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Implementing AOPs use for pesticide neurotoxicity assessment

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Due to the unique characteristics of the nervous system, chemical exposures pose significant challenges in establishing definitive links to neurological adverse health effects. The key obstacles to neurotoxicity assessment include the complexity of the nervous system, species differences, extended developmental time, and cumulative exposure risks. Regulatory agencies have long required neurotoxicity testing, which has relied primarily on animal models. With increased mechanistic understanding and technological progress, other approaches become possible that could address some of the limitations of traditional neurotoxicity testing (e.g. high cost, ethical concerns, low precision, or human relevance). Recent international efforts to develop adverse outcome pathways (AOPs) and AOP-informed Integrated Approaches to Testing and Assessment offer new possibilities for neurotoxicity testing and assessment. This mini review highlights the advances, while addressing limitations, in the development and application of AOPs for both adult and developmental neurotoxicity as a way to address shortcomings in regulatory neurotoxicity assessment. Case studies are provided to illustrate the recent successful integration of AOPs into regulatory decision-making for pesticide risk assessment.

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Abbreviations
AD, Alzheimer's Disease; ANT, Adult Neurotoxicity; AOP, Adverse Outcome Pathways; AOPNs, Adverse Outcome Pathway Networks; CNS, Central Nervous System; DNT, Developmental Neurotoxicity; EFSA, European Food Safety Authority; FPB, Functional Observational Battery; IATA, Integrated Approaches to Testing and Assessment; KE, Key Event; KER, Key Event Relationship; MA, Motor Activity; MoA, Mode of Action; MPTP, 1-methyl-4-phenyl-tetrahydropyridine; NS, Nervous System; NT, Neurotoxicity; OECD, Organization for Economic Co-operation and Development; PD, Parkinson's Disease; PNS, Peripheral Nervous System; PPR, Plant Protection products; WoE, Weight of Evidence.

Introduction

Neurotoxicologists encounter substantial obstacles when trying to definitively associate a substance with adverse health effects, assess the probability of its occurrence, and determine a safe exposure limit. In humans, neurotoxicity (NT) after chemical exposure can affect several domains (for example, neurotransmission inhibition or stimulation, oxidative stress, mitochondrial dysfunction, altered calcium signaling, cytoskeletal modifications) that can lead to alteration of neurotransmitters system, axonal dysfunction/degeneration, neuroinflammation, and neurodegeneration. NT can appear rapidly following a single, acute exposure and may be functional and reversible (for example, acute NT associated with exposure to organophosphate insecticides due to acetylcholinesterase inhibition in the central nervous system [CNS]) [1]. Alternatively, NT may evolve gradually over months or years, possibly causing permanent structural or functional damage that could resemble symptoms of various neurological and psychiatric disorders (for example, developmental NT [DNT] from lead exposure or chronic parkinsonian motor deficits caused by the loss of nigrostriatal neurons induced by 1-methyl-4-phenyl-tetrahydropyridine [MPTP] poisoning or Alzheimer's disease, which has been linked in human observational studies to long-term exposure to organophosphate or organochlorine pesticides) [2–5].

There are some recognized inherent difficulties of assessing NT of chemicals; among them are the

Box 1. Pesticide EU regulations.

Pesticide active substances are approved at the EU level under regulation (EC) No 1107/2009 and following evaluation against a set of agreed data requirements detailed in the Regulation 283/2013. Both regulations address the potential hazards and risks associated with active substances. The potential neurotoxic effects must be carefully considered and documented in the required routine toxicological studies. Clinical signs and routine pathological endpoints should be measured in a range of studies from acute (OECD TG 402, 403, 423, 425) to short-term and optionally in long-term toxicity studies (OECD TG 407, OECD TG 408, and OECD TG 452). These evaluations, conducted on young adult rats, do not indicate DNT, which is instead assessed through OECD reproductive/developmental toxicity tests (OECD TG 421, 422, 414, 443). Specific NT studies in rodents (OECD TG 424, 418, 419, adult; TG 426, 443 with DNT cohort, development) are required under the following circumstances:

- if routine toxicity studies of the active substance reveal signs of NT, such as clinical and pathological findings;
- if the active substance shares a structural similarity with a compound known to be neurotoxic;
- if the active substance operates through a neurotoxic mechanism to exert its pesticidal effect.

