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ABSTRACT

Investigation of the toxicity triggered by chemicals on the human brain has traditionally relied on approaches using rodent *in vivo* models and *in vitro* cell models including primary neuronal cultures and cell lines from rodents. The issues of species differences between humans and rodents, the animal ethical concerns and the time and cost required for neurotoxicity studies on *in vivo* animal models, do limit the use of animal-based models in neurotoxicology. In this context, human cell models appear relevant in elucidating cellular and molecular impacts of neurotoxicants and facilitating prioritization of *in vivo* testing.

The SH-SY5Y human neuroblastoma cell line (ATCC® CRL-2266™) is one of the most used cell lines in neurosciences, either undifferentiated or differentiated into neuron-like cells. This review presents the characteristics of the SH-SY5Y cell line and proposes the results of a systematic review of literature on the use of this *in vitro* cell model for neurotoxicity research by focusing on organic environmental pollutants including pesticides, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), flame retardants, PFASs, parabens, bisphenols, phthalates, and PAHs. Organic environmental pollutants are widely present in the environment and increasingly known to cause clinical neurotoxic effects during fetal & child development and adulthood. Their effects

on cultured SH-SY5Y cells include autophagy, cell death (apoptosis, pyroptosis, necroptosis, or necrosis), increased oxidative stress, mitochondrial dysfunction, disruption of neurotransmitter homeostasis, and alteration of neuritic length. Finally, the inherent advantages and limitations of the SH-SY5Y cell model are discussed in the context of chemical testing.

1. INTRODUCTION

The nervous system is a complex structure that regulates most of the body functions and responses by means of highly complex processes based on electrical and chemical signaling. It can be divided into the central nervous system (CNS), comprising the brain and the spinal cord, and the peripheral nervous system (PNS) comprising the nerves and ganglia that connect the body to the CNS. The main cells that form the nervous system structure are neurons, responsible for the transmission of information through electrochemical signaling, and glial cells, with diverse supporting roles.
The intricacy of the nervous system makes it often difficult to characterize the causes, effects, and extension of any kind of insult or disease, and there is no exception when trying to portray the potential toxicology of any kind of agent.

Neurotoxicity (NT) can be defined as the disruption of any of the functions and/or structures of the nervous system by an exogenous compound or a mixture of exogenous compounds. There is large evidence that in this category, industrial chemicals can have a negative impact on the well-functioning of the nervous system. They can cause acute or/and chronic toxicities and these effects can be even more dramatic if they take place during the developmental period. This developmental neurotoxicity (DNT) can cause permanent impairment and lead to neurodevelopmental diseases [1, 2]. Grandjean and Landrigan, based on published clinical and epidemiological data, identified 12 industrial chemicals or families of chemicals, as developmental neurotoxicants (i.e., lead, methylmercury, arsenic, fluoride, manganese, poly-chlorinated biphenyls, brominated diphenyl ethers, chlorpyrifos, DDT/DDE, trichloroethylene, ethanol and toluene), 214 chemicals susceptible to cause injuries to the nervous system in adults, while on the experimental side, more than 1000 chemicals have been reported to be neurotoxic in animals in laboratory studies [1, 3].

Regulatory assessment of adult and developmental neurotoxicity is mostly based on rodents' models with three primary OECD guidelines covering life stage-dependent neurotoxicity: OECD 424 (neurotoxicity study in rodents), OECD 426 (study of developmental neurotoxicity), OECD 443 (study of extended one-generation reproductive toxicity). These tests require a high volume of animals, are expensive and time-consuming. Putting it together with the vast amount of industrial chemicals currently in use and constantly newly released, ends in a lot of chemicals without any neurotoxicity information, and these numbers increase regarding DNT, as shown by Grandjean and Landrigan.
The high pressure to test large sets of compounds responding to legislation requirements, together with animal welfare concerns, are leading to a change of paradigm from analysis of

animal-based observational toxicities to a more mechanistic-based and predictive assessment of toxicities including *in vitro* testing [3, 4].
In vitro models are, indeed, good allies for neurotoxicity screening. Although they cannot recapitulate all the physiology and complexity of the *in vivo* state, specially concerning the evaluation of any sensory, cognitive, or behavioral disruption, they are the most useful tool to identify the mechanisms of toxicity. Moreover, they can constitute a first line screening tool to raise alarms for potential neurotoxicants, classify compounds by their potential to induce damage to the nervous system and help to prioritize regulatory testing [5].
Currently, there is a wide variety of *in vitro* models for neurotoxicity and DNT based on primary cell cultures (mostly of rodent origin), immortalized cell lines and induced or embryonic stem cells (reviewed in [4, 6]). All these systems have advantages and disadvantages and the choice of one or the other must consider them and what endpoints want to be assessed.

The use of cells of human origin is preferable to avoid interspecific differences in the effects of chemicals. Among the human options, cell lines have a reduced capability to mimic early development and cytoarchitecture of the nervous system compared with stem cells-derived cultures, but they have the advantage of having shorter and easier culture protocols and less variability between cultures.

The SH-SY5Y human neuroblastoma cell line has been one of the first *in vitro* models to be developed and has been intensively used to carry on neurotoxicity experiments. Cells can be maintained as neuroblasts or be induced to differentiate into more neuron-like morphologies, which renders them a suitable model to study toxicity respectively, on proliferating cells or on differentiated cells.

This review presents an overview of the SH-SY5Y cell model and proposes the results of a systematic review of literature on the use of this cell model to study the effects of selected environmental pollutants.

2. THE SH-SY5Y HUMAN NEUROBLASTOMA CELL LINE

2.1 - Characteristics

Human commercial cell lines are mostly immortalized cell lines derived from tumorigenic cells, that have the advantage over primary cell cultures of allowing unlimited and relatively easy culture, not subjected to the ethical concerns of human primary cell cultures [7, 8].

Probably the most broadly used human cell line in research on neuronal cell biology is the SH-SY5Y cell line (ATCC® CRL-2266™). This neuroblastic cell line is a thrice-cloned subline of the SK-N-SH cell line (ATCC® HTB-11™) that was established in the 1970s from a metastatic bone marrow biopsy from a 4-years-old girl suffering from neuroblastoma, a neural crest derived tumor [9]. The parental SK-N-SH cell line comprises two morphologically distinct phenotypes:

neuroblast-like cells (N-type) and epithelial-like cells (S-type), which can undergo interconversion [10].

Cultured SH-SY5Y cells can continuously proliferate, mainly as adherent neuroblast-like cells although the cell population contains a low proportion of epithelial-like cells [8, 11]. ~~ or~~. ~~N~~SH-SY5Y neuroblast-like cells present polygonal cell bodies with short processes and, although they can have some reminiscent activities of tyrosine hydroxylase (TH) and dopamine-β-hydroxylase from their sympathetic adrenergic ganglial origin [9, 12, 10], they do not present the morphological, biochemical, and functional characteristics of mature neurons. They do present, however, immature neuronal markers such as nestin and transcriptional regulators such as differentiation-inhibiting members of the DNA-binding protein inhibitor family (ID family), ID1, ID2 and ID3 [13]. Moreover, although they show excitability to acetylcholine and potassium, undifferentiated SH-SY5Y cells lack excitability to NMDA treatment as compared to cultured primary neurons and, as it the case for other catecholaminergic cell lines, lack excitability to glutamate [14, 15]. These features of undifferentiated SH-SY5Y cells may not make them the more suitable model to study neurotoxicity or neurodevelopmental toxicity in all its extension. However, undifferentiated cells can offer a good model to study the effects of neurotoxicants on the immature nervous system.

SH-SY5Y cells can also be induced to differentiate into neuron-like cells. The differentiation of SH-SY5Y cells using differentiation-inducing agents, causes the exit of the cells from the cell cycle [16, 17] and their shift to a morphology similar to that of primary neurons, with smaller cell bodies, which are frequently polarized, present extended neurites [18–20] and excitable membranes [20, 21] with distinct properties (such as potassium conductance) as compared to undifferentiated cells [22]. Upon differentiation, expression of several mature neuronal markers is induced or increased such as NeuN (neuronal nuclei antigen, also known as RBFOX3, RNA binding fox-1 homolog 3), a marker of neuron nuclei, and neuron specific enolase (NSE), an enzyme found in mature neurons and cells of neuronal origin [11, 17, 18, 23–26], but also mature neuron cytoskeleton components and associated proteins such as tubulin beta-III (TUBB3), neurofilament proteins, microtubule associated protein 2 (MAP2), and microtubule associated protein Tau (Tau, MAPT) [16, 19, 23, 27–32], proteins involved in neurite outgrowth such as growth associated protein 43 (GAP43) [11, 13, 16, 28, 29] and proteins associated with synaptogenesis and synaptic vesicles such as synaptophysin, synaptic vesicle protein Sv2 and synaptic associated protein 97 (SAP97) [18, 23]. Other markers indicating that cells have been differentiated, are the decreased expression of ID1, ID2 and ID3 and of the neuroblast-specific bHLH transcription factor achaete-scute family bHLH transcription factor 1 (ASLC1) and the increased expression of the differentiation-promoting bHLH factors neuronal differentiation 1 (NEUROD1) and 6 (NEUROD6) [13, 18].

The neurotransmitter signature of the differentiated cells depends on the differentiation-agent used. For example, differentiation with retinoic acid (RA) could give rise to a more cholinergic phenotype [33], whereas the use of other agents such as 12-O-tetradecancyl-phorbol-13-acetate (TPA), alone or in combination with RA, and dibutyryl cyclic adenosine monophosphate (dbcAMP) can lead to more dopaminergic-adrenergic and adrenergic phenotypes, respectively [17, 19, 26, 34].

2.2 - Retinoic acid as a differentiating agent

RA is, by far, the most used agent for differentiation of SH-SY5Y cells [35, 36]. The addition of RA to the culture medium (usually 10 µM) causes a quick differentiation including morphological transformation from neuroblastic- to neuron-like cells, and a decrease in cell proliferation [20].

Despite being the differentiation agent that has the strongest effect on cell proliferation arrest [20, 32], differentiation with RA has some important drawbacks. Some studies have reported that RA can increase cell survival and therefore resistance to neurotoxicity [13, 18, 37] but its ability to increase cell resistance to toxicants is still controversial. In the case of Parkinson's disease (PD) research, a field in which SH-SY5Y cells are extensively used for *in vitro* testing, some studies report that undifferentiated cells are more susceptible as compared to RA-differentiated cells to oxidative stress caused by toxins (6-hydroxydopamine, 6-OHDA, and 1-methyl-4-phenylpyridinium, MPP^+) used to mimic PD *in vitro* [18]. Conversely, other studies report differentiated cells as a more suitable model to study neurotoxicity as they present an increased vulnerability as mature neurons do [19, 24, 25]. Moreover, in some studies that did report increased oxidative stress resistance in differentiated cells related it to an increased reserve energetic capacity, a property also observed in mature neurons [31, 38]. Finally, it has also been observed that RA treatment can increase the expression of Bcl-2 (B-cell leukemia/lymphoma 2), an anti-apoptotic protein, that nonetheless is always strongly expressed in the nervous system and seems to be naturally increased in neurons upon neurotransmitter receptor stimulation [13, 39].

Another problem of differentiation, not only associated with the use of RA but also with other differentiating agents, and that could be of more concern, is the purity of the cell cultures. Despite the SH-SY5Y cell line was selected as a homogenous neuroblastic clone [12], some cells still present an epithelial morphology (**Figure 1**), and even some cells can undergo interconversion from one type to the other [10, 11, 20]. These epithelial-like cells can become an important percentage in prolonged cell culture, as this cell type does not seem to be affected by postmitotic effects of RA and, after long periods of culture (from 7-10 days of treatment), the cell count on the cultures increases due to an increased proliferation of epithelial-like cells [11].

Aiming to avoid the proliferation of epithelial-like cells in the neuronal cultures, different approaches have been applied. In fact, the addition of several factors to RA during differentiation such as cholesterol [20] or neurotrophic factors as brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) [11, 23] have proved to enhance differentiating properties of RA obtaining more neuronal characteristics in the cultures with reduced or non-observed cells presenting an epithelial morphology.

2.3 - Retinoic acid and brain-derived neurotrophic factor differentiation protocol

Of special interest is the differentiation protocol developed by Encinas and collaborators [11] based on sequential cell treatments with RA and BDNF. This protocol results in almost undetectable levels of epithelial-like cells even during prolonged culture periods (**Figure 1**).

SH-SY5Y undifferentiated cells do not express the BDNF membrane receptor, tropomyosin receptor kinase B (TrkB), also known as neurotrophic tyrosine kinase receptor type 2 (Ntrk2). Treatment with RA (10 μM, in Dulbecco's modified Eagle's medium, DMEM, containing 15% fetal bovine serum, FBS) induces TrkB expression, being maximal after 5 days of treatment, the moment when RA is then withdrawn from the medium, and BDNF (50 ng/mL) is added into serum free DMEM. BDNF through TrkB signaling, enhances the differentiating properties stimulated initially by RA [11, 23, 32, 35, 40], resulting, after 7 days of BDNF treatment, in cell cultures with neuron-like cells resembling those observed in neuron primary cell cultures, with polarized cell bodies with profuse branching, cytoskeleton neuron-specific markers such as MAP2, neurofilament heavy chain protein (NEFH) and Tau localized in the cell soma and neuritic processes (**Figure 2**).

Forster and collaborators proposed a differentiation protocol adapted from Encinas and collaborators and based on RA and BDNF successive treatments to induce differentiation [24]. They treated cells with RA only for 3 days in low serum (5% FBS) DMEM. According to the authors, this is enough time to induce the expression of the BDNF receptor. Further differentiation was obtained with BDNF in neurobasal medium with N2 supplements for 3 more days. Morphological and transcriptional analysis showed cells with characteristics of mature neurons including higher energetic stress levels.

Combined treatment of cells with RA and BDNF has proven to enhance RA-induced neuronal differentiation. RA and BDNF differentiated cultures show higher neuronal morphological characteristics and biomarkers [11, 23, 32], expression of gene clusters related to neuronal functionality and morphology [35], and electrophysiological properties of a neuronal model [41].

3. METHODOLOGY OF THE SYSTEMATIC REVIEW OF LITERATURE

The initial selection of the environmental pollutants for this review was done based on the European and American epidemiological studies HELIX [42], ExpoSOMICS [43], DEMOCOHES [44, 45] and LIFE [46]. Regarding pesticides, as a sample of the European use of pesticides, the most used pesticides in France [47] and the ones studied in two epidemiological studies in France, ELFE [48] and PELAGIE [49] were also added.

The list of chemicals was further enlarged using some of the compounds from the Fourth National Report of Human Exposure to Environmental Chemicals (NHANES, https://www.cdc.gov/exposurereport) from the American Center for Disease Control and prevention (CDC) and the list of cataloged neurotoxicants from two reviews produced by Grandjean and Landrigan [1, 3]. The list of compounds for the polycyclic aromatic hydrocarbons was extracted from the *Polycyclic Aromatic Hydrocarbons (PAHs): Factsheet* (4th Edition, JRC 66955) [50].

For pesticides, three compounds that did not belong to the original search list, were deemed relevant and later added: alpha-hexachlorocyclohexane (□HCH) and propanil belonging to organochlorine pesticides, and a third one corresponding to a triazole fungicide, propiconazole.

For polychlorinated biphenyls (PCBs), some congeners that appeared in the bibliographic search were added.

Medical drugs, nicotine and other specific tobacco compounds and cooking and food processing by-products were not included in this review.

The methodology used is summarized in **Figure 3** and the organic environmental pollutants used in the search are listed in **Table 1.** The selected organic pollutants belong to one of the seven following groups: 1/ pesticides including organochlorine pesticides (OCs), organophosphate pesticides (OPs), carbamates, pyrethroids, neonicotinoids and other compounds; 2/ polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs); 3/ flame retardants including polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), polybrominated diphenyl ethers (PBDEs), and phosphate flame retardants (PFRs); 4/ perfluorinated alkylated substances (PFASs); 5/ parabens and bisphenols; 6/ phthalates; 7/ polycyclic aromatic hydrocarbons (PAHs).

The search was performed in two different databases, PubMed and Web of Science, and the search included preferentially the MeSH (Medical Subject Headings) term of the compound with the construct “AND SH-SY5Y”, although other entry names or synonyms were used. The list of all the search terms used can be found in **Supplementary Table 1**. For some groups, rather than searching individually for each compound, the group MeSH term was used since all the congeners of the group contained the key words, this is the case for PCBs, PBBs, PBDEs, and parabens.

The articles found in the two databases were merged eliminating duplicates. Only articles written in English and original studies (no reviews) were selected. A first selection was done based on the titles, and a second one based on the abstracts of the articles. From the retained articles, a third selection was done after reading the whole text. To keep an article in the final selection, SH-SY5Y cells must have been used to test the toxicity of the compound of interest.

The last actualization of the search was done between January the 7th and January the 13th 2022.

