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Neurospheres as a 3D *in vitro* model for DNT testing: studying endocrine disruption of thyroid hormone signaling during brain development in a species-specific context.

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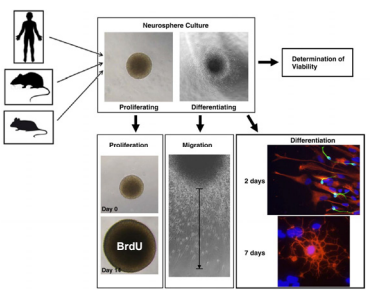
INTRODUCTION

Developmental neurotoxicity (DNT) of chemicals is a serious threat for society. Because the OECD426 guideline for DNT testing is very resource-intensive (time, costs, animals) and often difficult to interpret, alternative testing strategies are needed. For this reason, we established the 'Neurosphere Assay', a 3D *in vitro* cell culture model based on primary neural progenitor cells (NPCs) grown as neurospheres. Species-specific susceptibility towards chemicals and involved pathways can be evaluated with neurospheres from three different species: human, mouse and rat. We scientifically evaluated this model for studying endocrine disruption of thyroid hormone (TH) (triiodothyronine (T3) and thyroxine (T4)) signaling since thyroid hormones are crucial for proper brain development. Moreover, we investigated the TH disrupting potential of the flame retardant brominated diphenyl ether-99 (BDE-99) in NPCs. This compound causes DNT in rodents and epidemiological studies suggest that the compound class of polybrominated diphenyl ethers (PBDEs) impacts also human brain development. Although their mode of action for causing DNT is unknown , *in vivo* studies in rats suggest that they might exert TH disrupting properties thus affecting neurodevelopmental processes. This work demonstrates with the example of BDE-99 that the neurosphere model is able to identify TH disrupting potential of chemicals, potentially applicable to petroleum products.

The Neurosphere Assay



- Neurospheres consist of neural progenitor cells (NPCs) of human, mouse or rat origin.
- The assay mimics basic processes of brain development *in vitro* in a species-specific context.
- DNT effects of compounds and their underlying molecular mechanisms can be studied.

RESULTS

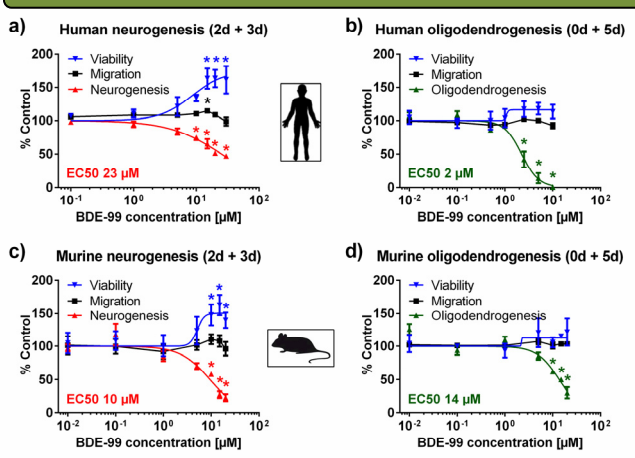
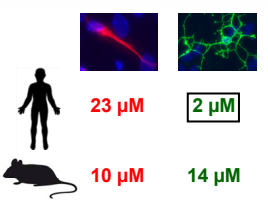


Figure 1: Human and murine NPCs were differentiated in presence/absence of 0.01-30 µM BDE-99. After immunocytochemical stainings for a+c) ß(III)-tubulin (neurons) or b+d) O4 (oligodendrocytes) labeled cells were counted, conc.-response curves plotted and IC_{50} values determined.



- **BDE-99 inhibits human and murine neurogenesis and oligodendrogenesis without affecting cell viability.**
- **Human oligodendrogenesis is the most sensitive endpoint.**

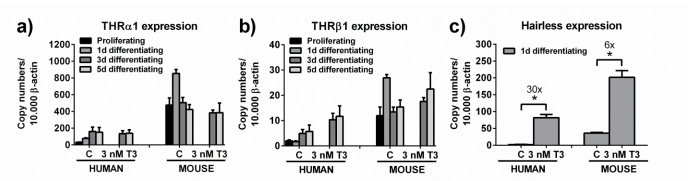


Figure 2: Expressions of thyroid hormone receptor (THR) α1 (a) and β1 (b) as well as the THRα regulated gene *hairless* (c) in hNPCs and mNPCs. NPCs were treated with 3 nM thyroid hormone (T3), then RNA was prepared, cDNAs transcribed and real time RT-PCR performed. Specific gene copy numbers (CN) were determined by product-specific copy number standards.

- **THR levels in mNPCs are higher than in hNPCs.**
- **THR levels are higher in differentiating than in proliferating hNPCs, while in mNPCs a maximal expression is reached at day 1 of differentiation.**
- **T3 does not affect THR expression.**
- **THR receptors are functional: T3 induces the expression of the THRα regulated gene *hairless*.**

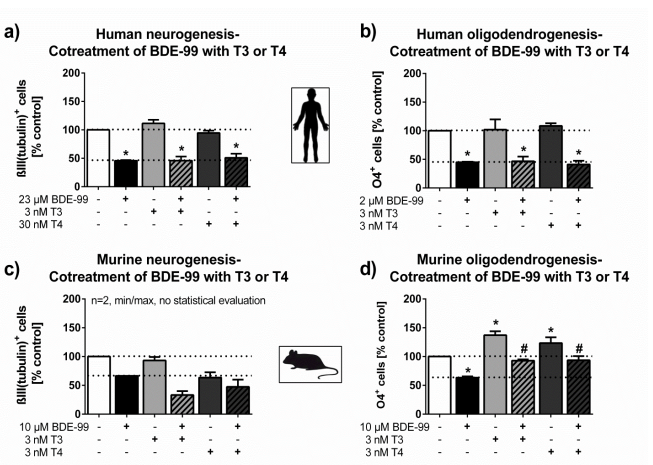


Figure 3: HNPCs and mNPCs were differentiated in presence of BDE-99 and/or TH, fixed and immunocytochemically stained for a+c) ß(III)-tubulin or b+d) O4, positive cells counted and normalized to number of total nuclei.