OECD TG 424 outlines neurotoxicity studies in rodents that include exposure durations from acute or 28 days, 90 days, or chronic (1 year or more), involving both male and female young adult rats (three dosage levels plus a control). This testing approach can be integrated with repeated dose toxicity studies or conducted independently. It measures various endpoints, including behavioral changes, motor and sensory functions, and the histopathology of representative sections of the central and peripheral nervous system. Stand-alone studies may also include neurobehavioral, neurochemical, and neuropathological examinations, along with tests for learning and memory.

OECD 418 (acute exposure) and 419 (28-day repeated dose study) focus on the assessment of delayed neurotoxicity of organophosphorus compounds. These studies involve young adult hens as the test subjects. The key observations include behavioral abnormalities, motor skills such as the ability to climb a ladder, along with biochemistry, assessment of neuropathy target esterase activity in brain and lumbar spinal cord, and post-mortem histopathological examination of specific areas of the brain.

OECD TG 426 focuses on DNT, exposing pregnant rats from gestation day 6 and their offspring until postnatal day 21 (three dosage levels plus a control). The study concludes at postnatal day 70, offering comprehensive clinical observations and functional tests such as behavioral development, motor activity, motor and sensory functions, and learning and memory assessments, in addition to measuring brain weights and conducting neuropathological examination and morphometric analyses of the brain's most representative areas.

OECD 443, the Extended One Generation Reproductive Toxicity Study, which includes cohorts designated for NT assessment, may serve as an alternative approach.

following: (i) the nervous system (NS) exhibits a greater degree of cellular, structural, and chemical heterogeneity than other organ systems; (ii) toxic chemicals could affect any functional or structural component of the NS (e.g. sensory and motor functions, memory processes, behavioral and neurologic abnormalities) with species differences and at different developmental windows of exposure; (iii) the NS is well known for its long developmental period, which also continues after birth, and several NS functions can only be tested after a certain postnatal age as sufficient maturity must be reached before a meaningful test can be performed; (iv) the long human lifespan increases exposure to various chemicals and factors able to modulate the final response; (v) NS development is more vulnerable to exposure to environmental chemicals during fetal and early postnatal periods [4]. Moreover, the toxic impacts of chemical exposure can interact with other risk factors like prenatal stress, and the persistence of some chemicals in the brain over time may result in cumulative toxicity.

Along these lines, there is a growing concern that certain chemicals may increase the prevalence of neurodevelopmental disorders and the incidence of brain diseases like Parkinson's disease (PD) and Alzheimer's disease (AD) [5,6].

Regulatory requirements

Regulatory bodies have long acknowledged the concept of testing chemicals for NT potential [7]. The hazards related to DNT and adult neurotoxicity (ANT) are critical factors in the risk assessment of chemical substances. For instance, in the human health risk assessment of pesticide active ingredients in the EU, potential neurotoxic effects shall be carefully addressed and reported in routine toxicological studies. Specific tests for neurotoxic effects are required for crop protection (Box 1). In addition, NT is considered to categorize active substances: (i) an active ingredient with neurotoxic effects cannot be deemed a low-risk or basic substance; and (ii) an active ingredient should be flagged for substitution if concerns about DNT effects arise.

Presently, rodent *in vivo* testing guidelines serve as the primary reference data for NT potential assessment in humans (refer to Box 1). The range of tests covers three main elements: the "functional observational battery", motor activity, and morphometric evaluation and neuropathology. In addition, DNT guideline-based testing could also require learning and memory and auditory startle response evaluations (see Box 1).

However, the current Organization for Economic Cooperation and Development (OECD) NT test guidelines have recognized the limitations of interpretative

uncertainties, cost, and resource constraints. Various case studies in the last years have shown the potential of robust adverse outcome pathways (AOPs) and AOP-informed Integrated Approaches to Testing and Assessment (IATA) as a new framework for assessing NT and addressing some of these limitations [8–12].

