Novel *in vitro* approaches for the detection of acute neurotoxicity using emerging technologies

Dissertation

zur Erlangung des akademischen Grades des Doktors
der Naturwissenschaften

an der Universität Konstanz (Fachbereich Biologie)
vorgelegt von

Erwin van Vliet

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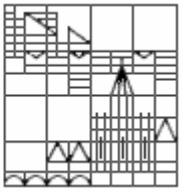

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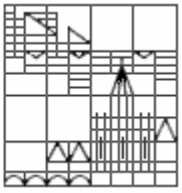

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List of publications

Manuscripts which are part of this thesis:

- **Development of a mechanistically-based genetically engineered PC12 cell system to detect p53-mediated cytotoxicity. Erwin van Vliet**, Chantra Eskes, Silvia Stingele, Joanne Gartlon, Anna Price, Massimo, Farina, Jessica Ponti, Thomas Hartung, Enrico Sabbioni, Sandra Coecke, (2007), *Toxicology in Vitro,* Jun; 21 (4): 698-705.

- **Electrophysiological recording of re-aggregating brain cell cultures on multi-electrode arrays to detect acute neurotoxic effects. Erwin van Vliet,** Luc Stoppini, Maurizio Balestrino, Chantra Eskes, Claudius Griesinger, Tomasz Sobanski, Maurice Whelan, Thomas Hartung, Sandra Coecke, (2007), *Neurotoxicology*, Nov; 28 (6):1136-46.

- **A novel *in vitro* metabolomics approach for neurotoxicity testing, proof of principle for methyl mercury chloride and caffeine. Erwin van Vliet,** Siegfried Morath, Jens Linge, Juri Rappsilber, Chantra Eskes, Paul Honegger, Thomas Hartung & Sandra Coecke, (2007), *Neurotoxicology,* (In press).

Significant contributions have been made to:

- **Metabolism-mediated neurotoxicity: the significance of genetically engineered cell lines and new three dimensional cell cultures.** Sandra Coecke, Chantra Eskes, Joanne Gartlon, **Erwin van Vliet**, Agnieszka Kinsner, Alesia Bogni, Laura Raimondo, Nicholaos Parissis, Ingrid Langezaal, (2002), *Altern Lab Anim*, 30 Suppl 2:115-118.

- **The value of alternative testing for neurotoxicity in the context of regulatory needs.**
  Sandra Coecke, Chantra Eskes, Joanne Gartlon, Agnieszka Kinsner, Anna Price, **Erwin van Vliet**, Pilar Prieto, Monica Boveri, Susanne Bremer, Sarah Adler, Christian Pellizzer, Albrecht Wendel, Thomas Hartung, (2005), *Environmental Toxicology and Pharmacology,* 21, No. 2: 153-167.

Scientific presentations relevant for this thesis:

- **Electrophysiological recording of re-aggregating brain cell cultures on multi-electrode arrays to detect acute neurotoxic effects. Erwin van Vliet,** Luc Stoppini, Maurizio Balestrino, Chantra Eskes, Claudius Griesinger, Tomasz Sobanski, Thomas Hartung, Sandra Coecke, *Oral and* p*oster presentation at the 11th Biennial Meeting of the International Neurotoxicology Association, 10-15 June, 2007, Pacific Grove, Montery, California U.S.A.*

- **An electrophysiological approach for neurotoxicity screening. Erwin van Vliet,** Luc Stoppini, Maurizio Balestrino, Chantra Eskes, Claudius Griesinger, Tomasz Sobanski, Thomas Hartung, Sandra Coecke, *Oral presentation at the ECOPA Science Initiative workshop, 29-30 September, 2006, Alicante, Spain.*

- **A novel test *in vitro* test system for the detection of p53-mediated toxicity.**

  Erwin van Vliet, Chantra Eskes, Silvia Stingele, Joanne Gartlon, Anna Price, Massimo, Farina, Jessica Ponti, Thomas Hartung, Enrico Sabbioni, Sandra Coecke, *Poster presentation at the international meeting on in vitro cytotoxicity mechanisms, 26-29 March, 2006*, *Verona, Italy.*

- **A novel metabonomics approach for *in vitro* neurotoxicity screening.**

  Erwin van Vliet, Siegfried Morath, Thomas Hartung, Sandra Coecke, *Oral presentation at the workshop on Metabonomics, 29-30, November, 2004,* Ispra*, Italy.*

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- Joint Research Centre innovative project competition 2005, “Toxfinder” *in vitro* metabonomics for neurotoxicity.

Abbreviations

AchE	Acetylcholine Esterase
aCSF	Artificial Cerebrospinal Fluid
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazole
ANOVA	Analysis of variances
APV	DL-2-Amino-5-Phosphonovaleric Acid
BSA	Bovine Serum Albumin
CaCl2	Calcium chloride
cDNA	Complementary DNA
CNQX	6-Cyano-7-Nitroquinoxaline-2,3-dione
CNS	Central Nervous System
ChaT	Choline acetyltransferase
COMET	Consortium for Metabonomic Toxicology
DMEM	Dulbecco's Modified Eagle's medium
DIV	Days *In Vitro*
DMSO	Dimethyl sulfoxide
DNA	Deoxyribosenucleic Acid
ECVAM	European Centre for the Validation of Alternative Methods
EPA	Environmental Protection Agency
EU	European Union
FDA	Food and Drug Administration
GABA	Gamma-aminobutyric acid
GAD	Glutamic Acid Decarboxylase
GCCP	Good Cell Culture Practice
GFAP	Glial Fibrillary Acidic Protein
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
IARC	International Agency for Research on Cancer
KA	Kainic Acid
LDH	Lactate dehydrogenase
MAB	Myeline basic protein

MAP2	Microtubule Associated Protein 2
MEA	Multi-Electrode Array
MeHgCl	Methyl mercury chloride
MS	Mass Spectrometry
MTT	(3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NGF	Nerve Growth Factor
NMDA	N-Methyl-D-Aspartate
NMR	Nuclear Magnetic Resonance
OECD	Organisation for Economic Co-operation and Development
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PCA	Principal Component Analysis
PC12	Pheochromocytoma 12
PI	Propidium Iodide
PVDF	Polyvinylidene
PTFE	Polytetrafluoroethylene
REACH	Registration Evaluation and Authorisation of Chemicals
RPMI	Roswell Park Memorial Institute
SD	Standard Deviation
Tc	Tetracycline
Tet-OFF	Tetracycline suppressing p53 expression
Tet-ON	Tetracycline absent allowing p53 expression
TMT	Trimethyltin chloride
TRE	Tetracycline Responsive Element
Triton x-100	t-octylphenoxypolyethoxyethanol
tTA	Tetracycline responsive transcriptional activator
USEPA	United States Environmental Protection Agency
wt	Wild type

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1 Introduction

"A blood sample of European Commissioner for environment M. Wallstrom was screened for 77 man-made chemicals. Of the 77 chemicals analysed 28 were found in the commissioner's blood". (EU, press release November 2003).

The 77 chemicals which were tested included man-made chemicals that can be found in everyday life products such as computers, TV's, curtains, carpets, furniture, cosmetics, and food.

Human society has undergone a chemical revolution in the last hundred years, reflected by the global production that has increased dramatically from 1 million tonnes in 1930 to 400 million tonnes today (EU, press release, 2004). Chemical regulators have not been able to keep up, which led to the situation that a lot of chemicals were never sufficiently assessed for their human and environmental safety (European Commission, 2006). Some of these chemicals like dioxins; PCB's can accumulate to persistent concentrations in the human body (Patandin et al., 1999). In the last years, the concern is progressively rising that there is a general lack of knowledge when it comes to the effects of environmental chemicals on human health (Yanez et al., 2002). This has led to upcoming changes in chemical regulation in the western societies in order to close the gap in knowledge of the toxic effects of chemicals. In Europe, the regulation, evaluation, assessment of chemicals (REACH) regulation was adopted by the European Parliament and Council in 2006 and will enter into force on the 1st June 2007. The REACH legislation includes the systematic examination of the chemicals of significant quantities within the European Union. Chemical producers and importers will be obliged to register the chemicals produced and imported in volumes greater than 1 tonne per year. This includes information on their properties, uses, risks, and safe ways of handling them. The chemicals of very high concern (e.g. bio-accumulative, carcinogenic, mutagenic and reproductive toxic compounds) will require specific authorizations for usage and

chemicals causing unmanageable risks will be phased out in the European Union by partial or total bans (European Commission, 2006).

Unfortunately, it appears that the upcoming REACH regulation comes rather too late, since in the last year's more and more toxicological and epidemiological evidence is brought up that long-term low level exposure to man-made chemicals is affecting human health (Landrigan, 2002). Some of the major concerns which were reported are decreased fertility by chemicals effecting sperm quality, and the female hormone system (Swan, 2006; Buck et al., 2006). Furthermore, a link suggested between the increasing incidences of testicular and breast cancer in developed countries and exposure of hormone disrupting chemicals and xenobiotics (Quinn et al., 2003; Kortenkamp, 2006). Moreover, epidemiologic evidence suggests an increase in neurological effects in children such as decreased intelligence and behavioral dysfunction (Schantz et al., 2003), as well as immune deregulating effects were observed (Dewailly et al., 2000). Though, many of the effects of environmental chemicals on human health are subtle and therefore remain hard to quantify. Thanks to advancements in technology and science, alternative approaches to conventional hazard assessment can contribute to fill the information gaps for single chemicals.

In reality, the situation is even more complex since the effects can be caused not only by single compounds but by mixtures of compounds since chemicals can interact with each other. Chemicals are often metabolized in the human body and become complex mixtures of many metabolites. The effect of one compound can change the metabolism of a second resulting in either an antagonistic or synergistic effect (Carpenter et al., 2002).

"The question of importance is not simply what chemical X does to the human health but rather what the impact is of all these chemicals acting together for long periods of time". (Carpenter et al., 2002).

However, since the mechanism of toxicity of many single compounds is not yet fully understood nowadays, the toxic effects of complex chemical mixtures are still far ahead. However, again science and technology opens new horizons for investigation of these complex effects.

“Especially the effect of chemicals on the health of developing infants and children has been a growing discussion in recent years. Since they are much more susceptible and at risk for environmental toxicants compared to adults”. (Landrigan, 2004).

Developing infants and children are more susceptible since some low molecular weight and lipophilic compounds were found to be capable of crossing protective barriers such as the placental, blood-milk and the blood-brain barrier which can lead to high risk exposure levels on the bases of body weight (Dorman et al., 2001). Children are more susceptible due to their behavior pattern which increases the risk of exposure to environmental toxicants. They move and play closer to the ground, and mouthing behavior increases the exposure to their environment. Besides this children breath more air, drink more water, and eat more food than adults related to their body weight which increases the dose levels of exposure. Once exposed, the metabolizing capacity which determines either the detoxification or excretion of the compound can still be developing which can make them either more or less sensitive for toxic effects (Moya et al., 2004). For these reasons the low concentrations of environmental compounds believed not to affect adults can induce toxicity in children.

"In the US one in every six children has a developmental disability and in most cases these disabilities affect the nervous system". (Rutter, 2005).

Especially neurodevelopmental toxicity has become a major issue of concern in the last years. Evidence has been accumulating which suggests that industrial chemicals play a role in the increase of neurodevelopmental effects (Hass, 2006; Grandjean and Landigan, 2006).

"Because of its extraordinary complexity the developing human brain is more susceptible to toxic interference than mature adult brain". (Dorman et al., 2001).

The developing brain is at risk since the blood-brain barrier is functionally not fully competent at birth, and there are ongoing discussions at what stage of life it is completely formed (Grandjean and Landigan, 2006; Plunkett, 2007).
Moreover, because of the brains long and complex developmental phase compared to other developing organs there is more susceptibility to environmental factors (Rice, 2000; Rodier, 2004).
The first link between environmental chemical exposure and widespread neurobehavioral changes was by studies showing lead induced neurodevelopment disorders such as neuropsychological dysfunction and reduction in intelligence in children in the absence of clinically visible symptoms of lead toxicity (Needleman et al., 1979; Baghurst et al., 1987; Dietrich et al., 1987; Chiodo et al., 2004). In response, the lead additives in petrol were banned which reduced the lead concentrations found in human blood by 90% (Landrigan, 2002). In recent times, data in the U.S suggest that of all the developmental disabilities 3% are caused by direct exposure to environmental chemicals and 25% are caused by interactions between environmental factors and genetic susceptibility of the individual (National Research Council, 2000).

"The true extend of the neurotoxic potential of many chemicals remains unknown". (Grandjean and Landigan, 2006).

In Europe, there is not a clear indication how many of the REACH compounds have neurotoxic potency because only few compounds were tested for their neurotoxic potential due to time and cost issues and no clear regulatory required systematic evaluation of the neurotoxic hazard. In the U.S. of the 80.000 existing chemicals on the market approximately 1000 chemicals are known to be neurotoxic in animal experiments. This number again refers to the compounds that are tested, which again is a restricted number. Of these known neurotoxic chemicals 201 were shown to be neurotoxic in human, and only 5 have been proven to cause neurodevelopment toxicity in human (Fig. 1), (Grandjean and Landigan, 2006). Although, species differences result in differences in these toxicity data, the differences in percentages clearly reflect the lack of systemic testing methods and strategies when it comes to neurotoxicity assessment of chemicals.

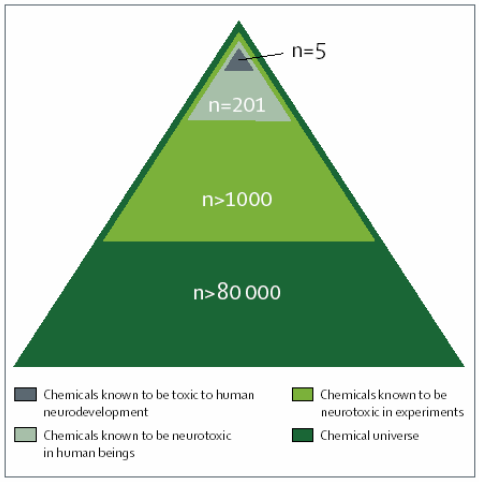

Fig. 1. Diagram of the extent of knowledge of neurotoxic chemicals (from Grandjean and Landrigan, 2006).

In order to get more knowledge and mechanistic understanding of how chemicals in our environment affect the developing or mature nervous system, experts in the field provide two recommendations. Firstly, the revision and optimization of existing tests methods and strategies for neurotoxicity assessment. Secondly the development of new testing methods, strategies and prediction models for better

and more efficient assessment of neurotoxicity as well showing mechanisms of toxicity (Harry and Tiffany-Castiglioni., 2005; Coecke et al., 2005; Grandjean and Landrigan, 2006, Tiffany-Castiglioni, 2006, Hass, 2006).

"Because behavior is the final functional output of the nervous system and the only one determinable in animals, it provides the most meaningful endpoint for studies of toxicant induced dysfunction". (Andreas and Ray, 1999).

Investigation for systemic / organ toxicity is to some extent undertaken as part of the repeated dose toxicity test according to the OECD test guidelines 407 and 408. These protocols include a number of nervous system endpoints based on functional observation and structure. Only in the case of neurotoxic indications specific test for neurotoxicity based on neurobehavioral and neuropathological studies (OECD, 2003) are performed.

Neurobehavioral studies decribed in the guidance document for neurotoxicity testing include the measurement of a variety of sensory, motor, cognitive, and autonomic functions (Tilson, 1993). The common methods to measure motor function include swimming performance, grip strength, rotation rod, rope climbing and tremor analysis. For sensory tests include vision, audition, taste, olfaction, thermoregulation, somatosensation, and nociception (painful stimuli). Tests to measure routinely chemical induced changes include tail flick, hot plate response, and sensory irritation. Methods to measure cognitive function or awareness include the perception, thinking, the learning capacity and memory of an animal. Tests include measurements of habituation, spatial mazes, ethologically based anxiety tests and active or passive avoidance. The interpretation of such behavioral tests must be done carefully, since tests measure often a motor response to a sensory stimulation and cognitive performance often depends on motor and sensory functions. Therefore the interpretation of the responses can be difficult and easily confused with one another. Furthermore, the outcome of a behavioral test is variable between

individual animals and between laboratories. Environmental factors (temperature, light, noise), the health and well being of the animal (sickness, nutrition), as well the handling practices (stress) can generate large differences in the final response of the animal in behavioral tests. Moreover, due to the functional reserve of the nervous system there is the possibility that induced dysfunctions are functionally compensated and therefore remain undetected (Slikker et al., 2005).
The use of behavioral studies has proven in many cases to be a good predictor for effects in human. However, the determination of which component in the nervous system has been damaged leading to behavioral changes can be a very difficult process (Andreas and Ray, 1999; Slikker et al., 2005).

Neuropathological studies are incorporated into test protocols when no prior indication of any neurotoxic effect is observed. The extreme complexity and functional diversity within the nervous system and the regional selectivity of neurotoxicants require the examination of a large range of tissue samples for exploratory investigations (Garman, 2003). Routine studies include hematoxyline and eosine stains to highlight tissue and cellular details. Observed lesions can be studied in more detail using special stains and immuno-histochemistry. Neuropathological studies are frequently inadequate to detect neurotoxicity. The major difficulty of neuropathology is that tissue and fixation artifacts can be confused with toxic effects (Garman, 2006). Moreover, functional defects can occur in the absence of histopathology.

In general, animal based behavioral and neurophatological studies are well accepted for the study of chemical induced neurotoxic effects. Even though, besides ethical considerations, these studies are time consuming, costly, and provide limited mechanistic information (Worth and Balls, 2002; Coecke et al., 2005; Harry and Tiffany-Castiglioni, 2005).
Considering the current gap of knowledge on the neurotoxic potential of chemicals it becomes clear that the current test methods for neurotoxicity assessment do not fulfill the needs. This is not surprising since many of the

current methods were developed decades ago in times when the need for regulatory chemical assessment was considerably lower.

"A chemical's risk is often assessed on the basis of assumptions and extrapolations rather than evidence". (Hoffmann and Hartung, 2006).

Due to the current high demand on large scale chemical risk assessment, toxicologists not only demand new tests but started to critically review the currently available toxicological methods. The so-called "evidence-based approach" considered normal in clinical medicine is starting to influence toxicological considerations (Eddy, 2005; Hoffmann and Hartung, 2006). It includes the critical review of traditional approaches on the basis of scientific knowledge providing the opportunity to renew and improve itself according to scientific progress. This could eventually lead to the development of new approaches and strategies in toxicology. However, regulators have good reasons to be conservative since consumer and patient safety is at stake and unmanageable risks can cause enormous problems, can create scandals.
Still it should not be neglected that some of the regulatory toxicological tests used nowadays have several problems of concern. Most of the used *in vivo* tests lack an assessment of performance criteria such as their relevance and reliability to predict effects in human (Hartung et al., 2004). Attempts to determine the variability and predictive capacity of *in vivo* test have been scarce since the finding that current toxicological classification could rely on weak information would cause enormous problems (Hoffmann et al, 2006).

"The neglecting of variability and relevance leads to an overestimation of the performance of traditional tests". (Hoffmann and Hartung, 2006).

The next question to address is how well the *in vivo* data correlate to the human situation since this is the actual risk that needs to be assessed. Moreover, there exists the possibility that *in vitro* test predict better the effects in human than *in vivo* tests. The main problem for such analysis is the availability of human and animal exposure data. Current validation studies of *in vitro* tests compare the *in vitro* test with the respective used animal test as its reference. But how well this animal test predicts human health effects is not yet considered (Fig. 2).

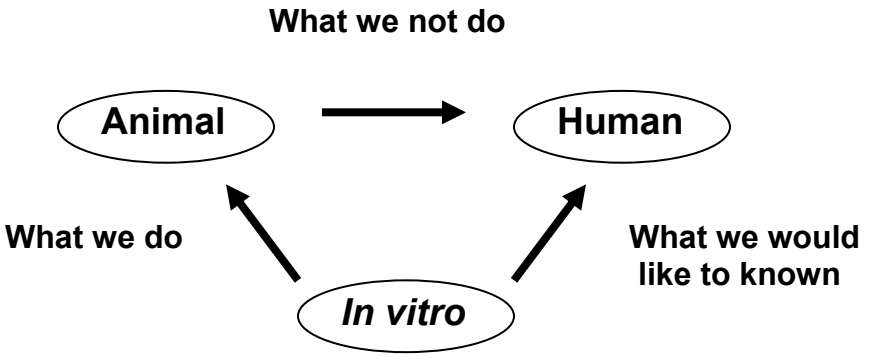

Fig. 2. The validation dilemma. (adapted from Hartung., 2007).

The evidence-based toxicology approach seems to be the basis of a critical analysis of the existing regulatory toxicological testing methods. In addition, the approach could help to give the emerging methods based on latest scientific and technological advances, such as e.g. *in vitro* test systems, the right place in current and future testing strategies (Hoffmann and Hartung, 2006).

1.1 *In vitro* test systems

The development of *in vitro* tests for neurotoxicity has been in response of three main requirements (OECD, 2003).

- To provide a means to systematically study the complex nervous system in order to address specific hypotheses in cell biology, nervous system functioning, and neurotoxicology.
- To define better the biological and chemical processes that contribute to the neurotoxic effects seen in animal and human based *in vivo* studies.
- To save time, money and reduce animal suffering.

In vitro systems consist of isolated tissue or cells grown in a controlled environment outside the living organism. *In vitro* systems are often applied in biological research given the opportunity to study specific biological mechanisms in organs, tissues, cells or cellular components under simplified conditions with fewer variables. Although *in vitro* systems represent a model of the in *vivo* situation, the better understanding of the biological mechanisms in such systems may contribute to determine whether the observed effect will take place in the whole organism.

"It is generally recognized that in vitro systems often provide only partial answers to more complex problems; therefore, they can supplement, but rarely replace, in vivo studies". (Harry an Tiffany-Castiglioni., 2005).

In toxicology, *in vitro* systems are becoming increasingly important since they represent a way to reduce time and costs for toxicity testing. Like in the *in vivo* situation, the toxic effect of a compound is dependent on pharmacokinetic parameters. These parameters include the absorption, distribution, metabolism, and excretion of chemicals and their metabolites. Since the *in vitro* system

represents an isolated part of the *in vivo* situation the observed toxic effects in *in vitro* and *in vivo* can vary due to the different pharmacokinetic parameters.

In vitro systems allow on the other hand the easy manipulation of the cellular environment creating the possibility to control the amount of test compound relevant for the *in vivo* situation and analyze precisely the related toxic effect. On the other hand, the chemical properties and behavior influence the bioavailability of the compounds and may change culture conditions affecting general cell viability. The absorption of a compound in the *in vivo* situation depends on route of exposure (digestion, inhalation or skin absorption). Its distribution depends on the quantity of the compound reaching the blood circulation. Metabolism within the organism may either bio-activate the compound or detoxify before it enters the blood circulation and ultimately is excreted. Most compounds bind reversible or irreversible to proteins and lipid within the organism. The differences in the above mentioned pharmacokinetic parameters are explaining the large species differences observed in toxicity of drugs and chemicals (Tozer and Rowland, 2006). In *in vitro* systems these differences are even bigger. Compounds are directly added to the cellular environment which greatly increases their cellular adsorption and distribution. Additionally, the metabolic competence of the *in vitro* system is often lacking or not comparable to the *in vivo* situation. Moreover, *in vitro* biokinetics such as binding to the plastic or componets of the culture media are often not considered (Guelden et al., 2006). This explains why compounds that are toxic in animals following bio-activation might induce no toxicity in some *in vitro* systems which lack this metabolic competence. In contrast compounds that are detoxified *in vivo* by metabolism can be toxic in the *in vitro* situation. To overcome this limitation, often metabolic-activating systems such as metabolic enzyme preparations or metabolically active cells are included in *in vitro* tests to include the biochemical activation and detoxification of compounds (Coecke et al., 2006). Although this can increase the predictive capacity of *in vitro* test systems, it is often questionable if the biotransformation processes are comparable to the *in vivo* situation.

Due to the differences in many of the pharmacokinetic parameters as described above, the extrapolation of *in vitro* results towards the animal or human data is a very difficult task. Nevertheless, a study determining the correlation between acute *in vivo* and *in vitro* toxicity by the comparison of oral LD_{50} data and IC_{50} cytotoxicity data in primary cell cultures showed a high reproducibility between the two (Halle, 2003). The results showed that of the 347 xenobiotics tested in rat a percentage of 72.6-73.5% was accurately predicted in the *in vitro* system, demonstrating that despite the pharmacokinetic differences *in vitro* systems can give a good prediction for acute oral toxicity in animals.

Of the 49 xenobiotics considered as negative outliers in the study (Halle, 2003) a number of 23 were found to be insecticides, neurotoxicants or compounds that require metabolic activation. This is due to the lack of metabolic capacity of used the *in vitro* system and the presence of the blood brain barrier which determines what concentration of a compound reaches the brain. Thus, this study reveals the applicability of *in vitro* test systems in regulatory toxicology. More and broader *in vitro* studies are necessary to further investigate their applicability, relevance and validity which could lead to a reduction in costs, time and animal suffering for toxicity testing. However, this study shows as well the limitations of *in vitro* cytotoxicity studies concerning metabolic capacity and the prediction of neurotoxic effects. Therefore, scientists suggest not the use of single *in vitro* test methods but the development of *in vitro* testing strategies using batteries of tests including cytotoxic and specific organ toxicity endpoints (Grindon et al., 2006). Moreover, studies are ongoing to determine the best testing strategy for acute systemic toxicity such as the ACuteTox project (Clemedson et al., 2006; Clemedson et al., 2007). The project aims to adapt new testing strategies based on new cell culture systems, new endpoints, and the implementation of pharmacokinetics and *in silico* models.

"In vitro systems have been proposed, but not yet demonstrated as a method to assess the neurotoxicity of compounds in an efficient and rapid manner". (Harry and Tiffany-Castiglioni, 2005).

The main reason why *in vitro* systems have not yet been demonstrated as valid methods for the assessment of neurotoxicity is the extreme complexity of the nervous system and the complex mechanisms that underlie neurotoxicity (Harry et al., 1998). Until now the developed *in vitro* screening models for neurotoxicity have been limited to measurements of cytotoxicity and did not usually include neuro-specific endpoints addressing mechanisms of neurotoxicity (Harry and Tiffany-Castiglioni, 2005). Scientists in the area stress the development of more focused approaches that are more relevant and complementary to the *in vivo* neurotoxicity methods by the use of latest advancements in cell culture and technological endpoints (Coecke, 2002; Harry and Tiffany-Castiglioni, 2005; Tiffany-Castiglioni, 2006).
Nowadays a range of *in vitro* systems with increasing biological complexity is available for toxicity testing that preserve better the biological structure and function as it occurs *in vivo.* Moreover, scientists are developing more advanced endpoints using the latest technologies (Zucco et al., 2004).

"One of the major advantages is the development of three-dimensional cell culture models that allow direct relationship among structure and function, and the possibilities to preserve the cellular interactions like they occur in vivo". (Zucco et al., 2004).

