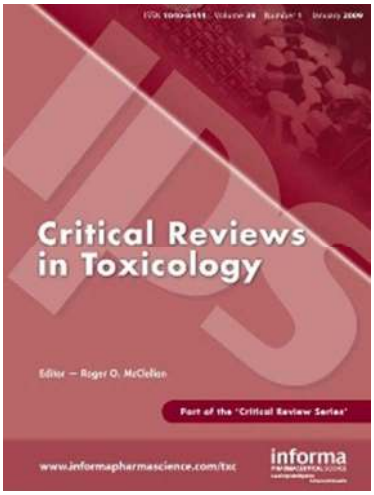

Workshop Report: Putative Adverse Outcome Pathways (AOP) Relevant to Neurotoxicity

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Workshop Report: Putative Adverse Outcome Pathways (AOP) Relevant to Neurotoxicity

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Abstract

The Adverse Outcome Pathway (AOP) framework provides a template that facilitates understanding of complex biological systems and the pathways of toxicity that result in adverse outcomes (AOs). The AOP starts with an molecular initiating event (MIE) in which a chemical interacts with a biological target(s), followed by a sequential series of KEs, which are cellular, anatomical and/or functional changes in biological processes, that ultimately result in an AO manifest in individual organisms and populations. It has been developed as a tool for a knowledge-based safety assessment that relies on understanding mechanisms of toxicity, rather than simply observing its adverse outcome.

A large number of cellular and molecular processes are known to be crucial to proper development and function of the central (CNS) and peripheral nervous systems (PNS). However, there are relatively few examples of well-documented pathways that include causally linked MIEs and KEs that result in adverse outcomes in the CNS or PNS. As a first step in applying the AOP framework to adverse health outcomes associated with exposure to exogenous neurotoxic substances, the EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) organized a workshop (March 2013, Ispra, Italy) to identify potential AOPs relevant to neurotoxic and developmental neurotoxic outcomes. Although the AOPs outlined during the workshop are not fully described, they could serve as a basis for further, more detailed AOP development by further identification of data gaps and application of modified Bradford-Hill criteria (Meek et al., 2014) to be useful for human health risk assessment.

Key words: *pathways of neurotoxicity, molecular initiating event, key events, adverse outcome pathway, in vitro testing, predictive toxicology*

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Introduction to AOP concept

Regulatory toxicology is undergoing a transformation from decision-making relying solely on apical animal testing to biological pathway-based approaches that include cellular, tissue and organismal dynamics. A key driver for this change is a collective desire of all stakeholders (academia, industry, and regulators) to better utilize the latest scientific thinking and tools within the safety assessment process (Vinken 2013). Success will improve protection of human health and the environment, considerably reduce animal testing, significantly increase the rate of data collection and provide opportunities for industrial innovation and competitiveness. Central to this transformation is a shift towards knowledge-based weigh-of-evidence paradigm for hazard assessment. This transformative process must include development of cost efficient and less time consuming toxicity testing methods that predict the impact of chemicals on human and ecological health (Collins et al., 2008). The ultimate goal would involve routine toxicity testing conducted in human-cell based test systems (NRC, 2007) to more accurately predict the potential adverse effects of chemicals rather than having to rely on in vivo animal models. Such predictive toxicology is still emerging and will require considerably more research and development before its principles and processes are mature enough to translate into mainstream regulatory practice (Thomas et al., 2013; Patlewicz and Lander, 2013; Adeleve et al., 2014). One area that needs particular attention is how to actually harvest, curate and manage relevant knowledge so that it informs AOP development that serves regulatory needs.

Extant literature contains an extraordinary amount of information on the mechanisms by which chemicals alter cellular signaling pathways mostly generated in the context of toxicological science or in relation to basic biomedical research. However, generation of this data is only one step in the process of use in risk decisions. The distillation of knowledge from such data requires

functional understanding of complex biological systems and underlying system dynamics, as well as xenobiotic-induced perturbations that lead to dysfunction and failure.

The Adverse Outcome Pathway (AOP) framework has been developed as a tool to organize data across multiple levels of biological organization to identify correlative and causal linkages between the events, that when sufficiently perturbed by chemical exposures, result in adverse outcomes. (Ankley et al, 2010). The AOP framework not only provides a means to adapt mechanistic understanding for regulatory decision making, but it also provides a useful tool for consolidating, managing and exchanging knowledge amongst the research community. The concept of the AOP structure encompasses functional systems biology/toxicology with the ultimate goal of predicting systems behaviour. It is important to appreciate, however, that an AOP is not a description of a biological system per se, but is a higher-level depiction of the sequence of toxicological events that lead to dysfunction or failure of the system, given a certain set of circumstances or boundary conditions.

AOPs can vary in resolution and expanse and can include both qualitative and quantitative descriptions of key events (KE) and their interlinking causal relationships (Fig. 1). Initial development and elucidation of an AOP begins with a proposed pathway (Ankely et al., 2010; Watanabe et al., 2011). Recent published guidance and template documents for development and assessment of AOPs (OECD, 2013) suggest whenever possible anchoring the AOP at the molecular initiating event (MIE) and the adverse outcome (AO) at the individual or population level (i.e. the regulatory effect/endpoint of concern). However, AOP development may originate at any step in the pathway, whether nearer to the initial chemical-biological interaction, the MIE, or somewhere in-between initiation and AO. The AOP is then further developed by identifying and describing the intermediate KEs and the causal or correlative relationships between them. An

additional aspect of this process is transitioning from a correlative-based AOP to one based on causative links between the MIE, KE, and AO within the pathway (Meek et al., 2014). This evidence can be drawn from various sources, but typically comprises relevant studies described in the literature, or results from experiments specifically designed and undertaken for the purpose of AOP development. Finally, an AOP can be enhanced with quantitative linkages that allow for more accuracy and surety in predicting outomes from biomakers of upstream key events or MIEs. This progress from correlative to causative and then quantitative is paralleled by concominant decreases in uncertainty in the model predictions and increases confidence for use by regulatory decision makers. In 2012 the Organisation for Economic Cooperation and Development (OECD) launched the AOP Development Program, which is coordinated by the OECD Extended Advisory Group on Molecular Screening and Toxicogenomics (EAG-MST). The OECD guidance document and template for developing and assessing AOPs aims to ensure consistency in approach and compliance with AOP standards related to content, structure and presentation (OECD, 2013). An AOP Knowledge Base (AOP-KB) has also been recently developed which includes an AOP-Wiki, slated for release later in 2014 (http:\\www.aopwiki.org). The AOP-Wiki is a web-accessible collaboration-space for AOP development teams to work together in an efficient and convenient manner for the capture, classification and evaluation of AOPs. It will also serve to crowd-source knowledge on a global scale to refine existing AOPs and trigger the development of new ones where gaps in the AOP landscape are identified. The AOP-KB is envisioned to be the primary hub for the regulatory science community to rapidly and efficiently access AOP knowledge to serve their needs.

As a first step in applying the AOP framework to adverse neurological outcomes caused by exposure to xenobiotics, the EU Reference Laboratory for Alternatives to Animal Testing

(EURL ECVAM) joined forces with the Safety Evaluation Ultimately Replacing Animal Testing (SEURAT-1) consortium to organize a workshop (March 2013, Ispra). The goal of the workshop was to identify potential AOPs relevant to neurotoxicity and developmental neurotoxicity. The outlined AOPs identified during this workshop are by no means comprehensive for neurotoxicity, however, they do provide a starting point to stimulate discussion and to which information can and should be added in the future.

Challenges for neurotoxicity AOP development

Development and use of the AOP framework for neurological outcomes following developmental or adult exposure has been hampered by a number of serious challenges. A major concern for neurotoxicity is a general lack of understanding of the MIEs that are causally responsible for altered KEs triggering AOs. For example, the relationship between developmental lead (Pb^{2+}) exposure and adverse cognitive outcomes in children is well described (Sanders et al., 2009); however, the initial molecular interactions between Pb^{2+} molecules and cellular targets that are causatively linked to adverse cognitive outcomes (e.g. IQ) are still not well understood. The lack of a known pathobiology of many neurodevelopmental disorders hampers AOPs development. For example, where is the locus of IQ loss from developmental lead exposure? The same can be said for a wide number of other well-known developmental and adult neurotoxicants (e.g., methylmercury, alcohol, polychlorinated biphenyls). Additionally, many human neurological disorders may have diverse pathophysiology that underlies similar clinical phenotypes, or conversely, diverse clinical outcomes that result from similar pathophysiology. For example, autism spectrum disorder (ASD) is a neurodevelopmental malady with an increasing incidence and is more prevalent in males (McDonald and Paul, 2010).

However, it is now clear that ASD is an umbrella term for multiple disorders with overlapping clinical symptoms, suggesting that there are shared and unique pathophysiological mechanisms which have yet to be identified.

There are, however, a limited number of neurotoxic outcomes that do have well defined pathophysiological outcomes and MIEs. One example, developed as an AOP below, is acute neuronal sodium channel disruption and consequent behavioural effects, exemplified by p,p'-DDT and pyrethroids (Shafer et al., 2005) (see AOP on *Acute neurotoxic effects of pyrethroids mediated by disruption of voltage-gated sodium channels*). Another example are the well-known peripheral neuropathies induced by a number of chemicals, including organophosphates, carbon disulfide, pyridoxine (Vitamin B6), 2,5-hexandione and acrylamide (LoPachin and DeCaprio, 2005; Rao et al., 2014) (see AOP on *Binding of certain organophosphates to NTE results in delayed neuropathy).*

In these cases the AOs are well-described AOs in the peripheral systems of multiple species correllatively and/or causatively linked to MIEs. For example, 2,5-hexandione forms irreversible covalent bonds (adducts) with proteins (LoPachin and DeCaprio, 2005; Graham et al., 2005).

The lack of known pathophysiology for a specific AO does not make it difficult to propose an AOP or to hypothesize MIEs. However, it does pose challenges in developing the empirical data needed to move from a proposed AOP to a causal or quantitative AOP. This has important consequences for the development and acceptance of more efficient and predictive testing methods for detecting chemicals that may lead to AOs of concern. An AOP that contains good correlative and/or causative links between the MIE or early KEs provides risk managers an increasing level of confidence to make regulatory decisions (Ankley et al., 2010). For example, the known causative relationships between estrogen receptor binding, activation of downstream

cellular ER-based signalling pathways, and adverse impacts on reproductive function facilitates the use of quantitative structure-activity relationships (QSAR) and chemical structure-based read-across models that can be used to make regulatory decisions (Schmeider et al., 2004; 2003). Indeed, this model is already in use as part of the OECD ToolBox (Mombeli, 2012).

Common key events for neurotoxic outcomes

Application of the AOP concept for hazard identification aims for faster, cheaper and more predictive neurotoxicity evaluation by including in vitro human-based systems in the testing strategy (NRC, 2007). Such a proposal was made for assessing the potency of compounds to induce skin sensitization, a complex procedure involving a variety of KE carried out by skin and immune cells. However, in the case of skin sensitization, the MIE triggering the AOP is the same for many skin sensitizers: covalent interaction of electrophilic substances with cellular proteins (MacKay et al., 2013). The functional and structural heterogeneity of the nervous system, coupled with the dynamics of brain development, suggests that a broad array of MIEs may be involved in adverse neurological outcomes. This complexity teamed with the previously mentioned dearth of well-accepted MIEs for developmental or adult neurotoxicity makes development of alternative test methods a serious challenge. One interim solution to this problem is the identification of downstream KEs that are common to multiple MIEs and pathways. On-going efforts to develop DNT screening methods based on common cellular phenotypes is an example of this approach (Coecke et al., 2007; Lein et al., 2007; Bal-Price et al., 2010; Crofton et al., 2011; Bal-Price et al. 2012). Alternatively, cellular signalling molecules common to multiple pathways could be utilized for assay development, which enables chemical testing in a medium- to high-throughput manner, enhancing the effectiveness and robustness of testing. This

approach might seem too reductionist for reflecting the complex issue of neurotoxicity with its diverse MIEs and AOs. However, scientific data is needed to understand the degree to which this type of testing strategy based on common KEs linked to the one or more AOs is able to be predictive.

For example, common KEs were identified that might possibly serve as endpoints for *in vitro* neurotoxicity testing with a high predictivity for hazard potential. These include cytoskeleton alterations (AOP V, VI, VII, VIII), impaired mitochondrial function (AOP II, V), increased oxidative stress (AOP V, VI, VIII, X) and altered neuronal firing rates (AOP I, III, IV, V, VI, VII, IX, X). It is important to note that some of these KEs are common to many cell and tissue types, not just nervous system tissues, (e.g, markers of oxidative stress, mitochondrial function). Data is needed to demonstrate how these non-neuronal specific KEs are linked to nervous system specific adverse outcomes. A classic example is the generation of the toxic cation MPP+ from MPTP by glial cells, the subsequent destruction of neural dopaminergic neurons in a specific brain region, the substantia nigra, that results in Parkinson's like symptoms. While the exact reasons for the specificity of MPTP for these neurons remains controversial, with one explanation that selective uptake of MPP+ by membrane transporters in dopaminergic cells is responsible for the targeting of dopamine neurons (Jenner and Marsden, 1986; Tipton and Singer, 1993).

Predictivity of assay endpoints based on common KEs, as well as variable combinations of them will need to be tested using a set of neurotoxic compounds (Crofton et al., 2011). This approach may lead to a defined test battery for neurotoxicity evaluation.

Within the proposed AOPs developed at the Workshop (see Appendices) two sub-groups can be identified. The AOPs I, II, III, IV, V and VI are strongly related to neurotransmitter receptor

MIEs located in the cell membrane. These targets all have critical functions for neurotransmission. The AOPs VIII, VII, IX and X are more related to events associated with general molecular and cellular support or defence mechanisms. Accordingly, there will be interlinks within these two groups as well, and likely across these two groups. The process of neurotransmission is a fine-tuned, multi-event process of various ion channels and receptors that finally depolarizes the membrane. Neurotoxicant-induced alterations in voltage-gated ion channels (see AOP IV) directly affects the functionality of neuronal N-methyl-D-aspartate (NMDA) receptors (see AOPs I, II). During depolarisation the influx of Ca^{2+} ions is a crucial cellular process for multiple physiological functions, including synaptic plasticity (e.g. AOP II and IX). It is also a common intracellular process that may adversely affect the integrity of neural cells (see AOP V, VI, VII, VIII, IX, X). Therefore, within the description of the AOPs there are commonalities between pathways caused by the complexity of the neurobiological processes related to normal functioning of the human brain.

Further development of the ten AOPs outlined here, as well as development of new AOPs for neurotoxicity require generation of new data as well as mining existing data. In this regard it is of utmost importance that the test systems mimic human physiology as closely as possible (NRC, 2007), i.e. co-culture of neurons and glia cells, expression and sensitivity of receptors, presence/absence of signalling molecules and pathways especially in a spacio-temporal context. Development and use of these models must also account for inter-species differences in brain physiology as responses to the same compounds may differ between human and rodent *in vitro* models (Gassmann et al., 2010; Harrill et al., 2011).

Considering life stage-specific susceptibility in neurotoxicity AOPs

Ideally, AOPs for neurotoxicity should consider specific life stages such as development or aging as significant age-related susceptibilities in response to chemical exposure are well documented (Rice and Barone, 2000; Landrigan et al., 2010). The development of AOPs that are based on life stage-specific KEs in nervous system development and aging, AOP-dependent hazard and risk assessment, should include not just the fetal and infants, but also juveniles and the elderly as major vulnerable subpopulations.

During brain development several processes occur primarily, or exclusively during this time. Key processes such as the commitment and differentiation of neural progenitor cells (NPCs) followed by glial and neuronal cell proliferation, migration, differentiation into specific neuronal and glial subtypes, axonal and dendritic outgrowth, formation and pruning of synapses, myelination, programmed cell death, ontogeny of neurotransmitters and receptors, and development of the blood brain barrier (BBB) are critical for functional brain development (Lein et al., 2005; Bal-Price et al., 2012; Stiles and Jernigan, 2010). Disruption of any of these processes by neurotoxic compounds may modify neuronal/glial cell function leading to adverse alterations in neuroanatomy, neurophysiology, neurochemistry and neurobehavior. Complex and dynamic glial/neuronal processes occur during discrete developmental windows that differ across brain regions, and this spatiotemporal variation influences the variable sensitivity of the developing brain to the same chemical exposure at different developmental stages (Barone et al., 2000; Rice et al., 2000; Lein et al., 2005). Insults that occur early during CNS development have the potential to cause more widespread impacts throughout the brain, while those occurring later in development may only affect a specific structure or structures. For example, methylmercury

has more widespread effects if exposure occurs early in CNS development, relative to exposures that occur later in development, which result in more focused insults in the cortex and cerebellum (Burbacher et al., 1990). Another critical instance with regard to life stage-specificities is given by the developmental switch of neuronal GABAergic responses from excitation to inhibition. This switch is dependent on GABA-induced $GABA_A$ receptor activation (Ganguly et al. 2001). Therefore, interference with GABA receptors during development and after brain maturation (see AOP III) is likely to cause distinct AOs (Westerholz et al., 2010)

Aging of the human brain is also characterised by processes that are unique or predominant at this life stage such as decreased neuronal cell volume, loss in cell numbers, reduced synaptic density/connectivity and declines in cognitive function (Walhovd et al., 2014). In addition, neurons show evidence of DNA damage, elevated reactive oxygen species (ROS) production, Ca^{2+}-signalling disturbances, mitochondrial dysfunction and increased neuroinflammation with increasing age (Bishop et al., 2010). Moreover, declining hippocampal neurogenesis associated with aging of hippocampal neural stem/progenitor cells has been proposed to contribute to aging-related cognitive decline (van Wijngaarden and Franklin, 2013). Identification of NPC dysfunction in the aging hippocampal regenerative niche suggests a parallel between aging and basic processes of neurodevelopment as NPC proliferation and neuronal differentiation, that are necessary for formation and maintenance of brain, function throughout life (see AOP X). Importantly, these molecular biomarkers and biological pathways associated with aging are also implicated in neurodegenerative diseases, suggesting an overlap between biological pathways associated with ageing and neurodegenerative brain disorders.

A representative example of such life stage-dependent neurotoxicity is anaesthetic exposure. There is growing concern that anaesthetics exposure causes learning impairment, memory

deficits and behavioural abnormalities in young subjects, and accelerated cognitive decline in the elderly (Rohan et al. 2005; Johnson et al. 2002; Moller et al. 1998). Although the MIE(s) responsible for the AO is (are) not clear, it is suggested that the extent of neuroapoptosis, neuronal network assembly, neuro- and synaptogenesis determine the qualitative and quantitative aspects of toxicity in the developing brain (reviewed in Jevtovic-Todorovic et al., 2013). In the elderly, clinical evidence for postoperative cognitive decline is present while underlying molecular mechanisms still need to be elucidated (Jevtovic-Todorovic et al., 2013).

An additional example of vulnerable life stages for neurotoxicity is adolescence. While developmental changes in the adolescent brain are more subtle than those in the first 4 years of life (Paus et al., 2001), magnetic resonance imaging (MRI) analyses of humans during juvenile and adolescence periods have clearly demonstrated structural and functional changes in synaptogenesis and connectivity in the human brain throughout adolescence and into adulthood (Giedd, 2004; 2008). Ethanol acts on multiple processes occurring during adolescent development (reviewed in Guerri and Pascual, 2010). It attenuates N-methyl-D-aspartate (NMDA)-mediated synaptic activity to a larger extent in the immature than in the mature hippocampus and thus, more potently inhibits the induction of long-term potentiation (LTP) in immature versus mature animals (Swartzwelder et al., 1995a; b). MIEs, cellular processes and pathways critical for brain development during adolescence may overlap with those important for development and aging of the nervous system.

What is the relevance of life stage-susceptibility for neurotoxicity AOPs development? First, AOP developers need to think in a broader context for incorporating specific aspects of brain development and brain aging into the neurotoxicity mode of action (MoA) portfolio. This means that besides ‘classical’ AOP for neurotoxicity like interference with neurotransmitter receptors or

inhibition of acetylcholinesterase (AOP I-V), other pathways specific for brain development and aging should be considered when developing AOPs for neurotoxicity. As a result, life stage-specific key events will increase the number of AOPs for neurotoxicity. One example for such life stage-specific issues in AOP development is the notable increase in brain oxidative stress and neuroinflammation with age (Perluigi et al., 2013; Hsieh and Yang, 2013). These suggest common key events in mitochondrial (AOP VII), inflammatory (AOP VIII) and epigenetic (AOP X) pathways within AOPs when addressing neurotoxicity in the elderly. Interestingly, two of the draft AOPs (AOP VII and AOP VIII) are interlinked and influence each other, rendering it difficult to separate them in a clear manner (Gemma et al., 2007). Importantly, in vitro models for key events should mimic the relevant stage of neural/glial differentiation and maturation for developing AOPs specific for the developing, adolescent, adult and aging nervous systems. As molecular targets and thus AOPs are numerous at all life stages and likely vary in importance across life stages, the AOPs presented here are just the initial step in a process that will continue to expand and be refined over the upcoming years as the science progresses.

Future directions for development of Neurotoxicity AOPs

With few exceptions, development of AOPs for developmental and adult neurotoxicity is a relatively recent concept. There are many different directions that could be taken as work in this area proceeds. A goal of the Workshop was to outline the directions that might prove most fruitful for the AOP concept to be employed effectively in environmental decision-making by risk assessors and others. The following directions should be considered as high priority:

- Cataloguing the current state of knowledge regarding known or putative AOPs for neurotoxicty
- Identifying AOPs specific for Developmental Neurotoxicology
- Prioritizing AOP development
- Identifying KEs that are amenable to High and Medium throughput screening
- Demonstrating the utility of the AOP approach to risk assessors using case studies

Each of these priorities is discussed briefly below.

Cataloguing the current state of knowledge regarding known or putative AOPs

This workshop report presents examples of 10 draft AOPs related to neurotoxicity and/or developmental neurotoxicity, but it was outside of the scope of the workshop to attempt to identify and catalog all of the known or putative AOPs that are related to neurotoxicity. Indeed, even the AOPs presented here are to be considered first drafts that require additional data, description and detail prior to use. These AOPs range from those that are mostly complete, such as those outlining pyrethroid effects on VGSCs or $GABA_A$ receptor mediated excitotoxicity and convulsions, to those that need substantial work to better establish linkages between proposed MIEs and KEs, such as the AOP for neuroinflammation leading to neurodegeneration. As mentioned previously, the AOP-Wiki will facilitate international collaboration in developing a knowledge base related to AOPs. As an important first step in utilizing AOPs for neurotoxicity and developmental neurotoxicity in risk decisions, it will be important for researchers to populate the AOP-Wiki with examples such as those developed at this Workshop. This will help to identify the most complete AOPs, and allow identification and prioritization of data gaps that require additional data collection. In a time when resources are limited and it is not possible to

address every data gap to have a "complete" AOP, prioritization of which data gaps are the most crucial for risk decision-making is necessary for effective and efficient prioritizing of research needs. Further, AOPs do not necessarily need to be complete to be useful and informative for decision-making, particularly if the data gaps and uncertainties are identified and understood.

Identifying AOPs for Developmental Neurotoxicology

As discussed above, it is challenging to develop AOPs for developmental neurotoxicity due to the complex symphony of events that occur during nervous system development. Still, development of AOPs following chemical exposure during development is crucial and should be a high priority given the increasing incidence of childhood neurological syndromes such as autism spectrum disorders, attention deficit hyperactivity disorder (ADHD) and others that have significant consequences for society (Bloom et al 2009; McDonald and Paul., 2010; Landrigan et al., 2012). However, as the etiology of these disorders, and in particular the role of environmental chemicals is not well understood, a more critical and useful initial goal would be to develop AOPs that are linked to adverse outcomes that traditionally have been used to make risk decisions for environmental chemicals. Doing so would allow researchers to take advantage of the existing databases in the public literature as well as publically available databases (e.g. ToxRefDB, http://www.epa.gov/ncct/toxrefdb/) to hypothesize and test putative AOPs for developmental neurotoxicity. Furthermore, it will facilitate adoption of the AOP concept by risk assessors, as they will be familiar with the described AOs.

Prioritizing AOP development

There are thousands of macromolecules (e.g. receptors, transporters, nucleic acids, lipids) in the brain that form a vast number of potential targets for xenobiotic chemicals. If even a small percentage of these function as MIEs, the task of developing AOPs for all of these poses an almost insurmountable hurdle. One way to address this challenge is to move beyond the linear approach to modelling AOPs that has dominated past research efforts. The initial models developed for the International Programme on Chemical Safety (IPCS) mode-of-action (MOA) framework (IPCS, 2007), as well as many AOPs (Crofton and Zoeller, 2005; Watanabe et al., 2010; Bushnell et al., 2010), describe a series of one-to-one relationships, in which the MIE initiates sequential KEs eventually leading to the AO. This linear sequence likely does not capture the whole complexity of molecular and cellular biology implicated in a neurotoxic response. Thus, systems approaches should be used to develop computational models of the networks of MIEs and KEs that collectively influence the AO. For example, Kleinstreuer et al. (2013) developed a novel multicellular agent-based model of vasculogenesis using the CompuCell3D (http://www.compucell3d.org/), which incorporates vascular endothelial growth factor signals, pro- and anti-angiogenic inflammatory chemokine signals, and the plasminogen activating system of enzymes and proteases to recapitulate disruption of vascular formation by environmental chemicals. Computational models of neurotoxicity that link networks of cellular and organ level systems provide one approach to reduce the complexity of the challenge of developing predictive models of neurotoxicity for use in regulatory decisions.

Identifying Key Events that lend themselves to High and Medium throughput screening

One of the advantages of understanding an AOP is that the knowledge can be used in a predictive manner. Whether it be a simple "read across" approach using structural information about a chemical and its interactions with a molecular target (MIE) or a more quantitative approach, AOPs foster the ability to predict potential AOs for chemicals that have not been evaluated for toxicity in a test system.

A high priority for neurotoxicity AOPs is to identify KEs that represent points of convergence across multiple AOPs and that are biological responses, which can be easily incorporated into high or medium throughput screening assays. There are clear advantages and disadvantages to this approach. KEs occurring at more apical points in AOPs (i.e., closer to the AO) will by definition give rise to screening assays that detect broad classes of chemicals, but may not have the capability of distinguishing which "upstream" AOP has been activated by a chemical or class of chemicals (Woodruff et al., 2008). By contrast, assays based on early MIEs in an AOP will be test for direct interaction between chemicals and the biological target (e.g., receptor, enzyme) and thus be more specific to individual chemicals or chemical classes. This will facilitate SAR and QSAR model development. In any event, approaches that yield rapid, reliable and high(er) throughput screening methods for detecting chemicals with the potential for neurotoxicity and developmental neurotoxicity are of high priority and data from screens that are based on AOPs will provide scientifically sound rationale to those making risk decisions about chemicals based on in vitro data.