Addressing shortcomings in regulatory NT evaluations: AOPs and AOP-informed IATA

It is acknowledged that (i) existing *in vivo* animal tests might not capture all human-relevant neurotoxic effects (e.g. pathological changes replicating human diseases such as PD, AD, and autistic spectrum disorders), (ii) the vast array of environmental chemicals could exceed our testing capabilities, considering the high costs, complexity, and the ethical imperative to minimize animal testing [13,14], (iii) with increased neurotoxicity mechanistic understanding and technological progress, other approaches have become possible [15–17].

This understanding has fostered a paradigm shift among international stakeholders, particularly for DNT testing, culminating in the OECD's initial recommendation document on how to integrate data from an *in vitro* battery of assays to provide reliable data on biomarkers of effect [18,8], moving toward its integration in an IATA framework with other *in vitro*, *in vivo*, *in silico*, and human observational studies data [19].

The AOP framework has been established to structure the evidence into a series of key events (KEs) that are causally and linearly connected to an adverse outcome of regulatory significance, assigning appropriate weight to the observed effect (weight of evidence, WoE) and incorporating any methodological approach appropriate for measuring each event. AOPs are pivotal in pinpointing data gaps and shaping test strategies that align more closely with human biology.

The increasing number of AOPs stored in the wiki has led to the organization of some of the most well-established linear AOPs into AOP networks (AOPNs), which bring together different AOPs that share one or more events (MIEs, KEs, Adverse Outcome). By integrating complex biological knowledge, AOPNs provide a more comprehensive understanding of biological systems, illustrating how different pathways can interact, overlap, or converge to produce a range of outcomes (For an extensive and recent review of the AOPNs topic, see Refs. [20,21]). AOPNs have the potential to support different aspects of hazard and risk assessment. For example, the identification of common biological mechanisms can highlight critical targets and support the grouping of chemicals with similar mode of action (MoA) or understand whether chemical exposure might lead to cumulative effects by activating multiple pathways. However, the actual application of AOPs/AOPNs in hazard and risk assessment processes remains limited and discussions on how to best use AOPs/AOPNs for regulatory purposes are still ongoing. The use of AOPs as a framework to develop AOP-informed IATA for NT hazard characterization, together with the use of AOPs for assessing MoA in the identification of endocrine-disrupting properties of pesticide active substances for human health, represents an ongoing area in the Next Generation Risk Assessment [22,3]. This has been exemplified in various European Food Safety Authority (EFSA) Scientific Opinions and IATA case studies [9–11,23–25].

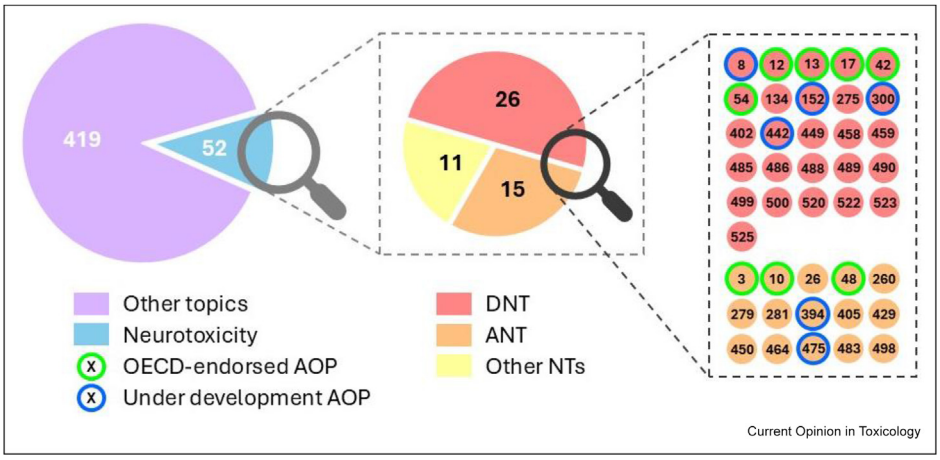
Progress of AOPs in targeting neurotoxicity

A comprehensive state-of-the-art assessment of existing AOPs for ANT and DNT has been recently published in Ref. [26]. From the total number of available AOPs in the AOP wiki (the website developed by the OECD which acts as an AOP's repository), more than 10% are for NT pathways as indicated in Figure 1, representing one of the most developed areas in the AOP wiki, updated to the end of May 2024, but still very limited for this complex endpoint.

