Establishment of *in vitro* assays for regulatory developmental neurotoxicity testing

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Background

- To assess developmental neurotoxicity (**DNT**) hazard, animal-free new approach methods (NAM) have been developed.
- NAM model certain key neurodevelopmental processes (KNDP) *in vitro.*
- For risk characterization, information from screen assays have to be combined with additional data like internal exposure.
- In this project, we use a set of pesticides to assess hits on **KNDP** using a variety of different **DNT** methods. Here, preliminary examples are shown.

Methods

1. Neurosphere Assay

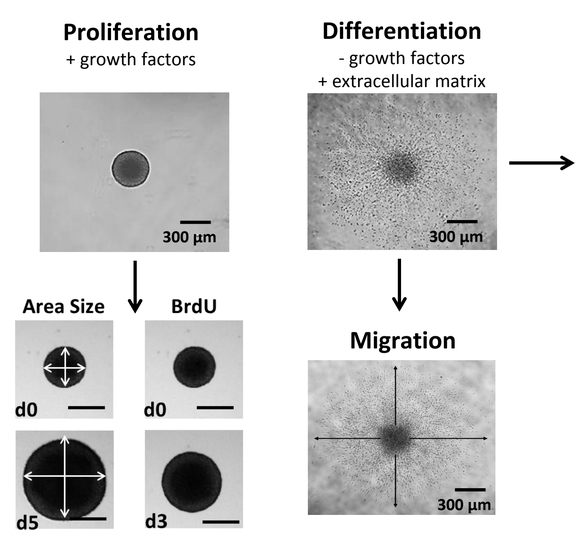

Fig. 1: The Neurosphere Assay
The assay is based on human neural progenitor cells (NPC) growing as neurospheres and covers the specific developmental neurotoxicity endpoints of proliferation, migration and differentiation into neurons, oligodendrocytes and astrocytes. Under proliferating conditions proliferation is assessed by measuring sphere diameter increase or performing a BrdU Cell Proliferation ELISA (Roche) (scale bars = 0.15 mm). Under differentiating conditions the migration distance of NPC is determined by taking phase-contrast pictures of the migration area and measuring the distance between the core of the neurosphere and the furthest migrated cells. After 5 days of differentiation immunocytochemical stainings are performed. Therefore, specific markers for astrocytes (GFAP, red), neurons (ßIII-tubulin, green) and oligodendrocytes (O4, pink) are used [1,2].

2. Neuronal Network Formation Assay

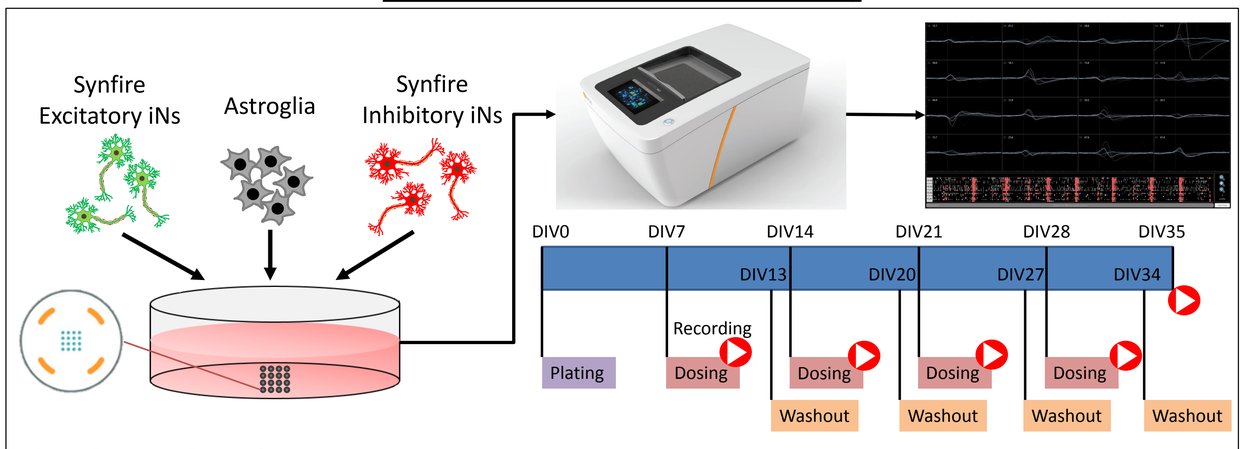

Fig. 2: The Neuronal Network Formation Assay
The assay is based on a co-culture containing iPSC-derived excitatory and inhibitory neurons (iNs) and primary Astroglia in 48-well (16 electrodes/well) MEA plates (NeuCyte, USA). Cultures are allowed to mature for 7 days until compound is added. On day 13; 20; 27 and 34 a washout of the compound is performed, followed by a 100 min recording (red dot) of spontaneous neuronal network activity on day 14; 21; 28 and 35 using the Axion Maestro Pro system. After recording cultures were re-dosed with the compound.

Results

3. Effects of CPFO on hNPC development

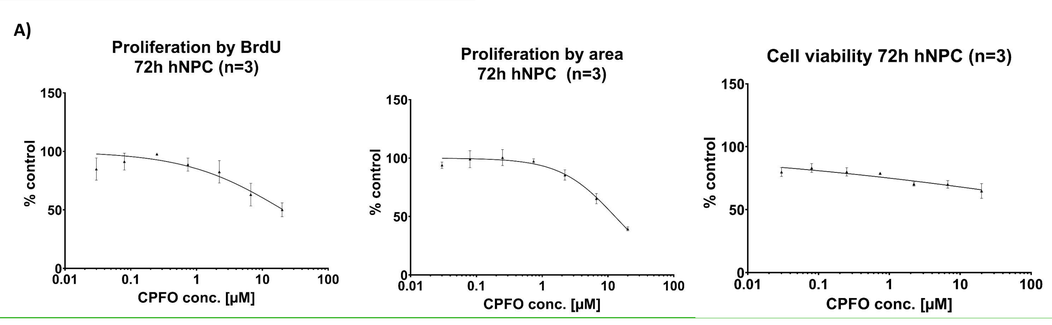

Fig. 3: Effects of CPFO on hNPC development
NPCs were treated with Chlorpyrifos-oxon (CPFO) under proliferative and differentiating conditions. Data are shown as mean of three independent experiments ± SEM **A)** Proliferation was assessed by measuring the diameter increase and by BrdU incorporation via ELISA (Roche) after 3 days. Additionally cell viability was assessed after 72h of proliferation with the Cell Titer Blue Assay (Cell viability assay from Promega, CTB) **B)** The migration distance was measured after 3 days and a Lactate Dehydrogenase Activity assay (CytoTox-ONE from Promega, LDH) was performed to detect cytotoxicity after 3 days. Data are shown as mean of two independent experiments ± MinMax

