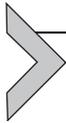Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Neuropathy target esterase (NTE/PNPLA6) and organophosphorus compound-induced delayed neurotoxicity (OPIDN)[☆]

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[☆]We dedicate this chapter to the memory of a cherished mentor, colleague, and friend—Dr. Martin Keith Johnson (1930–2018)—who discovered NTE and its connection to OPIDN (Lock et al., 2019).

1. Introduction

Systemic inhibition of a sufficient quantity of neuropathy target esterase (NTE) with certain organophosphorus (OP) compounds produces OP compound–induced delayed neurotoxicity (OPIDN), a distal degeneration of axons in the central nervous system (CNS) and peripheral nervous system (PNS), thereby providing a powerful model for studying a spectrum of neurodegenerative diseases (Richardson et al., 2013). Axonopathies are important medical entities in their own right (Watson and Dyck, 2015), but in addition, illnesses once considered primary neuronopathies are now thought to begin with axonal degeneration. These disorders include Alzheimer's disease (Liang et al., 2019; Stokin et al., 2005; Yuan et al., 2019),

Parkinson's disease (Burke and O'Malley, 2013; Pelzer et al., 2019; Tagliaferro et al., 2015), and motor neuron diseases such as amyotrophic lateral sclerosis (ALS) (Fischer et al., 2004; Moloney et al., 2014; Venkova et al., 2014). Moreover, conditional knockout of NTE in the mouse CNS produces vacuolation and other degenerative changes in large neurons in the hippocampus, thalamus, and cerebellum (Akassoglou et al., 2004), along with degeneration and swelling of axons in ascending and descending spinal cord tracts (Read et al., 2009). In humans, NTE mutations cause a variety of neurodegenerative conditions resulting in a range of deficits including spastic paraplegia and blindness (Hufnagel et al., 2015; Kmoch et al., 2015; Synofzik et al., 2014). Mutations in the *Drosophila* NTE orthologue SwissCheese (SWS) produce neurodegeneration characterized by vacuolization that can be partially rescued by expression of wild-type human NTE, suggesting a potential therapeutic approach for certain human neurological disorders (Sujkowski et al., 2015; Sunderhaus et al., 2019b).

This chapter defines NTE and OPIDN, presents an overview of OP compounds, provides a rationale for NTE research, and traces the history of discovery of NTE and its relationship to OPIDN. It then briefly describes subsequent studies of NTE, including practical applications of the assay; aspects of its domain structure, subcellular localization, and tissue expression; abnormalities associated with NTE mutations, knockdown, and conventional or conditional knockout; and hypothetical models to help guide future research on elucidating the role of NTE in OPIDN.
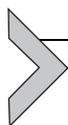
2. NTE definition: Gene, protein, and enzyme

Neuropathy target esterase (NTE), formerly called neurotoxic esterase (Johnson, 1970), is now also known as patatin-like phospholipase domain-containing protein 6 (PNPLA6), the sixth member of a nine-protein family of patatin domain lipid hydrolase proteins expressed in humans (Kienesberger et al., 2009).

NTE is encoded by the *PNPLA6* gene located on human chromosome 19p13.2 and containing 37 exons, giving rise to at least 4 splice variants of which the canonical protein sequence, UniProt isoform-4 or National Center for Biotechnology Information (NCBI) isoform-a, has 1375 amino acid residues. The *PNPLA6* gene is highly conserved evolutionarily, with orthologues in diverse species, including *Drosophila*, zebrafish, chicken, mouse, rat, and chimpanzee (NCBI, 2019; UniProt, 2019a).

The NTE protein is expressed ubiquitously in tissues and anchored in the endoplasmic reticulum (ER) of cells, with highest levels in lung, testis, spleen, brain, and kidney (NCBI, 2019). NTE has multiple roles in development, including neurite outgrowth and vasculogenesis (Moser et al., 2004). In the adult, the protein appears to be involved in axon maintenance in neurons (Read et al., 2009) and regulation of osmolarity in the kidney (Gallazzini et al., 2006).

Biochemically, NTE is a serine hydrolase with phospholipase B activity (UniProt, 2019a). As such, NTE possesses both phospholipase A1 and A2 activities, sequentially deacylating both acyl bonds of phosphatidylcholine (PtdCho), with cleavage of the *sn*-2 bond being rate-limiting, followed by rapid hydrolysis of the *sn*-1 bond to yield glycerophosphocholine (GroPCho) and 2 free fatty acids (FFA or RCOOH) (Glynn, 2013; van Tienhoven et al., 2002) (Fig. 1). Moreover, the enzymatic activity of NTE is enhanced in the presence of phospholipids—in particular, phosphatidylcholine (Atkins et al., 2002; Davis and Richardson, 1987; Pope and Padilla, 1989), and lysophospholipid hydrolysis by the catalytic domain of NTE in artificial bilayer membranes alters membrane fluidity (Greiner et al., 2010). Given its phospholipase capabilities and anchorage in the ER, it is likely that NTE plays a role in membrane lipid homeostasis (Zaccheo et al., 2004).
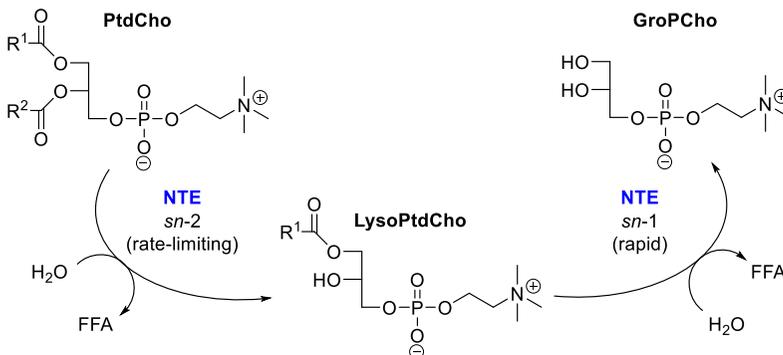
Fig. 1 Catalytic activity of NTE as a phospholipase B. The substrate, phosphatidylcholine (PtdCho), is sequentially hydrolyzed by NTE (shown in blue), first at the *sn*-2 position (rate-limiting) to yield lysophosphatidylcholine (LysoPtdCho) and a free fatty acid (FFA), followed by rapid cleavage at the *sn*-1 position to yield glycerophosphatidylcholine (GroPCho) and a FFA (Glynn, 2013; van Tienhoven et al., 2002). R^1 and R^2 represent the hydrocarbon chains of the FFA components of the phospholipid.
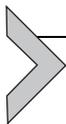
3. NTE and OPIDN

NTE was first identified by a systematic experimental search for the site of initiation of the delayed central–peripheral distal axonopathy (CPDA) brought about by sufficiently high single or repeated exposures to certain OP compounds—a syndrome called OPIDN (Abou-Donia, 1981; Carrington, 1989; Richardson et al., 2013; Wijeyesakere and Richardson, 2010). CPDA is a term used to describe the pattern of lesions found in OPIDN and a number of other neurodegenerative conditions, whereby distal degeneration of axons occurs in both the CNS and PNS (Moretto et al., 1987; Spencer and Schaumburg, 1977). OP compounds that can produce OPIDN are termed *neuropathic*.

Variations of the name ascribed to this chemically induced axonal degeneration include "organophosphorus," "organophosphorus ester," or "organophosphate" instead of "OP compound" and/or "neuropathy" instead of "neurotoxicity" (Abou-Donia, 1981; Cherniak, 1988; Emerick et al., 2010; Masoud and Sandhir, 2012). In addition, some authors favor "polyneuropathy" over "neuropathy" or "neurotoxicity," in which case the acronym becomes "OPIDP" (Jokanović et al., 2011, 2019; Lotti and Moretto, 2005; Monroy-Noyola et al., 2015).

In this chapter, we use OPIDN rather than OPIDP, and we understand the term to indicate "organophosphorus compound-induced delayed neurotoxicity" for two reasons. First, from a chemical standpoint, "organophosphorus" is the correct name for this generic class of chemicals. In contrast, "organophosphate" and "organophosphorus ester" refer to specific subclasses of OP compounds (Eto, 1974; IUPAC, 1978). Moreover, there are representatives from a number of other OP subclasses that can produce OPIDN (Richardson, 2010). Second, from a neurological perspective, "neuropathy" and "polyneuropathy" are rather imprecise terms that usually refer to pathology occurring in one or more peripheral nerves (Albers, 2005; Hutton et al., 2011; Karam and Dyck, 2015). However, OPIDN affects axons in ascending and descending spinal cord tracts as well as sensory and motor axons in peripheral nerves (Abou-Donia, 1981; Emerick et al., 2010; Richardson et al., 2013). Therefore, we regard "delayed neurotoxicity" within the context of exposure to neuropathic OP compounds as a better descriptor of this chemically produced condition than "delayed neuropathy" or "delayed polyneuropathy." The *delayed* aspect of OPIDN refers to a clinically quiescent period between exposure
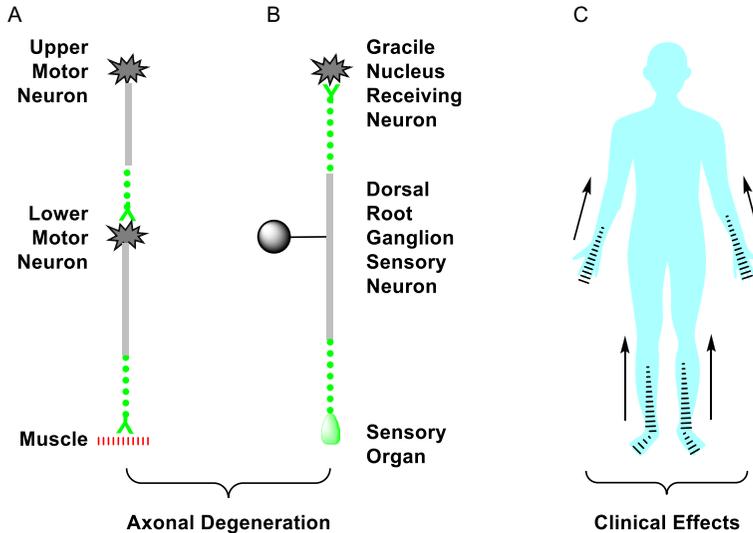
Fig. 2 Distribution of axonal lesions and sensorimotor deficits in OPIDN. (A) (Motor pathway). A descending axon from an upper motor neuron in the motor cortex of the brain passes along the corticospinal tract to synapse with a lower motor neuron in the anterior horn of the spinal cord. The lower motor neuron sends an axon through an anterior spinal root and peripheral nerve to synapse onto a muscle. (B) (Sensory pathway). A sensory neuron in the dorsal root ganglion sends the ascending branch of its bifurcated axon through a dorsal (posterior) column in the spinal cord to synapse with a receiving neuron in the gracile nucleus of the brainstem. The descending branch of the sensory neuron passes through a dorsal spinal root and peripheral nerve, terminating in a sensory organ, such as a Pacinian corpuscle (sensing changes in pressure) or a pain receptor. (C) (Clinical effects). Dashed lines depict the "stocking and glove" distribution of sensory effects (e.g., paresthesias) and motor deficits (e.g. paralysis); arrows indicate the spatial-temporal "dying back" progression from distal to proximal regions. Neurons and intact myelinated axons = gray; distal regions of degenerating axons = dotted green lines; green Y-shapes; sensory organ = green teardrop; muscle = dashed red rectangle (Abou-Donia, 1981; Bouldin and Cavanagh, 1979a,b; Davis and Richardson, 1980; Lotti and Moretto, 2005).

to a neuropathic OP compound and the appearance of clinical signs and symptoms. This hiatus is typically 8–14 days (Emerick et al., 2010), but periods of up to 4 weeks have been reported (Lotti and Moretto, 2005).

The motor and sensory pathways affected in OPIDN along with the distribution and progression of clinical signs and symptoms is shown schematically in Fig. 2. Motor effects include weakness and ataxia progressing to bilateral paralysis. Sensory manifestations comprise bilateral paresthesias such as numbness and tingling; in addition, cramping or lancinating muscle pain may be present. Histopathological findings reveal a primary distal

axonopathy with accumulation of tubulovesicular elements accompanied by secondary demyelination. Degeneration preferentially occurs in long, large-diameter axons in both sensory and motor modalities. Mild cases mainly affect the lower limbs, while more severe cases can also affect the trunk and upper limbs. The clinical course in humans consists initially of a stocking and glove distribution of sensory disturbances, attenuated reflexes, and flaccid paralysis. Gradually, some peripheral nerve regeneration occurs, but owing to the persistence of lesions in spinal cord tracts, hyperreflexia and spastic paralysis supervene. Electrophysiological findings over time are consistent with the clinical interpretation of CPDA followed by some degree of peripheral nerve regeneration accompanied by lingering central nervous system involvement (Abou-Donia, 1981; Bouldin and Cavanagh, 1979a,b; Davis and Richardson, 1980; Lotti and Moretto, 2005).

Animal studies and observations of human cases have revealed the following characteristics of OPIDN and its relationship to NTE (Carrington and Abou-Donia, 1984; Glynn, 2000; Johnson, 1982, 1988; Lotti and Moretto, 2005; Moretto et al., 1991; Richardson et al., 2013, 2020; Richardson and Makhaeva, 2014):

- Within a species, adults are vulnerable to OPIDN, while juveniles are resistant.
- Within a species, adults recover incompletely from OPIDN, while juveniles tend to recover completely.
- Within a species, OPIDN preferentially affects long, large-diameter axons in spinal cord and peripheral nerves.
- Within a species, the clinical course begins with paresthesias progressing to weakness, ataxia, hyporeflexia, and flaccid paralysis; mild cases mainly affect the lower limbs; severe cases affect lower limbs, upper limbs, trunk, and the left recurrent laryngeal nerve (which is longer than the right recurrent laryngeal nerve). Over time, some peripheral nerves regenerate while spinal lesions persist resulting in hyperreflexia and spasticity.
- Among species, large animals (e.g., humans, cattle, water buffaloes, sheep, pigs, cats, chickens) tend to be more susceptible to OPIDN than small animals (e.g., rats, mice, gerbils, guinea pigs).
- Among species, large animals tend to have higher NTE specific activity than small animals.
- Among species, those resistant to OPIDN tend to have higher NTE turnover rates than those susceptible to OPIDN.
- A threshold of >70% inhibition of nervous system NTE is necessary but not sufficient for OPIDN to ensue.

- Inhibited NTE must also undergo "aging" (loss of a secondary leaving group from the OP moiety, yielding a negatively charged organophosphyl group covalently attached to the active-site serine) in order for OPIDN to occur.
- NTE inhibition in brain has been used as a convenient surrogate for NTE inhibition in spinal cord or peripheral nerve.
- The animal model of choice for OPIDN studies is the adult hen.
- In hens, clinical signs of OPIDN are delayed by ∼8 days from the time of achieving >70% inhibition of brain NTE following a single or repeated dose of a neuropathic OP compound.