Out of the 52 AOPs listed, some of them cannot be distinctly categorized as either DNT or ANT. This is due to the author's use of vague descriptions and broad terms, making unclear the AOP life stage (DNT or ANT) they pertain to and representing a limitation. A key element that affects how the NS responds to a toxicant is its age. This is largely because the NS undergoes dynamic development through processes that are tightly controlled both temporally and spatially, and these processes diminish as the NS matures. Chemical exposure early in life can disrupt neurodevelopmental processes, potentially leading to lasting effects on neurodevelopment and/or clinical outcomes in later life, which would differ from those seen in a mature NS. This difference also impacts the methodologies required to evaluate the various KEs.

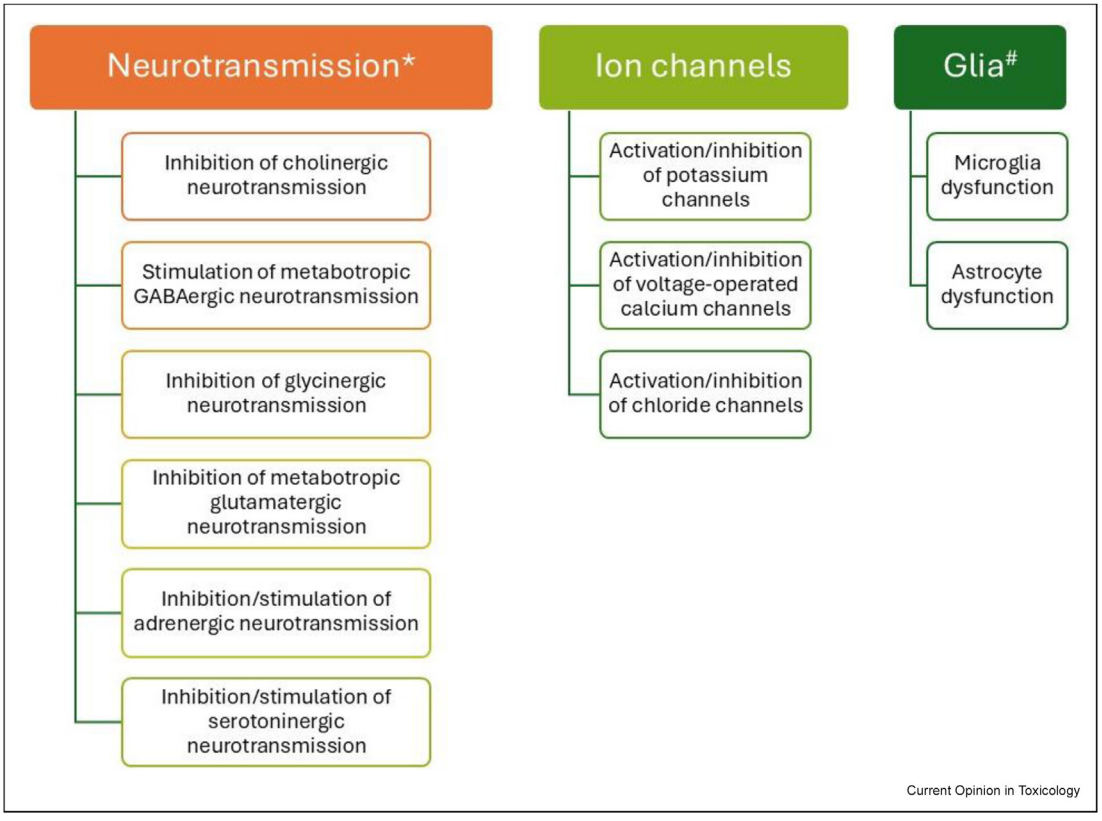
DNT AOPs account for half of the NT AOPs and are almost twice as many as ANT AOPs. The quantity of DNT AOPs that have received OECD endorsement or are in development surpasses that of ANT AOPs. This discrepancy is likely influenced by the scientific community's prioritization of DNT due to the recognition that the biological and toxicological consequences can be different when a molecular or cellular disruption occurs during NS development [27], as well as the advancement of new methodologies that focus on developmental stages [28,8]. The topics addressed by these 52 NT AOPs in the AOP wiki are diverse; however, certain areas are not as well represented. For instance, there is a significant disparity between the CNS and the peripheral nervous system (PNS), with the latter being considerably less focused on. Additionally, only a few AOPs properly consider the role of

