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Roles of NTE protein and encoding gene in development and neurodevelopmental toxicity

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ABSTRACT

Neuropathy Target Esterase (NTE) is a membrane protein codified by gene PNPLA6. NTE was initially discovered as a target of the so-called organophosphorus-induced delayed polyneuropathy triggered by the inhibition of the NTE-associated esterase center by neuropathic organophosphorus compounds (OPs). The physiological role of NTE might be related to membrane lipid homeostasis and seems to be involved in adult organisms in maintaining nervous system integrity. However, NTE is also involved in cell differentiation and embryonic development. NTE is expressed in embryonic and adult stem cells, and the silencing of *Pnpla6* by interference RNA in D3 mouse cells causes significant alterations in several genetic pathways related to respiratory tube and nervous system formation, and in vasculogenesis and angiogenesis. The silencing of gene PNPLA6 in human NT2 cells at the beginning of neurodifferentiation causes severe phenotypic alterations in neuron-like differentiated cells; e.g. reduced electrical activity and the virtual disappearance of markers of neural tissue, synapsis and glia. These phenotypic effects were not reproduced when NTE esterase activity was inhibited by neuropathic OP mipafox instead of being silenced at the genetic level. Neuropathic OP chlorpyrifos seems able to induce neurodevelopmental alterations in animals. However, the effects of chlorpyrifos in the expression of biomarker genes of differentiation in D3 cells differ considerably from the effects induced by *Pnpla6* silencing. In conclusion, available information suggests that PNPLA6 and/or the NTE protein play a role in early neurodifferentiation stages, although this role is not dependent upon the esterase NTE center. Therefore, impairments caused by OPs, such as chlorpyrifos, on neurodevelopment are not due to inhibition of NTE esterase enzymatic activity.

1. Introduction: neuropathy target esterase as a target of neurotoxicity and its role in maintaining the nervous system in adults

1.1. NTE as a target of organophosphorus delayed neurotoxicity

Neuropathy Target Esterase (NTE)[1,2] was first described in 1969 as the phosphorylable site where the organophosphorus compounds (OPs) inducers of a neurodegenerative syndrome called organophosphorus-induced delayed polyneuropathy (OPIDP) bind [1]. OPIDP is caused by OPs capable of inhibiting the esterase center of the protein and inducing a further non reversible change called “aging”, which involves loss of the inhibited enzyme's capability to be reactivated by nucleophilic chemicals [2,3].

Esterase activity has been associated with this phosphorylable site in NTE [4]. This esterase activity is usually measured with

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[1] Throughout the manuscript, the term NTE is always used to call the protein called neuropathy target esterase; the term NTE enzymatic activity is used to refer to the phenyl valerate esterase activity associated with NTE; *Pnpla6* and PNPLA6 are used to call the NTE-codifying genes in mouse and human, respectively.

[2] Abbreviations: CPS = Chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate); NT2 = Human embryonal carcinoma stem cells; NTE = Neuropathy Target Esterase; OP = Organophosphorus compound; OPIDP = Organophosphorus induced delayed polyneuropathy; PNPLA = Human patatin like phospholipase domain; PNPLA6 = Human patatin like phospholipase domain containing 6 gene (gene codifying for NTE in humans); Pnpla6 = Mouse patatin like phospholipase domain containing 6 gene (gene codifying for NTE in humans); PV = Phenyl valerate.

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phenylvalerate (PV) as a substrate [5]. However, PV is not a specific substrate of NTE because most assayed biological tissues display higher or lower capability to hydrolyze this carboxylester. This is the reason why NTE has to be discriminated and differentiated among the pool of esterases as the PV hydrolyzing activity that is resistant to nonneuropathic OP paraoxon and sensitive to neuropathic OP mipafox [6]. NTE is a member of a family of esterases called patatin-like phospholipase domain containing (PNPLA) and is codified by gene PNPLA6 [7]. There is high homology (50–80%) between human and avian PNPLA genes, which suggests the conservation of major enzymatic functions on a phylogenetic scale [8]. NTE is anchored to the endoplasmic reticulum with the esterase center facing the cytoplasmic part of the cell [9], and has been found to be expressed in several tissues: spinal cord, liver, kidney, placenta and spleen [10]. The highest NTE level has been usually considered in the brain [11], although even higher levels have been described in bovine adrenal medulla [12,13].

1.2. NTE phospholipase activity and role in nervous system maintenance

In addition to PV hydrolyzing activity, NTE is also able to hydrolyze membrane lipids, such as lysophospholipids or monoacylglycerols [14] and lysolecithin [15]. Experiments with yeasts and Neuro-2a and COS-7 cells have demonstrated that NTE and its associated lysophosphatidylcholine hydrolyzing activity play a critical role in lipid membrane homeostasis [16–18], and influence membrane fluidity [19].

Several experimental results have suggested that the physiological role of NTE is apparently related to nervous system integrity maintenance. Swiss cheese protein is a functional ortholog of mouse NTE. Drosophila flies deficient of this Swiss cheese protein show a progressive degeneration of the adult nervous system, glial hyperwrapping and neuronal apoptosis [20]. Another factor that favors NTE being involved in nervous system maintenance is the fact that its knockdown results in motor neuron defects in zebra fish [21]. The distal degeneration of the longest spinal axons in adult mutant mice that do not express NTE has been reported, which suggests that NTE is required for adult vertebrate axon maintenance [22]. Finally, this role of NTE seems conserved even for humans because gene mutations have been reported to cause motor neuron diseases [23].

Regardless of the above information on the role played by NTE in adult organisms, in the last 15 years other studies have suggested that NTE might also play an important role in embryonic development and cell differentiation. The present review attempts to sum up all the relevant findings, places special emphasis on the contributions of our group, and indicates a central role of NTE in development and neurodevelopment.

2. Role of NTE and encoding gene in development

2.1. NTE-deficient mice embryos

Winrow and coworkers [24] generated a transgenic mouse which expresses an NTE form that lacked the NTE esterase domain and two of the three cyclic nucleotide monophosphate-binding domains. They found that homozygous mice (which expressed muted NTE in both alleles) were not viable due to embryo lethality by day 9 of development, apparently caused by deficient closing in the neural tube. Heterozygous mice (which expressed one wild allele and one muted allele) were viable and expressed approximately 40% of wild animals' NTE esterase activity in the brain. Heterozygous animals were more sensitive to acute toxicity with octylphosphonofluoridate than wild animals, and presented a neurological phenotype of hyperactivity (mutant mice travelled a distance 1.5 times longer and experienced 4-fold more vertical events than wild animals) [24]. Such hyper-susceptibility and hyperactivity suggest that viable mutant animals develop an altered nervous system.

Another group also approached the challenge of clarifying the role of NTE in embryonic development by generating a mutant mice strain that expressed an NTE form truncated prior to the carboxyl-terminal catalytic domain by targeted mutagenesis in embryonic stem cells. The embryos that simultaneously carried a wild allele together with a mutant allele expressed around 50% of NTE esterasic activity, but underwent normal development [25]. However, those embryos that expressed two truncated alleles were not viable by day 9 of development, as previously reported by other authors [24]. These authors reported placental failure development and impaired vasculogenesis in yolk sacs and embryos by day 7.5 of development [25].

It is remarkable that the two above contributions have clearly contributed to establish a central role of NTE in embryo development through blood vessel formation [25], and respiratory tube and nervous system formation [24]. Yet neither of these studies has been supported by molecular approaches; indeed the molecular pathways in which NTE is involved still remain unknown. In this context, stem cells offer a good *in vitro* model to approach the discovery of molecular mechanisms of development. The following sections of this work present discoveries which help to clarify the role of NTE and its codifying gene in development and neurodevelopment.

2.2. NTE and Pnpla 6 expression in D3 mouse embryonic stem cells: role in early development

D3 mouse embryonic stem cells under spontaneous differentiation express PV esterase activities that are sensitive to paraoxon and resistant to mipafox [26]. Thus NTE activity could be assayed by using the same operative concept as that typically used for discriminating NTE among the pool of PV esterases found in different adult differentiated tissues. A sudden sharp peak of both NTE esterase activity and *Pnpla6* was recorded a few hours after differentiation began, which suggests that NTE and/or *Pnpla6* plays a critical role in early cellular differentiation stages [26].

In order to elucidate the relationships of *Pnpla6* with differentiation pathways, the gene was silenced using an interference-specific RNA in D3 cells just at the beginning of differentiation. Silencing did not alter cell viability and the aforementioned *Pnpla6* expression peak became a barely detectable increase of expression (*Pnpla6* expression was around 6-fold lower than the control cells at the critical point) [27]. The only gene altered after 24 h of silencing was the same *Pnpla6*. After 96 h of treatment, 545 genes presented an altered expression ($p < 0.05$) [27]. The analysis of these 545 genes revealed that they were involved mainly in the development of the nervous system, placenta development, vasculogenesis, membrane permeability and lipid homeostasis, including several biological processes (see Table 1) [27]. The analysis of the relationships and connectivity among the various altered genes showed that 38 of them had high interrelationships, and that myelocytomatosis oncogene (*Myc*), jun oncogene (*Jun*), B-cell leukemia/lymphoma 2 (*Bcl2*), intercellular adhesion molecule 1 (*Icam1*), vascular endothelial growth factor A (*Vegfa*) and interferon 1 (*Ifn1*) were the genes with the highest number of interrelationships [27].

In vivo experiments run with NTE knockout mice have reported defects in neural tube closing [24]. The microarray performed after *Pnpla6* silencing in D3 cells found impairments in vasculogenesis and respiratory tube development. All three alterations can be

Table 1
Biological process pathways altered 96 h after the silencing of gene PNPLA6 in D3 mouse embryonic stem cells and NT2 human embryonal carcinoma stem cells under differentiation. Gene PNPLA6 was silenced in both cases using specific interference RNAs at the beginning of differentiation. The whole transcriptome was analyzed 96 h after silencing by microarrays. Those genes with altered expression as regards the non-silenced control cells for at least $p < 0.05$ were analyzed using DAVID Bioinformatics Resources 6.7 to determine which pathways were altered by silencing. The Group Enrichment Score ranks the biological significance of the gene groups based on the overall EASE scores of all the enriched annotation terms. A higher score for a group indicates that group members were involved in more important (enriched) roles.

D3 cells*		NT2 cells**	
Biological process	Group enrichment score	Biological process	Group enrichment score
Respiratory tube development	3.88	Regulation of neurogenesis	0.85
Regulation of apoptosis	3.22	Regulation of cell proliferation	0.85
Regulation cell motion and migration	3.06	Epithelial tube morphogenesis	1.11
Regulation of vasculogenesis and angiogenesis	2.70	Positive regulation of developmental processes	1.11
Vesicle-mediated transport	2.28	Phospholipid metabolic process	1.52
Locomotors behavior, chemotaxis	2.09	Phosphorylation	0.98
Regulation of cell adhesion	2.03	Positive regulation of transcription	1.07
Neuron development	1.80	Regulation of protein complex assembly	1.09
Regulation developmental process	1.80	Cell-cell adhesion	1.24
		Rho protein signal transduction	1.46
		Cellular respiration	1.48
		Neurogenesis	1.56
		Actin cytoskeleton organization	1.85

*Data taken from Ref. [27]; **Data taken from Ref. [29].

joined according to a common factor: impairments in tubulogenesis. There are different models that explain tubulogenesis, but they all involve apical membrane biogenesis and vesicle coalescence and secretion [28], which are processes that involve strong cellular membrane-dependent interactions. At this point it is necessary to highlight that experiments run with D3 cells have also reported alterations in lipid homeostasis, regulation cell motion, migration and adhesion [27]. One possible mechanistic explanation for previously *in vivo* reported studies is depicted in Fig. 1. Loss of NTE by either *Pnpla6* silencing (*in vitro* experiments) or NTE truncation (*in vivo* experiments) causes alterations in lipid homeostasis which, in turn, causes tubulogenesis impairments (detected by microarrays experiments). Mice that do not express NTE (both truncated alleles) are not viable due to critical failures in vasculogenesis. Mice that express only one functional *Pnpla6* allele are viable, but with altered vasculogenesis, which would be responsible for the abnormal nervous system development observed in these animals (hyper-susceptible to acute toxicity of OPs and hyperactive animals).

2.3. NTE and PNPLA6 in NT2 human embryonal carcinoma stem cells

Human embryonal carcinoma stem cells (NT2) are able to differentiate after 9 weeks of culture in the presence of appropriate trophic factors in cells with neuronal morphology features; e.g. axons and dendrites, electrical activity, synapses, calcium channels and glia. This makes it a good model for studying alterations in neurodifferentiation.

The gene PNPLA6 was silenced with specific interference RNA in NT2 cells at the beginning of the differentiation. After 24 h, only four genes presented an altered expression (neurobeachin-like 1, neurogenin 1, NudC domain containing 2 and PNPLA6), and this number rose to 394 genes after 4 days of silencing [29]. The enrichment score revealed altered pathways, such as phospholipid metabolism, regulation of the developmental process and epithelial tube morphogenesis, and cytoskeleton organization (Table 1) [29]. It is remarkable that this silencing with another transcriptomic approach found common points between D3 and NT2 cellular lines, such as alterations in cell adhesion, developmental process and tube formation alterations (respiratory in D3 cells and epithelial in NT2 cells).

The PNPLA6 silencing of NT2 cells at the beginning of differentiation caused, apart from the transcriptomic alterations summarized in Table 1, major phenotypic alterations in culture with reductions of 87%, 55% and 67% in the number of differentiated neurons, the number of neurites and the branch points, respectively [29]. Electrical activity was also altered, with reductions of 79% and 44% in the number of spikes/min and their amplitude, respectively [29]. The immunostaining experiments also revealed

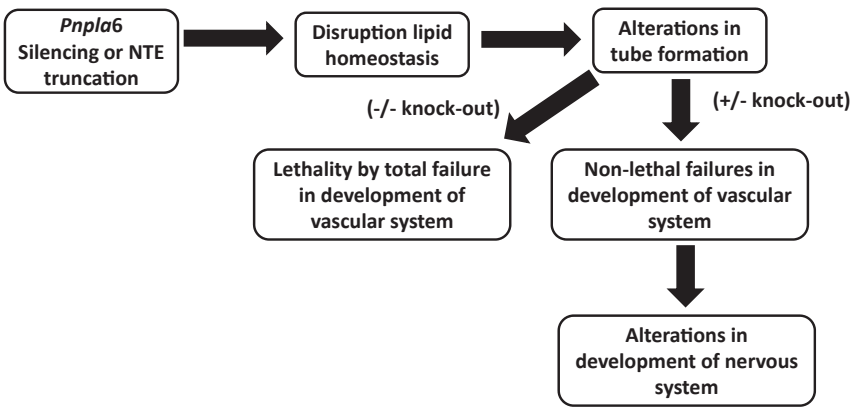

Fig. 1. A potential mechanistic explanation of the *in vivo* experiments with transgenic NTE-deficient mice.

Table 2
Neural markers recorded by immunohistochemistry in NT2-derived neurons after PNPLA6 silencing at two different time points of the differentiation process. A semi-quantitative estimation is offered where: 3 represents maximum staining; 2 represents medium staining; 1 represents barely detectable staining; 0 represents lack of staining. The control cells were not transfected, while the negative control represents a culture that was transfected with a nonspecific interference RNA, which should not alter the expression of any gene. Data were estimated using the immunochemical staining published in Ref. [29].

	Control	Negative control	Silencing performed on day:	
			Day 0	Day 35
Microtubule-associated protein 2 (Map 2)	3	3	0	1
Neurofilament heavy polypeptide (NF200)	3	3	1	2
β-tubulin III	3	3	0	2
Nestin	3	3	1	1
Glial fibrillary acidic protein (GFAP)	3	3	0	1
Synaptophysin	3	3	1	2

how the expression of neural markers drastically lowered, such as microtubule-associated protein 2, neurofilament heavy polypeptide and β-tubulin III, glial marker glial fibrillary acidic protein and synapsis marker synaptophysin (Table 2). However, the effect on the expression of these markers was not so drastic when silencing was performed on day 35 of neurodifferentiation (instead of day 0) because the staining used to record neurofilament heavy polypeptide, β-tubulin III and synaptophysin was lesser than that of the control, but was still significant (Table 2).

These experiments with NT2 cells demonstrated that NTE and/or PNPLA6 play a key role in early neurodifferentiation stages, although this role became less critical in later stages of the process. In this stage however, it was unclear whether esterase-related activity was responsible for this critical role, which is why we performed other studies using D3 and NT2 cells exposed to several OPs to study the effect of NTE inhibition on differentiation.

3. Role of NTE protein and encoding gene in neurodevelopmental toxicity

3.1. Effects of inhibition of NTE enzymatic activity by OPs on NT2 human embryonal carcinoma stem cells neurodifferentiation

Inhibition of NTE with the neuropathic OP mipafox at concentrations that did not alter cellular viability only significantly altered the expression of a long non-coding RNA, which caused no phenotypic alterations to the number of differentiated neurons, neurite length, the number of branch points, and the expression of neuronal, glial and synapsis markers, which were markedly altered by PNPLA6 silencing [30].

Yet despite the results obtained with mipafox, inhibition of other non-NTE PV esterases with the non-neuropathic OP paraoxon at concentrations that barely altered cell viability significantly altered the expression of 137 genes, mainly related to chromatin assembly and regulation, apoptosis or cell-cell signaling and differentiation [30]. No phenotypic feature was affected by paraoxon exposure during differentiation [30].

The above-stated experiments with NT2 cells suggest that the role of PNPLA6 and/or NTE in neurodifferentiation is not dependent on the esterase center, but other PV esterases might also be targets of neurodevelopmental toxicity.

3.2. Effects of chlorpyrifos on D3 mouse embryonic stem cells differentiation

Chlorpyrifos (CPS) is a neuropathic OP reported as being able to induce several neurodevelopmental effects in animals. Nonetheless, these effects have not been consistently documented in epidemiological terms. We previously reported how exposure to CPS in D3 cells under differentiation could cause similar alterations to those induced by valproic acid [31] (a chemical classically considered weak embryotoxicant) when we monitored alterations in differentiation by the expression of biomarker genes in a procedure proven affective for discriminating strong, weak and non-embryotoxicants [32].

Chronic exposure of D3 mouse embryonic stem cells under spontaneous differentiation to 20 μM CPS (a more than 6-fold lower concentration than the concentration that can reduce NTE enzymatic activity by 50%) caused statistically significant over-expressions of different biomarker genes; e.g. α-fetoprotein (11-fold); myosin heavy chain (3.5-fold); nanog homeobox (22-fold) and neurofilament medium polypeptide (29-fold) [33].