4. CELL CULTURE METHODOLOGY

The search of all the compounds selected for this review (**Table 1**) provided a total number of articles of 163 after eliminating duplicates. Cell culture conditions were studied to assess any trends in the culture conditions regarding SH-SY5Y cells.
The most used culture medium was DMEM (63 studies), followed of DMEM/F12 (1:1) (47), MEM or EMEM/F12 (1:1) (22), F12 (19), MEM or EMEM (8) and RPMI-1640 (7). It must be considered that some studies used different culture medium for differentiated and undifferentiated cells: some studies used MEM medium for undifferentiated cells and DMEM/F12 for differentiated cells [51–53], another study used F12 for undifferentiated cells and DMEM/F12 for differentiated cells [54], and some studies used mediums or protocols that did not fit any of the above categories. In one study, DMEM was used in combination with F12 as

follows: DMEM:DMEM-F12 (1:1) [55]. Xie and collaborators used a particular differentiation methodology comprising a first step with EMEM medium supplemented with 2.5% FBS and RA and a second step with Neurobasal medium supplemented with B-27, BDNF, dibutyryl cyclic AMP and RA [56]. Most of the studies used fetal bovine serum (FBS) in concentrations ranging 10-15%, and in some, the FBS was totally withdrawn before treatment to avoid interactions with the pesticides or before differentiation to favor it. This was particularly true for the studies with flame retardants due to the high affinity of polybrominated and polychlorinated compounds to the lipophilic compounds of the serum.

Most studies used undifferentiated cells, and only 27 used differentiated cells in combination or not, with undifferentiated cells. In most of the cases, the used differentiating agent was RA. Differentiation protocols varied in concentration used, duration and onset, and a brief description of the agents used, and the time of treatment can be found on **Table 2**.
Only two studies used coating to favor differentiation: one study used coverslips with poly-L-lysine [57] and the other laminin coated wells [30].

Chapters 5 to 11 describe the use of SH-SY5Y cells to assess neurotoxicity in the case of the 7 selected groups of organic environmental pollutants. **Supplementary Tables 2 to 6** list the effects of the selected pollutants assessed using general markers of cellular toxicity including 1) cell viability readouts such as cellular metabolic activities (measured with CKK-8, MTT, MTS, XTT, WST-1 assays), neutral red assay, protein content, cell counts, BrdU assay, mitochondrial membrane potential, respiratory chain activity, and ATP levels; 2) cytotoxicity readouts such as LDH-release assay, annexin V/PI staining, chromatin condensation, DNA damage (comet assay); 3) oxidative stress readouts such as ROS and nitrite formation, and lipid peroxidation.

5. PESTICIDES

Of originally 125 pesticides and important known metabolites (**Table 1**), the search retrieved results for 41 substances and 103 articles. 31 substances can be grouped in organochlorine pesticides, organophosphate pesticides, carbamates, pyrethroids, and neonicotinoids. 10 other pesticides include atrazine, glyphosate and alachlor herbicides, fipronil, which was the only representative of phenylpyrazole insecticides, and the antiparasitic agents abamectin and emamectin. These 10 pesticides were grouped in the section named “other compounds” in **Table 1**.
The 103 articles found span from 1993 until 2021. The group with more studies on SH-SY5Y cells corresponds to organophosphate pesticides (55 articles), this is in accordance with their broad and worldwide use after progressive banning of most classical organochlorine pesticides.

Regarding individual compounds, the organophosphate pesticides chlorpyrifos and parathion, and their metabolites are the most studied compounds with 25 and 26 articles, respectively. Until recently chlorpyrifos has been one of the most used pesticides, to control insect pests in a range of crops, before its progressive banning in Europe and US (European Commission, January 2020, and Environmental protection agency, August 2021, respectively). The intensive use of chlorpyrifos increases the interest of the study of its toxicity on humans. Likewise,

parathion, or its metabolite paraoxon, and mipafox are among the most tested organophosphate pesticides on SH-SY5Y cells. Both compounds are frequently used in the study of esterase inhibition by organophosphates. Paraoxon is a well-known cholinergic neurotoxicant and used *in vitro* as a model of non-neuropathic compound. On the other side, mipafox, is a known organophosphate that does not affect the activity of acetylcholine esterase but can cause delayed neuropathy and is also quite used in *in vitro* models (20 articles).

Finally, the increased popularity of insecticides such as fipronil and pyrethroids make them also an interesting object of study, with 7 articles for fipronil, 7 more for cypermethrin and 6 for deltamethrin for instance.

Differentiated cells were preferentially used in neuron-specific endpoints that need phenotypic characteristic of mature neurons such as cell membrane potential [51, 53], acetylcholine receptor (AChR) signaling [51–53], voltage-gated calcium channel (VOCC) flux [51–53], intracellular calcium homeostasis [51, 52, 58], neurite outgrowth [51, 52, 57–61], and expression of genes involved in functional properties of neuronal cells such as the gene encoding TH, an enzyme involved in dopaminergic function [62]. Finally, in some studies that analyzed the activities of acetylcholine and neuropathy target esterases (AChE, NTE), cells were differentiated to increase expression of these enzymes [63–66, 58].

Several cellular and molecular processes disrupted by pesticides were identified using the SH-SY5Y cells. Oxidative stress is commonly studied and observed, but generally with high concentrations of pesticides. It is characterized by an overproduction of reactive oxygen species (ROS) and represents one of the most important causes of neurotoxicity that eventually leads to neuronal cell death. This is the case for most of the studied pesticides. As most processes are linked, we decided to present the effect of pesticides per family. The effects of pesticides assessed using cell viability and oxidative stress read-outs are detailed in **Supplementary Table 2**.

5.1 - Organochlorine pesticides (OCs)

5.1.1 - Impact of OCs on general markers of cellular toxicity

Organochlorine pesticides (OCs including dieldrin, endosulfan, heptachlor, α-hexachlorocyclohexane, lindane, pentachlorophenol and propanil) decrease cellular metabolic activities and/or growth rate in a concentration-dependent [51, 52, 67–75] (**Supplementary Table 2**). Different isomers or mixture of isomers of the same compound may display differential effects, as in the case of endosulfan [68, 70]. Complementary data suggest an alteration of cell viability (e.g., increased LDH release) as in the case of endosulfan and heptachlor [76, 77].

Mechanisms of action classically associated with alteration of cell metabolic activities include alterations in the levels of antioxidant molecules, ROS generation and subsequent lipid peroxidation in the case of several OCs [67–69, 71, 75, 77, 78] (**Supplementary Table 2**).

One key contributor of ROS generation is the mitochondria. Disruption of mitochondrial functions can be observed after cell treatment with OCs. Dysfunction of the respiratory chain (composed of four complexes) is a common cause of ROS production due to a misusage of electrons and O_2. For example, heptachlor decreases complex III activity, ATP levels and causes consequently mitochondrial fragmentation [77]. Disruption of the activities of the respiratory chain also leads to anomalies of the mitochondrial membrane potential as observed with pentachlorophenol and its metabolites [69], and with □-hexachlorocyclohexane and propanil [75].

This oxidative stress could be at least in part responsible for apoptotic cell death caused by OCs [67, 77, 78]. Indeed, several markers of apoptosis including DNA fragmentation, increased levels of exposed phosphatidylserine at the cell surface and/or increased caspase-3 and -9 pro-apoptotic enzymatic activities are observed upon treatment with dieldrin, endosulfan, heptachlor and/or pentachlorophenol [67, 69, 76, 77] (**Supplementary Table 2**). Alteration of these markers is completed, in the case of endosulfan, by a characterization of 1) a decreased transcriptomic expression of anti-apoptotic genes (apoptosis inhibitor 5, *API5,* and huntingtin, *HTT*) and proteins (zinc finger protein 224, ZNF244), 2) an increased expression of pro-apoptotic genes such as those coding for apoptosis inducer factor mitochondria associated 2 (AIFM2), fos proto-oncogene (FOS), Bcl-2 associated X (Bax) and fas-associated protein with death domain (FADD), and 3) an increased expression of genes involved in inflammation such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [78, 70].

5.1.2 - Impact of OCs on markers of neuronal function

OCs also have effects on crucial characteristics for neuronal function. Glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) release is decreased upon dieldrin treatment [67] and the amount of granulocyte-colony stimulating factor (G-CSF) is decreased by endosulfan [70], suggesting an inhibition of neuronal differentiation by these compounds. Endosulfan also increases the amount of G protein-regulated inducer of neurite outgrowth 1 (GRIN1) protein, one subunit of N-methyl-D-aspartate (NMDA) receptors [70], suggesting an impact on neurotransmitter signaling. A 72h exposure to hexachlorocyclohexane (also named lindane), decreases intracellular calcium concentration (IC_{20}=32-35µM), inhibits voltage-operated calcium channel (VOCC, IC_{20}=3.4µM), and causes swelling and deformation of the neurites in differentiated SH-SY5Y cells [51, 52]. AChE activity is decreased by pentachlorophenol in differentiated SH-SY5Y cells exposed during 24h this OC compound [74] and this inhibition appears milder or absent in undifferentiated cells exposed during 1h or less [53, 69].

Later studies have explored the mechanisms that could link Parkinson's disease (PD) and exposure to OCs. Treatment with propanil causes aggregation of □-synuclein, an effect

observed in several neurodegenerative diseases including PD [75]. Using CRISPR/Cas9 genome wide gene disruption, Russo and collaborators found modified sensitivity to dieldrin cytotoxicity with the disruption of genes belonging to ubiquitin proteasome system, mTOR pathway and autophagy and mitophagy related genes [72]. Among others, they found that decreased expression of PARK2 and increased expression of mTOR, increased sensitivity to dieldrin. Authors suggested that disruption of the ubiquitin proteasome system could be related with increased □-synuclein aggregates and increased mTOR activity, which has been associated to a decrease in the processes of autophagy and mitophagy, could preclude the cell repair mechanisms, finding therefore a link between environmental exposure to OCs and PD.

Dieldrin has also been linked to immune dysfunction. It can interfere with the cellular immune response in SH-SY5Y cells challenged with high-molecular weight poly I:C (Poly I:C), which is a viral insult mimic that increases the expression of multiple antiviral response genes [71]. The interferon pathway is part of the signals activated upon a viral insult; mRNA up-regulation of two key genes of innate immune response, IFIT2 and MX1, upon Poly I:C incubation (used to mimic such insult), is observed in SH-SY5Y cells and this increased expression is significantly diminished when cells are pre-treated with dieldrin. These effects on gene expression were related with ROS generation since pretreatment with antioxidant NAC attenuated the effects of dieldrin on gene expression.

5.2 - Organophosphate pesticides (OPs)

5.2.1 - Impact of OPs on general markers of cellular toxicity

With **organophosphate pesticides** (OPs), induction of oxidative stress and apoptosis is also a common pathway of cytotoxicity (**Supplementary Table 2**).
One of the most used OPs is chlorpyrifos (CPF), it is therefore one of the most studied too. CPF decreases cellular metabolic activities and/or growth rate in a concentration-dependent manner and upon a general model of action similarly observed with other OPs [79–82, 64, 83–86, 62, 87–90] (**Supplementary Table 2**).
Mechanisms of cell death caused by CPF are well characterized, mainly represented by oxidative stress-induced caspase-dependent apoptosis with morphological apoptosis hallmarks such as heterophagosomes, nuclei fragmentation and chromatin condensation and an over-representation of oxidative stress markers [79–86, 62, 88] (**Supplementary Table 2**). Other CPF-induced apoptotic pathways may exist, since inhibition of the caspase-associated pathway does not totally arrest apoptosis in CPF-treated cells [88].
CPF causes mitochondrial damage: mitochondrial swelling and alterations in the pattern of the crests [83] and mitochondrial fragmentation [62, 81, 91]. Moreover, CPF decreases the mitochondrial membrane potential [79–82, 91, 92], disrupts the activity of complex I-III and V [92] and decreases ATP levels [79, 80, 82, 92]. Recently, the effects of CPF treatment (50 and 100 µM for 5d) on mitochondria have been linked to a loss of coenzyme Q_{10} status, and post-treatment of cells with coenzyme Q_{10} partially recovered the effects of CPF on cell viability, and the decreased activities of citrate synthase and of complexes II/III [89].

Beside the disruption of mitochondria functions, the involvement of inflammatory processes in CPF-triggered apoptosis was also observed, as CPF treatment increased the amount of cyclooxygenase-2 (COX-2), tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and NF-κB proteins [80, 84, 85]. The mitogen-activated protein kinases (MAPK) c-jun N-terminal kinase (JNK) and p38 MAPK seem to be implicated in the inflammation process and generation of ROS since the use of specific inhibitors of these kinases attenuates COX-2 expression and ROS production [84]. Consequently, CPF treatment can also cause pyroptosis (inflammatory cell death), as seen by an increase in the amount of NOD-like receptor family, pyrin domain containing 3 (NLRP3), caspase-1, IL-1β, and interleukin-18 (IL-18) proteins, known to be involved in pyroptosis. This increase is regulated by microRNA 181 (has miR-181-5p) via SIRT1/PGC-1α/Nrf2 signaling pathway [90].
CPF causes a decrease of the mitochondrial protectant and anti-inflammatory enzyme HO-1 which could be partially behind the effects of CPF on mitochondrial malfunction since pretreatment with pinocembrin or thransinone I, plant and propolis extracts respectively, can effectively increase HO-1 levels, and partially decrease CPF effects on oxidative stress, mitochondrial malfunction and inflammatory signaling [79, 80].
Finally, adaptive mechanisms can be triggered by the SH-SY5Y cells to avoid death: indeed, CPF-induced autophagy identified by the increased expression of microtubule-associated protein 1A/1B-light chain 3 (LC3-II) mRNA and protein, a marker of autophagosomes, was observed [86, 92]. CPF induces more precisely mitophagy, as shown by decrease of FUN14 domain containing 1 (FUNDC1), an outer mitochondrial membrane protein, co-localization of autophagosomes and mitochondria, and observation of autophagic vesicles engulfing mitochondria [81, 92]. This mitophagy is mediated by PTN induced kinase (PINK1)/parkin signaling and regulated by the JNKs and ERK1/2 MAPKs [81, 92].

SH-SY5Y treatment with other OPs, such as parathion and its metabolite paraoxon, leptophos and its metabolite leptophos-oxon, methyl parathion and its metabolite methyl parathion oxon, monocrotophos, dichlorvos, mipafox, glyphosate and trichlorfon, also show general cytotoxicity mainly associated with mitochondrial disruption, oxidative stress and apoptosis [58–60, 89, 93–104] (**Supplementary Table 2**) [58, 59, 94–96, 98–100, 103].
Moreover, OPs can modify peptides by making adducts to tyrosine, lysine, threonine, and serine residues. Onder and collaborators studied the effects of CPF oxon at 10 µM (during 2d) on diethoxyphosphotyrosine peptides (tyrosine with diethoxyphospho adducts), which are the more stable [105]. They observed that CPF oxon modified up to 51 peptides and suggested these modifications as an alternative toxicity mechanism of low doses of OP. Further study of this effect, characterization of the modified proteins and assessment of their function must be done, but authors provided a new tool to study effects of neurotoxicants on SH-SY5Y cells.

5.2.2 - Impact of OPs on the activity of neuronal esterases

Despite oxidative stress has a proven role on OPs neurotoxicity, another important side of the neurotoxicity triggered by this group of compounds is their effects on two neuronal enzymes, acetylcholine esterase (AChE) and neuropathy target esterase (NTE, also known as neurotoxic

esterase) which can lead to 1) excessive extracellular concentrations of acetylcholine (ACh) and subsequent cholinergic neurotoxicity and 2) organophosphate-induced delayed neuropathy (OPIDN), respectively. SH-SY5Y cells are a good model to study the disruptions in these enzymes since they express both AChE and NTE, and most of the studies agreed in that their expression is maximal after RA differentiation of the cells [58, 63, 64] with only one exception in which the higher expression of AChE in undifferentiated cells is most probably due to higher cell density [53]. Moreover, interspecies differences in esterases levels and sensitivity to OPs make even more desirable the use of a human *in vitro* model to test effects of OP compounds [104, 106]. AChE is a key enzyme to clear the levels of the neurotransmitter, acetylcholine, from the synaptic cleft (**Figure 4A**). OPs that strongly inhibit this enzyme, such as paraoxon (AChE IC_{50} = 2.3 to 57.3 nM in RA-differentiated cells) [58, 63, 64] or chlorpyrifos-oxon (AChE IC_{50} = 0.34 nM in RA-differentiated cells) [64] cause accumulation of the neurotransmitter and overexcitation, which can lead to neurotoxicity (cholinergic syndrome) and eventually individuals' death. By comparison, the compounds that do not cause a strong inhibition of AChE but, on the contrary, strongly inhibit NTE are the ones which are susceptible to cause OPIDN, a delayed neurotoxic effect related to NTE permanent inhibition (**Figure 4B**).

SH-SY5Y cells have been tested and used as an *in vitro* model to study OPs effects on esterase activity for some time now [107–111]. A method to elucidate potential neuropathic compounds is to compare the inhibition of both activities (AChE and NTE). Ehrich and collaborators tested SH-SY5Y cells as an *in vitro* model to distinguish between those OPs that most likely cause an acute neurotoxicity by inhibiting AChE activity and causing a cholinergic syndrome, from those that cause OPIDN by inhibiting NTE [64]. AChE activity was measured by quantification of the hydrolyzed substrate (acetylthiocholine) with 5,5'-dithiobis(nitrobenzoic acid), also named DTNB or Ellman's reagent, a chemical used to quantify thiol groups. NTE activity was evaluated by comparison of the amount of hydrolyzed substrate (phenyl valerate) in incubates containing only paraoxon (which is known to inhibit other esterases but NTE) and incubates containing paraoxon and mipafox (which inhibit all inhibitable esterases). The comparative ability of an OP to inhibit NTE or AChE was determined by dividing NTE IC_{50} value by AChE IC_{50} value. This ratio was very high (>40, NTE IC_{50} >> AChE IC_{50}, characteristic of compounds which are specific of AChE) for those compounds that do not cause OPIDN, such as paraoxon and malaoxon, and those unlikely to cause it, like chlorpyrifos-oxon, dichlorvos and trichlorfon. The ratios were lower for OPs capable of causing OPIDN like mipafox. Using SH-SY5Y cells, the authors were capable of correctly classifying all the tested OPs, validating the use of SH-SY5Y cells to study OP-induced inhibition of AChE and NTE.