Demonstrate the utility of the AOP approach to risk assessors using case studies

This workshop presents 10 proposed AOPs related to neurotoxicity, but it was beyond the scope of the workshop to finalize comprehensive AOPs or demonstrate how use for risk decision-making. Demonstration of the real-world applicability of neurotoxicity AOPs is paramount to having this concept more readily accepted and utilized by risk assessors. As such, an important goal for the neurotoxicology research community should be to identify a small number of case studies that demonstrate how applying an AOP approach to a risk-decision problem can improve the speed and/or confidence in a risk decision, or allows information from one chemical to be applied more broadly to an entire class of chemicals. Candidate AOPs for case studies may include the pyrethroids/sodium channels, thyroid hormone AOP, or the $GABA_A$ or NMDA-related AOPs; however, this has to be proven by empirical data.

Further development of the outlined AOPs to neurotoxicity

In this report, using the OECD template and information from literature searches, initial work has been conducted to identify and develop AOPs relevant to neurological outcomes. In all cases, the authors were told to identify an MIE or a putative MIE, followed by responses at the cellular, tissue, organ, organism and population level, with each AOP summarized in a flow diagram. The presented AOPs are often based on a few well-studied model neurotoxicants and a summary of the qualitative understanding of each AOP has been briefly described. The KEs have been further evaluated in a correlative manner based on the available published data and the subjective interpretation of the strength of the scientific evidence.

The AOP descriptions are based on the OECD guidelines following the rule that two anchors should be identified: one MIE linked in a causative manner to one AO. Indeed, in most of the

described AOPs this principle has been followed. However, available scientific knowledge for some complex AOPs (e.g AOP VIII: *Neuroinflammation*) suggests more than one putative MIE and AO. These draft AOPs will need further development following the OECD AOP Framework, and at thus a decision will be needed to identify the most salient MIE to link with the AO.

The main aim of the workshop was to identify a set of putative AOPs related to neurotoxicity and developmental neurotoxicity that could be further elaborated. The next step for development of these AOPs will be application of the modified Bradford Hill considerations following the full OECD Template and Guidance on Developing and Assessing the Completeness of Adverse Outcome Pathway (OECD, 2013). This implies evaluation of biological plausibility, concordance of dose-response, temporal concordance, consistency, and specificity of association between MIE and AO in a quantitative manner (Meek et al, 2014). These issues were outside the scope of this Workshop and are thus not included in the proposed AOPs. In the near future, the ultimate goal for the listed AOPs is their submission to the OECD AOP Development Program.

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Declaration of Interest (DOI)

The employment affiliation of the authors is shown on the cover page. The authors have sole responsibility for the writing and content of this paper. The contributing authors were participants of the workshop organized by the EURL ECVAM. The external workshop participants, were invited on the basis of a survey by EURL ECVAM neurotoxicity experts of the latest literature to identify those with specific expertise in the relevant research fields. The workshop organization was financially supported by the European Commission, including the cost of travelling and per diem of all invited external experts. The strategy for preparing the report, the literature selected for review, the conclusions drawn and the recommendations made are exclusively the collective scientific output of the workshop participants and do not necessarily represent the views of the participants' employers.

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Appendix: Examples of the putative AOPs for neurotoxicity

I. Adverse Outcome Pathway on: *Binding of antagonist to an NMDAR during synaptogenesis contributes to impairment of learning and memory abilities*

Cristina Suñol

1. Introduction

Learning and memory are processes that rely on functioning of the glutamate receptor N-methyl-D-aspartate (NMDAR), which is a postsynaptic channel protein permeable to Na^+ and Ca^{2+}. Pre-synaptically released glutamate binds to α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/glutamate receptor, which when repetitively activated depolarizes the postsynaptic cell, eventually leading to the relief of the Mg^{2+} block of the NMDAR. Activation of the NMDAR regulates neurodevelopment, results in long-term potentiation (LTP) and long-term depression (LTD) and affects neuronal synaptic plasticity, thought to be dependent on different receptor subunits. The crucial role of the NMDAR in synaptic plasticity is supported by the general scientific consensus on the effect of NMDAR blockade/deletion on LTP (Hassel, 2006).

All functional NMDARs are tetrameric complexes, containing the essential subunit NR1 and one or more different NR2 types (NR2A, B, C and D). The necessary subunit NR1 needs to associate with other NR2 subunits that regulate channel gating and Mg^{2+} dependency. During synaptogenesis there is a switch from NR2B to NR2A expression in the cortex and hippocampus that will be relevant for the duration of channel opening. Switching from the NR2B to the NR2A subunit is thought to underlie functional alteration of the NMDAR during synaptic maturation, and it is generally believed that activation of the NR2A and NR2B subunits results in LTP and LTD, respectively (Miwa et al., 2008). Consequently, alterations in the expression of the NR2A

and NR2B subunits during synaptogenesis could affect the learning and memory processes (Morris et al., 1986).

2. Characterization of the exposure to the chemicals relevant to the selected AOP

Pb^{2+} is an environmental neurotoxicant that interferes with neurotransmission during central nervous system (CNS) development. It passes the human placenta and accumulates in fetal tissue during gestation (David et al., 1972). Even low levels of exposure to Pb^{2+} seem to cause surprisingly significant functional damage to children's CNS (Lanphear et al., 2005). A RfD (reference dose) of 10 microgram/dL in blood has been considered to be protective for years however, subtle neurological effects in children have been observed at lower doses (Chiodo et al., 2004; Min et al., 2007, 2009). This suggests that a definitive RfD still needs to be established for Pb^{2+}. Therefore, preventing Pb^{2+} exposure is crucial. Exposure to Pb^{2+} has been reduced during the past decades (Braun et al., 2012) however, blood levels of lead still pose a warning mark as the values reported in several cohorts exceed 5 microgram/dL (Kim et al., 2013; Palaniappan et al., 2011). Recent studies define the critical period window for Pb^{2+} exposure to induce developmental deficits as being at prenatal and early childhood period (before two-year old) (Braun et al., 2012; Ethier et al., 2012; Wasserman et al., 2000).

3. Identification of the molecular initiating event (MIE)

The MIE in this AOP is the binding of Pb^{2+} to NMDAR during synaptogenesis. Pb^{2+} acts as non-competitive, voltage-independent, NMDAR antagonist inhibiting NMDA-induced Ca^{2+} currents (IC_{50} around 1- 10 µM) with similar potency for NR2A- and NR2B-subunits of the receptors (Alkondon et al., 1990). It has been shown that Pb^{2+} inhibits [^{3}H] MK801 binding to the NMDAR in brain homogenates (IC_{50}: 0.55 µM) (Lasley and Gilbert, 1999).

NMDAR sensitivity to Pb^{2+} binding is higher at the earlier developmental stages when compared to mature neurons (Guilarte and Miceli, 1992; Rajanna et al., 1997), indicating the relevance of this MIE to developmental neurotoxicity.

4. Identification of the responses on the cellular/tissue level that may be an adverse outcome or linked to the final adverse outcome

Key cellular effects of the sustained exposure of primary cultures of hippocampal neurons to Pb^{2+} during synaptogenesis is manifested by the decreased expression of NR2A-subunit of NMDARs at synapses and in an increased targeting of NR2B-NMDARs to dendritic spines (without increased NR2B-NMDARs expression) (Neal et al., 2011; Zhang et al., 2002). It has been reported that NR2A-containing NMDAR are critical for protein synthesis in dendrites, which may have relevance in the control of synaptic plasticity (Tran et al., 2007). Decreased expression of NR2A-containing NMDAR leads to the reduced calcium currents and decreased release of glutamate in the hippocampus of Pb^{2+}-exposed rats as observed using microdialysis (Lasley and Gilbert, 2002) resulting in lowered NMDAR activation. Postsynaptic NMDAR activation regulates the generation and release of brain-derived neurotrophic factor (BDNF) in a retrograde trans-synaptic way. Due to the reduced NMDAR receptor activation observed after exposure of hippocampal cultured neurons to Pb^{2+} resulted in a reduction of both cellular proBDNF protein synthesis and BDNF release (Neal et al., 2010) and reduced BDNF levels in the rat cortex and hippocampus (Baranowska-Bosiacka et al., 2013). The decreased release of BDNF is a critical key event as it affect neuronal processes such as survival, growth, differentiation and synaptogenesis (Acheson et al., 1995) affecting the learning and memory processes (Yamada et al., 2003).

Exposure to Pb^{2+} also reduced the levels of the presynaptic proteins synaptophysin and synaptobrevin, which are proteins involved in vesicular neurotransmitter release (Neal et al., 2010). The key cellular events triggered by binding of antagonist to NMDA receptor during synaptogenesis are summarized in Fig. 2.

Moreover, chronic Pb^{2+} exposure of rats increases the threshold for LTP induction in the hippocampus at non- maximal train stimulation (Gilbert et al., 1996). This could be linked to the decreased expression of the NR2A that is involved in LTP response. The alterations in the expression ratio of these two subunits (NR2A and NR2B) during synaptogenesis could affect the learning and memory processes as it is suggested based on in vivo studies (Morris et al., 1986). Indeed, Pb^{2+}-exposed rats exhibit deficits in acquisition of a water maze spatial learning task that correlates with the reduction in the maintenance of in vivo hippocampal LTP (Nihei et al., 2000). Some of these effects are mimicked by other NMDAR antagonists, like the decrease in NR2A subunit expression by exposure to memantine during synaptogenesis in vitro (Maler et al., 2005) and the reduced levels of presynaptic proteins by DL-2-amino-5-phosphonovalerate (APV) (Neal et al., 2010). The modulation of hippocampus-prefrontal cortex synaptic transmission and disruption of executive cognitive functions was also observed in animal model induced by noncompetitive NMDAR antagonist MK-801 (Blot et al., 2013).

5. Identification of the responses on the organ level that may be an adverse outcome or linked to the final adverse outcome

Rats exposed to Pb^{2+} during development and resulting in blood lead concentrations close to those found in exposed children showed reduced protein and mRNA expression levels of NR2A subunit in the hippocampus, whereas those of NR2B were unchanged (Nihei and Guilarte, 1999; Zhang et al., 2002). Rats chronically exposed to Pb^{2+} during the postweaning period showed

decreased MK-801 binding to the NMDAR, particularly in hippocampal regions (Cory-Slechta et al., 1997a; b). A decrease in the number of pyramidal and granule cells in the hippocampus and reduced adult neurogenesis of hippocampal granule cells displaying aberrant dendritic morphology was also found in vivo (Baranowska-Bosiacka et al., 2013; Verina et al., 2007; Gilbert et al., 2005; Jaako-Movits et al., 2005). Prenatal exposure to the NMDAR antagonist phencyclidine resulted in reduced proliferation of neuronal progenitors and decreased density of glutamatergic neurons in the hippocampus decreasing glutamatergic neurotransmission showing behavioral eficits in cognitive memory and sensorimotor gating until adulthood (Toriumi et al., 2012).

6. Identification of the responses on the organism level that may be an adverse outcome or linked to the final adverse outcome

Neonatal treatment with NMDAR antagonists has been reported to affect learning and memory and to produce hyperlocomotion (Harris LW, et al., 2003; Kawabe et al., 2007; Kawabe and Miyamoto, 2008). Likewise, Pb^{2+} exposure during development has been reported to produce deficits in acquisition and retention tasks, learning and memory functions (Bijoor et al., 2012; Massaro et al., 1986), delayed synaptogenesis (McCauley et al., 1982), impaired capacity for LTP (Gilbert et al., 1999) and for short-term and long-term depression (Ruan et al., 2000) . Schematic representation of MIEs, cellular key events and organ/organism effects is described in Fig. 2.

7. Identification of the overall effects on the population

Epidemiological studies show association of Pb^{2+} exposure with cognitive and motor deficits (Braun et al., 2012; Hornung et al., 2009), visual brain development (Ethier et al., 2012), ADHD (Min et al., 2007), behavioral signature (Chiodo et al., 2004), antisocial behavior (Dietrich et al.,

2001) and children intelligence measured by various techniques at age 3 and 4, 5 and 7 respectively (McCarthy GCI; Wechsler Preschool and Primary Scale of Intelligence-Revised, WPPSI-R IQ and Wechsler Intelligence Scale for Children-version III, WISC-III IQ) (Wasserman et al., 2000). A magnetic resonance spectroscopy case report study reported alterations in brain metabolism compatible with neuronal loss and decline in intellectual functioning in a child with blood lead levels about 40 microgram/dl, which showed inappropriate school learning (Trope et al., 1998) while magnetic resonance imaging studies found reduced adult brain volume (Brubaker et al., 2010). Follow-up analysis of the Cincinnati Lead Study Cohort (253 children) suggested that averaged lifetime blood lead concentrations in excess of 20 micrograms/dL were associated with deficits in performance IQ (PIQ) on the order of approximately 7 points when compared to children with mean concentrations less or equal to 10 micrograms/dL (Dietrich et al., 1993) showing that link between developmental consequences of low to moderate prenatal and postnatal lead exposure and decreased intellectual capacity of children.

8. Is the AOP specific to certain life stages (DNT or aging)?

This AOP is specific to neurodevelopmental life stage, especially when there is a shift in the expression of the NR2 subunits of the NMDAR increasing the expression of the NR2A subunit with respect to the NR2B subunit.

9. How much are initiating and key events conserved across species?

This is not known. The conservation of the MIE and key events across species would depend on the phylogenetic conservation of the NMDAR where Pb^{2+}interacts and the NR2B/A switch produced during neuronal development.

10. Challenges for further AOP development: strength, data gaps and uncertainties to be considered

As mentioned above solid epidemiological data in humans demonstrate unequivocally that learning and memory are impaired in children exposed to Pb^{2+} during development. Learning and memory are processes that rely on the right expression and functioning of NMDAR in the hippocampus and cortex during development. Several laboratories provided evidence that hippocampal LTP is affected after treatment of laboratory animals undergoing development with Pb^{2+} (reviewed in Toscano and Guilarte, 2005). Strong in vitro data suggest that Pb^{2+} could act as an antagonist of the NMDAR. However, the exact molecular mechanism by which Pb^{2+} inhibits NMDAR is not completely clear. A proposed mechanism involves the Pb^{2+} interaction at a divalent cation binding site that is responsible for glycine binding (Toscano and Guilarte, 2005). In rodents, Pb^{2+} binds to NMDAR of developing brain with higher affinity than does in adult brain. Whether in humans this is the case remains to be elucidated.

Furthermore, exposure to Pb^{2+} during development results in reduced expression of NMDAR subunits in the hippocampus based on animal models (Toscano and Guilarte, 2005). It is not clear whether the decreased expression of NR2A subunit by Pb^{2+} is linked to the entiered inhibition of the NMDAR activity or to the selective reduced expression of the NR2A subunit. Indeed, it has been shown that the reduction in NR2A subunit presence in dendritic spines induced by Pb^{2+} exposure is possibly due to entire inhibition of NMDAR function (not selective decrease in NR2A subunit expression) as treatment with a specific NMDAR inhibitor (APV) caused similar effects (Toscano and Guilarte, 2005). However, the mechanism behind this observation has not been fully elucidated yet. It has been found that under physiological conditions the developmental increase in NR2A subunits is mediated by calcium influx derived

by both NMDAR and L-type Ca^{2+} channels, pointing out the complexity of NMDAR subunit expression, which is not only genetically-mediated but also activity-dependent (Toscano and Guilarte, 2005).

Further studies are also needed to determine to what extent decreased expression of NR2A subunit is causality linked to impairment of learning and memory in human. Specifically needed are studies that characterize both the anatomical and physiological changes that caused by altered NR2A expression, and importantly, exactly how these changes result in decreased cognitive function. While, knockout studies in mice showing similar effects to Pb^{2+}-exposed rats, it is unlikely that Pb+ exposure in children is equivalent to knocking out or knocking down a gene. Thus, critical dose dynamic studies are needed to better mimic the level of alternations in NR2A likely to be found in Pb+ exposed children. Future elaboration of this AOP requires quantitative description of the cellular KEs that should be shown to be linked with AO in a causative manner.

II. Adverse Outcome Pathway on: *Binding of agonist to NMDA receptor causes excitotoxicity that mediates neuronal cell death, contributing to reduction (or loss) of cognitive, sensory and motor function*

André Schrattenholz

1. Introduction

NMDAR-mediated excitotoxicity is upstream of neuroinflammation and apoptosis (see AOP VIII on *Multiple molecular initiating events trigger neuroinflammation leading to neurodegeneration*) and thus has been implicated in many important human pathologies, ranging from amyotrophic lateral sclerosis, Alzheimer's and Parkinson's diseases, depression, epilepsy, trauma and stroke to schizophrenia.

The major excitatory neurotransmitter in the mammalian CNS is L-glutamate (Glu), activating a variety of ionotropic and metabotropic receptors. The ionotropic NMDARs are ligand-gated calcium channels tightly regulated by a complex set of endogenous ligands and ions like Mg^{2+}. They are activated by Glu, glycine (Gly) and depolarization (relief of voltage-dependent magnesium block). They are redox sensors (dithiol-bridges) and allosteric proteins regulated by a variety of factors like polyamines, zinc, lipid environment and phosphorylation. The four subunits composing the receptor are coded by seven genes (NR1, NR2A-D and NR3A-B), and the eventual architecture is shaped by alternative splicing, RNA-editing and extensive posttranslational modifications, like phosphorylation and glycosylation.

The underlying functional regulation of NMDARs depends on interacting proteins and cofactors, like membrane-bound receptor tyrosine kinases, cholesterol-rich membrane domains (lipid rafts), Ca^{2+}-related mitochondrial feedback-loops and sub-synaptic structural elements like post-synaptic density protein of 95 kD (PSD-95). Thus, NMDARs are at the core of highly dynamic molecular systems relevant for cognition and memory (see AOP I on: *Binding of antagonist to an NMDAR during synaptogenesis contributes to impairment of learning and memory abilities)* and in particular responsive to alterations of access of compounds to the CNS by dysfunction of the BBB (Schrattenholz and Soskic, 2006).

For toxicology, it is important that the NMDAR-dependent molecular machinery initiates and stabilizes neuronal plasticity and thus is tightly connected to brain energy metabolism (ATP/NADH) (see AOP VII *Binding of inhibitors to the mitochondrial respiration chain complex I, II, III or IV or interaction of uncouplers with oxidative phosphorylation decreases or blocks ATP production resulting in neurodegeneration).*

Under a variety of stressful conditions it can derail towards increasingly irreversible pathophysiological conditions, all converging on Ca^{2+} homoeostasis, but with different MIE's, ranging from epigenetic events (Chandrasekar, 2013), secondary neuronal damage after e.g. organophosphate poisoning (Chen, 2012) to direct interaction of neurotoxic compounds, e.g. domoic acid (DA), methyl mercury, with glutamate transport (Khandare et al., 2013; Liu et al., 2013) or certain biphenyls with related ion channels (Westerink, 2013).

2. Characterization of the exposure to the chemicals relevant to the selected AOP

The routes of exposure leading to excitotoxicity can be via inhalation, passage of the gastrointestinal system or by skin penetration, but the toxicant has to pass the BBB, usually by passive transport by appropriate hydrophobic and moderate polar properties (Grumetto et al., 2013) or by complex (co)transport mechanisms involving e.g. certain viral proteins (Silverstein et al., 2012). The permeability of the BBB also changes under oxidative stress, ischemia and during ageing (Enciu et al., 2013). Domoic acid (DA) a natural toxin that accumulates in mussels and shellfish is an analogue of L-glutamate. Prenatal exposure to DA has been associated with damage to neurons in different brain regions, decreased levels of brain gamma-aminobutyric acid (GABA) and increased glutamate levels (Hogberg et al., 2011). It has also been shown to cross the placenta reaching the brain tissue of the fetus and accumulate in the amniotic fluid. Moreover, a higher quantity of DA remains in the milk as compared to the plasma and therefore a new born baby can be more exposed than the mother (Maucher and Ramsdell, 2005).

3. Identification of the Molecular Initiating Event (MIE)

The MIE is the prolonged binding of Glu or an analogue (plus co-agonist glycine and plus depolarization via non-NMDARs) to NMDAR leading to long lasting opening of the channel resulting in the excessive intracellular Ca^{2+} concentrations. Any chemicals that can directly

activate NMDAR or indirectly via triggering increased level of endogenous glutamate could initiate this AOP. Over activation of NMDAR leads to Ca^{2+}-dependent kinases activation triggering cascades of events resulting in neuronal cell death (Fig. 3A). If certain thresholds of intracellular Ca^{2+} are breached, intrinsic apoptosis and mitochondrial transition follow downstream leading to neurodegeneration as an adverse outcome (Schrattenholz and Soskic, 2006).

4. Identification of the responses on the cellular/tissue level that may be an adverse outcome or linked to the final adverse outcome

NMDAR over activation results in excitotoxicity, by which neurons are damaged and finally killed. It is caused by excessive stimulation of NMDARs by Glu or its analogues allowing high levels of Ca^{2+} to enter the cell. Ca^{2+} influx into cells activates a number of enzymes, including phospholipases, endonucleases, and proteases such as calpain. These enzymes go on to damage cell structures such as components of the cytoskeleton, membrane, and DNA. At the same time Ca^{+2} overload leads to mitochondrial damage, overproduction of free radicals, opening of the mitochondrial permeability transition pore, energy depletion, poly-ADP ribosylation, activation of apoptotic signaling pathways (Fig. 3A and B) (see AOP on *Binding of inhibitors to the mitochondrial respiration chain complex I, II, III or IV or interaction of uncouplers with oxidative phosphorylation decreases or blocks ATP production resulting in neurodegeneration)* leading to apoptosis, autophagy and up regulation of inflammatory mediators (see AOP on *Multiple molecular initiating events trigger neuroinflammation leading to neurodegeneration*). Additionally, the mitochondrial production of reactive oxygen species (ROS) inhibits glial EAAT2 function leading to further increases in the glutamate concentration at the synaptic cleft and further rises in postsynaptic Ca^{2+} levels.

In the mammalian brain NMDA receptors are also responsible for activity-dependent Hebbian behavior of synapses and the formation of synaptic plasticity via LTP of postsynaptic excitatory potentials. Thus, they represent the key protein necessary for the formation of memory and cognition.

5. Identification of the responses on the organ level that may be an adverse outcome or linked to the final adverse outcome

The consequence of excitotoxicity and activation of the intrinsic pathway of apoptosis is neurodegeneration. Depending on the overall individual genetic, epigenetic and ontological predisposition, the brain regions where neurodegeneration occurs first can differ dramatically, producing different adverse outcomes. For example, organophosphate poisoning will primarily affect the cholinergic system and predominantly brain regions like the hippocampus. In other cases other brain regions like substantial nigra or spinal cord neurons may be affected more severely (Rahn et al., 2012; Gonda, 2012).

6. Identification of the responses on the organism level that may be the final adverse outcome or linked to the final adverse outcome

Depending on the CNS region primarily affected, there can be cognitive, behavioral or motor deficits due to exicitotoxicity induced neurodegeneration. Exicitotoxicity been implicated in many important human neurodegenerative diseases such as amyotrophic lateral sclerosis (Spalloni et al., 2013), Alzheimer's (Lakhan et al., 2013) and Parkinson's diseases (Mehta et al., 2013), depression, epilepsy, trauma, stroke and schizophrenia (Beal, 1992; Deutsch et al., 2001, Duman, 2009; Cho, 2013). Another important site of neurodegeneration e.g. due to exposure to antibiotics or cytostatics are the sensory neurons of the inner ear with subsequent hearing loss

(Deavall et al., 2012; Langer et al., 2013). Schematic representation of MIE, cellular key events and organ/organism effects is described in Fig. 3B.

7. Identification of the overall effects on the population

There is broad agreement that oxidative stress/excitotoxicity is one of the major contributing factors underlying neurodegenerative disorders like Alzheimer's (Lakhan et al., 2013) and Parkinson's diseases (Mehta et al., 2013), Amyotrophic lateral sclerosis (ALS) (Spalloni et al., 2013), but also it has been involved in conditions such as autism (Essa et al., 2013), neuropathic pain and others (Lipton, 2005).

8. Is the AOP specific to certain life stages (DNT or aging)?

Indeed, the physiological roles of glutamate and other neurotransmitter receptors are changing dramatically throughout development, brain maturation and aging (Paradies et al., 2013; Groebe et al., 2010; Oh et al., 2013; Crompton, 2004). At different life stages they undergo structural changes and define in a very dynamic way the relatively narrow threshold window of intracellular Ca^{2+}concentration changes which are essential for cognitive processes, but also integrate epigenetic, nutritional, individual life time histories of toxic exposures and infections and their role in excitotoxicity and neurodegeneration. NMDAR Ca^{2+}/-related threshold triggers neuronal apoptosis induced by excessive Ca^{2+} leading to neurodegeneration (adverse outcome).

9. How much are initiating and key events conserved across species?

They are relatively well conserved in mammals with similar physiological roles. In lower species there are still the same conserved pathways, but have different physiological roles: glutamate receptors are e.g. peripheral in muscle of insects and not expressed in the CNS as in the case of mammals (Di Antonio, 2006).

10. Challenges for further AOP development: strength, data gaps and uncertainties to be considered

Based on the cited references in the text it can be concluded that there is a general agreement that the excitotoxicity triggered by over activation of NMDAR is capable of inducing neurodegeneration. Indeed, NMDA agonists caused excitotoxic neurodegeneration in a dose-dependent manner that was also proportional to the increase in intracellular Ca^{2+} assessed in cultured cortical neurons (Sattler et al., 1998).

However, the individual genetic risk profiles for neurodegeneration, the complexity of contributing factors over the life time of mammalian brains and in particular the dynamic role of the blood brain barrier (damaged by chronic inflammation) make it difficult to extrapolate quantitative results from in vitro and in vivo models to human neuropathologies. The corresponding systems biology and modeling of the complex interactions and feedback signaling, typical for neurotransmission, is still at an early stage. However, the computational and data acquisition tools are developing fast in a context of personalized medicine (OMICS technologies, Next Generation Sequencing).

Also, it is important to note that glutamate, like each and every other neurotransmitter not only activates ionotropic receptors like the NMDA receptor, but at the same time, in the neighboring synaptic structures, activates metabotropic G-protein-coupled receptors. These receptors trigger a variety of downstream effects with completely different time scales and contribute to the complex regulation of cellular responses. Furthermore, recent findings suggest that increased voltage-gated calcium channels (VGCCs) density or activity in specific brain regions can also augment intracellular calcium levels leading to the increased glutamate release, causing over activation of NMDA receptor, promoting neurodegeneration (Cataldi, 2013).