Re-aggregating brain cell cultures represent one of the closest *in vivo*-like complex *in vitro* systems regarding the brain (Honegger et al., 1979). The cultures comprise an integrated population of neurons and glial cells arranged in a three-dimensional way. The cells undergo morphological differentiation including synaptogenesis and myelination (Zurich et al., 2000; Eskes et al.,

2002). The cell cultures can be maintained by rotation under continuous incubation conditions using chemically defined media for prolonged periods of time (maximum reported >6 months). Re-aggregating brain cell cultures were firstly proposed for toxicological studies to screen and classify toxic compounds by mechanistic criteria (Honegger et al., 1988). Over the last years, studies have demonstrated the importance of close cell-cell interaction in neurotoxicity induced by metals and organophosphates (Monnet-Tschudi et al., 1995; Eskes et al., 2002; Eskes et al., 2003; Zurich et al., 2004). Furthermore, maturation and developmental stage dependent effects were shown of trimethyltin (Monnet-Tschudi et al., 1995), lead acetate (Zurich et al., 2002), and organophosphates (Monnet-Tschudi et al., 2000) neurotoxicity. Besides toxicology, aggregating brain cell cultures provide a useful system for biochemical and morphological analysis of myelin and the process of demyelination and remyelination (Zurich et al., 1993; Duvanel et al., 2004).

"Genetic engineering has opened new interesting fields of application in biochemical toxicology". (Wiebel et al., 1997).

Engineered cells create possibilities to study specific targets or mechanism of toxicity. In general, the established cell lines express a low content of metabolizing enzymes, which severely reduces their predictive capacity for *in vivo* effects. The cDNA of several metabolizing enzymes can be introduced e.g. P450 (also humans) to give cells the metabolic capacity for the biotransformation of compounds (Sawada and Kamataki, 1998). Genetically engineered cells can also be used as a sensitive endpoint of toxicity by the introduction of the luciferase gene which can quantify toxic effects by the dose-dependent emission of light (Sanderson et al., 1996). Genes known to be involved in a toxic mechanism can be either introduced or knocked out. This way a cell becomes either more sensitive or resistant towards the toxic action of a certain compound giving information on the involvement in the mechanism of toxicity. Such

sensitization models can be created to study the specific effects compounds at low concentration levels. For example the p53 gene known to induce apoptosis after cellular stress or a toxic insult was introduced into a pheochromocytoma 12 (PC12) cell line to create a sensitization model for p53-mediated toxicity (Stingle et al., 1999). In this study the created p53-genetically engineered PC12 cell system was used to study metal induced p53-mediated toxicity (Vliet et al., 2006). Besides applications in toxicology, genetically engineered cell models are often used in cancer research, e.g. to make tumor cells more sensitive for chemotherapy (Hanania and Deisseroth, 1997).

"Electrophysiological recordings from both rodent and human brain tissue using microelectrode arrays can reveal sensitively neurotoxic actions of compounds". (Kohling et al., 2005).

One of the promising approaches for the screening of neurotoxicology is the use of electrophysiological measurements of more complex cell and tissue cultures by using multi-electrode arrays (Noraberg, 2004; Sundstrom et al., 2005). The first recordings using MEA were performed in the 1980's on neurons taken from spinal cords of mice (Gross et al., 1982). Since then the field of electrophysiology using multi-electrode array (MEA) recordings has evolved rapidly giving rise to better and easier recording systems. Nowadays, the most common *in vitro* systems used are hippocampal slices and primary dissociated cultures. Studies have demonstrated the response of these neuronal cultures to neurotransmitters and their specific agonists and antagonists (Keefer et al., 2001; Martinoia et al., 2005). Moreover, the extracellular electrophysiological responses from these in vitro models have been used to evaluate the effects of neuro-pharmacological and neurotoxic compounds on neuronal activity. Some of the compounds tested using MEA recordings are ethanol (Xia and Gross, 2003), trimethyltin chloride (Gramowski et al., 2000; Kohling et al., 2005), mercury (Gopal, 2003), kainic acid (Melani et al., 2005), and chloroquine (O'Shaughnessy et al., 2002). The main

advantage is that electrophysiological recordings enable the detection of neurotoxicity of compounds at a very early stage before the induction of severe toxic effects, e.g. structural damage. Moreover, the responses of neuronal networks to compounds was found to be reproducible, quantifiable, and in general are in agreement with published data derived from *in vivo* methods (Sundstrom et al., 2005). These properties led to an interest in using MEA technology as broadband biosensors for pharmacological screening and drug development to reduce time, costs and animal tests.

"Within preclinical toxicology metabonomics is having and will continue to have significant impact in the area of screening, biomarkers of safety and mechanism of action". (Robertson, 2005).

Technological advances in the biological sciences over the past few years have forged a new area of science so called the "omics cascade" which includes genomics, transcriptomics, proteomics, and metabolomics. The omics science deals with the comprehensive study of systems biology. Principally, the comprehensive analysis of an organism's response to a perturbation on the genome, transcriptome, proteome and metabolome level could lead to a better understanding of the mechanisms in complex systems (Dettmer et al., 2006). Of the omics cascade metabolomics is the closest to the phenotype (Fig. 3). Metabolomics / metabonomics is the systematic study of the chemical finger- or foot-prints that specific cellular processes leave behind, specifically the study of their small-molecule metabolite profiles which give information on alterations in cell physiology. The metabonomic approach and its applications are emerging the most promising areas where relevant information can be provided using metabolomics include toxicology (Lindon, 2005; Craig et al., 2006), disease, (Lindon et al., 2004; Griffin, 2006), aging (Wang et al., 2007), and drug development (Lindon et al., 2007).

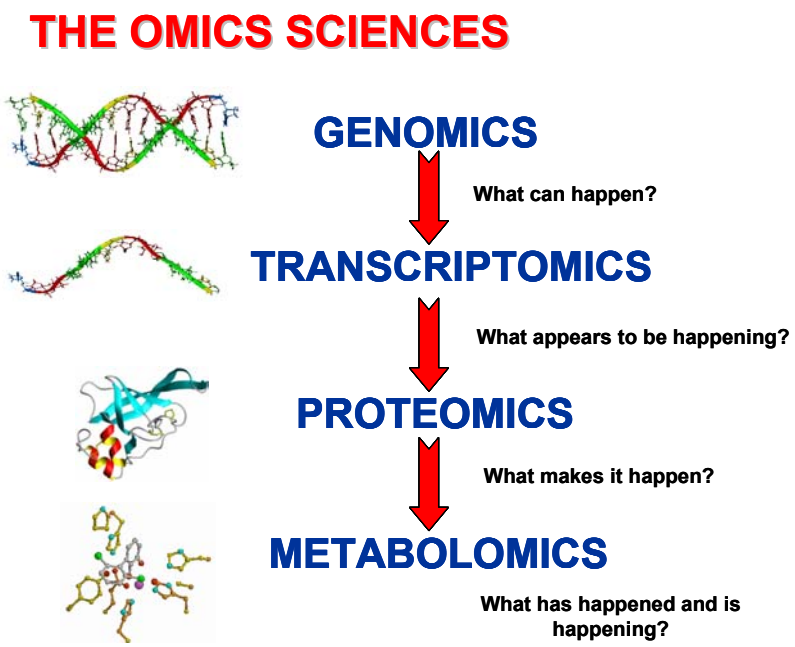

Fig. 3. The omics sciences. (adapted from Dettmer et al., 2006).

Until now, metabolomics based toxicity studies are mainly performed using samples from *in vivo* studies, including blood plasma, urine, saliva, or cells. These samples undergo a sample preparation and are then analysed by either nuclear magnetic resonances (NMR), or mass spectroscopy (MS) technologies. The data consist of metabolic profiles that are analysed by a multivariate statistical analysis showing the significant differences of markers in the dataset e.g. by principle component analysis (PCA) and postulate markers. Using MS/MS or NMR, the identities of the putative markers can be identified. The potential application of metabonomics in toxicology is mainly for screening purposes, the identification of biomarkers, and to reveal mechanisms of toxic action.

Toxicologists have been searching for a screening method that is fast, requires minimal compound, and is reflective for potential human effects (Robertson, 2005). For pre-clinical toxicology, *in vitro* approaches fullfil some of these requirements (human cell lines) and can be useful when the *in vivo* target link

has been established. The utility of metabolomics in the evaluation of xenobiotic toxicity has been comprehensively assessed by the Consortium for Metabonomic Toxicology a group of six pharmaceutical companies and the Imperial College of Science, Technology and Medicine, London, UK (Lindon et al., 2005). Although urine and blood samples have demonstrated to give relevant information on liver, kidney, brain toxicity and disease (Robertson et al., 2000; Pears et al., 2005; Viant et al., 2005, Griffin, 2006) this approach is limited for the brain due to presence of the blood-brain barrier. The barrier determines whether and what concentration of the compound reaches the brain, and the metabolites that can pass to the blood. For these reasons the application of *in vitro* based metabolomics approaches could have potential in the field of neurotoxicology.

The present study, explores the use of the described *in vitro* systems and emmerging technologies for the development of novel *in vitro* approaches detecting acute neurotoxicity. Approaches aim to support the design of *in vitro* testing strategies that intend to close the current gap of knowledge on the neurotoxic potential of chemicals.

2 Aims of the study

There is a lack of systemic testing methods and strategies when it comes to neurotoxicity assessment of chemicals. Scientists have recommended the development of testing strategies making use of *in vitro* systems for more efficient and accurate evaluation of the neurotoxic potential of chemicals. This study aimed to support the design of such testing strategies by the development of novel approaches based on the most promising *in vitro* models and emerging technologies.

The more specific aims of this study were:

1) To develop a sensitive *in vitro* model to detect p53-mediated cytotoxicity by the introduction of a controllable p53 gene into a PC12 cell line.
    a. Study the sensitivity of the p53-transfected PC12 cell line towards apoptotic cell death.
    b. Study the p53-dependence of metal-induced cytotoxicity.

2) To explore the extra-cellular electrophysiological recording of re-aggregating brain cell cultures on multi-electrode arrays to detect acute pharmacological and neurotoxic effects.
    a. Characterize the electrophysiological responses recorded in re-aggregating brain cell cultures.
    b. Evaluate whether the recordings can be used as a test system to detect the effect of chemicals on neuronal function.

3) To evaluate the use of *in vitro* based metabolomics for the comprehensive detection and prediction of neurotoxicity.
    a. Study the effects of methyl mercury chloride and caffeine in re-aggregating brain cell cultures by metabolic fingerprinting.
    b. To elucidate and identify putative biomarkers for their neurotoxicity.
    c. Evaluated whether the approach can be used to identify the neurotoxic potential of compounds.

3 Development of a mechanistically-based genetically engineered PC12 cell system to detect p53-mediated cytotoxicity

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3.1 Abstract

The human wild type p53 gene, key for apoptosis, was introduced into the pheochromocytoma (PC12) cell line, to create a mechanistically-based *in vitro* test model for the detection of p53-mediated toxicity. Expression of the wt p53 gene was regulated by a system, which allowed or blocked expression p53 by absence or presence of tetracycline in the culture media. Western blot analyses confirmed an inducible and tetracycline-dependent expression of the wt p53 protein. Functionality of the p53 protein was verified by camptothecin treatment, known to induce p53-dependent apoptosis. Results showed that p53-expressing cells were significantly more sensitive to camptothecin induced cytotoxicity compared to non-expressing cells, and presented a significantly higher incidence of apoptosis. A screening study on 31 metal compounds, showed that the classified human carcinogens ($NaAsO_2$, $CdSO_4 \cdot 8H_2O$, $Na_2CrO_4 \cdot 4H_2O$, $MnCl_2$, $(NH_4)_2PtCl_6$) significantly increased cytotoxicity in p53-expressing cells compared to non-expressing cells, suggesting that their cytotoxicity was p53-mediated. Finally, acute and subchronic treatment with methyl mercury showed no significant differences in cytotoxicity and the percentage of apoptosis or necrosis between p53-expressing and non-expressing differentiated cells, suggesting that methyl mercury cytotoxicity was p53-independent.

3.2 Introduction

The p53 tumor suppressor gene is known to induce apoptosis in reaction to cellular stress or damage (May and May, 1999, Striteska et al., 2005). There is accumulating evidence that p53 is perturbed in the central nervous system in a number of neurodegenerative disorders (Miller et al., 2000). In particular, the involvement of p53- dependent apoptosis have been shown in neurodegenerative diseases such as Parkinson, multiple sclerosis and Alzheimer (Biswas et al., 2005, Ohyagi et al., 2005, Wosik et al., 2003). Furthermore, the p53 tumor suppressor protein was identified to be a critical mediator of programmed cell death in response to DNA damage and genotoxic carcinogens (Kaiser et al., 2000; Schulte-Hermann et al., 1999), and has been shown to be involved in the carcinogenic effect of metals (Valko et al., 2005).

Previous studies showed that toxicity of chemicals can be dependent on the interaction with the p53 gene (Chen et al., 2006; Vanlandingham et al., 2005). Low levels of toxicity may affect p53-expression which can lead to apoptosis, while high toxicity levels affect homeostasis and finally induce necrosis (Nicotera et al., 1996). As a consequence, p53-mediated apoptosis could be used as a sensitive and early endpoint for *in vitro* toxicity of chemicals at low concentrations (Anselmi et al., 2002). Perhaps for that reason several biotechnology companies show interest in focusing on ways to control apoptosis with new therapeutics (Potera, 1998).

The recent proposal for a new European Union policy on chemicals, i.e. the REACH Regulation, will require information on the human health effects of around 30'000 existing chemicals currently marketed in volumes greater than 1 tone per year. If alternative methods are not used, this could result in a substantial increase of animal use for toxicity testing (Hofer et al., 2004). Integrated testing strategies including genetically engineered cell lines and more complex *in vitro* systems could play an important role within this new regulatory context such as for the assessment of neurotoxicity (Coecke et al., 2002, 2006).

As a consequence, the present study aimed at developing a genetically-modified rat pheochromocytoma cell line (PC12) to detect p53-mediated toxic effects

induced by chemicals. The PC12 cell line is known to respond to nerve growth factor (NGF) by extending long, branching neuron like processes and is commonly used in neurobiological research (Greene and Tischler, 1976). This cell line also constitutes a useful model for studying the mechanisms of apoptosis, its prevention or induction (Wang, et al., 2005; Raza, et al., 2006; Zhao, et al., 2002).

One approach to study the role of specific genes, such as p53, is to use a tetracycline regulated expression system. Such approach has the advantage to provide with an easily inducible “genetic switch” that tightly regulates gene expression in a reversible and quantitative way (Gossen et al., 1992). The tetracycline-responsive element (TRE) is located upstream of the promoter of minimal immediate early cytomegalovirus (PminCMV) and the gene of interest (Fig. 4). The presence of tetracycline (Tc) blocks the binding of the tetracycline responsive transcriptional activator (tTA) and thereby expression. In the absence of tetracycline the transcriptional activator can bind to the tet-responsive element and thereby activates the transcription of the gene of interest. As a consequence, presence of low and non-toxic tetracycline concentrations suppresses gene expression (Tet-Off), but in the absence of tetracycline, expression is fully re-stored within a few hours (Tet-On).

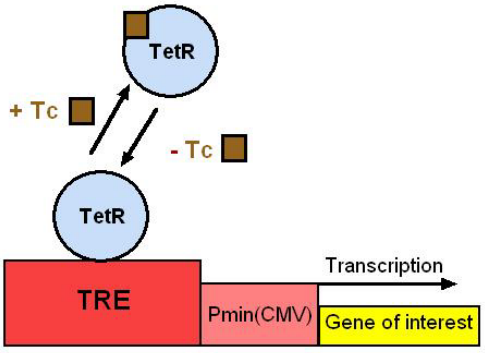

Fig. 4. Gene regulation in the Tet-On / Tet Off gene expression system. (Modified from Clontech Laboratories, Inc, USA).

Further to the creation of the genetically modified PC12 cell lines, the tetracycline inducible expression of the wt p53 protein was verified, as well as the functional state of the expressed human wt p53 protein by treating the undifferentiated cells with the topoisomerase I inhibitor camptothecin known to mediate apoptosis via a p-53 mediated mechanism (Lesuisse and Martin, 2002). Cells were then used for screening p53-mediated toxicity with trace metals which could interact with the p53 gene (Valko et al., 2005). In addition the acute and sub chronic toxicity of the well known neurotoxic compound methyl mercury (Clarkson, 2002) was investigated using undifferentiated and neuron-like differentiated PC12 cells.

3.3 Materials and Methods

3.3.1 Compounds

$AgNO_3$, $AuCl_3$, H_3BO_3, $CdSO_4$, $CoCl_2{\cdot}6H_2O$, $Ga(NO_3)_3{\cdot}6H_2O$, $HgCl_2$, $La(NO_3)_3{\cdot}6H_2O$, LiCl, $KMnO_4$, $MnCl_2$, $NaNO_3$, $(NH_4)_2PdCl_6$, $(NH_4)_2PtCl_6$, $(NH_4)_2PtCl_4$, RbCl, $(NH_4)_3RhCl_6{\cdot}H_2O$, Na_2TeO_3, Na_2TeO_4, $TlSO_4$, Ph_4As, $C_5H_{11}AsO_2$, $NaVO_3$, and $ZnSO_4{\cdot}7H_2O$ were from Alfachem, (Cologno Monzese, Milan, Italy); $(CH_3)_2AsOOH$, K_2MoO_4, $NaAsO_2$, $Na_2CrO_4{\cdot}4H_2O$ were from Fluka (Milan, Italy); $(C_6H_5)_4AsCl{\cdot}H_2O$, MeHgCl (methyl mercury chloride), and $C_{20}H_{16}N_2O_4$ (camptothecin) from Sigma-Aldrich (Milan, Italy); $SnCl_2$, $Na_2WO_4{\cdot}2H_2O$ were from BDH (Milan, Italy); $CH_3AsO(OH)_2$ or MMA (monomethylarsonate), $(CH_3)_3AsCH_2COO$ or DMA (dimethylarsonate) were from Tri Chemical Laboratory (Yamanashi, Japan). Metal compounds were freshly dissolved in water at 30 ºC for 1 h at concentrations of 10^{-2} or 10^{-3} M. Aliquots of mother solutions were added to the culture media to reach a final concentration of 100 µM (50 µM in the case of $(NH_4)_2PdCl_6$). Methyl mercury chloride was diluted in culture media at concentrations ranging from 0.01 to 5 µM. Camptothecin was dissolved in dimethyl sulfoxide (DMSO) at concentrations ranging from 0.1 to 150 µM. The final concentrations of DMSO in the medium were 0.01% to prevent cytotoxic effects.

3.3.2 Cell culture materials and reagents

Cell culture flasks were from Corning (Milan, Italy); RPMI (Roswell Park Memorial Institute Medium), PBS, penicillin G sodium, and streptomycin sulphate were from Gibco, (Milan, Italy); horse serum was from Biochrom (Berlin, Germany); Vitrogen 100 was from Collagen (Ismaning, Germany); fetal calf serum and hygromicin B were from Genzyme (Cinisello Balsamo, Milan, Italy). Restriction enzymes were from Boehringer Mannheim (Monza, Italy). The polyvinylidene (PVDF) membrane and protein assay kit were from BioRad (Segrate, Italy). The ECL™ system was from Amersham (Milan, Italy). The human wt p53 specific mouse monoclonal anti-body was from Inalco (Milan, Italy). The BSA, MTT, Annexin-V Apoptosis detection kit, propidium iodide (PI), RNAse and all other reagents not further specified were from Sigma-Aldrich (Milan, Italy).

3.3.3 Cell line and vectors

The PC12 Tet-Off cell line (stably transformed with plasmid vector pTet-Off) was from Clontech Laboratories (Mountain View, CA, USA). The plasmid vectors pTRE-p53 and pTK-Hyg were from Clontech Laboratories (Mountain View, CA, USA). The vector pTRE contains the tetracycline responsive element (TRE) described by (Gossen et al., 1992). In the vector pTRE-p53 the full encoding sequence of human wt p53 cDNA was inserted in “sense” orientation into the *Bam* HI sites of the multiple cloning site. The pTK-Hyg selection vector contains the ampicillin resistance gene, which confers resistance to ampicillin in bacterial cells and the hygromycin resistance gene, which hygromycin resistance in mammalian cells. The cell line is co-transfected with pTRE-derived plasmids to allow selection of stably transformed cell lines in the presence of hygromycin.

3.3.4 Culture of PC12 cells

Freshly thawed PC12 Tet-Off cells were seeded on culture flasks pre-coated with Vitrogen 100, 0.1% BSA. Cells were maintained in the presence of 2 μg/ml

tetracycline in RPMI supplemented with 10% (v/v) horse serum, 5% (v/v) fetal calf serum, 100 units/ml penicillin-G sodium, 100 µg/ml streptomycin sulphate, and 150 µg/ml geneticin at 37 °C in a humidified incubator with 5% CO_2. To induced neuronal differentiation we supplemented the culture media with 10 ng/ml of nerve growth factor (NGF). Every two days fresh medium was supplied and cells were sub cultured at confluency with a frequency of ten days. Experiments were performed using cells with passage numbers within the range of 20-30. Cells were maintained under good cell culture practice (GCCP) (Coecke et al., 2005).

3.3.5 Transfection of the PC12 Tet-Off cells with the human wt p53 gene

Both pTRE and pTK-Hyg vectors were linearised by *Hind* III. 40 µg of TRE-p53 and 2 µg of pTK-Hyg were co-precipitated with 0.2 M sodium chloride and three volumes of ethanol. The DNA pellet was dried and 42 µg of total DNA was resuspended in 0.25 ml of electroporation buffer (137 mM NaCl, 5 KCl, 0.7 mM Na_2HPO_4, 6 mM D-glucose, 21 mM Hepes: pH 7.1). The DNA solution was added to 0.25 ml of PC12 Tet-off cell suspension of 40.000 cells/ml in electroporation buffer, and electroporation was performed in a gene pulser system (BioRad, Milan, Italy) at 960 µF and 0.25 kV/cm, with a registered time constant of 15-30 ms. Electroporated PC12 cells were plated and grown in selection media containing 150 µg/ml hygromycin-B and 2 µg/ml of tetracycline. Clones resistant to hygromycin-B were isolated using trypsine and tested for tetracycline controlled expression of the human wt p53 gene by Western blot analyses.

3.3.6 Western blot analyses

Cell lyses was performed in lyses buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml Aprotonin, 1% Nindinet P-40 and 0.5% sodium deoxycholate. Protein content was quantified using the protein assay. 50 µg of protein was loaded into each lane of a 12% SDS-PAGE gel and left to run for 1 h at 100V. The proteins were then transferred to a polyvinylidene membrane using the transfer apparatus (BioRad, Milan, Italy). The membranes were probed using specific mouse anti-human wt p53 monoclonal antibodies (dilution 1:1000) and detected using the enhanced chemoluminescence (ECL) system.

3.3.7 Cell viability

Cell viability was determined using the (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) MTT test (Denziot and Lang, 1986). Two hours prior to the end of the exposure period 0.5mg/ml MTT was added to the incubated cells. The medium was then removed, and the formazan crystals formed were dissolved in DMSO by 15 minutes shaking at room temperature. The optical densities were read at λ=550 nm and λ=630 nm.

3.3.8 Flow cytometry

The detection of early apoptosis and necrosis was performed by flow cytometric analyses (Beckman Coulter, Inc, USA) using an Annexin-V and Propidium Iodide (PI) apoptosis detection kit, which allows living and early apoptotic cells to be distinguished from necrotic cells. After treatment the cells were washed with PBS, harvested by trypsin and re-suspended in binding buffer. Cell suspension was stained using Annexin-V and PI according to the kit's instructions, and the fluorescence was immediately measured by flow cytometry. Cells that were in the early stages of apoptosis were stained with the Annexin-V only, living cells showed no staining and necrotic cells showed staining of both Annexin-V and PI.

3.3.9 Treatment of the PC12 cells

For treatment the PC12 cells were transferred to pre-coated 96 well plates. After 6 h of cell attachment, medium was replaced by 100 µl of RPMI medium containing 2 µg/ml of tetracycline or tetracycline-free medium to inhibit or induce wt p53-expression respectively. Cells were cultured for 24 h to allow gene expression or inhibition. The medium was then replaced with 100 µl of RPMI medium containing the concentration range of the chemicals in tetracycline-containing medium or tetracycline-free medium.

3.3.10 Statistical analyses

The results are expressed as mean ± SD. Statistical significances were evaluated using the student's t-test and a two-way ANOVA followed by the Bonferroni post test.

3.4 Results

3.4.1 Human wt p53 protein expression

After the introduction of the human wt p53 gene into the PC12 cell line, the efficacy of the tetracycline-regulated wt p53-expression was investigated. Undifferentiated PC12 cells were cultured for 48 h in the presence of different concentrations of tetracycline. Western blot analyses showed that wt p53-expression could be controlled by tetracycline in a concentration-dependent manner. High tetracycline concentrations clearly reduced wt p53-expression and 2 µg/ml tetracycline inhibited wt p53-expression completely (Fig. 5).

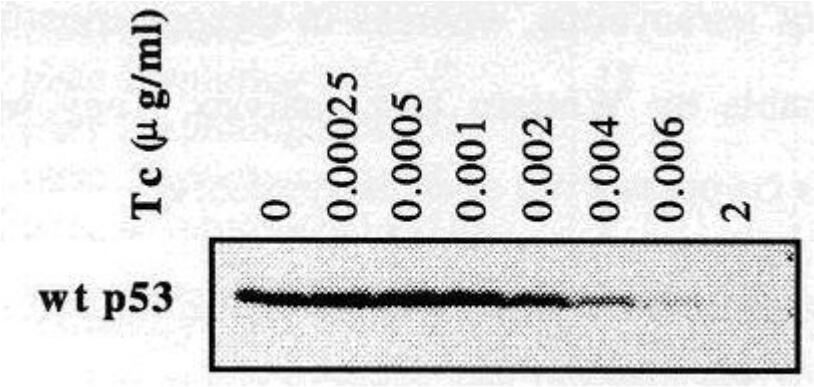

Fig. 5. Quantitative control of wt p53 protein expression by Western Blotting of PC12 cells cultures for 48 h in RPMI medium supplemented with 0, 0.00025, 0.0005, 0.001, 0.002, 0.004, 0.006 or 2 µg/ml tetracycline.

3.4.2 Functional state of the p53 protein

To investigate the functional state of the expressed wt p53 protein we exposure the undifferentiated PC12 cell line to 0.1 to 150 µM camptothecin for 24 h. Results showed a concentration-response effect, and statistically significant differences in cell survival between p53-expressing and non-expressing cells (37.1% and 51.3% of the control, respectively), (Fig. 6 and table 1).