More recently, Sogorb and collaborators reviewed the method used to study the potential of OPs to cause OPIDN on SH-SY5Y cells using several neuropathic and non-neuropathic compounds [112]. The measure of NTE activity was corrected by using higher concentrations of mipafox, as they observed that the traditional dose used (50 µM) was only inhibiting half of the mipafox-phenyl valerate sensitivity activity, which suggests that previous studies using this concentration infra-estimated NTE activity. They also studied NTE reactivation after OP-inhibition by incubation with fluoride ion immediately after and 2 hours later. According to the authors protocol, if NTE IC_{50} / AChE IC_{50} ratio is higher than 5, most probably the compound will never reach the *in vivo* concentrations needed to inhibit NTE without causing first a cholinergic

syndrome. If this ratio is lower than 5, this is indicative of a probable neuropathic compound, but NTE permanent inactivation must be also studied. If this compound irreversibly inactivates NTE then it can be concluded that it causes OPIDN *in vivo*. If not, it can be cataloged as a probable neuropathic compound and *in vivo* testing must be performed. In this study, mipafox had a ratio lower than 1, chlorpyrifos-oxon, diazoxon, leptophos-oxon and dichlorvos had all ratios higher than 5 despite most of them caused NTE inactivation immediately before and 2 h after treatment.

Santillo and Liu further investigated the suitability of SH-SY5Y cells to detect toxic effects on AChE activity with a fluorescence assay, the Amplex Red assay. This method has the advantage over DTNB to be based on resorufin, a fluorophore with a high quantum yield, allowing for the detection of AChE activity from small numbers of cells making it more fit to high-throughput analysis. For instance, authors were able to study permanent inactivation of AChE by paraoxon and chlorpyrifos-oxon with this method [113].

As regards methamidophos, its neuropathic potential is controversial. Methamidophos has a stronger inhibition of AChE than NTE, however, differences in efficiency of NTE inhibition of the enantiomers allow for a possible induction of OPIDN in humans [66].

Further validation of SH-SY5Y cell line for the study of OPs inhibition of esterases was the study of Gonzalez-Gonzalez and collaborators. Different pools of carboxylesterase enzymes were distinguished in the SH-SY5Y cell line, positioning this cell line as a good model to discriminate and identify esterases in the human nervous system other than AChE and NTE that could be also targets of toxicity [114].

Despite OPs effect on esterase activity is an acute effect that takes place within the first 30-60 min, a study of the chronic exposure to OPs on the activities of AChE and NTE, was performed [54]. Authors treated SH-SY5Y cells with OPs including parathion and chlorpyrifos and their oxon metabolites (0.01-100 nM) for 28 days, corresponding to the same period used in the OPIDN tests in hens (the animal model for OPIDN testing, OECD 419). Cell treatment consisted of 4 successive cycles of 7 days, including 5 days with OPs treatment and 2 days without treatment in medium containing serum to preserve cell detachment. A dose-dependent AChE inhibition by chlorpyrifos-oxon and paraoxon was observed and this effect was greater than inhibition from a single exposure of the same concentration. The protoxicants chlorpyrifos and parathion also inhibited AChE activity in the 28 days exposure and these effects were much greater than in a single exposure, where protoxicants had no effect at those concentrations. This suggests a certain degree of conversion of the pesticides to their active form (their metabolites: chlorpyrifos-oxon and paraoxon) during the treatment.

Regarding CPF, this effect was also observed in the study published by Hinojosa and collaborators: whereas a 60 min incubation with the parental compound had no effect on AChE activity (according to [63]), an incubation of 24 h did inhibit AChE activity [83].

The effects of organophosphate pesticides on AChE activity could be partially due to oxidative stress, since the use of antioxidant fullerene derivatives significantly improved AChE activity after paraoxon treatment [65].

5.2.3 - Impact of OPs on other features associated with neuropathy

Aside from NTE inhibition, other effects are associated with delayed neuropathy such as neurite degeneration, alterations in calcium homeostasis and increase of calpain, a calcium-dependent protease. Therefore, these endpoints are also studied when assessing the neuropathic potential of a compound.

Paraoxon can cause a decrease in neurite length, but not in the number of cells with neurites, and in the amount of cytoskeleton proteins growth-associated protein 43 (GAP-43), synapsin I, synaptophysin and NEFH, although its effects seem to be milder than those of neuropathic OPs [60]. At the concentrations that inhibit 70% NTE, paraoxon causes a decrease in cell viability and has no effect on calpain activity and intracellular calcium homeostasis [58, 59].

Mipafox, causes a decrease in neurite length and in the percentage of cells with neurites. It decreases the levels of cytoskeleton proteins GAP-43, synapsin I, synaptophysin, NEFH, f-actin and TUBB3 [60, 98]. It also causes an increase in calpain and an increase of intracellular calcium concentration, in which L-type and T-type VOCC are implicated [61]. At the concentrations needed to cause 70% inhibition of NTE, mipafox did not cause any effects on cell viability [58, 59].

Trichlorfon treatment presents similar characteristics as those observed in mipafox in SH-SY5Y cells: reduced percentage of cells with neurites, reduced neurite length, decrease in the amount of GAP-43, synapsin I, synaptophysin and NEFH proteins, increase in intracellular calcium concentration and no significant cytotoxicity at the concentration needed to cause 70% inhibition of NTE [59, 60]. However, it exerts its effect at much higher concentration than mipafox, and at this concentration it also affects AChE, which could lead to a cholinergic syndrome before showing neuropathic symptoms [61].

Hong and collaborators studied the effects on neurite length and disruption in intracellular calcium levels of mipafox and paraoxon at different stages of differentiation in NGF-differentiated cells [57]. No effects of paraoxon were observed in differentiated or undifferentiated SH-SY5Y cells regarding neurite length. On the contrary, mipafox causes a significant decrease of neurite growth following 0.05 mM daily treatment during 4-day periods at different points of differentiation (at day 4-8 and 8-12) but had no effect on days 0-4 of differentiation and on undifferentiated cells, suggesting a window of developmental susceptibility. Regarding intracellular calcium concentrations, acute response was measured immediately after treatment with either mipafox or paraoxon and delayed response was measured during 4-days (from days 4 to 8 of differentiation) of 0.05 mM daily treatment on differentiated cells. Surprisingly, mipafox had no effect on delayed or acute treatment. Paraoxon, on the contrary, caused a dose-dependent acute response characterized by a transient peak of intracellular calcium immediately after treatment and a delayed response after 4-days treatment consisting in depletion of intracellular calcium stores and blunted calcium peak in response to carbachol (a muscarinic receptor agonist) stimulation.

Stimulation of muscarinic receptors may contribute to OPs cytotoxicity as shown for CPF and paraoxon cytotoxicity [88, 96, 115]. Besides its cytotoxic and esterase-inhibitor effects, CPF treatment can also act on neuronal and dopaminergic markers since it can cause a decrease in TH expression, in dopamine (DA) levels [62, 85] and in TUBB3 expression [85] but does not affect glutamic acid decarboxylase 65-kilodalton isoform (GAD65) protein expression which is a marker of GABAergic signaling [62]. Parathion and paraoxon alter the cell cycle status and have effects on f-actin, that may be related to alterations on neurite extension and secretion of neurotransmitters [116, 117].

Finally, metrifonate (trichlorfon) was tested as a therapeutic treatment for Alzheimer's disease (AD). Metrifonate and its hydrolytic degradation product, dichlorvos (100 and 1 µM, respectively) cause an increase of soluble amyloid precursor protein alpha (sAPPα) release, which forms part of APP metabolism and can counteract amyloid beta (Aβ) release. This effect is inversely related with trichlorfon and dichlorvos inhibition of AChE, which suggests that these components could regulate APP metabolism through increasing ACh availability and therefore muscarinic stimulation. This effect, however, seems to be acute (2h) and, despite metrifonate permanently inhibiting AChE, incubation periods of 24h and 48h did not change *APP* mRNA expression or APP metabolism. Moreover, 7-day treatment caused upregulation of AChE expression [118].

5.3 - Pyrethroids

5.3.1 - Impact of pyrethroids on general markers of cellular toxicity

As seen for the other pesticides, **pyrethroids** (such as cyfluthrin, cypermethrin and deltamethrin) cause a dose-dependent cytotoxicity due to caspases-dependent apoptosis [119–123, 88, 124–128] (**Supplementary Table 2**).

Similarly to OCs and OPs, oxidative stress represents the main mechanism of cytotoxicity for a variety of pyrethroids [119, 122, 123, 125–127] (**Supplementary Table 2**). This oxidative stress leads to increased expression of apoptotic-related genes, oxidative stress response genes and neuroinflammation-related genes such as *BAX, Bcl-2,* Bcl-2 interacting protein 3 *(BNIP3),* AKT serine/threonine kinase 1 *(AKT1),* apoptotic peptidase activating factor 1 *(APAF1),* nuclear factor kappa B subunit 1 *(NFKB1),* TNF-α*, Nrf2,* caspase-3 *(CASP3)* and tumor protein 53 (*P53)* in cells treated with cyfluthrin and cypermethrin [122]. The two main metabolites of deltamethrin, 2'-OH-deltamethrin and 4'-OH-deltamethrin show higher cytotoxicity and stronger effect on lipid peroxidation and NO production on SH-SY5Y than deltamethrin [125]. The mixture of six pyrethroids (including cypermethrin, cyfluthrin, deltamethrin and cyhalothrin) caused oxidative stress at concentrations lower than the highest no-effect concentrations of individual compounds [126].

The effects of pyrethroids on oxidative stress and programmed cell death could be caused by their effects on mitochondria. Indeed, cyfluthrin, deltamethrin and permethrin cause a decrease of ATP levels [120, 121], and under deltamethrin treatment, a decrease of complex I activity and

mitochondrial membrane potential are observed, as well as the translocation of PINK1 to the mitochondria [121].

A decrease of protein levels of peroxisome proliferator-activated receptor gamma (PPAR-γ) is also observed, and in this study, it is also suggested that PPAR-γ might have a role in blocking the translocation of PINK1 and therefore apoptosis [121].

Finally, as was also observed for the OP chlorpyrifos, increased expression of mRNA related to autophagy *Microtubule-associated protein 1 light chain 3 alpha* (*MAP1LC3A*), *autophagy-related 5 homolog* (*ATG5*), *ATG7* and *ATG12* upon α-cypermethrin treatment suggests that the enhancement of autophagy could be a protective mechanism of SH-SY5Y cells [127].

5.3.2 - Impact of pyrethroids on markers of neuronal function

Regarding neuron-specific effects of pyrethroids, pyrethroids could cause a disrupted mRNA profile related to neural cell development and cell growth. Cyfluthrin and cypermethrin decrease mRNA levels of neurofilament light chain protein (*NEFL)* and synapsin-1 *(SYN1)* and increase mRNA levels of tubulin beta-III *(TUBB3),* neurofilament heavy chain protein *(NEFH), GAP43,* calcium/calmodulin dependent protein kinase II alpha *(CAMK2A),* calcium/calmodulin dependent protein kinase II beta *(CAMK2B),* Wnt family member 3A *(WNT3A),* Wnt family member 5A *(WNT5A),* Wnt family member 7A *(WNT7A)* and phosphatidylinositol 3-kinase catalytic subunit type 3 (*PI3KC3)* [123].
Pyrethroids could also disrupt neuronal cell functions. Cyfluthrin and deltamethrin cause the loss of the cell projections [120, 121]. Deltamethrin decreases the expression of TH protein, which could impact the production of neurotransmitters [121]. Moreover, several pyrethroids affect the function of neurotransmitter receptors or ion channels: stimulation of muscarinic receptors could contribute to cypermethrin toxicity [88] and deltamethrin prolongs the open state of $Na_v1.8$ sodium subunit channel transfected in SH-SY5Y cells [129]. Beside sodium, calcium homeostasis can be affected as Mukerjee and collaborators showed that cypermethrin and permethrin inhibit the phosphatase calcineurin [130].

5.4 - Neonicotinoids

5.4.1 - Impact of neonicotinoids on general markers of cellular toxicity

Acetamiprid and imidacloprid, among other **neonicotinoids**, caused a concentration-dependent decrease in cell metabolic activities and/or growth rate (due to the triggering of apoptosis) through several mechanisms shared by other pesticides: oxidative stress (increased intracellular ROS levels) and DNA damage [73, 131, 132] (**Supplementary Table 2**). Effects of neonicotinoids on growth rate might be potentiated by the commercial formulation of the compounds, as shown in the case of imidacloprid [73].
In addition, neonicotinoids could trigger ER-stress as shown in the case of acetamiprid inducing, at the mRNA level, ER-stress related proteins inositol requiring enzyme 1-α (IRE1-α) and binding immunoglobulin protein 90 (GRP90) [131].

5.4.2 - Impact of neonicotinoids on markers of neuronal function

Tomizawa and Casida studied the different affinity of nicotinic AChRs for neonicotinoids, including imidacloprid and acetamiprid, using SH-SY5Y cells, which express several subunits of the receptors. Results showed that neonicotinoids could exert their effect through different nicotinic AChR subunits, including those expressed in SH-SY5Y cells [133]. More recently, Loser and collaborators showed that acetamiprid and imidacloprid among other neonicotinoids act as agonists of the □7-nAChR subunit, triggering an increase in intracellular calcium (at 1-10µM) in RA-differentiated SH-SY5Y cells [134].

5.5 - Other pesticides

The herbicide **atrazine** causes cytotoxicity and reduces cell viability in a concentration-dependent manner, triggering oxidative stress [135–139, 55] (**Supplementary Table 2**). Effects of atrazine associated with oxidative stress include an increase in the production of ROS and carbonylated proteins. It also causes lipid peroxidation and increases the expression of the antioxidant enzymes catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) and decreases the levels of reduced glutathione (GSH) [135, 137]. At the molecular level, atrazine acts as a mitochondrial stressor, decreasing ATP levels, dioxygen consumption, Na^+ /K^+ -ATPase activity and mitochondrial membrane potential [139]. The effects of atrazine on mitochondria function are also confirmed by a microarray analysis showing that it downregulates the expression of several gene-s encoding subunits of respiratory chain complexes involved in oxidative phosphorylation [137]. Moreover, the mitochondrial dysfunction caused by atrazine could lead to insulin resistance because mitochondrial dysfunction is implicated as a central cause of insulin resistance and because atrazine alters the phosphorylation of proteins (IRS-1, insulin receptor substrate-1 and Akt) involved in insulin signaling [137].

At the cellular level, the oxidative stress caused by atrazine leads to caspase-3-dependent apoptosis, and subsequently to an increase in condensed nuclei and phosphatidylserine exposure [135, 136, 138]. These processes can also be explained by an alteration in the expression of apoptosis-related genes (mRNA and protein levels of p21, p53, Bax and Bcl-2) by atrazine [135, 136]. Finally, atrazine treatment also causes a decrease in expression of genes related to autophagy, *BEX2* (*Brain Expressed X-Linked 2*) and *LC3-II*, and protein expression of BNIP3 and NIX, which could suggest that it impedes the neuroprotective mechanism. Also, it causes a decrease in TH protein expression, indicating a potential alteration of dopaminergic signaling [138, 139].

The influence of the cellular microenvironment might also be key to relay the effects of OP: Ma and collaborators co-cultured SH-SY5Y cells with rat BV-2 microglia cells to assess the role of microglia in atrazine toxicity. Microglia undergoes oxidative stress upon atrazine treatment, releasing TNF-α and IL-1β neuroinflammatory cytokines to the medium and exacerbating atrazine effects on viability and ROS generation on the SH-SY5Y cells [55].

Recently, Xie and collaborators studied the effects of low, non-cytotoxic concentrations of atrazine (0.3-30 ppb) [56]. Atrazine treatment for 96h before the differentiation of the cells causes increased MAP2 positive cells (30 ppb) and longer and more branched neurites (0.3

ppb), suggesting that atrazine can modify neuron morphology and cytoskeleton. Atrazine pre-treatment also causes increased expression of □-synuclein and triggered aggregate formation upon treatment of cells with dopamine and MPP+, which could mean increased susceptibility to disease such as PD. Authors also studied the epigenetic effects of atrazine treatment showing alterations in silencing markers. After atrazine treatment, increased cytosine methylation and decreased levels of histone H3 methylation (specifically H3K9me3 and H3K27me markers) are observed. Cytosine methylation levels are almost completely recovered at the end of differentiation (after 14d), H3k9me3 levels are increased but remain lower as compared to untreated control, and H3K27me levels are increased compared to the untreated control, showing overcompensation.

The herbicide **alachlor** causes a concentration-dependent decrease of cell viability, assessed by total protein content and lysosomal function [140] (**Supplementary Table 2**). However, alachlor also caused an increase or a decrease of NAD(P)H-dependent dehydrogenase enzymes activity (MTS assay) as well as of AChE activity depending on the concentration and period of exposure. Authors read the observed increase of these two parameters as a possible compensatory mechanism in response to oxidative stress [140].

A study with the herbicide **3-amino1,2,4-triazole** (amitrole) showed that pretreatment (at concentration above 10 mM) with the herbicide exacerbates cytotoxic effects of amyloid peptides on SH-SY5Y cells assessed by MTT assay. In this study cells were induced to overexpress catalase and the effects 3-amino1,2,4-triazole were thought to be caused by inhibition of catalase and consequently of H_2O_2 degradation [141].

