Toxicology and Applied Pharmacology

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Stemcell based assay for *in vitro* developmental neurotoxicity testing

Current regulatory protocols to test the developmental neurotoxic potential of industrial chemicals and agricultural pesticides [ref. 1,2] require hundreds of animals in the *in vivo* tests to fulfill statistical requirements. Successful implementation of an adequate and validated *in vitro* method in first tier screening of chemicals (e.g. REACH) and drugs for global toxicity testing on neural cell level ultimately results in a reduced number of compounds selected for further study *in vivo* and, hence, a strong reduction of animal use. Development of a new method is in progress as part of a research project funded by the Dutch authorities on the development of new alternative methods.

In vivo neuronal development and *in vitro* neurotoxicity testing

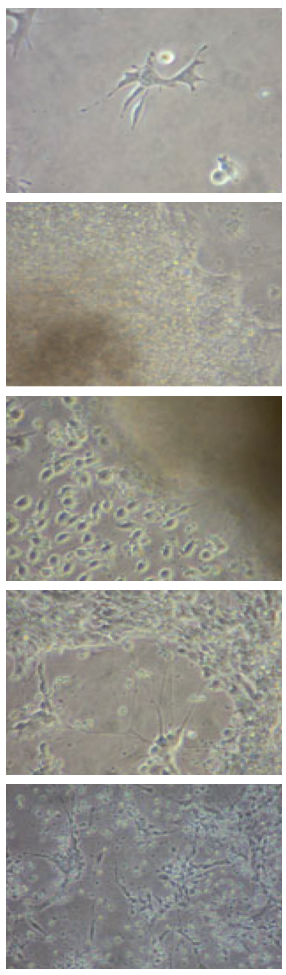
Effects of chemicals on any neurodevelopmental process like proliferation, migration, differentiation or apoptosis will likely have neurotoxic consequen-ces for the CNS as an organ. Current cell based *in vitro* models for neurotoxicity testing only mimic part of the *in vivo* neuronal development. Therefore, these *in vitro* models only detect the class of chemicals that specifically interferes with the specific *in vivo* process that is mimicked in the *in vitro* system.

ES-D3 mouse stem cell based assay for *in vitro* developmental neurotoxicity testing

Stem cells can be forced to undergo differentiation into a mixed culture of the three main cell types in the CNS: neurons, astrocytes and oligodendrocytes. In the *in vitro* system that is being set up with ES-D3 mouse stem cells, development towards neuronal destiny seems to be driven by processes like **proliferation**, **migration**, and **differentiation**. We hypothesize that the *in vitro* neuronal development from stem cells mimics the *in vivo* situation to such extent that chemicals that interfere with any process in *in vivo* neuronal development also interfere with neuronal development in this *in vitro* system. If true, this *in vitro* test system can be used as a model to detect developmental neurotoxic effects of a broad range of chemicals.

Differentiation from ES-D3 stem cells into neuronal cells (*Figure 1*)

The protocol used for differentiation of the stem cells into the cell types present in the CNS is based on a protocol described by Kim *et al.*, [ref.3], with some modifications.



(A) Day 1: undifferentiated ES-D3 cells on gelatin coated flasks.

(B) Day 7: cell clusters (Embryonic Bodies) just after transfer to 75 cm² flasks.

(C) Day 14: neuronal precursors migrating from the Embryonic Bodies.

(D) Day 14: Neuronal precursors (?) connected through processes.

(E) Day 23: differentiated cells on fibronectin/ poly-L-lysin coated coverslips.

Figure 1. Micrographs of ES-D3 stem cells in vitro during different phases of development.

Cell characterization: microscopy and immunohistochemistry

Critical aspect of the test is to <u>repeatedly</u> obtain cell cultures showing constant ratios of neurons, astrocytes and oligodendrocytes. During culturing, cell types can be distinguished morphologically by visual inspection of unstained or generally (H&E) stained cultures (see Figure 2). Further, attempts are being made to selectively stain the different cell types using immunochemical and immunofluor-escent stains. After day 23 of the culture period, cells are characterized using the antibodies α-β-(III)-tubulin, α-GFAP and O4, to specifically label the cells as neurons, astrocytes or oligodendrocytes, respectively (see Figure 3).