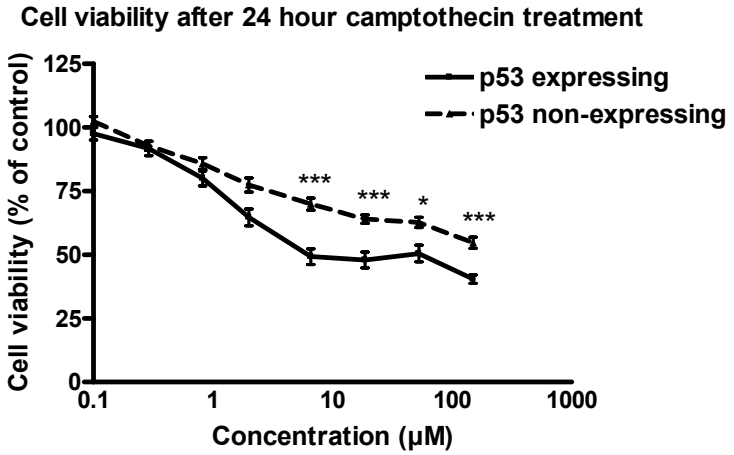

Fig. 6. Cell viability of p53-expressing and non-expressing PC12 cells treated 24 h with camptothecin. (n=18 from 3 independent experiments) * p <0.05, *** p <0.001.

In addition flow cytometry analyses after Annexin-V and PI co-staining showed that cells expressing the p53 protein were more sensitive and presented a significantly higher incidence of apoptotic cell death when compared with the p53 non-expressing cells (Fig. 7A). Under the same conditions the number of necrotic cells remained unchanged in both cases (Fig. 7B). Exposure to the negative control, 100 µM $NaNO_3$ led to no statistically significant differences in cell survival between p53-expressing and p53 non-expressing cells (Table 1).

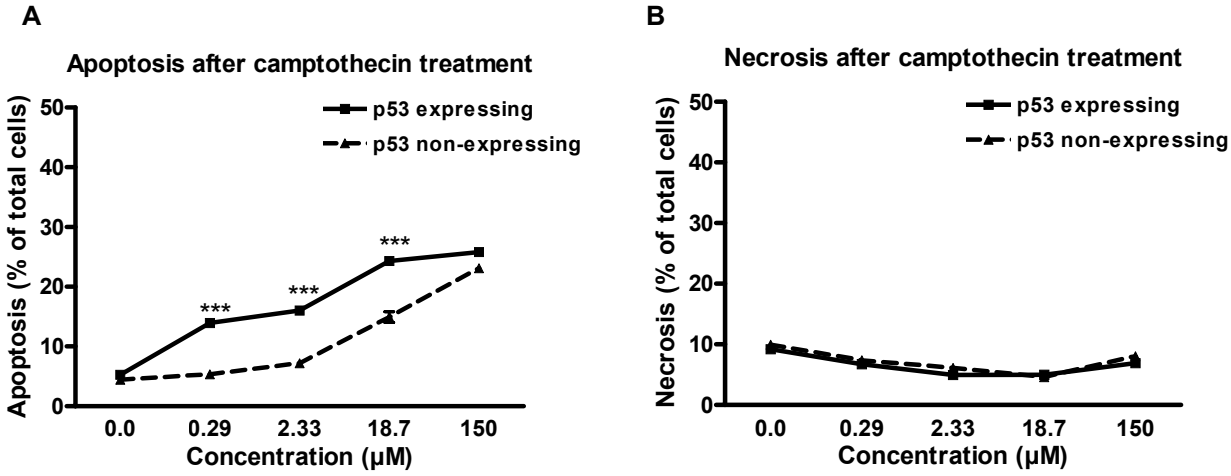

Fig. 7. Apoptotic **(A)** and necrotic death **(B)** induced by a 24 h camptothecin treatment analysed by flow cytometry of Annexin-V and propidium iodide co-staining. Values are presented as mean ± SD and are representative of 3 independent experiments *** p <0.001.

3.4.3 Screening for p53 mediated toxicity

To challenge the novel PC12 test system to detect p53-mediated toxicity a screening study using 31 metal compounds was performed. Table 1 shows the results concerning the cell viability in undifferentiated p53-expressing and non-expressing PC12 cells exposed for 24 h to 100 µM of 31 metal compounds. Due to solubility difficulties, $(NH_4)_2PdCl_6$ was tested at a top concentration of 50 µM, and due to its high toxicity 5 µM of methyl mercury. According to the cell viability results, the metal compounds can be arbitrarily classified into three groups:

Group one contained 18 metal species which showed cell viability higher than fifty percent with no statistically significant differences between p53-expressing and non-expressing cells. In this group the analytical values ranged from 108% (H_3BO_3) to 63% ($CoCl_2 \cdot 6H_2O$) in p53-expressing cells compared to unexposed controls. This group also includes Ph_4As, RbCl and K_2MoO_4.

Group two contained eight metal species which showed a decrease in cell viability below fifty percent values compared to controls without inducing statistically significant differences between p53-expressing and non-expressing cells ranged from 43.1% (Na_2TeO_4) to 0.27% (MeHgCl).

Group three contained five metal species which induced a decrease in cell viability, with statistically significant differences between p53-expressing and non-expressing cells. These metals were ($NaAsO_2$, $CdSO_4 \cdot 8H_2O$, $Na_2CrO_4 \cdot 4H_2O$, $MnCl_2$, $(NH_4)_2PtCl_6$).

Compound	Cell viability (% of the control)[a] ± SD	
	p53-expressing cells	p53 non-expressing cells
Control	100	100
Camptothecin	37.1 ± 2.0	51.3 ± 8.1 *
$NaNO_3$	95.6 ± 6.8	95.3 ± 9.0
H_3BO_3	108 ± 10	101± 7.2
$C_5H_{11}AsO_2$	103 ± 7.5	99 ± 10
Ph_4As	102 ± 7.0	105 ± 9.0
RbCl	102 ± 7.1	105 ± 13
Tl_2SO_4	102 ± 11	103 ± 9.0
$Na_2WO_4 \cdot 2H_20$	101 ± 9.2	98 ± 13
DMA	100 ± 1.5	97 ± 3.3
$(NH_4)_2PdCl_6$[b]	100 ± 7.6	95 ± 11
$SnCl_2$	100 ± 9.0	87 ± 14
LiCl	99± 8.4	102± 7.5
MMA	97± 4.8	93 ± 9.4
$(NH_4)_2RhCl_6 \cdot H_2O$	96 ± 10	95 ± 12
$AuCl_3$	94 ± 6.5	90 ± 5.6
K_2MoO_4	94 ± 8.6	93 ± 5.6
$Ga(NO_3)_3 \cdot 6H_2O$	88 ± 6.0	91 ± 4.2
$(NH_4)_2PtCl_4$	81± 9.2	87± 5.7
$ZnSO_4 \cdot 7H_2O$	77.4 ± 8.6	81.5 ± 11
$CoCl_2 \cdot 6H_2O$	63 ± 8.1	69 ± 9.7
Na_2TeO_4	43.1 ± 3.7	39.6 ± 7.5
$NaVO_3$	35 ± 6.8	35.4 ± 9.1
$La(NO_3)_3 \cdot 6H_2O$	34 ± 6.6	38 ± 4.9
$KMnO_4$	23.3 ± 6.2	20 ± 4.0
Na_2TeO_3	20.4 ± 2.9	21.6 ± 3.5
$AgN0_3$	15.3 ± 3.5	19.3 ± 6.0
$HgCl_2$	8.7 ± 2.3	11.5 ± 5.0
MeHgCl	0.27 ± 0.15	0.36 ± 0.20
$(NH_4)_2PtCl_6$	63.4 ± 11.0	92 ± 6.8 *
$MnCl_2$	61.4 ± 6.6	85 ± 10.0 *
$CdSO_4 \cdot 8H_2O$	37.1 ± 9.0	63.2 ± 4.0 *
$NaAsO_2$	18.7 ± 4.8	37.9 ± 3.3 *
$Na_2CrO_4 \cdot 4H_2O$	9.7 ± 3.3	21.3 ± 5.1 *

Table 1. Cell viability of p53-expressing and p53 non-expressing PC12 cells after exposure to 31 individual metal compounds as well as to camptothecin for 24 hours. [a] Data represents mean of 3 independent experiments and * $p<0.05$, [b] 50 µM, [c] 5 µM.

3.4.4 Study of sodium arsenite and methyl mercury toxicity

Transfected PC12 cells in both expression states were exposed to a concentration range of sodium arsenite 0.1 µM to 100 µM, and methyl mercury 0.01 µM to 5 µM for 24 h. Results showed a concentration-response effect for sodium arsenite with statistically differences between p53-expressing and non-expressing cells, their IC_{50} values were 21.81 ± 3.06 and 74.43 ± 2.0 µM respectively (Fig. 8A). For methyl mercury we observed a concentration-response effect with no statistically significant differences between p53-expressing and non-expressing cells, their IC_{50} were 1.92 ± 0.16 and 1.91 ± 0.09 µM respectively (Fig. 8B).

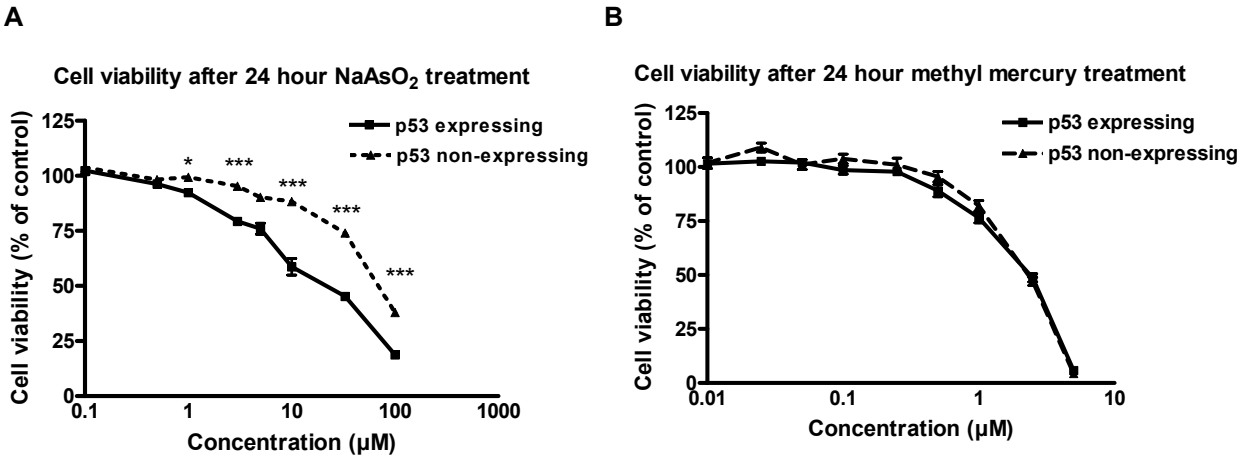

Fig. 8. Cell viability of p53-expressing and non-expressing PC12 cells treated 24 h with sodium arsenite **(A)** and methyl mercury **(B)**, (n=3 from 3 independent experiments). * $p < 0.05$, *** $p < 0.001$.

Flow cytometry analyses of methyl mercury treated cells showed a concentration-dependent increase in the rate of apoptotic cell death with no statistical differences observed between p53-expressing and non-expressing cells (Fig. 9A). At the highest tested concentration of 5 µM methyl mercury an increase in the rate necrotic cell death was observed with no statistical differences observed between p53-expressing and non-expressing cells (Fig. 9B). Results observed with MeHgCl were confirmed in neuron-like differentiated

PC12 cells treated with 0.001 µM to 1 µM for 3 to 10 days. Small but again no significant differences in cytotoxicity were observed between p53-expressing and non-expressing cells (data not shown). Flow cytometry analyses performed at day 8 confirmed that there were small but no significant differences observed in the rate of apoptotic and necrotic cell death between p53-expressing and non-expressing cells (data not shown).

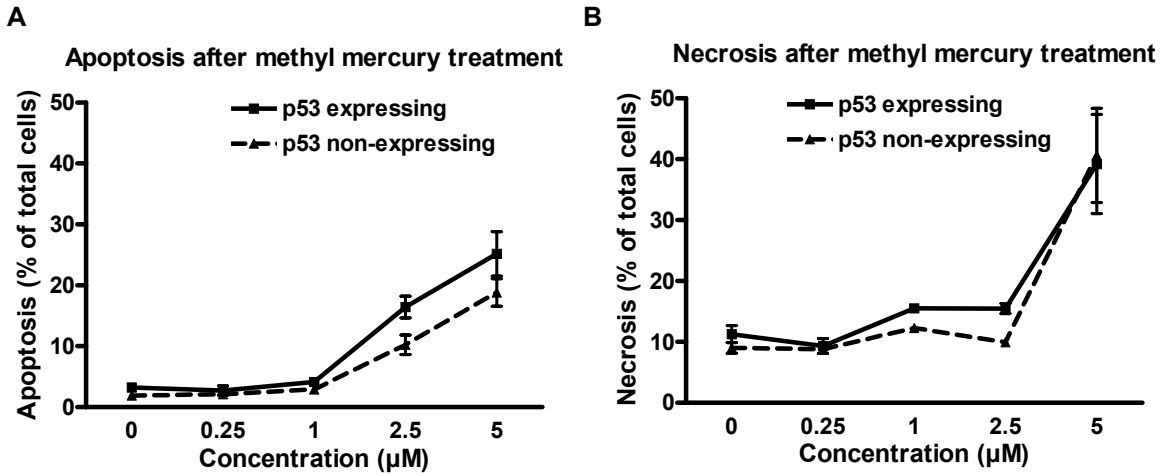

Fig. 9. Apoptotic **(A)** and necrotic death **(B)** induced by a 24 h methyl mercury treatment analysed by flow cytometry of Annexin-V and propidium iodide co-staining. Values are presented as mean ± SEM and are representative of 3 independent experiments.

3.5 Discussion

In order to develop a mechanistically-based *in vitro* model for detecting p53-mediated toxicity the human wt p53 gene was introduced into the PC12 cell line under control of a tetracycline responsive expression vector. Results from Western blot analyses showed an inducible and high tetracycline-dependent expression of the wt p53 protein in PC12 (Fig. 5). Exposure to camptothecin, which was known to induce p53-dependent apoptosis, showed that the transfected p53 protein was functional (Magrini et al*.,* 2002). The results showed a statistically significant increase in the cytotoxic effects induced by camptothecin in p53-expressing cells in comparison to non-expressing cells (Table 1, Fig. 6). A significantly higher incidence of apoptotic cell death in p53-expressing cells compared to non-expressing cells after camptothecin treatments was observed (Fig. 7A, B). This suggested that the increase of apoptosis in the p53-expressing cells was due to over expression of wt p53 protein. These findings were in agreement with previous studies which showed that camptothecin induced apoptosis was mediated by the up regulation of the p53 protein (Lesuisse and Martin, 2002; Nakajima et al., 2002).

As previously reported $NaNO_3$ exposure induces no cytotoxicity and no increase in cellular p53 in mouse fibroblasts (Duerksen-Hughes et al., 1999), therefore NaNO3 was used as a negative control. In our test system a concentration of 100 µM $NaNO_3$ induced no loss in cell viability in both p53-expressing and non-expressing cells (Table 1). In summary these findings indicated that the introduced human wt p53 gene was able to be controlled and functioned correctly, indicating that this PC12 cell line combined with a simple cell viability assay (MTT) could be used to detect compounds whose toxicity is mediated via a p53-related mechanism. To challenge the mechanistically-based test system to detect p53-mediated toxicity a screening study on 31 metal compounds was carried-out to determine the possible role of the human wt p53 protein in metal-induced cytotoxicity. Although many other classes of compounds can interact with the p53 gene, metal compounds were applied since they are constituents of our natural environment, and widespread used in industry, agriculture and

medicine. Numerous health risks may be associated due to exposure especially on neurotoxicity, genotoxicity and carcinogenicity (Florea et al., 2006). Various studies have confirmed and reported that metals activate signalling pathways and the carcinogenic effects has been related to mainly redox-sensitive transcription factors like the p53 gene (Valko et al., 2005). A standard concentration of these compounds was applied which by taking into account *in vitro* pharmacokinetics represents a realistic acute *in vivo* exposure. The results showed that among the metal compounds tested ($NaAsO_2$, $CdSO_4 \cdot 8H_2O$, $Na_2CrO_4 \cdot 4H_2O$, $MnCl_2$, and $(NH_4)_2PtCl_6$ showed obvious differences in the cytotoxic effect induced in the p53-expressing in comparison to the p53 non-expressing PC12 cells (Table 1). More detailed study of sodium arsenite showed as well concentration-response effects with a significant increase in cytotoxicity in p53-epressing cells compared to non-expressing cells (Fig. 8A). Interestingly, the arsenic, cadmium, and chromium compounds are classified as human carcinogens, on the basis of epidemiological evidence, and are therefore likely to interact with the p53 tumor suppressor gene (IARC, 2006). Despite this classification, data from the literature are contradictory and inconclusive regarding regulation of p53-expression following exposure to arsenic in human fibroblasts (Vogt et al., 2001), (Bode et al., 2002), cadmium, in Balb/3T3 (Fang et al., 2002; Waisberg et al., 2003) and chromium in human lung epithelial cells (A549) (Ye et al., 1999). The results presented here showed that the induced cytotoxicity of arsenic, cadmium, and chromium trace metals was p53-mediated in the transfected PC12 cells.

Although manganese is not classified as a human carcinogen (USEPA, 2006), it was found to induce p53-mediated cytotoxicity in the transfected PC12 cells. A previous study reported p53 up regulation after manganese induced apoptosis in human hepatocytes (Suzuki et al., 2005).

Concerning the p53-dependent toxicity of platinum, literature reported that the potency of platinum complexes based drugs in ovarian cancer cell lines depended upon the p53 tumor suppressor gene (Hagopian et al., 1999). All other metal compounds that induced cytotoxicity in this screening study showed no

p53-dependence. The metals tellurium, silver, vanadium, and mercury induced cytotoxicity but without significant differences between p53-expressing and non-expressing cells (Table 1). For these metal compounds other p53-independent mechanisms, e.g. formation of reactive intermediates that can bind DNA, oxidative stress, or DNA methylation, could be involved in their cytotoxicity.

In a speculative way it is interesting to consider our findings in terms of the relationship between cell viability and p53-expression and the possible implications concerning carcinogenic process. It is known that one of the mechanisms of chemical-induced carcinogenesis involves the inhibition of the p53 pathway with consequent apoptosis inhibition leading to increases in cell viability (Greenblatt et al., 1994). The results presented here for the well known carcinogenic chemicals arsenic, cadmium and chromium showed that in cells with a strong p53 stimulus, which are more sensitive to apoptotic response, the cell viability is lower in comparison to p53 non-expressing cells (Table 1). Further studies are necessary to establish the mechanism of p53-dependent metal-induced cytotoxicity.

Besides the screening of the metal compounds we used our model to study the p53-dependence of methyl mercury a well known neurotoxic compound. The results showed that the cytotoxic concentrations of methyl mercury towards the p53 genetically engineered PC12 cells corresponded to those found in literature in primary neural cells (Monnet-Tschudi et al., 1996). Furthermore, no statistical differences in concentration-dependent cytotoxic effects and on the rate of apoptotic and necrotic cell death were found between p53 expressing and p53 non-expressing cells after 24h of exposure (Fig. 8B and Fig. 9 A, B). Longer treatment period on differentiated cells from 3 up to 10 days showed as well small but no significant differences of methyl mercury cytotoxic effects between p53 expressing and non-expressing cells (data not shown). These findings are in agreement with Miura et al. (1999) who suggested methyl mercury to induce apoptosis via a p53-independent pathway in PC12 cell lines.

In conclusion, the present study confirmed that expression of wt p53 protein by the genetically modified PC12 cells was tetracycline inducible and regulated. The expressed wt p53 protein proved to be functional as shown with camptothecin. Furthermore, the developed p53 transfected PC12 cells appeared to be a useful test system for screening p53-mediated cytotoxicity. Investigation with additional neurotoxic compounds is further recommended in order to determine the relevance and reliability of the assay as a component of a test battery, together with more complex *in vitro* systems, for the assessment of neurotoxicity.

3.6 Acknowledgements

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4 Electrophysiological recording of re-aggregating brain cell cultures on multi-electrode arrays to detect acute neurotoxic effects

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4.1 Abstract

Neurotoxicity aims to understand how xenobiotics interfere with the function of the nervous system and to unravel their mechanisms of action. Neuronal activity is the primary functional output of the nervous system and deviations from its resting level may indicate toxicity. Consequently, the monitoring of electrophysiological activity in complex cell culture systems appears particularly promising for neurotoxicity assessment. To detect acute neurotoxic effects of chemicals we developed a test system based on the electrophysiological recordings from neural networks in re-aggregating brain cell cultures using multi-electrode arrays. We characterised the electrophysiological properties of the cultures and, using known neurotoxicants, evaluated their usefulness to predict neurotoxic effects. Aggregates displayed evoked field potentials and spontaneous neural activity involving glutamatergic and GABAergic synaptic transmission. Paired pulse inhibition indicated the presence of short-term synaptic plasticity via functional inhibitory networks. Cultures were treated with 0.1 – 100 µM of trimethyltin chloride (TMT), methyl mercury chloride (MeHgCl), parathion or paraoxon, and with 0.1 - 100 mM of ethanol for up to 100 minutes. TMT (10 µM), MeHgCl (1 µM) and ethanol (100 mM) all decreased the amplitude of evoked field potential. The effect of ethanol was reversible. In contrast paraoxon (10 µM) increased the amplitudes of evoked field potentials while parathion showed no significant effects. The effects of TMT and ethanol on the frequency of spontaneous activity were consistent with those obtained for evoked field potentials. All effects occurred at levels at which cytotoxic injuries were not detectable. Taken together our system expressed electrophysiological properties similar to those of established slice culture preparations. It detected known neurotoxicants at subcytotoxic levels and therefore appears suitable for the assessment of toxic insults specifically interfering with nervous system function, e.g. neuronal activity, synaptic transmission and short-term plasticity. If incorporated into testing strategies, it might represent a valuable tool for the mechanistic assessment of neurotoxic effects.

4.2 Introduction

There is a urgent need for efficient and cost effective toxicological assessment methods within the regulatory framework that provide mechanistic insight, crucial for a modern toxicology aiming to reveal the mechanisms and pathways by which certain chemicals exert their adverse effects on cells, organs and organisms. In particular, two recent EU legislations have increased the testing needs and the time pressure for the development of more effective frameworks for chemical safety assessments. The new chemicals legislation REACH and the 7th amendment of the cosmetics directive call for the increased use of *in vitro* methods for ethical but also scientific reasons. REACH (Registration, Evaluation and Authorisation of Chemicals) (European Commission, 2001) will require the testing of more than 30.000 existing chemicals and encourages, whenever possible, the use of *in vitro* methods as an alternative experimental means to animal experiments for the establishment of hazards. REACH will also be used for any new chemicals intended to enter the market. The 7th Amendment of the Cosmetics Directive (76/768/ECC) requires the phasing out of animal testing for cosmetics by 2009. Although testing for neurotoxicity is not a primary endpoint in both legislations, certain effects may trigger neurotoxicity testing and hence the area of neurotoxicity is affected by this pressure. It therefore needs to respond with improved scientific concepts. This will in particular concern the advancement of assessment methods currently in use. One key problem associated with the present methodologies for neurotoxicity accepted for regulatory purposes (OECD 2003) is that they comprise only behavioral and neuropathological studies. This has three disadvantages: the tests are costly, time consuming and provide very limited mechanistic insight (Coecke et al., 2005). In contrast, basic research in neurotoxicology employs a wide variety of methods to understand how chemical substances interfere either reversibly or irreversibly with the proper functioning of the nervous system and therefore may provide detailed mechanistic insight. A way to make use of such methodologies while maintaining the desired robustness of safety testing might be the replacement of current tests by testing strategies comprised of several *in vitro* tests or combinations of *in vivo* and in

vitro tests. Such strategies might finally meet the requirements of providing all necessary scientific endpoints while returning a wealth of mechanistic information. As a first step it is however necessary to identify individual building blocks of such a testing strategy, i.e. tests that are suitable to address one specific aspect or mechanisms of neurotoxicity.

The European Commission has favoured the implementation of *in vitro* and *in silico* based approaches and their combination in testing strategies to reduce the time scale, animal use and costs where they deliver reliable results. However, the development of *in vitro* methods for neurotoxicity assessment has been hampered by the complexity of neurotoxic mechanisms (Coecke et al*.,* 2005). One particularly promising approach is the electrophysiological analysis of neural activity in complex cell culture systems. Since neural activity is the functional output of the processes in the CNS it could provide a sensitive endpoint to detect the early effects of chemicals. It has been shown that functional impairments can be detected by electrophysiological measurements before any morphological changes occur (Melani et al., 2005). Novel technologies like multi-electrode arrays (MEA) provide a simpler and less time-consuming alternative to conventional techniques (Pancrazio et al., 2003; Kohling et al., 2005). Studies have shown the use of neuronal networks cultured on MEA to detect effects induced by compounds like trimethyltin (Gramowski et al., 2000), mercury chloride (Gopal, 2003), ethanol (Xia et al., 2003), or pharmacological inhibitors (Morefield et al., 2000; Keefer et al., 2001). These studies use dissociated primary cell cultures from either spinal cord or cortex grown directly on the MEA. For a more appropriate representation of the *in vivo* cytoarchitecture and neuronal connectivity, hippocampal slices have also been suggested as tools to study effects on the CNS (Sundstrum et al., 2005; Noraberg et al., 2005; Hakkoum et al., 2006). Furthermore, preliminary measurements in hen embryo brain spheroids were reported (Uroukov et al., 2006).

In this study, we perform for the first time extra-cellular electrophysiological recordings of the neural network activity of three-dimensional rat re-aggregating brain cell cultures (Honegger et al., 1979). This type of primary culture was shown to comprise a population of neurons and glial cells in a three dimensional arrangement, resulting in the development of *in vivo*-like neuronal processes which exhibit close cell-cell interactions, myelination and synaptogenesis (Eskes et al., 2003). To combine *in vivo*-like complexity with a maximised capacity for testing a high number of substances in a short time, pre-differentiated re-aggregating brain cell cultures were grown on membranes to allow their transfer to multi-electrode arrays for medium-throughput recording. A similar method was shown to be suitable for hippocampal slice cultures (Stoppini et al., 1991). With this test system, we aim to develop an efficient and sensitive *in vitro* system to detect acute effects of chemicals on CNS function.