4. Effects of BIS-I on neuronal network formation

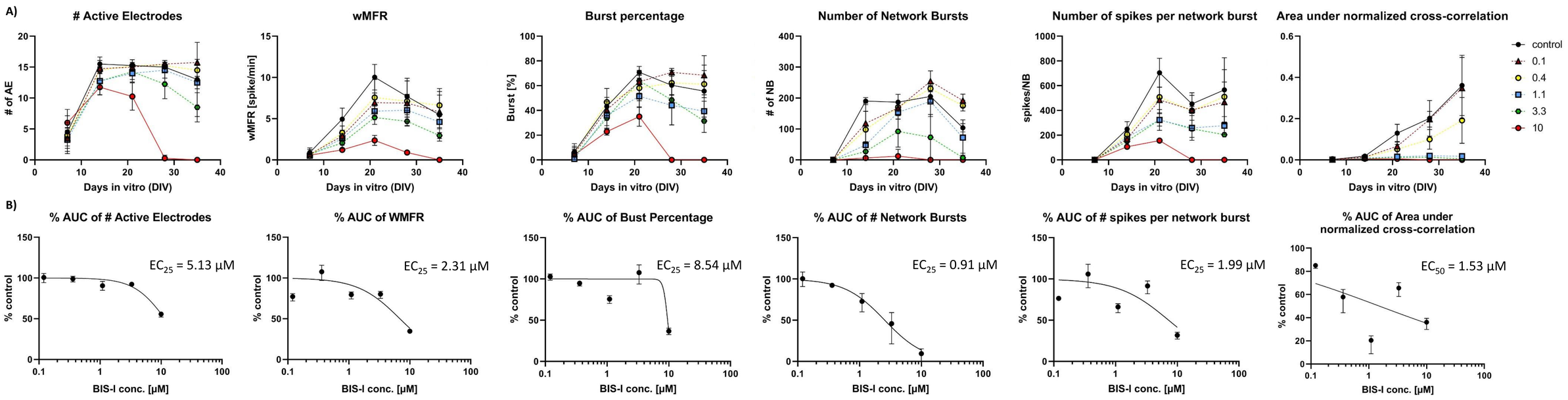

Fig. 4: Establishment of an endpoint-specific control using BIS-I on Neuronal Network Formation
After 7 days of differentiation cells were treated with different concentrations of the protein kinase C inhibitor Bisindolylmaleimide I (BIS-I). 20 min recordings were performed one day after total washout of the compound. **A)** # of active electrodes, weighted mean firing rate, burst percentage, network burst frequency, number of network bursts, number of spikes per network burst and are under normalized cross-correlation were assessed under different conditions. n=1 **B)** Determination of EC25/50 values for every parameter stated in A) based on Area Under the Curve (AUC) measurements (% of solvent control). n=1 **C)** a Lactate Dehydrogenase Activity (CytoTox-ONE from Promega, LDH) was performed to detect cytotoxicity on day 21; 28 and 35. n=1

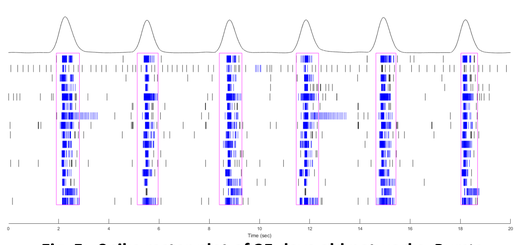

Fig. 5: Spike raster plot of 35 days old networks. Bursts are marked in blue. Rectangles indicate network bursts

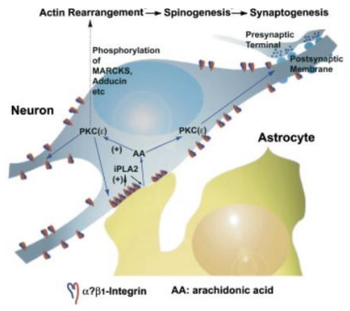

Fig. 6: Schematic of the intracellular signaling cascade progressing from local astrocyte contact to the global facilitation of synaptogenesis. There is evidence of global activation of PKC signaling within individual neurons after contact with astrocytes. PKC activation appears to be responsible for facilitation of excitatory synaptogenesis (Hama et al., 2004[3]).

Summary & Outlook

- **The Neurosphere Assay detects pesticide's effects on NPC proliferation, migration and differentiation.**
- **The SynFire Kit (NeuCyte) provides a standardized platform of human excitatory and inhibitory neurons as well as astrocytes that produces reproducible neuronal networks on MEAs *in vitro*.**
- **PKC signalling is crucial for synaptogenesis and therefore the PKC-inhibitor BIS-I was successfully established as an endpoint-specific control for the neuronal network formation (NNF) assay.**

- **35 selected pesticides will be tested in the Neurosphere Assay and in the NNF assay (IUF) as well as in two additional assays measuring neurite outgrowth (University of Konstanz).**
  Hit confirmation testing will be performed with an alternative, orthogonal assay based on hiPSC-derived NPC assessing the same endpoints.
  An interlaboratory transfer of the methods between the IUF and the University of Konstanz will improve readiness and robustness of tests.

Improvement of data analysis and interpretation of concentration-response toxicity data by creating an R-based data evaluation tool. (Poster P12-029)

Literature & Funding

[1]Baumann, J. et al. 2015. Application of the Neurosphere Assay for DNT Hazard Assessment: Challenges and Limitations. Methods Pharmacol. Toxicol. 1–29.
[2]Fritsche, E. (2017). Workshop Report: OECD/EFSA Workshop on Developmental Neurotoxicity (DNT): The Use of Non-Animal Test Methods for Regulatory Purposes. *ALTEX* 34, 2 (May 2017), 311-315
[3]Hama, H. (2004) PKC Signaling Mediates Global Enhancement of Excitatory Synaptogenesis in Neurons Triggered by Local Contact with Astrocytes

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