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REVIEW

Neurotoxicity of pesticides

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Abstract

Pesticides are unique environmental contaminants that are specifically introduced into the environment to control pests, often by killing them. Although pesticide application serves many important purposes, including protection against crop loss and against vector-borne diseases, there are significant concerns over the potential toxic effects of pesticides to non-target organisms, including humans. In many cases, the molecular target of a pesticide is shared by non-target species, leading to the potential for untoward effects. Here, we review the history of pesticide usage and the neurotoxicity of selected classes of pesticides, including insecticides, herbicides, and fungicides, to humans and experimental animals. Specific emphasis is given to linkages between exposure to pesticides and risk of neurological disease and dysfunction in humans coupled with mechanistic findings in humans and animal models. Finally, we discuss emerging techniques and strategies to improve translation from animal models to humans.

Keywords Pesticide · Neurotoxicity · Neurodegeneration · Organophosphate · Pyrethroid · Rotenone · Pyridaben · Mitochondrial Complex I · Glyphosate · Organochlorine · Dieldrin · Dichlorodiphenyltrichloroethane · Endosulfan · Microelectrode array · Zebrafish · iPSC · Paraquat · Fungicide

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AOP	Adverse outcome pathway
APOE	Apolipoprotein E
BBB	Blood–brain barrier
BHC	Beta-hexachlorocyclohexane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DUMBBELS	Diarrhea, urination miosis/muscle weakness, broncorrhea, bradycardia, emesis, lacrimation, salivation/sweating
EPA	US Environmental Protection Agency
FDA	US Food and Drug Administration
GSH	Glutathione
IL1β	Interleukin-1β
IL6	Interleukin 6
iNOS	Inducible nitric oxide synthase
iPSC	Induced pluripotent stem cell
MEA	Microelectrode array
MRI	Magnetic resonance imaging
MN-EBCD	Manganese ethylene-bis-dithiocarbamate
MN/ZN-EBCD	Manganese/Zinc ethylene-bis-dithiocarbamate
MPP^+	1-Methyl-4-phylpyridinium
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NOX2	NADPH oxidase 2
NSC	Neural stem cell
OP	Organophosphate
OPIDN	Organophosphate-induced delayed neuropathy
PD	Parkinson's disease
PKC	Protein kinase C

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SCG2	Secretogranin II
SOD	Superoxide dismutase
SNPc	Substantia nigra pars compacta
SLUD	Salivation, lacrimation, urination, diarrhea
TOCP	Tri-ortho-cresyl phosphate
TNFα	Tumor necrosis factor alpha
UPS	Ubiquitin–proteasome system
WHO	World health organization

Introduction

Pesticides are substances or preparations that repel, destroy or control pests [1]. As a class of compounds, pesticides are, in practice, composed of many subclasses that are generally divided based on their target pests. Although not an exhaustive list, the primary classes include pesticides targeting insects (insecticides), weeds (herbicides), fungi and molds (fungicides), and rodents (rodenticides), all of which may have several subclasses of their own based upon chemical identity, physical state/application method (i.e., fumigants), and origin of derivation (i.e., biopesticides, botanicals, etc.). While clearly diverse, the common goal of pesticide application results in a unique situation of environmental exposure since pesticides are specifically put into the environment with the intent to damage an organism.

Historically, pesticide use dates to long before the Common Era, where elemental sulfur was among the first known chemicals used as a pesticide, a practice that continues today in grape vineyards [1]. Moving into the Common Era, toxic metals, primarily arsenic, were used as pesticides in a practice that persisted into the twentieth century. In the nineteenth century, during which chemistry made major strides, pesticides derived from natural products, including pyrethrum from chrysanthemums and rotenone from legumes, became common in their use. It was not until the late nineteenth and early twentieth centuries, however, that synthetic organic chemistry became the backbone of modern pesticides, starting with the identification of the insecticidal properties of organochlorine compounds [dichlorodiphenyltrichloroethane (DDT) and β-hexachlorocyclohexane (BHC)] in the 1930s, which had been first synthesized in the 1820–1870s. The identification of DDT as a cheap, broad-spectrum insecticide by Muller in 1939 led to the first demonstration of the ability of an insecticide to have a significant positive impact on human health; it showed outstanding efficacy in suppressing typhus, malaria, and other insect-borne diseases. At the same time, synthetic organic chemistry was also being used to develop nerve agents in parallel with insecticide development. Through this research, several nerve agents were identified, which also launched the introduction of the organophosphate insecticides that became the dominant insecticide class used in the twentieth century. During this same time, synthetic organic techniques were used to develop herbicides and fungicides, including 2,4-dichlorophenoxyacetic acid (2,4-D) and the dithiocarbamates, both of which are still heavily used. Today, worldwide pesticide usage approaches almost 8 billion pounds (3.6 billion kg) of active ingredient per year [2].

Pesticides have proven to be an essential tool in agriculture and public health [3]. The fact, however, is that molecular targets of pesticides are often shared between pest and non-target species, including humans. This is particularly true for the neurotoxic organochlorine, organophosphate, and pyrethroid pesticides. Similarly, pesticides that inhibit mitochondrial complex I are increasingly being used as miticides (mites) and acaricides (mites and ticks). Although herbicides and fungicides theoretically should not have shared targets with mammals, several have been demonstrated to affect the mammalian brain. This review will focus on major classes of pesticides that have been demonstrated to or are suspected of causing neurotoxicity based on clinical, epidemiological, and experimental studies. Finally, new and emerging technology for translating neurotoxic effects from the laboratory to humans will be discussed.

Organochlorine insecticides

Owing to their slow degradation, organochlorine pesticides are notoriously persistent chemicals in the tissues of mammals, especially those occupying higher trophic levels. Direct human exposure to organochlorine pesticides occurs through contaminated fruits, vegetables, grains, dairy products, and meats, as well as through agricultural settings [4]. In this section, we will discuss three organochlorine insecticides, DDT along with the cyclodienes, dieldrin and endosulfan, that have been linked to neurotoxicity and human neurodegenerative disease.

DDT

DDT was used extensively from the 1940s through 1972 in the United States (US) in agriculture as a broad-spectrum insecticide and for control of vector-borne diseases [5]. Although the use of DDT was banned in the US in 1972 over concerns regarding its environmental persistence and potential effects on wildlife, it was still used throughout the 1980s around the world for various reasons, e.g., mosquito control. Significant decreases in serum levels of DDT and its metabolite dichlorodiphenyldichloroethylene (DDE) levels have been observed in adults over the past three decades in the US. DDE, however, is still found in almost all serum samples from the centers for disease control and prevention's cross-sectional National Health and Nutrition Examination

Survey (NHANES) [6]. Current exposure sources likely include the import of food from countries where DDT is still used, or from legacy contamination of soil and waterways [7]. Indeed, the US Food and Drug Administration (FDA) reported that 15% of food samples surveyed contained measurable DDT levels in 2011. Internationally, DDT has been recommended by the World Health Organization (WHO) for malaria eradication, which has increased its usage in recent years.