Figure 1



Pie charts representing the details of AOPs addressing NT in the AOP wiki. The number of AOPs addressing DNT and ANT is shown, together with their OECD status. ANT: adult neurotoxicity; AOPs: adverse outcome pathways; DNT: developmental neurotoxicity; NT: neurotoxicity; OECD: Organization for Economic Co-operation and Development.

Figure 2



Endpoint categories not covered in the AOP wiki database as of October 1, 2024. Gaps were selected from fully or partially described AOPs. *KE 2151 provides a general description of the overall process of neurotransmission. #Glia in the AOP wiki is only covered in the context of neuroinflammation, and there are no AOPs that define pathways in specific populations of glial cells. AOP: adverse outcome pathway.

glial cells (Figure 2). While numerous adverse outcomes concentrate on issues like memory loss, learning impairments, and cognitive deficits, those about motor functions and sensory abilities are less frequently covered.

One of the main outcomes of this study shows the large difference in curation and quality of the NT AOPs currently available in the AOP wiki. Incomplete or poorly reported AOPs, KEs, and key event relationships (KERs) are of more limited regulatory use than the ones reviewed and with robust WoE. Scientifically, the development of AOPs for complex endpoints is challenging and resource-demanding. It requires an interdisciplinary team with expertise at all levels of biological organization and methodology, and this is a complex undertaking, normally beyond the scope of a typical research project. This can be achieved through an independent, conflict-of-interest-free review as, for example, within the OECD program [29] or EFSA Panels. Nevertheless, AOPs with knowledge gaps and uncertainties can still be useful since they can map future research areas and evolve as new data and knowledge are generated. Figure 2 highlights the gaps in NT endpoint categories that are not yet covered in the AOP wiki database as of October 1, 2024.

AOPs are living documents that reflect the current state of science and knowledge as well as the expertise and investment of the authors in organizing and communicating the current state. Addressing some of these challenges will require enhancement in cooperation and collaboration between academic and regulatory bodies in this area as a recognized key success factor. In the following section, we will discuss two AOPs developed for addressing a regulatory need for NT assessment as an example of academic and regulatory bodies' collaborative effort: one associated with DNT and another with ANT. Both integrated into decision-making using AOP-informed IATA and demonstrated useful for filling information gaps.

Developmental Neurotoxicity (DNT)–Adverse Outcome Pathway (AOP) 442 [27]: binding to Voltage Gate Sodium Channels (VGSC) during development leads to cognitive function decrease

The Plant Protection products of EFSA (PPR-EFSA) developed this AOP originally as a stressor evidence–based, AOP-informed IATA [9] designed in response to a defined question in a specific regulatory context. The purpose of the IATA case study, specifically, was to provide an example of the applicability of an *in vitro* battery for DNT testing for hazard assessment in the context of the European Pesticide Regulation. For this IATA, a systematic literature review was done for a specific chemical and 3776 references were screened. For *in vitro* evidence, 31 papers were selected measuring 60 DNT endpoints; for human observational studies, 8 publications were selected measuring 11 DNT endpoints; for *in vivo*, 17 publications were selected measuring 52 DNT endpoints (details on methods for conducting the assessment, including eligibility criteria for study selection, study selection process, and data extraction are reported in Ref. [30]). A chemically agnostic AOP, detailed in the AOP wiki, was then developed by conducting both systematic broad and focused literature searches (i.e. searching literature using systematic search terms and, most importantly, providing a transparent description of how the literature was searched and selected) to collect empirical evidence in support of proposed KERs, all KERs of the AOP are now adjacent. The systematic methodology allowed for a structured, evidence-based, and transparent approach that allows for reproducibility of the work done. Despite the limited quantitative information at all KER levels, regulatory applications of this AOP for DNT assessment have been identified since robust and reliable *in vitro* methods exist mapping downstream KEs.