Another complexity of this AOP is the presence of three AOs linked to the same MIE instead of one. However, this has been done intentionally so future developers could choose one of the three AOs and elaborate further connecting a specific brain structure with a defined neurodegenerative disorder. The largest limitation of this AOP is linked to the lack of temporal concordance (i.e. KE_{up} precedes KE_{down}). Mitochondrial dysfunction and ER stress are ubiquitous cellular process that influence and perpetuate a wide array of cellular dysfunction, including facilitating the development of neurodegenerative diseases (Mei et al., 2013). Similarly, just as collapse of Ca^{2+} homeostasis can lead to ER stress, ER stress also contribute to increased intracellular Ca^{2+} (Mei et al., 2013). These complex cellular KEs have to be further described in a causative and quantitative manner to provide confidence that they are responsible for specific neurodegenerative pathologies (AO).

III. Adverse Outcome Pathway on: *Binding of antagonist to $GABA_A$ receptor results in hyperexcitability and convulsions*

Cristina Suñol

1. Introduction

The amino acid γ-aminobutyric acid (GABA) is ubiquitously present in the mammalian CNS where it is in charge of the inhibitory transmission signals between neurons. The GABAergic system mediates a series of physiological functions. Altered GABAergic function, mainly related to hyperexcitability, has a role in neurological and psychiatric disorders in humans. The most prominent GABA receptor is the $GABA_A$ receptor ($GABA_AR$), which is a ligand-gated Cl^- channel belonging to the family of ionotropic receptors. The $GABA_AR$ is the site of action of GABA released from presynaptic neurons as well as of many neuroactive drugs, among them

benzodiazepines, barbiturates, ethanol, neurosteroids and anesthetics. The activation of the $GABA_AR$ by GABA or other agonists leads to an increased membrane conductance to Cl^- that generally induces a membrane hyperpolarization and the consequent reduction in the probability of action potential firing, eventually causing neuronal inhibition (Olsen and Betz, 2006). The Cl^- flux is inhibited by convulsant agents like bicuculline and picrotoxin, which act as competitive and non-competitive $GABA_AR$ antagonists, respectively (Krishek et al., 1996). While bicuculline reduces Cl^- flux by decreasing the opening frequency and mean open time of the channel by binding at the GABA recognition binding, picrotoxin decreases the channel opening probability by binding at separate sites that block the chloride channel.

2. Characterization of the exposure to the chemicals relevant to the selected AOP

Hyperexcitability symptoms like anxiety and convulsions have been reported to occur after acute exposure to chlorinated pesticides belonging to the family of cyclodienes and hexachlorocyclohexane, produced by both intentional and non-intentional ingestion (Moses and Peter, 2010; Parbhu et al., 2009; Durukan et al., 2009). Although some of these pesticides are not in use nowadays in developed countries, they are still found in human fluids and tissues (Cassidy et al., 2005; Shen et al., 2007; Vizcaino et al., 2011) due to their high lipophilicity and body accumulation. These lipophilic compounds easily cross the BBB and target the $GABA_AR$ in the CNS.

Besides picrotoxin, the chemicals that block Cl^- conductance through the ion channel of $GABA_A$ receptor include: pentylenetetrazol, plant toxins like cicutoxin and oenanthotoxin, and pesticides such as lindane and cyclodienes (Wyrembek et al., 2010; Allan and Harris 1986; Vale et al., 2003).

3. Identification of the molecular initiating event (MIE)

MIE is triggered by binding of antagonist to $GABA_AR$ leading to the inhibition of GABA transmission (Fig. 4A). Organochlorine cyclodienes and γ-hexachlocyclohexane potently interact with the $GABA_AR$ at the picrotoxin recognition site. Competitive inhibition of [^{35}S]TBPS binding by these compounds is induced at concentrations in the nanomolar-micromolar range (Lawrence and Casida, 1984; Pomés et al., 1993; Huang and Casida, 1996; Ratra et al., 2001). Finally, they do not directly interact with the recognition sites for GABA and benzodiazepine at the $GABA_AR$ (Vale et al., 1997). Altogether these data suggest that organochlorine cyclodienes and γ-hexachlorocyclohexane bind to the picrotoxin recognition site at the $GABA_AR$. It is also noticeable that pentylenetetrazole inhibits [^{35}S]TBPS binding (Maksay and Ticku, 1985). This drug is used as a chemoconvulsant in animal seizure models for the screening of drugs effective against myoclonic seizures in humans (Mcnamara, 2006). Finally, cicutoxin and related compounds inhibited [^{3}H]EBOB binding at the $GABA_A$ receptor (Uwai et al., 2000).

4. Identification of the responses on the cellular/tissue level that may be an adverse outcome or linked to the final adverse outcome

The binding of the organochlorine pesticides to the picrotoxin recognition site results in the blocking of the chloride channel operated by GABA. Inhibition of GABA-induced currents and of GABA-induced Cl^- flux has been demonstrated by means of electrophysiological and Cl^- uptake ($^{36}Cl^-$) methods in cultured neuronal cells and neural preparations (Gant et al., 1987a; Obata et al., 1988; Pomés et al., 1994; Nagata et al., 1996; Vale et al., 2003; Ikeda et al., 1998; Galofré et al., 2010). A significant correlation was found for inhibition of [^{35}S]TBPS binding and GABA-induced Cl^- flux for these compounds (Obata et al., 1988; Pomés et al., 1994). Reduced or blocked Cl^- influx is leading to inhibition or decreased GABA transmission resulting in

increased excitability. Induced alterations in neuronal network function are contributing to the anxiety and can cause seizures and convulsions (Fig. 4A).

5. Identification of the responses on the organ level that may be an adverse outcome or linked to the final adverse outcome

The inhibition of the major inhibitory neurotransmission system alters the balance between excitation and inhibition in the brain, as a result of changes in network activity (Zhang and Sun, 2011). In this regard, an acute dose of γ-hexachlorocyclohexane induced an increase in the innate excitability of the granule cells in the hippocampus (Joy and Albertson 1988) and of glucose uptake in several regions of the brain (Sanfeliu et al., 1989) that is compatible with its excitatory activity. On the other hand, no major histologic alterations in the CNS have been reported after acute exposure to cyclodiene and γ-hexachlorocyclohexane organochlorine pesticides in animal models (Omer, 1970; Castro et al., 1992). This is not unexpected as in neurotoxicity many potent neurotoxic agents produce no morphological but functional changes (Ray, 1997).

6. Identification of the responses on the organism level that may be an adverse outcome or linked to the final adverse outcome

Inhibition or decrease of GABA neurotransmission induces alterations in neuronal network function (Fig. 4A) as the balance between excitory and inhibitory neuronal activity is disrupted resulting in seizure or convulsions observed in animal experimental models and in humans (Omer, 1970; Carvalho et al., 1991; Suñol et al., 1989; Moses and Peter, 2010; Parbhu et al., 2009; Durukan et al., 2009). Furthermore, subconvulsant doses of these pesticides reduced the intensity of electrical stimulation required to evoke seizures in amygdala kindled animals (Gilbert 1995; Gilbert and Mack 1995). Schematic representation of MIE, cellular key events and organ/organism effects is described in Fig. 4A.

7. Identification of the overall effects on the population

Several case reports have been published for the convulsant activity of organochlorine cyclodiene and γ-hexachlorocyclohexane pesticides. Although these effects are mainly induced after ingestion of relatively high doses of the toxicant, which is a rare condition nowadays, recent reports still attest to the existence of this exposure in humans and its toxicological consequences (Moses and Peter, 2010; Parbhu et al., 2009; Durukan et al., 2009).

8. Is the AOP specific to certain life stages (DNT or aging)?

There is no evidence suggesting that this AOP might be specific to certain life stages. However, there is a lack of studies regarding the effect during neurodevelopment especially at low concentrations.

9. How much are initiating and key events conserved across species?

The $GABA_AR$ is phylogenetically conserved across species (Garcia-Reyero et al., 2011) and the ligand binding properties at the $GABA_AR$ are similar among human, mammal, avian and fish brain (Cole et al., 1984).

10. Challenges for further AOP development: strength, data gaps and uncertainties to be considered

A reasonable causative correlation can be depicted for the inhibition of $GABA_AR$ (measured as inhibition of [^{35}S]TBPS binding and of GABA-induced Cl^- flux and the toxic doses (LD_{50}) in experimental animals exposed to cyclodienes and γ-hexachlorocyclohexane (Fig. 4B). Also, a causative correlation has been reported for the inhibition of [^{35}S]TBPS binding and the convulsant dose of several pentylenetetrazole analogues (Squires et al., 1984). Data on absorption distribution metabolism and excretion (ADME) of GABA antagonists are not available. In addition, it is not well-established that different expression (increase/decrease) and

localization of specific $GABA_AR$s subunits that form the receptors render these same receptors more sensitive (or not) to $GABA_AR$ antagonists, something that has been demonstrated to be important in animal models of epilepsy (Sperk et al., 2004). It is also not entirely clear whether the altered neuronal network function (increased excitability) is the only GABA-related mechanism leading to anxiety. Indeed, the $GABA_AR$-mediated currents are also known to be controlled by ion-regulatory molecules such as the neuronal Cl^- and/or HCO_3^- transporters (e.g. K-Cl co-transporter isoform 2, KCC2 and Na^+-independent and Na^+-dependent Cl-HCO_3 exchangers AE3 and NDCBE, respectively) and cytosolic carbonic anhydrases (e.g. CA2 and CA7). Furthermore, TrkB and calpain have emerged as important factors in GABAergic signaling during epileptogenesis and epilepsis. The above regulators of ionic plasticity that seem to be implicated in seizures and convulsions induction due to disturbance of GABAergic transmission should be also included in this AOP. Future elaboration of this AOP requires quantitative description of the cellular KEs that should be shown to be linked with AO in a causative manner.

IV. Adverse Outcome Pathway on: *Binding of Pyrethroids to Voltage-gated Sodium Channels induces acute neurotoxicity*

Timothy Shafer

1. Introduction

Pyrethroid insecticides have been used for pest control for over 50 years. Consequently, much is known regarding their acute neurotoxicity. In insects as well as mammals, two distinct poisoning syndromes (Adverse Outcomes) have been identified. Type I or T type syndrome, is characterized by hyperreactivity, aggressive sparing and tremor, while type II or CS syndrome is

characterized by pawing and burrowing, choreoathetosis, salivation (Verschoyle and Barnes, 1972; Verschoyle and Aldridge, 1980). These signs and symptoms, as well as the effects of pyrethroids on behavior have been demonstrated in many different laboratories and have been extensively reviewed (Gammon et al., 1981; Lawrence and Casida, 1982; Soderlund et al., 2002; Wolansky and Harrill, 2008). This class of compounds has been well studied at several different levels of biological organization and there is a solid database of literature to support development of an adverse outcome pathway for neurotoxicity following acute exposure. By contrast, the developmental neurotoxicity of pyrethroids as a class is not as well understood, and there is not a sufficient database for the development of an AOP (Shafer et al., 2005). Thus, the AOP described below applies only to acute neurotoxicity.

2. Short characterization of the exposure to the chemicals relevant to the selected AOP

Exposure to pyrethroids can occur via a variety of routes, due to the fact that these compounds are used for pest control on a variety of food crops, as well as for indoor pest control. Primary routes of exposure are therefore oral and dermal. Because of discontinued uses for organophosphorous (OP) pesticides in the last decade, uses of pyrethroids have increased, thus exposure potential for this class of compounds has increased.

3. Identification of the Molecular Initiating Event (MIE)

The molecular initiating event for pyrethroid neurotoxicity is binding of these compounds to voltage-gated sodium channels (VGSC) in neurons of the central and peripheral nervous system. This has been well documented in numerous laboratories and different types of preparations from insects, arthropods, mammals and other species. This topic has been extensively reviewed (Soderlund and Bloomquist, 1989; Vijverberg and van den Bercken, 1990; Narahashi, 1982; 1992; 1996; Bloomquist, 1993; 1996; Ray, 2001; Bradberry et al., 2005), and thus will only be

briefly summarized here. Mammalian VGSCs are a multimetric protein comprised of an alpha and two beta subunits. The alpha subunit is sufficient for channel function, and multiple isoforms have been identified in mammalian neurons.VGSCs in neurons are critical for the generation and propagation of action potentials, electrical signals that transmit information from one end of the nerve cell to the other. These channels open in response to slight changes in voltage across the neuronal cell membrane, and allow sodium ions to enter the nerve cell and depolarize the membrane further. The channels then close (or "inactivate") even in the presence of ongoing depolarization. The alpha subunit of VGSCs has distinct binding sites for a variety of neurotoxins, including saxitoxin, batrachotoxin, scorpion toxin and others (Ogata and Ohishi, 2002). Pyrethroids bind to a site on the alpha subunit of VGSC (Trainer et al., 1997) that is distinct from these other binding sites (O'Reilly et al., 2006), and this binding interferes with the open and closing of VGSC by delaying the kinetics, or transitions, between different open, closed and inactivated states, of the channel. When measured at the level of the entire population of VGSC in an individual cell, this results in short to long-lasting "tail currents" through VGSC when a depolarizing stimulus is ended. If membrane voltage is examined, depolarization under normal circumstances generates a single action potential. VGSCs modified by type I compounds depolarize the cell membrane above the threshold for action potential generation, resulting in a series of action potentials (repetitive firing). Type II compounds cause greater membrane depolarization, diminishing the sodium electrochemical gradient and subsequent action potential amplitude. Eventually, membrane potential becomes depolarized above the threshold for action potential generation (depolarization-dependent block). This is the result of sodium continuing to enter the cell through those channels that have been modified by pyrethroids. Several lines of evidence link this MIE to the adverse outcomes described above, including chemical structure

(Type I and II pyrethroids have different structures and alter VGSC differently), stereospecificity (pyrethroids exist as stereospecific isomers and the more toxic isomers have greater effects on VGSC), and the presence of mutations of sodium channels that are related to pyrethroid resistance.

4. Identification of the responses on the cellular/tissue level that may be an adverse outcome or linked to the final adverse outcome

The net result of the alterations in VGSC kinetics described above is an alteration of neuronal excitability. If the membrane voltage of a neuronal cell is examined, a depolarization event that generates a single action potential under normal circumstances, results in a series of action potentials (repetitive firing) when VGSCs are modified by type I compounds. This is the result of the pyrethroid modified channels in the membrane causing sufficient depolarization to trigger additional action potentials. By contrast, type II compounds cause greater membrane depolarization, which results in a diminution of the sodium electrochemical gradient and subsequent action potential amplitude. Eventually, membrane potential becomes depolarized above the threshold for action potential generation (depolarization-dependent block). These effects of pyrethroids have been well characterized in a number of different types of preparations from insects, mammals and other species. In many of these cases, it is possible to record both the VGSC currents as well as the action potential firing in the same preparation thus, the changes in neuronal excitability are well-linked to the MIE. The relationship between pyrethroid-induced alterations in VGSC function and disrupted membrane excitability (membrane depolarization and/or changes in firing of action potentials) is well established and has been demonstrated in a number of species in vitro. Using crayfish giant axon, Salgado and co-workers (Salgado et al., 1989) demonstrated temperature-dependent actions of fenvalerate on VGSC function as well as

membrane excitability. In addition, voltage-dependent potassium currents were not altered by fenvalerate. Lund and Narahashi (1981) demonstrated that cyphenothrin disrupts sodium channel function, depolarized the membrane, and upon stimulation of the nerve, induces repetitive firing that is blocked by TTX. These same authors demonstrated that tetramethrin altered VGSC function and induced repetitive firing in squid axon membranes (Lund and Narahashi, 1982). Similarly pyrethroids alter sodium current at the node of Ranvier and depolarization and/or induce repetitive in peripheral nerves from frogs (Vijverberg and Van den Bercken, 1979, reviewed in Vijverberg and Van den Bercken, 1982). Similar findings have also been reported in mammalian neurons in vitro, wherein pyrethroids modify VGSC function and produce changes in membrane excitability in dorsal rood ganglion neurons (Tabarean and Narahashi, 1998) and cerebellar Purkinje neurons (Song and Narahashi, 1996).

5. Identification of the responses on the organ level that may be an adverse outcome or linked to the final adverse outcome

The alterations in neuronal excitability are consistent with reports from in vivo measurements of modified neurophysiological endpoints. Following deltamethrin administration the typical period of supranormal nerve excitability of compound action potentials (CAP) in rat tail nerve was increased from ~30 msec to ~400 msec (Parkin and Le Quesne, 1982). Similar changes in CAP were reported following administration of fenvalerate or allethrin (Nozaki et al., 1995). When dorsal root potentials were recorded from urethane anesthesitized rats, cismethrin increased the amplitude of the potential by up to 142% (Smith, 1980). Recordings in the rat hippocampus demonstrate that a variety of pyrethroids disrupt hippocampal neurophysiology, including paired-pulse inhibition (Gilbert et al., 1989; Joy et al., 1990). Finally, EEG measurements demonstrated that administration of cypermethrin produced bursts of epileptic activity following

the first administration, and on subsequent days of administration these were accompanied by shaking, myoclones, and tonic-clonic seizures (Condés-Lara et al., 1999). Thus, data from in vitro studies indicate that pyrethroid modification of VGSC leads to changes in membrane excitability. In vivo, changes in neurophysiological measures occur that are consistent with alterations in action potentials, and correlate with behavioral changes.

6. Identification of the responses on the organism level that may be the final adverse outcome or linked to the final adverse outcome

As described above, there are two distinct syndromes of acute pyrethroid neurotoxicity that are observed after high-dose acute exposure to pyrethroids. Data from studies on auditory startle and functional observational battery protocols confirm this profile of toxicity based on structure--pyrethroids historically categorized as Type I and Type II cause different toxicity profiles in rodents. Auditory startle studies (Crofton and Reiter, 1984; Crofton and Reiter, 1988) are also consistent with two types of pyrethroid effects; with Type I compounds producing an increase in startle amplitude (permethrin, bifenthrin, cismethrin) and Type II compounds producing a decrease in startle amplitude (deltamethrin, cypermethrin, cyfluthrin, and flucythrinate). However, some compounds that produce alterations in the auditory startle reflex parameters are not predicted by the absence or presence of the α-cyano group (fenvalerate) (Wolansky and Harrill, 2008). Although this relationship between effects at the sodium channel level and behavioral changes is highly correlated, true causality is difficult to determine because of the lack of suitable methods to simultaneously assess sodium channel function at the cellular level in behaving animals. Schematic representation of MIE, cellular key events and organ/organism effects is described in Fig. 5.

7. Identification of the overall effects on the population

For humans, acute neurotoxicity following pyrethroid poisoning has little impact on the population level. Individuals who are affected typically recover if properly cared for and the most significant population effect is an association between poisoning severity and lost work time (Walters et al., 2009). There is no clear indication in the literature that there are permanent adverse effects following acute pyrethroid poisoning (Ray and Forshaw, 2000; Bradberry et al., 2005). From an ecological perspective, the most significant population effect is selection for resistant strains of insect pests. Several different point mutations have been found in the voltage-gated sodium channels of resistant insects, which supports the proposed AOP (Casida and Durkin, 2013).

8. Is the AOP specific to certain life stages (DNT or aging)?

Age-related differences to pyrethroid neurotoxicity have been documented in rodents (Sheets et al., 1994; Sheets, 2000; reviewed in Shafer et al., 2005). In the context of AOPs, these age related differences appear to be largely due to pharmacokinetics (Cantalamessa, 1993). With respect to the key initiating event, the functional and pharmacological diversity of sodium channel expression varies by tissue and stage of development (reviewed in Meacham et al., 2008; Mandel, 1992). What is currently unclear is whether or not these developmentally expressed subunits contribute to differential sensitivity of individuals to pyrethroids based on age. A study by Meacham et al using rodent $Na_v1.2$ and $Na_v1.3$ channels expressed in oocytes indicated that the developmentally expressed $Na_v1.3$ channel is more sensitive than the adult $Na_v1.2$ channel to modification by type II pyrethroids. However, a study by Tan and Soderlund (2009) found that the human $Na_v1.2$ and $Na_v1.3$ channels were not different in their sensitivity to pyrethroids.

Thus, additional work is needed in this area to understand whether pharmacodynamic differences contribute to age-related sensitivity to pyrethroids.

9. How much are initiating and key events conserved across species?

Voltage-gated sodium channels are highly conserved across species and play a key role in control of electrical excitability of the nervous system. As evidenced by the fact that both type I and type II syndromes of pyrethroid neurotoxicity can be described in insects, mammals and other species, the overall AOP is well conserved. However, there are known differences in the susceptibility to pyrethroid neurotoxicity between mammals and insects, which can be related to the AOP. First, there are differences in the pharmacokinetics of pyrethroid metabolism that can account for some of the differential toxicity between mammals and insects (Glickman and Casida, 1982; Godin et al., 2007; reviewed in Narahashi, 1996; Soderlund et al., 2002). Second, it is well known that actions of pyrethroids on VGSC are inversely temperature-dependent (Salgado et al., 1989); thus, insects, which are cold-blooded, are more susceptible than mammals due to greater modification of VGSC by pyrethroids in the former species. Finally, molecular biology studies have demonstrated that there are species differences in critical VGSC residues that are important for pyrethroid action. For example, mutation of mammalian VGSC channels to resemble more closely insect channels can increase the sensitivity of the channel to deltamethrin by 100 fold (Vais et al., 2000). In addition, more recent evidence indicates that there are differences in the sensitivity of VGSC from humans and rodents to pyrethroids (Meacham et al., 2008; Tan et al., 2009). Recently, a much larger cross-species comparison has been completed that demonstrated good cross-species predictivity of pyrethroid toxicity based on similarities in the VGSC alpha subunit (LaLone et al., 2013).

10. Challenges for further AOP development: strength, data gaps and uncertainties to be considered

In general this AOP is well documented. It is clear that interactions with VGSC is an MIE responsible for the acute neurotoxicity of pyrethroids. However, to better link experimental data to human, research is needed on which subtypes of VGSCs are the most susceptible. The compounds which trigger the MIE have been well studied and there is a solid database of literature to support development of an AOP for neurotoxicity following acute exposure.

While this AOP is well established, there are some important knowledge gaps, uncertainties and limitations. One of the greatest uncertainties is related to the heterogeneity of VGSC in mammals; mammalian VGSC are complex, and this creates uncertainty in correlating effects on specific VGSC isoforms in vitro to in vivo symptomology caused by pyrethroid toxicity in mammals. There are at least 10 distinct genes that encode sodium channel α subunit proteins (Nav1.1-Nav1.9; Nav1.X) in humans and mice (Plummer and Meisler, 1999; Goldin, 2001). In addition, four β subunits (β1-β4) (Yu et al., 2003) have been identified to date. The heterogeneity of the sodium channel can also be enhanced by the alternative mRNA splicing of some isoforms (Sarao et al., 1991; Schaller et al., 1992; Gustafson et al., 1993). Differential sensitivity of these channels has been demonstrated in mammalian neurons, in vitro (Ginsburg and Narahashi, 1993; Tatebayashi and Narahashi, 1994; Choi and Soderlund, 2006). While it is clear that interactions with VGSC are pivotal in the acute neurotoxicity of pyrethroids, exactly which subtypes are the most susceptible and their relative contributions are currently not completely understood. One of the limitations of the current data set is that there is no good method to measure directly VGSC function in vivo, thus the data describing pyrethroid interactions with VGSC are based on in vitro studies. Further, as there is no readily available

biomarker of pyrethroid effect on VGSC, it is difficult to compare directly between in vivo and in vitro concentrations. Another major data gap is the causative link between the known alterations in neuronal channel kinetics and the types of neurological outcomes. Type I and Type II pyrethroids produce vastly difference behavioral signs (Wolancky and Harrill, 2012). These difference may, or may not, be linked to differences in isoforms mentioned above. This gap includes a lack of studies examining pyrethroid effects on neurophysiological function in vivo. Thus it is this aspect of the AOP that has the largest uncertainty. Nevertheless, available data support a well-established AOP.

V. Adverse Outcome Pathway on: *Binding of certain organophosphates to NTE results in delayed neuropathy*

Alan Hargreaves, Anna Forsby, Mamta Behl and Magdalini Sachana

1. Introduction

OPs comprise a diverse group of compounds that are used extensively in pesticide formulations, aviation fluids, lubricants and flame retardants. Certain but not all OPs used in aviation fluids or as oil additives (e.g. tri-ortho-cresyl phosphate [TOCP]) and as insecticides (e.g. chlorpyrifos, dichlorvos, isofenphos, methamidophos, mipafox, trichlorfon, trichlornat, phosphamidon/mevinphos) have been shown to induce a central/peripheral sensory-motor distal axonopathy known as OP-induced delayed neuropathy (OPIDN), the clinical symptoms of which appear up to several weeks after exposure (Weiner and Jortner, 1999; Abou Donia and Lapadula, 1990; Lotti and Moretto, 2005). Pathological symptoms of OPIDN include numbness, cognitive dysfunction, ataxia and muscle weakness and the extent and severity of these symptoms greatly depend on the exposure level and intervention measures.

The primary target of OPs that is capable of inducing OPIDN is considered to be neuropathy target esterase (NTE) (Johnson, 1990). OPIDN inducers irreversibly inhibit NTE far more potent than they do for acetylcholinesterase (AChE), the primer target of OPs associated with acute neurotoxicity. This type of delayed neuropathy includes inhibition of axonal transport, impaired nerve regeneration and cytoskeletal disruption (reviewed in Hargreaves, 2012).

2. Characterization of the exposure to the chemicals relevant to the selected AOP

Exposure to OPIDN inducers can occur by multiple routes, including oral or dermal uptake and inhalation (e.g. contaminated dust and spray drift) (Lotti and Moretto, 2005). This can occur via a range of sources including occupational exposure, environmental pollution, intentional or unintentional contaminated food sources, dietary intake, self-poisoning (Jokanović and Kosanović, 2010). It is worth mentioning that some OPs may need to be metabolized into a neuropathic form, as it is in the case of TOCP.

3. Identification of the Molecular Initiating Event (MIE)

In the case of OPIDN, MIE has been identified as the covalent binding of inducers to the active site of the esterase NTE (Johnson, 1975; Johnson MK, 1990). However, not all OPs are capable of inducing OPIDN. It has been found that only those OPs that can cause not only inhibition but also aging of NTE can ultimately induce OPIDN (Johnson, 1975; Johnson MK, 1990). The aging process involves loss of an R-group from the phosphoryl moiety resulting in formation of a negatively charged phosphoryl group that is still covalently bonded to the active site serine of NTE (Richardson et al., 2013).

Several other OPs can inhibit the enzyme activity of NTE without causing its aging and consequently do not produce OPIDN (Richardson et al., 2013). Both in vitro and ex vivo studies using brain tissue from treated hens supported that OP inducers can age NTE whereas other OPs

do not (reviewed in Hargreaves, 2012). These non-ageable NTE inhibitors have been used in the past towards the protection against OPIDN from subsequently administered neuropathic OPs as they have been proved potent blockers of NTE (Richardson et al., 2013).