Table 1 summarizes chemical and genomic manipulations of NTE and their outcomes in various species. The most enigmatic and controversial aspect of NTE and its presumptive role in OPIDN is the apparent requirement for aging of the inhibited enzyme as the initiating event in the axonopathy. This theory proposes that inhibition of the catalytic activity of NTE is not enough to precipitate OPIDN and that a suitable chemical modification of the protein is also required. By this criterion, OPIDN results from combining a loss of physiological function with a gain of pathological/toxic function in NTE rather than arising from a loss of its physiological function alone. Decades of research focused on OPIDN have led toxicologists to this interim conclusion (Glynn, 2000; Richardson et al., 2013). On the other hand, as we shall see, recent studies directed toward understanding the physiological and pathogenic functions of NTE/PNPLA6 have challenged the gain of toxic function hypothesis, favoring instead the seemingly simpler idea that OPIDN is due to prolonged inactivation of the catalytic activity of the protein (loss of physiological function) (Glynn, 2007; Read et al., 2010). As we seek explanations that might reconcile the toxicological and genetic findings, we should keep two important facts in mind: (1) no drug or toxicant acts exclusively on a single target (Talevi, 2015), and (2) genetic or chemical manipulation of a given target elicits compensatory mechanisms (Schenone et al., 2013).
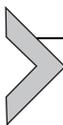
4. Overview of OP compounds

NTE was discovered using a chemical approach. This could not have been done without prior knowledge of the chemistry of OP compounds and some aspects of their biochemistry and toxicology. Therefore, a brief overview of these materials is in order.

Table 1 Summary of chemical and genetic manipulations of NTE and their outcomes.

Conditions	Species	NTE Status	Results	Selected References
Nonaging inhibitor—acute or chronic doses in adult	Hen	Inhibited	Loss of catalytic function	Baker et al. (1980)
	Cat		No gain of toxic function	Carrington (1989)
	Rat		No OPIDN	Johnson (1975c)
	Mouse		Protection	Richardson et al. (2013, 2020) Veronesi and Padilla (1985)
Aging inhibitor—acute or chronic doses in adult	Human	Inhibited + aged	Loss of catalytic function	Davis and Richardson (1980)
	Hen		Potential gain of toxic function	Howland et al. (1980)
	Cat		OPIDN (distal axonopathy in PNS and spinal cord)	Johnson (1990)
	Rat			Lapadula et al. (1985)
	Mouse			Richardson et al. (2013, 2020)
	+Others			Veronesi et al. (1991)
Mutation(s)—chronic throughout lifespan	Human	Mutated	Variable loss of catalytic function	Hein et al. (2010a,b) Hufnagel et al. (2015)
			Potential gain of toxic function	Kmoch et al. (2015) Rainier et al. (2008)
	Drosophila			Sunderhaus et al. (2019b)
			Variable types and severity of diseases	Topaloglu et al. (2014)

Continued

Table 1 Summary of chemical and genetic manipulations of NTE and their outcomes.—cont'd

Conditions	Species	NTE Status	Results	Selected References
Conventional KO (−/−) – chronic from embryonic day 0	Mouse	100% embryonic deletion	Lethality after embryonic day 7.5 due to placental failure and disrupted vasculogenesis	Moser et al. (2004) Winrow et al. (2003)
Conventional KO (−/+)—chronic from embryonic day 0	Mouse	~50% embryonic deletion	50% loss of catalytic function No gain in toxic function No apparent ill effects	Moser et al. (2004) Winrow et al. (2003)
Conditional KO (CNS)—chronic from embryonic day 11	Mouse	100% embryonic CNS deletion	100% loss of catalytic function No gain in toxic function Neurodegeneration in brain; distal axonopathy in spinal cord	Akassoglou et al. (2004) Read et al. (2009)
Knockdown (silencing)—chronic from 10h postfertilization	Zebrafish	Variable depression of NTE expression	Various developmental abnormalities including motor neuron defects	Song et al. (2013)

We tend to think of OP compounds as synthetic products of chemical, agrochemical, or pharmaceutical industry, but many of the most important natural constituents of life are OP compounds, including DNA, RNA, phospholipids in cellular membranes, and a number of important enzyme cofactors and metabolic intermediates (Berg et al., 2012). There are even naturally occurring OP compounds found in certain bacteria and marine sponges that exhibit high potency as acetylcholinesterase (AChE) inhibitors with consequent insecticidal activity (Neumann and Peter, 1987; Petroianu, 2012). Moreover, in the broadest chemical sense, OP compounds are either naturally occurring or synthetic organic molecules containing trivalent or pentavalent phosphorus. However, this chapter focuses on

synthetic substances containing pentavalent phosphorus, as these constitute the vast majority of exogenous OP compounds to which most people are likely to be exposed. In addition, the term "organophosphyl" is used to indicate a generic OP group attached to another entity such as the active site of an esterase, in recognition of the fact that the OP compound could be from any of a number of subclasses, e.g., organophosphate, organophosphonate, organophosphinate, or organophosphoramidate. Likewise, "organophosphylation" is used to indicate the generic process of adding an OP group to a protein.

Examples of some pentacovalent OP compounds are shown in Fig. 3. Note that the phosphorus atom is tetrahedral; consequently, it can be chiral, giving rise to optical isomers. For example, the nerve agent soman (GD; 3,3-dimethylbutan-2-yl methylphosphonofluoridate) has a chiral phosphorus and a chiral carbon, so that there are four possible stereoisomers. The P_SC_S isomer of soman has the highest inhibitory potency against AChE (Ordentlich et al., 1999; Sanson et al., 2009). Diisopropylphosphorofluoridate (DFP) and its analogue, N,N'-diisopropylphosphorodiamidic fluoride (mipafox; MIP) are aging inhibitors of NTE and can produce OPIDN. Diethyl 4-nitrophenyl phosphate
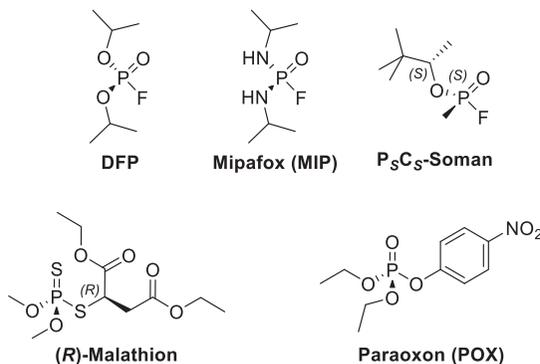
Fig. 3 Examples of pentacovalent OP compounds. The pentacovalent phosphorus atom is tetrahedral; consequently, it can be chiral, yielding optical isomers. Soman has a chiral phosphorus and a chiral carbon, yielding four stereoisomers; the most potent anti-AChE isomer (P_SC_S) is shown. Malathion has a chiral carbon; the (R)-isomer is shown, but the commercial insecticide is supplied as the racemic mixture. DFP and MIP are analogues; both are aging inhibitors of NTE and are neuropathic. Paraoxon (POX) is the active metabolite of the insecticide parathion; it is not an effective inhibitor of NTE and is not neuropathic. Mipafox (MIP) is employed in the NTE assay as the selective neuropathic inhibitor of NTE, whereas POX is used as the nonneuropathic inhibitor (Fig. 11) (Jianmongkol et al., 1996; Johnson, 1977; Kayyali et al., 1991; Richardson et al., 2020).

(paraoxon) is the active metabolite of the insecticide, diethyl 4-nitrophenyl phosphorothioate (parathion; POX); it is a potent anti–AChE compound but not an effective inhibitor of NTE, and it is not neuropathic (Lotti and Johnson, 1978). Diethyl 2-[(dimethoxyphosphorothioyl)sulfanyl]butanedioate (malathion) is a widely used insecticide that requires metabolic activation from the parent P=S (thion) form to the active P=O (oxon) form in order to be an effective inhibitor of AChE. Malathion has a chiral carbon; the (R)-isomer is shown here, but the commercial insecticide is supplied as the racemic mixture. Neither malathion nor its active metabolite, diethyl 2-(dimethoxyphosphorylsulfanyl)butanedioate, (malaoxon) is an NTE inhibitor and neither compound is neuropathic (Jianmongkol et al., 1996; Lotti and Moretto, 2005).

4.1 OP compounds: Uses and exposures

OP compounds are employed in myriad forms as flame retardants, fuel additives, hydraulic fluids, lubricants, pesticides, pharmaceuticals, and plasticizers (Davis and Richardson, 1980; Richardson et al., 2020). However, they are perhaps best known in the US for their use as insecticides in domestic and agricultural applications (CDC, 2019a). OP insecticides are also recognized throughout the world for the part they play in the control of vector-borne diseases such as African trypanosomiasis, Chagas disease, dengue, leishmaniasis, lymphatic filariasis, and malaria (WHO, 2011). Recently, OP insecticides have been pressed into increased service as a component of integrated vector management amid growing concern over the spread of mosquito-borne viruses such as Eastern Equine Encephalitis, West Nile, and Zika (CDC, 2019b; USEPA, 2019; WHO, 2016).

A consequence of widespread use of OP compounds is that some degree of exposure is inevitable throughout the population, thus prompting the need to understand potential adverse health outcomes so that proper risk assessments can be done and weighed against the societal benefits of using these products. Episodically increased exposures through such well-intended measures as vector control can generate controversy and alarm (Reuters, 2016), heightening the need for trustworthy data on health effects that can be effectively communicated to populations potentially at risk. Public apprehension surrounding exposures to OP compounds is understandable in view of the notoriety of highly toxic OP nerve agents such as (RS)-propan-2-yl methylphosphonofluoridate (GD; sarin), which have

been used as chemical weapons in a number of conflicts (Jamal, 1995; Page, 2003), reportedly including the Syrian civil war (Brooks et al., 2018; John et al., 2018). Moreover, these compounds have been used against civilian populations as agents of chemical terrorism (Nozaki et al., 1995; Okamura et al., 1996; Suzuki et al., 1995; Worek et al., 2016), and they continue to pose such a threat (Richardson et al., 2020). However, it is reassuring to note that nonoccupational exposures to low-level environmental levels of OP insecticides have not thus far been conclusively linked to adverse neurological outcomes in humans (Reiss et al., 2015).

4.2 Interactions of OP inhibitors with serine esterases

Fig. 4 shows the interactions of a generic OP inhibitor of serine esterases with a target enzyme. Except for the formation of an aged enzyme, the steps in the process are analogous to hydrolysis of a carboxylic acid ester substrate. The initial interaction is reversible, whereby the inhibitor forms a Michaelis-type complex with the enzyme, shown in blue as "EOH" to emphasize the
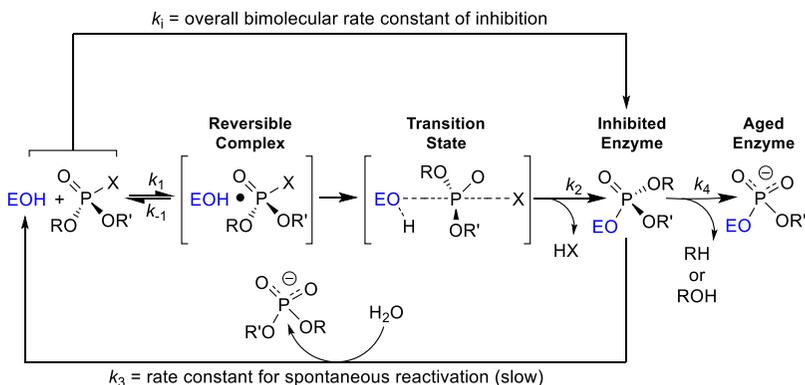
Fig. 4 Interactions of an OP inhibitor with a serine esterase. The esterase (blue) is depicted as EOH to emphasize the active site serine hydroxyl group that engages in a nucleophilic attack on the electropositive phosphorus atom, displacing the leaving group, X, and becoming organophosphylated. Kinetic rate constants: k_1, k_{-1} = formation and dissociation, respectively, of the reversible OP-EOH complex; k_2 = phosphylation; k_3 = spontaneous reactivation; k_4 = aging; k_i = overall bimolecular rate constant of inhibition. The tetrahedral OP inhibitor progresses through a trigonal bipyramidal transition state, which collapses to a tetrahedral species covalently attached to the active site serine residue, yielding inhibited enzyme, which can undergo spontaneous reactivation or aging. The aged enzyme is intractable to reactivation, even by powerful nucleophiles such as oximes (Richardson, 1992; Richardson et al., 2020). R and R' are substituted or unsubstituted alkyl or aryl groups that can be the same or different. X is a primary leaving group such as fluorine or 4-nitrophenoxyl.

serine hydroxyl nucleophile that attacks the electropositive phosphorus atom in the OP compound. The rate constants k_1 and k_{-1} indicate the forward and reverse reactions of formation and dissociation, respectively, of the reversible complex (Richardson, 1992; Richardson et al., 2020).

On its way to covalently inhibiting the enzyme, the OP compound morphs from a tetrahedral geometry to a trigonal bipyramidal transition state. Covalent acylation (phosphylation) occurs as the leaving group, X, is rapidly displaced while the transition state collapses back to a tetrahedral geometry; this step is characterized by the rate constant of phosphylation, k_2, and yields the covalently inhibited enzyme. If the OP inhibitor were chiral, in the absence of pseudorotation, inhibition would be expected to result in inversion of chirality at phosphorus (Eto, 1997), although the (R) or (S) designation could change depending on the assignment of group priorities according to the Cahn-Ingold-Prelog (CIP) rules (Carbonell et al., 2013; Epstein et al., 2009).

The dissociation constant of the reversible EOH-OP complex can be approximated by Eq. (1).

$$K_d \cong (k_{-1}/k_1) \tag{1}$$

This simplification is justified for most OP inhibitors of esterases because k_3 and k_4 are often orders of magnitude smaller than k_2, and k_2 is usually much smaller than k_{-1}. K_d is a Michaelis-type equilibrium constant in molar units that represents the concentration required to achieve 50% of the maximum rate of production of the acylated (phosphylated) enzyme (see Fig. 6 below). K_d is also regarded as a measure of the affinity of an OP inhibitor for binding to the active site of the enzyme. Thus, low values of K_d correspond to high affinity, and high values of K_d correspond to low affinity (Aldridge and Reiner, 1972; Main, 1980; Richardson et al., 2020).

The overall progress of the reaction from enzyme (EOH) and inhibitor (AX) to phosphylated (inhibited) enzyme (EOA) with expulsion of the primary leaving group (X) is characterized by the bimolecular rate constant of inhibition, k_i. This important measure of inhibitory potency is determined by measuring the activity remaining as a function of time of preincubation of the enzyme with various concentrations of inhibitor, where $[AX] > 10$ $[EOH]$. The ester substrate is added after the preincubation interval for a further incubation period to determine the residual activity (Richardson, 1992; Richardson et al., 2020). When $[AX] << K_d$, k_i is given by Eq. (2):

$$k_i = k_2/K_d \tag{2}$$

It is important to realize that k_i is a composite of the acylation (phosphylation) rate constant with units of min^{-1} and the dissociation equilibrium constant, K_d, with units of M. Therefore, k_i has the units of a second-order (bimolecular) rate constant, $M^{-1} min^{-1}$.

It is straightforward to determine k_i when pseudo-first-order kinetics is obtained. These conditions commonly occur when the concentration of the Michaelis-type reversible EOH-AX complex is low, k_2 is high, $k_3 << k_2$, and $[AX] > 10[EOH]$ (Richardson, 1992; Richardson et al., 2020). In this event, Eq. (3) applies:

$$\ln(v/v_0) = k_2[AX]t/([AX] + K_d) \qquad (3)$$

where v is the velocity (i.e., rate) of enzymatic hydrolysis of its ester substrate at time $= t$, and v_0 is the rate at time zero. Substituting (% *activity*/100) for (v/v_0), letting $k' = k_2[AX]/([AX] + K_d)$, and rearranging yields Eq. (4):

$$\ln(\%activity) = -k't + \ln(100) \qquad (4)$$

Accordingly, plots of ln(% *activity*) versus t will be straight lines with slopes $= -k'$ and y-intercept $= \ln(100) \approx 4.605$, as shown in Fig. 5A. These are the primary kinetic plots. Moreover, the experimentally determined dependence of ln(% *activity*) on the preincubation time (t) and inhibitor concentration $[AX]$ is given by Eq. (5):

$$\ln(\%activity) = -k_i[AX]t + \ln(100) \qquad (5)$$

Setting Eqs. (4) and (5) equal to each other gives Eq. (6):

$$k' = k_i[AX] \qquad (6)$$

A plot of $-k'$ vs. $[AX]$ will be a straight line with slope $= k_i$, as shown in Fig. 5B. This is the secondary kinetic plot. The k_i value is a measure of the overall inhibitory potency of a given compound (such as a direct–acting OP inhibitor) against a particular serine esterase, such as AChE or NTE. Again, it is important to realize that k_i is a composite quantity that includes K_d, an indication of the affinity of the inhibitor for the enzyme, and k_2, the rate of organophosphylation of the enzyme by the inhibitor (Eq. 2), so that the units of k_i are $[AX]^{-1} t^{-1}$. The k_i obtained for chlorpyrifos methyl oxon (CPMO) against hen brain AChE (microsomal fraction, pH 7.6 phosphate buffer, 37 °C) is $1.09 \times 10^7 M^{-1} min^{-1}$ (Kropp and Richardson, 2003). Note that the extrapolated primary plots exhibited a small amount of zero-time inhibition, indicating a low concentration of a reversible Michaelis-type complex.
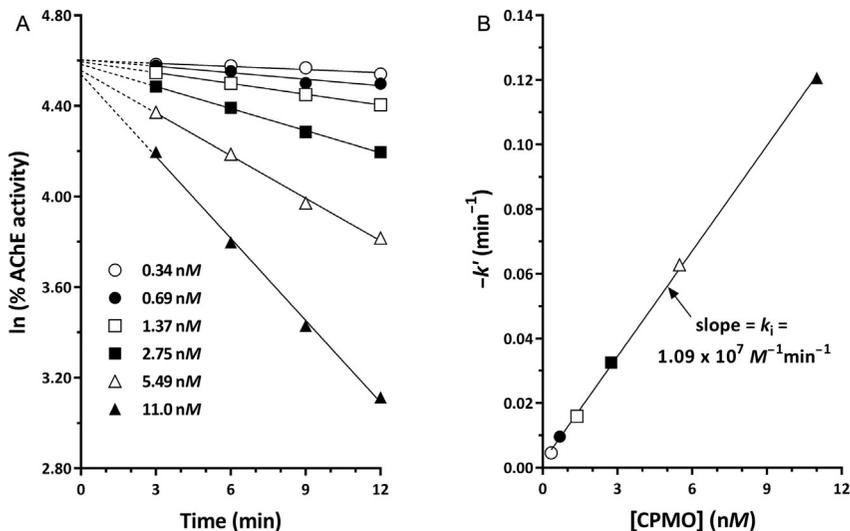
Fig. 5 Kinetics of inhibition of hen brain AChE by chlorpyrifos methyl oxon (CPMO). (A) Primary kinetic plots of ln(% activity remaining) after preincubating AChE with various concentrations of CPMO for timed intervals before adding substrate. Note that the extrapolated lines show a small amount of zero-time inhibition, indicating the presence of a low concentration of a reversible Michaelis-type complex (B) Secondary kinetic plot of −slopes (−k') of the primary plots vs. CPMO concentration; the slope of this line is the bimolecular rate constant of inhibition, $k_i = 1.09 \times 10^7$ M^{-1} min^{-1} (Kropp and Richardson, 2003).

Substituting a percent activity of interest into Eq. (5) gives the inhibitor concentration at a given time of preincubation with enzyme that would yield the particular percent activity. For example, for $[AX]_{50} = IC_{50}$, the inhibitor concentration required to produce 50% inhibition of the enzyme at a given time, t, of preincubation of enzyme and inhibitor at defined conditions of pH, temperature, and ionic strength before adding substrate, yields Eq. (7):

$$IC_{50} = \ln(2)/k_i t \cong 0.693/k_i t \qquad (7)$$

Note from Eq. (7) that k_i and IC_{50} are reciprocally related, and that IC_{50} is time dependent. It is valid to calculate an IC_{50} from a k_i value when pseudo-first-order kinetics is obtained, but it is not valid to calculate a k_i from an experimentally determined fixed–time IC_{50}, because the IC_{50} alone contains no information about the kinetics of the reaction. Moreover, if inhibitory potency is assessed by measuring fixed–time IC_{50} values directly, it is essential to report the time of preincubation along with the

concentration, because the IC_{50} decreases as the preincubation time increases. For example, the 20-min IC_{50} for CPMO against hen brain microsomal AChE at pH 7.6 and 37 °C is $0.693/[(1.09 \times 10^7 \, M^{-1} \, min^{-1}) (20 \, min)] = 3.18 \, nM$. However, if we were to choose $t = 40 \, min$, the 40-min IC_{50} decreases to 1.59 nM, giving an unwarranted impression of greater potency. It is preferable to assess inhibitory potency of an OP compound against an esterase kinetically rather than by fixed-time IC_{50} values, but the latter can be easier to conceptualize (Richardson, 1992; Thompson and Richardson, 2004).

For even greater ease of conceptualization, the pIC_{50} is recommended (Navre, 2019). After converting the IC_{50} to M units, $pIC_{50} = -\log IC_{50}$. For example, $IC_{50} = 3.18 \, nM = 3.18 \times 10^{-9} \, M$; $-\log(3.18 \times 10^{-9}) = pIC_{50} = 8.50$. The pIC_{50} has some advantages over the IC_{50}. For example, because IC_{50} values in M units require exponents, it is less cumbersome to present pIC_{50} data. Moreover, pIC_{50} values are more intuitive than IC_{50} data for conveying relative potencies—the pIC_{50} is directly related to inhibitory potency. In addition, when concentrations are equally spaced on a log scale, pIC_{50} confidence intervals and standard errors are symmetrical on a log scale (GraphPad, 2016a). However, as with the IC_{50}, the time of preincubation should be stated along with the pIC_{50}. Overall, it is preferable to use the pIC_{50} rather than the IC_{50} as a fixed-time measure of inhibitory potency, but the kinetically determined bimolecular rate constant of inhibition, k_i, is preferable to either fixed-time value (Kropp and Richardson, 2003; Mortensen et al., 1998; Strelow, 2017).

Under certain conditions, the K_d and k_2 components of k_i can be determined. For example, if the primary kinetic plots do not pass through the origin or if the secondary plot is not linear, there may be an appreciable concentration of a Michaelis-type complex. Consequently, the K_d term must be explicitly included. Combining Eqs. (3) and (4) yields Eq. (8):

$$k' = \ln(v_0/v) = k_2/(1 + K_d/[AX]) \tag{8}$$

Eq. (8) has the same form as the Michaelis-Menten equation, and a plot of k' vs. $[AX]$ is hyperbolic, as shown in Fig. 6A. The hyperbolic plot can be linearized as shown in Fig. 6B, but double-reciprocal plots magnify errors; therefore, values of k_2 and K_d should ideally be determined using nonlinear regression on Eq. (8) (GraphPad, 2016b; Ritchie and Prvan, 1996). In this example, $k_2 = 1.0 \, min^{-1}$ and $K_d = 3.0 \times 10^{-6} \, M$; therefore, $k_i = k_2/K_d = 1.0 \, min^{-1}/(3.0 \times 10^{-6}) M = 3.3 \times 10^5 \, M^{-1} \, min^{-1}$.
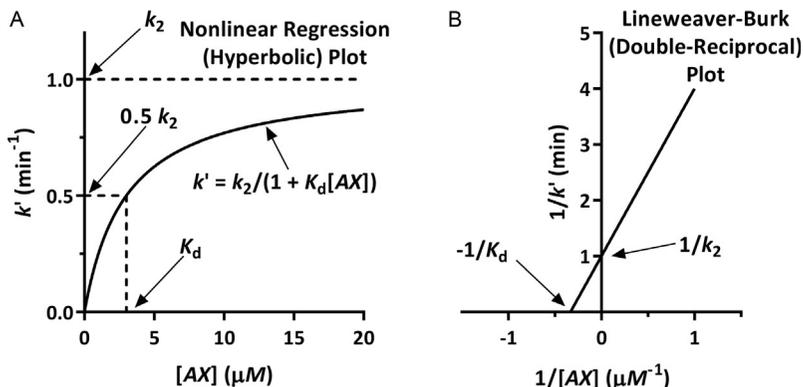
Fig. 6 Plots of Eq. (8) to determine k_2 and K_d values. (A) Direct nonlinear (hyperbolic) plot. (B) Linearized Lineweaver-Burk (double-reciprocal) plot. In this hypothetical example, $K_d = 3.0\,\mu M$ and $k_2 = 1.0\,min^{-1}$; therefore, $k_i = k_2/K_d = 3.3 \times 10^5\,M^{-1}\,min^{-1}$ (Main, 1980).

Whenever possible, it is good to determine k_2 and K_d, as these values provide information about the rate of phosphylation and the affinity of the inhibitor for the enzyme, respectively, whereas k_i alone provides information only about the overall potency of the inhibitor for the enzyme (Aldridge and Reiner, 1972; Main, 1980; Richardson et al., 2020).

Spontaneous reactivation is described by a pseudo–first-order rate constant, k_3, because the concentration of the reactivator, H_2O, is essentially constant and in great excess. The spontaneous reactivation rate constant is determined by preincubating the enzyme with a concentration of inhibitor that produces >90% inhibition within a short interval. Inhibitor is then removed or diluted to prevent ongoing inhibition, and the return of enzyme activity is measured in aliquots at timed intervals. A plot of ln(%activity) vs. time yields a straight line with slope $= -k_3$. The corresponding half-life can be calculated via Eq. (9):

$$t_{1/2} = \ln(2)/k_3 \cong 0.693/k_3 \qquad (9)$$

AChE phosphylated by most OP compounds reactivates quite slowly. A notable exception is dimethylphosphoryl–AChE, which reactivates much faster than the diethylphosphorylated enzyme. For example, rat dimethylphosphoryl–AChE $k_3 = 5.7 \times 10^{-3}\,min^{-1}$ ($t_{1/2} = 2\,h$), whereas rat diethylphosphoryl–AChE $k_3 = 2.5 \times 10^{-4}\,min^{-1}$ ($t_{1/2} = 44\,h$). Human diethylphosphoryl–AChE reactivates at a similar rate as the rat enzyme ($k_3 = 2.0 \times 10^{-4}\,min^{-1}$; $t_{1/2} = 58\,h$). Reactivation can be accelerated by strong nucleophiles such as certain oximes (Fig. 8). (Main, 1980; Richardson, 1992; Thompson and Richardson, 2004).

Serine esterases inhibited by OP compounds containing certain labile linkages, such as P–O–R or P–NH–R, can undergo a further reaction (aging), whereby a net loss of the R-group occurs, yielding a negatively charged organophosphyl group still covalently attached to the active-site serine residue of the enzyme. The rate constant for aging is denoted by k_4. Depending on the esterase and the inhibitor, aging can proceed via an S_N1 mechanism (e.g., unimolecular scission of an R–O bond to yield RH) or an S_N2 process (e.g., nucleophilic displacement of RO– by H_2O to yield ROH) (Richardson et al., 2020). If the original inhibitor contains a chiral phosphorus atom, the aged species produced by either mechanism becomes achiral, because the negative charge is delocalized over two oxygen atoms, rendering them equivalent. The charged phosphyl group is stabilized by hydrogen bonding to the oxyanion hole of the enzyme, and the negative charge repels nucleophilic reactivators (Masson and Nachon, 2017). The result is that the aged enzyme is intractable to reactivation. Thus, k_4 can be determined by rapidly inactivating the enzyme via preincubation with an OP inhibitor, removing or diluting the inhibitor to prevent ongoing inhibition, taking aliquots at timed intervals for reactivation by adding fluoride ion or an oxime, and measuring the residual activity (Richardson, 1992).

As aging progresses, the enzyme becomes increasingly resistant to reactivation. Because the process is either first-order or pseudo-first-order, a plot of $\ln$(% activity restored) vs. t is linear, with slope $= -k_4$. As with reactivation, the half-life may be calculated using Eq. (9), substituting k_4 for k_3. For example, k_4 for human AChE inhibited by DFP is 2.5×10^{-3} min^{-1}; $t_{1/2} = 4.6$ h. In contrast, k_4 for human AChE inhibited by paraoxon k_4 is 2.8×10^{-4} min^{-1}; $t_{1/2} = 41$ h. The diisopropylphosphorylated enzyme ages faster than the diethylphosphorylated enzyme because branched alkyl chains favor stabilization of the carbocation produced by S_N1 displacement (Main, 1980). This phenomenon has been exploited in the design of nerve agents such as soman, which has a highly branched ageable pinacolyl group, yielding an aging $t_{1/2}$ of approximately 2 min (Shafferman et al., 1996). Because aged enzyme cannot be reactivated by oximes, rapid aging following exposure to nerve agents precludes treatment with these antidotes (Thompson and Richardson, 2004).

4.3 Inhibition of AChE and cholinergic toxicity

The previous section dealt with interactions of OP inhibitors of serine esterases in general, although many examples were drawn from OP-AChE

interactions in particular. Therefore, this section will discuss AChE inhibition and aging only briefly, mainly in the context of toxicity.

Exposure to sufficiently high doses of OP insecticides or nerve agents produces cholinergic toxicity via inhibition of AChE in the central and/or peripheral nervous systems (Richardson, 2010; Thompson and Richardson, 2004). Such anti-AChE compounds are termed *cholinergic*. Because the physiological function of AChE is to terminate the action of the neurotransmitter, acetylcholine (ACh), in cholinergic synapses throughout the central and peripheral nervous systems, a relatively high level of inhibition of AChE can result in an accumulation of excess ACh. Consequently, postsynaptic muscarinic and nicotinic ACh receptors undergo hyperstimulation followed by fatigue, ultimately resulting in muscle paralysis, central depression of respiration, coma, and death.

4.4 Aging of OP-inhibited AChE

As noted above regarding OP interactions with serine esterases, AChE inhibited by OP compounds containing linkages such as P–O–R or P–NH–R can undergo aging, a postinhibitory reaction whereby a net loss of an R–group occurs, yielding a negatively charged organophosphyl group still covalently attached to the active-site serine residue of the enzyme. For AChE inhibition, this secondary reaction does not change the type or severity of toxicity—it only alters the kind of antidotal therapy that can be used. Whether or not aging occurs with inhibited AChE, the outcome of a sufficient level of inhibition is the same—cholinergic neurotoxicity. Aging of phosphylated AChE changes only the types of treatments that are available (Richardson, 2010). These distinctions are illustrated in Fig. 7.

To counteract the excess ACh resulting from inhibition or inhibition plus aging of AChE, atropine is given to attenuate muscarinic effects. If the dose of OP compound were high enough to cause convulsions or seizures, anti-convulsants such as benzodiazepines are also administered. In addition, if aging of inhibited AChE has not occurred, the oxime 2-[(hydroxyimino)methyl]-1-methylpyridin-1-ium (2-pyridine aldoxime methyl; 2-PAM) is given to reactivate the inhibited enzyme (Fig. 8) (Thompson and Richardson, 2004). However, if aging has occurred, 2-PAM is ineffective for two reasons: the negative charge of the aged phosphyl group repels the nucleophilic oxime, and the aged phosphyl group is strongly stabilized by hydrogen bonding to a region of the active site of the enzyme called the oxyanion hole (Masson and Nachon, 2017;

Fig. 7 Toxicological consequences of AChE inhibition and aging. The upper reaction of AChE (shown in red) with an organophosphate first yields an inhibited enzyme resulting in cholinergic toxicity, treatable with atropine. Before the inhibited enzyme ages, reactivation is possible, so that 2-PAM can also be administered (Fig. 8). After aging occurs, AChE is still inhibited but cannot be reactivated; therefore, 2-PAM will be ineffective and treatment is limited to atropine alone (although anticonvulsants could be given if convulsions were present). The lower reaction with an organophosphinate yields an inhibited enzyme resulting in cholinergic toxicity. Aging cannot occur because of the stability of the carbon-phosphorus bonds; both atropine and 2-PAM can be given as treatments. Note that aging of AChE is depicted here as proceeding via S_N1 scission, yielding RH (Richardson and Makhaeva, 2014). R = substituted or unsubstituted aryl or alkyl groups.
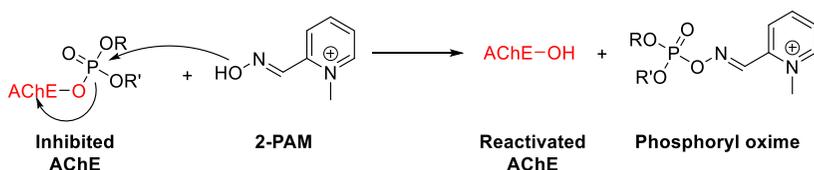
Fig. 8 Reactivation of inhibited AChE by 2-PAM. The oxime oxygen of 2-PAM attacks the phosphorus atom of the unaged organophosphoryl moiety that is covalently attached to the active-site serine of AChE, displacing reactivated AChE and producing a phosphoryl oxime compound. 2-PAM is ineffective if the inhibited enzyme has undergone the aging reaction (Thompson and Richardson, 2004). R, R′ = substituted or unsubstituted alkyl or aryl groups that may be the same or different.

Quinn et al., 2017). However, recent reports suggest that it might be possible to "resurrect" aged AChE using quinone methide precursors (QMPs) to realkylate the aged enzyme to render it reactivatable; the same compounds can also serve as reactivators (Franjesivic et al., 2019; Zhuang et al., 2018).

4.5 Inhibition and aging of NTE and OPIDN

Unlike cholinergic toxicity, which only depends upon inhibition of a critical amount of nervous system AChE, OPIDN appears to require inhibition of at least 70% of nervous system NTE along with aging of the inhibited enzyme (Richardson and Makhaeva, 2014). As noted above, OP compounds that can produce OPIDN are said to be neuropathic. The consequences of inhibiting NTE with an aging vs. a nonaging inhibitor are shown in Fig. 9.

It is possible for OPIDN to arise insidiously, because some neuropathic compounds are potent NTE inhibitors but poor AChE inhibitors. Accordingly, such compounds can trigger axonal degeneration well below the doses required to elicit cholinergic toxicity (Richardson, 2010; Richardson et al., 2020).

The aging process of inhibited NTE is similar to that for inhibited AChE—a postinhibitory loss of a secondary leaving group from the OP moiety resulting in the formation of a negatively charged organophosphyl group covalently attached to the active-site serine residue of the enzyme.

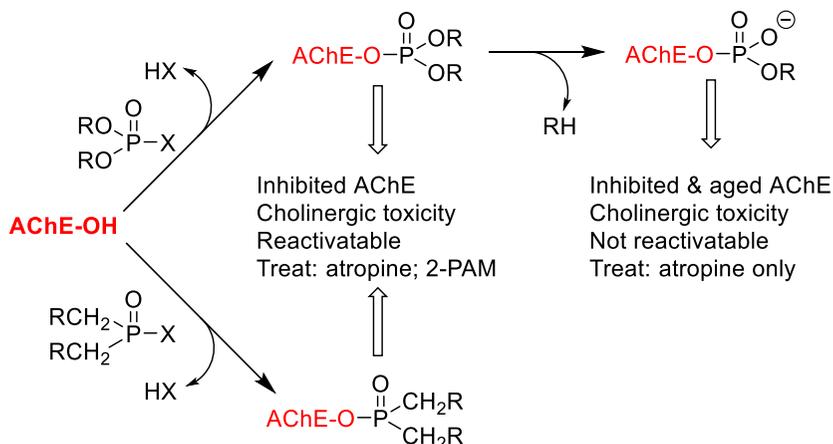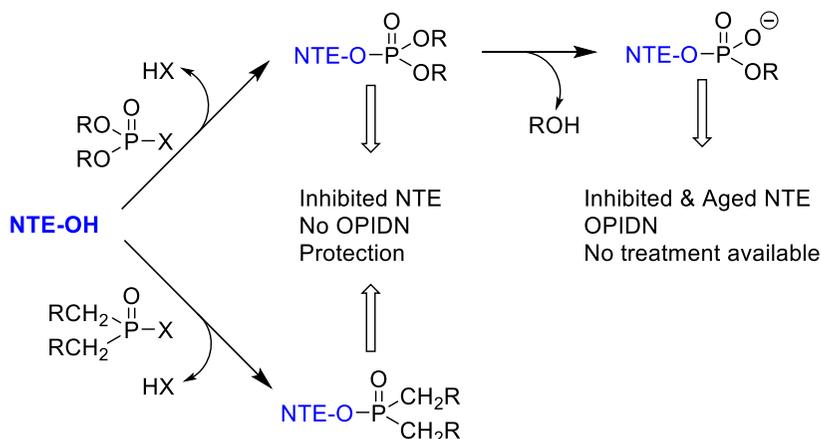**Fig. 9** Toxicological consequences of NTE inhibition and aging. The upper reaction of NTE (shown in blue) with an organophosphate yields inhibited enzyme, but OPIDN does not result until after aging occurs. Currently, there is no specific treatment for OPIDN. The lower reaction with an organophosphinate produces inhibited enzyme. Aging cannot occur because of the stability of the carbon-phosphorus bonds; there is no OPIDN, but there is protection against a subsequent exposure to a neuropathic OP compound. Note that aging of NTE is thought to proceed via an S_N2 reaction with H_2O, so that the R-group is displaced as ROH. Moreover, for most neuropathic NTE inhibitors, aging occurs within minutes, precluding postinhibitory treatment with reactivators (Clothier and Johnson, 1979, 1980; Kropp et al., 2004; Richardson and Makhaeva, 2014). R = substituted or unsubstituted alkyl or aryl groups; they may be the same or different.

However, rather than proceeding via an S_N1 mechanism, aging of NTE inhibited by neuropathic OP compounds is thought to occur by an S_N2 mechanism for organophosphates and organophosphonates, with aging $t_{1/2}$ values ranging from <1 to 10 min for compounds with R-groups that are straight-chain (e.g., *n*-butyl) or "slightly branched" (e.g., isopropyl) (Clothier and Johnson, 1979, 1980). NTE inhibited by the phosphoramidate mipafox appears to age instantaneously, possibly by deprotonation (Kropp et al., 2004). When the ageable R-group on organophospylated NTE is the highly branched pinacolyl moiety, the aging $t_{1/2}$ lengthens to 10 h, lending support to the proposed S_N2 mechanism for aging of organophosphylated NTE. Given these relatively rapid rates of aging of NTE inhibited by neuropathic OP compounds, aging is not the rate-limiting step in the delay of 1–4 weeks between inhibition of NTE and the onset of clinical signs of OPIDN. Likewise, inhibition of NTE is not the rate-limiting step, given that inhibition in experimental animals occurs within hours after dosing (Wijeyesakere and Richardson, 2010).

4.6 "Jake Leg" epidemic

While sporadic cases of OPIDN have been reported since the nineteenth century, the largest epidemic took place in the United States in the early 1930s, toward the end of the era of Prohibition, when the production, importation, transportation, and sale of alcoholic beverages was banned (Davis and Richardson, 1980). During this period, an extract of Jamaican ginger (colloquially called "Jake") was used widely as a means to obtain ethyl alcohol that was presumably potable. However, certain stocks of this extract were adulterated with solvents that contained tris(2-methylphenyl) phosphate (tri-*ortho*-cresyl phosphate; TOCP, also known as tri-*ortho*-tolyl phosphate, TOTP), which is metabolized to the neuropathic NTE inhibitor, 2-(*ortho*-cresyl)-4*H*-1,2,3-benzodioxaphosphoran-2-one (CBDP) (Fig. 10) (Carletti et al., 2013; Reinen et al., 2015).

Consumption of these TOCP-contaminated mixtures throughout the country resulted in a series of large-scale outbreaks of paralysis known by various names, including "Jake Leg," "Jake Walk, and "Ginger Paralysis" (Morgan and Tulloss, 1976; Smith et al., 1930a,b). The number of afflicted individuals has never been accurately determined, although figures as high as 50,000 have been cited (Morgan, 1982). A bulletin from the US Veteran's Administration states that there were 4837 officially reported cases
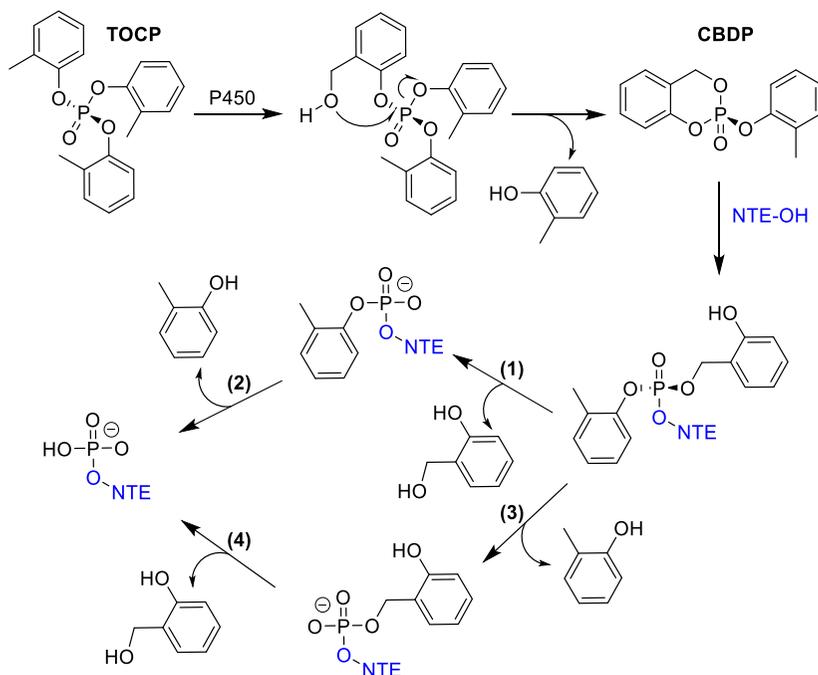
Fig. 10 Formation of CBDP from TOCP and its NTE inhibition and proposed alternative aging pathways. TOCP is metabolized by cytochromes P450 to a hydroxylated intermediate that undergoes internal cyclization with elimination of a cresyl group to form the active NTE inhibitor, CBDP. Inhibited NTE could then undergo aging via proposed alternative pathways with net loss of a saligenin (1) or cresyl (3) group. The aging reaction could stop after the first elimination or proceed with sequential loss of cresyl (2) or saligenin (4) to yield a final aged product with a phosphate group covalently attached to the NTE active site serine residue. (Carletti et al., 2013; Reinen et al., 2015). The (*S*)-isomer of CBDP is shown, as this enantiomer reacts faster with the catalytic domain of NTE (Wu and Casida, 1994); however, it is not yet known if the initial CBDP-NTE adduct has retained, inverted, or racemized stereochemistry. The aged products are achiral owing to delocalization of the negative charge between two equivalent oxygen atoms. NTE is shown in blue.

(Bevis, 1936), but the same article indicates that according to estimates from the Bureau of Prohibition, the true number was at least 20,000. Since then, smaller outbreaks of OPIDN following exposure to TOCP (usually in contaminated cooking oil) have been reported in several countries, including Germany (during World War II) (Karczmar, 2007), Morocco (1959) (Glynn, 2003; Smith and Spalding, 1959) and Sri Lanka (1977–1978) (Senanayake, 1981).
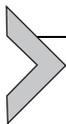
5. Rationale for NTE research

In recent times, reports of OPIDN cases worldwide have been infrequent and usually associated with failed suicide attempts involving ingestion of OP insecticides, although accidental and occupational exposures have also been involved (Jokanović et al., 2019; Lotti and Moretto, 2005). The global decline in OPIDN cases has been due in part to increasingly stringent regulation of OP compounds and the use of the NTE assay to screen OP compounds for their neuropathic potential.

Nevertheless, continued study of neuropathic OP compounds, OPIDN, and NTE is warranted for several reasons. For example, OP compounds continue to be employed in numerous applications, and their use as flame retardants is increasing owing to the phase-out of other classes of these agents, such as the polybrominated diphenyl ethers (PBDEs) (Ospina et al., 2018). Moreover, anticholinesterase OP compounds remain as important agents of chemical warfare and terrorism (Figueiredo et al., 2018), and neuropathic OP compounds pose a latent threat in this regard (Richardson et al., 2020). Exposures to OP compounds also continue to be implicated in the etiology of various conditions for which solid research is needed to resolve issues of causation vs. correlation amid complexity and controversy. These disorders include aerotoxic syndrome (Carletti et al., 2011; Hageman et al., 2019), Gulf War Illness (Michalovicz et al., 2019), thyroid and other endocrine disruptions (Yang et al., 2020), and Type 2 diabetes and obesity (Czajka et al., 2019). However, arguably the most compelling justifications for NTE research are embodied in the intriguing scientific questions concerning the mechanisms of axonal degeneration following changes in the structure and/or function of NTE brought about by chemical exposures and/or genetic mutations (Hufnagel et al., 2015; Kmoch et al., 2015; Read et al., 2009; Richardson et al., 2013; Sunderhaus et al., 2019a,b).
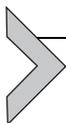
6. History of NTE

6.1 Importance of history

In 1810, Goethe wisely asserted, "… *die Geschichte der Wissenschaft die Wissenschaft selbst sei*." ("… the history of a science is the science itself.") (Goethe, 1810), and in 1905, Santayana famously observed, "Those who cannot remember the past are condemned to repeat it" (Santayana, 1905). Accordingly, to understand the science of NTE and to avoid

reinventing what has already been devised, it is prudent to retrace the story of its discovery and conceptual evolution. This story began with some clever chemical thinking about an important and intriguing toxicological problem long before the application of modern molecular biology and bioinformatics gave us our current view of this fascinating protein. Moreover, there is still much to be learned about NTE, and in the spirit of "… what's past is pro-logue …." (Shakespeare, 1623), its history is likely to provide valuable clues that can help us draw the blueprint for solving more of its mysteries in the future.

6.2 M.K. Johnson and the MRC toxicology unit

M.K. Johnson discovered NTE through work carried out during 1966–1969 in the Biochemical Mechanisms Section of the Medical Research Council (MRC) Toxicology Unit in Carshalton, England. Johnson was trained as a chemist and accustomed to solving intractable prob-lems. Before coming to the MRC, his former mentor would repeatedly exhort him with the challenge, "Johnson, this won't work—but you try it!" (M.K. Johnson, personal communication). Thus forearmed, Johnson was accustomed to undertaking experiments that others would consider impossibly difficult and fruitless.

6.3 MRC leadership and intellectual climate

When Johnson joined the MRC Toxicology Unit, it was primed with the most favorable leadership and attitudes imaginable to help ensure the success of the work he was to undertake. The Unit was under the directorship of John Barnes, a physician committed to fostering mechanistic research who, among many other notable achievements, had conducted the first tox-icology test on penicillin shortly before the onset of WWII (Witschi, 2002). Johnson reported directly to W.N. Aldridge, a highly respected biochemist in charge of the Biochemical Mechanisms Section. At this time, Aldridge was formulating the unifying thesis for much of his life's work—the notion that enzyme inhibitors resembled substrates. Aldridge had already done pioneering work on understanding the acute cholinergic effects of OP com-pounds by studying the kinetics of their binding to AChE. Somewhat later, in 1972, Aldridge collaborated with Elsa Reiner, a visiting scientist from the Institute for Medical Research in Zagreb, to produce the book, *Enzyme Inhibitors as Substrates: Interactions of Esterases with Esters of Organophosphorus and Carbamic Acids* (Aldridge and Reiner, 1972), which is now regarded as one of the great classics of mechanistic toxicology (Witschi and Lock,

2001). Aldridge kept his staff heading in the right direction for making important discoveries with the continual reminders, "What question are you asking?" and "Go after the anomalies — expected answers seldom tell us anything interesting; pursuing the unexpected will lead to the breakthroughs" (W. N. Aldridge, personal communication).

6.4 MRC philosophy

Barnes and Aldridge established the guiding philosophy of the MRC Toxicology Unit that defined the way its scientists would choose their research problems. There were two main criteria. First, the problem had to have been referred to the Toxicology Unit as a matter of practical public health or medical importance, usually arising from occupational exposures. Second, the problem had to be of intrinsic scientific interest and to show promise as something whose intensive and unrestricted study would yield mechanistic insights, thus advancing the science of toxicology (Witschi and Lock, 2001). These principles were to become official MRC doctrine by 1972, as evidenced by Johnson's quotation from the *MRC Handbook* in a review article on OPIDN:

"The aim of the (Toxicology) Unit is to establish the basic concepts necessary for explaining at a molecular level the mechanisms of toxicity and of chemical and physical injury" (Johnson, 1975b).

Several of the scientists who came to work at the Toxicology Unit had been associated during WWII with the Chemical Defence Establishment at Porton Down, where one of the chief concerns was to understand the acute cholinergic toxicity of OP nerve agents in order to develop prophylactic drugs or antidotes.

6.5 Mipafox incident

Although the delayed neurotoxicity of OP compounds had been well known since the major episodes of the early 1930s, this aspect of OP toxicity had been associated with a single noncholinergic compound, tri-*o*-cresyl phosphate (TOCP) (Bevis, 1936; Smith et al., 1930a,b). Then, in 1951 at Fisons PLC, three workers who were involved with the pilot plant production of a new insecticide candidate called mipafox presented with acute cholinergic toxicity (Bidstrup et al., 1953). Two of the three affected individuals were admitted to hospital for treatment and ultimately discharged. Subsequently, the two patients developed a delayed neurotoxicity that closely resembled poisoning by TOCP.

The mipafox incident demonstrated that an OP compound that was structurally dissimilar to TOCP or its active metabolite CBDP could produce OP delayed neurotoxicity (Figs. 3 and 10). The episode likewise raised concern that this form of insidious and permanently debilitating toxicity could extend to other OP compounds—either already in use or to be developed in the future. Here was a perfect problem to be taken on by the MRC Toxicology Unit. It had arisen from an industrial exposure, it was clearly of medical and public health importance, and it was an intriguing scientific mystery whose investigation held the promise of yielding mechanistic insights into neurotoxicity and neurodegenerative disease. Consequently, research was started at the Unit to investigate the mechanism of OP delayed neurotoxicity.

6.6 Discovery and naming of NTE

Barnes, together with Aldridge and E. Poulsen, laid the foundation for Johnson's ultimate discovery by initiating the search for a suitable target in hen brain (Aldridge, 1964; Aldridge and Barnes, 1966a,b; Poulsen and Aldridge, 1964).

By this time, earlier work had established adult hens as the vulnerable animal model of choice for OPIDN studies (Smith et al., 1930b). Rats, mice, and immature chickens appeared to be resistant; adult roosters were vulnerable, but less readily available and far less docile than hens. AChE and butyrylcholinesterase (BChE) had also been ruled out as potential targets for OPIDN (Cavanagh, 1954; Davison, 1953; Smith and Lillie, 1931).

Poulsen and Aldridge (1964) reasoned that neuropathic OP compounds should covalently bind to an unknown serine esterase in hen brain capable of hydrolyzing substrates such as phenyl phenylacetate (PPA) and phenyl phenylpropionate (PPP), whose structures somewhat resembled that of CBDP, the cyclic saligenin phosphate active metabolite of TOCP (Fig. 10). Initially, such enzyme activities were found, but a clear correlation between their inhibition and delayed neurotoxicity was lacking. In retrospect, these early experiments had come tantalizingly close to defining a relevant esterase activity, but they were hampered by the relative lack of sensitivity of the Warburg manometric assay method that required measuring small volumes of CO_2 gas liberated from a bicarbonate buffer through reaction of the acids produced by enzymatic cleavage of ester substrates. The true signal had been lost in the noise. Years later, Poulsen lamented his narrow miss in identifying NTE, but he was glad for Johnson's success (E. Poulsen, personal communication).

   Undaunted by these initial failures, Johnson was convinced that the theory of selective binding was correct. Using a sensitive radiometric approach employing [^{32}P]–diisopropylphosphorofluoridate (DFP), a radio-labeled neuropathic analogue of mipafox, he was able to identify a portion of labeled sites in hen brain tissue that was blocked by neuropathic mipafox, but not blocked by nonneuropathic tetraethylpyrophosphate (TEPP) (Aldridge et al., 1969; Johnson, 1968, 1969a). Furthermore, he was able to quantify the decrease in radiolabeling of the phosphorylation site both in vitro by preincubating hen brain homogenates with neuropathic OP compounds and ex vivo in brain homogenates from hens that had been dosed with neuropathic OP compounds.

   Johnson then returned to Aldridge's theory that the site identified by differential incubation with selective inhibitors and radiolabeling was likely a serine esterase. First, he was able to show that radiolabeling of this site could be blocked by preincubating hen brain homogenates with the carboxylic acid ester, PPA. Further study of the PPA hydrolase activity using a colorimetric assay showed that about 10% was resistant to inhibition by nonneuropathic OP compounds such as TEPP or paraoxon and that a portion of this residual activity was sensitive to inhibition by the neuropathic compound, mipafox (Johnson, 1969b,c). It was this relatively small proportion of mipafox-sensitive paraoxon–resistant activity that came to be known first as "neurotoxic esterase" (Johnson, 1973, 1974) and later as "neuropathy target esterase" (Johnson et al., 1985).

   Among the journals that were circulated to Johnson's desk from the MRC library was *Analytical Biochemistry*, reflecting his deep appreciation for the fact that while scientific advances required dreamy qualities such as a lively imagination, practical progress depended critically on methods. For example, he was fond of telling his coworkers in the lab that despite the astonishing success of the Lowry protein assay (Lowry et al., 1951) in being the most highly cited scientific paper of all time (Aksnes et al., 2019), there was still no perfect method for quantifying proteins in crude mixtures. Johnson predicted that fame if not fortune awaited the investigator who could devise—not a better mousetrap—but a better procedure than the Lowry method for assaying protein content in biological samples.

   Having lived through the privations in Britain imposed by WWII, Johnson was also keen to economize whenever possible. He made good use of his admiration and broad knowledge of techniques along with his desire to get the most out of sparse resources in at least two ways. First, recognizing that energetic electrons such as those emitted from ^{32}P emit visible

light when passing through a dielectric medium such as water (the Cherenkov Effect) (Cherenkov, 1934), he devised a method for measuring ^{32}P radiation using a standard scintillation counter and the simplifying economy of not requiring addition of a chemical scintillant or elaborate sample preparation (Johnson, 1969d). Second, in order to simplify and increase the sensitivity of esterase assays compared to the cumbersome and relatively insensitive Warburg manometric method, Johnson sought to develop a colorimetric assay. Realizing that his substrate of choice was PPA, which would be hydrolyzed by esterases to phenylacetic acid and phenol, he reached back to 1946 to retrieve a report on a quantitative colorimetric approach for determining concentrations of phenolic fungicides in fabrics (Gottlieb and Marsh, 1946) and adapted it for use in his esterase assays. The substrate was ultimately changed from PPA to phenyl valerate (PV), but the principle of the assay remained the same (see Fig. 11 below) (Johnson, 1977; Kayyali et al., 1991).

It is interesting to note that in Johnson's first published account—in abstract form—of this as-yet unnamed esterase activity, he inadvertently put together the right words but with a different meaning: "The phenyl esters of 1-phenylacetic acid (PPA) and 2-phenylpropionic acid (PPP) are structurally analogous to the *neurotoxic esterase* inhibitor phenylsaligenin phosphate" (Johnson, 1968; emphasis added). In this abstract, Johnson was referring to the fact that the esterase inhibitor was "neurotoxic," a term used then in context to indicate that a compound was neuropathic, i.e., capable of producing OPIDN, but it appears that he may have at that time unconsciously christened NTE with its initial name, "neurotoxic esterase." In his first full paper referring to neurotoxic esterase (Johnson, 1974), Johnson maintained a provisional status to the name by always putting quotation marks around it. Soon thereafter, at the urging of one of us (RJR), who was then a postdoc in his lab, Johnson became convinced that his new entity was fully fledged and allowed its name to stand undiminished by, as he would say, "inverted commas" (Johnson, 1975a,b,c).

During the next few years, in private correspondence and conversations at scientific conferences, a number of scientists expressed confusion over the name of Johnson's enzyme, saying that the nomenclature suggested that the esterase itself was neurotoxic. This seemed untenable under the premise that the protein surely must have a normal physiological function and would therefore not be neurotoxic in its own right. Johnson acquiesced to this logic and began referring to NTE as "neuropathy target esterase" (Johnson et al., 1985), a new name that seemed more descriptive of its role as the esterase site
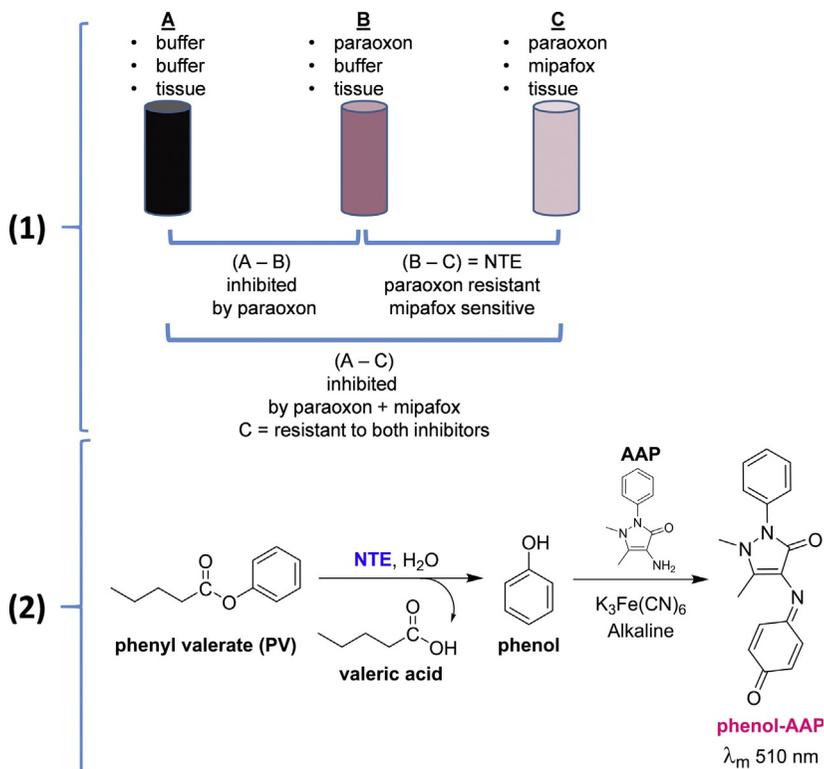
Fig. 11 Principle of the NTE assay. (1) Tissue aliquots are preincubated for a timed interval at a set temperature and pH with (A) buffer only, (B) paraoxon (nonneuropathic), and (C) paraoxon (nonneuropathic) + mipafox (neuropathic). (2) At the end of the preincubation interval, substrate (PV) is added for a timed incubation period, during which NTE and other esterases hydrolyze PV to valeric acid and phenol. The colorimetric reagent 4-aminoantipyrine (AAP) is then added along with $K_3Fe(CN)_6$ to form the plum-red phenol-AAP chromophore. Absorbances (A, B, and C) are read at 486 nm and converted to molar units of phenol formed by calibration against a phenol standard. NTE activity is proportional to $(A–C) – (A–B) = (B–C)$. Absorbance A is not required; it is included here for illustrative purposes. The blank is self-correcting, but it is good practice to include a tissue blank to check for nonenzymatic generation of phenol and a reagent blank to check for decomposition of the substrate into phenol and valeric acid (Johnson, 1977; Kayyali et al., 1991). Structures of the neuropathic inhibitor mipafox and the nonneuropathic inhibitor paraoxon are shown in Fig. 3. The structure of the phenol-AAP chromophore was deduced correctly by Gottlieb and Marsh (1946), and later confirmed by P.F. Jones and K. E., Johnson using proton NMR (Jones and Johnson, 1973). The AAP-phenol reaction has come to be widely used in numerous assays employing colorimetric or electrochemical endpoints (Varadaraju et al., 2018).

that was attacked by a neuropathic OP compound to produce axonopathy. Moreover, the refreshed moniker preserved the same set of initials that had originally been used for the protein. To paraphrase the proclamation of continuity echoed down the centuries by the British (and loyal subjects of other kingdoms) at the concerted passing and accession of their monarchs—NTE is dead! Long live NTE!

6.7 Biochemical lesion and pathogenesis

Sir Rudolph Peters was a renowned biochemist at Oxford who led the effort during WWII to develop British Anti-Lewisite (BAL), an effective antidote against the organoarsenic chemical warfare agent, lewisite (Peters et al., 1945). His subsequent work showing that the toxicity of fluoroacetate was due to its biotransformation to fluorocitrate generated his concept of "lethal synthesis," a powerful idea that had a major influence on toxicological thinking. Equally potent was his notion of the "biochemical lesion," a term he coined in 1931 to refer to the initial molecular perturbation in a chain of events leading to eventual tissue degeneration before any histopathological changes could be detected (Gavrilescu and Peters, 1931; Peters, 1963, 1969).

The concept of lethal synthesis and the biochemical lesion clearly had an impact on Johnson, who launched the theory of "neurotoxic esterase" with his seminal paper titled, "The primary *biochemical lesion* leading to the delayed neurotoxic effects of some organophosphorus esters" (Johnson, 1974; emphasis added). In this publication, Johnson recounted the key observation that not all inhibitors of NTE produced OPIDN. However, the non-neuropathic inhibitors were not biologically inert—they protected chickens (adult hens) against OPIDN from subsequent challenge by neuropathic NTE inhibitors (Johnson and Lauwerys, 1969). The critical molecular difference between the two types of inhibitors was that nonneuropathic protective compounds were incapable of forming aged NTE, whereas neuropathic OP compounds were capable of producing aged NTE—a protein covalently linked to a negatively charged organophosphyl group. Thus, here was a perfect example of lethal synthesis giving rise to a biochemical lesion.

Johnson and colleagues established that the concerted molecular reactions of inhibition and aging of NTE can take place within hours of dosing, well before histological lesions or clinical signs of OPIDN appear, typically 2–3 weeks later (Clothier and Johnson, 1979, 1980; Johnson, 1975a,b,c, 1982, 1990; Makhaeva et al., 2007). At present, this specific chemical

modification of NTE is still the earliest known stage in the pathogenesis of OPIDN and thereby regarded as the initiating event. Nevertheless, we have yet to elucidate the steps involved in the ensuing axonal degeneration and to determine how organophosphylation and aging of NTE trigger these degenerative changes.

However, important clues about pathogenesis were uncovered by J.B. Cavanagh, a distinguished neuropathologist at the Institute of Neurology in London who worked closely with scientists at the MRC Toxicology Unit throughout his career. Cavanagh and coworkers helped establish that OPIDN was a primary axonopathy (with demyelination a secondary process) especially affecting long axons of large diameter in peripheral nerves and spinal cord tracts. In addition, they determined that AChE and BChE were not involved, and observed that neuropathic OP compounds acted like chemical scalpels to produce a "chemical transection" of the axon causing the region distal to the initial lesion to undergo Wallerian degeneration. In addition, Cavanagh noted that the lesions resembled those produced by thiamine deficiency, which was thought to decrease energy metabolism, leading to degeneration of distal axons—the regions of neurons most distant from their cell bodies (Bouldin and Cavanagh, 1979a,b; Cavanagh, 1954; Cavanagh and Holland, 1961). We will return later to these observations in a suggested model of OPIDN as Wallerian degeneration.

6.8 Passing the torch

Just as Johnson had been inspired by the scientists around him, he had the gift of instilling others with a fascination for NTE research. He frequently attended international conferences and made visits to other laboratories around the world, taking a keen interest in the many people he met. These activities resulted in his attracting visiting workers to the MRC Toxicology Unit who would later take up NTE research in their home institutions. In addition, he would make a point of introducing one scientist to another, thereby engendering new and fruitful collaborations in the field.

7. Applications of NTE

7.1 NTE Assay and neuropathic potential

Since its inception (Johnson, 1969b), the NTE assay has enjoyed a number of relatively minor improvements (Correll and Ehrich, 1991; Johnson, 1977;

Johnson and Richardson, 1983; Kayyali et al., 1991). However, it is still based on differential inhibition of the hydrolase activity toward an artificial ester substrate (e.g., phenyl valerate) by neuropathic (e.g., mipafox) and nonneuropathic (e.g., paraoxon) inhibitors, and detecting the difference in product formation (e.g., phenol production) colorimetrically or electrochemically (Sigolaeva et al., 2001). A differential assay is required in cells or tissues containing esterases in addition to NTE because inhibition by both paraoxon and mipafox still leaves a residual hydrolase activity that is resistant to both inhibitors. The principle of the NTE assay is shown in Fig. 11.

The NTE assay made it possible to screen OP compounds for a quantitative assessment of their neuropathic potential using in vitro preparations or ex vivo following dosing in experimental animals. It was soon discovered that there was an apparent threshold of $\sim$70% inhibition (and presumably concomitant aging) of nervous system NTE activity in order to precipitate OPIDN (Johnson, 1970).

Moreover, the 70% NTE inhibition threshold was found to apply to chronic dosing as well as to an acute dose regimen. It had been shown in several independent experiments carried out between 1932 and 1975 that there was a dose threshold for OPIDN induced by TOCP given daily for 26 to 140 days (Barnes, 1975; Henschler, 1958; Smith et al., 1932). Using the potent neuropathic OP compound, mono-o-cresyl diphenyl phosphate (MOCP), Lotti and Johnson (1980) found that daily dosing at 2.5 mg/kg/day for 8 weeks resulted in a chronic inhibition of brain and spinal cord NTE of 60% and 45%, respectively, with no clinical signs of OPIDN. However, OPIDN could be precipitated by superimposing a single high dose of 50 mg/kg or by increasing the repeated dose to 5 mg/kg/day, which resulted in elevating NTE inhibition to >80%. These experiments also demonstrated that the effects of inhibiting NTE below the threshold were not cumulative.

For those compounds that have anti–AChE as well as anti–NTE activity, it is possible to calculate the relative inhibitory potency (RIP), defined as the ratio of bimolecular rate constants of inhibition (k_i) against each enzyme. Alternatively, the RIP can be defined using fixed–time IC_{50} values (concentration of inhibitor required to inhibit enzyme activity by 50% at a given time of preincubation of the inhibitor with the enzyme). Because the IC_{50} and k_i are reciprocally (see Eq. 7), the RIP based on IC_{50} values is the reciprocal of the ratio based on k_i values, as shown in Eq. (10).

$$RIP\ (cholinergic\ toxicity) = k_i(AChE)/k_i(NTE)$$
$$= IC_{50}(NTE)/IC_{50}(AChE) \qquad (10)$$

Defined in this way, if *RIP (cholinergic toxicity)* > 1, then the compound is more likely to produce cholinergic toxicity and death than to produce OPIDN (Kropp and Richardson, 2003; Lotti and Johnson, 1978; Richardson, 1992).

Note that some studies have preferred to emphasize the neuropathic potential of an OP compound and to define the *RIP* with the potency against NTE in the numerator, as in Eq. (11).

$$RIP(OPIDN) = k_i(NTE)/k_i(AChE) = IC_{50}(AChE)/IC_{50}(NTE) \quad (11)$$

Defined in this reciprocal manner, if *RIP(OPIDN)* > 1, then the compound is more likely to produce OPIDN than cholinergic toxicity (Makhaeva et al., 2014).

7.2 Structure-activity relationships (SARs)

In the earlier years of NTE research at the MRC, much of the important SAR data that was to be accumulated on NTE inhibitors was made possible by chemical industries supplying Johnson's lab with hundreds of OP compounds for NTE testing. This work was undertaken based upon the understanding that the data could be freely published in the open literature (Davis et al., 1985; Johnson, 1975a,b,c, 1988). The results also made it possible to quantify differences in potency among NTE inhibitors, thus giving rise to quantitative SAR (QSAR) data.

This SAR and QSAR information proved quite useful in making predictions about the neuropathic potential of a given compound. For example, the results of a hen brain NTE assay 24–48 hours after dosing enabled the following conclusions to be drawn:
1. If the compound failed to produce >70% inhibition, it would not cause OPIDN.
2. If the compound produced >70% inhibition, it would cause OPIDN if it belonged to a structural class (Type A) capable of aging (such as phosphates, phosphonates, or phosphoramidates).
3. If the compound produced >70% inhibition, it would not cause OPIDN if it belonged to a structural class (Type B) incapable of aging (such as carbamates, sulfonyl fluorides, or phosphinates).

The generic structures of Type A and Type B NTE inhibitors are shown in Fig. 12.

NTE Inhibitors

Type A
Aging and Neuropathic

$$X-\overset{\overset{O}{\|}}{P}\overset{OR^1}{\underset{OR^2}{}}$$

Phosphate

$$X-\overset{\overset{O}{\|}}{P}\overset{O-R^1}{\underset{CH_2R^2}{}}$$

Phosphonate

$$X-\overset{\overset{O}{\|}}{P}\overset{\overset{R^1}{|}}{\underset{\underset{R^4}{|}}{N-R^3}}$$

Phosphoramidate

Type B
Non-aging and Protective

$$X-\overset{\overset{O}{\|}}{P}\overset{CH_2R^1}{\underset{CH_2R^2}{}}$$

Phosphinate

$$X-\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}-R$$

Sulfonate

$$X-\overset{\overset{O}{\|}}{C}-\overset{\overset{R^1}{|}}{N}-R^2$$

Carbamate
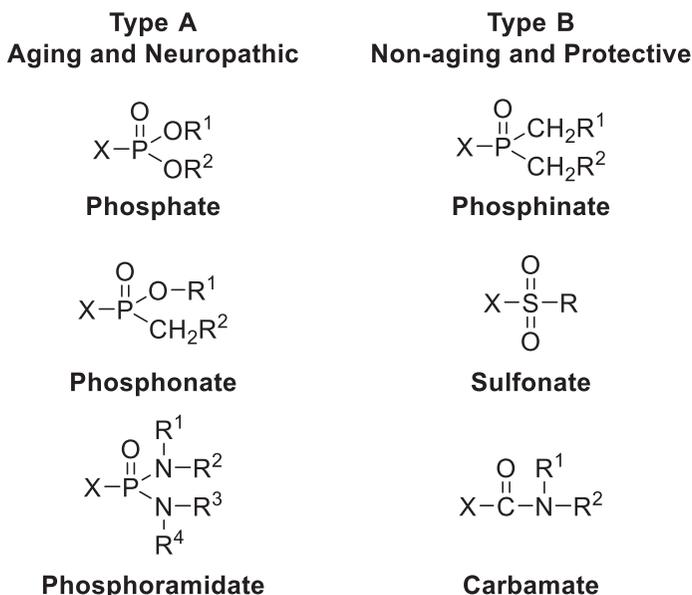
Fig. 12 Generic structures of class A and B NTE inhibitors. Type A inhibitors include phosphates, phosphonates and phosphoramidates. Type A inhibitors are capable of aging, and inhibition of >70% of nervous system NTE results in OPIDN. Type B inhibitors include phosphinates, sulfonates, and carbamates. Type B inhibitors cannot age, and inhibition of >70% of nervous system NTE does not result in OPIDN; however, inhibition of >30% of nervous system NTE with a nonaging inhibitor confers protection against OPIDN from subsequent administration of an otherwise neuropathic dose of a Type A inhibitor. The R-groups are substituted or unsubstituted alkyl or aryl moieties, and for phosphoramidates and carbamates, R can also be hydrogen. X is the primary leaving group in each case.

7.3 Protection (prophylaxis) against OPIDN

Although the extensive SAR and QSAR studies on NTE inhibitors pro–vided partial support for the theory that both inhibition and aging of NTE were required to precipitate OPIDN, the more convincing evidence came from experiments showing that pretreatment with nonageable NTE inhibitors protected against OPIDN from subsequent challenge with an ageable NTE inhibitor. Much of the early work on protection against OPIDN has been summarized in an excellent review by Carrington (1989).

The seminal report on protection showed that phenyl benzylcarbamate inhibited hen brain NTE in vitro and in vivo (Johnson and Lauwerys, 1969). However, the carbamoylated enzyme undergoes relatively rapid

spontaneous reactivation, with a half-life in vivo between 6 and 10 hours postdosing. This reactivation was fortuitous, as it permitted determination of the dose–response and time course of protection. Thus, pretreatment of hens with phenyl benzylcarbamate conferred complete protection against the clinical and histopathological manifestations of OPIDN from a challenge dose of the delayed neurotoxicant, DFP, as long as the level of NTE inhibition produced by the carbamate was >30% at the time when the DFP challenge was given. Positive controls pretreated only with the DMSO vehicle developed severe OPIDN from DFP administration. Parallel experiments with [^{32}P]-DFP showed that the carbamate pretreatment did not affect entry of DFP into the brain.

A follow-up study showed that protection against OPIDN from DFP was also conferred by pretreatment with phenyl N-benzyl–N-methyl carbamate, phenyl n-butylcarbamate, phenylmethanesulfonyl fluoride (PMSF), and n-butanesulfonyl fluoride (Johnson, 1970). Again, dose–response and time course experiments demonstrated that protection required at least 30% inhibition of NTE at the time that the challenge dose of DFP was administered, whereas dosing with DFP alone required >70% inhibition of NTE to produce OPIDN. Prolonged inhibition of NTE at a level of 80% for 2 weeks with PMSF or 85% inhibition with n-butanesulfonyl fluoride did not produce OPIDN. Finally, protection by PMSF against OPIDN from DFP could be blocked by pretreatment with phenyl benzylcarbamate.

Next, Johnson found that phosphinates (e.g., 4-nitrophenyl di-n–butylphosphinate; 4-nitrophenyl di-n–pentylphosphinate; and 2,2-dichlorovinyl di-n–pentylphosphinate) with high structural similarity to neuropathic phosphates and phosphonates inhibited NTE but were not neuropathic (Johnson, 1974). Upon testing these compounds for their ability to protect against OPIDN induced by DFP, he found complete protection that was afforded for 4–6 days, which matched the time for brain NTE inhibition to fall to 30%, thus again suggesting a threshold of about 70% inhibition and aging of brain NTE in order to precipitate OPIDN.

Subsequent experiments in other laboratories confirmed Johnson's observations of protection against OPIDN. These included a study of the time course of protection of PMSF against OPIDN induced by TOCP in hens (Carrington and Abou-Donia, 1983), as well as PMSF protection against an intra-arterially produced unilateral DFP-induced OPIDN in cats (Baker et al., 1980; Drakontides and Baker, 1983) and hens

(Caroldi et al., 1984). Moreover, an important conclusion from the unilateral experiments was that OPIDN could be produced as a direct effect of OP compounds on axons.

Whereas rats are resistant to clinical signs of OPIDN, they exhibit histopathological findings of axonal degeneration following administration of neuropathic OP compounds. Accordingly, Veronesi and Padilla (1985) were able to show protection by PMSF against spinal cord lesions produced by mipafox in rats. Likewise, protection was conferred by the thiocarbamate, molinate, against OPIDN induced by di-*n*-butyl dichlorvos (DBDCV) in rats as well as in hens (Moretto et al., 2001).

Further work in Johnson's lab extended protective agents in hens to inhibitory but nonaging isomers of the insecticide, EPN (protective against OPIDN from the opposite EPN isomer that both inhibited NTE and aged) (Johnson and Read, 1987). Partial protection was observed from pretreatment of hens with the nerve agent soman against challenge with DFP (Johnson et al., 1988a). Soman-inhibited hen brain NTE was found to age negligibly. Owing to the extremely high acute cholinergic toxicity of soman and NTE data on only a single animal from each group, it was not possible to assess accurately the level of nonaged NTE in soman-predosed hens.

Finally, Johnson had found that the phosphinates he had identified earlier as nonneuropathic NTE inhibitors that were protective against neuropathic OP compounds also had shortcomings for NTE/OPIDN studies; in addition to being difficult to synthesize, these phosphinates exhibited high acute cholinergic toxicity and chemical instability. Ultimately, he discovered that phenyl di-*n*-pentylphosphinate was easy to synthesize and that it displayed high potency against NTE, high chemical stability, and low potency against AChE. Moreover, this new phosphinate conferred complete protection against clinical and histopathological signs of OPIDN induced by DFP challenge at either 2 or 4 days after the prophylactic treatment (Johnson and Read, 1993; Johnson et al., 1988b).

7.4 Potentiation or promotion of OPIDN

In 1990, C.N. Pope and S. Padilla demonstrated that nonaging NTE inhibitors protected against OPIDN only if given prior to dosing with a neuropathic (aging) inhibitor—when the order of dosing was reversed, the nonaging NTE inhibitor potentiated the neuropathic effect of the aging inhibitor (Pope and Padilla, 1990). This surprising phenomenon was studied

further by M. Lotti and colleagues, who changed the name in their publications from potentiation to promotion. This terminology was based on the absence of clinical signs of OPIDN following dosing with an aging inhibitor of NTE (i.e., an initiator) vs. the appearance of clinical signs of OPIDN following dosing with an initiator and subsequent dosing with a nonaging inhibitor of NTE (i.e., a promoter) (Lotti and Moretto, 1999).

Potentiation had actually been noticed as early as 1985 in an experiment designed to demonstrate protection afforded by PMSF against mipafox-induced spinal cord lesions in rats (Veronesi and Padilla, 1985). As noted above, although rats and mice are highly resistant to the clinical manifestations of OPIDN, they express NTE/PNPLA6 in their nervous systems (UniProt, 2019b,c) and develop axonal lesions that are particularly evident in the dorsal columns of the spinal cord in response to treatment with neuropathic OP compounds (Lapadula et al., 1985; Padilla and Veronesi, 1985; Veronesi, 1984; Veronesi and Padilla, 1985).

PMSF protected rats against axonopathy when given 4 h prior to mipafox, but protection was absent when PMSF was given 14 days before mipafox. In contrast, when mipafox was given 4 h before PMSF, there was an exacerbation of the axonopathy. However, the authors noted that the lesions in the exacerbated case differed somewhat in appearance and distribution from those produced by mipafox alone (Veronesi and Padilla, 1985). In any event, this experiment added support to Johnson's theory that OPIDN requires both inhibition and aging of NTE, and it was the first report of the potentiation of OPIDN by a nonaging inhibitor of NTE.

The specific basis for potentiation or promotion of OPIDN has not been elucidated, but in view of the fact that potentiators or promoters delay recovery from nerve crush (Moretto et al., 1993), a plausible general explanation involves the notion of interference with mechanisms of repair of axonal injury (Lotti, 2002). In addition, although the first reports of potentiation/promotion indicated that the effective compounds were NTE inhibitors, subsequent work by Lotti and colleagues strongly suggested that the target for promotion/potentiation is not NTE (Lotti, 2002; Lotti and Moretto, 1993; Moretto et al., 1994, 2001).

7.5 NTE-based biomarkers and biosensors

The discovery and characterization of NTE activity in circulating lymphocytes and platelets (Bertoncin et al., 1985; Bleecker et al., 1983; Dudek and Richardson, 1982; Maroni and Bleecker, 1986) made it possible to use

blood samples for biomonitoring exposures to neuropathic OP compounds (Lotti et al., 1983; Schwab and Richardson, 1986). With the advent of electrochemical biosensors, NTE activity could be determined in whole blood (Makhaeva et al., 2003; Sigolaeva et al., 2001), thus obviating the need to isolate leukocyte fractions prior to analysis. Moreover, biosensor assays permitted real-time detection of NTE activity and could be extended for the simultaneous assay of several esterases (Kohli et al., 2007; Sigolaeva et al., 2010). Because different OP compounds have varying inhibitory potencies against different esterases, this differential inhibition creates an "esterase profile" for a given inhibitor that serves to predict the type of toxicity or therapeutic effect that it will exert (Makhaeva et al., 2013, 2016a,b).
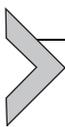
8. Cellular and molecular biology of NTE

8.1 NTE: The next generation

During the decades of research on NTE since its discovery, most of the studies were focused on the inhibition and aging of its esterase function, the association of these events with OPIDN, and the development of biomarkers and biosensors of exposure to neuropathic OP compounds (Makhaeva et al., 2016a,b; Richardson et al., 2013). Only relatively recently have investigations expanded to include noncatalytic regions of the protein, factors that may regulate its hydrolase function or turnover, interactions with other proteins, NTE knockouts or silencing, and NTE mutations associated with disease (Huang et al., 2016; Hufnagel et al., 2015; Sogorb et al., 2016; Synofzik and Schule, 2017; Synofzik et al., 2014, 2015). These new developments have of course resulted from engaging a new generation of researchers (along with some from earlier days) to continue the exploration of NTE by applying the modern tools of molecular biology, bioinformatics, and computational molecular modeling. Many of the advances in understanding NTE have stemmed from the discovery of SWS ("Swiss Cheese") the *Drosophila* orthologue of human NTE/ PNPLA6, so named because of the vacuolated appearance of fruit fly brains arising from certain mutations in the *sws* gene encoding the SWS protein (Kretzschmar et al., 1997; Sunderhaus et al., 2019a,b).

8.2 NTE protein sequences and domains

8.2.1 Canonical and isoform sequences

In the early 1990s, during M.K. Johnson's phased retirement and the move of the MRC Toxicology Unit from Carshalton to the University

of Leicester, important new developments in NTE research were realized, including the affinity purification, cloning, and sequencing of the human protein (Glynn, 2006; Glynn et al., 1994, 1999; Lush et al., 1998).

Screening of a human brain cDNA library yielded the first reported sequence for NTE (clone D16) comprising 1327 amino acids, with the catalytic center (defined by Ser966) located within a hydrophobic region (Lush et al., 1998). This sequence is now categorized by UniProt as isoform-2, with isoform-4 regarded as the canonical NTE sequence out of 4 isoforms arising from alternative splicing that have been identified thus far (UniProt, 2019a). The canonical isoform-4 comprises 1375 residues with Ser1014 as the active-site serine residue. Note that the National Center for Biotechnology Information (NCBI) refers to this sequence as isoform-a, accession/version number NP_001159583.1 (NCBI, 2019).

Throughout the remainder of this chapter, except where otherwise indicated, the numbering of the canonical isoform-4 sequence will be used. It should also be noted that despite our knowledge of the sequence and domains of NTE, its three-dimensional structure has still not been determined experimentally.

8.2.2 Four types of domains

Subsequent analysis of the primary amino acid sequence of NTE suggested it to be a multi-domain protein with three distinct regions (Fig. 13): (1) an N-terminal transmembrane (TM) domain; (2) a series of three tandem cyclic nucleotide binding (CNB) homology domains (CNB1, CNB2, and CNB3); and (3) a C-terminal catalytic domain, the patatin NTE
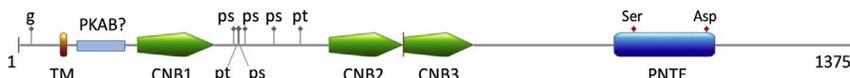
Fig. 13 Domains of the NTE/PNPLA6 protein. The canonical protein sequence-a (NCBI, 2019) or isoform-4 (UniProt, 2019a) comprises 1375 amino acid residues. The established three types of domains and their approximate residue number boundaries include the following: a transmembrane domain (TM, 60–80); three putative cyclic nucleotide binding domains (CNB1, 195–322), (CNB2, 511–633), and (CNB3, 629–749); and a catalytic domain (patatin-homology NTE; PNTE, 981–1147). By analogy to the *Drosophila* homologue, SWS (Wentzell et al., 2014), a potential fourth type of domain (PKA binding, PKAB) is proposed to reside between the TM domain and CNB1. Some specific sites of interest include g (*N*-glycosylation site, Asn20); ps and pt. (phosphorylation sites on serine and threonine, respectively; Ser354, Thr361, Ser362, Ser372, Ser420, and Thr464); and the active site residues in the catalytic domain (Ser1014 and Asp1134) (UniProt, 2019a).

(PNTE) homology domain (Glynn, 2013; Wijeyesakere et al., 2007). More recently, in the *Drosophila* NTE orthologue, SWS, a fourth domain (4) has been described that can bind a catalytic subunit (C3) of protein kinase A (PKA) (Bettencourt da Cruz et al., 2008; Sunderhaus et al., 2019a,b); here, we refer to this region as PKAB. In the following subsections, we will briefly discuss the current state of knowledge of these domains as well as the sub-cellular localization of NTE, highlighting areas where new research is needed to address gaps in knowledge and understanding. Note that protein domains are often not precisely demarcated. The residues assigned a given domain will sometimes overlap with an adjoining domain, and residue assignments to domains may differ somewhat between data sources, e.g., UniProt and NCBI.

8.2.3 Transmembrane (TM) domain

The N-terminal TM domain of NTE anchors the protein to the cytoplasmic face of the endoplasmic reticulum (ER). Consistent with the nature of mammalian transmembrane domains, its structure is predicted to be an α-helix spanning residues 9–31 in NTE isoform-2. In the case of isoform-4, the TM region consists of residues 60–80, with residues 1–59 being localized within the ER lumen and the remaining residues (81–1375) on the cytoplasmic side (UniProt, 2019a). Interestingly, analysis of the primary sequence of NTE isoform-4 suggests the presence of an *N*-linked glycosylation site at residue Asn20 that is absent in isoform-2 (denoted by "g" in Fig. 13). Virtually nothing is known about a possible role for this potential *N*-linked glycosylation site; however, in other ER-folded proteins such a site serves to direct the nascent polypeptide to the calnexin/calreticulin chaperone cycle (Lamriben et al., 2016; Wijeyesakere et al., 2013). Thus, the role of these glycan-binding ER chaperones in binding or promoting the folding of a subset of NTE molecules represents an intriguing area for further investigation.

8.2.4 Cyclic nucleotide binding (CNB) homology domains

The CNB domains of NTE encompass the following residues by isoform-2 numbering: 163–262 (CNB1), 480–573 (CNB2), and 597–689 (CNB3) (UniProt, 2019a; Wijeyesakere et al., 2007). By the canonical isoform-4 numbering, these domains are contained within the following residues (Fig. 13): 195–322 (CNB1), 511–633 (CNB2), and 629–749 (CNB3) (UniProt, 2019a).

Cyclic nucleotides such as cyclic AMP (cAMP) and cyclic GMP (cGMP) play crucial roles as second messengers that transduce signals in a variety of cellular signaling systems throughout life forms ranging from bacteria to humans (Gancedo, 2013). Thus, the presence of a tandem series of putative CNB domains within the N-terminal locale of NTE suggests that this region may play a role in regulating its hydrolase activity.

When the current domain organization of NTE was first published (Wijeyesakere et al., 2007), very little was known about the structure or function of the three CNB domains. Indeed, in the previous decade, there had been only a single study hinting that these domains might be vestigial remnants incapable of engaging cyclic nucleotides (Dremier et al., 2003). Nevertheless, we have been unwilling to accept that an apparent CNB region could be devoid of purpose, and the existence of three such domains suggests the possibility that NTE requires an exquisite tunability of control. Accordingly, using homology models, we have carried out preliminary molecular docking simulations and found that cyclic nucleotides (cAMP and cGMP) can be docked to the putative binding pockets of all three CNB domains with favorable free energies of binding (S.J.W. and R.J.R., unpublished data).

Recent elegant experiments in *Drosophila* have shown that certain mutations in SWS, the fruit-fly orthologue of NTE, produce neurodegenerative disease and disruption of phospholipid homeostasis that can be rescued by expressing wild-type NTE in the flies (Sunderhaus et al., 2019b). However, expression of NTE that contained disease-causing mutations in either the catalytic domain or the CNB domains suppressed neurological deficits but did not restore lipid homeostasis. These findings provided support for two important conclusions: (1) disruption of the catalytic function of NTE is not sufficient to produce neurodegeneration; and (2) the CNB domains participate in the regulation of NTE catalytic activity.

8.2.5 Catalytic (patatin-homology) domain (PNTE)

By far, the best-characterized aspect of NTE is its hydrolase activity, which resides in the catalytic domain (residues 981–1147) (UniProt, 2019a). While the structure of this region has not been determined experimentally, bioinformatics analyses of the primary sequence of NTE show that the catalytic Ser1014 nucleophile (isoform-4 numbering) is located within a region that has some homology to the plant protein, patatin. Accordingly, one of the names ascribed to this region is the patatin-homology domain of NTE (PNTE) (Fig. 13). Based on the initial published structure for patatin

isoform-17 (pat17) (PDB ID 1OXW) (Rydel et al., 2003), the first structural homology model for PNTE was presented (Wijeyesakere et al., 2007). In this paper, it was proposed that PNTE consists of a modified α/β hydrolase fold with a central parallel β-sheet flanked by seven α-helices. Subsequently, a somewhat higher resolution structure of pat17 was published (PDB 4PK9) along with its aged DFP complex (PDB 4PKA) and nonaged complex with methyl arachidonylfluorophosphonate (MAFP) (PDB 4PKB) (Wijeyesakere et al., 2014).

Previously, based on findings from site-directed mutagenesis, the catalytic machinery of NTE had been thought to consist of a novel Ser966-Asp1086-Asp960 catalytic triad (isoform-2 numbering) (Atkins and Glynn, 2000) that was distinct from the canonical Ser-His–Asp/Glu triads found in other α/β hydrolases. However, the current view is that akin to patatin and cPLA$_2$, the catalytic center of NTE consists of a Ser1014-Asp1134 catalytic dyad (isoform-4 numbering) (Fig. 13), with the Asp1134 residue serving as both the general base and general acid during the catalytic process.

Thus, the proposed hydrolysis mechanism for NTE involves Asp1134 serving as a general base at the initial stage of the catalytic process to activate the Ser1014 nucleophile by abstracting a proton. This allows the Ser1014 hydroxyl oxygen to perform a nucleophilic attack on the substrate. The ensuing tetrahedral intermediate, whose negative charge is expected to be stabilized by the oxyanion hole of NTE (formed by Gly986, Gly987, Ala988 and Arg989), would collapse to yield an acylated enzyme intermediate, with Asp1134 donating a proton to the leaving group, thereby serving as a general acid. Subsequent engagement and activation of a water molecule would allow the formation of a second tetrahedral intermediate transition state, resulting in the release of the hydrolyzed substrate and regeneration of the enzyme (Rydel et al., 2003; Wijeyesakere et al., 2007).