Avermectins are macrocyclic organic compounds with potent anthelmintic and insecticidal properties. Interactions between avermectins and ATP-binding cassette (ABC) transporters were studied. The expression of these transporters in the blood-brain barrier and in neural cells prevent the accumulation of xenobiotics into the brain. Emamectin and abamectin inhibit some of these efflux transporters, human multidrug resistance protein 1 (MDR1) and human multidrug resistance-associated protein (MRP), in SH-SY5Y cells [142]. This result indicates that avermectins could trigger accumulation of xenobiotics in the central nervous system.

Fipronil (a phytosanitary and veterinary antiparasitic product, which has an insecticide and acaricide effect) causes a concentration- and time-dependent cytotoxicity of undifferentiated SH-SY5Y cells increasing oxidative stress [143, 144] that leads to inflammation, apoptosis [143, 145–148, 144] and/or autophagy, potentially acting as an adaptative process [146, 148]. Fipronil also causes shortening and loss of cell projections [143, 145, 148] and increase of the protein levels of vimentin, an intermediate filament implicated in neurite outgrowth in immature neurons, changes in its isoforms, and translocation of this protein from the soma to the neurite outgrowths, and finally loss of its fibrillization [148]. Fipronil decreases protein levels of neuron specific markers (TUBB3) [143] and alters dopaminergic signaling by decreasing TH protein levels, DA content and increasing DA metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) [143, 145]. It has no effect on the glutamic acid decarboxylase 65 (GAD65) GABAergic marker [145]. MAPKs and Akt/GSK3β pathways seem to be involved in

apoptosis triggered by fipronil [143, 145]. Romero and collaborators tested the effects of fipronol fulgone, the main metabolite of fipronil, showing that this metabolite was 37 times more toxic to cells and caused oxidative stress at two orders or magnitude lower concentrations than the parental compound [149].

Hao et al. showed that **glyphosate** (a world-widely used herbicide) causes cytotoxicity and that ethoxylated additives used in the commercial glyphosate formulations significantly increase the cytotoxicity triggered by glyphosate [100, 150]. The mechanisms of cytotoxicity are shared with other pesticides (oxidative stress, apoptosis) and include changes in apoptosis related genes (mRNA) with an increase in the expression of genes such as *APAF1*, *BAX*, *caspase-3*, *caspase-7*, *caspase-9*, *myeloid cell leukemia 1* (*MCL1*), *nucleolar protein 3* (*NOL3*), *synaptonemal complex protein 2* (*SYCP2*), *TNF-α* and *P53*, and a decrease in the expression of *AKT1* and *Bcl-2*.

The study of the expression of important genes related to neural development and function by RT-qPCR showed an increased expression of *Wnt3a*, *Wnt5a*, *Wnt7a*, *CAMK2A* and *CAMK2B*, and a decreased expression of *GAP43* and *TUBB3* indicating that glyphosate can induce neurodevelopmental toxicity [100].

Finally, a recent study including the triazole fungicides **tebuconazole** and **propiconazole**. Tebuconazole treatment (from 200 to 300 µM during 24- and 48-h) showed a significant reduction of ATP levels and a disruption of the mitochondrial membrane potential for the higher doses (200 and 300 µM for 24h) [151]. Propiconazole showed stronger effects with reduction of ATP levels from 100 µM and mitochondrial membrane potential reduction from 50 µM. Lipidomic analysis showed that propiconazole caused alterations of the lipid profile with altered expression of 137 of the 1192 lipids identified. Among the modified lipids, the upregulation of the fatty acid esters of hydroxy fatty acids (FAHFA) suggests that this group of bioactive lipids could have an antioxidant role according to authors.

5.6 - Mixtures of pesticides

There are only a few studies testing effects of mixtures of pesticides: Romero and collaborators tested a mixture of 6 different pyrethroids, since pyrethroids can be commercialized as mixtures of more than one kind, finding synergic effects [126]. Raszewski and collaborators observed a synergic cytotoxic effect of chlorpyrifos and cypermethrin mixture, a mixture used to prevent plant pests [88]. Hinojosa and collaborators tested the effects of chlorpyrifos with the algae toxin cylindrospermopsin, as they can coexist in water, finding synergic or antagonistic effects on cytotoxicity depending on concentration [83]. Tang and collaborators found that pentachlorophenol exacerbates the AChE inhibition by mipafox [74]. Xu and collaborators tested effects of cadmium on cytotoxicity of chlorpyrifos finding a potential antagonistic interaction [91]. Qiao and collaborators tested effects of combined treatment of chlorpyrifos and nicotine finding that despite its own cytotoxic effect, nicotine could attenuate chlorpyrifos toxic effects [87]. Despite aldicarb inhibits NTE activity [104, 110] its inactivation is reversible and in co-treatment with mipafox, it was shown to protect NTE from the permanent inactivation caused by mipafox [111]. Marinovich and collaborators treated the cells with a combination of diazinon, dimethoate

and azinphos, finding additive effects on the amount of protein but not on AChE inhibition for which a maximum effect of the mixture was equal to the effect of the most potent agent which was diazinon [152]. Finally, Jia and Misra found additive and synergic effects of zineb and endosulfan mixture on SH-SY5Y cells in triggering apoptosis and cell death [76, 78].

5.7 - Endpoints assessment

Some studies used pesticides among other chemicals to investigate the neurotoxicity prediction potential of neurofunctional endpoints, setting the focus on the validity of the endpoints rather than the specific results. Gustafsson et al. use several neurotoxicants such as lindane and 2,4-dichlorophenoxyacetic acid (an herbicide) to test different neuronal endpoints in SH-SY5Y cells to predict acute toxicity in humans that act *via* neurotoxic mechanisms. Several neurofunctional endpoints were assessed: changes in cell membrane potential (CMP), AChR signaling and voltage-operated calcium channel (VOCC) functioning, noradrenaline uptake, AChE activity and general cytotoxicity. CMP measurement was proved to be the best assay to predict acute neurotoxicity in humans in part due to being a general neuronal endpoint in contraposition of more specific endpoints used [53]. Forsby and Blaauboer used a battery of *in vitro* tests to cover the complexity of the nervous system: neurochemical functions (VOCC and AChR function), physiological functions (intracellular calcium concentration and protein synthesis rate), morphology (number of neurites per cell) and general cytotoxicity (total cellular protein in 72h as a measure of inhibited cell growth and cell death). EC_{20} values were used as surrogates for the lowest observed neurotoxic levels (LOAEL) at the target site *in vivo* (rats) with physiologically based biokinetic (PBBK) modeling. The validity of the model was checked by comparison of simulated biokinetic data with *in vitro* kinetic data found in literature [52].

6. TCDD

Of all the polyhalogenated organic compounds and polychlorinated dibenzofurans searched, the search only retrieved results for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or Seveso dioxin). Five articles were obtained from TCDD search and two more were obtained from the citations of one of the articles.

The only mode of action known for TCDD, is the binding and activation of the Aryl hydrocarbon Receptor (AhR), a transcription factor which regulates the expression of xenobiotic-metabolizing enzymes such as cytochromes P450 (CYP) 1A1, 1A2 and 1B1 or phase 2-detoxification enzymes [153]. In humans, TCDD is associated with chloracne, several forms of cancer and immunotoxicity. However, its impact on the nervous system is poorly investigated.

TCDD has several impacts on the SH-SY5Y cellular functions: it causes a concentration-dependent decrease in cell viability [154, 155]: TCDD doses from 100 to 250 nM (more than what is needed to activate the AhR whose binding affinity in humans is less than 10 nM) for 90 min to up to 24h cause apoptotic cell death as supported by increased activated caspase-3 and PARP1, decrease protein levels of Bcl-2 and increase of Bax and PARP1, chromatin condensation, nuclear fragmentation and DNA laddering [154, 155]. This apoptosis is ROS-mediated [155] and could be associated with disruption of calcium homeostasis and decrease in

the mitochondrial membrane potential [154]. At these concentrations, ROS generation also triggers autophagy shown by an increase of LC3II, Beclin-1 and p62 protein levels, as well as the existence of autophagosomes [155]. Autophagy occurs at similar timing than apoptosis and it is regarded as a protective mechanism, since blocking it exacerbates apoptotic effects of TCDD. Luukonen and collaborators used the same range of concentrations in an acute (48h) and delayed (8 and 15 days) study [156]. Contrarily to that observed in Morales-Hernandez et al. study, Luukonen and collaborators only observed an acute increase of superoxide anion but no effects on mitochondrial activity and membrane potential. No effects on chromatin integrity were observed either. At lower concentrations (10-50 nM during 72h) TCDD could cause a premature senescence response in SH-SY5Y cells, as a modulation of the following senescence markers was observed: increase of β-galactosidase, p21 and p16 protein levels and decrease of phosphorylated Rb protein [157]. This premature senescence is associated with increased ROS generation, since the use of the ROS scavenger N-acetyl cysteine (NAC), attenuates TCDD effects. One hypothesis that could explain the increase of ROS levels associated with TCDD exposure is the induction of CYP which can produce hydrogen peroxide in presence of uncoupling substrates. Morales-Hernández and collaborators showed that TCDD treatment, at doses from 1 to 20 nM during 24- and 48-h, decreases cell viability (EC_{50} = 3 ± 0.5 nM) without chromatin condensation, nuclei fragmentation, and caspase-3 activation [158]. AhR knockdown protects cells against cell death triggered by TCDD. This protection is complete against a treatment with TCDD at 20 nM during 24 h but incomplete against a treatment with TCDD at 250 nM during 24 h (which could be considered as a very high dose). Taken together, the results obtained by Morales-Hernández and collaborators suggest that TCDD-dependent cell death is non-apoptotic and AhR-dependent at relatively low nanomolar concentrations and, at least in part, apoptotic and AhR-independent at higher nanomolar concentrations [154, 158].

More interestingly, sublethal doses of TCDD (1-5 nM during 48h) cause an inhibition of neurite outgrowth in cells in co-treatment with RA, but this inhibition seems to have a timeframe of action, since it was significant when TCDD was applied 6 h after RA addition to the medium but was not significant when applied 12- and 24-h after the onset of RA treatment. Moreover, TCDD treatment causes a decrease of tissue transglutaminase (TGase) levels, an enzyme strongly up regulated by RA and that seems to be crucial for cell differentiation. Therefore, the effects of TCDD on neurite outgrowth could be mediated by the decrease of TGase levels and occurs only during the first phases of RA-induced differentiation [159].

Since TCDD is a strong activator of AhR, it was used to study the suitability of SH-SY5Y for testing AhR disruptors [30]. In Imran et al. study, RA treatment was combined with BDNF (10 µM RA in 10% FBS medium for 5 days followed by 50 mg/mL of BDNF for 5 more days in serum-free medium) to obtain differentiated cells. TCDD treatment (2.5-5-10 nM during 24 h) caused changes in gene expression of AhR target genes including *CYP1A1* (*Cytochrome P450 1A1*), *CYP1B1* (*Cytochrome P450 1B1*) and *AhRR* (*AhR repressor*) and these changes were different in undifferentiated and differentiated cells. In undifferentiated cells, low TCDD concentrations (2.5 and 5 nM) decreased *CYP1A1* and *AhRR* mRNA amounts whereas the highest TCDD concentration used (10 nM) increased *CYP1A1* and *CYP1B1* mRNA amounts. In differentiated cells, *CYP1A1* mRNA was upregulated by TCDD at 2.5 and 5 nM and *CYP1B1*

mRNA was upregulated at all the concentrations used. The amount of *AhRR* mRNA only decreased at 10 nM TCDD treatment. Surprisingly, authors could not detect expression of AhR neither in differentiated cells, nor in undifferentiated cells. To confirm the reliability of the RT-qPCR protocol, it was tested in primary human hepatocytes, where the expression of AhR was detected. Authors could not explain the effects of TCDD on AhR targets without AhR expression.

7. FLAME RETARDANTS

A total of 13 different flame retardants (9 PCBs, 2 PBDEs and 2 PFRs), including the **Aroclor 1254** (A1254) commercial mixture of PCB congeners with 54% chlorine, retrieved results for SH-SY5Y cells: the search retrieved 27 articles from 2005 to 2018. The effects of flame retardants assessed using general markers of cellular toxicity are detailed in **Supplementary Table 3**.

7.1 - Polychlorinated biphenyls (PCBs)

Most of the studies performed with PCBs on SH-SY5Y cells used the A1254 commercial mixture. Several PCBs (PCB-3, -8, -11, -52, -95, -99, -126) and the A1254 mixture display a concentration-dependent decrease in cellular metabolic activities and increase cytotoxicity and cell death through mechanisms shared by other pesticides including oxidative stress (mainly characterized by increased intracellular ROS levels) and/or apoptosis [160–170] (**Supplementary Table 3**). However, several original modes of action were also highlighted and thus described below.

A1254 cytotoxicity can be linked to its concentration-dependent inhibition of mitochondrial activity as this mixture decreases mitochondrial membrane potential, the activities of mitochondrial complexes I, II, and IV, oxidative phosphorylation and ATP production, as well as glycolysis [161]. –Treatment with A1254 also causes a concentration-dependent increase (from 30 µg/mL corresponding to 91.9 µM) in intracellular calcium, that is prevented in the absence of extracellular calcium and that seems partially caused by the sodium-calcium plasma membrane exchanger (NCX) [160, 167]. A1254-induced calcium increase is also favored by an increased expression of NMDA receptors [169]. This spike of intracellular calcium causes an increased protein expression of neuronal NOS (nNOS), which leads to NO production that causes cell damage through cGMP-protein kinase G signaling [160].
Moreover, several cell death pathways have been highlighted upon exposure to A1254. Whereas Ndountse and Chan found A1254-induced cell death to be caspase-3-dependent, Formisano and collaborators found that associated A1254 cell death was calpain-dependent without activation of caspase-3 [162, 169]. Both studies used similar doses (between 25 and 50 µM) only differing in the treatment duration (4h for Ndountse and Chan *versus* 24- and 48-h for Formisano and collaborators) which could mean that longer exposures favor calpain-dependent cell death.

Finally, A1254 treatment causes a decrease on BDNF levels [168] and affects neural differentiation by impairing the transcription of the *synapsin 1* gene encoding a synaptic vesicle

phosphoprotein implicated in neurotransmitter release. This effect is mediated by the overexpression of the transcription factor REST, which is mediated by a decrease in phosphorylated ERK2 and consequential increase and binding of Sp1 and Sp3 to the *repressor element 1-silencing transcription factor* (*REST*) promoter [162, 164]. REST inhibits *synapsin 1* transcription by increasing the protein expression of histone deacetylase 3 (HDAC3) and subsequent formation of the REST/HDAC3 complex that binds to *synapsin 1* promoter [162, 163]. This complex has epigenetic effects, being histone 3 and 4 acetylation significantly decreased by the HDAC activity upon A1254 treatment [162].

The effects of other types of PCBs have been studied on SH-SY5Y cells. Similarly to A1254, increased *REST* mRNA and protein expression is also caused by the PCB congener **PCB95**, which also causes a concentration-dependent cell death but has no effect on ROS production and phosphorylated p65, suggesting that PCB95 has no effects on oxidative stress and inflammation at the concentrations tested [166]. PCB-95-induced REST increase represses CREB expression, which controls the expression of necroptosis regulators, causing upregulation of positive necroptosis regulators receptor interacting serine/threonine 1 (RIPK1), RIPK3 and mixed lineage kinase domain like pseudokinase (MLKL) and downregulation of the negative regulator caspase-8, leading to necroptosis, which is the inflammatory cell death [165].
PCB99, a non-coplanar di-ortho-substituted PCB, and **PCB126**, a coplanar dioxin-like PCB with affinity for AhR, cause both a concentration-dependent cytotoxicity and an increase in caspase-3 activity observed after 10-50 µM treatments for 24 h [169]. They also cause an increased expression in NMDA receptors that are responsible for an intracellular calcium increase. Having the same amount of chlorine atoms, PCB99 was more cytotoxic than its PCB126 congener, suggesting that the position of the chlorine atoms in the benzene ring and the shape of the molecule play an important role in PCB toxicity [169].
In line with the previous study, **low-chlorinated PCBs PCB3**, **PCB8**, **PCB11** and **PCB52** and their hydroxyl- and sulphate- metabolites were tested in SH-SY5Y cells. Hydroxylated metabolites were more toxic than the parental compounds and the sulfate metabolite. Ortho-substituted compounds PCB8 and PCB52 hydroxyl-metabolites were more toxic than the non-ortho-substituted compounds PCB3 and PCB11. Moreover, di-ortho-substituted PCB, PCB52 metabolites had the highest toxicity [170]. This study suggests that the metabolism of low-chlorinated PCBs by hydroxylation and subsequent sulfation may influence their toxicity.

7.2 - Polybrominated diphenyl ethers (PBDEs)

All the studies of PBDEs in SH-SY5Y cells used the same congener, PBDE-47 except for one using PBDE-209. This is not surprising since PBDE-47 is one of the most predominant congeners detected in environment and human samples [171].