DDT exerts its insecticidal activity by causing voltage-gated sodium channels (Na_v) to remain open, leading to persistent depolarization and hyperactivity in the nervous system. DDT is only moderately toxic (rat oral $LD_{50} = 113$ mg/kg) [8] and oral administration of 3.5 or 35 mg DDT/day to humans for up to 18 months did not cause overt toxicity or neurotoxicity [9, 10]. However, the potential for chronic exposure to DDT has raised concerns about a variety of potential adverse health effects [11, 12]. Unfortunately, only a few human studies have explored the potential neurotoxicity of DDT. Two studies found that workers engaged in spraying DDT displayed cognitive dysfunction, although no measurements of DDT or DDE were available for either study [13, 14]. One small study reported that DDT was found more often in brains from patients with Alzheimer's disease (AD; $n = 7$) compared to controls ($n = 14$) [15]. Most recently, a study using data from the NHANES population reported that serum DDE levels were associated with decreased cognitive function in elderly people in the US, suggesting that non-occupational exposures to DDT can also cause cognitive deficits [16]. Recently, Richardson and co-workers [17] reported that serum levels of DDE are approximately fourfold higher in AD patients, and this was associated with increased risk of AD diagnosis [odds ratio (OR) = 4.18]. Furthermore, high DDE levels and the presence of an apolipoprotein E (APOE) $\varepsilon 4$ allele resulted in greater cognitive impairment when compared to those without an APOE $\varepsilon 4$ allele. Concentrations of DDE and its parent compound DDT similar to those observed in highly exposed individuals in the general population of the US [10, 18, 19] also increased amyloid precursor protein levels in cultured neuronal cells.

Dieldrin

Based on a number of epidemiological and experimental studies, the organochlorine pesticide dieldrin is of particular concern, [20–22]. This insecticide was widely used throughout the US until the late 1980s when it was banned. It still persists heavily in the environment, particularly in soil sediments [23], due to its long half-life ranging between 141 and 592 days. Elevated levels of dieldrin persist in the serum of humans who have been previously exposed [22, 24]. For example, workers in India tied to the manufacture or use of dieldrin were found to have blood dieldrin levels up to 50 ng/ml [25]. Most importantly, high dieldrin levels have been detected in postmortem human Parkinson's disease (PD) brains as compared to age-matched human control brains [15, 21, 26].

Exposure to dieldrin has a strong inhibitory effect on the central nervous system of insects such as flies and cockroaches [27] because of its effects on $GABA_A$ receptors.

Dieldrin-induced neurotoxicity has been reported in many in vitro studies using dopaminergic neuronal cells [21, 28]. Exposure of these cells to dieldrin promoted severe oxidative stress, mainly due to mitochondrial dysfunction [21, 28], and was accompanied by significant upregulation and activation of caspases, thereby leading to apoptosis [29, 30]. Caspase-3-dependent proteolytic cleavage of protein kinase C (PKCδ) plays a major role in dieldrin-induced dopaminergic neurotoxicity [21, 30]. Studies have also highlighted the ability of dieldrin to induce mitochondrial impairment and ubiquitin–proteasomal dysfunction [31, 32]. Overexpression of Bcl2 attenuated dieldrin-induced poly (ADP-ribose) polymerase cleavage and apoptosis, suggesting mitochondrial redox signaling is an upstream event of dieldrin-induced dopaminergic neurotoxicity [33].

Dieldrin-induced neurotoxicity has also been well studied using mouse models. Pivotal research showed that exposing perinatal mice to low doses of dieldrin (0.3, 1, or 3 mg/kg every 3 days) during their gestation and lactation period significantly altered dopaminergic neurochemistry and accentuated the severity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity. This study also suggested that developing dopaminergic neurons might become more vulnerable later in life to chronic oxidative stress events [34]. A similar study demonstrated that another chlorinated cyclodiene, heptachlor, produced similar effects [35] and heptachlor epoxide levels have been associated with Lewy body pathology in postmortem PD brain [36]. Other studies highlighted the neurochemical deficits and oxidative damage, including in the nigrostriatal region, induced by chronic low-dose dieldrin exposure in animal models [20]. It was also reported that dieldrin induces epigenetic dysregulation through hyperacetylation of core histones in cell and mouse models of dieldrin neurotoxicity [37]. Collectively, these studies identify potential molecular mechanism by which dieldrin exposure may contribute to increased PD risk.

Endosulfan

Endosulfan is an organochlorine insecticide widely used in agriculture and forestry around the world. The US environmental protection agency (EPA) includes certain organochlorines including endosulfan, on its "extremely hazardous substances" list that was generated in 1987. However, despite the proposed ban in the US, endosulfan is still used

worldwide [38]. About one billion tons of organochlorine pesticides, including endosulfan, have been manufactured and used commercially for insect control on fruits, vegetables, grains, cotton, and tea crops [21, 38]. Mounting evidence documents adverse effects of endosulfan on both the environment and non-target organisms due to its long persistence and high bioaccumulation in the tissues of animals and humans. It is known to be highly neurotoxic, causing both developmental neurotoxicity and chronic neurodegeneration in rodents [39, 40]. Furthermore, acute exposure in agricultural workers has been shown to lead to epilepsy, memory impairment, and hyperactivity disorders [40, 41].

In addition to inducing apoptotic cell death via mitochondrial dysfunction and oxidative stress [42], endosulfan treatments also promote inflammatory responses and glial activation in both cell culture and animal models [39, 43]. Furthermore, cell culture and animal models of PD reveal that dopaminergic neurons are more sensitive than other cell types to endosulfan-mediated neurotoxicity [44]. Animal studies further highlight the role of endosulfan in mediating PD-related neuropathological changes and neurochemical deficits, including dopamine depletion and increased levels of both normal and aggregated α-synuclein in mice [45]. Endosulfan-exposed mice also exhibits stunted central nervous system development with severe disruptions to synaptic trafficking events and synaptogenesis, which negatively modulates behavior, memory, and learning [46, 47]. A recent study [48] showed that rats gavaged with 2 mg/kg endosulfan for 6 days exhibited damaged brain mitochondria marked by significantly reduced levels of catalase, glutathione (GSH), and superoxide dismutase (SOD), and increased lipid peroxidation.

Organophosphate insecticides

Organophosphorus (OP) insecticides were developed in the 1930–1940s, but the first OP compound synthesis dates to the 1800s. Gerhard Schrader was the first to propose that organophosphate compounds could potentially be used as an insecticide. He continued to work on OPs at IG Farben in Germany, where he discovered several new insecticides, including parathion (E605), and accidentally the nerve agent tabun. Subsequent work by his group resulted in the synthesis of additional G-series nerve agents including sarin, soman, and cyclosarin, the formulas of which were taken over by the German government for large scale production [49]. In this section, we will review several OP insecticides, focusing on their chemistry, mechanism of action, and neurotoxicity in animal and human studies.

For both OP insecticides and nerve agents, the general chemical structure consists of a phosphorus atom bound to an oxygen or a sulfur atom [50]. The remaining bonds include a wide variety of groups that often consist of two alkoxy moieties and another moiety, which is often the "leaving group". In the case of most insecticidal OPs, the phosphorus atom is bound to a sulfur atom that must be metabolically activated through a desulfuration reaction to form an oxon. This reaction is mediated through multiple cytochrome P450 enzymes primarily in the liver, but there is also evidence of target organ bioactivation (i.e., brain, lung, etc.). The desulfuration reaction proceeds through a phosphooxythiran intermediate that results in various ratios of oxon formation (via desulfuration), or another reaction generically termed diarylation which serves as a detoxication mechanism [51].

