NEUROTOXICITY TESTS:

HOW TO INTERPRET DATA

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IN VITRO NEUROTOXICITY TESTING

HOW TO INTERPRET DATA

PESTICIDE EXPOSURE AND PARKINSON’S DISEASE
INTRODUCTION

• Several epidemiological studies suggest an association between pesticide exposure and Parkinson’s disease (Priyadarshi et al., 2000; Brekenridge et al., 2016; Hernandez et al., 2016)

• Use of insecticides and herbicide is associated with statistically significant increase in Parkinson’s disease (Breckenridge et al., 2016)

• Early Environmental Origins of Neurodegenerative Diseases in Later life (Landrigan et al., 2005)

• The herbicide paraquat may be positively associated to Parkinson’s disease by mechanisms having a role in Parkinson’s pathophysiology (Tanner et al., 2011)
Which are the mechanisms that are involved in neurodegenerative diseases?

- Oxidative stress
- Accumulation of aggregated proteins
- Neuroinflammation
Neuroinflammation is involved in neurodegenerative diseases

- Trauma
- Infection
- Stroke/hypoxia
- Protein aggregation
- Toxic exposure

Modified from Ceulemans AG et al., 2010

- Insult
- Microglial activation
- Release of pro-/anti-inflammatory mediators
- Astroglial reactivity (increased GFAP expression)
- Tissue repair / tissue damage
- Neuronal damage
The dual role of microglia in neuroinflammation

Pro-inflammatory signals:
- e.g. free radicals, IL-6, IL-1β, TNFα

Anti-inflammatory signals:
- e.g. IL-4, IL-10, growth-factors

Classical activation
- e.g. LPS, IFN-γ

Alternative activation
- IL-4
- IL-10
- IL-13

M1
- Neurodegeneration

M2
- Tissue repair

Does paraquat induce a neuroinflammatory response?
The model: 3D cultures of rat brain cells

- **E16 rat forebrain**
- **Mechanical dissociation**
- **Defined medium**
- **Spontaneous aggregation**

**Culture start (day)**

- **Proliferation**
- **Differentiation**

**Neurons**
- NF

**Astrocytes**
- GFAP

**Oligodendrocytes**
- MBP

**Microglia**
- IB4

**Tissue-like organization – with cell-cell interactions**

**Functional – spontaneous and induced electric activity**

**Metabolic activity**
Experimental Setup

Subchronical exposure with low concentrations of paraquat of immature 3D rat brain cell cultures

Immediate effects

Paraquat exposure

Culture start (day) 0 5 7 9 11 13 15

Sample

Long term effects

Paraquat exposure

Culture start (day) 0 5 7 9 11 13 15

Recovery period 35

Sample

Minimal cell death

A

Total protein

Protein Content
Fold change of Ctrl ± SEM

DIV15 DIV35

B

Enzymatic activity
fold change of Ctrl ± SEM

DIV15 DIV35

CTR

0.5 μM PQ

1.0 μM PQ

Experimental Setup

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CTR

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1.0 μM PQ
Paraquat-induced effects on dopaminergic neurons

Immediate (DIV 15) and long term/delayed (DIV 35) effects of paraquat subchronical exposure on neurons

Effects of paraquat treatment on neurons are more pronounced immediately at the end of the treatment period, and a partial recovery is observed after treatment arrest.

Sandström et al., 2014
Paraquat-induced microglial reactivity

Long term/delayed paraquat-induced microglial reactivity.
Expression of the M1 neurodegenerative phenotype.