Several studies showed that **PBDE-47** treatment causes a concentration-dependent decrease in cellular metabolic activities assessed by MTS-assay and an increase in cytotoxicity and cell death [172–175] (**Supplementary Table 3**). The mechanisms linked to these processes are mitochondrial damage, ROS generation, genotoxicity and apoptotic cell death [176, 172–174, 177–179, 175]. Apoptosis is positively correlated with PBDE-47-associated intracellular calcium increase observed from 1 µM for a 24h exposure and from 5 µM for a 1h exposure [173, 179]

and might be mediated by the mitochondrial p53 pathway, as mRNA and protein levels of p53 are upregulated upon PBDE-47 treatment [178, 179]. Moreover, PBDE-47 increases the Bax/Bcl-2 ratio, causing mitochondrial membrane permeability and release of cytochrome c in cytoplasm, which in turn causes activation of caspase-12 and caspase-3 [173, 177–179].

Other adaptive mechanisms (potentially linked to cytotoxicity) are upregulated by PBDE-47. The unfolded protein response (UPR) is, for example, activated by PBDE-47 which causes upregulation of iron regulatory protein 1 (IRP1), an ER-resident transmembrane kinase and ER-stress sensor. This mechanism can work towards apoptosis or to protein repair depending on cell damage. PBDE-47 treatment effects were exacerbated on inositol requiring protein 1 (IRE1) silenced cells, suggesting a role for IRE1 and UPR mechanism in PBDE-47 toxicity [177]. Autophagy could also be implicated in PBDE-47 mediated cell death. PBDE-47 causes an increase of autophagic vacuoles and an increase in the amount of autophagy-related proteins LC3, Beclin-1 and p62 after 24 h of treatment. This generation of autophagosomes could be caused by oxidative stress, since the cotreatment with the antioxidant NAC significantly reduced PBDE-47-induced autophagic vacuoles and autophagy-related protein levels. Finally, pre-treatment with autophagy inhibitor 3-MA increased cell viability, therefore, autophagy may be a mechanism for cell death caused by PBDE-47 [175].

PBDE-47-observed effects on cell viability and proliferation, intracellular calcium, oxidative stress, apoptosis and DNA and chromosomal damage were increased in combination with PCB153, suggesting synergic effects in most of the cases [173, 174, 176].

Similarly to the effects observed with PBDE-47, PBDE-209 treatment induces mitochondrial damage, ROS generation, genotoxicity and apoptosis in SH-SY5Y cells, as shows a dose-dependent increase of caspase-3/7 activity. Exposure to PBDE-209 also increases levels of Aβ-42 which could be linked to an intracellular calcium increase (observed upon exposure to 20 µM PBDE-209) [180].

7.3 - Phosphate flame retardants (PFRs)

PFRs can cause similar effects than the ones observed with PBDEs on SH-SY5Y cells: TDCPP (Tris (1,3-dichloro-2-propyl)phosphate) and TOCP (Tri *o*-cresyl phosphate involved in the aerotoxic syndrome) cause a concentration-dependent decrease in cellular metabolic activities assessed by MTT assay and/or LDH release assay [181–184] (**Supplementary Table 3**). TDCPP increases intracellular ROS levels and chromatin condensation, decreases the Bcl-2/Bax ratio and triggers caspase-3-dependent apoptosis [181–183]. TOCP triggers cell cycle arrest through mRNA and protein downregulation of cyclin D1, upregulation of the CDK inhibitor p21 [184]. Both molecules also trigger autophagy in undifferentiated cells (at 100 µM for 24h or 2.5 µM for 3- or 5-d in the case of TDCPP and 1000 µM for 24h or 500 µM for 48h in the case of TOCP) and RA-differentiated cells (from 500 µM for 24h in the case of TOCP). This autophagy is characterized by increased LC3I conversion to LC3II and LC3 positive puncta and which seems to play a protective role against PFRs-induced apoptosis [181, 183–187].

Some interesting mechanisms observed upon TDCPP treatment can be highlighted: first, TDCPP-dependent cell death is associated with ROS triggered ER-stress, since ER stress related proteins (eukaryotic translation initiation factor 2a (p-EIF2a), glucose regulatory protein 78 (GRP78), C/EBP homologous protein (CHOP) and activating transcription factor 4 (ATF-4)) are up-regulated upon TDCPP treatment [181]. Second, TDCPP-related autophagy is mediated by activation of 5'-AMP-activated protein kinase (AMPK)-mediated signaling pathway, which in turn is activated by TDCPP-induced ROS generation linking both mechanisms [181]. Non-cytotoxic doses of TDCPP (2.5 µM for 3- and 5-d) cause SH-SY5Y cell differentiation in addition to autophagy. Finally, TDCPP treatment causes increased neurite-bearing cells and neurite length, and increased MAP2 expression [183]. Cytoskeletal components NEFL, NEFH and TUBB3 are also increased at transcript and protein levels upon TDCPP treatment. This event seems to be dependent on TDCPP-induced autophagy as its inhibition with the autophagy inhibitor 3-MA reduces cell differentiation and cytoskeletal components expression [183]. TDCPP also causes a concentration- and time-dependent increase of intracellular calcium concentration [182].

Regarding TOCP, the mitochondrial degradation linked to autophagy [185–187] might be related to onset of TOCP-associated OPIDN. Indeed, upon TOCP treatment on SH-SY5Y cells, there is colocalization of autophagosomes with mitochondria, parkin recruitment to mitochondria (key step for mitochondria engulfing by autophagosomes) and loss of mitochondrial inner and outer membrane markers including translocase of outer mitochondrial membrane 20 (Tom20) and translocase of inner mitochondrial membrane 23 (Tim23) [187]. Finally, TOCP-induced autophagy in differentiated cells causes inhibition of neurite outgrowth that might result from the degradation of the cytoskeletal proteins NFEH, NFEL and TUBB3 [185].

8. PERFLUORINATED ALKYLATED SUBSTANCES (PFASs)

Of all the perfluorinated alkylated substances (PFASs), the search retrieved results for studies using PFOA and PFOS. The final number of accessible articles retrieved was 10. The effects of PFASs assessed using general markers of cellular toxicity are detailed in **Supplementary Table 4.**

PFASs are synthetic chemicals used in a wide range of industrial and consumer products. They are found particularly in textiles (clothing, shoes, fabrics, carpets), paper and cardboard packaging for food contact and kitchen utensils (non-stick coating). They are also used in fire-fighting foams, electrical wire insulation, floor waxes, varnishes and paints, cleaning products and some pesticides. Perfluorinated substances can be released into the environment during production or use. They migrate from consumer products into the air, house dust, food, soil, groundwater, and surface water and into drinking water. Highly prevalent in the environment, persistent, and bioaccumulative, some PFASs fall into the category of persistent organic compounds, the best known of which are PFOA (perfluorooctanoic acid) and PFOS (perfluorooctanesulfonic acid). They are considered "Forever chemicals", i.e. substances that are very slowly eliminated by organisms [188].
Viability of RA-differentiated SH-SY5Y cells is not altered upon exposure to 100 µM **PFOA** within a 48h period. **PFOA** treatment decreases cell viability at 250 and 500 µM (24-48h) but

does not modify caspase-3 and -7 activation within a 24h period and exerts an opposite effect in term of caspase-3 and -7 activation depending on the concentration used during a 48h exposure. PFOA also impacts the mitochondria: it decreases the ATP levels (400µM, 24-48h; 100-400µM for 72h), the mitochondrial membrane potential (100-250 µM, 24h; 10-100-250 µM, 48h), and the basal respiration at 250 µM (48h). ATP synthase activity is also decreased upon exposure to 250µM (24h) PFOA. Moreover, metabolomics analysis showed an increase of ATP-related metabolites and a decrease of neurotransmitter precursor and oxidative stress-protectant metabolites triggered by PFOA treatment (100 µM for 24h) [189].

Exposure of undifferentiated and RA-differentiated SH-SY5Y cells to **PFOS** also causes a concentration- and time-dependent decrease in cell viability [190–196], and an increase in apoptotic cell death, which may be due to PFOS-induced oxidative stress [191–196] (**Supplementary Table 4**). Indeed, exposure to PFOS increases ROS and lipid peroxidation, and decreases the antioxidant enzyme activity GPx, leading to cell apoptosis [191, 195, 196]. Increased ROS accumulation upon PFOS treatment leads to JNK phosphorylation and translocation to the nucleus and mitochondria, inducing apoptosis by modulating Bcl-2 and Bax levels (respectively decreased and increased), causing the release of cytochrome c and the activation of caspase-3 [195, 196]. The Nrf2 pathway is also activated upon PFOS treatment as a response to the induced oxidative stress [195].

A recent study found an increased expression of two Alzheimer's disease biomarkers, APP and Tau proteins. Phosphorylation of Tau (on ser-404 and thr-181) also increases as well as Tau-related kinase GSK3β levels upon treatment with low doses of PFOS (0.001 µM during 24h) suggesting a potential implication of PFOS on Alzheimer's pathogenic pathways [190].

PFOS might also impact key factors of the differentiating process. Indeed, it decreases mRNA and protein levels of BDNF and subsequently disrupts the BDNF/TrkB/CREB signaling. This decrease in BDNF levels could be related to PFOS-mediated increased expression of miR-22, a known regulator or BDNF which may repress the translation of the BDNF mRNA [193].

The role of glial cells on PFOS neurotoxicity was also investigated using indirect approaches (treatment of SH-SY5Y cells with conditioned medium from low non cytotoxic doses-exposed astrocytes or microglia cells) [192, 197, 198]. Treatment of SH-SY5Y cells with conditioned medium from microglia cells treated by PFOS (20 nM during 6h) causes an increase in cell apoptosis related to microglial activation. This seems to be due to the secretion into the medium of the inflammatory factor TNF-α as well as NO, which would be responsible of the activation of apoptosis [197, 198]. PFOS treatment (20 nM during 24h) also activates astrocytes which also release of TNF-α inducing apoptosis of SH-SY5Y cells exposed to the corresponding conditioned medium [192].

In conclusion, most studies on the effects of PFASs were performed on undifferentiated SH-SY5Y cells but some of them suggest that PFOS could impact the differentiating process of these cells [193]. Therefore, it would be interesting to test the effects of PFOS but also of other PFASs during the differentiating process or after differentiation. Additionally, the inflammatory

response (TNF-α) triggered by PFOS on astrocytes and microglia can contribute to PFOS cytotoxicity on neurons and this dialog should be investigated in more details.

9. PHENOLS (PARABENS and BISPHENOLS)

8 articles were retrieved from the search of phenols, comprising one paraben (methylparaben) and one bisphenol (bisphenol A). The effects of phenols assessed using general markers of cellular toxicity are detailed in **Supplementary Table 5.**

9.1 - Parabens

Parabens are a series of parahydroxybenzoates extensively used in cosmetic and pharmaceutical products due to their antibacterial and antifungal properties. Paraben's main health concern is their potential action as xenoestrogens which makes it not surprising that there are no studies assessing their toxicity on SH-SY5Y cells. Indeed, only one study was found in which SH-SY5Y cells were treated with methylparaben (MP): Kopalli and collaborators tested the potential neuroprotective effect of MP against oxidative damage caused by 6-OHDA. MP treatment is not cytotoxic and has no effect on intracellular ROS levels (**Supplementary Table 5**) and MP pretreatment during 4h (doses from 0.01 to 1 nM) significatively ameliorates cytotoxic effects and generation of ROS induced by 6-OHDA on undifferentiated SH-SY5Y cells [199].

9.2 - Bisphenol A (BPA)

Bisphenol A (BPA) is an organic compound, used mainly in the manufacture of plastics and resins. It is used as a monomer in epoxy resins and polycarbonates. It has also been used extensively as a developer in thermal printing with a presence in free form in many cash register receipts and credit card receipts printed on thermal paper. It has been recently classified as an endocrine disruptor.

BPA can have opposite effects on cell viability depending on the concentrations and the time of exposure (**Supplementary Table 5**). Low doses of BPA have a proliferative effect, whereas higher doses decrease the number of cells and cause cell death in undifferentiated SH-SY5Y cells. The range of doses and time of exposure, however, vary depending on the study. Thus, in Kafi et al. study, the proliferative effect of BPA was observed with doses ranging 50 to 300 nM for 24h, and doses from 400 to 1200 nM decreased the cell number assessed by cell-based sensory array and MTT assay [200]. Senyildiz and collaborators described proliferative effects of BPA at 0.1 and 10 µM with longer exposure times (96h) using the BrdU assay and observed decreased cell viability after a 24h exposure to BPA concentrations starting from 31.25 µM using the MTT and neutral red assays [201]. Another study described a reduction on cell viability and an increase of cell death at very low doses of BPA, 1 pM during 48h in a MTT-based analysis and 1 nM during 48h in a LDH- assay [202]. The same authors, however, reported that concentrations from 0.1 to 10 µM of BPA increased cell viability for incubations of 8- and 72-h, but not for 24h, whereas at 100 µM BPA decreased cell viability after an 8h

incubation but increased cell viability after a 72h incubation [203]. Ayazgök and Küçükkilinç also reported that BPA (at 1 pM and 1 nM) caused an increase of apoptosis assessed by Annexin V detection after 12- and 24-h and that this apoptosis switched to necrosis after 48h [202]. A correlation between the cytotoxic effect of BPA assessed by MTT-based assays and the NO concentration found in the medium of the cells was also determined, suggesting that the neurotoxic effects of BPA may be explained by increased levels of NO [202, 203]. This concentration-dependent effect of BPA is consistent with recent changes in the regulation of this compound such as the reduction by EFSA of the tolerable daily intake from 50 to 4 µg/kg bw/d, whose effects at low concentrations were among the first documented.

Beside the effects on proliferation, BPA also affects differentiating processes as assessed by the study of DA or esterases status. BPA treatment (200-400 µM during 2-24h) causes an increase of DA uptake and of dopamine transporter (DAT) protein levels [204]. BPA also affects AChE activity although its effects are diverse, depending on concentration and time [202].

Other cellular processes could be impacted by BPA: BPA increases cell migration and invasion acting as an agonist of estrogen receptor alpha (ERα) and enhancing its activity [205].

Beside estrogen signaling, other relevant signals could be disrupted by BPA. BPA treatment (20 nM) causes a transient increase of intracellular calcium, increases ROS levels, decreases ATP levels and mitochondrial membrane potential [206]. BPA treatment (20-2000 nM during 12h) also hampers insulin signaling in SH-SY5Y cells: it diminishes phosphorylation of insulin receptor (IR) and consequential activation of several key downstream components. Moreover, some of the effects on these components, such as the reduced phosphorylation of mechanistic target of rapamycin kinase (mTOR) and the methylation of protein phosphatase 2 A (PP2A) are related with Tau phosphorylation (significant from 20 to 2000 nM during 12h, with maximum dose response at 20 nM). Moreover, the expression of APP protein was increased upon BPA treatment (from 2 nM during 12h). As for Tau, APP is involved in the pathogenesis of Alzheimer's disease. Effects of BPA on insulin signaling and Tau phosphorylation were partially rescued with cotreatment with insulin or rosiglitazone [206].

Finally, BPA also causes epigenetic changes in SH-SY5Y cells: indeed, a 10 µM treatment for 96h causes an increase of global DNA methylation and global levels of H3K9me3, H3K9ac and H3K4me3 histone markers. This treatment also increases the expression of genes encoding several histone methyltransferases, acetyltransferases, and deacetylases such as G9a (or EHMT2), SETD8, SETD1A, RIZ1, EZH2, HAT1, DNMT1 and SIRT1 and decreases the expression of *Suv39h1* gene [201].

10. PHTHALATES

Phthalates are a group of chemical compounds used to amend certain products, usually plastics, making them softer and more flexible. They are sometimes called 'plasticizers',

although many other chemicals are also called 'plasticizers'. Phthalates have a wide range of applications, they are found, for example, in medical devices and food packaging.

The search of studies on SH-SY5Y cells with the phthalates listed in **Table 1**, retrieved 7 studies with a total of 8 compounds tested.

10.1 - Impact of phthalates on general markers of cellular toxicity

The effects of phthalates assessed using general markers of cellular toxicity are detailed in **Supplementary Table 6.**

Phthalates as well as bisphenols seem also to display opposite effects on cell viability, depending on the type of compound, the concentrations, and the time of exposure (**Supplementary Table 6**). For example, proliferative and/or anti-apoptotic effects have been characterized for exposure to relatively low doses (0.01 and 0.1µM during 24h) of **N-butyl benzyl phthalate** (BBzP) whereas a higher dose (300 µM during 24h) inhibits cell proliferation [207]. The proliferative and anti-apoptotic effects of BBzP are associated with an increase in the expression of cyclin-D, proliferating cell nuclear antigen (PCNA) and Bcl-2 and a decrease in the expression of p53 observed upon a 24h exposure to 0.1µM BBzP [207]. Additionally, **di-(2-ethylhexyl) phthalate** (DEHP) treatment (0.1 µM during 24-48h) does not change cell viability whereas a higher dose (5-200 µM during 24 and/or 48h) causes a dose-dependent decrease of cell viability and increases apoptotic cells assessed by caspase-3 activation and Annexin V positive cells [208, 209]. Contrary to DEHP, exposure to **mono(2-ethylhexyl) phthalate** (MEHP), a metabolite of DEHP, increases cell proliferation (2-1000 µM during 12-24h) and decreases apoptosis with an increase in the expression of C-MYC, cyclin-D1 and Bcl-2 [210, 211]. MEHP treatment also increases cell migration and invasiveness as well as the amounts of PCNA, matrix metallopeptidase 2 (MMP-2) and 9 (MMP-9) mRNAs and proteins whereas it decreases those of tissue inhibitor of metalloproteinases 2 (TIMP-2) [210].