OP compounds exert neurotoxicity primarily through the inhibition of acetylcholinesterase (AChE). At the molecular level, the oxon moiety phosphorylates a serine hydroxyl found in the active site of AChE [52]. Once bound, the phosphorylated serine can be generated through spontaneous hydrolysis. This is a slow process with a rate that is generally determined by the nature of the leaving group, with less alkylated (i.e., dimethyl) compounds being hydrolyzed more rapidly than more alkylated (i.e., diethyl) compounds. In some cases, AChE can become "aged", which represents a permanent inactivation caused by loss of one of the alkyl groups. Inhibition of AChE leads to a buildup of acetylcholine in the synapse, and hyperstimulation of cholinergic receptors in the central (primarily muscarinic receptors and some nicotinic) and peripheral nervous system (primarily nicotinic receptors and some muscarinic). This hyperstimulation leads to the classic signs of OP intoxication that is often clinically termed as SLUD syndrome (salivation, lacrimation, urination, and diarrhea), or the more encompassing DUMBBELS (diarrhea, urination, miosis/muscle weakness, bronchorrhea, bradycardia, emesis, lacrimation, salivation/sweating). Typically, these signs are observed in acute poisoning situations that result in over 70% inhibition of AChE [53]. If inhibition is severe and sustained, death may occur through depression of respiratory centers in the brainstem and paralysis of the diaphragm. Current countermeasures to acute OP intoxication include administration of atropine that blocks muscarinic receptors, oximes (i.e., 2-PAM) that assist with reactivation of AChE, and benzodiazepines (i.e., diazepam or midazolam) to control seizures.

Two additional syndromes have also been observed in some humans (~ 20%) poisoned with OP insecticides. The first is termed as the "intermediate syndrome," which was first described in the 1980s. It typically begins days following a poisoning incident, and after the point when the patient appears to be recovering from acute cholinergic crisis [54]. This syndrome manifests as weakness of the respiratory muscles in the diaphragm, intercostals, and neck, along with weakness in the proximal limb muscles. At the present, there is no established relationship between specific

OP pesticides and development of the syndrome. Clinical management involves maintaining respiratory function, and most patients exhibit full recovery. The second syndrome is termed as organophosphate-induced delayed polyneuropathy (OPIDN) [55]. OPIDN is relatively rare and occurs days to weeks after OP intoxication. The most famous example of OPIDN occurred during the Prohibition Era in the United States, when a large number of men (estimated in the tens of thousands) developed signs of OPIDN, including arm and leg weakness, following consumption of "Ginger Jake", a patent medicine containing a large amount of alcohol. Subsequent investigation determined that the preparation was adulterated with tri-ortho cresyl phosphate (TOCP). The exposure caused axonal damage, particularly in the spinal cord, and led to the characteristic "jake leg" that presented with the toes touching the ground before the foot due to loss of motor control from muscles that moved the toes [56]. The molecular targets of OPIDN do not appear to be AChE, but rather a protein termed neuropathy target esterase (NTE) that involves an aging phenomenon similar to that observed with AChE [57]. Indeed, it is estimated that over 70% of NTE must be inhibited and aged for OPIDN to occur; however, not all inhibitors of NTE lead to OPIDN and structure activity relationships have revealed that only certain phosphates, phosphonates, and phosphoramidates can produce this effect [57]. Based on these findings, all OP insecticides must undergo screening for delayed neuropathy as part of regulatory approval and no pesticides that produce OPIDN are approved for use in the US.

Occupational exposures to OP insecticides at levels that do not result in the overt toxicities described above have become a major focus of research since they have the potential to result in long-term neurological impairment. Munoz-Quezada and co-workers recently reviewed the effects of chronic exposure to OP insecticides on neuropsychological function in farm workers [58]. Out of over 1000 articles, 33 met eligibility criteria for inclusion in the analysis, and 24 found significant associations between chronic occupational exposure to OP insecticides and reduced neuropsychological performance. The reduced performance spanned a variety of domains, including executive function, visuospatial ability, working and visual memory. Similarly, Ross and co-workers performed a meta-analysis of 14 studies related to low-level occupational exposure of OP insecticides [59]. The majority of these studies also found significant associations between OP exposure and impaired neurobehavioral function in the same domains mentioned previously. While there are numerous caveats to systematic reviews and meta-analyses, including the limited number of high-quality studies, the general consensus is that occupational exposure to OP insecticides leads to neurological impairment. Sanchez-Santed and colleagues have also reviewed potential links between OP exposure and overt neurodegeneration, which revealed a potential relationship between OP exposure and AD risk, mixed evidence for relationships to PD, and weak evidence for amyotrophic lateral sclerosis [60]. Although research in this area is limited, there have been tentative links established between OP exposure, particularly occupational exposures, and risk of AD [61, 62]. With regard to PD, the evidence for OP involvement is mainly negative, and positive associations are weak at best [60]. Similarly, there have been reported links between occupational OP exposures and amyotrophic lateral sclerosis, but these are, for the most part, inconsistent and weak [63]. Thus, occupational OP exposure appears to lead to disruption of neurological function, but much work remains to establish links with specific diseases.

With regard to non-occupational exposures, the 1990s represented a time of intensive investigation of potential developmental neurotoxicity following the National Academy of Science's report entitled Pesticides in the Diets of Infants and Children (NAS, 1992). This report raised significant concerns regarding the potential for children to represent a uniquely susceptible population to OP neurotoxicity and identified dietary and non-dietary oral exposures as major routes of exposure. Specifically, the report identified a higher potential of exposure based on child-specific activities, the presence of critical periods of vulnerability in the developing nervous system, and that developing children do not have a full complement of enzymes involved in detoxication of OP insecticides [64]. To date, the majority of research on developmental neurotoxicity of OP insecticides is dominated by chlorpyrifos, which has been the most widely used of the OP insecticides [65]. Although much work continues in this area, the EPA effectively banned the household use of chlorpyrifos and most other OP insecticides in the early 2000s based on concerns over developmental neurotoxicity. Epidemiological studies have found that developmental exposure to chlorpyrifos is associated with a variety of neurodevelopmental endpoints in populations where there were higher exposures based on proximity to agriculture or extensive pest control spraying [66].

Mechanistically, it is unclear at this time whether all of the reported effects of developmental neurotoxicity resulting from OP insecticide exposure, and particularly chlorpyrifos, is the result of AChE inhibition or alternate targets [53]. In the past decade, additional targets of OP insecticides have been proposed, including axonal transport, axonal outgrowth, direct binding to acetylcholine receptors, disruption of neurotrophin levels, and, most recently, inhibition of other serine hydrolases such as fatty acid amide hydrolase [65]. The disruption of axonal transport has been ascribed to potential direct binding of OPs to tubulin and kinesin, which may provide a mechanistic link to observed changes in neurite outgrowth [67]. A recent study by Kanthasamy and colleagues reported that chlorpyrifos impairs STAT1 signaling to induce dopaminergic neurotoxicity in cell culture

and animal models, suggesting potential non-cholinergic mechanisms of OP neurotoxicity in certain cell types and brain regions [68].

Pyrethroid insecticides

Pyrethroid insecticides were first used in the form of chrysanthemum crude extracts in the eighteenth century. Over 30 years ago, however, the first synthetic pyrethroids were introduced based on the structure of natural pyrethrins from chrysanthemum. Today, pyrethroid insecticides are one of the most widely used agricultural and household insecticides, accounting for about 25% of the worldwide insecticide market [69]. Further, pyrethroids are used extensively for public health and commercial concerns, including mosquito control following outbreaks of the West Nile, Ross River, and Dengue viruses, malaria, and for bed bug infestations [70, 71]. This increased usage has led to significant increases in the levels of pyrethroid residues in the environment worldwide [72]. In this section, we review the mechanism of action and studies of neurotoxicity in animals and humans.

Pyrethroids exert neurotoxicity primarily through modification of the kinetics of voltage-gated sodium channels (Na_v), resulting in prolongation of the deactivation of sodium channels [73], similar to what is observed for DDT. Studies on pyrethroid neurotoxicity were initially focused on acute neurotoxicity and the identification of different syndromes produced by various pyrethroid insecticides. These studies revealed two major types of acute pyrethroid neurotoxicity [74]. The first, designated the T syndrome, showed dominant signs of neurotoxicity that included tremor, twitching, coma, and death. With the discovery of deltamethrin, the first pyrethroid containing a cyano group, a different acute syndrome was observed that presented as salivation, jerking leg movements, and choreoathetosis. This was termed as the CS syndrome to reflect choreoathetosis and salivation. Subsequent work by Casida's laboratory proposed an alternative nomenclature to describe pyrethroid-induced neurotoxicity that included symptoms of intoxication, chemical structure, and electrophysiological actions in insects. Casida's group labeled them Type I or Type II pyrethroids and the acute toxic effects were similar to those described for T syndrome and CS syndrome, respectively [75]. It should be noted that this classification is not perfect, and different pyrethroids can display aspects of both Type I and Type II syndromes. From a mechanistic standpoint, Type I compounds generally prolong action potentials for a shorter period of time compared with the Type II, which results in repetitive firing of the action potential and a depolarizing block, respectively [74].

More recently, research has been focused on the potential neurotoxicity of longer term, but lower level pyrethroid exposures that do not result in overt intoxication in humans and animals. Although exposure of the general population to pyrethroids is often thought to be low, humans lack serum carboxylesterases [76], a primary mechanism of pyrethroid detoxication through hydrolysis. This could potentially lead to humans having a reduced capacity to metabolize pyrethroids. Data from a recent physiologically based pharmacokinetic (PBPK) study predicted that exposure to the Type II pyrethroid deltamethrin was predicted to result in a two-fold greater peak brain concentration in humans compared to rats [77]. Neurological effects, including cognitive impairment, have been observed following pyrethroid exposure of pesticide applicators and their families [78, 79]. Further, recent data demonstrate neurotoxic effects following pyrethroid exposure, particularly in sensitive populations such as children [80, 81].

Age-related sensitivity to pyrethroids has been a focus of intense investigation based on early findings that younger animals were much more sensitive to higher levels of pyrethroids [82]. This led to concerns that exposure of pregnant women and children to these compounds may lead to neurotoxic effects in children during development due to decreased metabolic capacity [83, 84]. More recently, PBPK modeling techniques have been employed to address age-related differences of pyrethroid metabolism [85, 86]. These support the previously identified differences in toxic susceptibility. Further, incomplete development of the blood–brain barrier (BBB) at early ages may lead to enhanced brain accumulation [87]. Experimentally, pyrethroid exposure in rats or mice during development has been reported to have a broad range of potentially neurotoxic effects on various neurotransmitter systems, the BBB and neurobehavior [69].

Adult exposure of rodents to pyrethroids at doses that do not result in the classic T or CS syndrome has been found to elicit a variety of neurotoxic effects [74]. Wolansky and Harrill [88] reviewed studies on 20 pyrethroids as they related to adult neurobehavioral toxicity following acute exposure and found that Type I pyrethroids generally increased acoustic-evoked startle response amplitude. This was in contrast to Type II pyrethroids that decreased this response. Other studies demonstrated the ability of pyrethroids, particularly deltamethrin, to cause apoptosis through the endoplasmic reticulum (ER) stress pathway in cultured neuronal cells and animals [89–91]. Repeated exposure to other Type II pyrethroids has been demonstrated to cause dopaminergic neurodegeneration and alter mitochondrial function in vitro and in vivo [92]. More recently, repeated pyrethroid exposure was linked to direct effects on glial cells and to cause neuroinflammation in vivo [93–95] and anti-inflammatory treatment reduced cypermethrin-induced dopaminergic neurodegeneration [96]. This may be particularly relevant to human neurotoxicity, as postmortem analysis of Parkinson's disease brain revealed enhanced microglial expression of Na_v 1.6, which was targeted by pyrethroids [97]. Pyrethroid

exposure, primarily in the form of permethrin, alone or in combination with exposure to other neurotoxicants or prophylactic treatments (i.e., pyridostigmine bromide) has also been linked to Gulf War illness, perhaps through a pro-inflammatory mechanism [98].

Mitochondrial Complex I inhibitors as insecticides

Pesticides that inhibit mitochondrial Complex I are increasingly being used as miticides and acaricides. Mammalian mitochondrial Complex I is a large membrane-bound enzyme consisting of 45 protein subunits that oxidizes matrix NADH and transfers electrons to the lipid-soluble carrier ubiquinone. Complex I also translocates protons across the inner mitochondrial membrane against a proton motive force, thus establishing a proton gradient that drives ATP synthesis and leads to the generation of a mitochondrial electrochemical membrane potential. Neurons are extremely sensitive to mitochondrial dysfunction due to their considerable energy demand, and for this reason, defects in Complex I are expected to contribute to several human neurological disorders. Indeed, the inhibition of Complex I has become a widely accepted pathway contributing to the pathogenesis of PD. Loss of Complex I catalytic activity within the mitochondrial electron transport chain has been detected in multiple brain and peripheral tissues from individuals with sporadic PD, including the substantia nigra *pars compacta* (SNpc), frontal cortex, skeletal muscle, lymphocytes, and platelets, suggesting a systemic Complex I defect in PD [99, 100]. Schapira et al. [101] reported a distinct reduction of 15–30% in Complex I activity in non-familial, sporadic PD cases. This impaired Complex I activity in PD brains may be associated with increased oxidation of the catalytic subunits and disrupted assembly of Complex I subunits [102].

Further evidence for the involvement of mitochondrial Complex I inhibition in PD emerged with the discovery of the synthetic heroin analog MPTP in 1982 when several drug users in California developed subacute onset of severe Parkinsonism [103]. MPTP is a lipophilic neurotoxin that can easily cross the blood–brain barrier and be metabolized to the active toxin 1-methyl-4-phenylpyridinium (MPP^+). MPP^+ is a Complex I inhibitor that can be selectively taken up by the dopamine neurons [104]. In addition to MPTP, a variety of mitochondrial Complex I inhibitors, including the pesticide rotenone, induce Parkinsonism in rodents and non-human primates, providing important insight into the role of Complex I deficiency in PD pathogenesis. Mechanistically, rotenone and pyridaben are potent inhibitors of mitochondrial Complex I activity, and thereby they reduce oxygen consumption and bioenergy deficits resulting in structural damage to mitochondria. In this section, we focus on two Complex I inhibitor pesticides, rotenone and pyridaben. Current knowledge regarding their neurotoxicity and relation to PD etiopathogenesis will also be summarized.

Rotenone

Among the neurotoxic pesticides associated with PD, rotenone is a well characterized, highly selective inhibitor of mitochondrial Complex I that occurs naturally in the roots of several plant species [105]. Rotenone is frequently used worldwide to control fish populations, thus raising the potential for environmental exposure in humans and the need to address its toxicological and pathological profile [106, 107]. Rotenone is highly hydrophobic and readily crosses the cell membrane without the aid of specific transport mechanisms (unlike MPP^+). An adverse outcome pathway (AOP) was recently constructed to highlight mitochondrial Complex I inhibition as the molecular initiating event for plausibly linking rotenone exposure to the risk of developing PD based on mitochondrial dysfunction, impaired proteostasis, neuroinflammation and the degeneration of dopaminergic neurons as the key events indicative of disease onset [108].

Although rotenone has a relatively short half-life in the environment and limited commercial use, epidemiological studies using rigorous case-control data over several decades of reported PD cases have linked chronic, environmental rotenone exposure to a higher risk of developing PD in specific populations [107, 109]. Another noteworthy study was the recent French AGRICAN cohort study, which after controlling for crop type, livestock, sex, age, education, smoking status, and alcohol consumption, logistically regressed PD risk against the duration of pesticide exposure. The results suggest that the risk of PD increases in farmers exposed to certain pesticides including rotenone [110].

With the link established between Complex I inhibition and PD, rotenone has gained significant attention as both a potentially causative agent of and possible modeling tool for PD.

Although mixed reports on the extent of the pathology induced by rotenone have been produced, there is now compelling experimental evidence showing that chronic treatment of rats with rotenone is capable of inducing many key pathological features and neurochemical hallmarks of PD [106, 111, 112]. The most prominent study, by Betarbet and colleagues, used a chronic, systemic infusion protocol via a jugular vein cannula attached to an osmotic minipump, to show that systemic inhibition of Complex I by rotenone caused selective degeneration of dopaminergic neurons and terminals in the SNpc and striatum, respectively, accumulation of ubiquitin- and α-synuclein-positive cytoplasmic inclusions, as well as the development of motor and postural deficits characteristic of PD [106]. So far, various routes of administration of rotenone have been used to establish

animal models of PD, and the most common delivery regimen to induce PD symptoms in rats is through a chronic, systemic administration of the compound. Subcutaneous, intravenous, and intraperitoneal injections of rotenone have all been shown to induce chronic progressive degeneration of the nigrostriatal pathway and α-synuclein pathology [113]. The apparent disadvantage, however, of systemic administration of rotenone is the substantial variability in neuropathological changes and high mortality of animal populations. It was also reported that the deleterious effects induced by chronic systemic rotenone treatment are not specific to the dopaminergic system [114], suggesting that rotenone-induced neurodegeneration seen in experimental rats more closely resembles an atypical Parkinsonism rather than idiopathic PD itself.

The neurotoxic mechanisms underlying rotenone-induced Parkinsonism are thought to involve the inhibition of oxidative phosphorylation, heightened oxidative damage, interruption of the mitochondrial electron transport chain, and Ca^{2+}-mediated cellular excitotoxicity [111, 112]. Rotenone treatment produces abundant oxidative damage to cellular macromolecules such as proteins, lipids, and nucleic acids [115]. A chronic, 48-h exposure of PC12 cells (derived from a pheochromocytoma from rat adrenal medulla) to rotenone leads to intracellular dopamine oxidation-mediated cell death [116]. In in vitro models, rotenone exposure potently disrupts autophagy and promotes α-synuclein aggregation [117, 118]. As a Complex I inhibitor, rotenone can dysregulate multiple mitochondrial functions that are essential for mitochondrial maintenance. For example, rotenone-treated neuronal cells exhibit mitophagy involving the externalization of the mitochondrial lipid cardiolipin (LC) to the mitochondrial surface and the subsequent recruitment of the autophagic machinery through an interaction between cardiolipin and LC3 [119]. Mitophagy is a specialized autophagic process that selectively degrades damaged mitochondria. A similar study by the same group reinforced the importance of the cardiolipin–LC3 interaction as a signal for rotenone-induced mitophagy in SH-SY5Y cells, a neuroblastoma human line [120]. Similar to MPTP, rotenone neurotoxicity may include a neuroinflammatory component. Rotenone has been shown to induce microglial activation in BV2 microglial cell line leading to production of inducible nitric oxide synthase (iNOS) and other pro-inflammatory factors like TNFα and IL-1β [121]. N-acetylcysteine, a reactive oxygen species (ROS) scavenger, was shown to reduce this rotenone-induced inflammation by reducing NFκB and p38 MAPK activation [121]. Rotenone was also shown to directly interact with membrane-bound NADPH oxidase 2 (NOX2), the catalytic subunit of NOX2 enzyme complex, thereby playing an important role in SOD and ROS generation [122]. Recently, the Kanthasamy laboratory demonstrated that rotenone exposure leads to NLRP3 inflammasome activation in microglial cells in cell culture and animal models [123]. It was further shown that mitochondrial SOD formation plays an important role in rotenone-induced inflammasome activation [123].

Pyridaben

Pyridaben is a commonly used acaricide in vineyards and commercial greenhouses [124]. Chemically classified as a pyridazinone, pyridaben has also been shown to function as a mitochondrial Complex I inhibitor, according to the Insecticide Resistance Action Committee (IRAC), similar to rotenone. Moreover, the Washington State Department of Agriculture recently documented neurological, ocular, and gastrointestinal symptoms in farm workers poisoned by an off-target exposure to pyridaben [125].

Like rotenone, pyridaben 1) can significantly inhibit mitochondrial respiration, as demonstrated in *Caenorhabditis elegans* (*C. elegans*) acutely exposed for just 1 h to either 50 μM rotenone or 25 μM pyridaben [124], and 2) is highly lipophilic and can thus easily cross the BBB [126]. Despite the link between Complex I dysfunction and PD, the epidemiological evidence linking pyridaben exposure to PD risk is currently lacking. Recent research has demonstrated that exposure to low, nano- or micro-molar concentrations of pyridaben induces significant neurotoxicity in cultured neuronal cells and organotypic midbrain slices [127, 128]. In fact, pyridaben proved to have higher toxicity in SK-N-MC human neuroblastoma cells than did rotenone [128]. In another study, Gollamudi et al. [129] found a strong correlation between pyridaben exposure and both dopaminergic neuronal loss and increased α-synuclein immunoreactivity in pyridaben-treated C57BL mice. The authors also performed an RNA sequencing analysis that revealed gene expression patterns bearing significant correspondence to pathways that are well known in human PD cases. Besides mitochondrial Complex I impairment, thus far only a few putative pathogenic mechanisms have been proposed to explain pyridaben-induced neurotoxicity, including oxidative stress and ubiquitin–proteasome system (UPS) dysfunction [130, 131], which are highly interrelated molecular pathways that could synergistically culminate in neuronal death [132].

Neurotoxicity of herbicides

Historically, assessing the general or neurotoxic mechanisms of herbicides in non-target organisms, such as humans or other mammals, has not been a high priority in pesticide research. As mentioned previously, the primary reason is that most herbicides exert their action in plants via pathways not found in mammals. For example, the most widely applied herbicide in the world uses glyphosate as its active

ingredient [133]. Glyphosate's herbicidal mechanism of action is through its ability to inhibit the shikimic acid pathway, which is responsible for the synthesis of aromatic amino acids (e.g., phenylalanine, tyrosine, and tryptophan) in plants. Since this pathway does not exist in mammals, the potential toxicity associated with chronic glyphosate exposure was largely ignored until the early 2000s [134]. Similarly, atrazine, the second most applied herbicide in the US, kills plants by inhibiting the photosystem II complex (PS II) protein D2 [135]. Furthermore, designing toxicity studies is complicated by the fact that herbicides, and pesticides in general, are a mixture of one or more active ingredients with multiple adjuvants that are included in the commercial formulation to increase the potency and/or efficacy of the active ingredient. The exact nature of these adjuvants can also vary by country and manufacturer. Notably, the chemicals used as adjuvants are generally not disclosed due to their proprietary nature. While these issues are not unique to any of the top herbicides in use today, further research is required to address the gaps in our knowledge related to mechanisms of neurotoxicity and neuropathology in humans, and to identify the nature of the toxic components in commercial formulations. Addressing this latter issue will also provide information that could lead to herbicide formulations that have decreased neurotoxicity in humans and other mammals.

Glyphosate-containing herbicides

Glyphosate-containing herbicides (e.g., Roundup, Touchdown) are by far the most widely used pesticides in the world. Between 2012 and 2015, the amount of these herbicides applied each year was over four times greater than that of the second-place pesticide [64, 92]. For many years, the active ingredient, glyphosate, was largely considered relatively non-toxic due to its high oral LD_{50} in both rats (5.6 g/kg) and mice (10 g/kg) in acute studies [136]. This low toxicity is consistent with recent studies demonstrating that commercial formulations are generally more toxic than glyphosate alone [137, 138]. Studies evaluating the neurotoxicity of glyphosate (or the commercial formulation) are much less well documented or emphasized. While some research suggests that glyphosate inhibits AChE [139], the IC_{50} in human serum was calculated to be 714 mM [140], which is much higher than blood concentrations associated with indirect exposures (< 0.05 mM) or acute poisoning (0.05–5.0 mM) [139]. As such, this seems unlikely to be a mechanism of neurotoxicity. In terms of neuropathology, dopaminergic and γ-aminobutyric acidergic neurons preferentially undergo neurodegeneration in *C. elegans* treated with commercial glyphosate formulations at concentrations used by pesticide applicators [141, 142]. This neurodegeneration was attributed to mitochondrial inhibition and increased oxidative stress [143]. Other studies demonstrate that zebrafish exposed to glyphosate formulations show abnormal brain development [144, 145], which may be attributable to glutamate excitotoxicity [146, 147] observed in developing rats exposed to glyphosate. More recently, rats treated with a commercial formulation showed increased anxiety and depression that correlated with changes in gut microbiota number and diversity [148]. Since many bacteria also rely on the shikimic acid pathway to produce cyclic amino acids, inhibition of this pathway by glyphosate is hypothesized to decrease tryptophan catabolism. This decrease is potentially important since tryptophan is the precursor for serotonin, which plays an important role in both anxiety and depression. Taken together, these recent studies strongly support further research into the potential neurotoxic effects of glyphosate-based herbicides.

Paraquat-containing herbicides

Data regarding paraquat's neurotoxicity are probably the most conclusive of all herbicides. Paraquat exposure has been linked to an increased risk for PD [107], and has been used in animals to model aspects of PD pathology, including dopamine neuron loss and synuclein aggregation through its induction of oxidative stress and neuroinflammation [149–152]. Exposure to paraquat also promotes tyrosine phosphorylation of parkin in SH-SY5Y cells [153]. This post-translational modification of parkin, which inhibits the protein's function, facilitates disease progression. Paraquat exposure has been shown to induce hyperacetylation in cell models of Parkinson's disease, suggesting that prolonged exposure of this pesticide may promote epigenetic reprograming [154]. More recently, data from induced pluripotent human stem cells treated with paraquat showed increases in the proinflammatory cytokine, interleukin-6 (IL-6). This cytokine is also part of the senescence-associated secretory phenotype in both astrocytes and fibroblasts [155]. Additionally, conditioned media from paraquat-treated astrocytes induced dopaminergic cell death. Paraquat incubation also resulted in an increase in secretogranin II (SCG2) production in astrocytes [156], which is associated with large, dense core vesicles that co-localized with IL-6. Taken together, these data suggest that exposure to paraquat, in addition to increasing oxidative stress, may also increase the release of IL-6 to promote neuroinflammation. Future studies may provide additional insight as to how paraquat and other herbicides modulate inflammatory pathways and initiate processes associated with neurodegeneration.

Neurotoxicity of fungicides

Early fungus control (1700s) was achieved by adding arsenic to fields where important crops were grown. Later (1800s), lime (calcium carbonate) or dolomite (magnesium calcium carbonate) was used, followed by the application of copper

sulfate. Eventually, methylmercury (1900s) was applied to plants and seeds to prevent fungal outbreaks in seed stocks intended for use in breads and cereals [157]. Using heavy metals to protect food crops, however, came with the risk of poisoning human populations. Perhaps one of the most infamous occurrences of human toxicity from fungicide exposure happened in Iraq in the early 1970s [158–160]. Grain treated with methylmercury and intended for planting was instead consumed by entire communities. This resulted in methylmercury intoxication in hundreds of people, and the death of over 400. Despite this, heavy metals are still among the most widely used active ingredients in fungicides in the US. Many fungicides exert their effects via multimodal mechanisms, meaning that they cause fungal death through multiple pathways. Some of the more common pathways involve the production of oxidative stress by increasing ROS and depleting available antioxidant enzymes (e.g., SOD, catalase, glutathione-*S*-transferase) or molecules (i.e., GSH). In other cases, inhibition of mitochondria results in fungal death. Finally, these pesticides may exert their fungicidal action through the chelation of essential metals in the organism [161]. Regrettably, many of these pathways, enzymes, and molecules are also found in several species of mammals, including humans. Furthermore, all of these targets are found in the brain. It is, therefore, not surprising that accidental, chronic, or indirect exposure to fungicides may lead to neurotoxicity.