Sandström et al., 2014
Paraquat-induced expression of cytokines and of molecules involved in the triggering of neuroinflammation

Paraquat-induced cytokine expression

INFLAMMATION | Div15 | Div35 |
---|---|---|
ANXA1_RAT | Annexin A1 | - | 1.32 |
ANXA2_RAT | Annexin A2 (PAP-IV) | - | 1.26 |
LEG3_RAT | Galectin-3 (Gal-3) (CBP 35) | - | 1.37 |
TOLIP_RAT | Toll-interacting protein | 1.36 | 1.14 |

Sandström et al., 2014
Paraquat-induced astrocyte reactivity

Paraquat induced an astrocyte reactivity (astrogliosis) of long duration

Sandström et al., 2014
Paraquat treatment is able to induce a delayed neuroinflammatory response, that acquires the M1 neurodegenerative phenotype even when a partial recovery of adverse effects on neurons is observed.

Long term neuroinflammation may be a link between early environmental-induced adverse effects and late onset of neurodegenerative disease.
Paraquat exposure should be considered as a risk factor for Parkinson’s disease, since it is able to trigger Neuroinflammation expressing the M1 neurodegenerative phenotype, a mechanism involved in the disease pathophysiology.

As support:

Purisai et al., 2007, showing that if microglial reactivity is blocked after a first exposure to paraquat, no deleterious effect on dopaminergic neurons is observed after several re-exposures.
Mechanistically-driven regulation

The concept of Adverse Outcome Pathway (AOP)

Development of AOP

Molecular Initiating Event (MIE)

Intermediate Key Events (KE) linked by Key Events Relationships (KER)

Adverse Outcome (AO) relevant for risk assessment

Anchor 1

Evidence in the literature?

Anchor 2

Figure 2. A Schematic Diagram for the development of an AOP Starting at any of the Three Main Blocks of Information.

Modified from OECD. 2010
Definition of Toxicity Pathway, MOA and AOP

MOA = Chemical **specific** toxicity pathway (WHO/IPCS, 2002)

AOP = Chemical **agnostic** toxicity pathway (OECD, 2012)
Mechanistically-driven regulation

AOPs on: Mitochondria-dependent ROS overproduction in nigra-striatal neurons leading to parkinsonian motor deficits

Molecular Initiating Event (MIE)

Redox-cycling (of a chemical) initiated by electrons of the mitochondrial respiratory chain

Cellular effects

KE 1

Reactive oxygen species (ROS) formation

KE 2

Mitochondrial dysfunction

KE 3

Impaired proteostasis

Organ effects

 KE 4

Neuronal degeneration of nigrostriatal pathway

KE 5

Neuro-inflammation

AO

Motor symptoms of PD (Bradykinesia, rigor, tremor)

Viviani et al., AOP wiki, in preparation
Proteomic analysis for global analysis of changes induced by a 10-day paraquat treatment of differentiated 3D rat brain cell cultures

<table>
<thead>
<tr>
<th>KE1</th>
<th>KE2</th>
<th>KE3</th>
<th>KE4</th>
<th>KE5</th>
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</thead>
<tbody>
<tr>
<td>ROS formation</td>
<td>Mitochondrial dysfunction</td>
<td>Impaired proteostasis</td>
<td>Neuronal degeneration of the nigrostriatal pathway</td>
<td>Neuroinflammation</td>
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<tr>
<td>GST alpha3 (1.31)</td>
<td>cytochrome c oxidase (1.40)</td>
<td>Huntingtin-1 (0.76)</td>
<td>GABAR subunit (0.62)</td>
<td>Microglia</td>
</tr>
<tr>
<td>NADPH dehydro (NADPH oxidase (1.27)</td>
<td>NADPH dehydro ubiquinone (0.78)</td>
<td>amyloid A4 precursor (0.78)</td>
<td>synaptogamin (0.72)</td>
<td>neuromodulin (GAP-43) (0.80)</td>
</tr>
<tr>
<td>NADPH dehydro quinone (1.27)</td>
<td>ubiquitin (0.80)</td>
<td>GluR2 (0.79)</td>
<td>NADPH dehydro quinone (NADPH oxidase) (1.27)</td>
<td></td>
</tr>
<tr>
<td>stathmin-3 (microtubule destabilizing activity) (0.76)</td>
<td>mGluR5 (0.80)</td>
<td>Cam kinase-like vesicle associated (0.63)</td>
<td>Astrocyes</td>
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<tr>
<td>neurofilaments -L (NF-L) (0.68)</td>
<td>MAP 1A/1B (0.70)</td>
<td>synaptogamin 1 (0.74)</td>
<td>neuromodulin (GAP-43) (Parkinson) (0.80)</td>
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<tr>
<td>ankyrin-repeat (0.70)</td>
<td>N-CAM (0.75)</td>
<td>synapsin 1 (0.76)</td>
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<tr>
<td>neurofilament-M (NF-M) (0.71)</td>
<td>-synuclein (0.76)</td>
<td>neuroplastin (adhesion) (0.76)</td>
<td>Adaptation in pathology</td>
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<tr>
<td>-internexin (0.77)</td>
<td>MAP-6 (0.77)</td>
<td>OX-2 (CD200) (role in microglial activation) (0.80)</td>
<td>neuronal growth regulator (0.79)</td>
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<tr>
<td>MAP-2 (0.80)</td>
<td></td>
<td>B crystallin (1.24)</td>
<td></td>
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</tbody>
</table>
CONCLUSIONS