The growth-promoting effects of BBzP and MEHP seem at least in part to be mediated by ER signaling since BBzP treatment increases the release of estradiol (E2) by SH-SY5Y cells and the protein levels and activity of aromatase, which is the key enzyme for estradiol synthesis [207], and the knock-down of the ERα in SH-SY5Y cells decreases or totally inhibits the effects of MEHP [211]. MEHP treatment also causes an increase in ERα expression [211] and the effects of E2 are similar to those of MEHP on PCNA, MMP-2, MMP-9 and TIMP-2 mRNA and proteins [210].

Moreover, the effects of MEHP on cell cycle and apoptosis correlate well with increased expression of Notch-1, Notch-2, Notch-3, Jagged-2 and DII-4 proteins indicating that the Notch pathway is involved in mediating these effects.

On the other side, DEHP reduces the protein level of the pro-survival transcription factor Sp3 (but it does not affect the mRNA expression), probably through the up-regulation of class II deacetylase HDAC4 which deacetylates Sp3 and facilitates its ubiquitination [209]. Indeed, the overexpression of Sp3 and the knock-down of HDAC4 significantly reduce the number of apoptotic cells [209]. In addition to promoting apoptosis, DEHP favors ROS generation, mitochondrial malfunction (reduced activity of all the complexes and membrane potential) and ER stress (increased RNA and protein levels of GRP78 and CHOP) [208]. Treatment of SH-SY5Y cells with the chemical chaperone 4-phenylbutyric acid (PBA), a known protector against ER stress, alleviates the promoting effects of DEHP on cell death [208]. These results indicate that mitochondrial and ER malfunction trigger apoptosis on SH-SY5Y treated with DEHP.

Taken together, these results suggest that the effects of phthalates on SH-SY5Y cells could vary depending on the congener and, moreover, the effects could differ from parental compounds to metabolites. Some compounds could cause mitochondria and ER malfunction and apoptosis, some others may have a tumorigenic effect increasing cell proliferation and migration most probably through ER signaling.

10.2 - Impact of phthalates on markers of neuronal function

Phthalates such as BBzP, DEHP, Di(n-butyl) phthalate (DnBP), Dicyclohexyl phthalate (DCHP), Monobenzyl phthalate (MBzP), Diethyl phthalate (DEP) and Mono-n-butyl phthalate (MnBP) can inhibit the calcium signaling coupled with nAChR signaling through non-competitive blocking of the receptor [212, 213]. The suppression potency depended on the amount of alkyl groups. The phthalates with residues with 4 to 6 carbons are more efficient in suppressing the signaling than those with shorter or longer chains [212]. The longer the number of carbons, the more hydrophobicity and more suppression potency, but high number of carbons can also cause steric hindrances in fitting the binding site [212]. Also, the observation that BBzP (IC_{50} = 0.28 µM) and DnBP (IC_{50} = 0.44 µM) had a higher suppression power than their metabolites MBzP (IC_{50} = 58 µM), MnBP (IC_{50} = 116 µM) and phthalic acid (PA, IC_{50} = 505 µM) suggests that diesters are more toxic than monoesters [213]. Finally, this antagonistic effect persists over time, DnBP treatment over 5 days (10 µM) maintained the suppression characteristics on nAChR [213].

11. POLYCYCLIC AROMATIC HYDROCARBONS (PAHs)

Of all the PAHs searched only one study was retrieved assessing cytotoxic effects of several PAHs, namely benzo(a)pyrene (B(a)P), chrysene, and anthracene, and one halogenated aromatic hydrocarbon, pentachlorophenol [74]. All compounds were assessed at concentrations ranging from 0.03 to 30 µM for 1 and 4 days. B(a)P was the compound showing more toxic effects, decreasing amino acid incorporation, total protein levels and number of live cells, and these effects were stronger after metabolic activation with rat liver microsomes. Chrysene had only effects after metabolic activation while anthracene had no effect in either condition.

Coexposure of cells with B(a)P and chrysene or pentachlorophenol had similar results than B(a)P alone. Finally, AChE inhibition was tested for B(a)P and chrysene, showing no effect.

12. SH-SY5Y CELLS IN HIGH-THROUGHPUT SCREENING

Of special interest is the study of Li and collaborators [214]. Framed in the American project Tox21, with the main aim to develop high-throughput screening for toxicity of chemical compounds, Li and collaborators tested more than 8,000 compounds (Tox21 10K compounds collection) for the ability to inhibit AChE activity. They used enzyme- and cell-based approaches with monolayer and spheroid culture of SH-SY5Y cells and neural stem cells for the cell-based testing. SH-SY5Y cells proved to be a good model for the screening of AChE inhibitors, with modest differences of sensitivity to certain compounds between monolayer cultures and spheroids. With the large number of compounds tested covering most, if not all, of the compounds used in this review, a first screening detected 187 compounds (2.25%) capable of inhibiting AChE more than 50%. From these chemicals, they selected 111 compounds that inhibited 50% of AChE activity (IC_{50}) at concentrations lower than 20 μM for further analysis, comprising several organophosphate pesticides, including bromophos and chlorpyrifos among others, several carbamates (for which no other studies with SH-SY5Y cells were found) and the organochlorine pesticide endosulfan. Clustering analysis of the Tox21 compounds library based on the chemical structure revealed clusters of compounds enriched with AChE inhibitors, including a cluster with 21 carbamates and another one with OPs comprising chlorpyrifos and chlorpyrifos-oxon. Enzyme-based assays revealed that most of the OPs were more potent with metabolic activation by incubation with human microsomes. Further docking studies characterized structural interactions of the compounds with AChE. Overall, high-throughput analysis of AChE inhibition on SH-SY5Y provided data to characterize potential neurotoxic compounds and, together with enzyme-based tests and structural analysis, this data can be used in predictive models of toxicity.

13. CONCLUSION AND PERSPECTIVES

In conclusion, the studies included in this review illustrate the relevance of the SH-SY5Y cell model, either undifferentiated or differentiated into neuron-like cells, in deciphering the mechanisms involved in the toxicity of chemicals on neuronal cells. The review focused on several classes of organic pollutants and the main effects of these chemicals on SH-SY5Y cells are summarized in **Figure 5**. Many organic pollutants, if not all, induce oxidative stress, inflammation, and apoptotic cell death, which are features of general cytotoxicity. Oxidative stress is mainly highlighted by ROS and NO generation, lipid peroxidation and by decreased levels of antioxidant proteins. Apoptotic cell death is, at least in part, caspase-dependent and caused by oxidative stress and/or inflammatory processes. Apoptotic hallmarks are observed such as chromatin condensation and nucleus fragmentation. In addition to apoptosis, other modes of programmed cell death (pyroptosis, necroptosis), necrosis or autophagy can be triggered by pollutants, depending on the pollutant or the conditions of exposure. Organic pollutants also affect general cell functioning and, in this context, mitochondrion is strongly

impacted as demonstrated by changes in its morphology (fragmentation and alteration in the pattern of crests) and its functions (decreased mitochondrial membrane potential, respiratory chain activity, ATP synthesis). Endoplasmic reticulum is also targeted as some chemicals are shown to trigger the unfolded protein response. Impact on the cytoskeleton is featured by alteration in its components including neuron-specific proteins found in neurites (dendrites, axons) and by changes in neuritic length and shape. In addition, beyond neuritic homeostasis, specific neuronal functioning is targeted by organic pollutants as these chemicals disrupt several processes involved in neurotransmission such as neurotransmitter synthesis, uptake and degradation, activity of neurotransmitter receptors and calcium homeostasis.

The SH-SY5Y human neuroblastoma cell line, established in the 1970s from a metastatic bone marrow biopsy of a neuroblastoma patient, has been widely used as a neuronal model since the 1980s. As well as other *in vitro* cellular systems in monolayers used in the context of neurotoxicological studies, it is featured by a limited cost, is an easy-to-use model, allows a rapid evaluation of toxicity on a single cell type, allows a tight control of the neurotoxicant concentration and exposure time, and is not associated with ethical concerns. Of human origin, the SH-SY5Y cell line represents a relevant tool to evaluate the neurotoxic potential of compounds in humans. This cell line may differentiate upon treatment with various agents such as retinoic acid and neurotrophins such as NGF and BDNF. Therefore, it can be used at a proliferative and undifferentiated stage, during differentiation and at various differentiated stages recapitulating the whole phenotype of a neuron, featured by a neuron-like morphology and function, and expressing a variety of neuronal-specific markers. Depending on the considered stage of differentiation, this model allows evaluation of cellular neurotoxicity on proliferative neuroblasts, on the late-differentiating neurons or on mature neurons. The vast majority of the 163 published neurotoxicological studies from 1992 and January 2022 used undifferentiated SH-SY5Y cells (146 studies including 10 studies using both undifferentiated and differentiated cells) to evaluate the toxicity triggered by selected organic pollutants. The results obtained in these studies illustrate that the undifferentiated SH-SY5Y cell line is an appropriate model to measure general endpoints of neurotoxicity such as cell viability, mitochondrial function, and oxidative stress. In addition, neuroblastic cells are a good model to study potential carcinogenicity with endpoints such as cell proliferation and migration. However, even if a compound can impact these endpoints in SH-SY5Y cells, it does not prove that this compound is neurotoxic, but only that it is cytotoxic in this model (and at a specific stage of differentiation). Likewise, a compound at a specific concentration that does not influence general toxicity or function of the cell, cannot be discarded as neurotoxicant. Indeed, effects on neuron-specific endpoints can be observed at concentrations far below cytotoxic levels. A good quality assessment in the *in vitro* neurotoxicity field should always include evaluation of 1/ general cytotoxicity, 2/ general, but essential, cell functioning, and finally 3/ specific neural endpoints such as electrical excitability or synaptogenesis [5].

Undifferentiated SH-SY5Y cells can be used to evaluate the effect of neurotoxicants on gene expression analyzed by quantification of mRNA and/or protein of interest in targeted studies or by large-scale techniques. Several organic pollutants analyzed in this review were shown to affect expression of genes related to neural and neuronal functions in undifferentiated SH-

SY5Y, illustrating a possible use of this model to evaluate this specific aspect of neurotoxicity in the context of neurotoxicity screening.

Exposure of SH-SY5Y cells during the differentiation process or exposure of differentiated cells to neurotoxicants allows to measure specific endpoints of neurotoxicity such as neuritogenesis, synaptogenesis, neurotransmitter, and calcium homeostasis. Few studies among those included in this review (27 out of 163) describe exposure of SH-SY5Y cells to potential neurotoxicants during or after the differentiation process. These studies showed that organic pollutants of interest affect neuronal specific features such as neurite outgrowth, intracellular calcium levels, and neurotransmitter signaling.

Several studies performed in other models including integrated *in vivo* models and *in vitro* neuronal cell models, suggest that the results obtained with the SH-SY5Y cell line, are transposable and may be eventually helpful for regulatory purposes. For example, regarding the impact of pesticides on signaling pathways or the levels of biomarkers is also confirmed by several studies using rats, mouse cells (N2a), or chicken-derived neuronal cell cultures, including CPF and lipid peroxidation [91], deltamethrin and ROS production [119], avermectins and MDR activity [142], paraoxon and the activities of several mitochondrial complexes [101]. Beside pesticides, pollutants such as flame retardants (PBDE47, TOCP) also display similar effects *in vivo* (rats, hens) and in SH-SY5Y cells, on the activation of the apoptosis and autophagy pathways [179, 187]. Moreover, the caspase-3 dependent apoptosis induced by dieldrin, CPF, TCDD, and PFOS observed with undifferentiated SH-SY5Y was confirmed in many studies performed in more complex models such as dopaminergic cells in cultures (SN4741) and rodents' brains [215–219]. Altogether, these studies suggest that undifferentiated SH-SY5Y cells may represent a good model for studying neurotoxicity.

In addition to these non-neuronal specific endpoints, the effects of many of the environmental pollutants described in our review on neuronal endpoints were also observed in several animal models (neuronal cells in culture or *in vivo* models) such as rodents, hen, chicken as well as in human neuronal precursors. This is the case, for example, of chlorpyrifos, methamidophos, mipafox, paraoxon, trichlorfon, leptophos, dichlorvos, diazinon, endosulfan, lindane, TCDD and neonicotinoids on 1) the inhibition of AchE and/or NTE [65, 66, 110, 220–223]; 2) the expression and functions of neurotransmitter receptors such as AChR and GluR [133, 134, 224, 225]; or 3) the neurite arborization complexity [224, 226]. A comparison of SH-SY5Y cells to other neuronal cell models such as the precursor cells generating dopaminergic neurons, LUHMES (a human embryonic neuronal precursor cells that can be maintained as proliferating cells or differentiated to postmitotic neurons), indicates that SH-SY5Y cells could be considered as a consistent neuronal model [134].

We can conclude, based on this review of literature, that the SH-SY5Y cell model, especially in the differentiating or differentiated stage, is a relevant cell model to evaluate cellular neurotoxicity.

However, it is important to keep in mind the limitations of this model. The SH-SY5Y cell line derives from a tumor and therefore contains genetic peculiarities that can, potentially, deviate

the expected response of the cells to a determined insult. Additionally, cell lines do not allow to mimic microenvironment perturbations or to test integrative processes. Indeed, the nervous system is a highly complex structure composed of many cell types (neurons and glial cells) with multiple functions. The development and functioning of this system depend on general cell functions such as energy metabolism and calcium homeostasis, and specific processes such as electrical activity, synaptogenesis and interactions between neurons and glial cells. Among other limitations to keep in mind while working with undifferentiated SH-SY5Y cells is their glycolytic phenotype which differs significantly from the one observed with primary neuronal cells. This limitation seems to be partially overcome, using differentiated cells in which the mitochondrial function (respiration) appears to be increased [31]. Excitability of the cells should also be considered, depending on the studied endpoint: even if they show excitability to some neuroactive molecules such as ACh, undifferentiated SH-SY5Y cells lack excitability to other ones such as glutamate and NMDA [14, 15]. This lack of excitability may be attenuated in differentiated SH-SY5Y cells regarding their electrophysiological properties [41] and expression of neurotransmitter receptors such as glutamate [35, 227]. Moreover, the central nervous system is insulated from the bloodstream by the blood-brain barrier that limits the passage of compounds such as neurotoxicants between both blood and neural tissues. In this context, cell lines such as the SH-SY5Y cell line, cultured as monolayers should be considered as a part of a large set of complementary *in vitro* models that can be used for neurotoxicity evaluation including primary cultures, co-cultures, 3-dimensional systems, organotypic cultures, neural stem cells, and blood-brain barrier models [6, 228, 229]. All these models are featured by specific advantages and limitations that should be carefully considered in the context of the endpoints to investigate. Moreover, SH-SY5Y cells can be used as a part of blood-brain barrier models. As an example, in Balbuena et al. SH-SY5Y cells were used as the parenchyma layer in a comparative study of two blood-brain barrier *in vitro* models. Rat astrocytes and bovine or rat endothelial cells were used to mimic the blood-brain barrier in both sides of an insert cultured in wells with the SH-SY5Y cells at the bottom part of the culture system. The trespassing capability of malathion, malaoxon and lead of the two systems was tested [230].

Furthermore, usually, the *in vitro* experiments, such as those described in this review, often use a high concentration approach (in the µM or mM range), as compared to the expected brain levels of neurotoxicants, to better appraise the mechanistic basis of neurotoxicity. Many studied compounds display cytotoxic effects due to the use of very high concentrations stimulating cell death pathways, which cannot be reached *in vivo*. This is a classical approach in toxicology especially when the cells cannot be maintained for long periods of culture. In this review, we have summarized in tables the processes related to cellular toxicity while trying to highlight the ones related to neurodevelopment for example. Several of these latter issues use relatively low doses (e.g., PFASs). Moreover, cells are classically exposed to a single compound whereas, humans are traditionally exposed to mixtures of compounds (drugs, environmental pollutants). Finally, rather than an acute single exposure to a determined substance, humans are chronically exposed to a wide range of compounds sometimes over long time periods. It could be interesting to consider low concentrations corresponding to expected concentrations in the brain, longer treatments, and combined exposures to mimic more environmentally relevant scenari.

Humans are potentially exposed to thousands of industrial chemicals and evidence suggests that an increasing number of these compounds are neurotoxicants causing neurodevelopmental and/or neurodegenerative disorders. This pandemic of neurotoxicity and impaired neurodevelopment causes human, social and economic costs. Indeed, the share of chronic diseases and disorders (including neurobehavioral disorders) attributable to pollutants, endocrine disruptors, and contaminants, costs the European Union (EU) each year more than 157 billion euros, or approximately 1.23% of the EU's gross domestic product. For example, attention deficit hyperactivity disorder (ADHD) is among the most common neurodevelopmental disorders in childhood (5%). In this context, there is an urgent need to extend neurotoxicity testing to guide regulatory agencies and high-throughput methods are welcome. SH-SY5Y cells have recently been used, in classical monolayer cultures or as spheroids, in a quantitative high-throughput screening aimed to identify compounds able to inhibit AChE activity by using the Toxicology in the 21st Century (Tox21) 10K compound library [214]. This study illustrates the relevance of the SH-SY5Y cell line in high-throughput testing of neurotoxicants and "neuro-disruptors".