4.3 Materials and Methods

4.3.1 Materials

DL-2-amino-5-phosphonovaleric acid (APV), 3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX), γ-aminobutyric acid (GABA), bicuculline methiobromide, kainic acid (KA), ethanol, trimethyltin chloride (TMT), methyl mercury chloride (MeHgCl), parathion, paraoxon, L-glutamine, NaCl, KCl, NaH_2PO_4, $NaHCO_3$, KCl, glucose, HEPES, NaCl, CaCl, and MgCl were purchased from Sigma-Aldrich (Milan, Italy). Mouse anti-microtubule associated protein (MAP2) was from Neomarker, (Fremont, CA), rabbit anti-glial fibrillary acidic protein (GFAP) was from Dakocytomation, (Glostrup, Denmark), Alexa 546 rabbit anti-mouse IgG and Alexa 546 mouse anti-rabbit were from Molecular Probes (Leiden, The Netherlands). Culture plate inserts (0.4 μM) Millicell-CM were from Millipore (Milan Italy). The circular polyvinylidene difluoride (PVDF) membranes were from BioCell Interface (La Chaux-de-Fonds, Switzerland). Nylon mesh filters were from Swiss Silk Bolting Cloth (Zurich, Switzerland). Six well plates were from Nunc (Milan, Italy). Milli-Q water, HEPES, Neurobasal

medium A, modified serum free DMEM 041-95366M, B27 supplement, phosphate buffered saline (PBS), and donkey serum were from Invitrogen (Milan, Italy). The lactate dehydrogenase (LDH) cytotoxicity assay kit was from Promega (Milan, Italy).

4.3.2 Electrophysiological analyses

Electrophysiological recordings were performed using a multi-electrode array system (BioCell-Interface S.A., La Chaux-de-Fonds, Switzerland).

The BioCell multi-electrode array (MEA) consists of a network of 40 gold-plated microelectrodes (30 μM thick, impedance of 100 Ohms) laminated onto a perforated polymide support so that perfusion solutions from the lower chamber can reach the tissue placed onto the array. The console unit consisted of a thermo-regulated electronic module housing a disposable cartridge and which included the multi-electrode array, a series of signal preamplifiers and conditioners, as well as multiplexed channels. An analogue/digital interface (National Instrument Corporation, Austin TX, U.S.A) was installed inside a computer and digitised the recorded signals. On- or offline analyses were carried out using dedicated software (BioCell-Interface S.A).

4.3.3 Cell culture

Aggregating brain cell cultures were prepared from 16-day old fetal rat telencephalon as previously described (Honegger et al., 1979; Honegger and Monnet-Tschudi, 2001). The dissected tissue was mechanically dissociated using nylon sieves with 200 and 115 μM pores. The dissociated cells were washed and re-suspended at a density of $7.5*10^6$ cells/ml in modified serum-free DMEM supplemented according to (Honegger and Monnet-Tschudi, 2001). Cells were maintained in 15 ml flasks at an initial shaking speed of 68 rpm, which was increased over the days *in vitro* to a maximum of 80 rpm, at 37 ºC in an atmosphere of 10% CO_2 and 90% humidity.

After 10 days *in vitro* (after isolation) aggregates were placed on 8mm (diameter) circular hydrophilic membranes (3 aggregates per membrane) which were positioned on 30 mm inserts with a pore size of 0.4 µM in Neurobasal A medium supplemented with B27 supplement and 0.5 mM L-glutamine in a six-well plate. Aggregates on membranes were maintained at 37 ºC in an atmosphere of 5% CO_2 in air and 90% humidity and medium was replenished every second day. Extracellular recordings were performed between day 14-28 *in vitro (*after isolation), by placing the neural tissue directly on the electrodes of the MEA. Cell cultures were obtained and maintained according to good cell culture practice (GCCP) standards (Coecke et al., 2005).

4.3.4 Immunohistochemistry

Aggregates on membranes were fixed for 30 minutes in 4% paraformaldehyde in PBS at room temperature. Cells were permeabilized using 0.2% Triton X-100 in PBS for 45 minutes. After sufficient rinsing, the aggregates were incubated with primary antibody mouse anti-MAP2 overnight at 4ºC. Cells were washed 3 times with PBS and non-specific binding sites were blocked using 5% donkey serum in PBS for 30 min at 37 ºC. Secondary antibody rabbit anti-mouse IgG (1:400) was incubated for 2 h at 37 ºC in the dark. After washing 3 times with PBS, aggregates were transferred to a glass slide and mounted. Images were taken using a laser scanning confocal microscope (BioRad, Milan, Italy).

4.3.5 Extracellular recording conditions

For perfusion of the tissue by capillary forces the perfusion chamber of the MEA was filled with pre-warmed artificial cerebrospinal fluid (aCSF) (124 mM NaCl, 3 mM KCl, 1.2 mM NaH_2PO_4, 10 mM glucose, 5 mM Tris, 25 mM HEPES, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, at pH 7.4). One hydrophilic membrane carrying the neural tissue was transferred from the culture insert onto the MEA. The membrane was placed upside down so that the neural tissue made direct contact with the

recording electrodes. Temperature of the recording console was kept constant at 37 ºC.

4.3.6 Evoked field potentials

To evoke field potentials the tissue was electrically stimulated by bipolar stimulation electrodes. Although the MEA contains 40 electrodes (see above), the recording software allows the simultaneous recording from 8 channels.

Hence the 8 recording electrodes and the 2 stimulation electrodes could be freely chosen from 40 electrodes of the array. Optimal combinations that yielded maximal amplitudes upon stimulation were tested for each aggregate individually using the BioCell software. Once optimal electrode settings were found, an input/output study was performed to determine the maximum amplitude of the field potentials that could be evoked in the aggregate. The stimulation strength was then set to 50% of the maximum to avoid saturation of the signal and thus to be able to detect either a decrease or increase of evoked amplitudes. Only aggregates having field potential amplitudes ≥ 0.3 mV remaining stable over a baseline recording of 20 minutes were accepted. Prior to each recording session, a medium change was performed to check for possible artifacts due to movement. Cultures having low or unstable field potential amplitudes were discarded (less than 10 % of all cultures). During the experiment, field potential amplitudes were continuously evoked by bipolar stimulation every 10 seconds. The stimulation strengths ranged from 1000 to 3000 mV, with pulse duration of 100 µs.

4.3.7 Spontaneous activity

The occurrence of spontaneous spikes and burst formations was recorded using the 8 selected electrodes which had the highest and most stable frequencies using the software (Biocell interface). Cultures with low spontaneous activity (frequency <2 spike / second) were discarded (less than 10 % of all cultures). After changing the medium to check for mechanical stability, we performed a

baseline recording for 20 minutes to determine the mean frequency of spontaneous activity. The aggregates were then treated with the respective neurotoxicant and spontaneous activity was recorded for another 20 minutes to determine any effect of the test compound on the mean frequency. Aggregates were then washed and spontaneous activity was again recorded for 20 minutes to assess whether the effects were reversible.

The cultures were found to have the highest and most stable evoked field potential amplitudes and spontaneous activity frequencies between 14 to 28 days *in vitro* (after isolation) and all experiments were performed within this time period.

4.3.8 Assessment of acute neurotoxic effects

For the assessment of acute neurotoxic effects, compounds were dissolved in aCSF. The neural tissue was exposed by perfusion through the pores of the MEA. A volume of 1.5 ml was used to saturate the 150 µl volume of the MEA's perfusion chamber with the new conditions.

For the study of concentration dependent effects, cultures were perfused with increasing concentrations of the neurotoxic test substances as performed in (Keefer et al., 2001; Gopal, 2003). When the effects of one concentration reached equilibrium (i.e. a plateau in the field potential amplitude) the next incremental concentration was applied. To assess reversibility, a minimum 5 ml of aCSF was used for the wash. Wash-out conditions lasted at least 3 h (of which the first 30 minutes are shown in the figures). Preliminary data (not shown) demonstrated that a time period of 6 h was a reasonable limit for viability and stability of the culture on the MEA. Therefore, the experimental time including transferring the aggregate to the MEA, field potential detection, optimisation of stimulus intensity, stability check, and assessment of test compound was performed within a 6 h time period.

4.3.9 Cytotoxicity

Cell viability was evaluated using a commercially available lactate dehydrogenase (LDH) cell viability assay based on the release of cytosolic LDH in the culture media. Aggregates were distributed into 6 well plates in such way that each well contained 2 ml of medium with approximately 50 aggregates. Aggregates were treated with the test compounds (TMT, MeHgCl, ethanol, parathion, paraoxon) and synaptic modulators (NBQX, APV and KA) at the concentrations used for electrophysiological experiments. Medium samples were taken after 1, 2, and 3 h of treatment to maximise the detection of possible cytotoxic effects since treatments in the electrophysiological experiments lasted up to 100 min. For the quantification of LDH, culture medium samples were transferred to a 96 well plate (50 µl/well). 50 µl of substrate mix were added to the medium samples and incubated for 30 minutes at room temperature. After incubation, 50 µl of stop solution was added and optical density was read at 490 nm. Background readings of medium alone were subtracted from sample readings. Maximal LDH release was determined by total cell lysis using the provided lysis solution. The extent of cytotoxicity was calculated and expressed as a percentage of total release. The provided positive control (Bovine LDH) was used to evaluate the correct detection of LDH by the assay.

4.3.10 Statistical analysis

All values are presented as mean ± standard deviation. Data on spontaneous activity was analysed using a students-T test. *P< 0.05 was considered significant. The cytotoxicity data was analysed by a one-way ANOVA followed by a post-hoc Bonferroni's multiple comparison test. *P< 0.05 was considered significant. For the analysis of the field potential amplitudes we used a non linear curve fit spline LOWESS analysis using Prism 4 statistical software (GraphPad Inc). The mean change in amplitude after treatment was analysed using a one-way ANOVA followed by a Dunnett post-test versus the mean amplitude of the initial baseline recording. *P< 0.05 was considered significant.

4.4 Results

4.4.1 Morphological features and neural activity

In a first step towards exploring the usefulness of the re-aggregating brain cultures for the detection of neurotoxicants, we explored whether re-aggregating brain cell cultures can be used for MEA recordings. To be able to use aggregate cultures for MEA recordings they had to be grown on hydrophilic membranes so that the cultures could be easily transferred to the MEA with the membrane facing up and the culture facing down in contact with the MEA electrodes. The pre-differentiated aggregates placed onto hydrophilic membranes attached and grew to a culture with a tissue-like organisation. Fig. 10A shows a microscopy photo of an 11-day pre-differentiated aggregate culture, grown for 10 days on a hydrophilic membrane (total 21 days *in vitro*) after placement on the multi-electrode array. At this time point cultures showed similar morphological features as re-aggregating brain cultures grown under gyratory culture conditions: a dense network of neurons with cell bodies and elongated axons stained for MAP2 (Fig. 10B.), a network of astrocytes stained for GFAP (Fig. 10C). Using Annexin-V and propidium iodide we assessed the extent of cell death occurring in the cultures grown on membranes. Apoptosis and necrosis were comparable to cultures under gyratory conditions (data not shown).

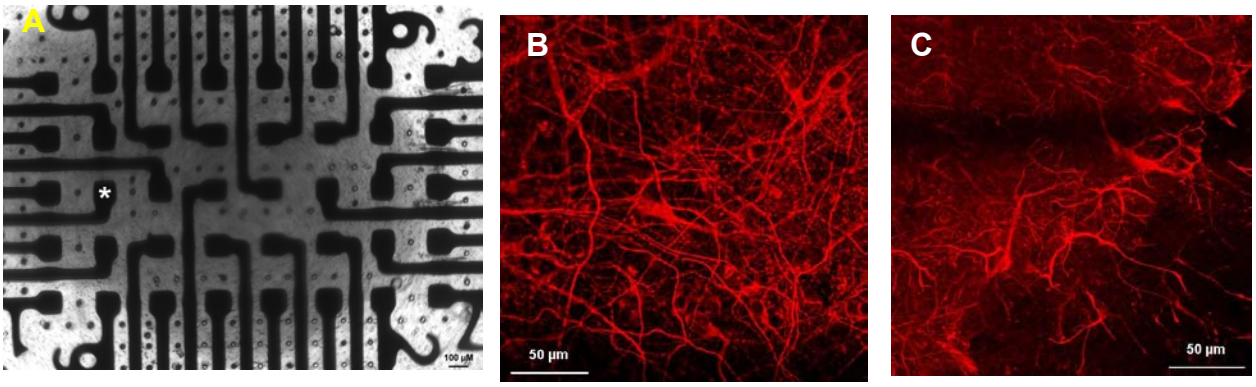

Fig. 10. (A) Microscopy photo of an aggregating brain cell culture (21 days *in vitro*) after placement on the multi-electrode array where the white asterisk indicates one of the electrodes. **(B)** MAP2 staining for neurons of an aggregating brain cell culture revealing the neuronal network with cell bodies and elongated axons. **(C)** GFAP staining revealing the network of astrocytes.

Two types of electrical activity were assessed in re-aggregating brain cell cultures: evoked field potentials upon electrical stimulation (Fig. 1D) and spontaneous neural firing consisting of spikes and burst formations (Fig. 1E). In conclusion, re-aggregated brain cell cultures can be grown on membranes and transferred to MEA allowing electrophysiological recordings.

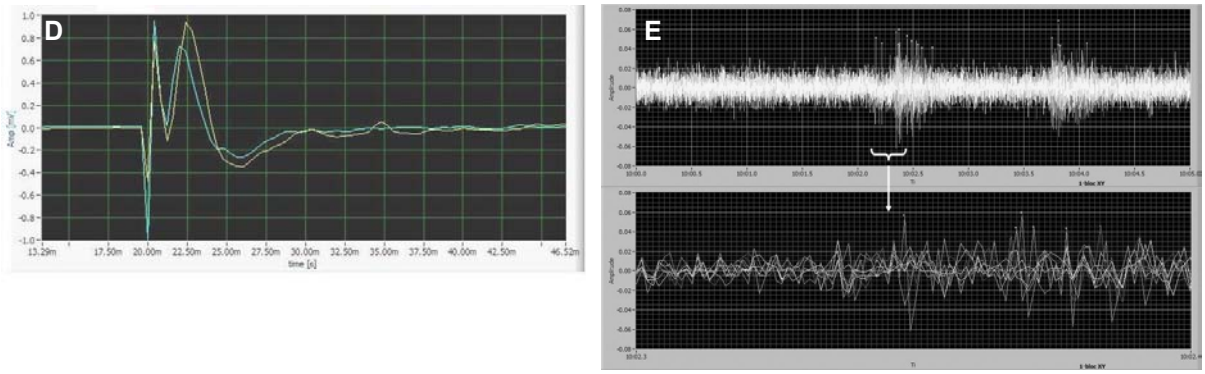

Fig. 10. (D) The electrophysiological recording of field potentials over two channels evoked by bipolar stimulation. **(E)**The electrophysiological recording of spontaneous neural activity over eight channels showing two burst formations and the magnification of single spikes within the burst (below).

4.4.2 Cytotoxicity

We used an LDH leakage cytotoxicity assay to study the possible cytotoxic effects induced by the selected antagonists and agonists APV (100 µM), NBQX (10 µM), KA (50 µM), GABA (10µM) and bicuculline (5µM), and the selected neurotoxicants TMT, MeHgCl, parathion / paraoxon (0.1 to 100 µM concentration range), ethanol (0.1 to 100 mM concentration range). After 1 or 2 h of exposure none of synaptic modulators and compounds induced any significant leakage of LDH into the culture medium (data not shown). The first significant release of LDH was observed after 3 h treatment with MeHgCl (100 µM) and ethanol (100 mM) (data not shown). The positive control consisting of bovine LDH showed that LDH was easily detected by the assay. Thus, none of the synaptic modulators or neurotoxicants induces cytotoxic effects in the timeframe and concentration ranges of the electrophysiological experiments.

4.4.3 Pharmacological effects of glutamatergic and GABAergic receptor blockers on evoked field potentials

To be used for the broad and robust assessment of neurotoxic effects on neurons from CNS, the model system needs to contain the broadest possible range of neuronal populations and certainly, glutamatergtic and gabaergic neurons, being the most prominent neurotransmitter systems in the brain. Using specific antagonists and agonists we therefore, evaluated whether and to which extent the evoked field potentials were dependent on glutamatergic and/or GABAergic synaptic transmission. When treating aggregates with the N-methyl-D-aspartate (NMDA) receptor antagonist APV at 100 µM for 1 h we observed a 20.3% decrease in field potential amplitudes (Fig. 11, triangles), (control: 0.63 mV, APV: 0.54 mV). Application of both APV at 100 µM and the alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor antagonist NBQX at 10 µM for 1 h resulted in a 75.2 % decrease in field potential amplitudes (dots), (APV&NBQX: 0.15 mV). This decease was found to be irreversible by wash-out (diamonds), (wash out: 0.11 mV).

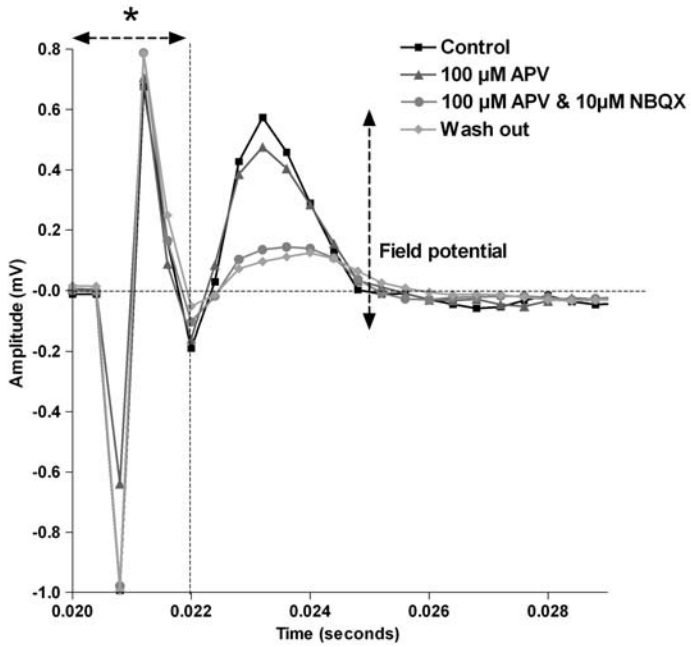

Fig. 11. The effects of glutamatergic agonists on the evoked field potential amplitude. The artifact of stimulation is indicated by the asterisk (*).The squares (■) show the control situation. The triangles (▲) show the decrease in amplitude after treatment with 100 µM of APV for 1h. The dots (●) show the decrease in amplitude after treatment with 100 µM APV and 10 µM NBQX in combination for 1h. The diamonds (♦) shows the irreversibility of the observed effect 3 h after wash out.

Treating aggregates with the kainate / AMPA receptor agonist kainic acid at 50 µM for 1 h (concentration that depolarises neurons) resulted in a 87.3% decrease in the field potential amplitudes (Fig.12, triangles), (control: 0.50 mV, KA: 0.06 mV). This decrease was partly reversible upon wash-out (diamonds), (wash out: 0.28 mV).

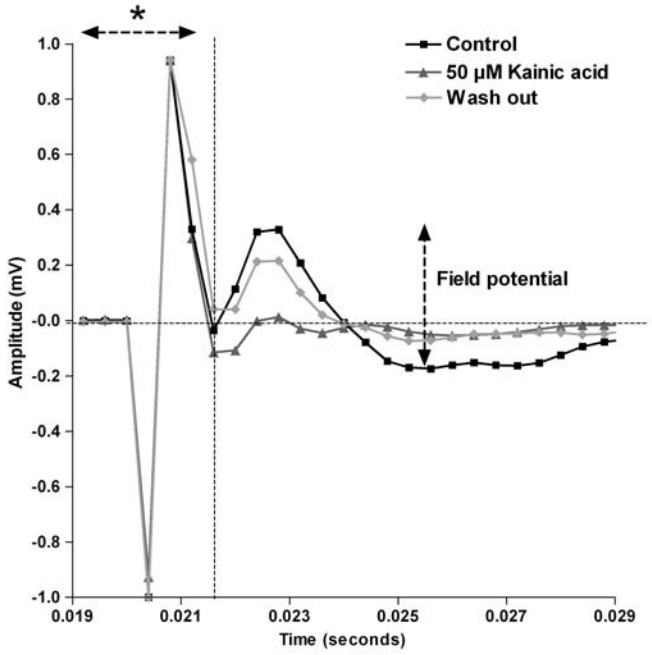

Fig. 12. The effects of the glutamatergic agonist kainic acid on the evoked field potential amplitude. The artifact of stimulation is indicated by the asterisk (*). The squares (■) show the field potential amplitude in the control situation. The dots (●) show the decrease in amplitude after 1h treatment with 50 µM kainic acid. The diamonds (♦) show the partial recovery 3 h after wash out.

The involvement of the GABAergic inhibitory system was studied by application of GABA and its competitive antagonist bicuculline methiobromide. Treatment with GABA at 10 µM for 30 minutes resulted in a 97.4 % decrease in the field potential amplitudes (Fig. 13, dots), (control: 0.56 mV, GABA: 0.01 mV). The same GABA treatment, however in the presence of 5 µM bicuculline resulted in no inhibition in field potential amplitudes suggesting the involvement of GABAergic receptors (triangles). The decrease in evoked field potential

amplitudes by GABA was partly reversible by wash-out (diamonds) (wash out: 0.07 mV). Thus, electrophysiological recordings of evoked field potentials in re-aggregating brain cell cultures mainly involve glutamatergic and GABergic synaptic transmission reflecting the essential characteristics of the CNS physiology.

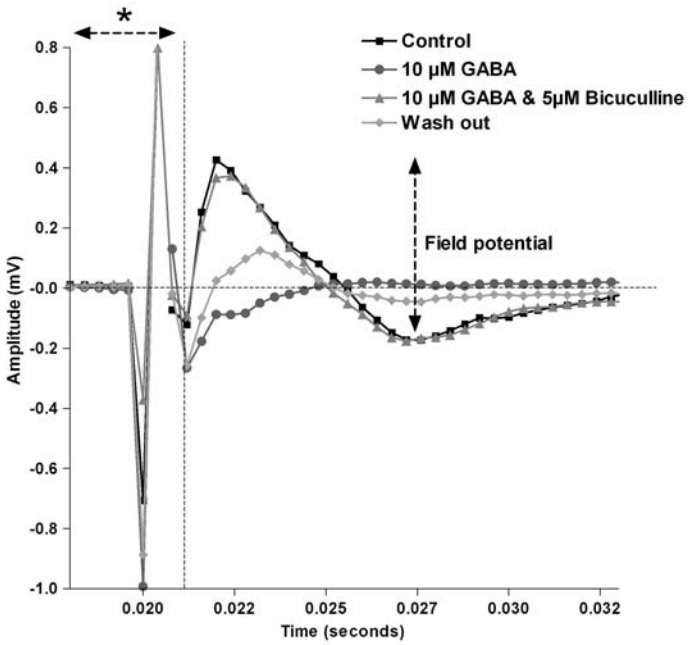

Fig. 13. The effects of GABA and its competitive antagonist bicuculline methiobromide on the evoked field potential amplitude. The artifact of stimulation is indicated by the asterisk (*). The squares (■) show the field potential in the control situation. The dots (●) show the inhibitory effect on the field potential amplitude after treatment with 10 µM GABA for 30 minutes. The triangles (▲) show the block of this effect by 5 µM bicuculline methiobromide. The diamonds (♦) show the partial recovery of GABA treatment 3 h after wash out.

4.4.4 Paired pulse stimulations

We used paired pulse stimulation to assess the presence of an inhibitory system and the existence of synaptic plasticity in aggregating brain cell cultures. We used paired-pulse stimulations with inter-pulse intervals of 15, 20, and 50 milliseconds. Results showed an inhibitory effect of the first pulse on the second one using inter-stimulation intervals of 15 ms (Fig. 14A) and 20 ms (Fig. 14B). For inter-pulse interval longer than 50 ms no paired pulse inhibition was observed (Fig. 14C). Thus, paired pulse inhibition can be observed in re-aggregating brain cell cultures.

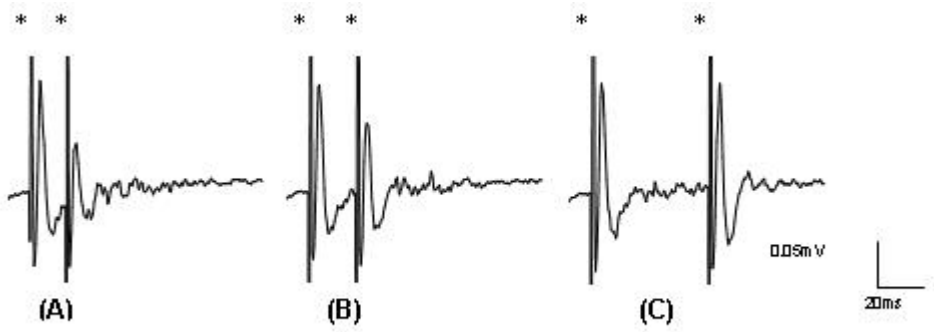

Fig. 14. Paired pulse inhibition. At inter-pulse intervals of 15ms **(A)** and 20ms **(B)** the second pulse decreases in amplitude compared with the first one. No effect is seen at inter-stimulus interval of 50 ms **(C)**. The asterisk (*) indicates the artifact of bipolar stimulation.

4.4.5 Pharmacological effects synaptic modulators on spontaneous activity

Next, we assessed whether the spontaneous firing of neural networks within the cultures depended on the same neurotransmitter/receptor systems as those necessary for evoked field potentials upon bipolar stimulation. Treatment of aggregates with APV at 100 μM for 1 h induced no significant effect on the frequency of the recorded spontaneous activity (control: 4.75±1.51; APV: 3.72±1.33; wash-out: 3.8±2.65 spikes/sec), (Fig. 15, open bars). The treatment with both APV at 100 μM and NBQX at 10 μM for 1 h induced a significant reversible 66.3% decrease in spontaneous activity frequency (control: 4.96±1.58; APV&NBQX: 1.66±1.14; wash-out: 3.72±0.77 spikes/sec), (Fig. 15, dotted bars).

Treatment with GABA at 10 μM for 30 minutes induced a significant and irreversible 55.2% decrease in spontaneous activity frequency (control: 3.91±1.61; GABA: 1.75±0.31; wash-out: 0.94±0.92 spikes/sec), (Fig 15, small squared bars).Treatment with kainic acid at 1 μM for 30 minutes induced a significant and reversible 45% increase in spontaneous activity frequency (control: 2.75±0.81; KA 1 μM: 5.08±1.48; wash out: 1.59±0.49 spikes/sec), (Fig. 15, big squared bars). A higher concentration of kainic acid of 50 μM (concentration which depolarizes neurons) induced a significant irreversible 88.9 % decrease in spontaneous activity frequency (control 2.13±0.79, KA 50 μM; 0.237±0.15; wash-out 0.428±0.23 spikes/second), (Fig. 15, filled bars). In general and not surprisingly the frequency of spontaneous activity over aggregates and electrodes was very variable with activity levels ranging from 0.1 to 6 spikes per second for untreated controls. Despite this variability the application of synaptic modulators showed comparable results for spontaneous activity as for evoked field potentials suggesting the involvement of similar synaptic interactions.