Manganese ethylene-bis-dithiocarbamate (EBDC)-containing fungicides

With the exception of manganese (Mn)/zinc (Zn)-EBDC, no systematic neurotoxicity has been associated with, or reported for, the top four most widely used commercial pesticides in the US. While Mn/Zn-EBDC (mancozeb) is not as well studied as Mn-EBDC (maneb), the latter was voluntarily withdrawn from the US market in 2010. As a result of this removal, Mn/Zn-EBDC is now the second most common commercial fungicide [2]. Studies in humans [162], human-derived cell culture [163], and non-target animals [164] exposed to Mn/Zn-EBDC have shown increased blood, cell, and tissue Mn levels, respectively. These higher Mn concentrations, along with measurable amounts of ethylene thiourea (an EBDC metabolite), in children raised near banana plantations in Costa Rica are correlated with adverse neurobehavioral outcomes [162]. It is unclear whether Mn/Zn-EBDC has the same propensity to affect dopaminergic neurons as maneb [165] or increase a person's risk for PD [166, 167]. Studies in *C. elegans*, however, have shown that the dopaminergic system is one of the targets following either acute or chronic exposures to this fungicide [142, 168]. Furthermore, the toxicity appears to be blocked when worms are pretreated with a dopamine transporter antagonist [169], suggesting it may enter dopamine neurons via this presynaptic transporter. Since the neurotoxic mechanism of action of Mn/Zn-EBDC appears to involve oxidative stress [170] and mitochondrial inhibition [171], it is not unreasonable to hypothesize that neurons, among other cell types, would be vulnerable to Mn/Zn-EBDC exposure. It is currently unknown, however, how this fungicide might cross the blood–brain barrier and enter the brain. Additional research regarding the associated neurotoxicity should not only provide insight into the toxic mechanisms of organic metal fungicides in general, but also afford opportunities to better understand how metal complexes are transported to, and deposited in, specific regions of the brain.

Emerging techniques for translational research in neurotoxicity

The previous section has summarized historical and current knowledge on the relationship between pesticide exposure and neurotoxicity. However, there remains a significant challenge in translating mechanistic work in cells and animals to human populations. Further, epidemiological studies primarily reveal associations between exposure and neurotoxic outcomes, most of which are behavioral in nature and lack mechanistic or neuropathological insight. Finally, the large number of potentially neurotoxic compounds and mixtures of compounds that may not have been adequately studied requires a robust and high-throughput approach. Thus, there is a significant need to incorporate new and emerging techniques and models to aid in translational research.

In recent years, the effects of pesticide exposure on brain function and structural damage or degeneration have been investigated using a number of in vivo imaging techniques. For example, magnetic resonance imaging (MRI) studies demonstrated that glufosinate ammonium, the active component of many herbicides, causes dose-dependent structural alteration in the hippocampus and somatosensorial cortex of chronically exposed mice [172]. More recently, MRI has been used to demonstrate the correlation between OP-induced brain damage (cortical edema and brain metabolic dysfunction) and clinical outcomes (behavior and pathology), as well as to study the effectiveness of potential antidotes [173]. Similarly, MRI has also been used to show that repeated OP exposure at levels that do not induce acute toxicity and appear unrelated to inhibition of AChE can induce persistent inhibition of axonal transport [174]. As MRI allows visualization of effects in the intact living animal, it represents a useful tool to link functional and structural deficits in the brain to both in vivo neurobehavioral effects and in vitro mechanisms. Given the possibility to also apply these imaging techniques in human patients, and even in epidemiological studies, these techniques hold great promise

for improved diagnosis, biomarker identification, and investigation of neuroprotective treatments.

From a mechanistic standpoint, a relatively newer development that may speed up translational pesticide research is multi-well microelectrode array (mwMEA) recordings. MEAs typically consist of an electrode array containing 16–64 electrodes and allows for non-invasive recordings of neuronal activity in an in vitro neuronal network [175]. Neuronal networks grown on MEAs develop spontaneous electrical activity over time and are responsive to neurotransmitters and pharmacological agents comparable to in vivo neurons [176, 177]. While traditional single-well MEA systems have a relatively low throughput, the recent development of mwMEAs with 12-, 48-, or 96-wells has increased the throughput and its use in neurotoxicity testing considerably. MEA recordings are typically performed using heterogeneous neuronal networks, consisting of multiple types of neurons and supporting cells that span a wide range of potential targets (e.g., neurotransmitter receptors, ion channels, intracellular signaling pathways) that can all contribute to modulation of spontaneous neuronal activity.

Just over 10 years ago, these MEA recordings were used to investigate the effects of the pyrethroid insecticides deltamethrin and permethrin on neuronal activity in vitro in hippocampal cultures [178] and cortical cultures [179]. Both compounds reduced neuronal activity in a concentration-dependent manner, with the Type II pyrethroid deltamethrin being more potent than the Type I pyrethroid permethrin. Since then, these results have been reproduced by multiple laboratories [180, 181], highlighting the robustness of this innovative technique. In recent years, a large number of other types of pesticides have been investigated using MEA recordings. Other pyrethroids, like bifenthrin, cyhalothrin, cypermethrin, β-cyfluthrin, and esfenvalerate [180, 182, 183] as well as the carbamate carbaryl [182] and the OP chlorpyrifos/chlorpyrifos-oxon [184] have also been shown to concentration-dependently decrease neuronal activity. Interestingly, the herbicide glufosinate [185] and the organochlorines insecticides lindane [184, 186] and endosulfan [182] exert biphasic effects on neuronal activity. Neuronal activity is increased at low concentrations, due to *N*-methyl-D-aspartate subtype glutamate receptor agonism and γ-aminobutyric acid receptor antagonism, respectively. Neuronal activity is inhibited at higher concentrations, likely due to less-specific effects such as inhibition of voltage-gated calcium channels [187]. Collectively, this work provides important insight into the direct effects of pesticides on neuronal function that was previously only performed in isolated single neurons.

Due to their non-invasive nature, MEA recordings have recently also been used to investigate the effects of chronic exposure to different pesticides during neurodevelopment of the in vitro network [182]. The observed effects are largely comparable to acute exposure for chlorpyrifos, α-cypermethrin, and endosulfan, whereas chlorpyrifos oxon and carbaryl inhibited neuronal activity only during acute exposure suggesting some sort of adaptive capacity in the developing neuronal network. As mwMEA recordings have a considerable throughput, they are also suitable for assessing mixture effects. These mixture assessments have been mainly limited to binary [188] and more complex [183] mixtures of pyrethroids. These studies indicate that pyrethroid mixtures in vitro cause dose-additive effects on spontaneous neuronal network activity, consistent with in vivo assessments of pyrethroid mixtures.

As mentioned above, assessment of mixture effects is still often performed using (binary) mixtures of similarly acting pesticides. For example, additive effects of pyrethroid mixtures on increased sodium influx through Na_v have previously been shown in cortical neurons [189]. Similarly, mixtures of OP and/or carbamate insecticides generally show additive or even synergistic effects with respect to their presumed primary mode of action, i.e., inhibition of AChE [190, 191]. To better bridge the gap between in vitro and in vivo studies with the human exposure situation, however, experiments using complex mixtures that more closely resemble real life exposures are required. Additionally, such experiments should not focus on the distinct separate modes of action, but may need to take into account integrated endpoints, e.g., neurobehavior and brain imaging (in vivo) or neuronal activity measurements using MEA recordings (in vitro). Several more recent experiments, using complex mixtures of pesticides with different primary modes of action, have shown synergistic, agonistic, and antagonistic effects, depending on the type of endpoints, species, and sex studied [192]. The number of potential mixture combinations is immense, and the resulting effects can range from additive (most common for same mode of action) to antagonistic and synergistic (in particular for divergent modes of action that are upstream of a more integrated endpoint). Although essential to bridge the gap between in vivo and in vitro experimental research and the human exposure situation as studied in epidemiology, mixture toxicology will remain a huge challenge for future research.

Obviously, behavioral assessment in the in vivo situation provides a strong integrated endpoint for effects assessment of (mixtures of) pesticides. The major drawback of low-throughput animal studies may be largely circumvented using alternative species, such as *Drosophila melanogaster* or *C. elegans*. As *Drosophila* is an important target species, it has been used mainly to determine the effectiveness of pesticides. The nematode *C. elegans*, on the other hand, has been used for over 10 years to investigate acute behavioral toxicity of pesticides. *C. elegans* has become a well established model lab organism as it has a short life cycle (allowing for more throughput), is inexpensive, has a high degree of evolutionary conservation with mammals, and its nervous

system connectivity has been completely mapped. The possibility for automated assessment of effects in highly integrated endpoints, such as locomotion and feeding behavior, further increases its value as model for studying mammalian (developmental) neurotoxicity of pesticides [193, 194]. As potential target species, however, translatability to human neurotoxicity might be challenging, particularly for pesticides with high selectivity for *arthropoda* and *nematoda*.

The use of zebrafish, particularly in the embryo or larval stage, may bypass some of the translational challenges while maintaining throughput. Consequently, zebrafish has emerged over the last 15 years as an appreciated and complementary vertebrate model to study pesticide-induced (developmental) neurotoxicity [195]. Zebrafish have been extensively used to investigate effects of pesticides on parameters that are indicative for gross neurotoxicity. Likewise, zebrafish have been used as an experimental model to assess pyrethroid-induced developmental neurotoxicity [196–198]. Similar to observations in mammals, pyrethroid exposure in zebrafish results in behavioral disruption, including hyperactivity, particularly for deltamethrin [199].

Exposure to chlorpyrifos in a concentration-dependent manner increases the frequency and total duration of spontaneous tail coilings at 24–26 h post-fertilization (hpf), whereas swimming activity is decreased at 96 hpf, highlighting that effects may differ with developmental stage [200]. Additional methods exist to increase mechanistic insight, including neurotransmitter profiling, which has been used to demonstrate that levels of acetylcholine in zebrafish decrease following the exposure to the neonicotinoid imidacloprid and several carbamate and OP insecticides [201]. In particular, the recent use of fluorescent transgenic zebrafish lines may allow for further assessing of specific effects on distinct targets or pathways, such as insecticide-induced motor neuron degeneration [202].

As outlined above, current hazard characterization and risk assessment of pesticides still heavily relies on ethically debated, time- and resource-intensive animal experiments, which are not always fully predictive for human adverse health effects. At the same time, most in vitro test strategies are not sophisticated enough to be sufficiently predictive to replace animal experiments. This is at least partly due to the animal origin of many currently used in vitro models. With the development of human neural stem cell (NSC) lines and human-induced pluripotent stem cell (hiPSC)-derived neuronal cultures, this may now be feasible. These cell cultures can be maintained for weeks or months and can be grown as pure neuronal cultures or as co-cultures with astrocytes or other glial cells. As these models mature over time to form a complex network of spontaneously active cells [203, 204], it allows for assessing both developmental and acute neurotoxicity testing in vitro. Using mwMEA recordings, it was shown that the insecticide and γ-aminobutyric $acid_A$ receptor antagonist endosulfan, which evokes hyperexcitation in vivo, increases neuronal activity of commercially available hiPSC-derived pure neuronal cultures [204] and neuronal co-cultures with astrocytes [205].

Using a human stem cell-based model, it was shown that developmental chlorpyrifos exposure reduced the expression of several neural differentiation marker genes, decreased intracellular ATP levels, and induced mitochondrial fragmentation [206]. Importantly, co-culturing hiPSC-derived neuronal cultures with astrocytes, which have high expression of cytochrome P450 enzymes, allows for inclusion of biotransformation of chlorpyrifos to chlorpyrifos oxon and subsequently, the less neurotoxic end products. Chlorpyrifos was shown to concentration-dependently inhibit neurite length, neurite number, and branching in pure neuronal cultures more potently than in co-cultures with astrocytes, highlighting the protective role of astrocytes [207]. Addition of astrocytes to human dopaminergic neurons had been shown previously to increase metabolism, and thereby alter the sensitivity to chemical insults [208]. Inclusion of astrocytes in human neuronal cultures also enhances the development of neuronal networks and their spontaneous activity [209], as well as the synchrony of network activity [210], thus increasing the in vivo relevance of these models.

As a model compound for development of PD, the mitochondrial Complex I inhibitor rotenone is among the pesticides most studied in human neurons in vitro. Rotenone was shown to inhibit neurite growth in human dopaminergic neurons or human neuronal precursor cells differentiated towards dorsal root ganglia neurons [211]. In hiPSC-derived postmitotic mesencephalic dopamine neurons, rotenone also decreased neurite length. Additionally, rotenone decreased GSH levels and increased lipid peroxidation [212]. In mixed hiPSC-derived neuronal/glial cultures, rotenone exposure induces Nrf2 activation, resulting in astrocyte activation, decreased neurite length, and eventually cell death of dopaminergic neurons [213]. Recently, neurons differentiated from induced pluripotent stem cells derived from healthy and familial AD were used to investigate the effects of pyrazole insecticides, including fipronil, on generation of amyloid-β peptides, which play a key role in the development of AD. Interestingly, compared to wild-type neurons, neurons derived from the patient produced more amyloid-β_{42} peptide, which is associated with the onset of the disease [214].

Combined, these studies emphasize the potential of human (iPSC-derived) neuronal cultures for studying acute and developmental pesticide-induced neurotoxicity as well as pesticide-induced degeneration and disease development. While further characterization and toxicological validation are required for large-scale implementation of the use of human iPSC-based models for future neurotoxicity testing, the possibility to use patient-derived material holds great

promise and may allow for further translation to behavioral and neuropathological findings in human clinical and epidemiological studies.

Conclusions

Pesticides represent one of the oldest and most used environmental contaminants. Although pesticides serve a useful and necessary purpose, there is significant potential for human exposure and potential toxicity. Higher level exposures that result from occupational exposures and proximity to agricultural spraying are associated with several neurotoxicities in humans. However, much remains to be learned regarding the potential effects of longerterm lower-level exposures, particularly in sub-populations that may be uniquely sensitive. Further, there are several pesticides that have been introduced relatively recently compared to the ones covered here (i.e. neonicitinoids, pyrazoles, biopesticides, etc.) for which there are emerging data regarding potential neurotoxicity. With the rapidly increasing world population, the need to generate higher crop yields to accommodate population growth, and the emergence and re-emergence of new and old vector-borne diseases, it is clear that pesticides will continue to be common in our environment and continued study is needed to understand the potential risks of exposure, particularly as it relates to neurotoxicity. Unfortunately, there are few human neuropathological data linked with pesticide exposures, with the exception of the Honolulu-Aging Asian Study [36]. Future collaborative work will be needed to monitor ongoing and new human cohort studies such that an autopsy component might be added. In the meantime, imaging modalities show great promise for exploring functional and structural changes following pesticide exposure in humans [215].

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