Refinement of stem cell-based *in vitro* assays towards a regulatory use for developmental and adult neurotoxicity testing of chemicals

Ilka Scharkin[1], Kristina Bartmann[1,2], Sarah Delovec[1], Arif Dönmez[1,2] Julia Hartmann[1], Haribaskar Ramachandran[1], Etta Zühr[1], Volker Busskamp[4], Andrea Rossi[1], *Katharina Koch[1,2] and *Ellen Fritsche[1,2,3]

[1] IUF - Leibniz Research Institute for Environmental Medicine, Duesseldorf, Germany; [2]DNTOX GmbH, Duesseldorf, Germany; [3] Swiss Centre for Applied Human Toxicology (SCAHT), Basel, Switzerland; [4]Department of Ophthalmology, Medical Faculty, University of Bonn, Bonn, Germany

** Equal contribution*

BACKGROUND & OBJECTIVES

- The **EU Green Deal's Chemical Strategy for Sustainability** highlights developmental (DNT) and adult neurotoxicity (ANT) as critical outcomes.
- Currently, **regulatory DNT and ANT testing** of chemicals is conducted in rats following OECD or US-EPA *in vivo* guideline studies. The **PARC Project** *[1]*, initiated in 2022 under Horizon Europe, aims to implement **Next Generation Risk Assessment** using data from **New Approach Methods** (NAMs).
- An ***in vitro* battery (DNT-IVB)** has been established as an alternative for regulatory DNT testing *[2]*. However, **neural network formation** (NNF) in human-based systems was identified as a gap in the DNT-IVB v1.0, and there is currently **no available IVB for ANT**.
- To address this gap and to establish NAMs for an MoA-based first-generation ANT-IVB, two hiPSC-based assays have been developed: the **human Neural Network Formation Assay (hNNF)** for DNT and the **human Multi-Neurotransmitter Receptor Assay (hMNR)** for ANT, both using **microelectrode arrays (MEAs)**.
- Both assays are undergoing **refinement** to reduce costs, increase throughput, and **improve regulatory acceptance**.

TWO STEM CELL-BASED ASSAYS FOR DNT AND ANT TESTING

COVERING FUNCTIONAL NEURAL NETWORK ENDPOINTS

THE **hNNF ASSAY** CLOSES A GAP OF THE DNT-IVB

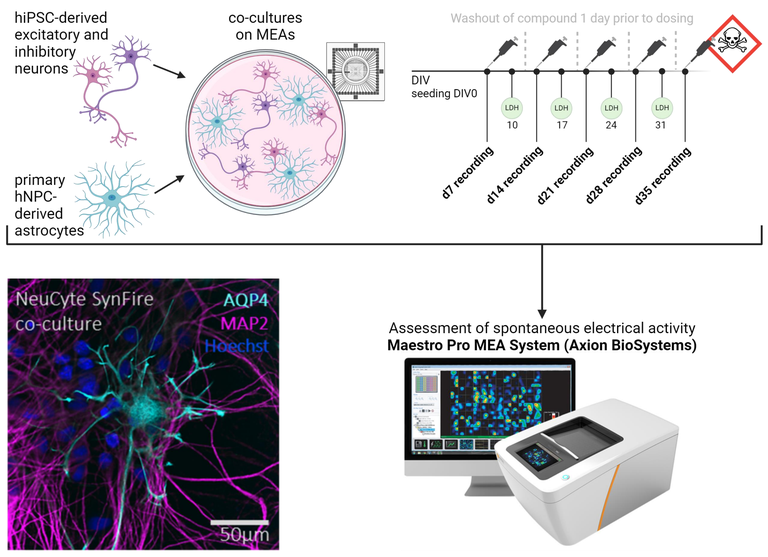

Figure 1. The hNNF Assay. Commercially available, pre-differentiated hiPSC-derived excitatory and inhibitory neurons and primary human astrocytes (NeuCyte, USA) form functional co-cultures in 48-well (16 electrodes/well) MEA plates (Axion BioSystems). Cultures differentiate for 7 days *in vitro* (7DIV) before exposure to the test compound. 24h before the weekly recording of spontaneous network activity with the Maestro Pro MEA system (Axion BioSystems), a washout of the compound is performed. Together with a weekly cytotoxicity assessment, this procedure is repeated until DIV35. *Corresponding publication: [3] Bartmann et al. (2023).*