NTE is known to have lipid hydrolase activity and belongs to the family called patatin-like phospholipase domain-containing proteins and seems to play an important role in axonal and synaptic integrity (Glynn, 2013). Interestingly, mutations in the catalytic domain of NTE in humans lead to the development of a condition that is comparable to OPIDN named NTE-related motor neuron disorder (NTE-MND) (Richardson et al., 2013).

4. Identification of the responses on the cellular/tissue level that may be an adverse outcome or linked to the final adverse outcome

The binding by OPs to NTE is resulting in inhibition of its enzyme activity and disrupted Ca^{2+} homeostasis, activation of Ca^{2+}/calmodulin kinase, altered phosphorylation, distribution and reduced levels of cytoskeletal proteins (Fig. 6). However, the cellular events that occur between NTE inhibition and the clinical manifestation of AO are not completely understood. Although there are experimental studies demonstrating that calcium channel blockers can ameliorate the signs and symptoms of OPIDN by restoring calcium balance, the same has not been noted for NTE enzymatic activity (Guilherme et al., 2012). Another KE is mitochondrial dysfunction resulting in oxidative stress and ATP depletion, which can potentiate the disruption of Ca^{2+} homeostasis leading to the inhibition of axonal transport both in vitro and in vivo that severely affects nerve regeneration processes (Gultekin et al., 2000; Zhang et al., 2007). OP-induced increase in the intracellular free Ca^{2+} concentration activates calpain, which degrades structural proteins, whose cleavage disrupts axonal transport, cell signaling and axonal integrity (Hargreaves, 2012).

5. Identification of the responses on the organ level that may be an adverse outcome or linked to the final adverse outcome

Energy depletion, elevated ROS formation, disruption of Ca^{2+} homeostasis, impaired synaptic signal transmission, cytoskeletal disruption and the ensuing impairment of axonal transport, ultimately result in distal to proximal degeneration of the nerve terminal and neuropathy. Large and thin nerve fibers in the PNS are typically damaged first but the CNS neurons are also affected in some instances (Dobbs, 2009). In the CNS, degeneration of myelin sheets and "swelling" of axons has been observed (Dobbs, 2009).

Histopathological analysis performed postmortem in humans and animals exposed to OPIDN inducers revealed axonal degeneration that initially involves focal but nonterminal areas of the axon and then spreads to damage the entire distal axon. Prior the onset of OPIDN aggregation and accumulation of neurofilaments in peripheral nerves have been described, whereas, after the appearance of OPIDN symptoms changes in mitochondria and their accumulation have been also detected (Jokanović and Kosanović, 2010), pointing out the importance of these two KEs in the pathology of the adverse outcome.

6. Identification of the responses on the organism level that may be the final adverse outcome or linked to the final adverse outcome

OPIDN is considered a rare neurodegenerative disorder in humans and is characterized mainly by the following symptoms due to damage in peripheral nerves (reviewed in Jokanović and Kosanović, 2010):

- Mild effects in sensory organs, e.g. "restless legs", tingling or lost sensation in hands and feet
- Pain in hands and feet, headache

- Muscle weakness / abnormal posture
- Ataxia and gait problems
- Cognitive dysfunction
- Behavioral effects

Physical examination of humans or animals exposed to OPIDN inducers reveled motor polyneuropathy with weakness of distal limb muscles. These findings have been further supported by electrophysiological evaluation of patients diagnosed with OPIDN showing acute denervation of affected muscles with abnormal spontaneous activity (Jokanović and Kosanović, 2010; Lotti and Moretto, 2005). Schematic representation of MIE, cellular key events and organ/organism effects is described in Fig. 6.

7. Identification of the overall effects on the population

Most documented cases of exposure are through food consumption, occupational exposure and environmental pollution. For example, the first major outbreak of OPIDN was found to be caused by the substitution of castor oil with TOCP in 'Ginger Jake' health remedy, resulting in a flaccid paralysis of the extremities in thousands of individuals in the USA during the 1930s (Weiner and Jortner, 1999). Since then a substantial number of clinical cases of OPIDN have been reported and reviewed in Jokanović and Kosanović, 2010 and Lotti and Moretto, 2005.

8. Is the AOP specific to certain life stages (DNT or aging)?

This AOP on OPIDN is specific to the adult life stage. Indeed, adult hens (18 weeks old) are used as animal model of choice to measure NTE and identify OPs that cause OPIDN (Doherty, 2006). In contrast, adult mice treated with known OPIDN inducers do not show clinical signs or axonal degeneration but only few swollen axons in the brain stem (Veronesi et al., 1991). Only

long time of exposure (9 months) to these compounds can cause axonal degeneration and paralysis in mice (Lapadula et al., 1985).

In humans, clinical cases of OPIDN have mainly been reported in adults (Jokanovic et al., 2011). In rare cases of young individuals diagnosed with OPIDN the clinical signs were considerably milder, whereas their recovery was faster and complete compared to adults (Lotti, 1992; Jokanović et al., 2011). It is believed that the reason behind this difference in sensitivity to develop OPIDN depending on the age is related to the repair mechanisms that seem to be more active in young rather than old individuals (Glynn, 2000).

9. How much are initiating and key events conserved across species?

NTE was identified initially in adult vertebrate neuronal tissue. However, NTE is also present in a variety of non-neuronal tissues such as intestine, placenta and lymphocytes. NTE is highly conserved across species including mammals, insects, nematodes, and yeast (Moser et al., 2000). The remaining KEs outlined in this AOP, are also conserved across species and observed in a variety of cellular models.

10. Challenges for further AOP development: strength, data gaps and uncertainties to be considered

Perhaps the clearest evidence for causal links between MIE and the final AO is that pretreatment of experimental animals with reversible inhibitors of NTE prevents the induction of OPIDN. For example, prior administration of certain carbamates, sulphonyl fluorides and phosphinates to hens, cats and/or rats has been found to prevent or significantly ameliorate the symptoms of OPIDN as determined by clinical, histopathological and/or electrophysiological measurements (Carrington, 1989). Although capable of binding to the active site of NTE and inhibiting its activity, these agents do not induce aging.

Studies in genetically modified mice have shown that NTE is important for normal blood vessel and placental development and that the death of embryos is not associated with the deletion of NTE (Moser et al., 2004). On the contrary, conditional mutant strains where NTE was deleted only in neuronal tissue appeared no embryo-lethality. These animals were sacrificed at the age of 3-4 months and histopathological examination revealed vacuolization and a dramatic redistribution of the rough endoplasmic reticulum in the hippocampus and the thalamus, whereas loss of Purkinje cells was also detected in the cerebellum (Akossoglou et al., 2004). Studies with heterozygous ($Nte^{+/-}$) showed that the presence of 40% less brain NTE than in normal mice results in clinical signs and lesions that are not detectable in cases of OPIDN (Winrow et al., 2003). For example, neurodegeneration and loss of endoplasmic reticulum are not encountered in the typical pathology of OPIDN. Similarly, hyperactivity presented in $Nte^{+/-}$ animals is not a clinical symptom related to OPIDN. However, these controversial findings may be attributed to known resistance of mice to OPIDN (Veronesi et al., 1991) and this is probably the reason why the studies with transgenic mice cannot confirm the direct association of NTE with the AO.

Protection to clinical symptoms of OPIDN can also be given by Ca^{2+} channel blockers. These blockers given prior to OP exposure relieve symptoms of OPIDN, most importantly these drugs given after the OP are still able to alleviate clinical symptoms (reviewed in Emerick et al., 2012). Although these experimental data shows that there is strong causal relationship between calcium levels and AO, the same is not true for the relationship between NTE inhibition and calcium. Indeed, no published data is available showing reversibility in NTE levels after treatment with Ca^{2+} channel blockers, rendering this KE relationship moderate.

Ca^{2+} channel blockers also prevent the increase of OP-induced calpain activity in the nerves of hens (reviewed in Song and Xie, 2012). Calpain activation has been suggested to be involved in

the onset and development of OPIDN and its reduced activation by Ca^{2+} channel blockers has been followed by attenuation of clinical symptoms and histopathological findings related to OPIDN (Song and Xie, 2012). However, the same review concluded that further experimental work is required on proteolytic pathways to shed light on the direct or indirect role of calpain in the pathogenesis of OPIDN (Song and Xie, 2012).

Oxidative stress has not been extensively studied in the development of OPIDN and although antioxidants are known to protect from OP acute neurotoxicity, no studies are available about their protective role from OPs that induce delayed neuropathy. The same is truth for some more KEs of this AOP, like mitochondrial and cytoskeletal dysfunction, where the causative relationship between these changes and the NTE inhibition remains to be established.

VI. Adverse Outcome Pathway on: *Impairment of learning and memory induced by binding of electrophilic chemicals to SH(thiol)-group of proteins and non-protein molecules in neuronal and glial cells during development*

Christoph van Thriel and Magdalini Sachana

1. Introduction

Several, chemically unrelated neurotoxins (acrylamide, methylmercury, acetaldehyde, acrolein, etc.) share the physicochemical property of being electrophilic (LoPachin and Barber, 2006). Thus, they share the ability to form adducts or in other words to modify nucleophilic sulfhydryl groups (SH- or thiol-groups) from either low- or high-molecular weight biomolecules (LoPachin and Barber, 2006). This interaction of chemicals with SH-groups has been found to be responsible, partially, for the disturbance of redox cell balance due to increase in reactive oxygen

species (ROS) and decrease in antioxidant capacity (Farina et al., 2011). The induced oxidative stress leads to damage of proteins, lipids and nucleic acids eventually resulting in cell death and neurodegeneration (Andersen, 2004). Furthermore, the binding to SH-groups of synaptic proteins by electrophilic neurotoxicants has also been shown to modulate many pre- and postsynaptic events of neurotransmission, disrupting Ca^{2+} signaling and finally, causing severe adverse neurobehavioral effects (LoPachin and Barber, 2006). During the development of this AOP particular emphasis was given to methylmercury (MeHg) exposure during brain development, as the key cellular events described below are involved in MeHg induced developmental neurotoxicity and its binding to and modification of SH-containing proteins.

2. Short characterization of the exposure to the chemicals relevant to the selected AOP

Mercury (Hg) is available in the environment in three distinct chemical forms (elemental mercury vapor, inorganic mercury salts and organic mercury) and thus, all routes of exposure (e.g. inhalation, dermal, oral) might contribute to its uptake. Mercury compounds are mainly released in the aquatic environment from anthropogenic activities, where they undergo biomethylation causing contamination of fish. MeHg-containing fish meat represents a major source of human exposure. MeHg has high affinity to -SH groups leading to the formation of a MeHg cysteine complex (Cys-SHgMe or MeHg-S-Cys) in fish meat that influence not only the bioavailability but also the neurochemical and neurobehavioral toxicity of the parent compound (Harris et al., 2003; Berntssen et al., 2004). By mimicking the essential proteinogenic amino acid methionine, cysteinyl-bound MeHg can initially be absorbed by an organism and then be taken up by cells and redistributed into subcellular compartments. This alikeness to methionine can facilitate the crossing of MeHg cysteine complex through specific barriers (e.g. BBB) via

specialized amino acid transporters (Kerper et al., 1992). Thus, this MIE can influence not only the toxicodynamics but also the toxicokinetics of the electrophilic neurotoxicants.

3. Identification of the Molecular Initiating Event (MIE)

MeHg reacts with specific cysteine residues (SH-groups) on astrocytic proteins like Glu transporters and small molecules such as glutathione (GSH) (LoPachin and Barber, 2006; Farina et al., 2011). MeHg interacts with SH-groups of GSH not only in astrocytes but also in neuronal cells as it is the most abundant intracellular low molecular weight molecule in all organs, including CNS and this interaction has been demonstrated both in vitro and in vivo (Farina et al., 2011). However, this is not the only target of MeHg that leads to increased levels of reactive oxygen/nitrogen species (ROS/RNS). The direct binding by MeHg to specific SH-containing proteins in mitochondria of neuronal cells, including respiratory chain complexes and mitochondrial creatine kinase found in vitro and in vivo further contributes to imbalances in oxidative metabolism and increased levels of ROS/RNS (Farina et al., 2011).

Furthermore, electrophilic compounds have been proposed to interact with SH-groups of proteins involved in pre- and post-synaptic processes related to neurotransmitter storage, release, uptake and binding in neurons (LoPachin and Barber, 2006), thus affecting both intra- and extra-cellular neurotransmitter homeostasis. In case of MeHg, Glu dyshomeostasis occurs not only by inhibiting Glu uptake into astrocytes but also by increasing the spontaneous release of Glu (Farina et al., 2011). Moreover, disturbance in neurotransmission by MeHg is not only limited to Glu but extents also to GABA neurotransmission (Sadiq et al., 2012).

Finally, MeHg and other electrophilic neurotoxicants form adducts with SH-groups that also function as acceptors for redox modulators such as nitric oxide (NO) (Taqatqeh et al., 2009) that is known to affect various protein complexes at synapse level (e.g. NMDARs) (LoPachin and

Barber, 2006). While some of the modulatory processes (e.g. NO redox modulation) are reversible and relevant for normal physiology (Lei et al. 1992), electrophilic neurotoxins (e.g. acrylamide, acrolein) might cause long-lasting SH-group binding that subsequently causes molecular and cellular events that are not reversible. Such an effect is also described for cyanide (Sun et al., 1999). Clearly, it can be stated that targeting of SH-containing proteins and non-protein molecules of neuronal and glial cells during development can be the MIE that can potential trigger subsequent key cellular events that are described below, ultimate resulting in impairment of learning and memory.

4. Identification of the responses on the cellular/tissue level that may be an adverse outcome or linked to the final adverse outcome

The described MIE can initiate a cascade of parallel events by targeting glutamatergic synapses, permanently blocking or modifying normal neurotransmission. MeHg has been found to interact with SH-groups from proteins involved in the modulation of intracellular Ca^{2+} levels such as ligand-, voltage-gated channels and transporters that can promote or block the release, storage and uptake of neurotransmitters (Farina et al., 2011). Importantly, MeHg exposure leads to postsynaptic intracellular increase of Ca^{2+} and activation of important pathways involved in cell death, which follows the increased levels of extracellular Glu (reviewed in Ni et al., 2012). In addition, the elevated Glu levels may result also in overactivation of NMDARs leading to neuronal cell death induced by excitotoxicity (Berliocchi et al., 2005) (see AOP II on *Binding of agonist to NMDA receptor causes excitotoxicity that mediates neuronal cell death, contributing to reduction (or loss) of cognitive, sensory and motor function*).

Moreover, it is well documented that MeHg can disrupt mitochondrial structure and function by binding to the specific SH-containing proteins of the respiratory chain complexes and

mitochondrial creatine kinase (reviewed in Farina et al., 2011), resulting in mitochondrial dysfunction and ROS overproduction. Mitochondrial damage contributes to many pathways of toxicity, potentially leading to neurodegeneration (see AOP VII on *Binding of inhibitors to the mitochondrial respiration chain complex I, II, III or IV or interaction of uncouplers with oxidative phosphorylation decreases or blocks ATP production resulting in neurodegeneration*). This mitochondrial dysfunction further causes Ca^{2+} overload and stimulation of neuronal nitric oxide synthase (nNOS) increasing production of NO that nitrosylates a specific cysteine thiolate on both the NR1 and NR2A subunits and final blockage of NMDA receptor (LoPachin and Barber, 2006) (see AOP I on *Binding of antagonist to an NMDAR during synaptogenesis contributes to impairment of learning and memory abilities*). It has been suggested that this nitrosylation of brain proteins may be involved in neurotoxicity and neurodegenerative disorders (Brown, 2010).

Although it is well documented that disruption of Ca^{2+} homeostasis and mitochondrial function play a central role in MeHg-induced neurotoxicity, it has not been yet elucidated whether these changes are primary or secondary events after interaction of MeHg with other cellular components. Furthermore, it is not clear if more mechanisms (e.g. ROS production) rather than direct binding of MeHg to astrocyte Glu transporters interferes with the oxidative and inhibitory effect on the same transporters.

Besides the induced oxidative stress, MeHg exposure can also directly bind to SH-group of low molecular weight compounds involved in the antioxidant defense mechanisms. MeHg has been found to bind to GSH reducing the functionality of this important antioxidant in ROS scavenging, contributing further to the imbalance in oxidative metabolism and the increased

levels of ROS. It is worth mentioning that CNS is particularly susceptible to oxidative insults and is, therefore, very dependent on its GSH content, especially during development.

Such multiple and interactive molecular and cellular events might cause disturbance in neuronal cell physiology and morphology, especially during development (e.g. reduce activity dependent neuronal plasticity), resulting in the neurobehavioral phenotype of MeHg exposure.

5. Identification of the responses on the organ level that may be an adverse outcome or linked to the final adverse outcome

Even without the presence of neurodegeneration, perturbation of synaptic neurotransmission by redox modulation of transmitter-gated ion channels can cause adverse effects as seen in the pathophysiology of seizures (Sanchez et al., 2000). Impaired GSH functioning is relevant in schizophrenia models and is an early biomarker of nigral neuron degeneration (Do et al., 2009). However, chronic exposure to SH-binding neurotoxins can lead to neurodegeneration caused by the downstream pathways triggered by altered intracellular Ca^{2+} concentrations as well as by the increased oxidation of cellular proteins, lipids and nucleic acids.

6. Identification of the responses on the organism level that may be the final adverse outcome or linked to the final adverse outcome

Some electrophilic compounds such as MeHg and acrylamide that are capable of interacting with functional cysteine residues of proteins are known or suspected human developmental neurotoxicants, respectively (Ko et al., 1999; Sorgel et al., 2002; Farina et al., 2011). Experimental evidence has shown that the developing CNS is more susceptible to neurotoxic effects of MeHg than the adult brain (Grandjean and Landrigan, 2006). These findings were further supported by epidemiological studies showing that prenatal or early postnatal exposure to this electrophile can cause decrease in IQ, mental retardation, dose-related impairments in

memory, attention, language and visuospatial perception (Bellinger, 2013) or impaired test scores in standardize neuropsychological tests (White et al., 2011). Schematic representation of MIEs, cellular KEs and organ/organism effects is described in Fig. 7.

7. Identification of the overall effects on the population

The population effects of MeHg exposure have recently been described in a review of several suspected DNT compounds (Bellinger, 2013). Apart from IQ loss, MeHg has also been found to contribute to neurodevelopmental disorders, including autism (Kern et al., 2012), defective learning and memory processes and Attention deficit hyperactivity disorder (ADHD) (Bellinger, 2013).

8. Is the AOP specific to certain life stages (DNT or aging)?

Since some functions of the various receptors change across certain life stages (e.g. GABAergic signaling in the developing brain) the AOP might be more relevant for particular stages of development. Aging is associated with a lot of neurobiological changes including the expression of glutamatergic receptors (Hof et al., 2002) and thus, this AOP might also be relevant during aging.

In astrocytes, GSH production is dependent not only on the l-γ-glutamyl-cysteine synthase but also on available cellular Glu levels (Wu et al., 2001; Mates et al., 2002). Interestingly, astrocytes are tightly related to glutamatergic transmission and antioxidant defense (Araque and Perea, 2004; Takuma et al., 2004) and play important role during different processes of brain development, including migration and synaptogenesis, pointing out their importance in MeHg-induced developmental neurotoxicity.

The decreased GSH levels in the CNS have been reported to be more pronounced in young rather than adult rodents exposed to MeHg (Farina et al., 2011). Indeed, it is well known that the

developing brain is even more vulnerable to oxidative stress than mature brain because it contains limited amounts of protective enzymes and antioxidants and is formed by higher number of neurons than glia, while at the same time it is characterized by increased metabolic demand related to growth.

9. How much are initiating and key events conserved across species?

As described in the AOP I *Binding of antagonist to an NMDAR during synaptogenesis contributes to impairment of learning and memory abilities* and AOP II. *Binding of agonist to NMDA receptor causes excitotoxicity that mediates neuronal cell death, contributing to reduction (or loss) of cognitive, sensory and motor function,* the cellular and molecular targets (e.g. NMDARs) are well conserved across species. The same is true for the antioxidant machinery that is affected by toxin-binding to GSH (Nava et al., 2009).

10. Challenges for further AOP development: strength, data gaps and uncertainties to be considered

For some aspects of this AOP (e.g. NMDA receptor mediated excitotoxicity) there is evidence for cause-effect relationship, however, the direct effects of electrophilic neurotoxins on regulatory sites within the synapses are not fully elucidated. Only for acrylamide solid evidence for neurotoxicity that is partly caused by redox signaling in the synaptic compartment exists (LoPachin and Gavin, 2012). For the same electrophile there also experimental studies showing that presynaptic buildup of cysteine adducts is progressive and closely correlated to the development of acrylamide neurological symptoms (LoPachin and Gavin, 2012). In the case of MeHg, although there is a good understanding of the KEs that mediate its neurotoxicity, our understanding of the primary critical targets of MeHg remains limited, meaning that it is not clear if mitochondrial dysfunction and Ca^{2+} homeostasis disruption occur prior or after the MeHg

interaction with key protein or no-protein molecules (Farina et al., 2011). As mentioned before, the initial idea of this AOP is based on organic chemistry and the general principle that electrophiles form covalent bonds with nucleophiles. Such an approach is relevant for computational toxicity but large batteries of test compounds have not been tested with respect to their affinity for regulatory cysteine residues yet. Moreover, the way in which such a covalent binding would affect the functionality of the synapse and subsequently the whole brain has not been studied in sufficient detail to identify causal links between the MIE and KEs.

It is important to emphasize that there are no available comparative studies among developing and adult brain on the differential expression of specific SH-containing targets for MeHg or other compounds that would be covered by this AOP. Additionally, studies are lacking that identify cellular events prone to MeHg electrophilicity that are specific to critical periods of brain development.

Besides the interaction of electrophilic neurotoxicants to SH-containing proteins and non-protein molecules, these compounds are known to bind to another group of proteins named selenoproteins much earlier and at a higher level (Farina et al., 2011), pointing out the possibility for multiple MIEs in the presented AOP that could potentially be explored in the future. Different organic selenocompounds have been proposed as potential therapeutic agents to MeHg-induced neurotoxicity as seleno-containing intermediates are produced and interact with MeHg in a higher affinity, protecting by this way the SH-containing biomolecules (Farina et al., 2013).

VII. Adverse Outcome Pathway on: *Binding of inhibitors to the mitochondrial respiration chain complex I, II, III or IV or interaction of uncouplers with oxidative phosphorylation decreases or blocks ATP production resulting in neurodegeneration.*

Alicia Paini, Brigitte Landesmann, Jochem Louisse and Anna Bal-Price

1. Introduction

In the CNS mitochondria play a pivotal role in neuronal and glial cell survival and cell death because they are regulators of both energy metabolism and apoptotic/necrotic pathways (Fiskum, 2000; Wieloch, 2001; Friberg and Wieloch, 2002). The production of the ATP via oxidative phosphorylation (OXPHOS) is critical for maintaining ionic gradients across the cell membranes necessary for neuronal excitability and for execution of complex processes such as neurotransmission and plasticity (Kann and Kovács, 2007; Nunnari and Suomalainen, 2012). In addition, mitochondria are also involved in numerous other cellular functions including Ca^{2+} signalling, steroid synthesis (Kang and Pervaiz, 2012), lipid and phospholipid metabolism, and the biosynthesis of essential intermediates, including heme and iron-sulfur clusters (Green, 1998; Hajnóczky et al., 2006; McBride et al., 2006). They also contribute to various cellular stress responses, such as deregulation of cellular Ca^{2+} homeostasis (Graier et al., 2007), ROS production and release of pro-apoptotic factors (Nunnari and Suomalainen, 2012). In general mitochondrial dysfunction is considered to be an early event in neurotoxicity. Exposure to xenobiotics can cause mitochondrial damage leading to decreased (or entirely blocked) ATP production triggering a cascade of events culminating in apoptotic and/or necrotic neuronal cell death.

The mitochondrion is formed by an outer and inner membrane, which create two separate compartments, the internal matrix and an intermembrane space. The electron transport chain (ETC) (Fig. 8A), embedded in the inner membrane, transfers electrons from the matrix into the complexes (I-IV). The electrons lose free energy at each step (complex I, III, and IV), generating a proton gradient used for the production of ATP in the matrix by complex V (Alberts et al., 2002). Due to their structural and functional complexity, mitochondria present multiple targets for compounds and numerous AOPs can be developed related to mitochondrial dysfunction. Mitochondrial dysfunction can be caused by direct interference with one of the ETC complexes (I-IV) (MIE) or as further downstream KE involved in different toxicity mechanisms induced by various mitochondria-independent MIEs (see AOP VIII on *Multiple molecular initiating events trigger neuroinflammation leading to neurodegeneration*). The described framework can serve as an initial base for further development of multiple AOPs related to mitochondrial dysfunction resulting in different adverse outcome depending on the affected brain structure.

2. Short characterization of the exposure to the chemical relevant to the selected AOP

Several classes of chemicals, entering the body via oral, dermal, or inhalation exposure, are known to induce neurotoxicity via mitochondrial dysfunction by different mechanisms. Well-studied examples of chemicals that induce neurotoxic adverse effects via mitochondrial dysfunction are pesticides such as rotenone, paraquat and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which inhibit complex I (Desplats et al., 2012; Lin et al., 2012; Sava et al., 2007). Also under pathological conditions (e.g. inflammation) endogenously formed increased amounts of nitric oxide can compromise mitochondrial respiration through inhibition of Complex IV (Bal-Price and Brown, 2001) causing excitotoxicity resulting in neurodegeneration (Brown and Bal-Price, 2003) (see AOP on *Binding of agonist to NMDA*

receptor causes excitotoxicity that mediates neuronal cell death, contributing to reduction (or loss) of cognitive, sensory and motor function). Certain drugs, such as gramicidins, act as uncouplers of OXPHOS (Katsu et al., 1987; Luvisetto and Azzone, 1989). These examples indicate that sources of exposure can be diverse, ranging for example from occupational exposure (pesticides) to food and drug intake.

3. Identification of the Molecular Initiating Event (MIE)

There are two main mechanisms underlying the MIEs that cause the common KE triggered by inhibition of mitochondrial respiration. The MIE can be induced by:

1) Chemicals that directly bind to the complexes of the respiratory chain leading to the inhibition of ATP production. They may inhibit each of the four complexes of the ETC or ATP synthase directly (Wallace and Starkov, 2000).

2) Chemicals that act through uncoupling of OXPHOS, by acting as alternative electron acceptors since they (a) accept electrons from the ETC and feed them back at the site of higher redox potential, or (b) become reduced by an electron carrier of the respiratory chain, producing heat (Rousset et al., 2004).

4. Identification of the response on the cellular/tissue level that may be an adverse outcome or linked to the final adverse outcome.