- **Thyroid hormones T3 and T4 only induce and antagonize the BDE-99 effect on oligodendrogenesis in mNPCs, but not in hNPCs.**

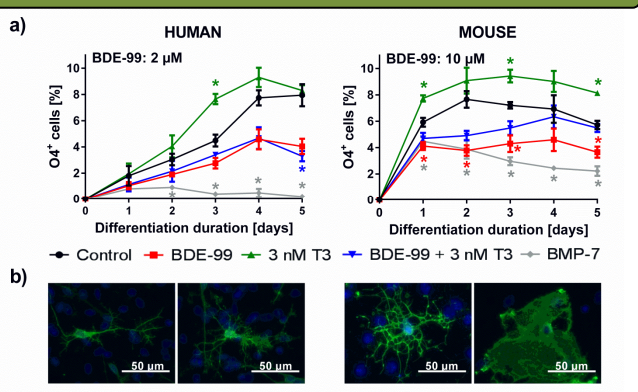


Figure 4: NPCs were differentiated in presence of BDE-99 (respective IC_{50} values for oligodendrogenesis) and/or T3 over a time course of 5 days and stained for O4. a) Quantification of $O4^+$ cells/nuclei, b) Representative $O4^+$ cells after 5 days differentiation.

- **Compared to human cells, murine NPC oligodendrocyte differentiation produces more mature oligodendrocytes after 5 days of differentiation.**
- **T3 accelerates human oligodendrogenesis (speed), but induces murine oligodendrogenesis (number).**
- **BDE-99-dependent reduction of murine, but not of human oligodendrogenesis is antagonized by T3.**

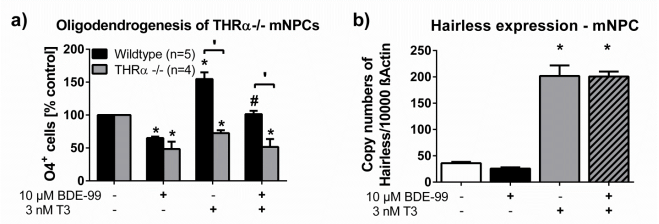


Figure 5: a) Wildtype (wt) and THRα-/- mNPCs were differentiated in presence of T3 ± BDE-99 for 5 days and $O4^+$ cells/nuclei were quantified. b) *Hairless* expression was measured and copy numbers (CN) quantified in wt mNPCs by real time RT-PCR.

- **In contrast to T3-reliant induction, BDE-99-dependent reduction of murine oligodendrogenesis is THRα-independent.**
- **BDE-99 exposure does not antagonize T3-induced, THRα-dependent *hairless* expression in mNPCs.**

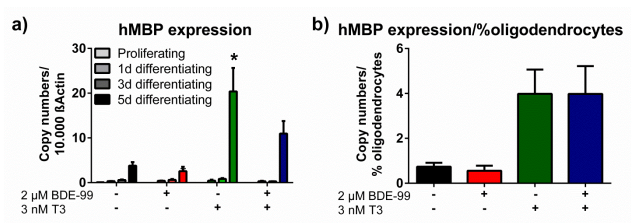


Figure 6: Expression of the human oligodendrocyte maturation marker MBP (myelin basic protein) was quantified during 5 days of differentiation (CN_{MBP}) (a) and CN_{MBP}/%oligodendrocytes (Q_M, Fig. 3a) were calculated after 5 days differentiation (b).

- **MBP expression of hNPCs increases during differentiation.**
- **BDE-99 reduces basal and T3-induced MBP expression in hNPCs.**
- **BDE-99 does not inhibit human oligodendrocyte maturation (copy numbers/%oligodendrocytes).**

Putative criterion for an endocrine disruptor reducing human oligodendrocyte maturation:

$$Q_M = \frac{CN_{MBP}}{\%\ oligodendrocytes}$$

$$Q_{M,\text{Control}} < Q_{M,\text{Chemical + T3}} < Q_{M,\text{T3}}$$

- **BDE-99 is no endocrine disruptor of human oligodendrocyte maturation, because:**

$$Q_{M,\text{Control}}\ (0.7) < Q_{M,\text{Chemical + T3}}\ (4) = Q_{M,\text{T3}}\ (4)$$

CONCLUSIONS

The "Neurosphere Assay" is a promising tool for ED in developmental neurotoxicity testing:

- **NPCs maintain species-specific signaling, e.g for oligodendrocyte formation and maturation.**
- **Transgenic NPCs help elucidating molecular pathways, like TH signaling.**
- **Gene to function analyses can be performed (gene expression vs. oligodendrocyte number).**
- **A putative human-specific strategy for identification of cellular TH disruptors was developed which takes cellular homeostasis into account.**

LITERATURE

- Breier JM..Fritsche et al., *Neurotoxicol Teratol*, 32(1), 2010: 4–15.
- Costa LG et al., *Tox Letters,* 2014, 230(2): 282-94.
- Horn S and Heuer H, *Mol Cell Endocrinol,* 2010, 315(1-2): 19-26.

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