)	1000 nM
NPC apoptosis (Cspase-3 activation)	ReN CX cells (Millipore)	**2**	24 hrs	2-fold induction	-	IC_{50}	-
NPC proliferation (BrdU incorp.)	ReN CX cells (Millipore)	**2**	24 hrs	IC_{50}	30,000 nM	IC_{50}	-
NPC proliferation (BrdU incorp.)	hNPC (Neurospheres)	**2**	72 hrs	IC_{50}	700 nM	IC_{50}	1,100 nM
Neural crest cell migration (MINC Assay)	hESC-der NCC	**3**	48 hrs	IC_{50} Stat. signif.	n.r. 50 nM	IC_{50}	n.r., >50 nM
Radial glia migration assay	hNPC (Neurospheres)	**4**	24 hrs	IC_{50} Stat. signif.	650 nM 30 nM	IC_{50}	n.r., ≥3000 nM

SENSITIVITY OF IN VITRO ASSAYS TOWARDS MEHGCL: SHORT-TERM ASSAYS UP TO 72 HRS

Cell Endpoint	Cultures	Exposure time	Measure	Effect. Conc. Endpoint	Measure	Effect. Conc. Viability	Literature
ReN CX cell BrdU	Myc-immort. NPC	24 hrs	IC_{50} Stat.	30,000 nM 3,000 nM	IC_{50} Stat.	- 3,000 nM	Culbreth et al. 2012 Breier et al. 2008
Radial glia migration	Primary NPC Neurosphere Assay	24 hrs	IC_{50} Stat.	650 nM 30 nM	IC_{50} Stat. signif.	n.r. ≥3000 nM	Baumann et al. 2015
Neurite Count	hN2	24 hrs	IC_{25}	70 nM	IC_{25}	90 nM	Harrill et al. 2011
Neurite Length	hN2	24 hrs	IC_{25}	200 nM	IC_{25}	90 nM	Harrill et al. 2011
Neurite Area	LUHMES	24 hrs	IC50 Stat. signif.	90 nM 40 nM	IC50 Stat. signif.	110 nM 100 nM	Stiegler et al. 2011
NCC migration	MINC Assay hESC-NCC	48 hrs	IC_{50} Stat.	n.r. 50 nM	IC_{50} Stat.	n.r. >50 nM	Zimmer et al., 2012
hNPC BrdU	Primary NPC Neurosphere Assay	72 hrs	IC_{50}	700 nM	IC_{50}	1,100 nM	Baumann et al. 2015
hNPC neuronal diff	Primary NPC Neurosphere Assay	72 hrs	IC_{50} Stat. signif.	60 nM 37 nM (>40% reduction)	IC_{50}	800nM 12 nM (<25% reduction 12 and 37 nM)	Baumann et al. 2015

SENSITIVITY OF IN VITRO ASSAYS TOWARDS MEHGCL

- **Long-term Assays 5 to 13 days**

Cell Endpoint	Cultures	Exposure time	Measure	Effect. Conc. Endpoint	Measure	Effect. Conc. Viability	Literature
Neuronal differentiation	hNPC (Neurospheres)	5 days	IC_{50} Stat. sign.	44 nM 100 nM	IC_{50} Stat. signif.	n.r. 330 nM (40% reduction)	Schmuck et al. 2016
Neurite outgrowth assay Neurite Count	hNPC (Neurospheres)	5 days	IC_{50} Stat. signif.	200 nM	IC_{50} Stat. signif.	n.r. 330 nM (40% reduction)	Schmuck et al. 2016
Neurite outgrowth assay Neurite Length	hNPC (Neurospheres)	5 days	IC_{50} Stat. signif.	200 nM	IC_{50} Stat. signif.	n.r. 330 nM (40% reduction)	Schmuck et al. 2016
Neurite outgrowth assay Neurite Branching points	hNPC (Neurospheres)	5 days	IC_{50} Stat. signif.	200 nM	IC_{50} Stat. signif.	n.r. 330 nM (40% reduction)	Schmuck et al. 2016
Neuron migration assay	hNPC (Neurospheres)	5 days	IC_{50} Stat. signif.	-	IC_{50} Stat. signif.	n.r. 330 nM (40% reduction)	Schmuck et al. 2016
NEP differentiation (global gene expression), UKN1	hESC	6 days	Endpoint determination	1500 nM	Benchmark concentration (10)	1500 nM	Shinde et al. 2016

SENSITIVITY OF IN VITRO ASSAYS TOWARDS MEHGCL

- **Long-term Assays 5 to 13 days (cont.)**

Cell Endpoint	Cultures	Exposure time	Measure	Effect. Conc. Endpoint	Measure	Effect. Conc. Viability	Literature
NEP differentiation (Map2 gene expression)	hESC	12 days	Endpoint determination	25 nM	NOAEC	25 nM	Stummann et al. 2009
Neuronal differentiation assay (Map2 gene expr)	hESC	12 days	IC_{50}	190 nM	IC_{50}	190 nM	Stummann et al. 2009
Neuronal differentiation assay ($Map2^+$ cells)	hESC-der. mixed cultures	13 days	IC_{50}	1 nM	IC_{50} Stat. sign.	ca. 400 nM 100 nM	He et al. 2012
Neurite outgrowth assay Neurite Length	hESC-der. mixed cultures	13 days	Stat. sign. <60%	1 nM	IC_{50} Stat. sign.	ca. 400 nM 100 nM	He et al. 2012
Neurite outgrowth assay Neurite Branching points	hESC-der. mixed cultures	13 days	Stat. sign.	100 nM	IC_{50} Stat. sign.	ca. 400 nM 100 nM	He et al. 2012