The above results suggest that CPS is able to alter the gene expression of D3 cells under differentiation through NTE inhibition. However, CPS can also inhibit other esterases apart from NTE, which is why we studied the effect of mipafox (a neuropathic OP that fails to alter the differentiation of NT2 cells [30]), paraoxon (a non-neuropathic OP that inhibits other PV esterases and that fails to alter the differentiation of NT2 cells, but can alter certain genetic pathways [30]) and CPS (a neuropathic OP that alters the gene expression of D3 cells and is suspected of being able to induce other neurodevelopmental alterations) on the expression of four different genes previously reported to present an altered expression after *Pnpla6* silencing in D3 cells.

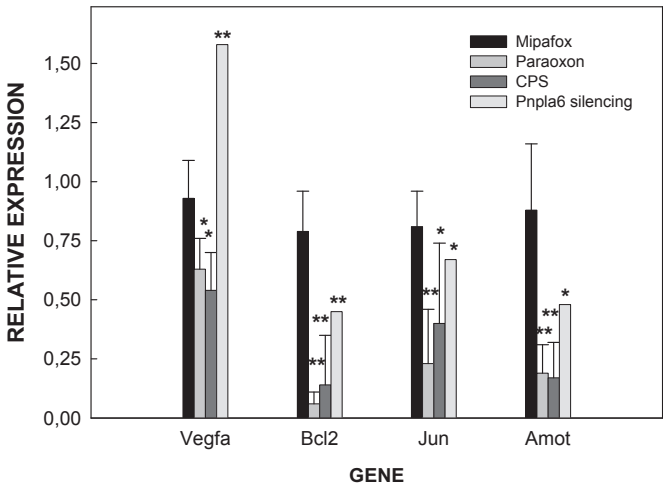

Fig. 2. Effect of different OPs on the expression of four different gene biomarkers of differentiation. The expression of the biomarker genes was tested after 3 days of chronic exposure of D3 cells under differentiation to 5 μM mipafox, 65 μM paraoxon and 50 μM CPS. Data were taken from Ref. [40]. Data on the effect of *Pnpla6* silencing were taken from Ref. [27]. Jun = jun oncogene; Amot = Angiomotin gene; Bcl2 = B-cell lymphoma-2-like 1 gene and Vegfa = vascular endothelial growth factor A gene. * = Statistically differed from the respective control for at least $p < 0.05$; ** = Statistically differed from the respective control for at least $p < 0.01$.

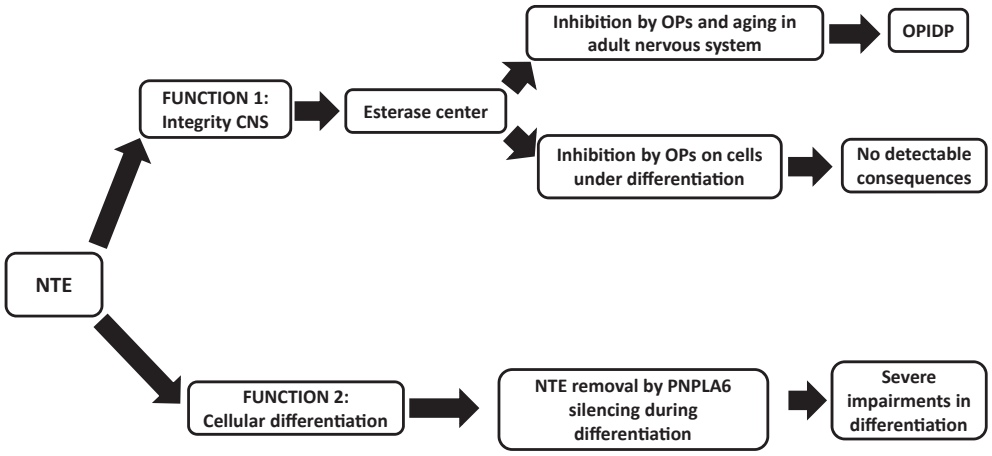

Fig. 3. Alterations of NTE and consequences.