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Abbreviations

2,4-D: 2,4-dichlorophenoxyacetic acid; 3-MA: 3-methyladenine; 6-OHDA: 6-hydroxydopamine; A1254: aroclor 1254; ABC: ATP-binding cassette; ACh: acetylcholine ; AChE: acetylcholine esterase; AChR: acetylcholine receptor; AD: Alzheimer's disease; αHCH: alpha-hexachlorocyclohexane ; AhR: Aryl hydrocarbon receptor; AhRR: AhR repressor; AIFM2: apoptosis inducer factor mitochondria associated 2; AKT1: AKT serine/threonine kinase 1 ; AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AMPK: 5'-AMP-activated protein kinase; APAF1: apoptotic peptidase activating factor 1; APE1: apurinic/apyrimidinic endonuclease 1 ; APIl5: apoptosis inhibitor 5; APP: amyloid precursor protein ; ASLC1: achaete-scute family bHLH transcription factor 1; ATF-4: activating transcription factor 4 ; ATG: autophagy-related homolog; ATP: adenosine triphosphate; Aβ: amyloid beta; Bax: Bcl-2 associated X; BBzP: butyl benzyl phthalate ; Bcl-2: B-cell leukemia/lymphoma 2; Bcl-xL: B-cell lymphoma-extralarge; BDNF: brain derived neurotrophic factor ; BEX2: brain expressed X-linked 2; bHLH: basic helix loop helix; BNIP3: Bcl-2 interacting protein; BPA: bisphenol A; CAMK2A: calcium/calmodulin dependent protein kinase II alpha; CAMK2B: calcium/calmodulin dependent protein kinase II beta ; CASP: caspase; CAT: catalase; CCK-8: cholecystokinin-8; cGMP: cyclic guanosine monophosphate; CHOP: C/EBP homologous protein; CMP: cell membrane potential; CNS: central nervous system; COX-2: cyclooxygenase-2; CPF: chlorpyrifos; CREB: cyclic AMP-responsive element-binding protein; CRISPR/CAS9: clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9; CYP: cytochrome P450; DA: dopamine; DAT: dopamine transporter; dbcAMP: dibutyryl cyclic adenosine monophosphate; DCHP: dicyclohexyl phthalate; DDE: dichlorodiphenyl dichloroethylene; DDT: dichlorodiphenyltrichloroethane; DEHP: bis(2-ethylhexyl)phthalate; DEP: diethyl phthalate; DMEM: Dulbecco's modified Eagle's medium; DNA: deoxyribonucleic acid; DNA pol β: DNA polymerase beta ; DnBP: di(n-butyl) phthalate; DNMT1: DNA methyltransferase 1; DNT: developmental neurotoxicity; DOP: dioctyl phthalate; DOPAC: 3,4-dihydroxyphenylacetic acid; DTNB: 5,5'-dithio-bis-(2-nitrobenzoic acid), Ellman's reagent; E2: estradiol; EC: effective concentration; ED: effective dose; EHMT2: Euchromatic histone-lysine N-methyltransferase 2; eIF2a: eukaryotic translation initiation factor 2a ; ER: endoplasmic reticulum; ERα: estrogen receptor alpha; ERK1/2: extracellular signal-regulated kinases 1 and 2; EZH2: enhancer of zeste 2 polycomb repressive complex 2 subunit; f-actin: actin filaments; F12: Nutrient Mixture F-12; FADD: fas-associated protein with death domain; FAHFA: fatty acid esters of hydroxy fatty acids ; FBS: fetal bovine serum; FUNDC1: FUN14 domain containing 1; γ-HCH: Hexachlorocyclohexane; G-CSF: granulocyte-colony stimulating factor ; G9a: euchromatic histone lysine methyltransferase 2 ; GABA: gamma-aminobutyric acid; GAD65: glutamic acid decarboxylase 65-kilodalton isoform; GAP43: growth associated protein 43; GDNF: glial cell line-derived neurotrophic factor; GPx: glutathione peroxidase; GR: glutathione reductase; GRIN1: G protein-regulated inducer of neurite outgrowth 1; GRP78: glucose regulatory protein 78; GRP90: binding immunoglobulin protein 90; GSK3β: glycogen synthase kinase 3 β; has miR-181-5: microRNA 181; HAT1: histone acetyltransferase 1; HCB: hexachlorobenzene; HDAC3: histone deacetylase 3; HO-1: heme oxygenase 1; HTT: huntingtin; HVA: homovanillic acid ; IC: inhibitory concentration; ID: inhibitory dose; ID: DNA-binding protein inhibitor; IFIT2: interferon induced protein with tetratricopeptide repeats 2; IL-18: interleukin-18; IL-1β: interleukin-1 beta; iNOS: inducible NO synthase; IR: insulin receptor; IRE1-α: inositol requiring enzyme 1-α; IRP1: iron regulatory protein 1; JNK: c-jun N-terminal

kinase; LC: lethal concentration; LD: lethal dose; LC3-II: microtubule-associated protein 1A/1B-light chain 3; LDH: lactate dehydrogenase; LOEC: lowest observed effects concentration; MAP1LC3A: microtubule-associated protein 1 light chain 3 alpha ; MAP2: microtubule associated protein 2 ; MAPK: mitogen-activated protein kinase; MAPT: microtubule associated protein Tau; MBzP: monobenzyl phthalate; MCL1: myeloid cell leukemia 1; MDA: malondialdehyde; MDR: multidrug resistance protein; MDR1: multidrug resistance protein 1; MEHHP: mono(2-ethyl-5-hydroxylhexyl) phthalate ; MEHP: mono(2-ethylhexyl) phthalate; MEM: modified Eagle's medium; MeSH: Medical Subject Headings; MLKL: mixed lineage kinase domain like pseudokinase; MMP: matrix metallopeptidase; MnBP: monobenzyl phthalate; MP: methylparaben; MPP+: 1-methyl-4-phenylpyridinium; mTOR: mechanistic target of rapamycin kinase ; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium ; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MX1: myxovirus resistance 1 ; NAC: N-acetyl cysteine ; NCX: sodium-calcium plasma membrane exchanger ; NEFH: neurofilament heavy chain ; NEFL: neurofilament light chain protein; NeuN: neuronal nuclei antigen; NEUROD: neuronal differentiation ; NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells; NFKB1: nuclear factor kappa B subunit 1; NGF: nerve growth factor; NHANES: National Report of Human Exposure to Environmental Chemicals; NIX: NIP-3-Like Protein X or BCL2 Interacting Protein 3 Like (BNIP3L); NLRP3: NOD-like receptor family, pyrin domain containing 3; NMDA: N-methyl-D-aspartate; nNOS: neuronal nitric oxide synthase; NO: nitric oxide; NOL3: nucleolar protein 3; NQO1: NAD(P)H dehydrogenase (quinone) 1; Nrf2: nuclear factor erythroid 2-related factor 2; NSE: neuron specific enolase; NT: neurotoxicity; NTE: neuropathy target esterase; Ntrk2: neurotrophic tyrosine kinase receptor type 2; OC: organochlorine pesticide; OECD: Organisation for Economic Co-operation and Development ; OP: organophosphate pesticide; OPIDN: organophosphate-induced delayed neuropathy ; P53: tumor protein 53; PAH: polycyclic aromatic hydrocarbon; PARK2: Parkinson's disease protein 2/Parkin; PARP: poly(ADP-Ribose) polymerase ; PARP1: poly (ADP-ribose) polymerase 1 ; PBA: phenylbutyric acid ; PBB: polybrominated biphenyl; PBBK: physiologically based biokinetic; PBDE: polybrominated diphenyl ether; PCB: polychlorinated biphenyl; PCDD: polychlorinated dibenzodioxin; PCDF: polychlorinated dibenzofuran; PCNA: proliferating cell nuclear antigen; PD: Parkinson's disease; PFAS: perfluorinated alkylated substance; PFOA: perfluorooctanoic acid; PFOS: perfluorooctanesulfonic acid; PFR: phosphate flame retardant; PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PIK3C3: phosphatidylinositol 3-kinase catalytic subunit type 3; PINK1: PTN induced kinase; PNS: peripheral nervous system; poly I:C: polyinosinic:polycytidylic acid; PP2A: protein phosphatase 2 A; PPAR-γ: peroxisome proliferator-activated receptor gamma; RA: retinoic acid; RBFOX3: RNA binding fox-1 homolog 3; REST: repressor element 1-silencing transcription factor; RIPK1: receptor interacting serine/threonine 1 ; RIPK3: receptor interacting serine/threonine 3; RIZ1: retinoblastoma protein-interacting zinc finger protein; ROS: reactive oxygen specy/ies; RPMI: Roswell Park Memorial Institute Medium; RT-qPCR: reverse transcription-quantitative polymerase chain reaction; SAP97: synaptic associated protein 97; sAPPα: soluble amyloid precursor protein alpha ; SETD1A: SET domain containing (histone lysine methyltransferase) 1A; SETD8: SET domain containing (histone lysine methyltransferase) 8; SIRT1: sirtuin 1; Suv39h1: suppressor of variegation 3-9 homolog 1 (histone lysine methyltransferase) ; Sv2:

synaptic vesicle protein 2; SYCP2: synaptonemal complex protein 2; SYN1: synapsin-1 ; TCDD: 2, 3, 7, 8- tetrachlorodibenzo-p-dioxin; TDCPP: Tris (1,3-dichloro-2-propyl)phosphate; TEM: transmission electron microscopy; TGase: tissue transglutaminase; TH: tyrosine hydroxylase; Tim23: translocase of inner mitochondrial membrane 23; TIMP-2: tissue inhibitor of metalloproteinases 2; TNF-α: tumor necrosis factor-alpha; TOCP: tri-o-cresyl phosphate; Tom20: translocase of outer mitochondrial membrane 20; TPA: 12-O-tetradecancyl-phorbol-13-acetate ; TrkB: tropomyosin receptor kinase B; TUBB3: tubulin beta-III; UPR: unfolded protein response; VOCC: voltage-gated calcium channel ; WNT: Wnt family member ; ZNF244: zinc finger protein 224.

Figure legends

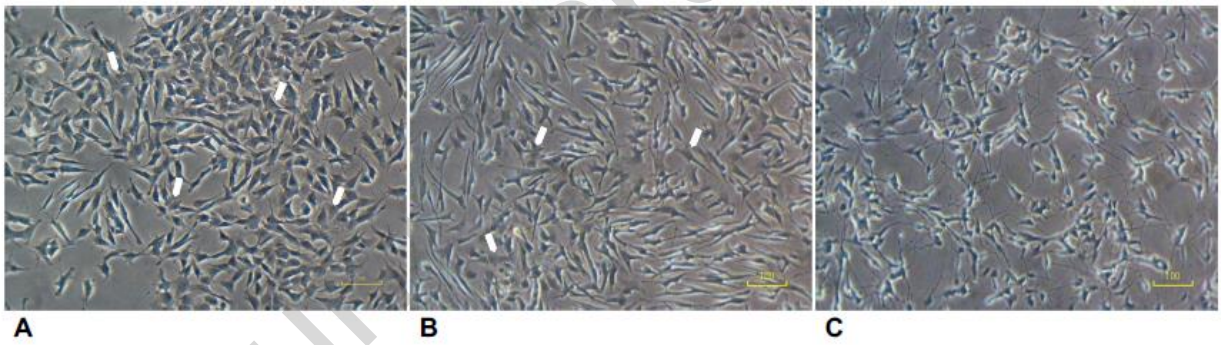

Figure 1. Undifferentiated and differentiated SH-SY5Y cells (phase-contrast microscopy). Despite SH-SY5Y cells are a third clone purified for neuron-like cells, some epithelial-like cells can be observed (white arrows) in undifferentiated cells (A). Treatment with retinoic acid (RA, 10 µM) for 5 days leads to differentiation of the cells, presenting longer processes (B). Epithelial-like cells are not affected by the postmitotic effects of RA and keep proliferating. 4 days treatment with brain-derived neurotrophic factor (BDNF, 50 ng/mL) in serum free medium after 5 days differentiation with RA results in polarized cells with long interconnected neurites. Cultures are selected for only neuron-like cells. Scale bars: 100 µm. Cultures and pictures were carried out by L. Lopez-Suarez.

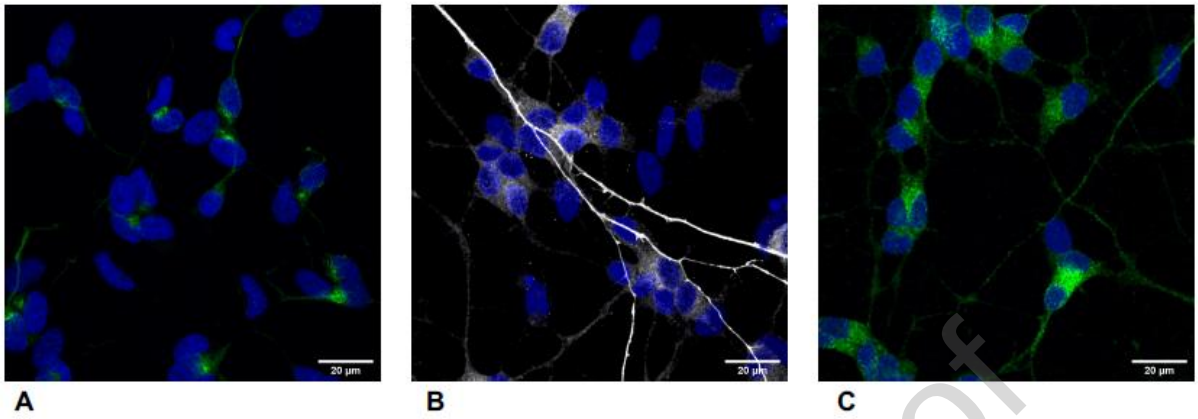

Figure 2. Immunocytochemistry staining of SH-SY5Y cells differentiated with retinoic acid and brain-derived neurotrophic factor. After the successive treatment of cells with retinoic acid (RA, 10 µM) for 5 days and brain-derived neurotrophic factor (BDNF, 50 ng/mL) for 4 more days, cells present mature neuron morphology, with well-developed dendrites and axons and neuron-specific cytoskeletal markers. A: neurofilament light chain (NEFL, green staining), B: Tau (grey staining), C: microtubule-associated protein 2 (MAP2, green staining), A-C: nuclei are stained by DAPI (blue staining). Scale bars: 20 µm. Cultures and pictures were carried out by L. Lopez-Suarez.

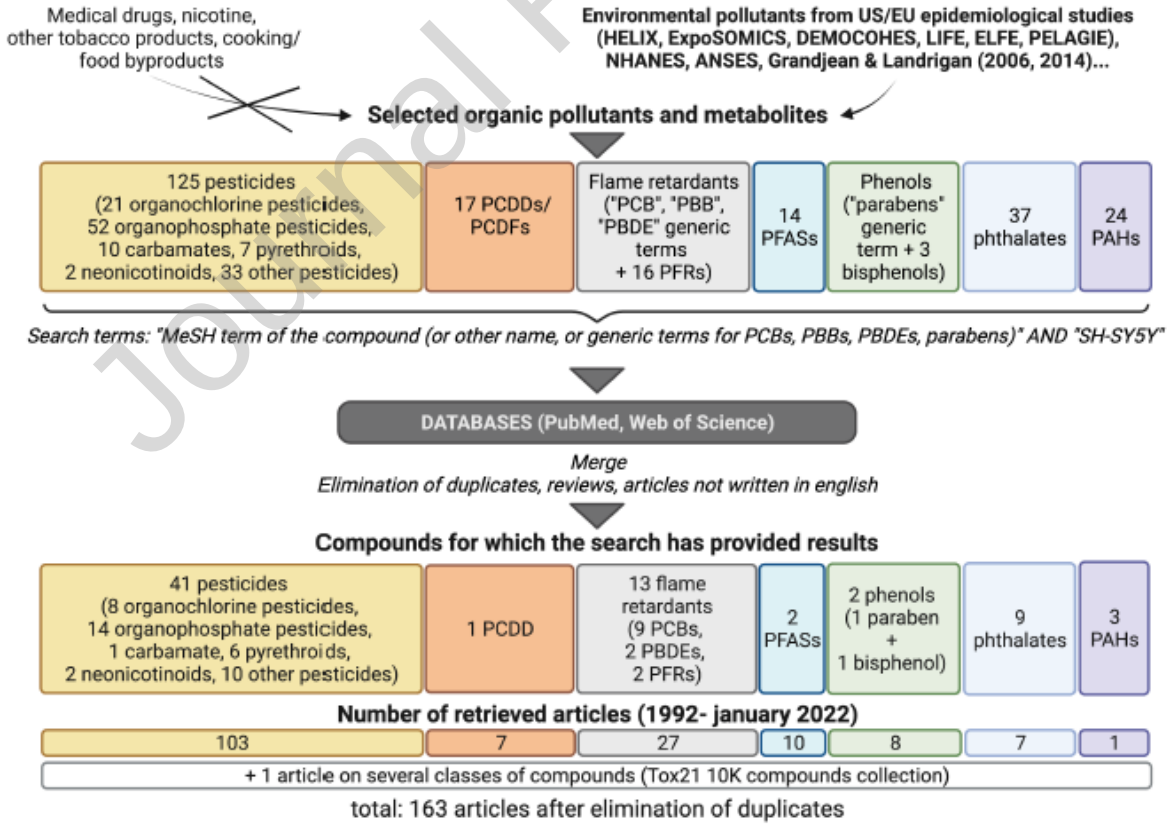

Figure 3. Methodology of the systematic research of studies including SH-SY5Y cells and selected organic pollutants. Organic pollutants and metabolites were selected based on several studies. 236 individual compounds as well as generic terms for compounds from seven classes of organic pollutants (pesticides, PCDDs/PCDFs, flame retardants, PFASs, phenols, phthalates, PAHs) were used as well as the “SH-SY5Y” term in PubMed and Web of Science databases. After a merge of the results obtained from the two databases and elimination of duplicates, reviews and articles not written in english, the search retrieved 163 articles (from 1992 to January 2022) for 71 organic pollutants. Created with BioRender.com.