1. Paraquat exposure should be considered as a risk factor for Parkinson’s disease because it is able to trigger a cascade of Key Events (of an AOP) involved in the disease pathophysiology.

1. 3D cultures containing all types of brain cells are good models to study toxicant-induced mechanisms of toxicity.

What about toxicokinetic / ADME for MOA

- In vitro models of BBB are available
- Absorption/Uptake
- Metabolism
Drug Uptake in vitro

$^{14}$C-labelled Paraquat uptake in 3D rat brain cell cultures

Dose- and time-dependent uptake of paraquat
Presence of several CYPs in 3D cultures of rat brain cells (Vichi et al., 2015)
**Xenobiotic Metabolism in vitro**

Cellular localization of several CYPs in 3D cultures of rat brain cells (Vichi et al., 2015)

THC metabolism (by CYP 2C for 11-OH-THC) in 3D rat brain cell cultures

Monnet-Tschudi et al., 2008
Human models to increase predictability

Sandström et al., submitted
Acknowledgements

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Igor Charvet
Adrien Roux

Michael Aschner
Paraquat-induced oxidative stress, an early KE

**Lipid peroxidation (48h)**

F$_2$-IsoP

<table>
<thead>
<tr>
<th></th>
<th>CTR</th>
<th>0.5</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIV7</td>
<td>§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIV35</td>
<td></td>
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</tr>
</tbody>
</table>

$^\S$ P = 0.078

**Heme oxygenase (hsp 32) mRNA expression (10d, long term)**

<table>
<thead>
<tr>
<th></th>
<th>CTR</th>
<th>0.5</th>
<th>1.0</th>
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</thead>
<tbody>
<tr>
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<td>DIV35</td>
<td></td>
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</tbody>
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<table>
<thead>
<tr>
<th><strong>OXIDATIVE STRESS / TOXIC METABOLISM</strong></th>
<th>Div15</th>
<th>Div35</th>
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</thead>
<tbody>
<tr>
<td>CAH2-RAT</td>
<td>1.26</td>
<td>1.09</td>
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<tr>
<td>SODM_RAT</td>
<td>1.16</td>
<td>1.09</td>
</tr>
<tr>
<td>TXNL1_RAT</td>
<td>1.25</td>
<td>1.24</td>
</tr>
<tr>
<td>GSTA4_RAT</td>
<td>1.10</td>
<td>1.02</td>
</tr>
<tr>
<td>GSTT2_RAT</td>
<td>1.26</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Sandström et al., 2014
Preliminary Results in 3D hESC

72 h exposure of differentiating 3D cultures prepared from hESC to methylmercury, trimethyltin, paraquat and ibuprofen as negative control

![Graphs showing mRNA fold change and LDH activity](image)