Inhibition of the complexes of the respiratory chain or uncoupling of OXPHOS primarily results in mitochondrial dysfunction eventually resulting in neuronal and/or glial cell death. Thereby, mitochondria dysfunction triggered by inhibition of mitochondrial respiration or uncoupling of oxidative phosphorylation results in the decreased ATP level that is linked in a causative manner to the following events observed at the cellular level: (a) the loss of the mitochondrial membrane potential, (b) the loss of mitochondrial protein import and protein biosynthesis, (c) reduced

activities of enzymes of the mitochondrial respiratory chain and the Krebs cycle, (d) elevated levels of reactive oxygen species (ROS) production, (e) the loss of mitochondrial motility, causing a fail to re-localize to the sites with increased energy demands, such as synapses (f) the destruction of the mitochondrial network, and (g) increased mitochondrial Ca^{2+} uptake, causing Ca^{2+} overload (Graier et al., 2007), (h) the rupture of the mitochondrial inner and outer membranes, leading (i) to the release of mitochondrial pro-death factors, including cytochrome *c* (Cyt. *c*), apoptosis-inducing factor, or endonuclease G (Braun, 2012; Martin, 2011; Correia et al., 2012; Cozzolino et al., 2013).

This leads to overproduction of free radicals, activation of cell death signalling pathways and up regulation of inflammatory mediators (see AOP VIII on *Multiple molecular initiating events trigger neuroinflammation leading to neurodegeneration*). Indeed, excessive Ca^{2+} uptake into mitochondria has been shown to inhibit ATP synthesis, breakdown and depletion of mitochondrial phospholipids in cellular membranes (Pieczenik and Neustadt, 2007) inducing mitochondrial permeability transitions pore (mPTP) opening. This results in mitochondrial swelling, which can lead to the release of proapoptotic proteins such as cytochrome c, Smac/Diablo, HtrA2/Omi, etc., triggering caspase-dependent apoptosis. Severe mitochondrial cytochrome c release is also a precursor of necrotic cell death (Lewen et al., 2000). Whether a cell dies by apoptosis or necrosis following a specific compound treatment, dependents on cell type and ATP levels (Bal-Price and Brown, 2000). Low levels of ATP (< 30% of control) inhibit apoptotic cell death in favour of necrosis.

5. Identification of the response on the organ level

Chemicals, that act as inhibitors of mitochondrial ETC complexes or as uncouplers of OXPHOS, can induce necrotic and/or apoptotic cell death. Induced cell death triggered by mitochondrial

dysfunction could contribute to the pathology of various neurodegenerative disorders depending on the extent and the affected brain structure where cell death takes place. Dopaminergic neurons in the substantia nigra (SN, pars compacta) have been found to be particularly sensitive to oxidative stress and inhibition of mitochondrial respiration is the key events that contribute to the pathological features of Parkinson's disease (PD) (Perfeito et al., 2013). Environmental chemicals such as rotenone or MPTP have been reported to induce selective degeneration of the nigrostriatal pathway leading to formation of alpha-synuclein-positive inclusions in dopaminergic neurons of the SN, possibly by inhibiting mitochondrial complex I and increasing oxidative stress (Alam and Schmidt, 2002; Fleming et al., 2004; Sherer et al., 2003). As a result, the dopamine release in the striatum is reduced affecting processes related to motor control and various cognitive functions. Recently, increased risk for PD was found in amphetamine users, possibly due to amphetamine induced mitochondrial dysfunction, oxidative stress and α-synuclein aggregation (Perfeito et al., 2012). Pesticides, such as paraquat and rotenone caused selective cell death of dopaminergic neurons, resulting in specific lesions in the substantia nigra and striatum (Costello et al. 2009, Wu et al. 2013), suggesting that these toxicants may be associated with Parkinson's disease.

Oxidative stress and diminished energy metabolism also occur in the brains of Alzheimer's disease (AD) patients, mainly in such structures as locus coeruleus, frontal, occipital and mid-temporal cortex as well as caudate. In particular, permeability transition pore formation is strongly linked with neurodegenerative disorders including AD (Rao et al., 2013).

6. Identification of the response on the organism

A neurotoxicant-induced decrease of dopaminergic neurons and the resulting decrease in motor control can lead to severe adverse responses in the organism. A decrease in locomotor activity

and an increase in catalepsy (muscular rigidity), resulting from the degeneration of dopaminergic neurons, have been observed in animals following rotenone treatment (Alam and Schmidt, 2002; Fleming et al., 2004; Sherer et al., 2003). Since many of the responses induced by rotenone (and other complex I inhibitors, such as MTPT) in rodents are similar to the symptoms of Parkinson's disease (Betarbet et al., 2000; 2002), chemicals that act as inhibitors or uncouplers of mitochondrial respiration could strongly contribute to the pathology of Parkinson disease. However, the specific AO caused by inhibition of mitochondrial respiration or uncoupling of oxidative phosphorylation will depend on the cell type of the brain structure where these MIEs will be triggered. Here the description of the AO is linked to PD as the dopaminergic neurons are one of the most sensitive to mitochondria damage and oxidative stress suggesting that the key events described at the cellular level (Fig. 8B) could contribute to the overall pathology of the Parkinson disease, however they are other neuropathologies lined to the same MIE and KEs described in this outlined AOP. Schematic representation of MIE, cellular key events and organ/organism effects is described in Fig. 8B.

7. Identification of the overall effects on the population

Mitochondrial damage is a hallmark for Alzheimer's, Parkinson's, Huntington's diseases, and amyotrophic lateral sclerosis (Martin, 2011; Correia et al., 2012; Cozzolino et al., 2013). These neurodegenerative diseases represent a real concern for public health and for health care costs. Particularly, the prevalence of patients with Alzheimer's and Parkinson's disease increases dramatically with aging (Kawas et al. 2000, de Lau and Breteler 2006). For both diseases, less than 5 percent of the cases are genetically transmitted, suggesting that environmental factors are involved in the most common idiopathic form (Tsang and Soong 2003). In humans, there is epidemiological evidence linking Parkinson's disease to exposure to pesticides such as rotenone

(Tanner et al., 2011) and paraquat (alone or in combination with other environmental contaminants, particularly maneb) (Costello et al. 2009, Berry et al. 2010, Wang et al. 2011a).

8. Is the AOP specific to certain life stages (DNT or aging)?

Mitochondrial dysfunction can lead to neuropathology at any life stage (development, maturation or aging). However, the kind of adverse outcome is life-stage dependent. Therefore the development of a specific AOP related to mitochondrial dysfunction must be life stage specific and for *in vitro* testing the applied cell model and endpoints must be selected accordingly. It is well documented that mitochondria play a central role in the process of brain development and aging across different species (Bratic and Larsson, 2013; Lee and Wei, 2012). During brain development high energy demanding processes are taking place (e.g. cell proliferation, migration differentiation etc.) and decreased level of ATP will affect these critical developmental processes. A wide spectrum of alterations in mitochondria are described in the course of human aging including (a) increased disorganization of mitochondrial structure, (b) decline in mitochondrial oxidative phosphorylation (OXPHOS) function, (c) accumulation of mtDNA mutations, (d) increased mitochondrial production of ROS and (e) increased extent of oxidative damage to DNA, proteins, and lipids. Therefore adverse outcomes from chemical-induced mitochondrial damage can be further potentiated with increasing age. This age-dependence is even more pronounced in women, as estrogens and estrogen receptors play a pivotal role in regulating energy expenditures and protecting against oxidative stress in the mitochondria. Estrogen, androgen and progesterone receptors are also found in human brain (Henderson and Diaz Brinton, 2010). Peroxide production by mitochondria (in particular brain synaptic mitochondria) from males is higher than that from females of the same age and the mitochondrial glutathione content is higher in females than in males (Borras et al., 2003). Hormonal deficit in

post-menopausal women has been proposed to be one risk factor in AD since two thirds of AD patients are women (Grimm et al 2012). There is a greater incidence of PD in men than in women (Baldereschi et al., 2000), persisting across age groups (Baldereschi et al., 2000; Bower et al., 1999). Further, age at onset tends to be later in women compared to men, though more data are needed in this area (Pavon et al., 2010).

9. How much are initiating and key events conserved across species?

The key events described in Fig. 8B are general mitochondria-related events, which can be observed in animal models used for toxicity testing, as well as in humans. However, the sensitivity of different species to these chemicals in relation to critical changes in key events and resulting adverse outcomes differ and have to be studied on a case by case basis, taking into consideration which brain structure is affected.

10. Challenges for further AOP development: strength, data gaps and uncertainties to be considered

The development of neurodegenerative diseases gives some evidence for the link between mitochondrial injury and human neuropathology. The information collected on mitochondrial respiration inhibitors and uncouplers of oxidative phosphorylation (MIEs) provide correlative evidence that the described cellular key events are linked to neuronal cell death (Fig. 8B). In animals, such mitochondrial damage-related neuronal cell death is linked to different types of adverse outcomes, dependent on the brain structure that is affected. However, the causative relationship between the same key events induced by described MIEs leading in a causative manner to a specific neurodegenerative disorders in humans is not that clear. Indeed, a major unknown is how alternations in ubiquitous no-specific cellular processes such as mitochondrial and oxidative phosphorylation lead to specific adverse neurological outcomes. Additional

complexity of the issue is added by the various possible windows of exposure from neurodevelopment through the aging, which might drive the neurodegenerative outcome, sometimes taking place many years later on. Further development of this AOP should be focused on providing dose- and time-and dynamic studies that demonstrate how these common cellular events are linked to cellular changes in the specific brain structure, potentially contributing to a defined neurodegenerative disorder relevant to a brain structure where neurodegeneration takes place.

VIII. Adverse Outcome Pathway on: *Multiple molecular initiating events trigger neuroinflammation leading to neurodegeneration.*

Florianne Monnet-Tschudi and Anna Bal-Price

1. Introduction

Neuroinflammation is can be triggered by several cellular key events (Wyss-Coray and Mucke, 2002), such as neuronal stress, injury, or death (Kreutzberg, 1995; Kreutzberg, 1996; Monnet-Tschudi et al., 2007), demyelination (Defaux et al., 2010), or direct activation of microglia and astrocytes by neurotoxicants (Eskes et al., 2002; Eskes et al., 2003). When neuroinflammation becomes chronic (Kraft and Harry, 2011) and/or acquires a neurodegenerative phenotype (Kigerl et al., 2009), it can lead to neurodegeneration, which is the adverse outcome. Indeed neuroinflammation is a component of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Multiple Sclerosis (Neumann, 2001), playing a secondary or an active primary role in the disease process (Hirsch and Hunot, 2009). As several classes of toxicants are able to induce neuroinflammation, the MIEs can be multiple.

Neuroinflammation is characterized by the activation of both microglial cells and astrocytes (Graeber and Streit, 1990; Aschner, 1998, Streit et al., 1999; Monnet-Tschudi et al., 2007). When activated, both glial cell types undergo changes in cell morphology and physiology, accompanied by increased expression and/or release of pro-inflammatory cytokines, chemokines, eicosanoids, metalloproteins, and stress proteins (Dong and Benveniste, 2001), as well as by the production of reactive oxygen (ROS) and nitrogen species (RNS) (Brown and Bal-Price, 2003).

Neuroinflammation can have both neuro-protective/-reparative and neurodegenerative consequences (Monnet-Tschudi et al., 2007). Under normal physiological conditions, microglial cells participate in surveillance of immune status (Kreutzberg, 1995; Kreutzberg, 1996; Aloisi, 2001; Rivest, 2009) and of neuronal integrity (Nimmerjahn et al., 2005). In the event of brain cell stress or injury, microglial and astrocytic cells are activated. Within the spectrum of microglial activation, two distinct activation states are described (Perego et al., 2011; Gordon 2003; Ponomarev et al., 2005; Maresz et al., 2008; Mosser and Edwards, 2008; Kigerl et al., 2009): The M1 activation state is induced by interferon-gamma and/or other pro-inflammatory cytokines and is characterized by increased expression of integrin alpha M (Itgam) and cluster of differentiation 86 (CD86) and the release of pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-6) and ED1 staining, and generally causes neurodegeneration. The M2 activation state depends on the presence of IL-4 and IL-13 (Ponomarev et al., 2007; Maresz et al., 2008; Perego et al., 2011) and induces the expression of mannose receptor 1 (MRC1), arginase1 (Arg 1) and Ym1/2; it is involved in repair processes.

Chronic neuroinflammation can induce secondary injury (Kraft and Harry, 2011), and appears to play a role in the onset and pathological outcome of Alzheimer's and Parkinson's diseases (McNaull et al., 2010; Tansey and Goldberg, 2009), motor neuron diseases, multiple sclerosis,

meningitis and AIDS dementia (Brown and Bal-Price, 2003). McNaull and coworkers (McNaull et al., 2010) suggested that early developmental onset of brain inflammation could be linked to late onset of Alzheimer's disease. Several reports suggest that exposure to heavy metals during development showed delayed symptoms, or cause silent damage, that are revealed only under conditions that challenge the functional capacities (Stern et al., 2001; Fortune and Lurie, 2009). Heavy metal exposure triggers a neuroinflammatory response (Tiffany-Castiglioni et al., 1989; Charleston et al., 1994; Pompili et al., 2004; Monnet-Tschudi et al., 2007; White et al., 2007), and is considered to be a risk factor in the etiology of Alzheimer's disease (Mutter et al., 2004; Wu et al., 2008). Similarly, neuroinflammation was also observed following pesticide exposure (Zurich et al., 2004; Binukumar et al., 2011; Mitra et al., 2011) and is associated with Parkinson's disease (Wang et al., 2011a).

2. Characterization of the exposure to the chemicals relevant to the selected AOP

The neurotoxic potential of environmental chemicals depends on their ability to cross the BBB. Entry into the brain depends on exchange between three compartments, the plasma, the cerebro-spinal fluid and the brain parenchyma, taking into account also possible efflux mechanisms, drug binding and drug metabolism (Liu et al., 2008). Toxicants can use amino acid or ion transporters to cross the BBB (Shimizu et al., 2001; Ose et al., 2010; Wang et al., 2011b; Corvino et al., 2013; Farina et al., 2013).

Regarding the characterization of the exposure itself, long-term exposure to low doses of toxicants is more relevant to human environmental exposure possibly linked to neurodegenerative disorders. Widespread neurodegeneration can take years to develop. It was shown in humans and monkeys that microglial activation and chronic neuron damage continues

for years after the initial exposure, suggesting that active pathology continues for a long time after the toxin has been metabolized and eliminated (Taetzsch and Block, 2013).

3. Identification of the Molecular Initiating Events (MIEs)

As several classes of toxicants are able to trigger a neuroinflammatory response, the molecular initiating events can be very diverse. Binding to thiol and/or selenol containing proteins (See AOP VI on *Impairment of learning and memory induced by binding of electrophilic chemicals to SH(thiol)-group of proteins and non-protein molecules in neuronal and glial cells during development*) leads to modification of the oxidation state of proteins and/or depletion of glutathione, or interferences with the respiratory chain in mitochondria (see AOP VII on *Binding of inhibitors to the mitochondrial respiration chain complex I, II, III or IV or interaction of uncouplers with oxidative phosphorylation decreases or blocks ATP production resulting in neurodegeneration)* are MIEs described for mercury and the herbicide paraquat, respectively (Farina et al., 2013). They lead to oxidative stress as an intermediate cellular key event. Binding of glutamate to NMDA receptors can induce either excitotoxicity (see AOP II on: *Binding of agonist to NMDA receptor causes excitotoxicity that mediates neuronal cell death, contributing to reduction (or loss) of cognitive, sensory and motor function)* such as described for the heavy metal trimethyl tin (TMT) and kainate (Jeong et al., 2011; Little et al., 2012; Corvino et al., 2013), or synapse impairment, as after lead (Pb^{2+}) exposure, which is a potent non-competitive NMDA receptor antagonist (see AOP I on *Binding of antagonist to an NMDAR during synaptogenesis contributes to impairment of learning and memory abilities*) (Neal and Guilarte, 2010). Inhibition of acetylcholinesterase (AChE), as triggered by the organophosphates soman and parathion (Zurich et al., 2004; Collombet et al., 2007), or other unknown molecular events leading to interferences with Sonic Hedgehog, or 15-deoxy-Δ prostaglandin J_2 (15d-PGJ_2)

signaling pathways following low dose exposure to the mycotoxin ochratoxin A (Hong et al., 2002; Zurich et al., 2005; Sandström et al., submitted), are also possible MIEs.

4. Responses on the cellular/tissue level

Effects on neurons

The intracellular calcium (Ca^{2+}) overload due to glutamate dyshomeostasis and excitotoxicity (see AOP II on *Binding of agonist to NMDA receptor causes excitotoxicity that mediates neuronal cell death, contributing to reduction (or loss) of cognitive, sensory and motor function*) can lead to cytoskeletal disruption or apoptosis. Such a cascade of key events has been described following mercury (Sanfeliu et al., 2003) or TMT exposure (Corvino et al., 2013). Cytoskeleton instability has also been observed following ochratoxin A treatment, as evidenced by a decrease in the expression of the non-phosphorylated and the phosphorylated form of neurofilaments heavy chain (Sandström et al., submitted). Oxidative stress caused by paraquat exposure, can lead to neuronal death by apoptosis and secondary induction of a neuroinflammatory response (Choi et al., 2010; Klintworth et al., 2009). Thus it is generally well accepted that neuronal stress or mild neuronal injury (Nimmerjahn et al., 2005), as well as changes in excitability (Janigro and Costa, 1987) are sufficient to trigger a neuroinflammatory response with a possible rescue process. However, when neuronal cell death is occurring, the neuroinflammatory response has the potential to exacerbate this state and lead to a sustained neurodegenerative process (Eskes et al., 2003).

Effects on oligodendrocytes

Although oligodendrocytes are very sensitive to oxidative stress and to excitotoxicity (Gonsette, 2008), demyelination is not often described as a primary target after a neurotoxic insult, but it may occur rather as secondary effect, due to neuronal/oligodendroglial interactions (Zoupi et al.,

2011). Ochratoxin A was shown to induce a demyelination by decreasing myelin basic protein and myelin oligodendrocyte glycoprotein content. It may also interfere with oligodendrocyte maturation by decreasing the expression of markers of maturation (Sandström et al., submitted).

Effects on microglial cells

The heavy metals mercury and TMT are able to activate directly microglial cells, modulating thus cytokine release (Eskes et al., 2002). Presence of microglial cells appeared to be necessary for paraquat-induced dopaminergic neurotoxicity (Wu et al., 2005; Taetzsch and Block, 2013), suggesting a direct effect of paraquat on microglia. In a histotypic environment, microglial cells may become reactive secondary to neuronal or oligodendroglial stress. Such observations were made *in vivo* following sub-clinical long term methyl mercury (Charleston et al., 1994; Charleston et al., 1996), TMT (Corvino et al., 2013) or paraquat exposure (Mangano et al., 2012) and *in vitro* after mercury and TMT treatments (Figiel and Dzwonek, 2007; Monnet-Tschudi et al., 1995a;b). Recently, ochratoxin A exposure was shown to cause microglial activation expressing the M1 degenerative phenotype with an upregulation of the pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and a downregulation of the anti-inflammatory cytokine (IL-4). This response was downstream of ochratoxin-induced effects on neurons, astrocytes and oligodendrocytes (Sandström et al., submitted).

Effects on astrocytes

It is generally accepted that cytokines (IL-1β, IL-6) released by reactive microglial cells induce astrocyte reactivity, called astrogliosis (Banati et al., 1993; Van Wagoner and Benveniste, 1999). Astrogliosis is characterized by an upregulation of glial fibrillary acidic protein (GFAP), a cytoskeleton protein (Eng et al., 2000). Astrogliosis was observed *in vivo* and *in vitro* after TMT (Monnet-Tschudi et al., 1995a; Monnet-Tschudi et al., 1995b; Pompili et al., 2004), mercury

(Charleston et al., 1996; Monnet-Tschudi et al., 1996; Roda et al., 2008) and paraquat exposure (McCormack et al., 2002). In addition, 27 compounds out of 86 with different chemical properties and undefined MIEs, tested in the EU project AcuteTox, were able to induce an upregulation of GFAP mRNA expression attesting for astrocyte reactivity (Zurich et al., 2013). Ochratoxin A caused an atypical astrocyte reaction with a decrease of GFAP expression (Zurich et al., 2005). This unusual astrocyte reactivity is accompanied by a decrease of metallothioneins (Sandström et al., submitted), localized in astrocytes (Miyazaki et al., 2011). A treatment with metallothioneins during ochratoxin exposure caused a decrease of microglial reactivity, suggesting that in the case of ochratoxin the astrocyte perturbations are upstream of the microglial reactivity (Sandström et al., submitted).

Following specific toxicant-induced cellular perturbations, cross-talk between the different neural cells play a crucial role in the triggering, control, evolution, and consequences of neuroinflammation. As example, microglial reactivity following neuronal stress can be due to a loss of chemokine control (Blank and Prinz, 2013; Chapman et al., 2000; Streit et al., 2001). Whereas demyelination- or neural cell death-induced neuroinflammation can be triggered by early cytokines/chemokines production by the different neural cells themselves (Peferoen et al., 2013) or the release of intracellular content acting on specific receptors such as DAMPS (Damage Associated Molecular Pathways) (Marin-Teva et al., 2011). The mechanisms by which activated microglia and astrocytes can kill neurons and induce/exacerbate the neurodegenerative process has been suggested to include the release of nitric oxide that causes inhibition of neuronal respiration, ROS and RNS production, and rapid glutamate release resulting in excitotoxic death of neurons (Brown and Bal-Price, 2003; Kraft and Harry, 2011; Taetzsch and

Block, 2013). These feedback loops amplify the inflammatory response and lead to a self-sustained neuroinflammation that exacerbates the neurodegenerative process.

5. Identification of the responses on the organ level that may be an adverse outcome or linked to the final adverse outcome

Cortical regions and hippocampus show a higher sensitivity to heavy metals (Fiedorowicz et al., 2001; Falluel-Morel et al., 2012; Schneider et al., 2012). This may be due to differential accumulation of mercury, as observed following exposure to high concentrations (Hamilton et al., 2011), or to differential vulnerability. Changes in genes involved in the amyloid cascade related to Alzheimer's disease were observed in the cortex of monkeys following Pb^{2+} exposure early in life (Zawia and Basha, 2005; Wu et al., 2008). In addition, aggregation of the β-amyloid peptide was particularly enhanced in these monkeys after re-exposure to Pb^{2+} (Basha et al., 2005). The particular sensitivity of cortical areas to heavy metal exposure together with the increased amyloid peptide deposition suggest a link between heavy metal exposure and Alzheimer's pathology (Mutter et al., 2004; Castoldi et al, 2008). Pesticides, such as paraquat and rotenone caused specific lesions in the substantia nigra and striatum (Costello et al., 2009; Wu et al., 2013), suggesting that these toxicants may be associated with Parkinson's disease.

6. Identification of the responses on the organism level that may be an adverse outcome or linked to the final adverse outcome

As consequence of neurotoxicant-induced alterations of brain specific regions, behavioral perturbations can occur. Heavy metal exposure is associated with memory deficits (Chen et al., 2012; Wang et al., 2012; Kaur and Nehru, 2013; Lam et al., 2013), which may be related to tau hyperphosphorylation (Olivieri et al., 2000; Rahman et al., 2012). Such alterations give weight to

the hypothesis that heavy metals increase the risk of developing a neurodegenerative disease of Alzheimer's type (Mutter et al., 2004).

Decrease in locomotor activity and lesion in the striatum were observed following paraquat and ochratoxin treatment (Sava et al., 2006; Prakash et al., 2013). In addition, paraquat exposure resulted in a robust accumulation of α-synuclein, reinforcing the link between pesticide exposure and the development of Parkinson's disease (Costello et al., 2009; Berry et al., 2010; Wang et al., 2011a). Schematic representation of MIEs, cellular key events and organ/organism effects is described in Fig. 9.

7. Identification of the overall effects on the population

Neuroinflammation is a hallmark of Alzheimer's and Parkinson's diseases and the prevalence of patients with these disease is increasing dramatically with aging (Kawas et al., 2000; de Lau and Breteler, 2006). Therefore the neurodegenerative disorders represent a real concern for public health. For both diseases, less than 5 percent of the cases have a genetic background, suggesting that environmental factors are involved in the most abundant, idiopathic form (Tsang and Soong, 2003). In humans, there is epidemiological evidence linking Parkinson's disease with exposure to paraquat alone or in combination with other environmental contaminants, particularly maneb (Costello et al., 2009; Berry et al., 2010; Wang et al., 2011a). In Parkinson's disease, inflammation is mainly associated with microglia activation that can underlie the neurodegeneration of neurons in the substantia nigra and anti-inflammatory treatment in PD patients exerts a neuroprotective effect.

8. Is the AOP specific to certain life stages (DNT or aging)?

Neuroinflammation is not specific to brain development or aging, as it happens at both life stages but it may be regulated differently during these two life time. There was some controversy about

the developmental regulation of neuroinflammation, with the final consensus that during development the glial reactivities depend on the extent of the insult (Morioka and Streit, 1991). The triggering of an inflammatory response during early development may be related to the hypothesis of Landrigan and coworkers (Landrigan et al., 2005) of early environmental origins of neurodegenerative disease in later life. Aging is associated with a low-grade chronic neuroinflammation (Giunta et al., 2008), which may be exacerbated in the presence of environmental toxicants.

9. How much are initiating and key events conserved across species?

Toxicant–induced neuroinflammation, tau hyperphosphorylation, and formation of insoluble amyloid peptides have been described *in vivo* in different mammalian species, mice, rats and monkeys (Charleston et al., 1994; Zawia and Basha, 2005; Wu et al., 2008; Blesa et al., 2012, Corvino et al., 2013), and *in vitro* in mice and rats and in neuroblastoma cells of human origin (Olivieri et al., 2000; Aschner et al., 2007; Monnet-Tschudi et al., 2007; Fiedorowicz et al., 2008).

10. Challenges for further AOP development: strength, data gaps and uncertainties to be considered

The fact that environmental toxicants trigger inflammatory responses is a robust observation. It is also accepted that neuroinflammation contributes to the pathogenesis of neurodegenerative diseases. However, The link between environmental exposures , neuroinflammation and human neurodegenerative diseases is weak. Because microglial/astroctyte activation and chronic neuron damage continues for years after initial exposure (Taetzsch and Block, 2013), and because in Parkinson's and Alzheimer's diseases related cognitive deficits appeared only when

neurodegeneration was widespread (Marsden, 1990; Lichtenstein et al., 2010), the link between toxicant exposure and neurodegenerative diseases is difficult to study.

A major main data gap is the elucidation of a causative link between toxicant-induced neuroinflammation and neurodegenerative diseases. There are some correlative links, such as the association of occupational exposure to paraquat and Parkinson's disease (Costello et al., 2009; Berry et al., 2010; Wang et al., 2011a), or early exposure to Pb^{2+} and the increased formation of amyloid plaques (Zawia and Basha, 2005; Wu et al., 2008), or the finding of increased level of mercury in brain and blood of Alzheimer's patients (Hock et al., 1998), however, it is still matter of controversy. The difficulty of studying such links is due to the fact that neurodegenerative diseases are complex, multifactorial, depends on gene-environment interactions and have a slow temporal evolution (Sherer et al., 2002; Steece-Collier et al., 2002; Tsang and Soong, 2003; Mutter et al., 2004). This AOP deserves further development which should focus on providing better correlative and causative links between exposure and activation of key-events, identification of MIEs, and linking changes to specific adverse outcomes through an more robust understanding of the celluar processes by which neuroinflammation leads to neurodegeneration.

IX. Adverse Outcome Pathway on: *The interaction of non-dioxin-like PCBs with ryanodine receptors (RyRs) causes their sensitization affecting neuronal connectivity that results in behavioral deficits (developmental neurotoxicity)*

Pamela J. Lein

1. Introduction

Polychlorinated biphenyls (PCBs) are synthetic chlorinated aromatic hydrocarbons that are non-flammable, chemically stable and have high boiling points. PCBs are often classified according to their molecular structure as dioxin-like or non-dioxin-like (NDL). PCB congeners with < 2 *ortho*-chlorine substituents are typically coplanar and exhibit dioxin-like toxicity while congeners with >1 *ortho*-substituted chlorine are often non-coplanar and classified as NDL (Safe, 1993). Commercial PCB mixtures were widely used in several industries for their insulation and heat dissipating properties and these compounds were broadly incorporated into a variety of common products such as pesticide extenders, plastics, varnishes, adhesives, carbonless copy paper, newsprint, fluorescent light ballasts and caulking compounds (Ross, 2004). In the late 1970s, commercial production of PCBs was banned in many countries because of their persistence in the environment coupled with growing concerns regarding their carcinogenic potential. While environmental and human PCB levels dropped significantly between 1970 and 1995 as a result of the production ban, recent studies of temporal trends show no or only a slight decrease in PCB levels since the mid-1990s (Hornbuckle et al., 2006),

Chronic low level PCB exposures remain a significant public health concern because human studies indicate an association between PCB body burdens and immune system dysfunction, cardiovascular disease, and impairment of the developing nervous system. Of these various adverse health effects, developmental neurotoxicity is considered a particularly vulnerable endpoint in PCB toxicity. Critical reviews and meta-analyses of epidemiological studies have concluded that the weight of evidence indicates a negative association between developmental exposure to environmental PCBs and measures of neuropsychological function in infancy or childhood. (Schantz et al., 2003; Carpenter, 2006; Korrick and Sagiv, 2008; Winneke, 2011).

Combined *in utero* and lactational PCB exposure correlates with decreased scores on IQ tests, impaired learning and memory, psychomotor difficulties, and attentional deficits. Because of discrepancies between studies with respect to the spectrum and persistence of adverse neurobehavioral outcomes, confounding co-exposures and differences in congener profiles that comprise the exposure, questions have been raised concerning the causative role of PCBs in human developmental neurotoxicity (Winneke, 2011). However, experimental findings in animal models confirm that developmental PCB exposure causes deficits in learning and memory (Schantz et al., 1989; Hany et al., 1999; Widholm et al., 2001; Sable et al., 2006; Yang et al., 2009) and sensorimotor functions (Roegge et al., 2004; Nguon et al., 2005; Powers et al., 2006). More recently, it has been posited that developmental exposure to PCBs contributes to increased risk of neurodevelopmental disorders (Grandjean and Landrigan, 2006; Landrigan, 2010; Landrigan et al., 2012; Stamou et al., 2013); however, there are as yet no epidemiological data to support this hypothesis.

2. Characterization of the exposure to the chemicals relevant to the selected AOP

Humans are exposed to PCBs via oral, inhalation and dermal routes of exposure, and since PCBs readily cross the placenta and are concentrated in breast milk, fetuses and infants are at risk from *in utero* and lactational exposures, respectively. Environmental PCB contamination is not static: mass flux studies demonstrate that large quantities of PCBs are deposited and volatilized every year from Lake Michigan (Hornbuckle et al., 2006). PCBs in the environment bioaccumulate and biomagnify up the food chain, thus, consumption of contaminated meat, fish, poultry and dairy products is a predominant route of human exposure. Exposure also occurs via secondary PCB sources, such as release from building materials including caulking and paint (Jamshidi et al., 2007; Thomas et al., 2012), as well as from contemporary unintentional sources, most

notably commercial paint pigments (Hu and Hornbuckle, 2010). There is still considerable risk of human exposure to PCBs as corroborated by recent reports from the United States of widespread exposure to PCBs among women of childbearing age (Thompson and Boekelheide, 2013) and levels of PCBs in the indoor air of elementary schools that exceed the EPA's 2009 public health guidelines (Thomas et al., 2012).

NDL PCBs with multiple *ortho* chlorine substitutions are particularly stable and predominate over dioxin-like congeners in environmental samples (Kostyniak et al., 2005; Hwang et al., 2006; Martinez and Hornbuckle, 2011) and in human tissues (DeCaprio et al., 2005; Schantz et al., 2010; Marek et al., 2013; Megson et al., 2013). *Ortho*-substituted congeners with the highest activity towards ryanodine receptors (RyRs) collectively represent 40-50% of total PCBs currently found in environmental and biotic samples and their net effects are likely to be additive (Pessah et al., 2006). Consistent with these reports, analyses of PCB levels in human brains obtained from the general adult population detected predominantly *ortho*-substituted congeners at concentrations ranging from 0.07 to 12-ng/g wet weight (Dewailly et al., 1999, Covaci et al., 2002, Chu et al., 2003).

3. Identification of the Molecular Initiating Event (MIE)

A molecular initiating event for PCB developmental neurotoxicity is the interaction of NDL PCBs with Ryanodine Receptors (RyRs) in neurons of the central nervous system. RyRs are microsomal Ca^{2+} channels that are broadly expressed throughout the mammalian brain and associate with cytosolic, endoplasmic reticulum (ER)-anchored and ER luminal proteins to form local Ca^{2+} release units (CRUs). These CRUs regulate Ca^{2+} release from the ER and modify gating responses and signal gain of plasma membrane ion channels, notably NMDA receptors and voltage-gated Ca^{2+} channels. Thus, RyRs function to modulate the amplitude and

spatiotemporal fluctuation of intracellular Ca^{2+} during cell activation (Pessah et al., 2010). Significant to the neurotoxic potential of PCBs, RyR channel activity regulates a variety of physiological and pathophysiological processes in the developing and mature central nervous system (Berridge, 2006; Pessah et al., 2010).

Nanomolar concentrations of NDL PCB congeners interact with RyRs to dramatically increase their sensitivity to activation by nanomolar Ca^{2+} and to attenuate their sensitivity to inhibitory feedback by millimolar Ca^{2+} and Mg^{2+} (Pessah and Wong, 2001), thereby stabilizing the RyR in its full open conformation (Samso et al., 2009). NDL PCBs potently and selectively sensitize both RyR1 and RyR2 channel activities, and PCB-triggered Ca^{2+} release from ER membrane vesicles can be selectively blocked by pretreatment with either FK506 or rapamycin without inhibiting responses to other RyR channel activators such as caffeine (Pessah et al., 2010). Rapamycin and FK506 interfere with NDL PCB actions in the same concentration range that promotes the dissociation of the FKBP12/RyR1 complex, suggesting that NDL PCBs interact with a binding site formed at the FKBP12/RyR1 complex interface to enhance channel open probability. However, an allosteric mechanism has not been ruled out.

A stringent structure-activity relationship has been identified for RyR sensitization by NDL PCBs with PCB 95 (2,2',3,5'6-pentachlorobiphenyl) being the most potent and efficacious congener identified to date (Pessah et al., 2010). Non-coplanar PCBs possessing 2-3 chlorine *ortho* substitutions are the most potent RyR activators (Pessah et al., 2006), which is consistent with findings from multiple laboratories that non-coplanar, but not coplanar, PCBs increase intracellular Ca^{2+} in neurons [reviewed in (Kodavanti, 2005)]. Two important aspects of the PCB structure-activity relationship towards RyR1 include: (1) chlorine substitutions at the *ortho*-positions which restrict the biphenyl rings to non-coplanarity; and (2) the contribution of *para*-

substituents which can reduce or eliminate activity (Pessah et al., 2010). The 2,3,6-Cl configuration on one ring with *ortho*- and *meta*-chloro substitutions on the other is optimal for recognizing a binding site within the RyR1 complex and for sensitizing channel activation. In general, PCBs lacking at least one *ortho*-substitution are inactive toward RyR1 and RyR2, regardless of the degree of chlorination, whereas *para*-chloro substitution lowers the efficacy towards RyR1 regardless of the presence of one or more *ortho*-substitutions. Since hydroxylated PCBs are appearing in human tissues, there is currently great interest in the biological activity of these metabolites relative to the corresponding parent structures. The 4-OH derivative of PCB 30 (4'OH-PCB 30) was found to be significantly more active toward RyR1 than the parent PCB 30 (2,4,6-Cl). Thus, a *para*-OH group on the phenyl ring that carries no other deactivating substitution confers potency and efficacy towards activating RyR1.

4. Identification of the response on the cellular/tissue level that may be an adverse outcome or linked to the final adverse outcome

Critical determinants of neuronal connectivity include neuronal apoptosis (Barone et al., 2000; Sastry and Rao, 2000, Martin, 2001) and dendritic morphogenesis (Kennedy, 2000; Matus, 2000), and both apoptosis and dendritic morphogenesis are regulated by Ca^{2+}-dependent signaling [reviewed in (Pessah et al., 2010)]. As determined by Ca^{2+} imaging of dissociated cultures of primary rat hippocampal neurons, PCB 95, a potent RyR sensitizer, enhanced synchronized Ca^{2+} oscillations in somata and dendrites. This effect was blocked by ryanodine, indicating that PCB 95 increases spontaneous Ca^{2+} oscillations in neurons by stabilizing RyR channels in the open configuration (Wayman et al., 2012a). Consistent with this observation, picomolar to nanomolar concentrations of PCB 95 activate two Ca^{2+}-dependent signaling pathways in cultured hippocampal neurons: (1) sequential activation of CaMKK, CaMKIα/γ, and

MEK/ERK and CREB, which increases transcription of Wnt2 (Wayman et al., 2012a); and (2) CREB-mediated miR132 upregulation, which suppresses the translation of p250GAP (Lesiak et al., 2013). In cultured hippocampal neurons, the former signaling pathway mediates PCB 95-induced dendritic growth (Wayman et al., 2012a), whereas the latter mediates PCB 95-induced synaptogenesis, which is evident as increased spine density and increased frequency of miniature excitatory post-synaptic currents (Lesiak et al., 2013).

The dendrite- and synapse-promoting activity of PCB 95 was blocked by pharmacologic antagonism of RyRs by FLA 365 or by siRNA knockdown of RyR1 or RyR2, and exposure of sister cultures to PCB 66 (2,3,4',4-tetrachloro-biphenyl), a congener that has negligible RyR activity, did not alter dendritic arborization relative to vehicle controls (Wayman et al., 2012b, Lesiak et al., 2013). Dendritic growth in hippocampal slice cultures was similarly enhanced by PCB 95 and inhibited by pharmacologic blockade or siRNA knockdown of RyRs (Wayman et al., 2012b). PCB 95, but not PCB 66, also promotes dendritic growth in primary cultures of cortical neurons via RyR-dependent mechanisms (Yang et al., 2009).

Aroclor 1254 (10 μM), a commercial PCB mixture comprised predominantly of NDL PCBs, as well as the NDL congener PCB 47 (1 μM) significantly increase caspase-dependent apoptosis in primary cultures of hippocampal but not cortical neurons (Howard et al., 2003). In contrast, PCB 77, a congener with little to no RyR activity, has no effect on apoptosis in either neuronal cell type. The pro-apoptotic activity of PCB 47 was inhibited by the RyR antagonist FLA 365 and by the antioxidant α-tocopherol but not by antagonists of the IP3 receptor (xestospongin C), L-type calcium channel (verapamil), or NMDA receptor (APV) (Howard et al., 2003).

There is also functional evidence that NDL PCBs interfere with neuronal connectivity at the cellular/tissue level. First, PCB 95 but not PCB 66 altered excitability in hippocampal slice

cultures (Wong et al., 1997). Second, two different NDL congeners, PCB 95 and PCB 170 altered the ratio of excitatory to inhibitory neurotransmission in hippocampal slice cultures, and this effect was blocked by the RyR antagonist dantrolene (Kim et al., 2009). Third, the NDL congener PCB 136, which enantiospecifically sensitizes RyRs (Pessah et al., 2009), exhibits the same enantiomeric specificity on dendritic arborization and synchronized Ca^{2+} oscillations in hippocampal neurons cultured on microelectrode arrays (Yang et al., 2013).

5. Identification of the responses on the organ level that may be an adverse outcome or linked to the final adverse outcome

Gestational and lactational exposure to Aroclor 1254 (A1254) in the maternal diet significantly increased dendritic arborization of pyramidal neurons in the CA1 region of the hippocampus of weanling rats (Wayman et al., 2012b). In a separate study of experience-dependent dendritic growth, gestational and lactational exposure to Aroclor 1254 promoted dendritic growth in cerebellar Purkinje cells and neocortical pyramidal neurons among untrained animals but attenuated or reversed experience-dependent dendritic growth among Morris water maze-trained littermates (Yang et al., 2009). A1254 is comprised predominantly of NDL PCB congeners (Kostyniak et al. 2005), and consistent with the hypothesis that these congeners are primarily responsible for the effects of A1254 on neuronal connectivity, developmental exposure to PCB 95 in the maternal diet significantly increased dendritic growth of CA1 pyramidal neurons in the hippocampus of untrained weanling rats at the lower doses tested (0.1 – 1.0 mg/kg/d in the maternal diet) but not at the highest dose tested (6.0 mg/kg/d in the maternal diet) (Wayman et al., 2012b).

PCBs have also been shown to increase apoptosis in the developing brain. Caspase-3 activity was significantly increased in the cortex, hippocampus and cerebellum of newborn but not

weanling rats exposed to Aroclor 1254 at 1.0 mg/kg/d in the maternal diet (Yang and Lein, 2010). The most prominent effect was observed in the cerebellum, and PCB-induced apoptosis in this brain region was confirmed by TUNEL. Further evidence that PCBs modulate development of neuronal networks at the organ level is the demonstration that developmental exposure to PCB 95 significantly enhanced the ratio of excitatory to inhibitory currents within the primary auditory cortex (A1) of weanling rats (Kenet et al., 2007). This effect was associated with irregularly shaped topographic organization of A1 and disruption of the critical period plasticity that underlies normal postnatal auditory system development.

6. Identification of the response on the organism level that may be the final adverse outcome or linked to the final adverse outcome

Structural plasticity of dendrites is considered the cellular substrate of learning and memory (Leuner and Shors, 2004), and developmental A1254 exposure causes subtle but statistically significant delays in learning and memory that exhibit an inverted dose-related response similar to that observed for experience-dependent dendritic plasticity in A1254-treated animals (Yang et al., 2009). Similarly, perinatal exposure to PCB 95 has been shown to persistently alter activity levels and behavior in the radial arm maze in adult rats (Schantz et al., 1997). Perinatal exposure to a mixture of the NDL PCB 47 and the dioxin-like PCB 77 has recently been reported to alter social behaviors in rats (Jolous-Jamshidi et al., 2010). Schematic representation of MIE, cellular key events and organ/organism effects is described in Fig. 10.

7. Identification of the overall effects on the population

Population-based studies have consistently demonstrated that PCBs negatively impact neuropsychological function in exposed children (Schantz et al., 2003; Carpenter, 2006; Korrick and Sagiv, 2008). Similar to the behavioral deficits observed in experimental models of PCB

developmental neurotoxicity, the subtle effects of PCBs on cognitive function in children may be overcome by training or increasing age (Schantz et al., 2003; Carpenter, 2006; Korrick and Sagiv, 2008). Such subtle effects may have significant biological and social costs when considered at the population level (Weiss, 2000; Grandjean et al., 2007). More recently, NDL PCBs have been implicated as environmental risk factors for complex neurodevelopmental disorders such as autism and ADHD (Grandjean and Landrigan, 2006; Eubig et al., 2010; Landrigan, 2010, Landrigan et al., 2012; Stamou et al., 2013). Abnormalities in dendritic shape and impaired experience-dependent dendritic plasticity are the most consistent pathologic correlate of behavioral deficits in heritable and environmentally triggered neurodevelopmental disorders (Fukuda et al., 2005; Bourgeron, 2009; Garey, 2010; Svitkina et al., 2010; Penzes et al., 2011). PCB 95 effects on dendritic arborization were blocked by pharmacological antagonism or siRNA knockdown of the Ca^{2+}/calmodulin kinase-I (CaMKI)-CREB-Wnt signaling pathway (Wayman et al., 2012a). Genes encoding these same Ca^{2+}-dependent signaling molecules are implicated as ASD susceptibility genes (Krey and Dolmetsch, 2007; Pessah and Lein, 2008), and the proteins encoded by these genes are upregulated in iPSC-derived neurons from Timothy syndrome patients (Pasca et al., 2011). Ca^{2+} imaging studies of cultured rat hippocampal neurons (Wayman et al., 2012a) revealed that acute exposure to the NDL congener PCB 95 promotes the same bursting type of Ca^{2+} activity as was reported in iPSC-derived neurons expressing gene mutations that confer ASD susceptibility, specifically the gain-of-function missense mutation in the L-type Ca^{2+} channel CaV1.2 that causes Timothy syndrome (Pasca et al., 2011) and the *FMR1* premutation (Cao et al., 2012, Liu et al., 2012) (see section 2). Collectively, these observations suggest that this Ca^{2+}-dependent signaling pathway represents a

potential convergent molecular target for both NDL PCBs and ASD risk genes that interfere with a final common path of activity-dependent dendritic arborization and plasticity.

8. Is the AOP specific to certain life stages (DNT or aging)?

The answer to this question is not known. Certainly there are age-related differences in vulnerability to the neurotoxicity of NDL PCBs (Winneke, 2011) but it is not clear if this is predominantly a reflection of age-related differences in toxicokinetic or toxicodynamic factors. RyRs are expressed in the mammalian brain throughout life, but their spatiotemporal expression patterns change as a function of developmental stage [reviewed in (Pessah et al., 2010)]. Functionally, RyRs not only regulate neurodevelopment and physiological processes in the central nervous system, but they are also implicated in Ca^{2+} dysregulation associated with aging and neurodegeneration [reviewed in (Thibault et al., 2007)]. With respect to the key cellular events downstream of RyR sensitization, altered patterns of neuronal apoptosis not only impact neuronal connectivity in the developing brain, but also influence the susceptibility of the adult brain to subsequent environmental insults or aging (Langston et al., 1999; Barlow et al. 2007), and altered patterns of dendritic growth and plasticity are thought to contribute to neurodegenerative diseases (de Ruiter and Uylings, 1987; Jagadha and Becker, 1989; Flood and Coleman, 1990). Thus, it is plausible that this AOP may be relevant to not only PCB toxicity in the developing brain, but also PCB toxicity in the adult and aging brain; however, additional work is needed in this area to determine whether this is the case.

9. How much are initiating and key events conserved across species?

Ryanodine receptors were first identified because of the pronounced actions of the plant alkaloid ryanodine on insects and vertebrate striated muscles; but subsequently they have been detected in a wide range of species including nematodes, mollusks, arthropods, fish, reptiles, amphibians

and birds (Rossi and Sorrentino, 2004). However, RyR expression profiles vary across species: in vertebrate, three isoforms of RyRs have been identified (RyR1, RyR2 and RyR3); whereas in in vertebrates, only one RyR isoform has been cloned. By contrast, in most avian, amphibian and fish skeletal muscles, two isoforms of RyRs, named α and β, that correspond to mammalian RyR1 and RyR3 are expressed. A third isoform, which is better recognized by antibodies against the mammalian RyR2 than against avian α and β isoforms and is likely to represent the homologous of mammalian RyR2, has been detected in chicken heart. Whether the action of NDL PCBs on RyR activity is conserved across species remains to be determined. However, it is known that the key events of Ca^{2+}-dependent dendritic arborization, synapse formation and neuronal apoptosis are conserved across species (Lein et al., 2005).

10. Challenges for further AOP development: strength, data gaps and uncertainties to be considered

In vitro studies establish a causal link between the MIE, RyR sensitization, and PCB effects on dendritic arborization and synaptogenesis. Dendritic growth in primary dissociated cortical or hippocampal cells or in hippocampal slice cultures is promoted by PCB 95, a congener with potent RyR activity, but not by PCB 66, a congener with negligible RyR activity (Yang et al., 2009; Wayman et al., 2012b). Blocking RyR activity using either pharmacological approaches or siRNA knockdown of RyR prevented PCB 95 enhancement of both synchronized Ca2+ oscillations (Wayman et al., 2012a) and dendritic growth (Yang et al., 2009; Wayman et al., 2012b), and blocked activation of the CaMK-CREB-Wnt2 signaling pathway (Wayman et al., 2012a). Similarly, PCB 95-induced synapse formation, as defined by increased spine density and increased mEPSCs in primary cultures of dissociated hippocampal cells or hippocampal slice cultures, was blocked by pharmacologic antagonism or siRNA knockdown of RyR (Lesiak et al.,

2013). Structure-activity relationship studies similarly support a causal link between RyR sensitization and PCB-induced neuronal apoptosis: the RyR active congener PCB 47, but not the RyR inactive congener PCB 77, induced apoptosis in primary cultures of dissociated hippocampal cells (Howard et al., 2003). Furthermore, the pro-apoptotic activity of PCB 47 was inhibited pharmacologic block of RyR but not by pharmacologic block of IP3 or voltage-gated Ca2+ channels (Howard et al., 2003).

In vivo, developmental exposure to A1254 enhanced basal levels of dendritic growth but blocked experience-dependent dendritic growth in weanling rats (Lein et al., 2007; Yang et al., 2009; Wayman et al., 2012b), while developmental exposure to PCB 95 increased basal levels of dendritic growth in the weanling rat hippocampus (Wayman et al., 2012b) and disrupted the balance of neuronal inhibition to excitation in the developing rat auditory cortex (Kenet et al., 2007). Several lines of evidence suggest that RyR sensitization contributed to these PCB effects. First, these changes in neuronal connectivity were associated with exposure to NDL PCBs with high affinity for the RyR. Second, PCB-induced changes in dendritic growth and plasticity were coincident with increased [3H]ryanodine binding sites and RyR expression in the brains of untrained animals and inhibition of training-induced RyR upregulation (Yang et al., 2009). Increased RyR expression in brain tissues has also been associated with PCB-induced changes in gene expression (Royland and Kodavanti, 2008; Royland et al., 2008) and locomotor activity (Roegge et al., 2006). Third, the dose relationship for PCB effects on dendritic growth and plasticity was similar to that of PCB effects on RyR expression but not to that of PCB effects on thyroid hormone levels or sex-steroid-dependent developmental endpoints (Yang et al., 2009). Fourth, the dose relationship for PCB effects on dendritic growth and plasticity was also similar to that of PCB effects on spatial learning and memory in the Morris water maze (Yang et al.,

2009). This is consistent with the extensive literature documenting the robust effect of activity or experience on the development and refinement of synaptic connections, which not only patterns neural circuitry during development but also underlies associative learning (Pittenger and Kandel, 2003). Furthermore, altered patterns of dendritic growth and plasticity are associated with impaired neuropsychological function in experimental models (Berger-Sweeney and Hohmann, 1997), and abnormalities in dendritic shape and experience-dependent plasticity are the most consistent pathologic correlate of behavioral deficits in heritable and environmentally triggered neurodevelopmental disorders (Fukuda et al., 2005; Bourgeron, 2009; Garey, 2010, Svitkina et al., 2010; Penzes et al., 2011).

While a causal link between the MIE and key events at the cellular/tissue level is well-established in vitro, and key events identified in vitro are recapitulated in vivo, there remain uncertainties as to whether the MIE is causally linked to altered in vivo neuronal connectivity and behavioral deficits. This uncertainty arises in part because other biological activities have been ascribed to non-coplanar PCBs, including increased intracellular levels of ROS (Fonnum et al., 2006; Mariussen and Fonnum, 2006), disruption of thyroid hormone signaling (Zoeller, 2007; Crofton, 2008) and decreased levels of dopamine (Mariussen and Fonnum, 2006). RyR activity has been implicated in these other biological activities [reviewed in (Pessah et al., 2010)], which raises the interesting question of whether these other biological activities are causally related to PCB developmental neurotoxicity, and if so, do they represent divergent or convergent mechanisms of PCB developmental neurotoxicity? The observation that RyRs play critical roles in diverse tissue types and in numerous cellular processes (Berridge, 2006) raises another interesting challenge associated with the identification of RyR sensitization as a MIE in PCB developmental neurotoxicity. What factor(s) determine the specificity of PCB toxicity?

Why do PCBs seem to preferentially target the developing nervous system? Certainly the timing of exposure will influence the biological outcomes of PCB exposures, as will pharmacokinetic parameters such as dosage, the metabolites produced, and distribution of PCBs within the body. But other factors that could be equally important include expression patterns of RyRs and the complement of accessory proteins that comprise the calcium release unit as well as the antioxidant capacity of the cell. An important data gap in this context is whether NDL PCBs exert comparable effects on all 3 RyR isoforms that are expressed in the brain. Another interesting data gap that emerges from consideration of the structure-activity relationship of PCB interactions with the RyR is whether the RyR functions as a target for other toxicants that possess non-coplanar structures. Obvious candidates include the polybrominated diphenyl ethers (Kim et al., 2011) and triclosan (Cherednichenko et al., 2012).

X. Adverse Outcome Pathway on: *The interaction of redox cycling chemicals with NADH cytochrome b5 reductase and NADH-quinone oxidoreductase results in NAD^+ formation causing reduced adult neurogenesis.*

Ellen Fritsche

1. Introduction

Adult neurogenesis of hippocampal neural progenitor cells (NPCs) takes place in human brains up to old age (Eriksson et al., 1998) and contributes to brain function in the adult mammal (Dupret et al., 2008). During the physiological process of aging, a decline in hippocampal NPC function is observed (Altman and Das, 1965; Kempermann et al., 1998; Kuhn et al., 1996; Seki and Arai, 1995; van Praag et al., 2005) correlating with a decline in learning and memory tasks (Aizawa et al., 2011; Chang et al., 2008; Drapeau et al., 2003; Driscoll et al., 2006; Kempermann

et al., 1998; Kempermann et al., 2002; Klempin et al., 2013; Kronenberg et al., 2006; Montaron et al., 2006; Shors et al., 2001; van Praag et al., 2005). To study adult neurogenesis the rodent has proven to be a valuable *in vivo* model. However, with regard to the mechanisms responsible for decreasing neurogenesis with age, it has to be kept in mind that there seem to be species differences between primates (possibly humans) (Aizawa et al., 2011) and rodents (Eisch and Petrik, 2012) regarding the extent of adult neurogenesis (Eisch and Petrik, 2012) and processes contributing to an aging hippocampus.

Oxidative stress contributes to loss-of-function during NPC aging (reviewed in van Wijngaarden and Franklin, 2013). Thereby, the intracellular redox state seems to govern cell fate during differentiation as an oxidized intracellular environment favours glial over neuronal differentiation of SGZ neural stem cells (Prozorovski et al., 2008). Hence, increased ROS generation might influence NPC proliferation, differentiation and fate determination (van Wijngaarden and Franklin, 2013).

2. Characterization of the exposure to the chemicals relevant to the selected AOP

ROS-producing chemicals are able to enter adult brains and cause oxidative stress. Routes of exposure can be via skin, lung or the gastro-intestinal tract. After entering the circulation, the compound has to pass the BBB either by passive diffusion or active uptake. ROS-producing compounds include paraoxon (Jafari et al., 2012b), diazinon (Jafari et al., 2012a), ammonium acetate (Satpute et al., 2012), acrylamide (Lakshmi et al., 2012), adriamycin (doxorubicin) (Joshi et al., 2005) and paraquat (Chen et al., 2010). In addition to chemical compounds, gamma-irradiation also produces oxidative stress in brain tissue (Riley, 1994; Zhao and Robbins, 2009).

3. Identification of the Molecular Initiating Event (MIE)

The MIE prompting this AOP is the interaction of redox cycling chemicals (R) with enzymes (NADH-cytochrome b_5 reductase, NADH-quinone oxidoreductase) catalysing electron transport from NADH to the chemical, thereby forming a radical $R^{\bullet}$ and the NADH oxidation product NAD^+ (Dinis-Oliveira et al., 2006; Shimada et al., 1998). These processes happen at the cytosolic side of the outer mitochondrial membrane and thus do not directly involve or alter the mitochondrial electron transport chain (Shimada et al., 1998). The radical $R^{\bullet}$ causes non-enzymatic generation of the reactive oxygen species (ROS) $^{\cdot}O_2^-$. Enzymatic detoxification of $^{\cdot}O_2^-$ involves superoxide dismutase and glutathione peroxidase, thereby producing the oxidized form of glutathione (GSH), GSSG (Andreyev et al., 2005; Zhu and Shan, 2009). Glutathione reductase then reconstitutes GSSG to reduced glutathione by forming $NADP^+$ (reviewed in Belenky et al., 2007; Bolton et al., 2000). The mitochondrial enzyme nicotinamide nucleotide transhydrogenase (NNT) is able to reconstitute $NADP^+$ to NADPH by generating NAD^+ (Olgun, 2009). Thus, NAD^+ is formed by two different mechanisms: as the MIE by direct chemical interference with electron-donating enzymes and secondly by the first series of key events, i.e. ROS formation, detoxification and reconstitution of GSH. One example of such a redox-cycling chemical is paraquat (PQ). PQ directly interacts with NADH-cytochrome b_5 reductase and NADH-quinone oxidoreductase, thereby accepting one electron from NADH reducing PQ^{2+} to $^{\cdot}PQ^+$ and forming NAD^+ (Belenky et al., 2007; Dinis-Oliveira et al., 2006; Shimada et al., 1998). ROS effects on cells, tissues and organs are multifarious and depend on the amount of intracellular redox active species. While a certain low level of ROS produced by mitochondrial respiration is nowadays considered 'physiological signalling molecules', large excess of ROS causes macromolecular and thus cellular damage with deathly consequences. ROS concentrations triggering this AOP

are meant to be below causing cell death, but function as signalling molecules by shifting the cellular redox state towards the oxidative side. Such ROS production occurs during aging in humans and experimental animals (reviewed in van Wijngaarden and Franklin, 2013) as well as in response to toxicant exposure (Cheng et al., 2009; Merzoug et al., 2011; Milatovic et al., 2009; Ojha et al., 2013) or radiation damage (Robbins and Zhao, 2004; Zhao et al., 2007). Thus, aging poses a higher susceptibility to adverse effects of exogenous ROS due to raised basal intracellular levels.

4. Identification of the responses on the cellular/tissue level that may be an adverse outcome or linked to the final adverse outcome

NAD^+ is a necessary co-factor and activator for the histone deacetylase (HDAC) Sirt-1 (Araki et al., 2004; Lin et al., 2004), the mammalian homologue of the yeast, drosophila and C. elegans Sir2 protein (Imai et al., 2000). Sirt-1 lacks a DNA binding domain and has to be recruited to target promoters by sequence-specific transcription factors to induce chromatin remodelling and regulate gene expression (Rosenberg and Parkhurst, 2002). One of the transcription factors associating with Sirt-1 is the transcriptional co-repressor Hes1 (Takata and Ishikawa, 2003), which is expressed in neural stem/progenitor cells (NS/PCs) and prevents premature neuronal differentiation into DCX (double cortin)$^+$ cells by repressing activation of the pro-neuronal basic helix-loop-helix (bHLH) transcription factor Mash1 (Ishibashi et al., 1995); reviewed by (Libert et al., 2008). Redox state contributes to NS/PC proliferation and neuronal differentiation: oxidation-mediated *Mash-1* repression in NS/PCs is blocked in the absence of Sirt-1 or Hes-1 *in vitro* (Prozorovski et al., 2008). It is highly likely that NAD^+ is the signalling molecule linking redox state and Sirt-1-dependent repression of neuronal differentiation.

5. Identification of the responses on the organ level that may be an adverse outcome or linked to the final adverse outcome

In vivo, Sirt-1 regulates the neurogenic potential of NS/PCs in the early postnatal as well as in the adult subventricular zone (SVZ) and adult hippocampus (Prozorovski et al., 2008; Saharan et al., 2013). In adult mouse SVZ and hippocampi, Sirt-1 knock down results in a significant increase in neuronal production, whereas Sirt-1 overexpression or activation by resveratrol, a potent chemical Sirt-1 activator, prevent adult neural precursors from differentiating into neurons (Saharan et al., 2013). A pro-oxidative state by systemic glutathione depletion decreases NS/PC proliferation and generation of young neurons and increases the number of $GFAP^+$ cells at the same time in the SVZ in a Sirt-1 dependent manner, demonstrating contribution of Sirt-1 to neural fate decision in the oxidative milieu (Prozorovski et al., 2008); reviewed by (Libert et al., 2008). One repression target of Sirt-1 is the pro-neural gene Mash-1 (Ishibashi et al., 1995) and Mash-1 regulates neural versus glial fate of embryonic and adult NS/PC (Nieto et al., 2001; Parras et al., 2004). During the aging process, oxidative damage occurs in the hippocampus of mice (Nicolle et al., 2001). Feeding middle aged mice with already impaired NS/PC proliferation and generation of DCX^+ young neurons with the NO-donor flurbiprofen, which amongst others has antioxidative properties, restores these NS/PC functions almost to levels of young controls (L'Episcopo et al., 2013) suggesting causal involvement of oxidative stress in age-related decline of NS/PC functions. These are related to altered wnt-signaling (L'Episcopo et al., 2013) and a crosstalk between Sirt-1- and wnt-signaling was reported in a different context earlier (Holloway et al., 2010). Whether this aging-related, oxidation state-dependent, wnt-mediated decline in NS/PC functions is determined by NAD^+-dependent Sirt-1 activation needs further clarification.

6. Identification of the responses on the organism level that may be the final adverse outcome or linked to the final adverse outcome

Decreased adult neurogenesis by waning NS/PC proliferation and/or generation of new, young neurons result in the adverse outcome of impaired cognitive function that mirrors changes characteristic of aging (Aizawa et al., 2011; Drapeau et al., 2003; Driscoll et al., 2006; Kempermann et al., 1998; Montaron et al., 2006; Shors et al., 2001). Two notions support this statement: (i) Interventions that enhance or reduce hippocampal neurogenesis cause improved (Chang et al., 2008; Kempermann et al., 1998; Kempermann et al., 2002; Klempin et al., 2013; Kronenberg et al., 2006; Montaron et al., 2006; van Praag et al., 2005) or impaired, aging-characteristic (Montaron et al., 2006; Shors et al., 2001) cognitive functions, respectively. (ii) Creating an inducible transgenic strategy allowing specific ablation of adult-born hippocampal neurons in mice causes impairment of spatial relational memory in these animals (Dupret et al., 2008).

Spatial learning and memory impairment in Sirt-1 knockout mice imply that Sirt-1 is involved in hippocampal-dependent cognitive functions (Michan et al., 2010). Feeding aged mice an antioxidative saffron diet that improves learning and memory measured by a passive avoidance task supports the notion that oxidative stress indeed triggers the impaired adverse outcome (Papandreou et al., 2011). Schematic representation of MIE, cellular key events and organ/organism effects is described in Fig. 11.

7. Identification of the overall effects on the population

In humans, cognitive deficits are attributed to reduced adult neurogenesis (reviewed in Knoth et al., 2010; van Wijngaarden and Franklin, 2013). By current knowledge, such stem cell exhaustion belongs to the nine identified hallmarks of aging (reviewed in Lopez-Otin et al.,

2013). Thereby, oxidative stress is one of the main contributors to NS/PC aging (reviewed in (van Wijngaarden and Franklin, 2013). Such age-related decline in NS/PC function can be accelerated by exogenous noxae, which increase the oxidative burden in the brain. In the human population, chemotherapy or gamma-irradiation for cancer treatment cause cognitive impairment probably due to compromised NC/PC function (reviewed in Gibson and Monje, 2012; Greene-Schloesser et al., 2012; Monje and Dietrich, 2012). One discussed mechanism of chemotherapy- or gamma-irradiation-induced cognitive changes is the generation of ROS (Ahles and Saykin, 2007; Greene-Schloesser et al., 2012). Such accelerated cognitive decline poses a large financial and social burden on society. Whether environmentally relevant compounds trigger the same AOP in humans is so far not known. However, accumulating data from animals and humans suggest that antioxidative strategies have the ability to improve cognition in the elderly (reviewed in Joseph et al., 2009).

8. Is the AOP specific to certain life stages (DNT or aging)?

The current body of literature supports the hypothesis that age-associated increases in the generation of ROS might influence CNS stem cell proliferation, differentiation and fate determination (reviewed in Takata and Ishikawa, 2003). While mitochondrial dysfunction increases as a function of age raising endogenous ROS formation (reviewed in Bishop et al., 2010), antioxidative defence capacities decrease during the aging process (Li et al., 2012; Saharan et al., 2013). Therefore, susceptibility to disturbance of the delicate cellular redox balance is likely to increase with age. Moreover, BBB permeation changes within the function of age, adding to the higher susceptibility for this AOP during aging (Simpson et al., 2010).

9. How much are initiating and key events conserved across species?

The general concept of adult neurogenesis is conserved between species. However, there are some uncertainties and some already known species differences. (I) MIE: Direct and indirect mechanisms contribute to the generation of NAD^+. It is not known if these are quantitatively conserved across species. (II) Cellular ROS detoxifying capacities determine $NAD(P)^+$/NAD(P)H ratios. Whether detoxification capacities of adult NS/PCs are comparable across species has never been studied. (III) Concerning gamma-irradiation of brains, rats tolerate a 5-times higher dose than humans (Monje and Palmer, 2003). The underlying molecular rationale is not known. (IV) In rodents, SVZ and hippocampal neurogenesis contribute to cognition. In humans, current evidence suggests that SVZ is negligible beyond late infancy (Sanai et al., 2011; Wang et al., 2011c). (V) Whether human Sirt-1 also regulates the human Mash-1 homologue Hash-1 and thus regulates adult NS/PC fate decision is not known. However, this pathway seems to be homologous in human embryonic stem cells (Zhang et al., 2011). (VI) Aging studies in non-human primates suggest that signalling pathways and NS/PC phenotypes during the physiological brain aging process might be different between primates (maybe including humans) and rodents (Aizawa et al., 2011).

10. Challenges for further AOP development: strength, data gaps and uncertainties to be considered

Causalities between the MIE, KEs 1-5 and the AO are very stringent. However, data linking the MIE directly and causally with the AO are missing. Moreover, human data on chemical exposure and affected hippocampal function neurogenesis is not available.

Although causality within this AOP is quite stringent, it has not yet been determined if the above-mentioned indeed increase NAD+ in regenerative zones of the brain and cause decreased

adult neurogenesis by this mechanism chemicals (Chen et al., 2010; Jafari et al., 2012a,b; Joshi et al., 2005; Lakshmi et al., 2012; Satpute et al., 2012). Moreover, the proposed higher susceptibility towards ROS-induced impairment in NS/PC function has not specifically been addressed experimentally.

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Legends of figures

Fig. 1. Relationships between the uncertainty in AOP models, the data and resources needed to develop the models and the regulatory domains of applicability.

Fig. 2. Schematic representation of the putative AOP on *Binding of antagonist to an NMDAR during synaptogenesis contributes to impairment of learning and memory abilities*. The binding of antagonist to NMDAR in hippocampus during synaptogenesis leads to inhibition of the receptor activity and to the delay in the ontogeny of the NR2A subunit, contributing to decreased Ca^{2+} influx into neurons and decreased glutamate release, causing a concomitant reduction of the BDNF release. The resulting cellular key events eventually lead to impaired capacity for processes underlying learning and memory. Brain-derived neurotrophic factor (BDNF); N-methyl-D-aspartate receptor (NMDAR); long-term potentiation (LTP); long-term depression (LTD).

Fig. 3A. Cascade of events taking place after NMDAR over activation leading to neuronal cell death. Over activation of the ligand-gated calcium channel NMDAR leads to various stress responses like endoplasmic reticulum (ER) stress, which is also termed as "unfolded protein" response and is related to protein aggregation (β-amyloid, α-synuclein in Alzheimer and Parkinson disease), mitochondrial apoptosis, oxidative stress, with subsequent activation of poly-ADP-ribose polymerase-1 (PARP-1), resulting in rapid energy depletion of apoptotic neurons. Calcium signaling via NMDAR and calcium-dependent kinases is in a normal "healthy" setting absolutely key to memory and cognition. NMDARs are allosteric proteins tightly regulated by activity and numerous endogenous factors maintaining cytoskeletal and nuclear integrity of the neuron and its dendritic contacts. One key aspect is maintenance of energy homeostasis which

explains the crucial role of mitochondria and NADH in neurodegenerative processes. PARG is the antagonistic enzyme to PARP-1 degrading poly-ADP-ribosylated modifications.

Fig. 3B. Schematic representation of the putative AOP on *Binding of agonist to NMDA receptor causes excitotoxicity that mediates neuronal cell death, contributing to reduction (or loss) of cognitive, sensory and motor function.* The binding of exogenous agonist to NMDAR causes over activation of the receptor and collapse of calcium homeostasis, a key regulator of synaptic plasticity underlying cognition. Intracellular calcium overload mediates chemical-induced neurodegeneration induced by key cellular events such as mitochondrial dysfunction, oxidative and ER stress. Depending on the degree, time of exposure and structure of the brain where neurodegeneration takes place, it can result in different adverse outcomes such as reduction (or loss) of cognitive, sensory or motor function. N-methyl-D-aspartate receptor (NMDAR); Adenosine triphosphate (ATP); endoplasmic reticulum (ER); reactive oxygen species (ROS). (*see AOP on *Binding of inhibitors to the mitochondrial respiration chain complex I, II, III or IV or interaction of uncouplers with oxidative phosphorylation decreases or blocks ATP production resulting in neurodegeneration*).

Fig. 4A. Schematic representation of the putative AOP on *Binding of antagonist to $GABA_A$ receptor results in hyperexcitability and convulsions.* By blocking the activity of the $GABA_AR$, the inhibitory GABAergic neurotransmission is reduced, due to the reduced Cl^- influx, resulting in increased excitatory activity in neuronal network. A severe deregulation of the balance between excitation and inhibition beyond physiological levels is involved in several pathologies of the central nervous system such as seizures and/or /convulsions (Adverse Outcome). γ-aminobutyric acid A receptor ($GABA_AR$).

Fig. 4B. Correlation between acute toxicity in vivo (LD50) and inhibition of the $GABA_AR$ induced by convulsant drugs and pesticides acting at the $GABA_AR$. Symbols (correlations): squares for the inhibition of GABA-induced Cl^- flux (R2 = 0.613; p = 0.004) and triangles for the inhibition of [^{35}S] TBPS binding (r2 = 0.371; p = 0.02). Values are extracted from references cited along the text (Obata et al., 1988; Pomés et al., 1994)

Fig. 5. Schematic representation of the putative AOP on *Binding of pyrethroids to voltage-gated sodium channels induces acute neurotoxicity.* This AOP is initiated by binding of pyrethroids to a distinct site on voltage-gated sodium channels (VGSCs). This binding interaction between pyrethroids and VGSCs results in alterations in the kinetics of VGSC activation and inactivation (opening and closing of the channel). The degree of perturbation of VGSC kinetics is structure-dependent, and a continuum of lengths of modification of kinetics have been observed in a variety of species, including mammals. At the cellular level, altered VGSC kinetics give rise to changes in membrane excitability that also depend on the length of modification, with short-lasting modifications resulting in repeated firing of action potentials and long lasting modifications resulting in membrane depolarization and ultimately, depolarization-dependent block of action potential propagation. In turn, these cellular changes result in changes in the activity at the organ level, resulting in changes in activity in different neuronal pathways that ultimately lead to the two different Adverse Outcomes that are observed as Type I (T) or II (CS) syndromes described in the main text.

Fig. 6. Schematic representation of the putative AOP on *Binding of certain organophosphates to NTE results in delayed neuropathy.* Binding of some specific organophosphates (OPs) to neuropathy target esterase (NTE) causes inhibition and aging of the enzyme followed by cellular key events such as disruption of Ca^{2+} homeostasis, mitochondrial dysfunction with subsequent

energy depletion and buildup of oxidative stress. One or more of the above cellular effects could disrupt the regulation of cell signaling, causing altered phosphorylation of cytoskeletal proteins that lead to cytoskeletal dysfunction. Energy depletion due to mitochondrial dysfunction would also impact on cytoskeletal motor protein activity and axonal transport with subsequent impairment of nerve regeneration or neurite development. Prolongation of these cellular key events causes degeneration of axons in the CNS and PNS, leading to peripheral neuropathy (adverse outcome).

Fig. 7. Schematic representation of the putative AOP on *Impairment of learning and memory induced by binding of electrophilic chemicals to SH(thiol)-group of proteins and non-protein molecules in neuronal and glial cells during development.* By binding of a compound to SH(thiol)-groups of proteins and non-protein molecules (Molecular Initiation Event) during brain development and the subsequent functional modification of their function leads to several distinct cellular key events that depend on the function and location of these proteins in the specific brain cell type and the brain structure. Mainly proteins and non-protein molecules associated with mitochondria, antioxidant defense, and glutamate storage, release and uptake are targeted. This binding leads to the depletion of reduced glutathione, increased of extracellular Glu levels inducing over activation of NMDAR, possible neuronal/glial dysfunction of respiratory chain complexes, triggering oxidative and nitrosative stress causing neuronal cell death. The induced neurodegeneration contributes to the decreased neuronal network formation and function responsible for the deficits in developmental learning and memory processes (AO). Glutathione (GSH); glutamate (Glu) N-methyl-D-aspartate receptor (NMDAR); neuronal nitric oxide synthase (nNOS); nitric oxide (NO); thiol- (SH-). (*see AOP on *Binding of inhibitors to the mitochondrial respiration chain complex I, II, III or IV or interaction of uncouplers with*

oxidative phosphorylation decreases or blocks ATP production resulting in neurodegeneration ; **see AOP on *Binding of agonist to NMDA receptor causes excitotoxicity that mediates neuronal cell death, contributing to reduction (or loss) of cognitive, sensory and motor function*; ***see AOP on *Binding of antagonist to an NMDAR during synaptogenesis contributes to impairment of learning and memory abilities*).

Fig. 8A. Schematic representation of the mitochondrial electron transport chain (ETC). Electrons are transferred from the matrix via NADH (nicotinamide adenine dinucleotide) reduction into the complex I and via FADH2 (FAD: flavin adenine dinucleotide) reduction into complex II. The Coenzyme Q transfers electrons from complex I and II to complex III. Cytochrome C further transfers these electrons from complex III to the final electron acceptor complex IV, where oxygen is reduced to water. A proton gradient is generated that is used for production of ATP by complex V (ATP synthase).

Fig. 8B. Schematic representation of the putative AOP on *Binding of inhibitors to the mitochondrial respiration chain complex I, II, III or IV or interaction of uncouplers with oxidative phosphorylation decreases or blocks ATP production resulting in neurodegeneration.* Binding of inhibitors to the ETC complexes or uncoupling of OXPHOS induces inhibition of mitochondrial respiration leading to reduction of the ATP production. The decreased level of ATP leads to the cellular effects such as disturbed calcium homeostasis, causing ROS production, disruption of mitochondrial membrane potential, cytochrome c release resulting in apoptotic or necrotic cell death. If the significant cell death of dopaminergic neurons takes place through these mechanisms in substantia nigra, the symptoms of Parkinson's disease might be triggered.

Adenosine triphosphate (ATP); cytosolic (c); Calcium (Ca^{2+}); cytochrome C (Cyt C); electron transport chain, (ETC); mitochondria (mt); oxidative phosphorylation (OXPHOS); reactive oxygen species (ROS); Substantia Nigra pars compacta (SNpc) *see AOP on *Binding of agonist to NMDA receptor causes excitotoxicity that mediates neuronal cell death, contributing to reduction (or loss) of cognitive, sensory and motor function*; **see AOP on *Multiple molecular initiating events trigger neuroinflammation leading to neurodegeneration.*

Fig. 9. Schematic representation of the putative AOP on *Multiple molecular initiating events trigger neuroinflammation leading to neurodegeneration.* Several MIEs can lead to intermediate cellular key events that cause cell type specific effects, followed by the neuroinflammatory response. The triggered cellular effects such as mitochondrial dysfunction, oxidative stress, depletion of reduced GSH and excitotoxicity lead to neuronal damage (axonal demyelination, synapse impairment and cytoskeleton disruption) and glia activation (microglia and astrocytes) causing neuroinflammation leading to neurodegeneration. Neurodegeneration mediated through these pathways are well documented in the brain structures which are linked to PD and AD. Molecular Initiating Event (MIE); Acetylcholinesterase (AChE); glial fibrillary acidic protein (GFAP); glutathione (GSH); N-methyl-D-aspartate receptor (NMDAR), Parkinson disease (PD); Alzheimer disease; (*see AOP on: *Binding of antagonist to an NMDAR during synaptogenesis contributes to impairment of learning and memory abilities*; ** AOP on: *Impairment of learning and memory induced by binding of electrophilic chemicals to SH(thiol)-group of proteins and non-protein molecules in neuronal and glial cells during development*; *** AOP on: *Binding of agonist to NMDA receptor causes excitotoxicity that mediates neuronal cell death, contributing to reduction (or loss) of cognitive, sensory and motor function*; **** AOP on: *Binding of inhibitors to the mitochondrial respiration chain complex I, II, III or IV or interaction of*

uncouplers with oxidative phosphorylation decreases or blocks ATP production resulting in neurodegeneration).

Fig. 10: Schematic representation of the putative AOP on *The interaction of non-dioxin-like PCBs with ryanodine receptors (RyRs) causes their sensitization affecting neuronal connectivity that results in behavioral deficits (developmental neurotoxicity).* NDL PCBs sensitize RyR activity and alter Ca^{2+}-dependent signaling mechanisms that link neuronal activity to dendritic growth and plasticity and to neuronal apoptosis. These cellular effects alter normal patterns of neuronal connectivity in the brain and contribute to behavioral and psychomotor deficits observed at the organismal level (adverse outcome). Ryanodine receptor (RyR).

Fig. 11. Schematic representation of the putative AOP on *The interaction of redox cycling chemicals with NADH cytochrome b5 reductase and NADH-quinone oxidoreductase results in NAD+ formation causing reduced adult neurogenesis.* The MIE of this AOP is the interaction of a redox-active chemical with NADH-cytochrome c reductase or NADH-quinone oxidoreductase on the cytoplasmic site of the outer mitochondrial membrane associated to mitochondrial complex I (**Fig. 8A**). NAD^+ is formed either directly by this MIE or secondary through formation of O2- by the redox cycler and subsequent oxidation of GSH to GSSG, which is reconstituted under generation of $NADH^+$ and finally NAD^+. NAD^+ is a necessary co-factor activating the HDAC Sirt-1. By recruiting Hes-1, Sirt-1 represses expression of the pro-neuronal gene Mash-1 thus shifting neural progenitor cell fate to the glial side. On the organ level, this causes diminished neuronal regeneration in the hippocampus with decreased cognitive performance as the AO. Glutathione (GSH); oxidized glutathione (GSSG); histone deacetylase (HDAC); nicotinamide adenine dinucleotide (NAD^+); reduced form of nicotinamide adenine dinucleotide (NADH); neural stem/progenitor cells (NS/PC).