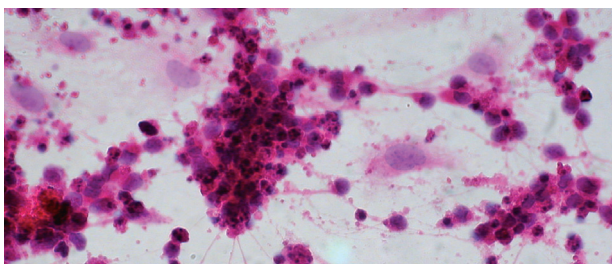
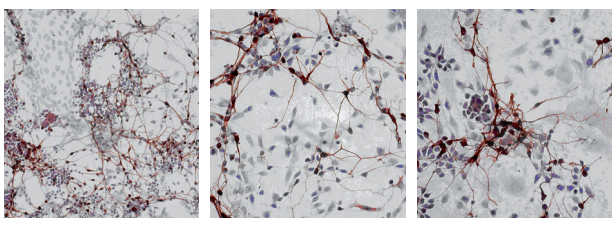


Figure 2. Cells in culture, fixated at day 23 with methanol. 200x, LM, H&E stain.



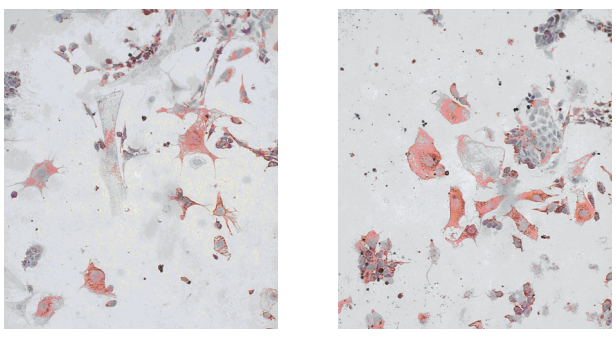
200x *400x* *400x*

LM, α-β-(III)-tubulin, counter-stained with haematoxiline.

Figure 3. Cells fixated at day 23 with paraformaldehyde. Stained with α-β-(III)-tubulin, which is specific for neurons. The specificity exceeds that of enolase and as such it can be used as a marker for neural tissues.

Effects of chemical exposure

The development of one or more of these specific cell types may be affected by chemical exposure and, thus, their numerical relationship in the culture may change. Hence, a clear shift in percentage of one of the cell types in an exposed culture indicates the (developmental) neurotoxic potential of a compound. In many cases, however, the changes induced by toxic substances will not lead to easily visible shifts in cell populations and a quantitative analysis of such subtle changes is required.



200x, LM, O4, counter-stained with haematoxilin.

Figure 4. Cells fixated at day 23 with formalin. Stained with O4, which is specific for oligodendrocytes. Oligodendrocyte Marker O4 is an antigen on the surface of oligodendrocyte progenitors.

Quantification of cell types: the CAST system

Changes in cell ratio's as indicators for toxicity don't tell us to which extent the nominator or the denominator in the ratio was affected. Therefore, a major goal of this project is to design an efficient way to characterize and quantify the different cell-types in absolute terms. To that end, stereological principles will be used to develop an efficient sampling design along the lines of *in vivo* develop-mental neuropathology testing, facilitated by a validated Computer Aided Stereology Toolbox (CAST) [ref.4]. Quantification of 3-D cell characteristics by stereological means is about to start with the development of an efficient sampling design to optimally analyze the cells in the culture with minimum effort.

Additional endpoints

Once well-characterized cultures with stable cell type ratios are achieved, selected test compounds will be used to validate the *in vitro* test. Then we will also gather information concerning additional endpoints including:

- Cytotoxicity: *in vitro* neurodevelopmental effects can only be considered relevant when they occur at a lower concentration than the cytotoxic concentration.
- Effects on proliferation/differentiation and migration: these processes play their role in *in vivo* development of the CNS, and are also witnessed in the developed *in vitro* system.
- Functionality of specific cell types: functionality tes-ting of the *in vitro* assay by electrophysiology (vesicu-lar dopamine release and cellular Ca2+ dynamics).

Conclusions

- Development, validation and implementation of an *in vitro* screening assay may improve regulatory testing for developmental neurotoxicity and reduce the number of animals used for risk assessment of chemicals.
- The stem cell based *in vitro* assay offers the possibility to test substances for selective effects on either proliferation, migration or differentiation, independently of each other. Animal experiments should only be performed with the test substances showing clear *in vitro* developmental neurotoxic effects.

References

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[2] EPA, Guidelines OPPTS 870.6300; 8600. http://www.cpn.gov/epahome/research.htm
[3] Kim *et al*. Methods in Enzymology, 2003;365:303-327.
[4] De Groot *et al*. Reproductive Toxicology 20 (2005) 417-432.

Acknowledgements

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