ANT–AOP 3: inhibition of the mitochondrial complex I of nigrostriatal neurons leads to Parkinsonian motor deficits

The AOP 3, created in 2016 by PPR-EFSA and detailed in the AOP wiki, was designed to explore the toxic effects of plant protection products in relation to PD. The aim was to support epidemiological findings linking pesticide exposure to an increased risk of developing PD and to help integrate these data into regulatory risk assessments. Developed according to the OECD guidelines, AOP 3 was based on a comprehensive analysis of existing literature, though not a systematic review. Despite this, the quality and comprehensiveness of the documentation led to its endorsement by the OECD.

Experimentally, AOP 3 has been tested against 21 substances known as mitochondrial inhibitors [31], which helped to enhance the AOP network by adding more MIEs that affect mitochondrial function and provided dose–response information up to KE4 for the pesticides tested. AOP 3 resulted effective in incorporating specific technologies and test systems that have supported the establishment of *in vitro* points of departure [12] and, along with toxicokinetic simulations, the read-across of structurally related substances [32]. AOP 3 is currently the only NT-related AOP that has been modeled and calibrated to predict dose–response curves for all KEs. However, AOP 3 still requires refinement to better support risk assessment. It identifies parkinsonian motor deficits as the adverse outcome, which is just one aspect of a multifaceted disease. Therefore, additional AOPs are needed to address the full complexity of the disease. In addition, AOP 3 does not account for modulatory events that may be crucial in determining the adverse outcome, as

shown by the lower concentrations affecting earlier KEs than the later ones [31]. Finally, refining the KE descriptions to reflect specific biological events representative of different compounds' actions, such as the various events leading to mitochondrial dysfunction, is necessary.

Conclusions

AOPs/AOPNs and AOP-informed IATA represent important tools toward an NT mechanistic-based risk assessment. Recent case studies have demonstrated AOPs relevance particularly when used to develop or map reliable *in vitro* methods and within an AOP-informed IATA to draw a regulatory conclusion. In this line, AOPs can be frameworks to develop IATAs (e.g. AOP 3) and AOP-informed IATAs can be a starting point to develop AOPs (e.g. AOP 422). This approach, when based on systematic methods and robust WoE, strengthens the scientific basis for risk assessment and supports regulatory decision-making. Looking ahead, it is critical to (i) develop more AOP-informed IATA case studies, (ii) improve the quality and completeness of the existing AOPs for NT endpoints, including addressing gaps in mechanisms and pathways relevant to the progression of NT to provide a more comprehensive overview of the different pathways leading to neurotoxicity and complex diseases, as such, (iii) implement linear AOPs in AOPNs to better address the complexity of chemical MoAs, and (iv) further develop more through interdisciplinary efforts and rigorous peer review. Addressing these challenges will lead to both to an understanding of how chemical exposure might affect brain health and to the translation of this knowledge into new solutions for regulatory science.

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No data was used for the research described in the article.

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** of outstanding interest

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This case study was developed to show the applicability of an in vitro battery (IVB) for developmental neurotoxicity (DNT) testing in the context of the European Pesticide Regulation. This represents an example of good practice for developing an Adverse Outcome Pathway (AOP) that is relevant from a regulatory perspective.

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This case study was developed to show the applicability of an in vitro battery (IVB) for developmental neurotoxicity (DNT) in the context of the European Pesticide Regulation. This represents an example of good practice for developing an Adverse Outcome Pathway (AOP) that is relevant from a regulatory perspective.

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This study aims at mapping biological knowledge within the AOP-Wiki represented by the already developed AOPs (with OECD-endorsed status or under validation). It is important since it provides a current and comprehensive overview of the AOP-Wiki database also considering the limitations and data gaps.

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