Inhibition of NTE by mipafox and CPS gave quite different results because mipafox, consistently with previously published results with NT2 cells [30], did not alter the expression of the tested genes, previously reported as being altered by *Pnpla6* silencing (Fig. 2). Paraoxon had similar effects to CPS (Fig. 2).

4. Discussion

The overall conclusion of the results reviewed in this manuscript experimentally supports the hypothesis that NTE is able to perform two different biological functions (Fig. 3). One of these functions would be related with its esterase activity, which is needed for nervous system maintenance, as suggested by the fact that the inhibition of its enzymatic activity by OPs induces a neurodegenerative syndrome in adults (Fig. 3). The other function would be a role in early embryonic development, as suggested by the fact that depleting the expression of the NTE-codifying gene causes severe alterations in the cellular differentiation of D3 mouse embryonic stem cells, and also in the neurodifferentiation of NT2 human embryonal carcinoma stem cells (Fig. 3).

One possible question that arises from this double biological role of NTE is whether the NTE esterase center is also involved in development and if, consequently, it might be a target of the developmental toxicity induced by CPS and potentially by other OPs. The information reviewed in this work suggests that the esterase center of NTE is not involved in the developmental role of NTE because it was not possible to reproduce the differentiation impairments induced by PNPLA6 silencing [27,29] after NTE inhibition by CPS [Fig. 2] and mipafox [28].

This conclusion (NTE is involved in development, but not through the esterase center) leads to a new question, that might be, what is the target of toxicity of the differentiation impairments induced by CPS? OPs are able to inhibit a wide variety of esterases, and in the pool of esterases found in the nervous system we can distinguish among NTE (as the target of OPIDP), acetylcholinesterase (as the target of cholinergic OP-induced signs) and other carboxylesterases that possess PVase activity of still unknown functions, whose inhibition does not apparently trigger neurotoxicity. All these three esterases are also expressed in developing systems [26,30,33].

It has been previously proven that cellular systems, such as human neuroblastoma SH-SY5Y, are able to differentiate between neuropathic and non-neuropathic OPs on the basis of the differential inhibition of NTE and acetylcholinesterase [34,35], with the subsequent predictable and distinct neurological consequences in exposed subjects. Thus it might also be possible that stem cells, such as D3 and NT2, can suffer distinct phenotypic consequences when esterase activities are differentially inhibited by OPs.

NT2 cells cultured with retinoic acid (which was done in the works reviewed herein) differentiate into post-mitotic cells that have cholinergic properties [36]. This allows postulating acetylcholinesterase as the potential targets of the developmental toxicity induced by CPS. Indeed inhibition of acetylcholinesterase by CPS in these NT2 cells differentiated by retinoic acid led to a variation in the intracellular localization of acetylcholinesterase, with potential unwished effects on developing embryos [37].

Other authors have postulated that other esterases, apart from NTE and acetylcholinesterase and most of which with PV esterase activities, might be targets of other neurological syndromes associated with OP exposures, such as long-term neurobehavioral and neuropsychological symptoms, and potentiation of neuropathy, for which the target/s of toxicity is/are still unknown [38]. These authors claimed that the identification and characterization of the whole group of PVases targets of OPs (some of them probably with shared acetylcholine hydrolyzing activity [39], at least in chicken brains) are necessary to clarify the importance of these other targets in the neurotoxicity of OPs [38]. Therefore, on a parallel track, we also request the clarification of the role that these PVases play in development because they are potential targets of the differentiation impairments induced by CPS, and potentially by other OPs.

In conclusion, the available information allows the refusal of NTE as the target of developmental toxicity induced by CPS, suggests that acetylcholinesterase might be a target of this developmental toxicity, but does not allow the determination of whether the inhibition of other non NTE PVases is involved, either alone or in conjunction with acetylcholinesterase, in the afore-mentioned toxicity.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Transparency document

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