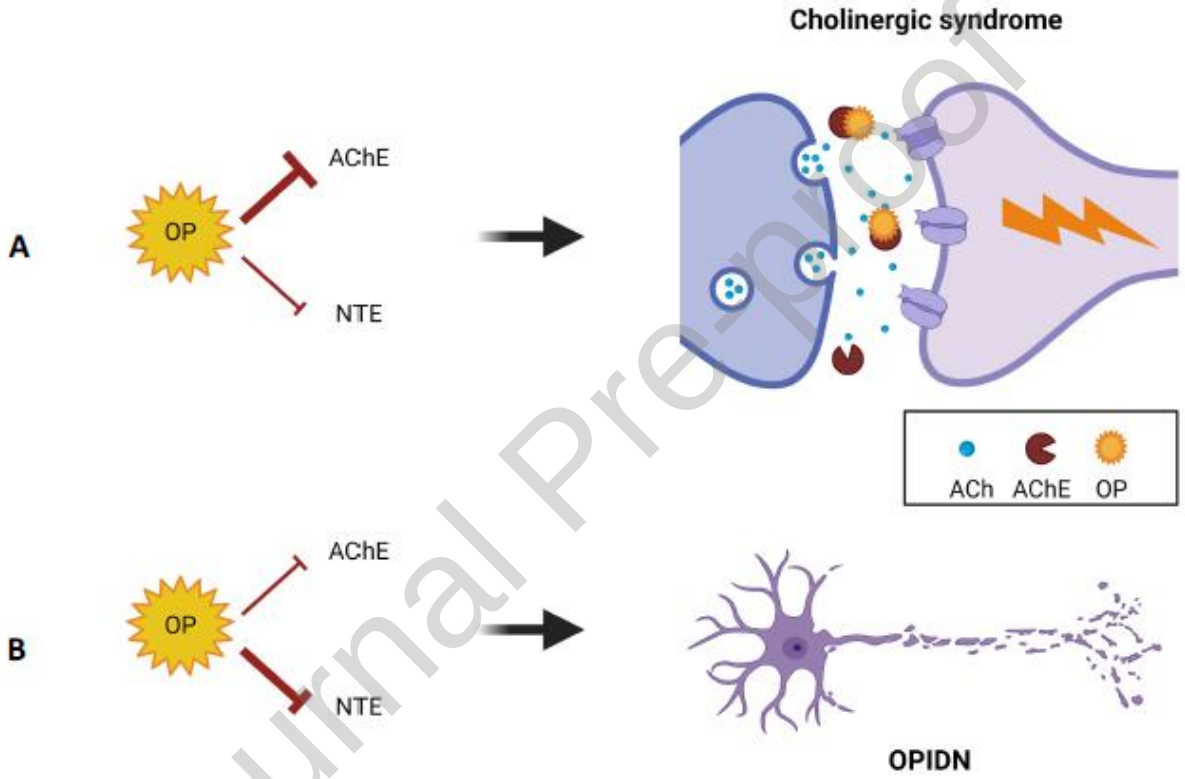

Figure 4. Effects of organophosphate pesticides on neuron esterases. Organophosphate pesticides (OP) can inhibit the activity of the neuronal esterases Acetylcholine (Ach) esterase (AChE) and neuropathy target esterase (NTE). Those compounds that strongly inhibit AChE cause the overstimulation of the cholinergic synapse since the AChE cannot effectively remove ACh from the synaptic cleft leading to cholinergic neurotoxicity (A). Those OPs that have a mild effect on AChE can cause organophosphate induced delayed neuropathy (OPIDN) by permanent inhibition of NTE leading to a delayed axonopathy and progressive associated ataxia (B). Created with BioRender.com.

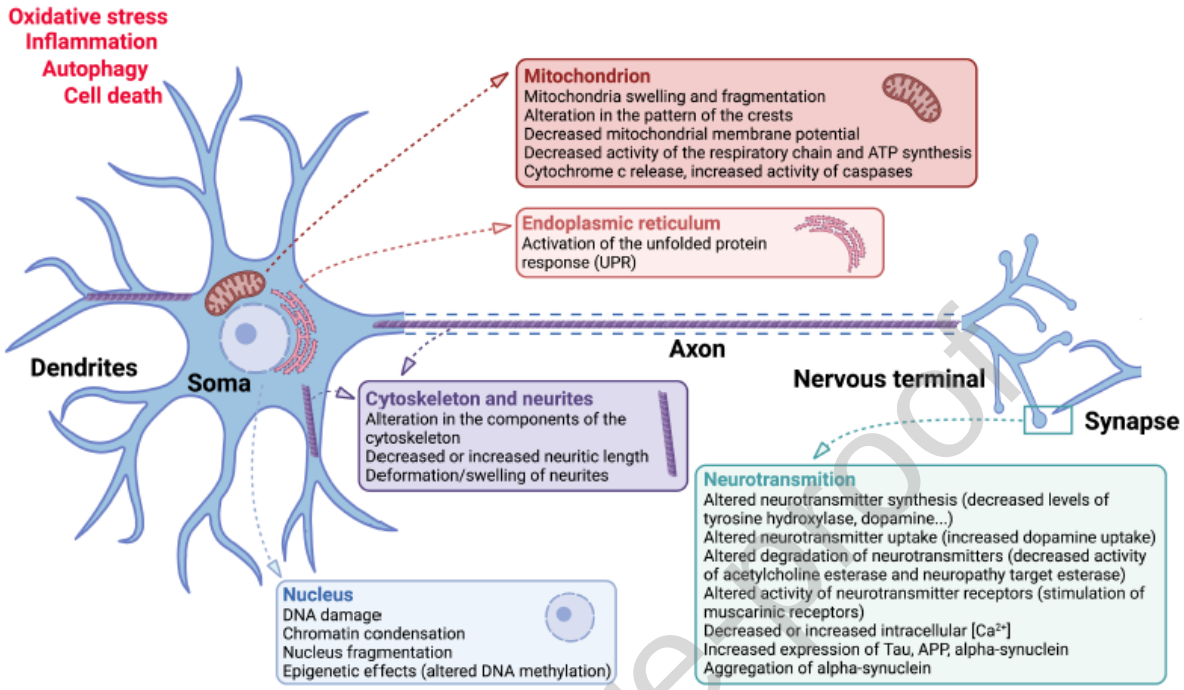

Figure 5. Summary of the main potential effects of organic pollutants on neuronal cells identified by using the SH-SY5Y model. The SH-SY5Y human neuroblastoma cell line either undifferentiated or differentiated into neuron-like cells allows the study of the mechanisms underlying the toxicity of chemical compounds on neuronal cells. The organic pollutants included in the review induce mainly oxidative stress, inflammation, autophagy and cell death (mainly apoptosis). They disturb mitochondrial morphology and function, affect endoplasmic reticulum, cytoskeleton, and neurites (dendrites, axons), nucleus, and impact several processes involved in neurotransmission. Created with BioRender.com.

Table 1. Organic pollutants and metabolites used in the bibliographic research in association with SH-SY5Y on PubMed and Web of Science databases (if they exist, MeSH terms are indicated, if MeSH term does not exist, the name is written in italic).

Pesticides	OC pesticides	Aldrin, chlordan, chlordecone, chlorothalonil, DDT (p,p'-DDT), dichlorodiphenyl dichloroethylene (p,p'-DDE), o,p'-DDT, dieldrin, endosulfan, endrin, heptachlor, hexachlorobenzene (HCB), α-hexachlorocyclohexane (α-HCH), β-hexachlorocyclohexane (β-HCH), hexachlorocyclohexane (Y-HCH, lindane), *isobenzan*, mirex, nonachlor, pentachlorophenol, propanil. **Metabolite:** oxychlordane.
	OP pesticides	Bensulide, bromofos, carbophenothion, chlorfenvinphos, *chlormephos*, chlorpyrifos, *chlorthion*, coumaphos, dialifor, diazinon, *dichlofenthion*, dichlorvos, dimefox, dimethoate, dioxathion, disulfoton, edifenphos, *endothion*, phenylphosphonothioic acid, 2-ethyl-2-(4-nitrophenyl) ester (EPN), ethion, ethoprop, fenitrothion, fensulfothion, fenthion, fonofos, formothion, fosetyl-al, heptenophos, *isoxathion*,

		leptophos, malathion, merphos, methamidophos, methidathion, methyl demeton, methyl parathion, mevinphos, mipafox, monocrotophos, naled, paraoxon, parathion (ethyl parathion), phorate, phosphamidon, phospholan, propaphos, *schradan*, sulprofos, *tebupirimphos*, terbufos, trichlorfon, *trichloronat*.
	Carbamates	Aldicarb, carbaryl, carbofuran, ethiofencarb, isolan, mancozeb, methomyl, mexacarbate, propoxur, thiram.
	Pyrethroids	Cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, fenvalerate, permethrin, 2,3,5,6-tetrafluoro-4-methylbenzyl (Z)-(1RS)-cis-3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclopropanecarboxylate (tefluthrin).
	Neonicoti--noids	Acetamiprid, imidacloprid.
	Other compounds	1,3-dichloropropene, 2-methyl-4-chlorophenoxyacetic acid (MCPA), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 4,6-dinitro-o-cresol (dinitrocresol, DNOC), acetochlor, aclonifen, alachlor, amitraz, amitrole, atrazine, abamectin, chloralose, chlormequat, chlortoluron, dinoseb, emamectin, fipronil, folpet, glyphosate, hexaconazole, isoproturon, metaldehyde, *metam zinc*, methyl bromide, methyldithiocarbamate, metolachlor, propiconazole, simazine, spiroxamine, tebuconazole, tetramethylenedisulfotetramine, trifluralin.
PCDDs & PCDFs	PCDDs	1,2,3,4,6,7,8-heptachlorodibenzodioxin (HpCDD), 1,2,3,4,7,8-hexachlorodibenzodioxin (HxCDD), 1,2,3,6,7,8-hexachlorodibenzodioxin (HxCDD), *1,2,3,7,8,9-hexachlorodibenzo-p-dioxin (HxCDD)*, *1,2,3,4,6,7,8,9-octachlorodibenzo-p-dioxin (OCDD)*, 1,2,3,7,8-pentachlorodibenzo-p-dioxin (PeCDD), *2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)*.
	PCDFs	1,2,3,4,6,7,8-heptachlorodibenzofuran (HpCDF), *1,2,3,4,7,8,9-heptachlorodibenzofuran (HpCDF)*, 1,2,3,4,7,8-hexachlorodibenzofuran (HxCDF), 1,2,3,6,7,8-hexachlorodibenzofuran (HxCDF), 1,2,3,7,8,9-hexachlorodibenzofuran (HxCDF), 2,3,4,6,7,8-hexachlorodibenzofuran (HxCDF), 1,2,3,4,6,7,8,9-octachlorodibenzofuran (OCDF), 1,2,3,7,8-pentachlorodibenzofuran (PeCDF), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF).
Flame retardants	PCBs	Polychlorinated biphenyls (PCBs) generic term.
	PBBs & PBDEs	Polybrominated biphenyls (PBBs), halogenated biphenyl ethers (polybrominated diphenyl ethers, PBDEs) generic terms**.**
	PFRs	Tri-(2-chloroisopropyl)phosphate (TCPP), *tris(2-chloroethyl)phosphate (TCEP)*, tris(1,3-dichloro-2-propyl)phosphate (TDCPP), tribenzyl phosphate (TBP), tri-o-cresyl phosphate (TOCP), *tri-p-cresylphosphate (TPCP)*, triphenyl phosphate (TMP). **Metabolites:** bis(1-chloro-2-propyl)phosphate (BCPP), *bis(2-chloroethyl)phosphate (BCEtP)*, bis(1,3-dichloro-2-propyl)phosphate (BDCPP), *dibenzyl phosphate (DBzP)*, di-n-butylphosphoric acid (DBuP), *di-o-cresylphosphate (DoCP)*, *di-p-cresylphosphate (DpCP)*, *diphenyl phosphate (DPhP)*, *2,3,4,5-tetrabromobenzoic*

		acid (TBBA).
PFASs		Perfluorobutanesulfonic acid (PFBS), perfluorododecanoic acid (PFDoA), perfluoro-n-heptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), branched perfluorooctanoic isomers (Sb-PFOA), perfluorononanoate (PFNA), perfluoromethylheptane sulfonic acid isomers (Sm-PFOS), perfluoroundecanoate (PFDeA), perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS), perfluorooctane sulfonamide (PFOSA), 2-(N-ethyl-perfluorooctane sulfonamido) acetate (Et-PFOSA-AcOH), 2-(N-methyl-perfluorooctane sulfonamido) acetate (Me-PFOSA-AcOH), perfluoroundecanoic acid (PFUnDA).
Phenols	Parabens	Parabens generic term.
	Bisphenols	Bisphenol A, bisphenol F, bis(4-hydroxyphenyl)sulfone (*bisphenol S*).
Phthalates		Butylbenzyl phthalate (BBzP), di-2-ethylhexyl terephthalate (DEHTP), *di-iso-nonyl phthalate (DiNP)*, dibutyl phthalate (DnBP), dicyclohexyl phthalate (DCHP), diethyl phthalate (DEP), diethylhexyl phthalate (DEHP and DOP), diisobutyl phthalate (DiBP), diisodecyl phthalate (DPP), diisononyl 1,2-cyclohexanedicarboxylic acid (DINCH), dimethyl phthalate (DMP). **Metabolites:** *cyclohexane-1,2-dicarboxylic acid mono carboxyisooctyl ester (MCOCH)*, *cyclohexane-1,2-dicaroxylic acid mono hydroxyisononyl ester (MHNCH)*, *mono [(2-carboxymethyl) hexyl] phthalate (MCMHP)*, *mono (3-carboxypropyl) phthalate (MCPP)*, mono-(2-ethylhexyl)phthalate (MEHP), *mono(2-ethyl-5-carboxyphentyl) phthalate (MECPP)*, *mono-2-ethyl-5-carboxypentyl terephthalate (MECPTP)*, *mono-2-ethyl-5-hydroxyhexyl terephthalate (MEHHTP)*, *mono-2-methyl-2-hydroxypropyl phthalate (MHiBP*), *mono-3-hydroxybutyl phthalate (MHBP)*, *mono-4-methyl-7-hydroxyoctyl phthalate (7-OHMMeOP*), *mono-4-methyl-7-oxoocyl phthalate (7oxo-MMeOP*), *mono-(carboxynonyl) phthalate (MCNP)*, *mono-(carboxyoctyl) phthalate (MCOP)*, mono-benzyl phthalate (MBzP), mono-isobutyl phthalate (MiBP), *mono-iso-nonyl phthalate (MiNP)*, mono-n-octyl phthalate (MNOP), *mono-oxoisononyl phthalate (MONP*), mono(2-ethyl-5-hydroxylhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), monobutyl phthalate (MnBP), *monocyclohexyl phthalate (MCHP)*, monoethyl phthalate (MEP), monomethyl phthalate (MMP).
PAHs		5-methyl chrysene, 1,12-benzoperylene, 1,2,5,6-dibenzanthracene, acenaphthene, acenaphthylene, anthracene, benz(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(c)fluorene, benzo(j)fluoranthene, benzo(k)fluoranthene, chrysene, cyclopenta(cd)pyrene, dibenzo(a,e)pyrene, dibenzo(a,h)pyrene, dibenzo(a,i)pyrene, dibenzo(a,l)pyrene, fluoranthene, fluorene, indeno[1,2,3-cd]pyrene, naphthalene, phenanthrene, pyrene.

Table 2. Studies that used differentiated SH-SY5Y cells in some of the endpoints studied. The concentration of the differentiating agent used, the duration of the differentiation treatment and its onset, when known, are listed as well as the associated references. BDNF: brain-derived growth factor; dbcAMP: dibutyryl cyclic adenosine monophosphate; NGF: nerve growth factor; RA: retinoic acid.

Differentiation conditions	**Associated references**
20 µM RA for 4 days	[63]
10 µM RA for 6 days, starting at 80% confluence	[190]
20 µM RA for 4 days	[185]
20 µM RA for 4 days, starting at 60-80% confluence	[64, 65]
10 µM RA for 4 days, starting at 60-80% confluence	[66]
10 µM RA for 3 days or 100 ng/mL NGF for 2 days, starting at 60-80% confluence	[58]
10 µM RA for 7 days	[59]
10 µM RA for 5 days, starting 24h after seeding	[59–61]
1 µM RA for 72 h, starting 24h after seeding	[51–53]
7 ng/mL NGF for up to 12 days, starting 24h after seeding	[57]
10 µM RA for 5 days followed by 50 mg/mL BDNF during 5 days	[30]
10 µM RA for 2 or 3 days	[159]
1 µM RA for 3 days, starting 24h after seeding	[134]
1 µg/mL NGF for 5 days	[110]
1 µg/mL NGF for 5-7 days	[111]
10 µM RA for 2 days, starting at 70-80% confluence	[105]
10 µM RA for 7 days, starting 24h after seeding	[62]
3 µM RA for 1 day or 10 µM RA for 4 or 8 days	[87]
10 µM RA for 6 days, starting 24h after seeding	[151, 189]

20 µM RA for ~~6~~5 days, starting 4 days after seeding	[74]
10 µM RA for 6 days followed of 10 µM RA, 50 ng/mL BDNF, 1x B-27 and 200 µM dbcAMP for 8 more days	[56]

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: