AIMT11 - ENHANCING IN VITRO DNT TESTING STRATEGY

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Background

- **Recently, the need for a refinement of developmental neurotoxicity (DNT) testing has been moving into the focus of the scientific community and regulatory authorities.**
- **The use of human-based in vitro new approach methodologies (NAMs) instead of animal studies can advance toxicity testing with regards to costs, testing throughput and predictivity of test results for humans.**
- **A DNT in vitro battery (DNT-IVB) has recently been assembled under regulatory guidance and challenged with 119 chemicals. Despite its broad coverage of neurodevelopmental key events, a gap analysis suggested that the implementation of test methods based on radial glia (RG), astrocytes (AC), and microglia (MG) could improve battery performance.**
- **This project therefore envisions the establishment of an expanded DNT-IVB including RG-, AC- and MG-based test systems.**

Work Package 1 – Radial Glia (RG)

- RG proliferation, migration and terminal differentiation are crucial for the generation of the different cells of the central nervous system (CNS), cortical expansion and the formation of a cortical scaffold for migrating neurons.
- In consequence chemical-induced changes in RG functionality and/or differentiation disturb cortical scaffold formation and thus impair subsequent neuronal migration.

Objectives:

- Set-up and characterization of test methods detecting chemical interference with key neurodevelopmental endpoints, e.g. proliferation, migration, morphology, and differentiation potential of RG.

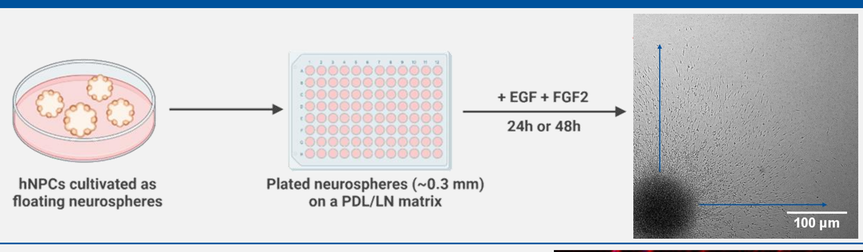


Fig. 1: Test method set-up for the simultaneous assessment of chemical interference with RG proliferation, migration, and morphology. Human neural progenitor cells (hNPC) plated in differentiation media containing EGF and FGF2 give rise to migrating, proliferating RG cells within 24h and 48h of differentiation.

Characterization of the RG cell system

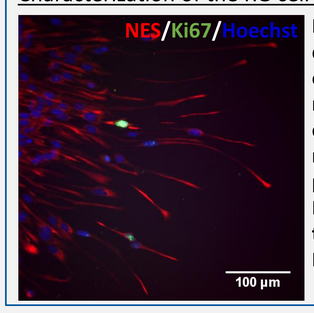
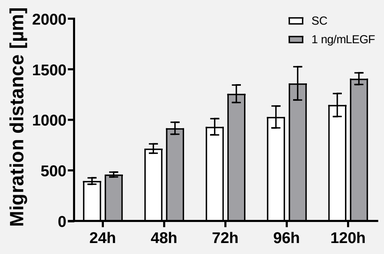


Fig. 2: After 24h of hNPC differentiation, migrated cells exhibit the characteristic RG morphology and express the cell type-specific marker nestin (NES) and the proliferation marker Ki67. Proliferation is enhanced by treatment with EGF and FGF2.



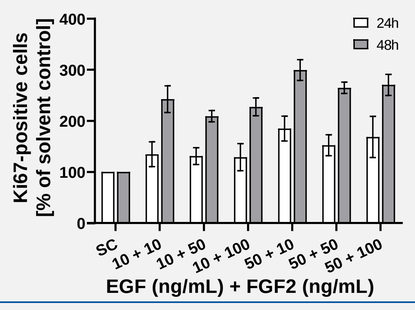


Fig. 3: Human NPC-derived RG obtain cell type-specific functions (migration, proliferation) during development. Cells respond to modulation of cell function-specific signalling pathways like EGF receptor activation with 1 ng/mL EGF (left). RG proliferation, assessed by Ki67-staining, can be enhanced by treatment with EGF and FGF2 combined (right).

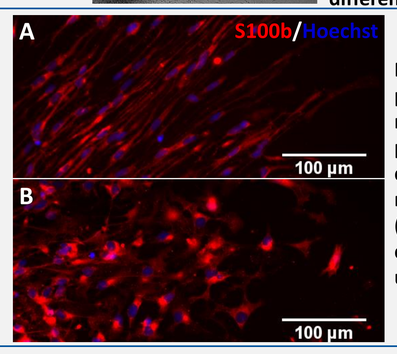


Fig. 4: Human NPC-derived RG are perturbed by a known chemical mode of action. Exposure of primary hNPCs to 1 µM MeHg (B) disrupted RG morphology and reduced the migration distance (not shown) after 48h of differentiation compared to the untreated control (A).

Work Package 2 – Astrocytes (AC)

- The different in vitro systems (e.g. primary vs. iPSC-based, human vs. mouse) give rise to different subtypes of ACs, which differ in morphology, physiological function, and their sensitivity to external stimuli/chemical exposure.
- Exposure of AC precursor cells to a DNT chemical can affect astrogenesis and the sensitivity of neurons to DNT chemical-triggered toxicity can be modulated by the presence of ACs in co-culture.

Objectives:

- Establishment and characterization of in vitro models to assess AC toxicity and AC roles in neuroprotection or neurotoxicity.
- For the detection of compound-induced disturbance of AC differentiation and maturation, AC differentiation from primary hNPC neurospheres or neural stem cells (NSC) will be performed in the presence or absence of test compounds. After 5 days of differentiation, AC will be quantified and their maturation assessed by immunocytochemistry and/or qPCR.
- Well-characterized neuronal test systems will be exposed to toxicants alone or in co-culture with ACs. Neurotoxicity and the modulation of toxic effects by ACs will be evaluated by assessing e.g. neuronal cell death, neurite outgrowth, and electrical activity.

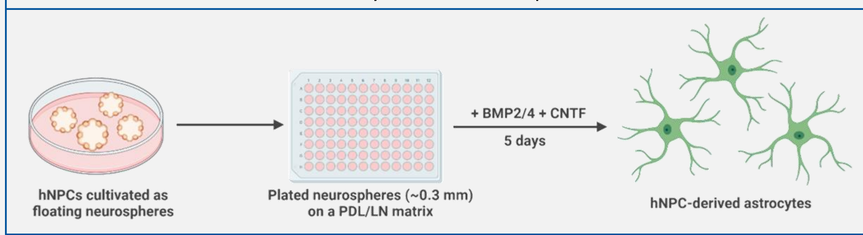


Fig. 5: Differentiation of ACs from hNPCs (IUF). Human NPCs plated on a PDL/LN-coated matrix are exposed to BMP2 or BMP4 in combination with CNTF for 5 days to promote AC differentiation.

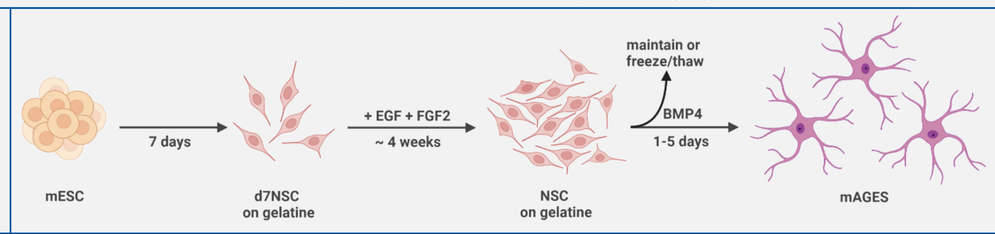


Fig. 6: Generation of murine astrocytes generated from embryonic stem cells (mAGES; UKN). After generation of a homogenous NSC population, transfer of cells to PDL/LN-coated dishes and exposure to BMP4 resulted in generation of mAGES.

Characterization of AC cell systems

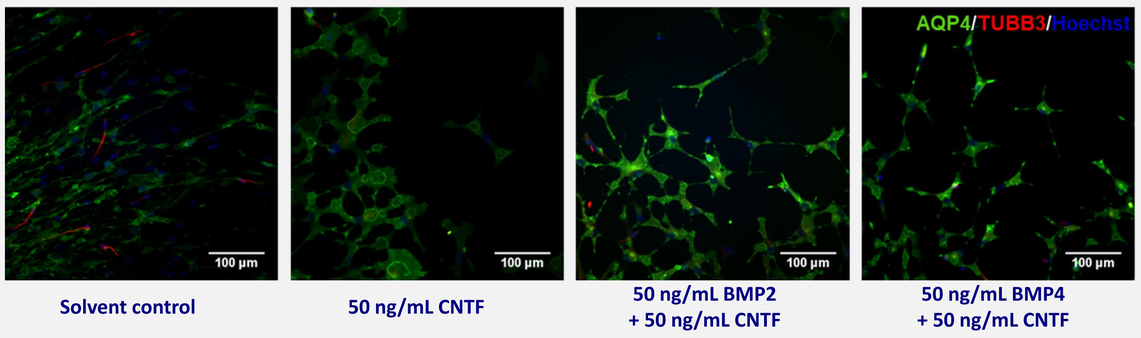


Fig. 7: Mature ACs are generated from hNPCs upon treatment with BMP2/4 + CNTF for 5 days (IUF). Immunocytochemical staining for AQP4 (green) and TUBB3 (red). Nuclei were counterstained with Hoechst33258 (blue).

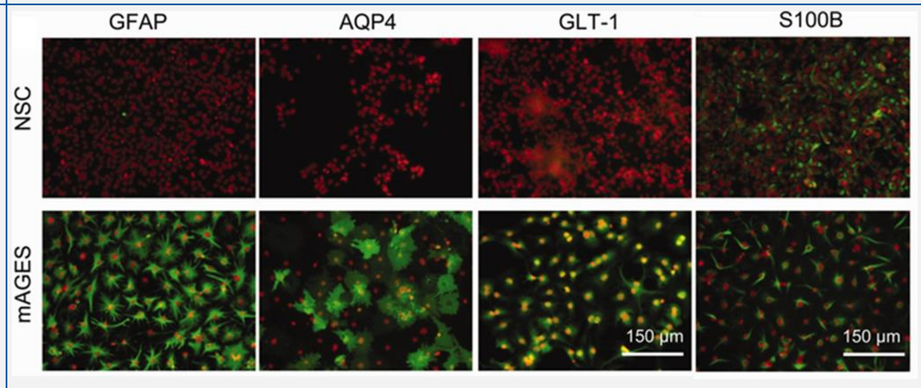


Fig. 7: Immunocytochemical characterization of marker expression of NSC and mAGES (UKN). Marker proteins (green) were visualized by immunocytochemistry and nuclei were counterstained with H-33342 (red; taken from Kleiderman et al., 2016).

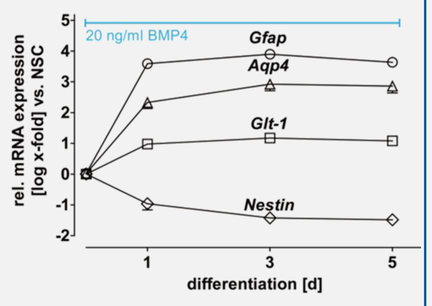


Fig. 8: Quantification of marker gene expression of mAGES by qPCR. Data is represented relative to the expression in NSC based on ΔΔCt (taken from Kleiderman et al., 2016).

Work Package 3 – Microglia (MG)

- Exposure of iPSC-derived MG to a toxicant can lead to microglial activation.
- MG can modulate the sensitivity of DNT models to chemical-triggered toxicity.

Objectives:

- Establishment and characterization of in vitro test systems to assess MG toxicity and the impact of MG co-culture on DNT effects on co-cultured cells.

Fig. 9: Generation of microglia-like cells from iPSC (UKN). Protocol by Haenseler et al., 2017.

Characterization of MG cell system

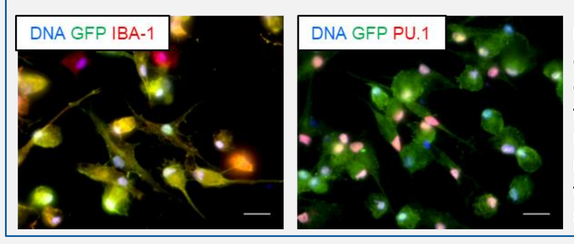
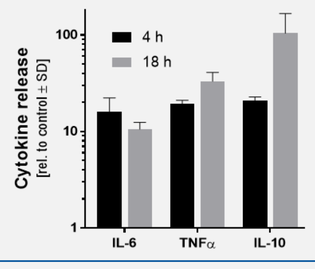


Fig. 10: Immunocytochemical characterization of microglia-like cells after 12 days of differentiation from macrophage precursor cells (MPCs). Cells were stained for the microglia markers IBA-1, PU.1 and the DNA intercalating dye H-33342 (taken from Brüll et al., 2020).



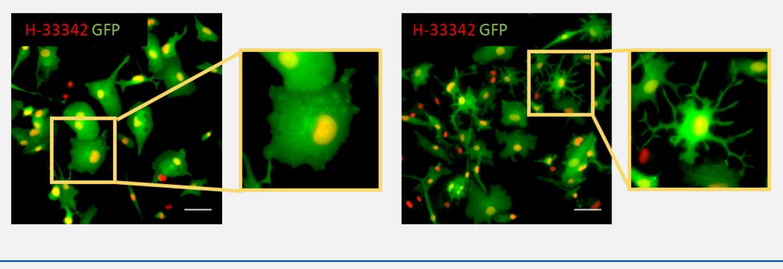


Fig. 11: Release of cytokines by microglia-like cells after stimulation with lipopolysaccharide (LPS; left). Microglia-like cells were differentiated from MPCs for 14 days and incubated with 100 ng/mL LPS (taken from Brüll et al., 2020). Microglia-like cells change morphology upon LPS treatment (right).

Summary and Outlook

- **Test systems based on RG, AC, and MG are available at the IUF and UKN.**
- **Further characterization of AC models will include gene expression analysis for developmental markers, functional characterization (glutamate uptake capacity), and assessment of astrocyte reactivity.**
- **The established cell models will be challenged with known DNT positive and negative compounds and especially false-negatives identified within the classical DNT-IVB.**
- **Results will be compared regarding cell source (primary vs. iPSC-derived) and species (human vs. mouse).**

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Literature:

- Brüll, Markus; Spreng, Anna-Sophie; Gutbier, Simon *et al.* (2020). Incorporation of stem cell-derived astrocytes into neuronal organoids to allow neuro-glial interactions in toxicological studies. ALTEX (3), S. 409–428. DOI: 10.14573/altex.1911111.
- Haenseler, Walther; Sansom, Stephen N.; Buchrieser, Julian *et al.* (2017). A Highly Efficient Human Pluripotent Stem Cell Microglia Model Displays a Neuronal-Co-culture-Specific Expression Profile and Inflammatory Response. Stem cell reports (6), S. 1727–1742. DOI: 10.1016/j.stemcr.2017.05.017.
- Kleiderman, Susanne; Sá, João V.; Teixeira, Ana P. *et al.* (2016). Functional and phenotypic differences of pure populations of stem cell-derived astrocytes and neuronal precursor cells. Glia (5), S. 695–715. DOI: 10.1002/glia.22954.