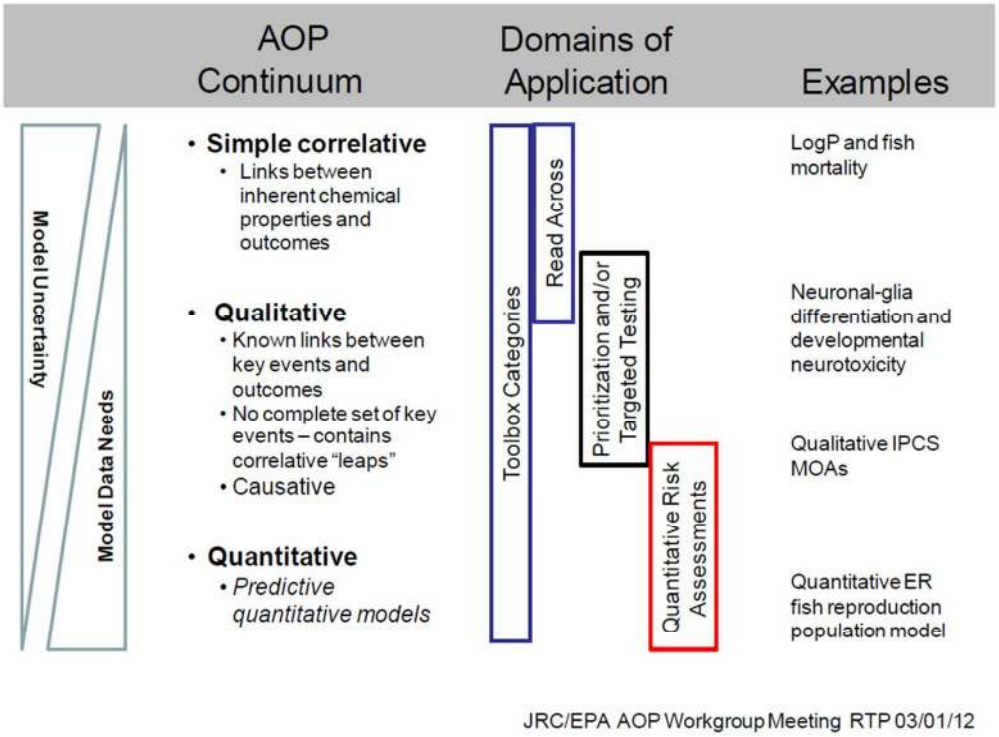

Fig.1.
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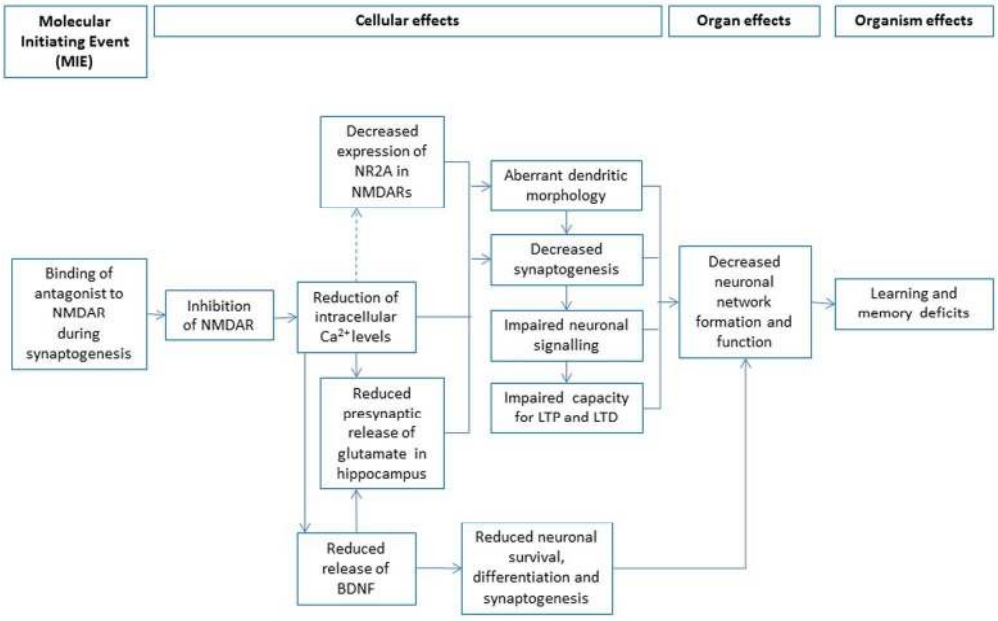

Fig. 2.
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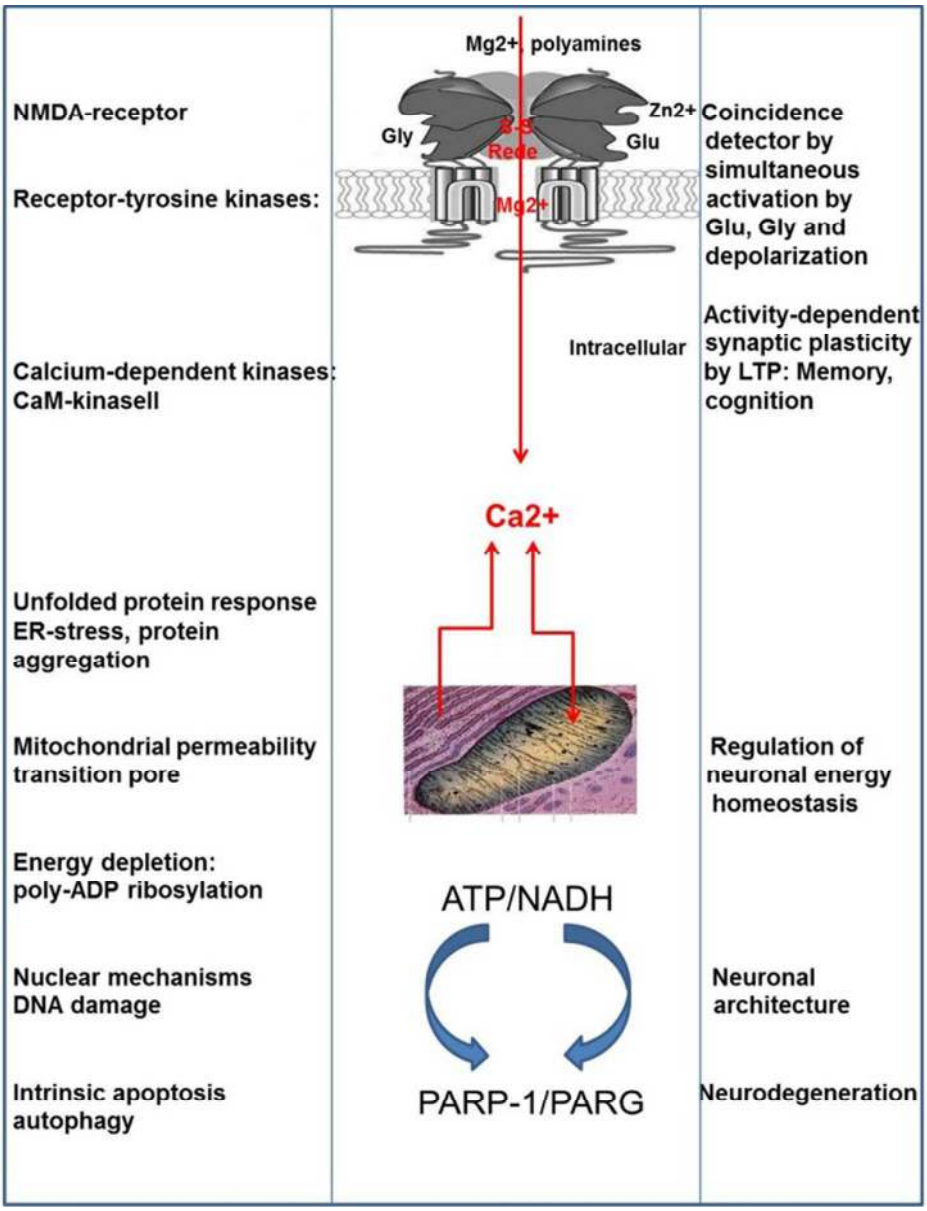

Fig. 3A.
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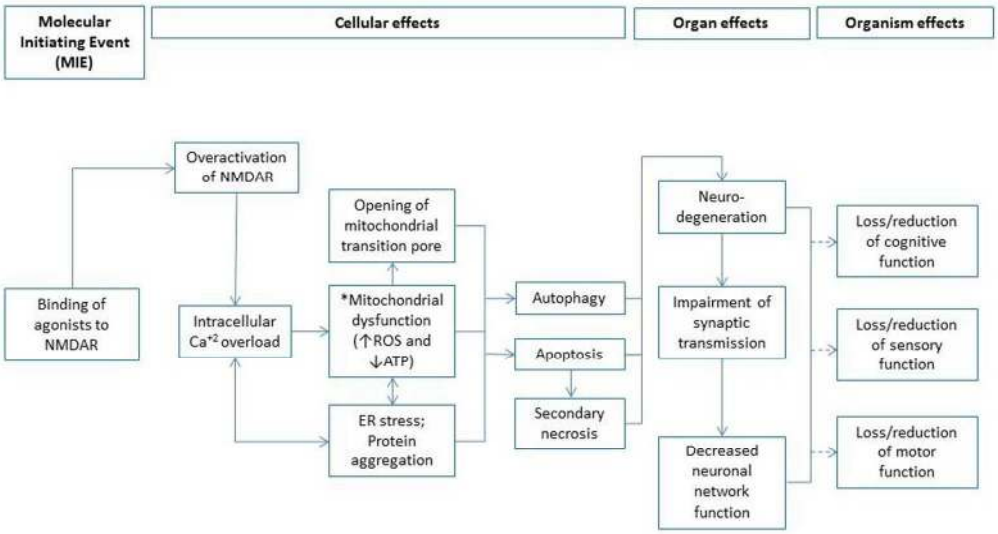

Fig. 3B.
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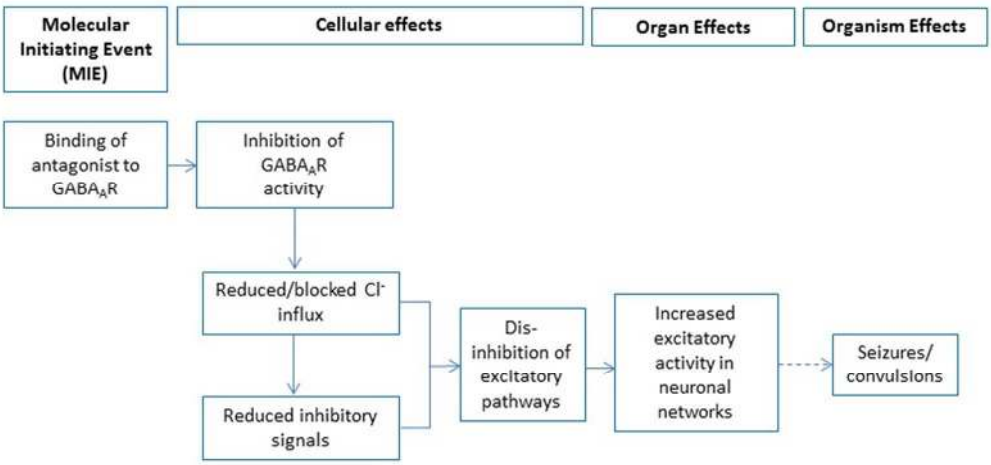

Fig. 4A.
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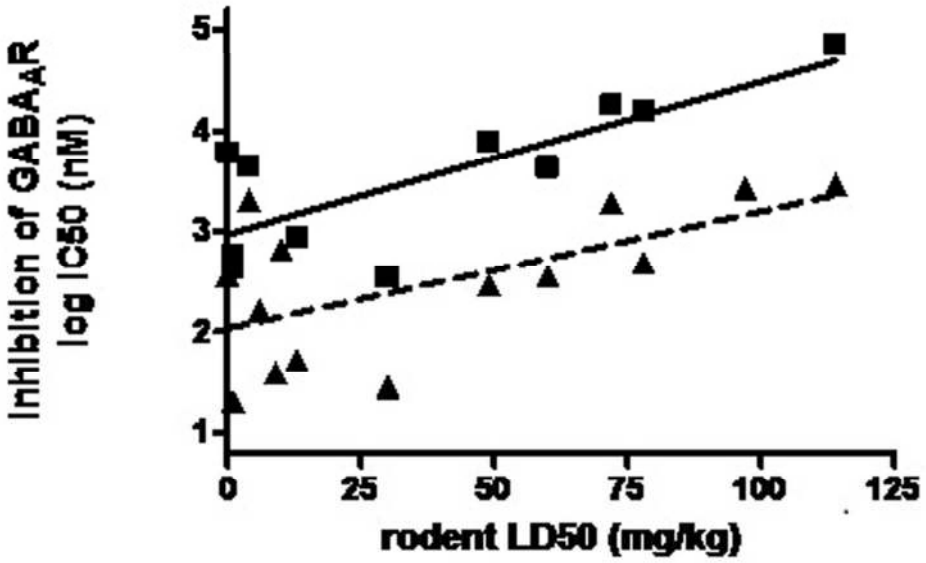

Fig. 4B.
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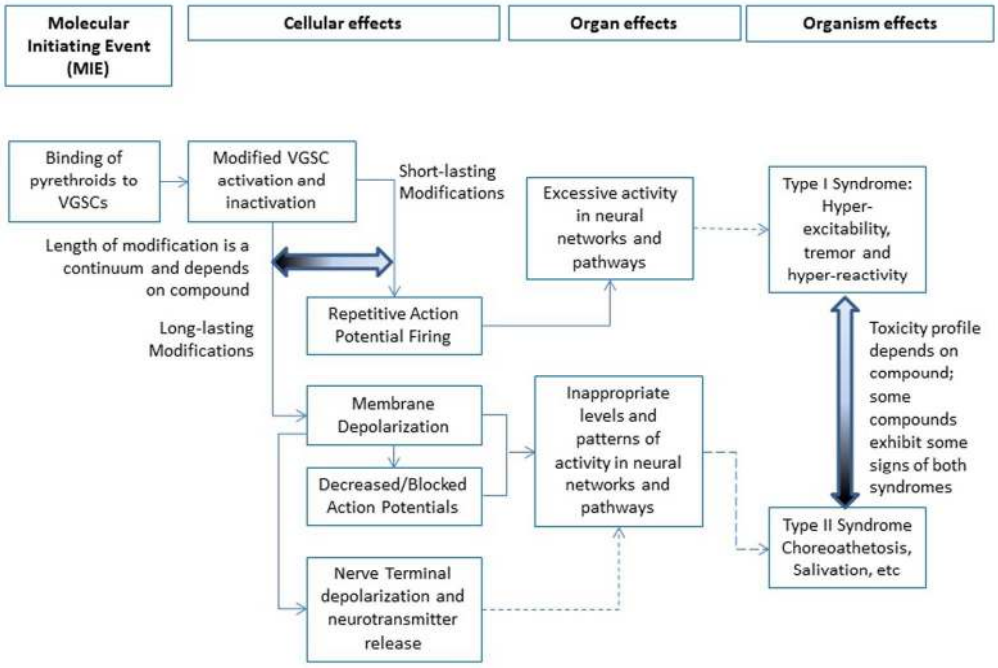

Fig. 5.
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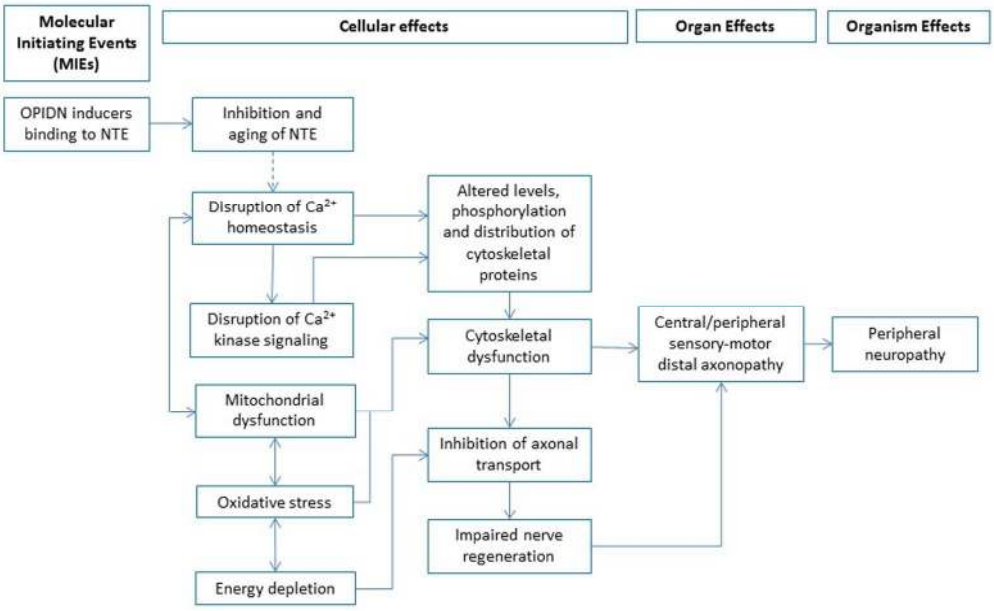

Fig. 6.
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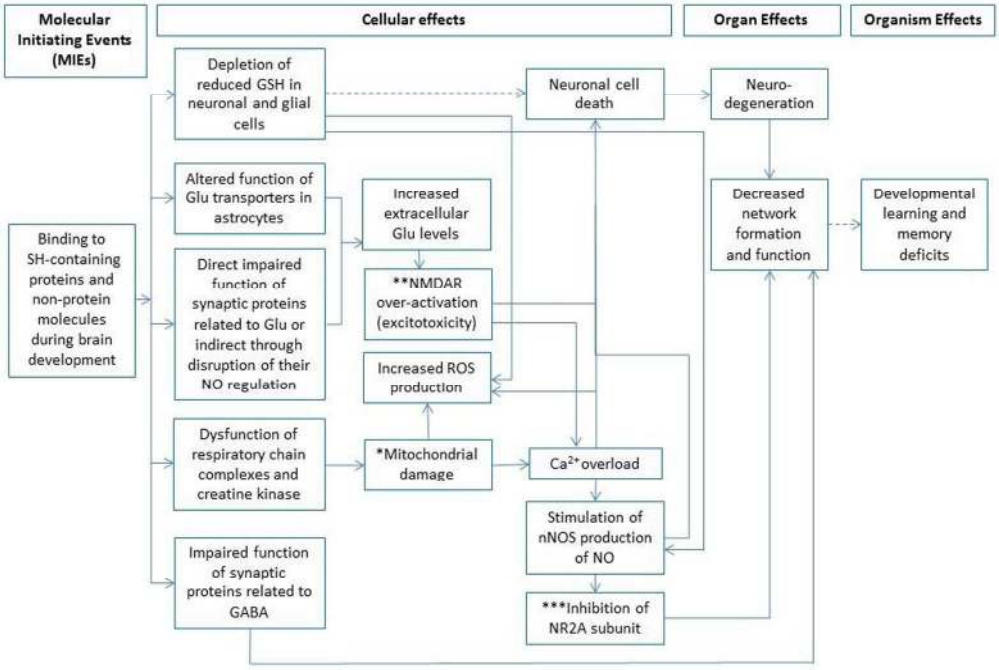

Fig. 7.
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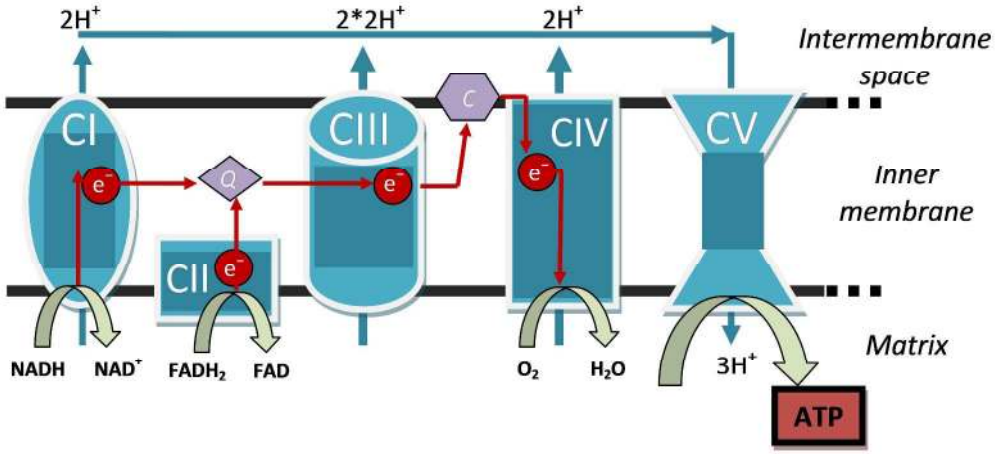

Fig. 8A.
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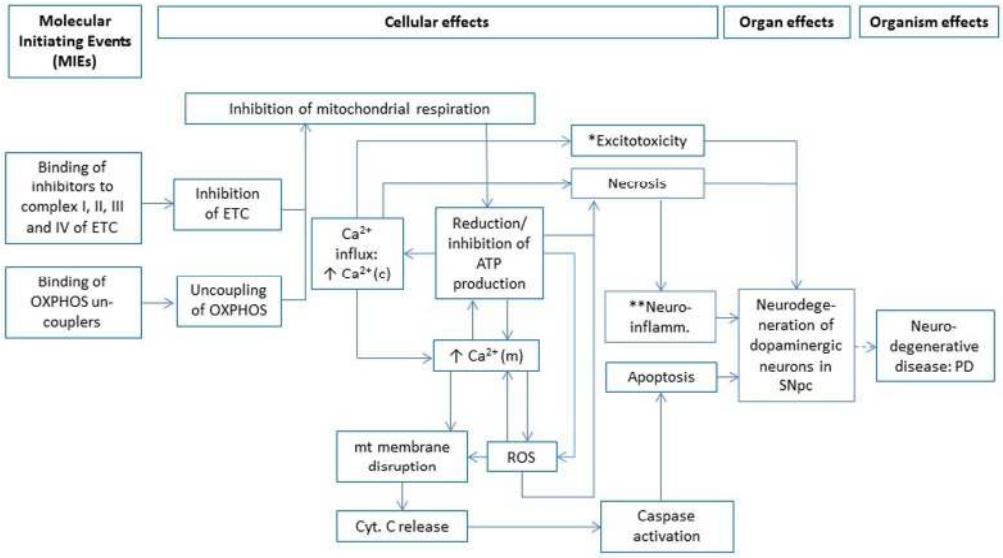

Fig. 8B.
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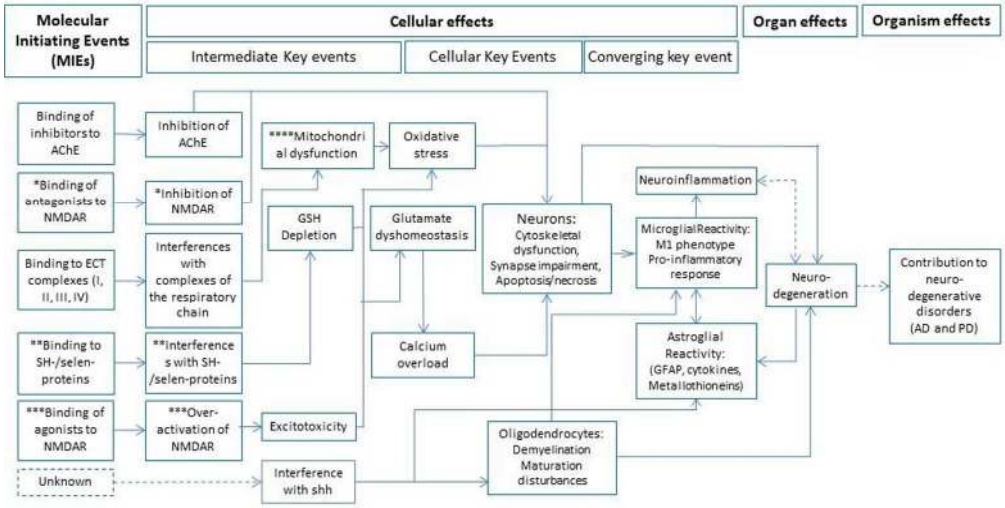

Fig. 9.
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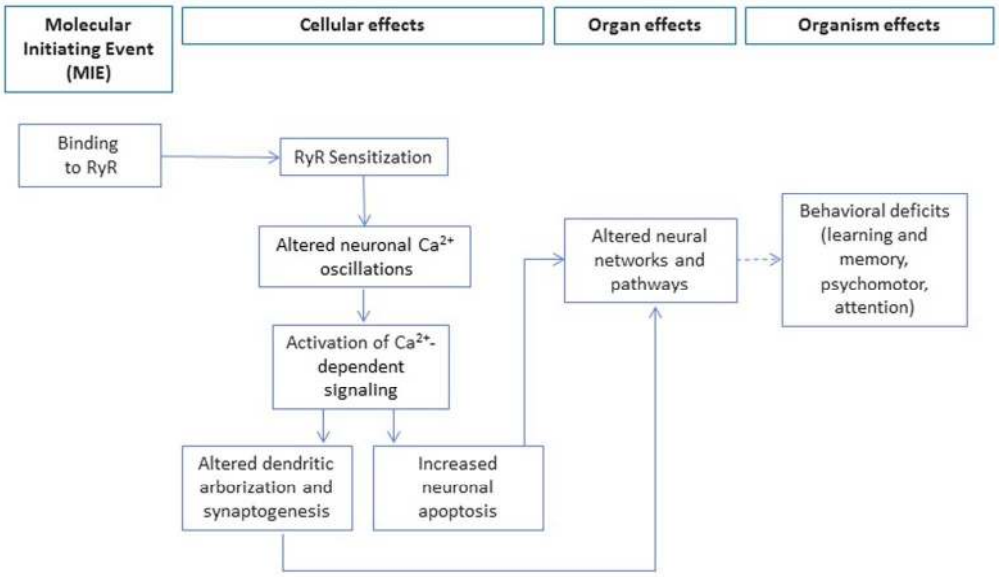

Fig. 10.
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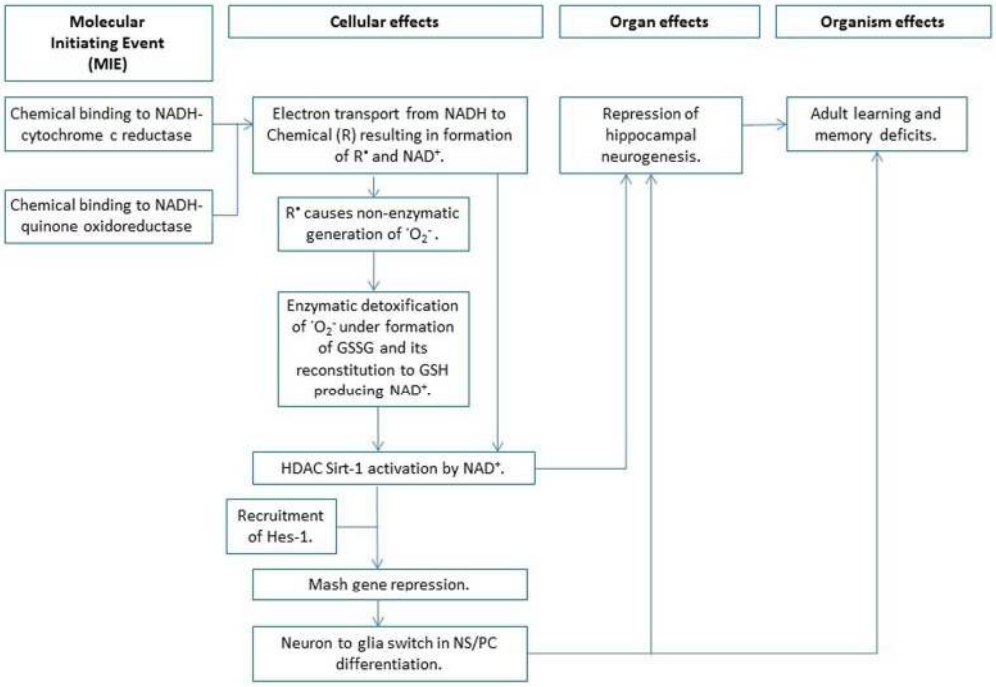

Fig. 11.
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