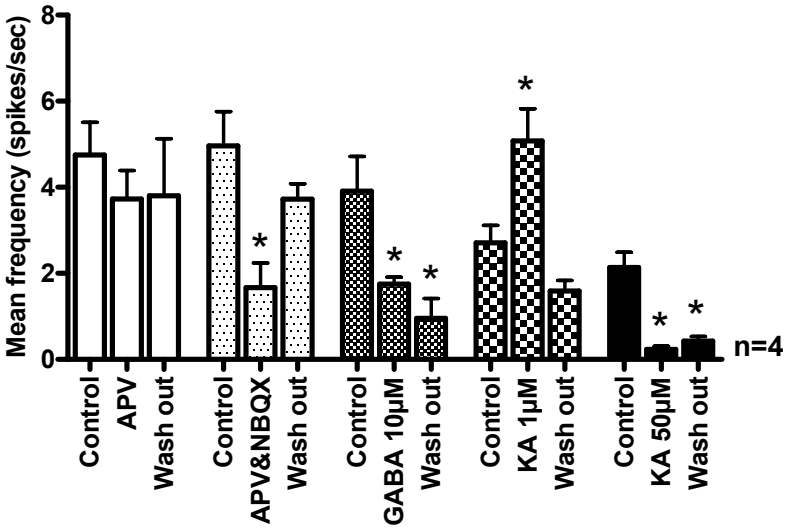

Fig. 15. The effects of synaptic modulators on the spontaneous activity mean frequency of aggregating brain cell cultures. Treatment with APV at 100 μM induced no significant effect (open bars), APV at 100 μM and NBQX at 10 μM in combination induced a significant reversible decrease (dotted bars), GABA at 10μM induced a significant irreversible decrease (small squared bars). Kainic acid at 1μM induced a significant reversible increase (big squared bars) and kainic acid at 50 μM induced a significant partial reversible decrease (filled bars). Mean (n=4 experiments) ± SD. * indicates $p<0.05$ versus control.

4.4.6 Assessment of neurotoxic effects on evoked field potentials

A number of well known neurotoxicants were tested in order to evaluate whether extracellular recordings of the electrical activity of neural networks in these cultures allow a sensitive detection of neurotoxic effects. Aggregates were treated with increasing concentrations of TMT, MeHgCl, ethanol, parathion and paraoxon while evoked field potential amplitudes were continuously monitored.

Treatment with TMT induced a significant concentration dependent decrease in field potential amplitudes at a concentration of 10 µM, and treatment with a concentration of 100 µM caused an irreversible disappearance of field potentials (Fig. 16A). MeHgCl also induced a significant concentration dependent decrease in field potential amplitudes at a concentration of 1 µM, and treatment with higher concentrations led to the total and irreversible disappearance of evoked potentials (Fig. 16B). Ethanol induced only a significant decrease in field potential amplitude at a concentration of 100 mM. The effect of ethanol was fully reversible upon wash-out (Fig. 16C). The treatment with parathion induced no significant decrease in field potential amplitudes. In contrast its active metabolite paraoxon increased the evoked field potential amplitude starting at 0.1 µM, being statistically significant at a concentration of 10 µM (Fig. 16D). At a concentration of 100 µM the evoked field potential amplitude starts to decline suggesting the occurrence of more severe toxicity leading to cell degeneration not detected by the LDH assay. Thus, the selected neurotoxicants either reduced or increased the recorded evoked field potential amplitudes at non-cytotoxic concentrations.

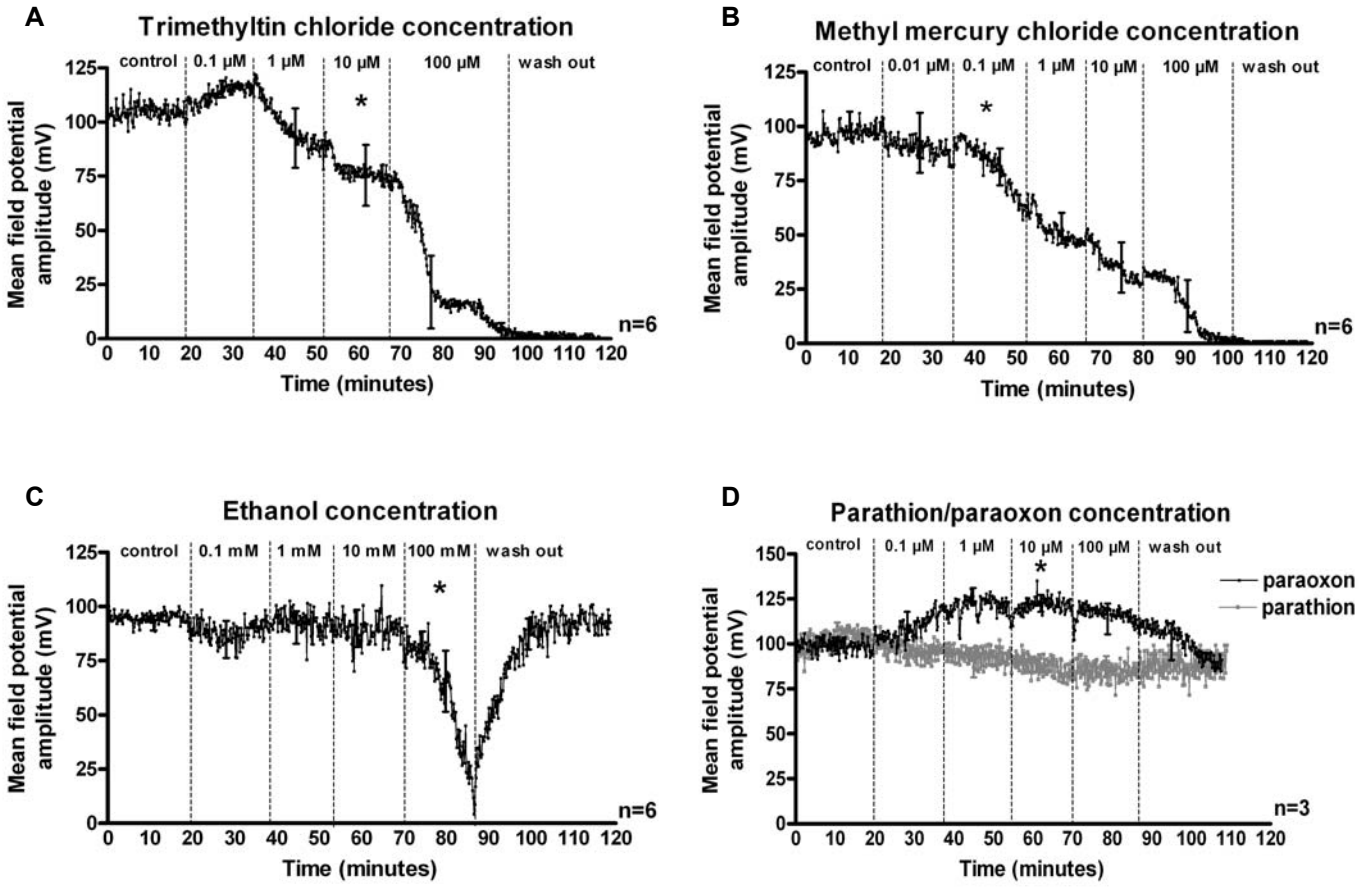

Fig. 16. The concentration dependent effect of TMT **(A)**, MeHgCl **(B)**, ethanol **(C)** and parathion (gray), paraoxon (black) **(D)** on the evoked field potential amplitude and reversibility evaluation by wash-out in aggregating brain cell cultures. Amplitudes are expressed as percentage of control. Mean (n=6 experiments) amplitudes ± SD, * $p < 0.05$ indicates mean change in treatment amplitude versus control amplitude.

4.4.7 Assessment of neurotoxic effects on spontaneous activity

Finally, we evaluated whether the spontaneous intrinsical activity of neurons within the cultures can also be used for the detection of neurotoxic effects. Concentrations of TMT and ethanol that significantly affected the amplitudes of evoked field potentials were used to study the effects on spontaneous neural firing. Treatment with TMT (10 µM) for 1 h induced an significant 86.6% decrease of spontaneous activity that was irreversible (control: 3.73±0.62; TMT: 0.5±0.47; wash out: 0.03±0.04 spikes/sec), (Fig. 17, filled bars). Treatment with ethanol (100 mM) for 20 minutes induced a significant 87.6% decrease in spontaneous activity frequency that was reversible (control: 4.15±0.99; ethanol: 0.57±0.22; wash out: 2.76±1.21 spikes/sec), (Fig. 17, open bars). Thus, spontaneous activity recordings showed comparable results to evoked field potentials for the detection of neurotoxic effects.

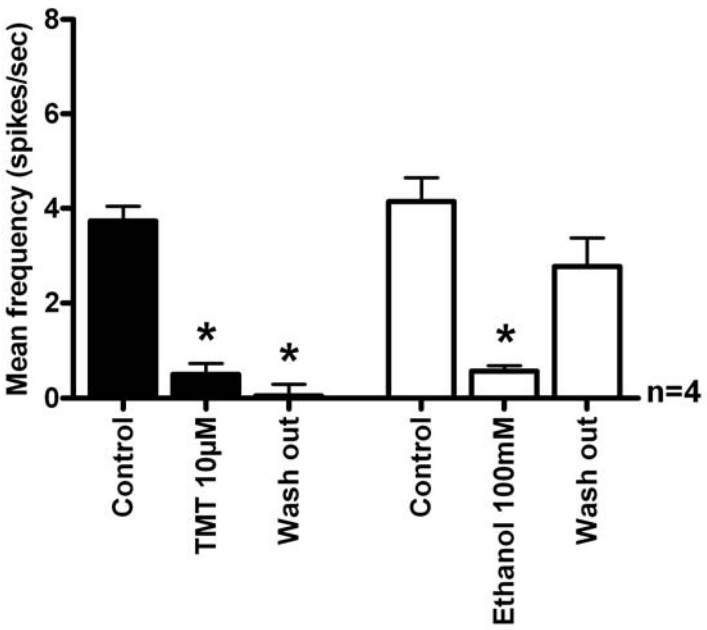

Fig. 17. The effect of TMT and ethanol on the spontaneous activity frequency recorded in aggregating brain cell cultures. Treatment with TMT using 10 µM for 1h induced a significant irreversible decrease (filled bars). Treatment with ethanol using 100 mM for 20 minutes induced a significant reversible decrease (open bars). The wash-out condition for TMT lasted 3 h and for ethanol 20 minutes. (n=4 experiments) ± SD. * indicates $p<0.05$ versus control.

4.5 Discussion

In this study we explored the use electrophysiological recordings in re-aggregating brain cell cultures on MEA for the detection of neurotoxic effects. To achieve both, *in vivo*-like complexity and maximised testing capacity, pre-differentiated brain aggregates were grown on membranes for easy transfer onto multi-electrode arrays. The preparation and culture conditions of this primary culture model are already well standardized and found to be reproducible (Honegger, 1979). One single preparation yields approximately 4000 aggregates and is sufficient for thousands of recordable membranes. This high yield of recordable cultures and the easy handling of membranes allow a good testing capacity, and make this model comply well with the 3R principles.

Microscopy and immunohistochemistry showed that pre-differentiated aggregating brain cell cultures grow well on the membranes and comprise a dense network of neurons and astrocytes. From an electrophysiological point of view, aggregating brain cell cultures displayed stable evoked field potentials and spontaneous firing activity which were shown to depend on the major glutamatergic and GABAergic synaptic transmission systems.

Moreover, paired pulse stimulation revealed a classical paired pulse inhibition of the second field potential indicating the presence of short-term synaptic plasticity via a functional inhibitory network. Thus, despite the lack of cytoarchitecture re-aggregating brain cell cultures displayed essential electrophysiological properties of neurons *in situ* (excitability, synaptic transmission) and also featured more complex neural networks (short-term plasticity). Re-aggregating brain-cultures thus show all prerequisites for exploiting the recording of electrophysiological activity for the assessment of neurotoxic effects. The assessment of other neurotransmitter systems and the possible use of paired pulse inhibition to study the neurotoxic effects of chemicals are still to be performed.

One major advantage of neural activity recordings for neurotoxicity assessment is its sensitivity to detect both reversible and irreversible effects. It is known that certain compounds e.g. barbiturates and benzodiazepines can affect neural function reversibly due to their pharmacological action without inducing

permanent damage. It is important to note that such effects would not be detected using e.g. morphological endpoints.

However, our test method can not distinguish between pharmacological actions e.g. the block of receptors and interactions with neurotransmitters, or neurotoxicity by e.g. a structural damage. Therefore, the suggested purpose of this model is to detect any adverse effects to neural function induced by chemical compounds. Exact mechanisms of toxicity may be studied later by multiple, more time consuming and expensive techniques. It is important to stress that in the case of toxicity screening high sensitivity is desirable.

In order to evaluated the usefulness of the model to predict neurotoxic effects the known neurotoxicants TMT, MeHgCl, ethanol and paraoxon were assessed for effects on neural activities. TMT is an anti fouling compound, which affects neural activity either pharmacologically by blockage of glutamatergic and GABAergic receptors (Kruger et al., 2005) or induces a total loss of neuronal activity (Gramowski et al*.,* 2000). The effect of TMT on the field potential amplitudes and spontaneous activity of aggregates occurred at similar concentrations as previously reported using dissociated cultures from spinal cord and auditory cortex on MEA (Gramowski et al*.,* 2000). The irreversibility of the observed effect suggests that TMT induced an adverse toxic effect affecting neuronal function.

The well know neurotoxicant MeHgCl was previously shown to reduce voltage activated calcium and potassium currents in rat dorsal root ganglion neurons (Leonhardt et al., 1996), and hippocampal slices (Szucs et al., 1997) at micro molar concentrations. Our results confirmed this acute action of MeHgCl on neuronal function. The irreversible loss in activity suggests that MeHgCl induces toxic effects.

Ethanol interacts with various neurotransmitters and receptors in the brain. It can depress neuronal activity by the potentiation of GABAergic receptors and block of glutamatergic receptors (McIntosh and Chick, 2004). In our model ethanol reversibly reduced field potential amplitudes at concentrations similar to those reported for frontal cortex cultures (Xia, 2003). A concentration of 100 mM induced an acute and almost complete loss in field potential amplitude and

spontaneous activity frequency. The full reversibility of the effect upon wash-out suggests that ethanol induces a pharmacological effect but no toxic effect as defined by permanent damage.

Paraoxon the active metabolite of the pesticide parathion is a well known acetylcholine esterase inhibitor (Garcia et al., 2003). Previous studies using biochemical assays showed the inhibition of acetylcholine esterase by paraoxon in re-aggregating brain cell cultures (Monnet-Tschudi, 2000). The fact that paraoxon and not parathion induced an electrophysiological increase in field potential amplitude reflects the epileptogenic effects of paraoxon (Harrison et al., 2004). It is interesting to note that such effects can be detected only by electrophysiology, and not by e.g. morphological methods. Although, acetylcholine esterase inhibition is irreversible, the observed epileptogenic effects of paraoxon decreased at higher concentrations. Such a decrease could be due to the occurrence of more severe neuronal damage as observed for other epileptogenic toxins like ouabain (Balestrino et al., 1999) and kainic acid (Kohling et al., 2005) which firstly increase, then decrease and ultimately abolish the evoked electrical potential of nervous tissue. It is however also conceivable that the epileptogenic effect is independent of acetylcholine receptor activation and actually acts by directly affecting membrane excitability as previously suggested (Vatanparast et al., 2006). Nevertheless, further studies are necessary to determine the mechanisms of paraoxon-induced epileptogenic effects in re-aggregating brain cell cultures.

In summary, the tested neurotoxicants were found to either reduce or increase the recorded evoked field potential amplitudes and spontaneous activity frequencies at non-cytotoxic concentrations, suggesting that the test system can be used as a sensitive functional endpoint to detect effects on neural function.

It is generally believed that well-designed *in vitro* test systems may serve as models of the *in vivo* situation or at least specific aspects thereof. To examine the relevance of the developed *in vitro* test system we largely compared the obtained results for TMT, MeHgCl, ethanol and paraoxon towards *in vivo* data in literature. Literature reported that single intraperitoneal injection of 8 mg/kg TMT

in rats caused behavioural and physiological effects within the first day (Segal et al., 1988). Intracranial injection of 0.3 mg/kg mercury in rat showed changes in behaviour and motor coordination after 24 hours (Venable et al., 1977). Ethanol concentrations of 122 mM lead to sleep and hypothermia in mice. In man, ethanol concentrations in the range of 50–100 mM lead to loss of consciousness and central nervous system activity depression while concentrations greater than 100 mM lead to coma and respiratory arrest (Charness et al., 1989 and Little, 1991). Intraperitoneal injection of three doses of 3 mg/kg paraoxon in rats inhibited plasma cholinesterase and induced behavioural changes during the first day (Carr and Chambers, 1991). In general these data suggests that all test compounds affecting neural activity in out test system also induce neurobehavioural effects *in vivo*. Moreover, neurobehavioural effects were observed at exposure levels not very different from *in vitro* treatments when taken into account *in vivo* pharmacokinetics. Therefore, *in vitro* electrophysiological recordings seem to be relevant for the prediction of neurotoxic effects and may lead to the reduction or replacement of more costly and elaborate animal based studies.

To continue the work presented we plan to carry out further experiments, screening a larger number of compounds to further confirm the applicability of this system to a broader array of neurotoxic compounds. This will be simplified by upcoming advances in the MEA recording system allowing simultaneous and longer recording of several aggregates. In conclusion, the developed test system was shown to represent relevant electrophysiological activity and to detect early effects of neurotoxicants on neural function. When incorporated into testing strategies, it could represent a valuable tool for the mechanistic assessment of neurotoxicity.

4.6 Acknowledgements

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5 A novel *in vitro* metabolomics approach for neurotoxicity testing, proof of principle for methyl mercury chloride and caffeine

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5.1 Abstract

There is a need for more efficient methods giving insight into the complex mechanisms of neurotoxicity. Testing strategies including *in vitro* methods have been proposed to comply with this requirement. With the present study we aimed to develop a novel *in vitro* approach which mimics *in vivo* complexity, detects neurotoxicity comprehensively, and provides mechanistic insight. For this purpose we combined rat primary re-aggregating brain cell cultures with a mass spectrometry (MS) based metabolomics approach. For the proof of principle we treated developing re-aggregating brain cell cultures for 48 hours with the neurotoxicant methyl mercury chloride (0.1 – 100 µM) and the brain stimulant caffeine (1-100 µM) and acquired cellular metabolic profiles. To detect toxicant induced metabolic alterations the profiles were analysed using commercial software which revealed patterns in the multi-parametric data set by principal component analyses (PCA), and recognised the most significantly altered metabolites. PCA revealed concentration dependent cluster formations for methyl mercury chloride (0.1-1 µM), and treatment dependent cluster formations for caffeine (1-100 µM) at sub-cytotoxic concentrations. Four relevant metabolites responsible for the concentration-dependent alterations following methyl mercury chloride treatment could be identified using MS-MS fragmentation analysis. These were γ-aminobutyric acid, choline, glutamine, creatine and spermine. Their respective mass ion intensities demonstrated metabolic alterations in line with the literature and suggest that the metabolites could be biomarkers for mechanisms of neurotoxicity or neuroprotection. In addition, we evaluated whether the approach could identify neurotoxic potential by testing eight compounds which have target organ toxicity in the liver, kidney or brain at sub-cytototoxic concentrations. PCA revealed cluster formations largely dependent on target organ toxicity indicating possible potential for the development of a neurotoxicity prediction model. With such results it could be useful to perform a validation study to determine the reliability, relevance and applicability of this approach to neurotoxicity screening. Thus, for the first time we show the benefits

and utility of *in vitro* metabolomics to comprehensively detect neurotoxicity and to discover new biomarkers.

5.2 Introduction

Metabolomics / metabonomics is the systematic study of the unique biochemical fingerprints that cellular processes leave behind, specifically the study of their small-molecule metabolite profiles (Nicholson et al., 1999). While gene expression and proteomics address the phenotypic adaptation of a cell, metabolic profiling can give an instantaneous snapshot of alterations in cell physiology. The metabolomics approach promises to provide information on toxicity, disease processes, gene function (Nicholson et al., 2002). Moreover, a recent study demonstrated the use of metabolomics for the discovery of new biomarkers for mechanisms of toxicity (van Ravenzwaay et al., 2007). Metabolomics studies are mostly based on data derived by either nuclear magnetic resonance spectroscopy (NMR) or mass spectrometry (MS). The non-selectivity, lack of sample bias, and reproducibility makes NMR suitable for screening applications. However, MS has the advantage over NMR based metabolomics because of its selectivity and sensitivity, particularly for the identification of new biomarkers (Robertson, 2005).

The utility of metabolomics in the evaluation of xenobiotic toxicity has been comprehensively assessed by the Consortium for Metabonomic Toxicology a group of six pharmaceutical companies and the Imperial College of Science, Technology and Medicine, London, UK (Lindon et al., 2005). Their main objectives were to define methodologies and to apply metabonomic data using ^{1}H-NMR spectroscopy of urine and blood serum for preclinical toxicological screening of drug candidates. This was achieved by generating a database of results for a wide range of model toxins as a basis for computer-based target organ toxicity prediction. Although, the analyses of urine and blood samples have given relevant information on liver and kidney toxicity (Robertson et al., 2000; Griffin, 2006), this approach is limited for neurotoxicity due to the presence of the blood-brain barrier. The blood brain barrier determines whether a compound

reaches the brain, at which concentration, and which metabolites can pass from the brain into the blood stream. Consequently, the application of *in vitro* based metabolomics approaches could play an important role in the area of neurotoxicology.

Current guidelines for neurotoxicity assessment are largely based on behavioural and neuropathological studies (OECD, 2003) which, although provide well accepted information, are time and cost consuming, limiting their use for high throughput screening programs. Moreover, it can be difficult to determine the mechanisms of toxicity that leads to a behavioural effect (Slikker et al., 2005). The use of testing strategies comprising *in vitro* methods addressing specific mechanistic questions has been proposed to provide more cost and time efficient information on the neurotoxic potential of compounds (Harry et al., 2005; Coecke et al., 2005; Lein et al., 2005).

The aim of this study was to develop an innovative *in vitro* approach for neurotoxicity screening by combining MS based metabolomics and the advanced neuronal cell culture model re-aggregating brain cell cultures (Honegger et al., 1979). Re-aggregating brain cell cultures are obtained from mechanically dissociated 16 day old rat fetal forebrains cultured in a serum free chemically defined medium and maintained under constant gyratory agitation (Honegger and Monnet-Tschudi, 2001). The dissociated cells coalesce into spherical aggregates of approximately 400 µm in diameter comprising a population of neurons, astrocytes, oligodendrocytes and glial cells (Honegger et al., 1979; Trapp et al., 1979, Almazan et al., 1985: Honegger and Werffeli, 1988). During the first two weeks, *in vitro* cell proliferation is predominant and aggregates are considered immature, whereas after 25 days, neurons, astrocytes and oligodendrocytes are characterised by a high degree of differentiation including myelination and synaptogenesis (Monnet-Tschudi et al., 1993; Monnet-Tschudi et al., 1995; Trapp et al., 1979). In particular, the cell culture model was shown to present morphological differentiation which resembles the *in vivo* situation (Trapp et al., 1979). The presence of cholinergic, glutamatergic, dopaminergic and serotonergic neurotransmitters has also been shown (Honegger and Richelson,

1979), and the occurrence of electrical activity was recently demonstrated (van Vliet et al., 2007). Cultures can be maintained over a period of two months where cell populations continue to mature consistently (Honegger and Schilter, 1992). Previous studies have demonstrated the occurrence of close cell-cell interactions between neurons and glial cells which play a role in either neurotoxic or neuroprotective mechanisms (Zurich et al., 2002; Eskes et al., 2002; Eskes et al., 2003). Finally, it was shown that toxic effects observed in re-aggregating brain cell cultures can occur at concentrations equivalent to the doses where *in vivo* effects are observed (Honegger and Schilter, 1992; Zurich et al., 2000).

For the proof of principle of *in vitro* metabolomics for neurotoxicity testing we selected two compounds with different mechanisms of action. Methyl mercury chloride is a well established neurotoxicant inducing neurotoxicity in the mature and developing CNS by various mechanisms including microtubule disruption, calcium signaling, oxidative stress, neurotransmitter deregulation, microglial activation and reactive gliosis (Castoldi et al., 2001; Eskes et al., 2002; Costa et al., 2007). Caffeine is a widely consumed brain stimulant which induces excitation by acting as an antagonist for adenosine A receptors and triggering voltage related calcium channels (Cauli and Morelli, 2005). While high concentrations of caffeine can increase oxygen consumption in neurons leading to ischemia and cell death (Gepdiremen et al., 1998), the concentrations used in this study were suggested to play a neuroprotective role in Parkinson related effects (Schwarzschild et al., 2003). Thus, for the first time we combined an *in vitro* model of neurodevelopment with a comprehensive metabolomics approach to create and evaluate a new tool for neurotoxicity screening.

5.3 Material and methods

5.3.1 Chemicals

The chemicals acetaminophen, acetic acid, acetonitrile, aminobutyric acid (GABA), caffeine, choline, creatine, dichlorophenoxy acetic acid, trifluoroacetic acid, dimethylformamide, glutamine, methyl mercury chloride, paraquat, spermine, trimethyltin chloride, and triton-X-100, were purchased from Sigma-Aldrich (Milan, Italy).

5.3.2 Re-aggregating brain cell cultures

Re-aggregating brain cell cultures were prepared from 16-day old fetal rat telencephalon as previously described (Honegger and Monnet-Tschudi, 2001). The dissected tissue was mechanically dissociated and cells were re-suspended at a density of $7.5*10^6$ cells/ml in modified serum-free DMEM (041-95366M, Gibco, Milan, Italy) with high glucose (25 mM) supplemented with insulin (0.8 µM), triiodothyronine (30 nM), hydrocortisone-21-phosphate (20 nM), transferrin (1µg/ml), biotin (4µM), vitamin B_{12} (1µM), linoleate (10 µM), lipoic acid (1µM), L-carnitine (10 µM), and trace elements (Honegger and Monnet-Tschudi, 2001). Cells were maintained in 15 ml flasks under an initial gyratory shaking speed of 68 rpm, which was increased to a constant speed of 80 rpm, at 37 ºC in an atmosphere of 10% CO_2, over five days. Culture medium was replenished every three days up to day 14 and every other day thereafter (5ml replaced of 8 ml total). For the humane treatment of pregnant rats we followed the European Commission Recommendations, Part 1 and Part 2 (Close et al., 1996; Close et al., 1997). Cell cultures were maintained according to good cell culture practice (GCCP) standards (Coecke et al., 2005).

5.3.3 Treatments

For toxic treatments, re-aggregating brain cell cultures (21 days after isolation) were pooled and distributed over six well plates (Nunc, Milan, Italy). Each well contained approximately 100 aggregates in 2 ml of culture medium. Stock

solutions of the compounds were prepared in ultra pure sterile water. A final concentration range from 0.1 to 100 µM of methyl mercury chloride and from 1 to 100 µM caffeine was added to the medium by adding 20 µl of stock solution to each well containing 2 ml of culture media. In untreated controls 20 µl of sterile water was added to the culture media.

5.3.4 Cell viability assay

Cell viability was evaluated using a lactate dehydrogenase (LDH) cell viability assay based on membrane leakage (Promega, Milan, Italy). After the treatment period, 50 µl samples of cell culture medium were transferred to a 96 well plate. The medium was incubated with 50 µl of substrate mix for 30 minutes at room temperature. After incubation, 50 µl of stop solution was added and the optical density was read at 490 nm. Background readings of medium alone were subtracted from sample readings. The extent of cytotoxicity was calculated and expressed as a percentage of total release after total cell lysis using 0.1% of triton-X-100 for 2 hours.

5.3.5 Sample preparation

After toxic treatment re-aggregating brain cell cultures were harvested and washed three times with 15 ml of ice-cold PBS (Gibco, Milan,Italy) to remove the culture media. For the study of hydrophilic brain metabolites (e.g. neurotransmitters) cell lysates were prepared using pulsing ultrasound sonication (Heat systems, New York, U.S.A) in ultrapure water on ice. The cell lysates were centrifuged at 4°C to remove debris and supernatants were collected. To ensure uniformity in measurements, the cell protein contents were standardised prior to the LC-MS measurements. For each sample the protein content was quantified using the Lowry assay (BioRad, Milan, Italy), and cell lysate samples with a standardised protein content of 2 µg/ml were prepared in ultrapure water before LC-MS measurement. The amount of protein obtained was further checked by

quantifying the protein content of samples after the LC-MS measurements.

5.3.6 Analytical measurements by LC-MS

Metabolic profiles ranging from 50 to 1000 Da of the cell lysates were acquired using an HPLC system (Alliance 2795, Waters, Milford, U.S.A) coupled to a mass spectrometer (Micromass Quattro Ultima, Waters, Milford, U.S.A). All the samples were acquired by operating the electrospray source of the mass spectrometer in positive mode. A source temperature of 120 ºC, a cone gas flow of 75 L/Hr and a desolvation gas flow of 750 L/Hr were employed. The capillary voltage was set to 3 kV and the cone voltage to 50 V. A scan duration of 0.5 second was used throughout with an interscan delay of 0.1 second. Data was collected in the continuum mode. A sample volume of 10 µl was injected into the mass spectrometer using direct infusion (flow rate 200 µl/min). The wash sequence “purge-wash-purge” was employed on the auto sampler, with water/acetonitrile (80:20) used as wash solvent and 0.1% trifluoroacetic acid in water as purge solvent to ensure that the carry-over between injections was minimized. Samples prepared in 96 well plates (Nunc, Milan, Italy) were measured using auto sampling. The total sample run time was 6 minutes.

5.3.7 Data analysis

The cellular metabolic profiles acquired by the LC-MS measurements were analysed using MarkerLynx 4.1 software (Waters, U.S.A). The application detects, integrates and normalises the intensities of the peaks to the sum of peaks within the sample. The resulting multivariate data-set consisting of the peak number (based on the retention time and m/z), sample name, and ion intensity was analysed by principal component analyses (PCA). This statistical tool transforms the large set of inter-correlated variables to a smaller set of independent uncorrelated variables (principal components). The first principal component describes the largest variation in the data-set in which the samples

spread the most in the variable space. The second component describes the next largest variation and is orthogonal to the first component. The number of principal components that can be generated is equal to the number of samples in the data-set. Two orthogonal components create a plane or score plot in which the data-points are projected. This plot highlights clustering or pattern formations in a two-dimensional space which provides a view of the similarities and dissimilarities between the samples. The most significant masses responsible for the variation in the multivariate dataset are displayed in a PCA loading plot and their significance and respective normalised ion intensities are listed.

5.3.8 Statistical analysis

Values are presented as mean ± SD. PCA was performed using MarkerLynx 4.1 software (Waters, Milford, U.S.A). Concentration dependent effects were analysed by a one-way ANOVA followed by a Bonferroni's Multiple Comparison Test comparing the increasing concentrations (*) indicates $p<0.05$, (**) indicates $p<0.01$, (***) indicates $p<0.001$.

5.4 Results

5.4.1 Cytotoxicity

As a first step we determined the cytotoxic effects induced by methyl mercury chloride and caffeine in order to discriminate between sub-cytotoxic and cytotoxic concentration ranges. Re-aggregating brain cell cultures were kept for 21 days *in vitro* to reach a high differentiation and maturation stage of the different cell types. Cultures were treated for 48 hours with a 0.1-100 µM concentration range of methyl mercury chloride and 1-100 µM of caffeine and the release of lactate dehydrogenase (LDH) in the cell culture media was quantified. Results showed that methyl mercury chloride has a no-effect level concentration of 1 µM. Treatment with higher concentrations of 3, 10, 33 and 100 µM induced a concentration dependent increase of LDH release with an IC_{50} value of 4.07 ± 0.30 µM (Fig. 18). In contrast, caffeine induced no significant release of LDH compared to the untreated controls in the used concentration range (data not shown).

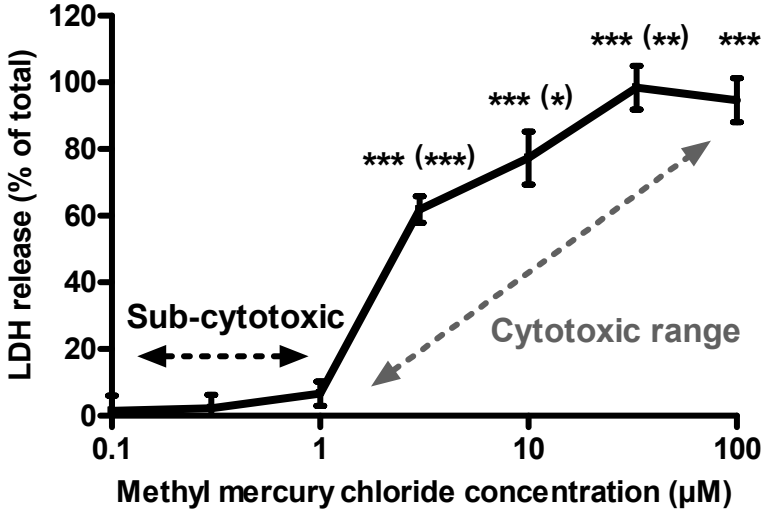


Fig. 18. Cytotoxicity concentration response curve for methyl mercury chloride after 48 hours of exposure, with an IC_{50} value of 4.07 ± 0.30 µM. (mean value n=9 ± SD, % of total release). One-way ANOVA followed by a Bonferoni Multiple Comparison Test comparing the control and all concentrations, (* $p<0.05$; ** $p< 0.01$, *** $p<0.001$). Asterisks indicate significantly different from control, and asterisks in brackets indicate significantly different from previous concentration.

5.4.2 Metabolic fingerprinting of neurotoxicity

For the metabolic fingerprinting of neurotoxic effects, cellular metabolic profiles of the methyl mercury chloride and caffeine treated aggregates were analysed using statistical software (see data analysis). The results of the analysis showed PCA score plots displaying the similarities and dissimilarities between the untreated control and treated cell lysate samples. The methyl mercury chloride samples showed clear concentration dependent cluster formations at sub-cytotoxic concentrations (Fig. 19a). On the other hand caffeine treated samples could be well differentiated from the untreated controls, but no clear concentration dependent clustering was observed (Fig. 19b). Testing with a larger data-set for caffeine might be needed to clarify the observed results. These results demonstrate nevertheless that mass spectrometry based metabolomics is able to detect both methyl mercury chloride and caffeine induced metabolic alterations at sub-cytotoxic concentrations. The concentration dependent effects induced by methyl mercury chloride were further investigated according to the PCA loading plot (Fig. 20). This plot displays the masses of the detected metabolites and their contribution to the cluster formations of sub-cytotoxic concentrations of methyl mercury chloride in the PCA score plot (Fig. 19a) according to their spread from the plots origin. The masses which contributed significantly to the concentration-dependent metabolic alterations were screened for their identity, to provide potential insight into the neurotoxic mechanisms of methyl mercury chloride and postulate its biomarkers.

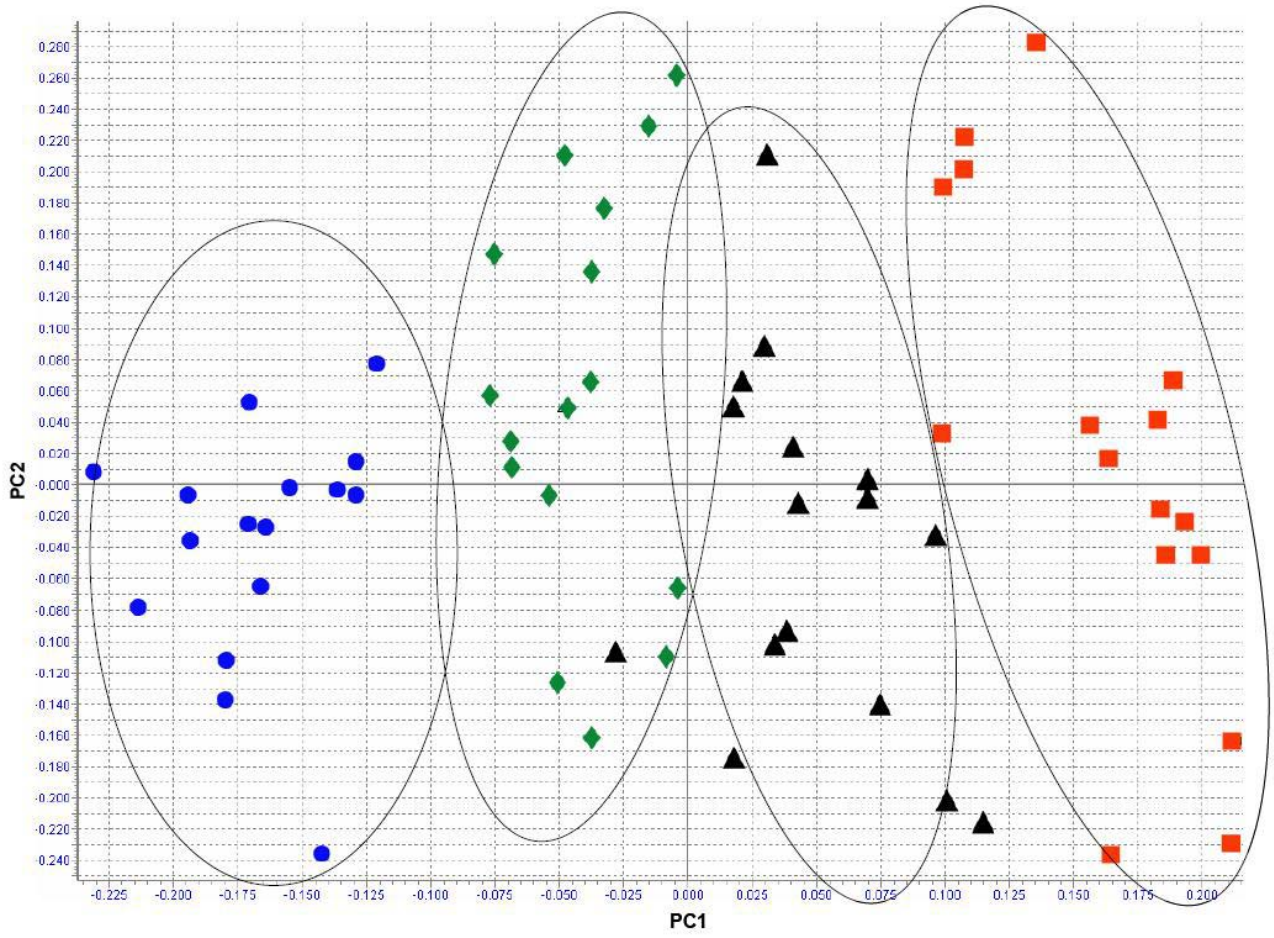


Fig. 19A. PCA score plot (PC1 versus PC2) of the analysed metabolic profiles of re-aggregating brain cell cultures exposed for 48 hours to a concentration range of methyl mercury chloride. The statistical data analysis resulted in a concentration dependent cluster formation at sub-cytotoxic concentration levels. The replicates (n=16) represent untreated controls (■) and methyl mercury chloride concentrations of 0.1 µM (▲), 0.3 µM (♦) and 1 µM (●).

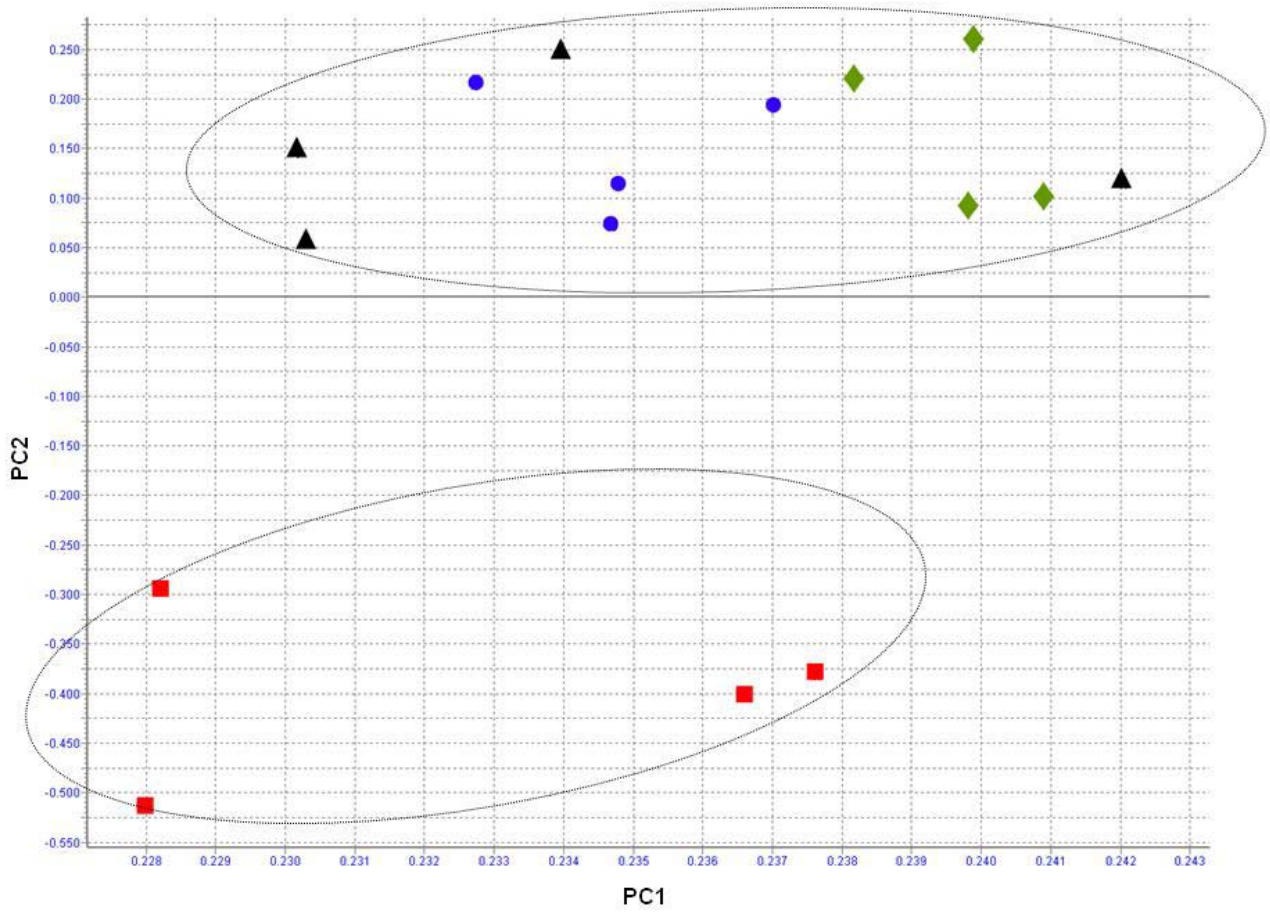


Fig. 19B. PCA score plot (PC1 versus PC2) of the analysed metabolic profiles of re-aggregating brain cell cultures exposed for 48 hours to a concentration range of caffeine. The statistical data analysis resulted in a cluster formation of controls and exposed samples at sub-cytotoxic concentration levels. The replicates (n=4) represent untreated controls (■) and caffeine concentrations of 1 µM (▲), 10 µM (♦) and 100 µM (●).

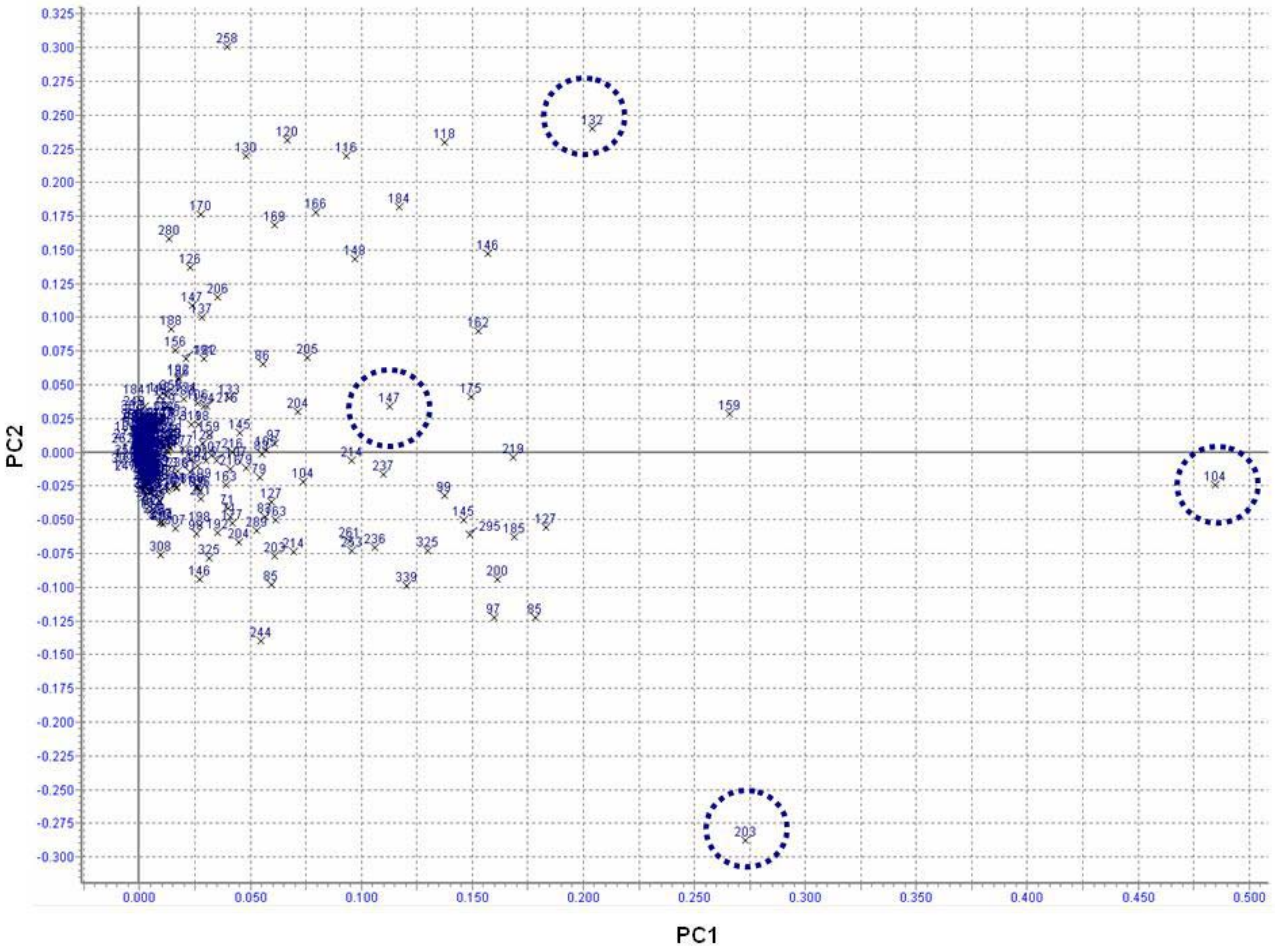


Fig. 20. PCA loading plot of the analysed metabolic profiles of re-aggregating brain cell cultures exposed for 48 hours to sub-cytotoxic concentrations of methyl mercury chloride. The plot revealed the masses of the putative biomarkers for the effects induced by methyl mercury chloride. The circles indicate the masses which were identified by MS-MS analysis.

5.4.3 Metabolite identification

To identify the metabolites which induced the most significant alterations within the metabolic profiles we obtained the exact masses of 58 putative biomarkers using two accurate mass spectrometers (Bruker Daltonics, apex-Qe 9,4, Bremen, Germany; Thermo Electron, LTQ FT 7.0, Waltham, U.S.A). The exact masses allowed the performance of elemental composition calculations of the empirical chemical formulas. The biochemicals corresponding to the deduced formulas that were biologically relevant and commercially available were purchased as reference compounds. The chemical identities of the putative biomarkers were confirmed using (MS/MS) analyses which compared the specific fragmentation patterns of the metabolites in the cell lysates to patterns of the purchased reference compounds. The putative biomarkers for methyl mercury chloride neurotoxicity that could be identified were GABA, choline, glutamine, creatine and spermine. Their position in the PCA loading plot (Fig. 20, circles) demonstrates they are amongst the most significant biomarkers. Their measured exact mass, calculated formula, and empirical chemical formula are listed in Table 2. The empirical elemental composition calculations for the accurate masses resulted in single putative identities. However, the 104.1 mass revealed that there were potentially two possible identities for both GABA and choline due to their similar masses. The result of MS-MS fragmentation pattern analysis demonstrated the presence of both GABA and choline compounds in the 104.1 mass peak by the presence of both specific fragmentation patterns. MS-MS analysis of the other masses demonstrated a single compound specific fragmentation pattern for creatine, glutamine and spermine. Although, the compounds GABA and choline were also present in the culture media, the extensive washing procedure of the re-aggregating brain cell cultures avoided any interference with the cell lysate sample measurements. The final wash fractions were checked and did not contain GABA or choline at the detection limit.

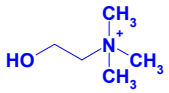
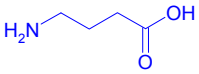
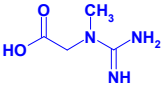
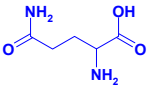
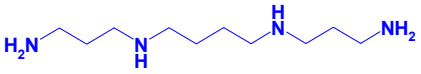
m/z	Accurate mass (postulated atomic composition)	Metabolite identity
104.1	104.10697 (C5H13NO)	choline 
	104.07120 (C4H19NO2)	GABA 
132.1	132.07676 (C4H9N3O2)	creatine 
147.1	147.07692 (C5H10N2O3)	glutamine 
203.2	203.22357 (C10H26N4)	spermine 

Table 2. Postulated atomic compositions and their metabolite identities.

5.4.4 Mass ion intensities of identified metabolites

To get more insight into the metabolic alterations induced by methyl mercury chloride and caffeine we quantified the mass ion intensities of the identified metabolites GABA, choline, glutamine, creatine and spermine in the metabolic profiles. The results showed that methyl mercury chloride induced a significant concentration dependent decrease in GABA and choline levels starting at sub-cytotoxic concentrations of 0.3 and 1 µM (Fig. 21a). The levels of creatine increased significantly at sub-cytotoxic of 0.1, 0.3 and 1 µM, but decreased significantly at cytotoxic concentrations (Fig. 21b). Glutamine levels increased significantly at sub-cytotoxic concentrations of 0.1 and 1 µM and decreased significantly at cytotoxic concentrations (Fig. 21c). Spermine levels remained unaltered at sub-cytotoxic concentrations, but increased significantly in a concentration dependent manner at cytotoxic concentrations (Fig. 21d). The effects of methyl mercury chloride at sub-cytotoxic concentrations are suggested to be neurotoxic effects, while the high losses of brain metabolites at cytotoxic

concentrations are likely to be due to cell death. Treatment with sub-cytotoxic concentrations of caffeine did not affect the levels of GABA, choline or glutamine levels (Fig. 22a, b), but significantly decreased creatine levels (Fig. 22c). The levels of spermine increased significantly in a concentration dependent manner (Fig. 22d). Overall, these results demonstrate that the developed metabolomics approach allows the detection of significant alterations in brain metabolites at both cytotoxic and sub-cytotoxic concentrations of methyl mercury chloride and sub-cytotoxic concentrations of caffeine.

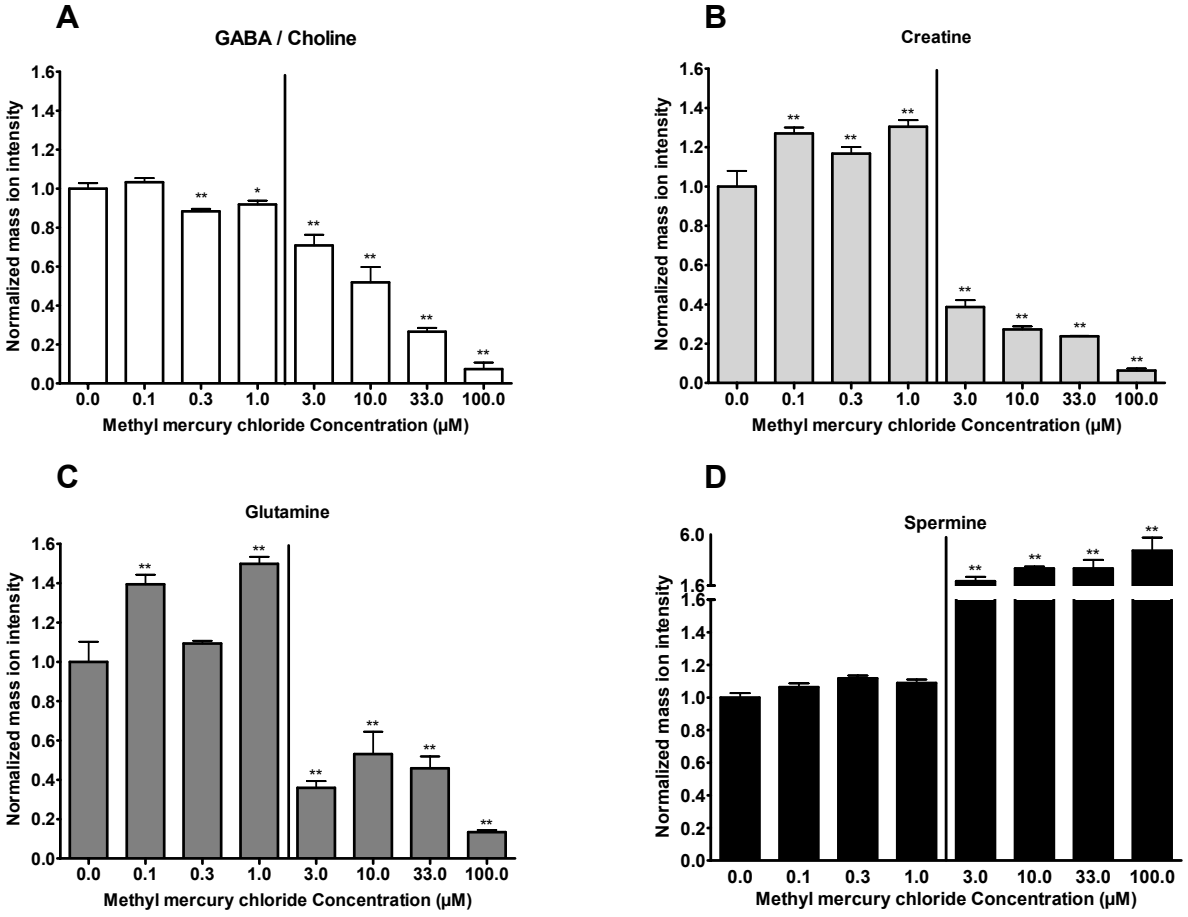


Fig. 21. Mass spectrometry-metabolic alterations induced by 48 hour methyl mercury chloride treatment on the mass ion intensities of the identified biomarkers GABA and choline **(A)**, creatine **(B)**, glutamine **(C)**, and spermine **(D)**, (mean value n=16 ± SD). The black vertical line indicates the separation between the sub-cytotoxic and cytotoxic concentrations. One-way ANOVA followed by a Dunnett's Multiple Comparison Test vs. control, (* $p<0.05$, ** $p< 0.01$).

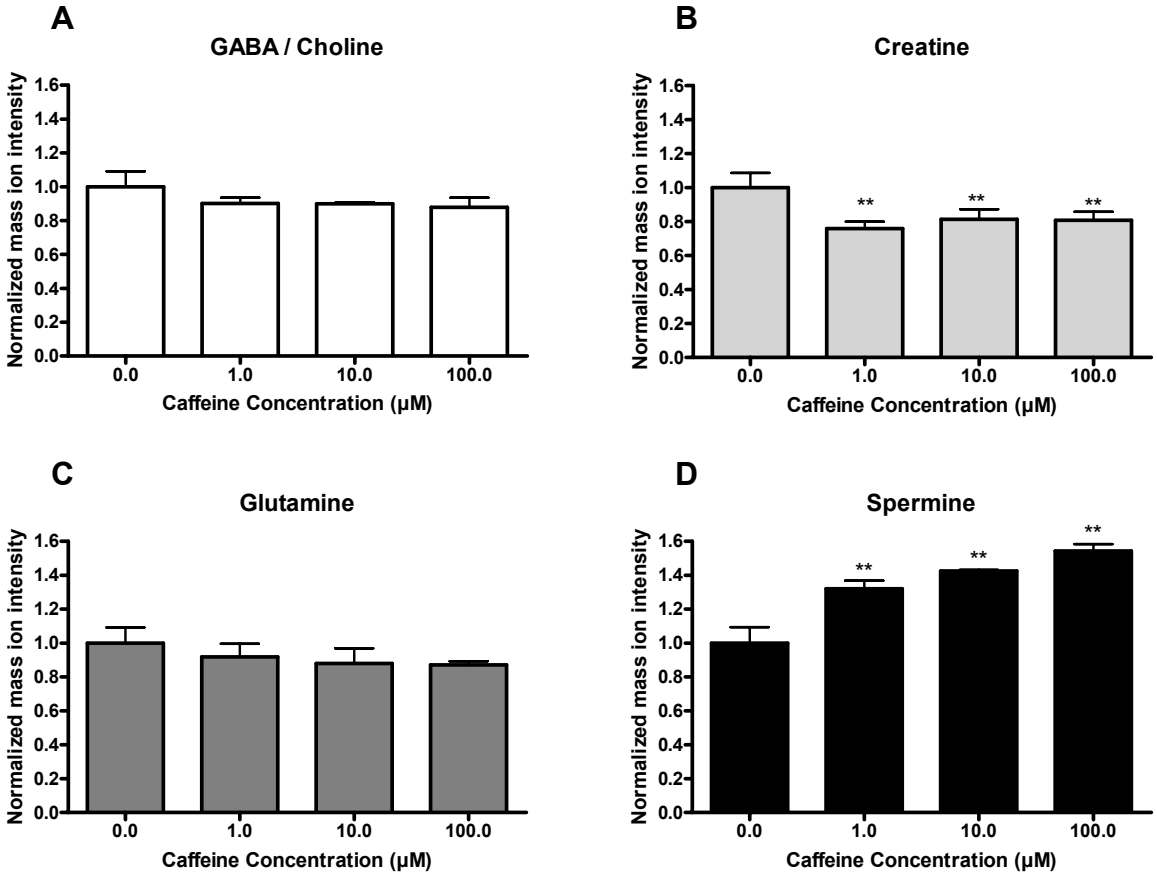


Fig. 22. Mass spectrometry-metabolic alterations showing concentration dependent effects induced by 48 hour caffeine treatment on the mass ion intensities of the identified biomarkers GABA and choline **(A)**, creatine **(B)**, glutamine **(C)**, and spermine **(D)**, (mean value n=4 ± SD). One-way ANOVA followed by a Dunnett's Multiple Comparison Test vs. control, (** $p < 0.01$).

5.4.5 Identification neurotoxic potential of compounds

In addition, we evaluated whether the *in vitro* metabolomics approach could be used to identify the neurotoxic potential of compounds in re-aggregating brain cell cultures. For this purpose we tested eight compounds with specific target organ toxicity for the brain, liver, kidney via different mechanisms of toxicity. The final design of the study is shown in Table 3. Re-aggregating brain cell cultures were treated for 48 hours with a sub-cytotoxic concentration of trimethyltin chloride, methyl mercury chloride, colchicine, paraquat, cycloheximide, dimethylformamide, dichlorophenoxy acetic acid, and acetaminophen as determined by an LDH release cytotoxicity assay (data not shown). We prepared cell lysate samples of the treated re-aggregating brain cell cultures and acquired metabolic profiles which were analysed using statistical software. The results showed a PCA score plot which largely separated the eight compounds into two clusters (Fig. 23). The first cluster includes the untreated control samples and the compounds with the liver and kidney as their target for toxicity. The second cluster includes the compounds with the brain as their target for toxicity, which are more widespread and separate from the control samples in the plot. This cluster formation suggests that compounds targeting the brain induce stronger and more extensive metabolic alterations compared to compounds with different target organ toxicity which remain more similar to the control. The PCA loading plot revealed that the previously identified putative biomarkers GABA, choline, spermine and creatine induced significant metabolic alterations within the multivariate data set of this preliminary study (Fig. 24, circles).

Nr	Compound	Concentration	Target organ
1	trimethyltin chloride	1 µM	brain
2	methyl mercury chloride	1 µM	brain
3	colchicine	1 µM	brain
4	paraquat	1 µM	brain
5	dimethylformamide	1 µM	liver
6	2,4-dichlorophenoxy acetic acid	1 µM	liver
7	cycloheximide	1 µM	liver, kidney
8	acetaminophen	1 mM	liver, kidney

Table 3. Compounds tested for their neurotoxic action using the *in vitro* metabolomics approach.

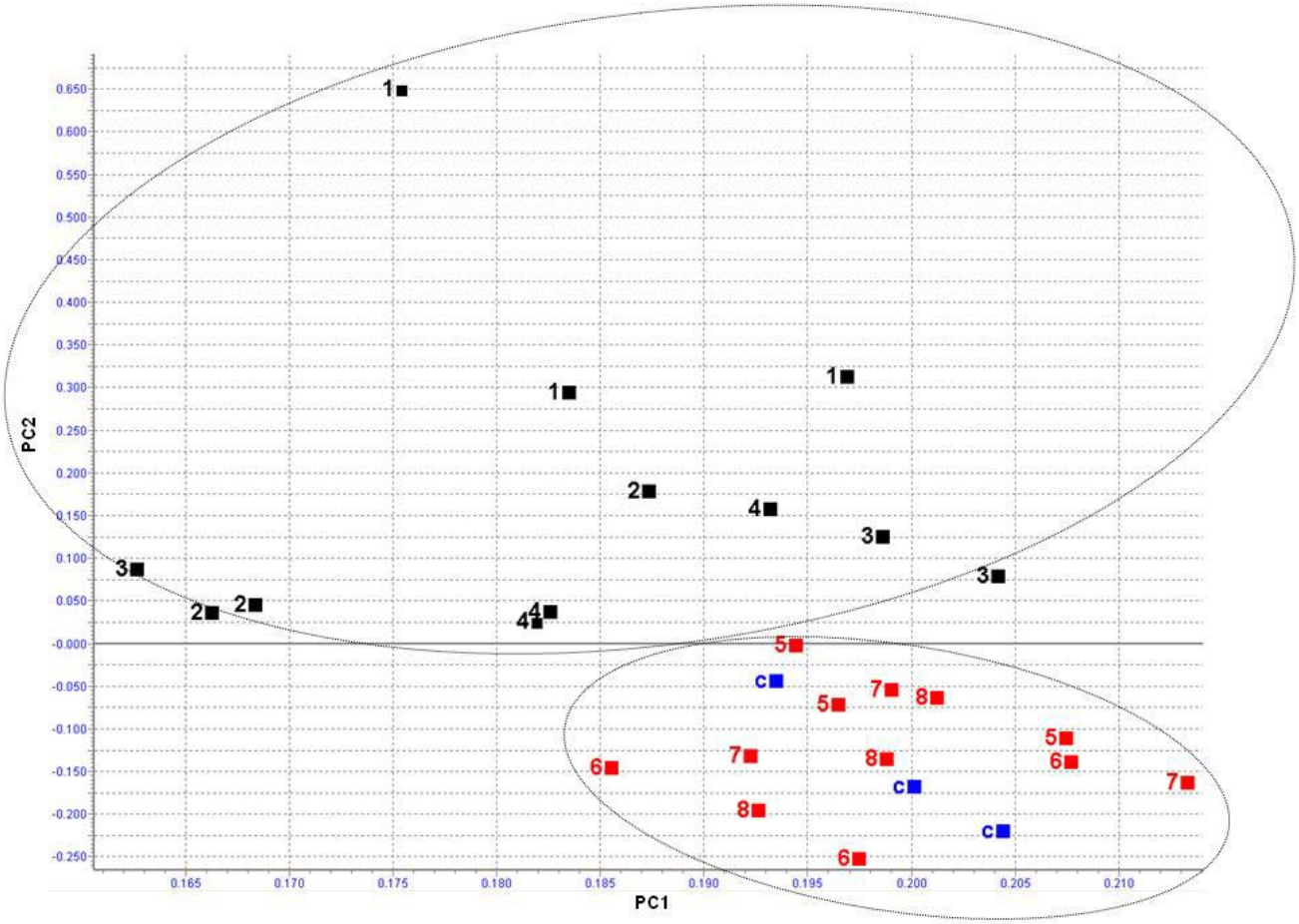


Fig. 23. PCA score plot (PC1 versus PC2) showing the metabolic deviation within the analysed metabolic profiles of re-aggregating brain cell cultures (triplicates) exposed for 48 hours to a sub-cytotoxic concentration of trimethyltin chloride (■1), methyl mercury chloride (■2), colchicine (■3), paraquat (■4), dimethylformamide (■5), 2,4 dichlorodiphenoxypropene (■6), cycloheximide (■7), acetaminophen (■8), and untreated controls (■c). The results revealed a cluster formation separating largely the compounds which have target organ toxicity in the brain from the compounds which have target organ toxicity in the liver and kidney.

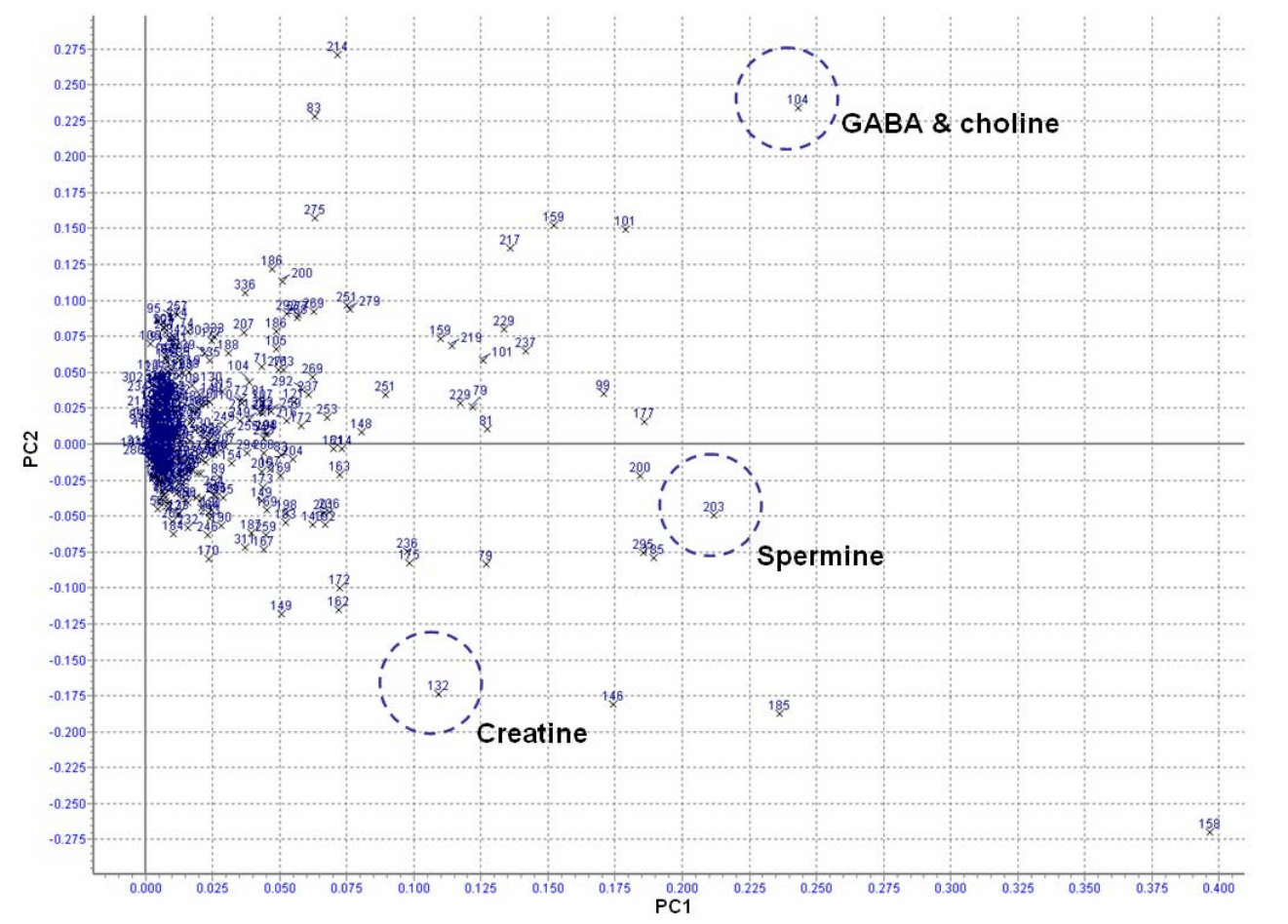


Fig. 24. PCA loading plot (PC1 versus PC2) revealing the putative biomarkers that induced the metabolic alterations in the analysed metabolic profiles of re-aggregating brain cell cultures exposed to compounds which have target organ toxicity in the liver, kidney or brain at sub-cytototoxic concentrations.

5.5 Discussion

In this study we combined for the first time an advanced three dimensional cell culture model of the developing brain with a sensitive mass-spectrometry based metabolomics approach to create a novel, efficient and comprehensive tool for the screening and prediction of neurotoxicity. For the proof of principle, re-aggregating brain cell cultures in an advanced developmental stage were treated with the environmental pollutant methyl mercury chloride, which causes neurotoxic effects in both the developing and mature central nervous system and was proven to be a source of human poisoning (Clarkson, 2002). In contrast, cultures were treated with the most widespread consumed CNS stimulant caffeine, which induces excitation (Schwarzschild et al., 2003). The effects of the different treatments were studied by MS-based metabolic fingerprinting.

The results showed concentration dependent metabolic alterations for methyl mercury chloride but not for caffeine at sub-cytotoxic concentrations. The metabolic alterations by methyl mercury chloride were further analysed to evaluate their identity and relevance for neurotoxicity. Of the 350 masses in the PCA loading plot which were responsible for the observed cluster, a total of 58 masses were selected based on their significance and presence in the lower mass range. Masses in the lower range have a reduced probability of isomers, which facilitates their possible identification. The selected masses were screened by accurate mass measurements and elemental composition calculations. Although some calculations revealed a high number of isomers, and reference compounds for MS-MS analysis were not always commercially available, four masses were identified to be potential biomarkers relevant for the CNS. Moreover, one mass demonstrated the presence of two biomarkers with a similar mass. The quantification of the mass ion intensities of the identified biomarkers GABA, choline, creatine and spermine showed significant decreases or increases at sub-cytotoxic concentrations of methyl mercury chloride. Interestingly, a previous study of methyl mercury chloride toxicity in re-aggregating brain cell cultures based on a battery of conventional biochemical assays (Monnet-Tschudi et al., 1996, Eskes et al., 2002) demonstrated highly

pronounced neuron-specific toxicity by a decrease in the neuro-specific enzyme activities of glutamine synthesis, choline acetyltransferase, and glutamic acid decarboxylase, which could explain the significant decrease in GABA and choline levels. Moreover, these studies reported distinct glia-specific reactions typical for gliosis in aggregates at sub-cytotoxic concentrations. The increase in creatine, which is thought to play a role in both energy homeostasis and osmoregulation of the brain (Bothwell et al., 2001), may reflect gliosis accompanied by the increase in metabolic activity of the glial cells (Bluml et al., 1998). In human studies using magnetic resonance spectroscopy, creatine is considered to be a measure of cellular density, and in patients with adrenoleukodystrophy the increase in creatine was considered to reflect gliosis (Eichler et al., 2002). Furthermore, methyl mercury chloride was found to inhibit creatine kinase activity which could possibly lead to an accumulation of creatine levels (Matsuoka et al., 1992). Interestingly, the increase in creatine could be related to glial cell dependent neuroprotective effects as shown earlier in re-aggregating brain cell culture treated with ammonia (Braissant et al., 2002). The mechanism behind the strong increase of spermine levels at cytotoxic concentrations of methyl mercury chloride remains to be clarified by further studies. Although the identified individual brain metabolites significantly altered at sub-cytotoxic concentrations no concentration response effects were observed in their normalized ion-intensities. This indicates that the observed concentration dependent cluster formations in the PCA score plot were not the result of the single metabolic alterations studied here, but most likely the result of combinations of alterations including other metabolites. This also shows the usefulness of the metabolomics approach, which allows a more comprehensive study of combined alterations induced by a toxicant.

Caffeine treatment did not alter GABA, choline or glutamine levels, however creatine levels were significantly decreased. Although caffeine is not known to reduce creatine it was previously shown to inhibit the metabolism of L-arginine, which is the metabolic substrate for the production of creatine and polyamines (Nikolic et al., 2003). The concentration dependent increase in spermine levels

could be related to the neuroprotective action of spermine towards excitotoxicity. Previous studies have shown that spermine blocks caffeine-induced increases in unidirectional calcium efflux (Chini et al., 1995) and NMDA-mediated excitotoxicity by blocking NMDA receptors and voltage-activated calcium channels in hippocampal slices (Ferchmin et al., 2000). Further mechanistic studies of creatine and spermine are necessary to understand their role in methyl mercury chloride and caffeine induced effects and their potential neuroprotective properties.

To evaluate whether the metabolomics approach could identify the neurotoxic potential of compounds, we tested eight compounds with different target organ toxicity (Table 2). The neurotoxicants trimethyltin chloride and methyl mercury chloride were shown to induce neurotoxic effects and gliosis in re-aggregating brain cell cultures (Monnet-Tschudi et al., 1995; Monnet-Tschudi et al., 1996). Colchicine induces structural neuronal damage by the disruption of microtubules (Kristensen et al., 2003) and the herbicide paraquat induces oxidative stress in neurons (McCormack et al., 2005). In contrast the other selected compounds target mainly the liver and kidney. The hepatotoxicant dimethylformamide affects the hepatic mitochondria membrane potential in mice (Whitby et al., 1984), and the protein synthesis inhibitor cycloheximide induces apoptosis in rat liver (Kumagai et al., 2006). The drug acetaminophen causes hepatotoxicity by oxidative stress and peroxynitrite formation in the liver (Jaeschke and Bajt, 2006). The mechanism of toxicity of the selected hepatotoxicant dichlorophenoxy acetic acid is not well understood (Bharadwaj et al., 2005).

The results indicate that neurotoxic compounds induce more significant metabolic variation in the metabolic profiles of re-aggregating brain cell cultures compared to compounds with target organ toxicity for liver or kidney. One hypothesis for this effect could be the presence of specific targets for neurotoxic mechanisms of compounds. To further address this hypothesis a large blinded screening study including compounds with various target organ toxicities and mechanisms should be undertaken. This could lead to the establishment of a database with metabolic fingerprints for various target organ toxicities including neurotoxicity. The

comparison of metabolic profiles of unknown compounds with the profiles in the database could provide a prediction of their target organ toxicity. The obtained results in this preliminary study give a first indication that a neurotoxicity prediction model could be developed. Another application of interest would be the more defined study of developmental neurotoxicity by the metabolic fingerprinting of treated re-aggregating brain cell cultures during both their early and advanced maturation period.

The multi-parameter unbiased approach of metabolomics makes it a good tool for providing information on the overall metabolic changes in a biological system. A possible problem is that small singular metabolic perturbations with low variation but of biological importance could be diminished if care is not taken. We demonstrated the potential of combining a complex *in vitro* model with the metabolomics approach, to create a novel screening tool which can provide a relevant basis for more mechanistic studies on neurotoxicity.

In conclusion, this study shows for the first time the promising benefits and utilities of *in vitro* metabolomics to comprehensively detect neurotoxicity and to discover new biomarkers.

5.6 Acknowledgements

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6 Summarizing discussion

There is an urgent need for efficient and cost effective toxicological assessment methods that provide mechanistic insight, crucial for a modern toxicology aiming to reveal the mechanisms by which chemicals exert their adverse effects on cells, organs and organisms. In particular, two recent EU legislations have increased the testing needs and the time pressure for the development of more effective methods for chemical safety assessments. The new chemicals legislation REACH (European Commission, 2006), and the 7th Amendment of the Cosmetics Directive of 2003 (76/768/ECC), call for the increased use of *in vitro* methods for ethical but also scientific reasons. REACH will require the testing of more than 30.000 existing chemicals and encourages, whenever possible, the use of *in vitro* methods as an alternative to animal experiments for the establishment of hazards. The 7th Amendment of the Cosmetics Directive requires the phasing out of animal testing for cosmetics ingredients by 2009. Although testing for neurotoxicity is not a primary endpoint in both legislations, certain effects may trigger neurotoxicity testing and therefore the area of neurotoxicity is affected by this pressure.

The assessment of neurotoxicity currently relies on animal based behavioral and neuropathological studies (OECD, 2003). Although well accepted they are not suitable for systematic testing due to their time consumption, costs and provision of limited mechanistic information (Worth and Balls, 2002; Coecke et al., 2005; Harry and Tiffany-Castiglioni, 2005).

A way to meet the requirements while maintaining the desired robustness of safety testing might be the replacement of current tests by testing strategies comprised of several *in vitro* tests or combinations of *in vivo* and *in vitro* tests (Veronesi, 1992; Atterwill et al., 1994; Williams et al., 1994; Abdulla et al., 1995; Costa, 1998; Balls and Walum, 1999; Coecke et al., 2005). However, consensus has not been reached on the design of such strategies due to the complexity of the nervous system, and the restrictions on strategy design to maintain feasibility. As a first step it is necessary to identify individual building blocks of such a

testing strategy, i.e. tests that are most suitable to address one specific aspect or mechanisms of neurotoxicity.

6.1 The design of testing strategies

In most cases a stand alone test cannot adequately address the different aspects of safety, costs and animal use. The best approach believed to achieve these aims is to combine the data from in *silico, in vitro* and *in vivo* models as strategic components in an intelligent way. The different components are systematically designed and combined based on scientific input from toxicologists as well on financial and political issues play a role. The design of such strategies is not an easy task as the combinations of tests gets very complex.

The simplest example of only two tests (A and B) believing either in the positive or negative result of the first test and applying first test A or test B can give already eight different results and a decision has to be made which one to choose (Fig. 25).

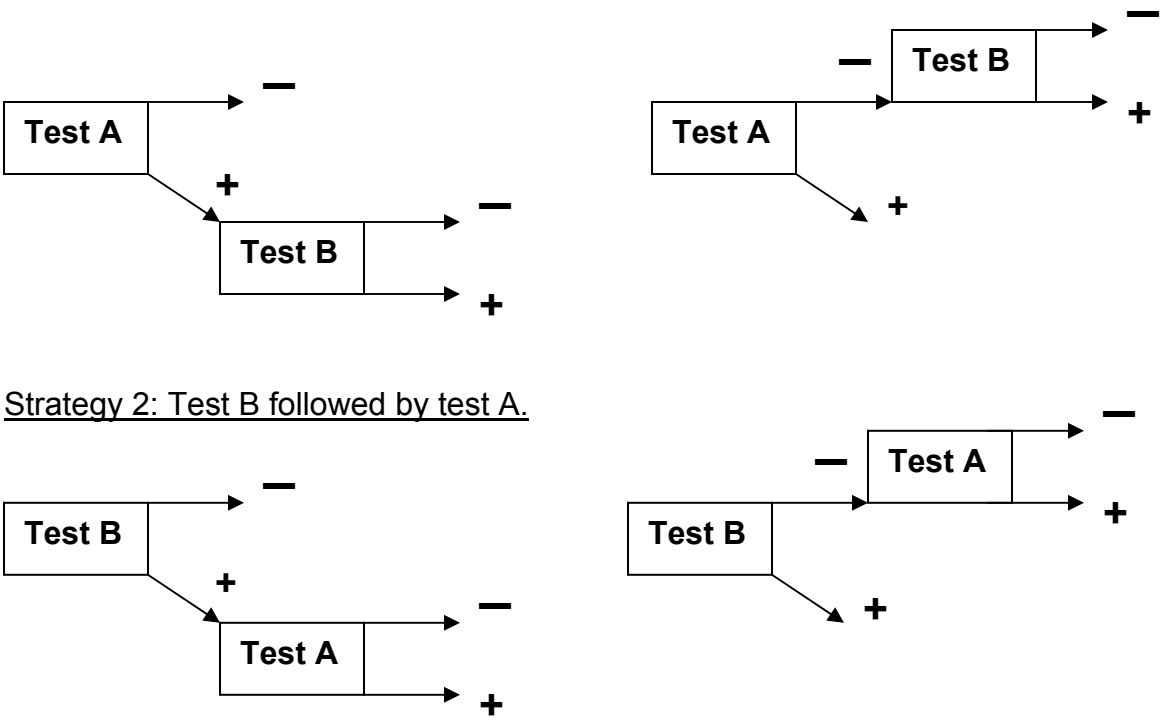

Fig. 25. The designs of testing strategies using two tests (adapted from Hoffmann and Hartung, 2005).

This demonstrates that the number of tests that can be incorporated in a testing strategy is restricted in order to maintain its feasibility. Therefore, to assess a wide range of toxic mechanisms in a feasible way, it is best to include complementary testing methods that give efficiently comprehensive mechanistic information.

In the case of neurotoxicity, scientists generally proposed the use of combinations of general and neurospecific endpoints in neural and non-neural *in vitro* models to distinguish the specific neurotoxicants from general toxicants (Veronesi, 1992; Atterwill et al., 1994; Williams et al., 1994; Abdulla et al., 1995; Costa, 1999). But first feasibility studies that addressed this hypothesis revealed that such approaches are rather limited to discriminate between neurotoxicants and general cytotoxicants (Xie and Harvey; Williams et al., 1996; Weis and Sawyer, 1993; Gartlon, 2006). Therefore, the use of neurospecific endpoints in complex neural models seems more promising to discriminate neurotocicity form general cytotoxicity and to distinguish different mechanism of neurotoxicity (Harry and Tiffany-Castiglioni., 2005; Coecke et al., 2005; Tiffany-Castiglioni, 2006). However, due to the complexity of neurotoxic mechanisms it is difficult to keep the number of mechanistic endpoints low a requirement to maintain testing strategy feasibility.

This study aimed to support the design of *in vitro* testing strategies for neurotoxicity by the development of novel *in vitro* approaches providing relevant mechanistic neurotoxicity data in an efficient and comprehensiveness manner. Moreover, the developed approaches are complementary to each other thereby covering a broad range of possible neurotoxic mechanisms.

6.2 A genetically modified cell system to detect p53 mediated toxicity

A PC12 cell line was used which is known to respond to nerve growth factor by extending long, branching neuron like processes and is commonly used in neurobiological research (Greene and Tischler, 1976). The cell line was found to be a useful model for studying the mechanisms of apoptosis, its prevention or induction (Wang, et al., 2005; Raza, et al., 2006; Zhao, et al., 2002). The p53 tumor suppressor gene known to induce apoptosis in reaction to cellular stress or damage (May and May, 1999; Striteska et al., 2005) was introduced into the PC12 cell line to make the cells more sensitive towards apoptotic cell death. The gene was put under the control of a tetracycline responsive expression vector to provide an easily inducible “genetic switch” that tightly regulates gene expression in a reversible and quantitative way (Gossen et al., 1992).

The p53 engineered PC12 cell line was previously created (Stingele et al., 1999) and used to study the hypothesis that neuron-like differentiated cells display greater sensitivity towards neurotoxicants compared to undifferentiated cells since they comprise specific targets for their toxic action (Gartlon et al., 2006). The results of this study did not support this hypothesis since a wide range of well established neurotoxicants was not consistently more toxic to neuron-like differentiated cells in comparison to undifferentiated cells.

For this reason, the present study aimed to evaluate p53-mediated cytotoxicity in undifferentiated PC12 cells. Results confirmed a controllable expression of the p53 protein which was proven to be in functional state by treatment with camptothecin known to induce p53-mediated apoptosis (Lesuisse and Martin, 2002; Nakajima et al., 2002).

To evaluate the usefulness of the mechanistically-based PC12 cell system to detect the p53-mediated cytotoxicity, a number of metal compounds were screened. Although many other classes of compounds can interact with the p53 gene, metal compounds may be associated with numerous health risks especially neurotoxicity, genotoxicity and carcinogenicity (Florea et al., 2006). Cells were treated with metal compounds at a standard concentration of these compounds which represented a realistic acute *in vivo* exposure when taking into

account *in vitro* pharmacokinetics. Results showed that the well known carcinogens arsenic, cadmium and chromium induced p53-mediated cytotoxicity by an increase in sensitivity of p53 expressing cells towards the apoptotic response compared to the non-expressing cells. Additionally, a more in depth study of the neurotoxicant methyl mercury chloride showed p53-independent cytotoxicity. Thus, this study showed that the developed p53 transfected PC12 cell model system appears to be a useful and sensitive system for the screening of p53-mediated cytotoxicity. The *in vitro* system could be implemented in a neurotoxicity testing strategy to sensitively determine the involvement of p53 in the toxic mechanisms of chemicals.

6.3 Re-aggregating brain cell cultures

One of the promising *in vitro* models proposed to be implemented into a testing strategy is three-dimensional rat re-aggregating brain cell cultures (Honegger et al., 1979). This primary cell culture model closely resembles the brains complexity by comprising a population of neurons, astrocytes and glial cells in a three dimensional arrangement allowing close *in vivo*-like processes to occur such as close cell-to-cell interactions, myelination and synaptogenesis (Eskes et al., 2003). Though, due to the dissociation step in the culture preparation the *in vivo* cytoarchitecture is not maintained. The major advantages for neurotoxicity testing are the models robustness, use for both acute and chronic studies, and data reproducibly showing neurotoxicity at similar concentrations to those observed *in vivo* (Honegger and Schilter, 1992; Kucera et al., 1993; Zurich et al., 2000). Moreover, this study demonstrated that despite its complexicity the culture has a good between- and within-laboratory transferability. In contrast, the model represents as well some restrictions which firstly are the need to work with primary cells making it a reduction model and not full replacement of *in vivo* tests. Though, drawbacks in the availability and distribution of the primary cultures can be overcome by the use of cryopreservation (Purcell et al., 2003). Secondly, due to the cultures tissue-like organization the application of certain standardized conventional endpoints is constrained. In order to overcome this disadvantage

we developed novel endpoints based on promising methodologies that allow such tissue-like organization.

6.4 The promise of human stem cells

In the long-term, embryonic and adult human stem cells are likely to be promising *in vitro* models for a broad range of purposes including toxicology. The major advantage of applying such systems in toxicology is that they avoid the difficulty of extrapolation between species (Davila et al., 2004). Moreover, embryonic stem cells are capable of renewing themselves and when differentiated can give rise to more specialized cells of the human body such as heart (Mummery, 2007), liver (Ong et al., 2006), and nerve cells (Buzanska et al., 2002; Lee, 2007). In the field of neurotoxicology, still little is reported on the use of human embryonic stem cells. First reportings describe the use of human stem cells in combination with proteomics for neurotoxicity screening (Klemm and Schrattenholz, 2004). The use of cord-blood-derived stem cells to study neurotoxicity (Buzanska et al., 2005), and the use of stem cell derived dopaminergic neurons to study the neurotoxic action of 1-methyl-4-phenylpyridinium which induces Parkinson's disease-like symptoms *in vivo* (Zeng et al., 2006). It is to be expected that the use of human stem cells for toxicological purposes will increase. It would be of great interest to combine the developed methods in this study with human stem cells. Still, the use of stem cells for safety assessment of chemicals represents some obstacles such as the availability of cells and ethical considerations.

6.5 Electrophysiological recordings of neuronal activity

One of the promising methodologies for neurotoxicity testing is the electrophysiological recording of neuronal activity in complex cell culture systems (Noraberg, 2004; Sundstrum et al., 2005). It has been shown for example that an electrophysiological endpoint detects functional impairment before any morphological changes occur (Melani et al., 2005). The existing technologies like multi-electrode arrays (MEA) provide a simpler and less time-consuming

alternative to conventional techniques (Pancrazio et al., 2003; Kohling et al., 2005). The most common *in vitro* models for electrophysiological recordings are dissociated primary cultures grown directly on the MEA which are rather limited in their *in vivo* complexity and slices of hippocampus which have *in vivo* complexity but are limited regarding testing throughput and still require a considerable animal use. In this study we explored the use of electrophysiological recordings in re-aggregating brain cell cultures on MEA representing a good compromise of testing capacity and *in vivo* like complexity. The preparation and culture conditions of this organotypic model are already well standardized and found to be reproducible (Honegger, 1979). Moreover, the model complies well with the 3R principles since, one single preparation is sufficient to obtain thousands of recordable cultures. For easy handling and transfer on the MEA pre-differentiated aggregates were grown on hydrophilic membranes. Aggregates were shown to grow well on the membranes and comprise a dense network of neurons and astrocytes. However, unlike slice cultures the brain's general cytoarchitecture is not maintained due to the dissociation step in the culture preparation. This non-fixed geometry of the neuronal populations does not allow neuronal type-specific recordings but the more simplified recording of various neuronal populations. Electrophysiological recordings in aggregates showed stable evoked field potentials and spontaneous firing activity depending on the major glutamatergic and GABAergic synaptic transmission systems. Moreover, paired electrical stimulation revealed paired pulse inhibition which is an indicator for the presence of synaptic plasticity occurring *in vivo*. These findings suggest that despite the lack of cytoarchitecture the general synaptic connections and interactions are re-established and are functionally similar to those in other complex and accepted organotypic models like e.g. hippocampus slices.

Therefore, the recording of electrophysiological parameters such as field potential amplitudes and spontaneous activity frequency provides a general indication of neuronal function and could be used as an endpoint for neurotoxicity. One major advantage of neural activity recordings for neurotoxicity

assessment is its sensitivity to detect both reversible and irreversible effects. It is known that certain compounds e.g. barbiturates and benzodiazepines can affect neural function reversibly due to their pharmacological action without inducing permanent damage. It is important to note that such effects would not be detected using e.g. morphological endpoints.

However, our test method can not distinguish between irreversible pharmacological mechanisms e.g. the block of receptors and interactions with neurotransmitters, or neurotoxicity by e.g. a structural damage. Therefore, the suggested purpose of this model is to detect any adverse effects to neural function induced by chemical compounds. Exact mechanisms of toxicity may be studied later by multiple, more time consuming and expensive techniques. It is important to stress that in the case of toxicity screening high sensitivity is desirable.

To evaluate if the developed *in vitro* test system detects acute effects on neuronal functioning, we tested the neurotoxicants trimethyltin chloride (Szucs et al., 1997; Gramowski et al*.,* 2000; Kruger et al., 2005), methyl mercury chloride (Venable et al., 1977; Segal et al., 1988; Leonhardt et al., 1996), and ethanol (McIntosh and Chick, 2004; Xia, 2003; Little, 1991) known to affect neuronal functioning *in vivo* and *in vitro*. Moreover, the well known acetylcholine esterase inhibitor paraoxon the active metabolite of the pesticide parathion was tested (Garcia et al., 2003).

Results demonstrated that evoked field potential amplitudes were irreversibly decreased by trimethyltin chloride and methyl mercury chloride, whereas such decrease was shown to be reversible with ethanol, in agreement with literature based on dissociated primary and slice cultures (Gramowski et al*.,* 2000; Kruger et al., 2005; Xia, 2003). Paraoxon induced elpileptogenic-like effects by an increased in evoked field potential amplitudes as previsously shown in hippocampal slices (Harrison et al., 2004), whereas parathion showed no significant effects. Recordings of spontaneous activity frequency showed similar results as demonstrated with trimethyltin and ethanol. The treatments affected neuronal activities without inducing cytotoxicity, demonstrating that neurotoxicity

was detected before general toxicity occurred. Moreover, the results indicate that *in vitro* electrophysiological recordings can detect an effect on neuronal function at similar concentrations where neurobehavioral effects are observed using *in vivo* studies. Thus, the developed test system was shown to present relevant electrophysiological activity and to detect early effects of neurotoxicants on neuronal function. The *in vivo*-like complexity and testing throughput of the system make it a valuable tool to be integrated into a neurotoxicity testing strategy.

6.6 The use of omics

To assess the complex processes of neurotoxicity, *in vitro* toxicologists propose the implementation of various biochemical endpoints. Such single parameter tests address the synthesis of a specific neurotransmitter or activity of a neural enzyme (Costa, 1998; Balls and Walum, 1999). Though, considering the high number of endpoints needed to address all of these neural parameters it will be impossible to maintain the feasibility and efficiency of a testing strategy.

To overcome this difficulty, this study aimed to develop a multi-parametric approach by making use of the latest technological advances in the biochemical sciences. Over, the last years, a new area of science so called the “omics” dealing with the study of complex biological systems has emerged. Metabonomics is the systematic study of the unique biochemical fingerprints that specific cellular processes leave behind, specifically the study of their small-molecule metabolite profiles. While gene expression and proteomics address the phenotypic adaptation of a cell, metabolic profiling can give information on the alterations in cell physiology.

Until now, metabonomic approaches have been primarily based on the study of *in vivo* metabolic profiles and little is reported on the application of metabonomics for the evaluation and interpretation of *in vitro* toxicology based on cell or tissue culture models. Thus, for the first time we combined a complex *in vitro* model with sensitive mass-spectrometry-based metabolomics to create a new efficient and

comprehensive approach to be implemented in a testing strategy for neurotoxicity.

For the proof of principle, cultures were treated with the neurotoxicant methyl mercury chloride and the brain stimulant caffeine. Cell lysate samples were prepared and their cellular metabolic profiles were obtained and analyzed by principal component analyses (PCA) a statistical tool showing the metabolic deviation in data sets and revealing significant changes by creating cluster formations in a two or three dimentional space of similar data points. The results showed PCA plots with concentration-dependent cluster formations at non-cytotoxic concentrations of methyl mercury chloride and caffeine. Moreover, the significant metabolic perturbations revealed were considered as putative biomarkers for their neurotoxic effects. Using MS fragmentation analyses the most prominent putative biomarkers were identified to be γ-aminobutyric acid, choline, glutamine, creatine and spermine. The quantification of their spectral mass ion intensities demonstrated concentration dependent alterations which were in agreement with previous studies and hypothesized mechanisms of neurotoxicity and neuroprotection by creatine and spermine. In addition, we evaluated whether the approach could identify neurotoxicity by testing eight compounds at non-cytototoxic concentrations having target organ toxicity for liver, kidney or brain. The results showed a PCA plot with cluster formations largely dependent on target organ toxicity indicating the potential of the approach to identify the neurotoxicity of compounds. Overall, results propose the performance of a validation study testing a large number of blinded compounds in a number of independent laboratories to determine its relevance and applicability for neurotoxicity screening. The multi-parameter unbiased approach of metabolomics makes it a good tool to provide information on the overall metabolic changes in a biological system due to a neurotoxic insult. However, the consequence of looking at the whole metabolome could be that small singular metabolic perturbations are not weighted as strong as by conventional single parameter approaches. Therefore, once validated the developed approach could be best implemented in a testing strategy as a comprehensive tool for the more

refined study of neurotoxicity. Thus, the performed study showed for the first time the benefits and utility of *in vitro* metabolomics to detect comprehensively neurotoxicity and its ability to discover new biomarkers.

This study was carried out to support the emerging development of testing strategies for neurotoxicity. Its major strength was combining advanced cell culture methods and promising endpoint methodologies to create enhanced *in vitro* approaches for neurotoxicity testing. The biological complexity, comprehensiveness, and efficiency of the developed approaches make them suitable for future integration into testing strategies. Moreover, since approaches are complementary to each other, they can cover a broad range of possible neurotoxic mechanisms. The continuation of this work would be the optimization and validation of the approaches to evaluate their applicability for regulatory testing. Thus, this study shows future prospective for the creation of testing strategies aiming to close the gap for information on the neurotoxic potential of compounds.

7 Summary

There is a gap in knowledge when it comes to the effects of chemicals on human health. In particular, the adverse effects on the developing and mature brain are of concern. Presently, neurotoxicity assessment of chemicals relies on behavioral and neuropathological studies. Although well accepted they are not suitable for systematic testing due to their time consumption, costs and provision of limited mechanistic information. To overcome such limitations, it is generally recommended to develop and make use of suitable tiered testing strategies including *in vitro* systems. However, consensus has not been reached on the design of such strategies due to the complexity of the nervous system, and the restrictions on endpoints to maintain feasibility.

The present study aimed at developing and assessing the suitability of novel and promising *in vitro* approaches and technologies to integrate *in vitro* testing strategies for neurotoxicity testing. Overall, three *in vitro* systems were studied which evaluated the effects of chemicals on mechanisms of cell death, neuronal function, and biochemical cellular processes in an efficient, comprehensive, and mechanistic manner.

First, a mechanistically-based *in vitro* test system was developed to detect p53-mediated cytotoxicity by introducing a controllable human wild type p53 gene into a pheochromocytoma 12 (PC12) cell line.

- Western blot analyses confirmed a controllable expression of the wt p53 protein.
- Cells over-expressing the p53 protein demonstrated a greater sensitivity towards the topoisomerase I inhibitor camptothecin, known to induce p53 mediated apoptosis, when compared to non-expressing cells. Thereby, confirming the functional state of the p53 protein.
- A screening test of 31 metal compounds showed that p53-mediated cytotoxicity was induced by arsenic, cadmium, chromium, and manganese, compounds well known for their carcinogenicity potential to humans.

As a consequence, the developed PC12 cell line demonstrated to be a useful test system to screen for p53-mediated cytotoxicity.

Secondly, a novel test system was developed to detect early effects of chemicals on neuronal function. The test system was based on the electrophysiological recording of neural activity in re-aggregating brain cell cultures using multi-electrode arrays (MEA). This complex primary cell culture is known to closely resemble the brain's complexity due to its tissue-like organization, be well standardized, and produce neurotoxicity data correlating well with effects observed *in vivo*.

- Re-aggregating brain cell cultures were shown to display both evoked field potentials and spontaneous neuronal firing involving glutamatergic and GABAergic synaptic transmission as shown by their respective synaptic agonists and antagonists.
- Treatments with trimethyltin chloride, methyl mercury chloride, ethanol and paraoxon affected neuronal activities without inducing cytotoxicity, demonstrating that neurotoxicity was detected before general toxicity occurred. Moreover, results were in agreement with data obtained in primary dissociated or hippocampal cultures.
- Since a single isolation yields about 4000 recordable cultures the method achieves both *in vivo*-like complexity and high testing throughput.

Thus, the developed test systems displayed relevant electrophysiological activities that allowed the sensitive detection of early effects on neuronal function by neurotoxicants. When incorporated into testing strategies, they could represent a valuable tool for the mechanistic assessment of neurotoxicity.

Thirdly, to evaluate whether metabolic finger-printing of treated cells could be used to detect neurotoxicity, by combining re-aggregating brain cell cultures with a mass-spectrometry-based metabolomics approach. Such approach is recognized to provide a comprehensive insight into complex biological systems

and therefore might comprise valuable biochemical endpoints for neurotoxicity assessment.

- Treatment with the neurotoxicant methyl mercury chloride and the brain stimulant caffeine induced concentration-dependent metabolicperturbations starting at non-cytotoxic concentrations.
- The most prominent perturbations induced by methyl mercury chloride and caffeine were identified to be γ-aminobutyric acid, choline, glutamine, creatine, spermine and are considered as putative biomarkers for their effects.
- Biomarker intensities in the metabolic profiles demonstrated concentration-dependent alterations which were in agreement with hypothesized mechanisms of neurotoxicity and neuroprotection.
- The evaluation of 8 compounds with target organ toxicity for liver, kidney and brain largely showed target organ-dependent metabolic perturbations indicating the potential of the approach to identify neurotoxic compounds.

Overall, the use of *in vitro* metabolomics showed to have great potential to detect comprehensively neurotoxicity and to identify new biomarkers involved in such effects.

In conclusion, this study showed the usefulness of combining advanced cell culture methods and novel methodologies to create relevant *in vitro* approaches for neurotoxicity testing. Their biological complexity, comprehensiveness, and efficiency make them mechanistically relevant for integration into testing strategies. Moreover, since approaches are complementary to each other they cover a broad range of possible neurotoxic mechanisms. The continuation of this work would be the optimization and validation of the approaches to evaluate their applicability for regulatory testing. Thus, this study shows future prospective of *in vitro* methods to be integrated into new testing strategies, to cover some of the currently existing gaps of traditional neurotoxicity testing, and to gain mechanistic information on the neurotoxic potential of compounds.

8 Zusammenfassung

Die Auswirkung von Chemikalien auf die menschliche Gesundheit ist immer noch eine Grauzone. Bedenklich sind im Besonderen die nachteiligen Effekte auf das sich entwickelnde und das voll ausgereifte Gehirn. Derzeit basieren neurotoxikologische Einstufungen von Chemikalien auf Verhaltens- und Neuropathologischen Untersuchungen. Obwohl diese Studien wissenschaftlich anerkannt sind, sind sie für eine systematische Überprüfung von Chemikalien nicht geeigent, da sie zeit- und kostenaufwendig sind und nur begrenzt mechanistische Information bieten. Um diese Einschränkungen zu überwinden, ist es notwendig, geeignete, stufenweise aufgebaute Teststrategien, die auf *in vitro*-Systemen basieren, zu entwickeln und einzusetzen. Auf Grund der Komplexität des Nervensystems und der für die Durchführbarkeit notwendigen Beschränkung auf ausgewählte Endpunkte, konnte bisher jedoch noch keine Einigkeit über das Design solcher Strategien erzielt werden.

Die hier vorliegende Studie hatte zum Ziel, neue und vielversprechende *in vitro* - Ansätze und Technologien zu entwickeln, zu bewerten und zu optimieren, um *in vitro*-Tests für die neurotoxikologische Untersuchung von Chemikalien zu vereinen. Insgesamt wurden drei *in vitro*-Systeme evaluiert und die Auswirkungen von Chemikalien auf die Mechanismen des Zelltodes (Apoptose), die spezifischen neuronalen Funktionen und die biochemischen, zellulären Prozesse wurden in umfangreicher und mechanistischer Weise bewertet.

Durch die Einführung eines kontrollierbaren, humanen Wildtyp (wt) p53-Gen in eine Pheochromocytom 12 (PC 12)-Zelllinie wurde ein mechanistisches *in vitro*-estsystem entwickelt, um die p53 vermittelte Zelltoxizität zu erfassen.

- Westernblot-Analysen bestätigten eine kontrollierbare Expression des wt p53-Proteins.
- Zellen, die das p53-Protein überexprimieren, zeigten eine grössere Sensitivität gegenüber des Topoisomerase-I-Inhibitors Camptothecin, welcher bekanntlich p53 vermittelte Apoptose induziert, im Vergleich zu

nicht überexprimierenden Zellen. Dadurch konnte der funktionelle Status des p53-Proteins bestätigt werden.

- Eine Überprüfung von 31 metallischen Verbindungen zeigte, dass p53 vermittelte Zelltoxizität durch Arsen, Kadmium, Chrom und Mangan verursacht wurde – Verbindungen die für ihr krebserregendes Potenzial im Menschen bekannt sind.

Es konnte gezeigt werden, dass die entwickelte PC12-Zelllinie ein geeignetes Testsystem ist, um p53 vermittelte Zelltoxizität festzustellen.

Zweitens wurde ein neues Testsystem entwickelt, um frühe Effekte von Chemikalien auf spezifische, neuronale Funktionen nachzuweisen. Das Testsystem basiert auf elektrophysiologischen Messungen der neuronalen Aktivität in „sich wieder zusammenlagernden Gehirn-Zellkulturen“ (brain-aggregates) auf Multielektroden-Arrays (MEA). Diese komplexe Primärzellkultur ist dafür bekannt, dass sie der Komplexizität des Gehirns besonders ähnlich ist. Wenn das System gut standardisiert ist, ist es in der Lage, neurotoxikologische Daten zu erheben, die in Übereinstimmung mit *in vivo* gefundenen Auswirkungen sind.

- Es konnte gezeigt werden, dass brain-aggregates in der Lage sind, induzierte Aktionspotentiale zu bilden und spontan neuronal zu feuern, was glutaminerge und GABAerge synaptische Übertragung erfordert. Dies konnte durch den Einsatz ihrer respektiven synaptischen Agonisten und Antagonisten bestätigt werden.
- Behandlungen mit Trimethylzinnchlorid und Methylquecksilberchlorid, Ethanol und Paraoxon beeinträchtigten die neuronale Aktivität ohne Zelltoxizität hervorzurufen, was darauf schliessen lässt, dass Neurotoxizität vor der generellen Toxizität auftritt. Desweiteren waren die Ergebnisse der Studie in Übereinstimmung mit Daten, die in primären, dissoziierten oder Hippocampus-Kulturen erhoben wurden.

- Da eine einzige Isolation über 4000 funktionelle Kulturen hervorbringt, enthält diese Methode beide Vorteile der *in vivo*-ähnlichen Komplexität und einer hohen Durchlaufkapazität.

Das entwickelte Testsystem zeigte relevante elektrophysiologische Aktivitäten, die eine sensitive Erfassung von frühen Effekten auf die neuronale Funktion durch nervenspezifische Giftstoffe möglich machten. Integriert in Teststrategien könnte es ein wertvolles Werkzeug sein, um eine mechanistische Neurotoxizität zu detektieren.

Drittens untersuchten wir, ob der metabolische Fingerabdruck (fingerprint) der behandelten Zellen benutzt werden könnte, um Neurotoxizität zu bestimmen. Die Kombination von brain-aggregates und einem Metabolomansatz, basierend auf Massenspektrometrie, ist dazu geeignet, eine reichhaltige Einsicht in komplexe biologische Systeme zu gewähren und könnte deshalb wertvolle biochemische Endpunkte für eine neurotoxikologische Einstufung liefern.

- Behandlung mit dem Nervengift Methylquecksilber Chlorid und dem Gehirnstimulans Caffein rief konzentrationsabhängig metabolische Veränderungen hervor, schon beginnend in noch nicht zelltoxischen Konzentrationen.
- Die bemerkenswertesten Veränderungen, hervorgerufen durch Methylquecksilberchlorid und Koffein wurden als γ-Aminobuttersäure, Cholin, Glutamin, Kreatin, Spermin identifiziert und somit als mögliche Biomarker für diese Auswirkungen erkannt.
- Biomarkerintensitäten im Stoffwechselprofil zeigten konzentrations-abhängige Veränderungen, die in Übereinstimmung mit dem hypothetischen Mechanismus der Neurotoxizität und der Neuroprotektion sind.

- Die Untersuchung von acht Chemikalien mit Toxizität für Leber, Nieren und Gehirn zeigten Zielorgan-abhängige metabolische Störungen, darauf hindeutend, dass das Potenzial dieser Methode darin liegt, neurotoxikologische Substanzen zu identifizieren.

Zusammen gesehen zeigte der Gebrauch von Metabolomuntersuchungen ein grosses Potenzial, um Neurotoxizität festzustellen und neue Biomarker zu entdecken.

Zusammenfassend demonstriert diese Studie die Nützlichkeit der Kombination von hochentwickelten Zellkulturmethoden und neuen Technologien, um relevante *in vitro*-Ansätze zur Feststellung von Neurotoxizität zu kreieren. Ihre biologische Komplexizität, ihr Umfang und ihre Effizienz machen sie mechanistisch relevant, um in Teststrategien integriert zu werden. Da die Ansätze komplementär zueinander sind, decken sie einen breiten Bereich von möglichen neurotoxischen Mechanismen ab. Die Weiterführung dieser Arbeit wäre die Optimierung und Validierung dieser Methoden, um ihre Anwendbarkeit für regulatorisches Testen zu zeigen. Folglich zeigt diese Studie die Zukunftsaussichten von *in vitro* Methoden, die in neue Teststrategien eingebaut werden können, um existierende Wissenslücken zu schliessen.

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