THE **hMNR ASSAY** AIMS AT THE INITIATION OF AN ANT-IVB BY MoA ANALYSIS

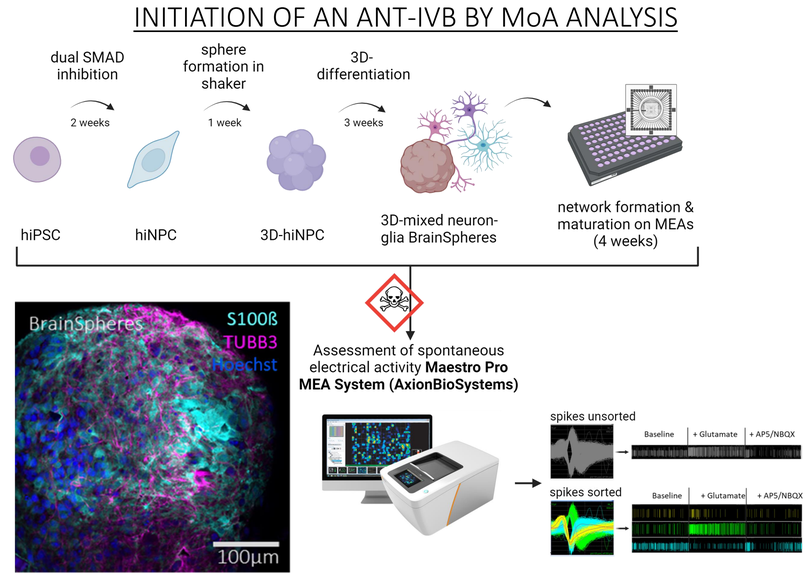

Figure 2. The hMNR Assay. Dual SMAD inhibition of hiPSCs generates hiNPCs. Shaking cultivation in CINDA+ differentiation medium yields 3D mixed neuron-glia BrainSpheres containing glutamatergic, GABA-ergic, dopaminergic, serotonergic and cholinergic neuronal subtypes that exhibit spontaneous electrical activity on 96-well MEAs. Detected spikes are sorted using the Plexon Offline Sorter software after treatment with neurotransmitter receptor agonists/antagonists. Compound effects on neuronal subtype receptors, including GABAergic, glutamatergic, and dopaminergic units, can be detected and quantified. *Corresponding publication: [4] Hartmann et al. (2023).*

Abbreviations: hiPSC - human induced pluripotent stem cell; hNPC - human neural progenitor cells; MEA - microelectrode array; Prol. - proliferation; S100ß - S100 calcium binding protein ß (Astrocyte marker); TUBB3 - ß-III-tubulin (Neuron marker); AQP4 - Aquaporin 4 (Astrocyte marker), MAP2 - Microtubule-associated protein 2 (Neuron marker)

RESULTS

ASSAY REFINEMENT TOWARDS ENHANCED THROUGHPUT AND COST REDUCTION

hNNF ASSAY REFINEMENT:

DOXYCYCLINE-INDUCIBLE GENERATION OF NEURONS - TWO APPROACHES

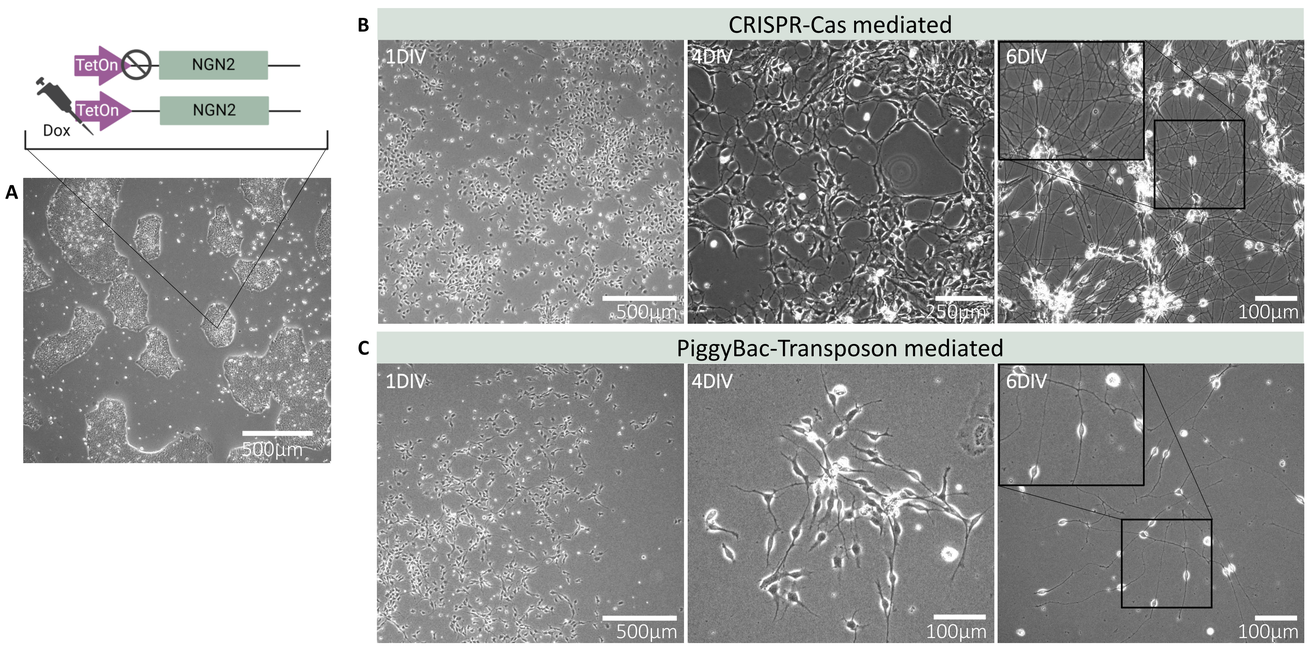

Figure 3. In-house-generated hiPSC lines differentiate into post-mitotic excitatory (*Ngn1/2*) neurons through doxycycline induction. Two approaches for generating inducible neurons are being tested: Commercially available IMR90 hiPSCs (**A**, WiCell, USA) are equipped with a doxycycline-inducible expression cassette for the excitatory neuronal transcription factor *Ngn1/2* by CRISPR-Cas (**B**) or the PiggyBac-Transposon System (**C**). With both methods, post-mitotic, network-forming neuronal cultures have been generated within six days in our laboratories. *Corresponding publication: [5] Busskamp et al. (2014).*

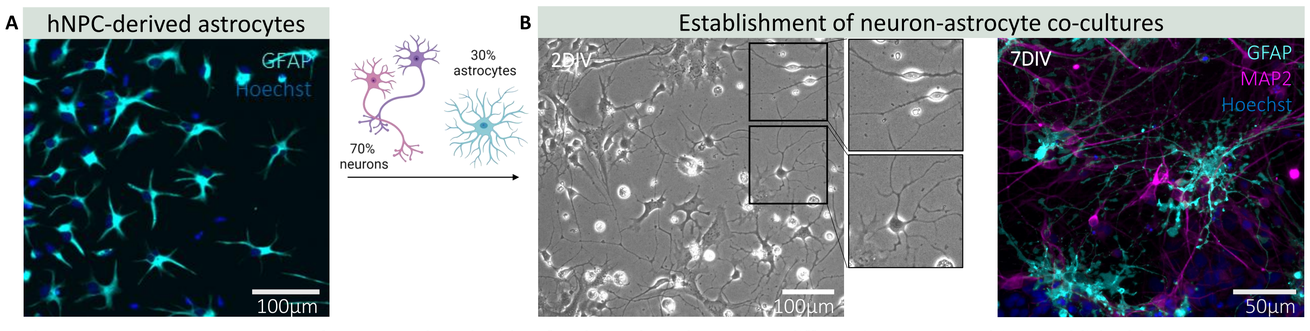

Figure 4. Neuron-astrocyte co-cultures. Combined with a fast hNPC-based astrocyte differentiation protocol (**A**, established by Etta Zühr, IUF), first successful defined co-cultures using the CRISPR-Cas-generated excitatory neurons (see **Fig. 3B**) have been established. Pre-differentiated hiPSC-based neurons together with cryopreserved and batched hNPC-based astrocytes were co-cultured in a 70%/30% ratio. (**B**) After two days of culture, both cell types present the characteristic morphologies (see enlarged boxes: top – neurons, bottom: astrocytes). Performing immunocytochemistry at day 7 in culture, both neurons and astrocytes could be identified by the corresponding markers MAP2 and GFAP.

hMNR REFINEMENT:

CASE STUDY - CHLOROPHENE DISRUPTS GABAergic SIGNALING *IN VITRO*

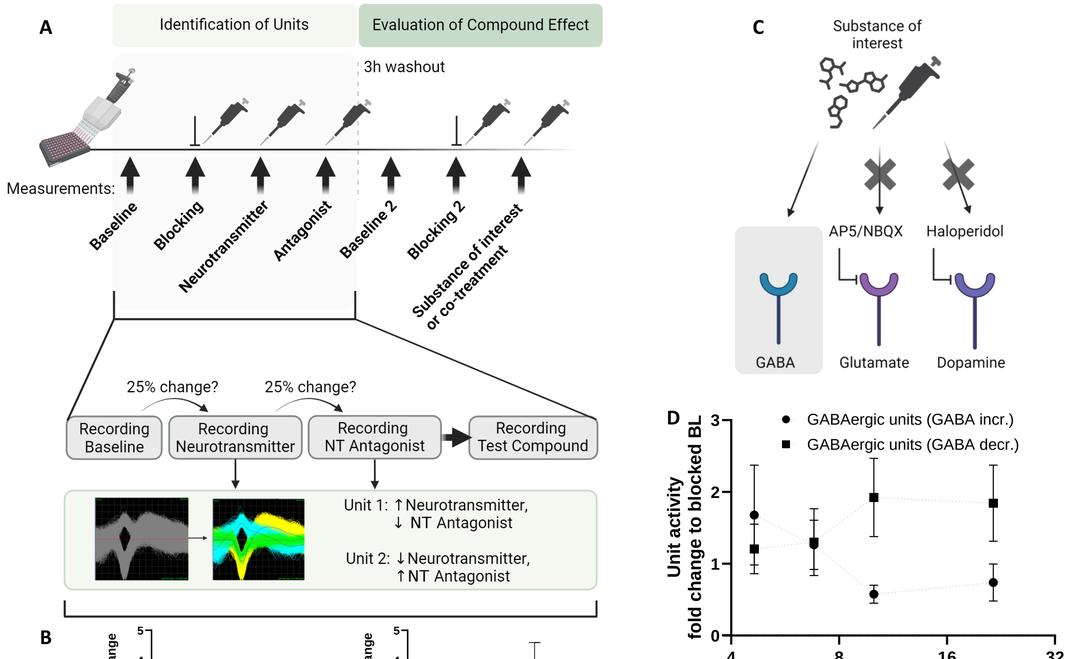

Figure 5. hMNR case study: Chlorophene disrupts GABAergic signaling *in vitro* using the refined hMNR-Assay. (**A**) The assay workflow involves spike sorting with the Plexon Offline Sorter (Plexon, USA) software to identify units reacting to receptor agonists and antagonists prior to compound effect analysis. A cutoff of 25% change in spiking between baseline and neurotransmitter addition determines unit identification. (**B**) This approach can discriminate early and later stages of neuronal subtype maturation, like pre- and post-GABA-switch neurons. Displayed as mean +- SEM. *n >= 21 (units) per response cluster*. (**C**) Refinements include an additional blocking step for precise measurement of signals from the neuronal subtype of interest, exemplified for GABAergic units. (**D**) Collaboration with the UFZ Leipzig using BrainSpheres treated with Chlorophene in the hMNR Assay (manuscript in prep.) reveals opposing responses in pre- and post-GABA switch-like units, with glutamatergic units unaffected. Displayed as mean +- SEM . *n >= 4 (units) per response cluster.* Ongoing refinement aims to develop a robust data evaluation strategy for higher-throughput analysis of MEA data.

Abbreviations: DIV - days in vitro; Ngn1/2 - Neurogenin1/2; CRISPR-Cas - Clustered regulalrly interspaced short palindromic repeats and CRISPR-associated protein; GFAP - glial fibrillary acidic protein; MAP2 - microtubule-associated protein; BIC - Bicuculline, GABA A receptor antagonist; AP5/NBQX - glutamate receptor antagonists; GLU - glutamate, incr. - increase; decr. - decrease.

CONCLUSION & OUTLOOK

- Set-up of the **hNNF assay** closed one identified gap of the DNT-IVB. The ongoing refinement using **in-house generated hiPSC-based cell lines** aims at providing a more **cost-efficient alternative**, enabling a higher throughput and extensive test system characterization.
- There is a need for setting up a first-generation **ANT-IVB** with test methods focusing on known **MoA of neurotoxicity**. The **refined hMNR assay** aims at **time-efficient and unified evaluation of large data sets from functional MEA readouts**, inclusion of male and female donor-derived hiPSC lines as well as further characterization of the cell system for more exact determination of applicability domains. Testing of model compounds demonstrated the suitability of the assay for the MoA identification for multiple neurotransmitter receptors [3].
- The described *in vitro* test methods will further contribute to the establishment and acceptance of DNT- and ANT-IVBs. Efforts continue to **refine both assays towards regulatory suitability and acceptance**, with future **validation planned** through testing of known positive and negative DNT and ANT model compounds.

LINKS & LITERATURE

[1] Marx-Stoelting, P. *et al.* (2023)
doi: 10.1007/s00204-022-03435-7.
[2] Blum, J. et al. (2022)
doi: 10.1016/j.chemosphere.2022.137035
[3] Bartmann, K. *et al.* (2023)
doi: 10.14573/altex.2206031.
[4] Hartmann, J. *et al.* (2023)
doi: 10.3390/cells12091270.
[5] Busskamp, V. *et al.* (2014)
doi: 10.15252/msb.20145508.

The PARC Project

*All figures created with **biorender.com.***

Contact: Ilka Scharkin (Ilka.Scharkin@iuf-duesseldorf.de)
IUF – Leibniz Research Institute for Environmental Medicine
Auf'm Hennekamp 50 , 40225 Düsseldorf

This work was funded by **Horizon Europe PARC** (Grant Agreement No. 101057014), the **Danish EPA** (Grant No. MST-66-00205) and **CERST Germany** (File No. 233-1.08.03.03-121972).