In the case of interactions between NTE and neuropathic OP compounds, a similar mechanism is proposed to be responsible for the formation of the organophosphylated enzyme (Wijeyesakere et al., 2007). However, an additional path (aging) is available for OP compounds containing linkages such as P–O–R or P–NH–R that involves loss of a side chain via an S_N2-mechanism (or possibly loss of a proton from a singly substituted nitrogen atom in a phosphoramidate such as mipafox). This reaction results in a permanently inhibited enzyme and, as Johnson postulated as early as 1974 (Johnson, 1974), is thought to be the initiating factor in the pathogenesis of OPIDN (Ehrlich and Jortner, 2001; Kropp et al., 2004; Richardson et al., 2013; Wijeyesakere and Richardson, 2010).

The catalytic serine residue also resides in the center of a serine lipase motif, GXSXG, as found, for example in enzymes of the PLA2 superfamily of phospholipases (Ma and Turk, 2001). The NTE sequence contains an additional GXSXG motif in residues 1256–1260; this region appears to be disordered, and it is likely that this second serine lipase motif is a false positive (Via et al., 2007); nevertheless, it would be of interest to determine if the serine residue in this locus were subject to organophosphylation.

8.2.6 Protein kinase A binding (PKAB) domain

Located between the TM domain and the first CNB domain, the PKAB domain in the *Drosophila* NTE orthologue, SWS, has been shown to bind PKA-C3, thereby localizing the kinase to the ER and inhibiting its activity. The PKAB domain also appears to regulate the esterase/phospholipase activity of SWS and to protect against the neurodegenerative effect of PKA-C3 hyperactivity (Bettencourt da Cruz et al., 2008).

However, in a *Drosophila* OPIDN model, TOCP produced 80% inhibition of SWS catalytic activity, resulting in neurodegeneration and motor deficits. Increasing SWS levels by overexpression was not protective, whereas decreasing SWS levels was protective against the neuropathic effects of TOCP, suggesting a gain of toxic function in OP-inhibited SWS. TOCP treatment resulted in a significant decrease in PKA activity. Using yeast two-hybrid assays, mouse NTE was shown to bind to *Drosophila* PKA-C3, and flies overexpressing PKA-C3 were protected against the behavioral deficits produced by TOCP (Wentzell et al., 2014).

Based on the *Drosophila* studies and the mouse NTE binding experiments, similar interactions between human NTE and PKA are expected, but preliminary investigations have not yet confirmed this interaction (J.K.F., unpublished observations). We have designated the provisional site of this domain, as "PKAB?" in Fig. 13; but currently the boundaries of this hypothetical region are unknown.

8.2.7 Posttranslational modifications (PTMs)

In addition to the predicted *N*-linked glycosylation site at Asn20 within the TM domain indicated above, there are phosphorylation sites at Ser354, Thr361, Ser362, Ser372, Ser420, and Thr464 (Isoform-4 numbering) (UniProt, 2019a) (Fig. 13). Such sites presumably would have regulatory functions (Hirabayashi et al., 2004; Walaas and Greengard, 1991), but these have not yet been characterized. However, it is of interest that exposure of HEK293 cells to chlorpyrifos oxon resulted in organophosphylation of Tyr residues in 73 proteins, and that many of these residues were

phosphorylation sites for protein kinases (Onder et al., 2018). Thus, it is possible that NTE could be organophospylated at sites other than the catalytic Ser residue, and that such chemical modifications of the protein could disrupt normal phosphorylation/dephosphorylation mediated by protein kinases and phosphoprotein phosphatases.

8.3 Subcellular localization of NTE

Given the presence of an N-terminal TM domain, NTE is, unsurprisingly, characterized as a membrane-bound phospholipase. Indeed, its association with the ER membrane had been evident given the early subcellular fractionation studies of hen brain homogenates showing the enzyme to be concentrated within the microsomal fraction (Richardson et al., 1979). These results were further confirmed by subsequent findings demonstrating the need for NTE to be associated with the membrane, which is facilitated by the N-terminal TM domain (Li et al., 2003). Furthermore, limited proteolysis of NTE-containing microsomes revealed the protein to be exposed to the cytoplasm (Li et al., 2003). These findings were further supported by subsequent colocalization studies in primary cultures of mouse hippocampal neurons (Akassoglou et al., 2004), bioinformatics analyses of the primary sequence of the N-terminal region of NTE (Wijeyesakere et al., 2007), and fluorescence microscopy studies of GFP-tagged NTE expressed in HEK 293T cells (Chang et al., 2009). In contrast to the ER localization of NTE in brain, a significant fraction of the NTE activity in peripheral nerve is found in the soluble faction (Vilanova et al., 1993, 1999). Currently, the role of this soluble form of apparent NTE activity is unclear, and it is not known whether it arises as a distinct isoform or if it is released from the ER and transported down the axon as a soluble protein.

8.4 Expression of NTE in human tissues

Early work indicating the presence of NTE in various species and tissues based on enzyme activity has more recently been augmented using next-generation sequencing of RNA transcripts in tissues (RNA-Seq). These results have confirmed that NTE/PNPLA6 is expressed in a wide variety of human tissues. In addition to nervous system and lymphatic tissues, expression of NTE is particular high in kidney, lung, and testis (NCBI, 2019).

8.5 NTE knockouts, silencing, and mutations

8.5.1 Conventional knockouts

Conventional knockout of NTE in mice proved to be lethal after embryonic day 8, but disruption of the *Nte* (*PNPLA6*) gene in a single allele permitted survival with NTE activity levels reduced to about 60% of normal and no apparent structural abnormalities in the nervous system (Winrow et al., 2003). Lethality due to complete inactivation of the *Nte* gene in mice was subsequently attributed to placental failure and impaired vasculogenesis, and again, heterozygous $Nte^{+/-}$ mice displayed normal organ development, despite possessing NTE activities of only 50% of normal levels (Moser et al., 2004). In connection with the role of NTE in placental function, pre-eclampsia in humans has been associated with decreased placental NTE expression (Zhong et al., 2018).

8.5.2 Silencing

Downregulation of NTE gene expression by RNA interference in D3 mouse embryonic stem cells was found to alter multiple pathways involved in development of respiratory, neural, and vascular systems, but such effects did not occur when NTE activity was inhibited by the neuropathic OP compound, mipafox (Sogorb et al., 2016).

A zebrafish model of PNPLA6 insufficiency has been created using a morpholino oligonucleotide knockdown technique in embryos that resulted in developmental abnormalities and motor neuron defects. The study also showed that signaling via the bone morphogenetic protein (BMP) was involved, as phosphorylation of Smad1/5/8 was markedly elevated in the knockdown fish. The phenotype could be rescued by human wild-type *PNPLA6* mRNA but not by mRNA containing active-site mutations that inactivated the catalytic activity of PNPLA6 (Song et al., 2013). The BMP pathway has also been implicated in the development of hereditary spastic paraplegia (HSP) (Zhao and Hedera, 2013).

8.5.3 Conditional knockouts

Conditional knockout (cKO) of NTE in CNS neurons of mice was carried out using the *nestin-cre/loxP* system, which deleted neuronal NTE as of embryonic day 11 (Akassoglou et al., 2004). Brain NTE activity in cKO mice was decreased by about 90% compared to wild type control mice. Brain morphology in cKO mice appeared normal at 2 weeks of age, but at 6 weeks, these mice developed ER disruption and vacuolation of neurons

in the hippocampus and thalamus; cerebellar Purkinje cells were also affected. Functionally, cKO mice exhibited significant decreases in rotarod staying times.

A second cKO study in mice was undertaken in order to investigate the role of NTE deficiency in spinal cord axonopathy, neuronal PtdCho homeostasis, and neuronal secretion (Read et al., 2009). The same *nestin-cre/loxP* protocol used by Akassoglou et al. (2004) was followed; therefore, NTE deficiency was induced at embryonic day 11. Axonal degeneration was found in cKO mice aged 18–19 days in the gracile nucleus of the medulla—the site of termination of long ascending tracts in the dorsal columns of the spinal cord. Spinal tract lesions in ascending and descending tracts consisting of swollen axons progressed in severity and number in cKO mice over a period of many months. Hindlimb dysfunction also progressed over the same period. Brain PtdCho levels were elevated by nearly 20% at 1–3 months and remained at this level when assessed at 6–12 months. The rate of secretion of two proteins, reelin and the secreted fragment of the amyloid precursor protein (sAPPa) was impaired by about 20% in cultured cerebellar granule cells from cKO mice, indicating moderate compromise of the constitutive secretory pathway (Read et al., 2009).

In order to compare the cKO results with OPIDN, normal C57BL/6J mice were dosed orally for 5 days with 500 mg/kg of the neuropathic OP compound, MOCP in arachis oil (Read et al., 2009). As noted previously, mice do not develop clinical signs of OPIDN. However, they possess neural NTE that is inhibited by acute dosing with neuropathic OP compounds (Makhaeva et al., 2014, 2016a,b), and they develop spinal cord lesions following chronic dosing with TOCP (Lapadula et al., 1985). Axonal degeneration was found in the gracile nucleus at 1 week after starting MOCP dosing. The type and number of lesions was similar to what was seen in one-month-old cKO mice. However, the lesions in the MOCP-dosed mice did not increase in number or severity over time, and clinical signs of hindlimb dysfunction did not appear. Brain PtdCho levels were modestly elevated by nearly 20% 1 week after the start of MOCP dosing and then returned to baseline during weeks 2–7 rather than remaining elevated as was the case with the cKO of NTE. Taken together, these results were interpreted to indicate that inactivation of NTE by either cKO or sustained inhibition by MOCP resulted in axonal degeneration that was likely due to a deficit in adult axonal maintenance rather than an effect on neurological development (Read et al., 2009).

8.5.4 NTE disease-causing mutations in humans

The first human disease-causing mutations in NTE were reported in 2008 (Rainier et al., 2008). These mutations occurred in the catalytic domain, producing an entity named NTE motor neuron disease (NTE-MND) characterized by progressive spastic paraplegia from childhood leading to muscle wasting in the lower legs and intrinsic muscles of the hands (Rainier et al., 2011). Because this condition is a subset of the hereditary spastic paraplegias (HSPs), the condition is also referred to as SPG39, SPG39/NTE-MND, or SPG39/NTE[PNPLA6]-MND. Constructs of the NTE catalytic domain containing these disease mutations were expressed in human fibroblasts and found to have altered enzymatic properties compared to wild-type controls (Hein et al., 2010a). Constructs from asymptomatic subjects who were heterozygous for a mutation predicted to lack 235 residues from the catalytic domain had only about 40% of control NTE activity (Hein et al., 2010b). This finding was consistent with results from other studies of $Nte^{+/-}$ mice that had 50–60% of control NTE activity with apparently normal nervous system development (Akassoglou et al., 2004; Moser et al., 2004).

Since the first reports of disease-causing mutations in human NTE/PNPLA6, numerous additional cases have been published, indicating that these mutations produce a broad spectrum of neurodegenerative conditions, including Boucher-Neuhauser and Gordon Holmes syndromes (Synofzik et al., 2014; Topaloglu et al., 2014) as well as Oliver-McFarlane and Laurence-Moon syndromes (Hufnagel et al., 2015). These conditions present clinically with a wide range of deficits, including hypogonadism, ataxia, and retinal photoreceptor degeneration with concomitant childhood blindness (Kmoch et al., 2015). Most of the mutations reported thus far have been located in the catalytic domain, but some have occurred in the CNB region of NTE.

When wild-type human NTE was expressed in *sws* null *Drosophila* mutants, both the movement and neurodegenerative phenotypes were suppressed; in addition, elevations of phospholipids were ameliorated to some extent. However, when human NTE containing disease-causing mutations was expressed in *sws* null *Drosophila*, movement and neurodegenerative phenotypes were suppressed to some degree, but none of the human mutants had any effect on lipid levels in the flies. The human NTE mutants included some with mutations in the catalytic domain and others with mutations in the region containing CNB domains. The results were interpreted to indicate that CNB regions could modulate NTE catalytic activity and that

the mutant forms of human NTE retained some degree of their biological function, suggesting that disruption of the catalytic function of SWS was not the only factor in disease pathogenesis (Sunderhaus et al., 2019b).

9. Reconciling apparent conflicts between toxicological and genetic data

It has been over 50 years since the discovery of NTE and nearly that long since publication of the theory that both inhibition and aging of >70% of NTE in the nervous system are required in order to produce OPIDN (Johnson, 1969b, 1974). SAR/QSAR data accumulated over this period have largely supported the idea that OPIDN results from a combination of a loss of physiological function (NTE inhibition) coupled with a gain of toxic function (formation of aged NTE with its negatively charged organophosphyl group covalently attached to the active-site serine residue) (Makhaeva et al., 2014; Richardson et al., 2013).

On the other hand, relatively recent molecular biology studies tend to suggest that neurodegenerative and other deleterious effects are produced by a loss of physiological function of NTE alone rather than a combined loss of function and gain of toxic function. These studies included those employing conventional (Winrow et al., 2003) and conditional (Akassoglou et al., 2004; Read et al., 2009) knockouts of the PNPLA6 gene, knockdown of PNPLA6 in zebrafish, and neurogenetics findings in human subjects with disease-causing NTE mutations (Topaloglu et al., 2014). In particular, these investigations indicate that sustained absence of NTE activity and/or other functions of the protein is/are sufficient to produce neurodegenerative effects.

However, the toxicological data and at least some of the genetics results are not necessarily mutually exclusive. In the case of disease-causing NTE mutations, there are varying degrees of inactivation of catalytic activity, but there is also the presence of mutant forms of NTE that might parallel the situation with inhibited and aged NTE in OPIDN.

Given that the catalytic function of NTE is thought to be concerted deacylation of PtdCho to yield GroPCho +2 FFA, systemic inhibition of NTE activity by neuropathic OP compounds would be expected to result in increases in brain PtdCho levels. However, only a modest and transient increase was produced by repeated dosing of MOCP in mice (Read et al., 2009). Similarly, there was no demonstrable change in brain PtdCho

concentrations following a single large dose of TOCP or PMSF in mice (Hou et al., 2009) or hens (Hou et al., 2008), although it is possible that the inhibition levels and/or the timing of phospholipid assays prevented detection of a transient and relatively small increase in PtdCho levels. Indeed, a follow-up study using enhanced lipidomics techniques found significant elevations of PtdCho and LysoPtdCho levels in hen spinal cord microsomal fractions 2 days after a single oral dose of 750 mg/kg of TOCP, and these changes, except for elevated PtdCho, were prevented by pretreatment with 60 mg/kg PMSF 24 h before dosing with TOCP. Interestingly, the largest increase in the lipid profile was seen with sphingomyelin, and there was a decrease in phosphatidylinositol (PtdIns) (Zhu et al., 2016). Substantial and persistent disruptions of lipid homeostasis were found in sciatic nerve of hens given a single dose of 750 mg/kg TOCP—PtdCho levels were significantly elevated as much as ∼44% on days 2, 7, and 14 and returned to control levels by day 21 (Xu et al., 2018). Whereas the axonal lesions in OPIDN have been described as Wallerian degeneration (Bouldin and Cavanagh, 1979a), early studies of lipid changes during Wallerian degeneration showed that synthesis of PtdCho was elevated in rat sciatic nerve starting 3 days after a crush injury (Natarajan et al., 1982). Thus, it is possible that increases in PtdCho concentrations during OPIDN or Wallerian degeneration reflect decreased NTE activity, increased PtdCho synthesis, or both.

It is much more difficult to reconcile the cKO NTE results with the toxicological findings. Although there have been a number of caveats raised about conditional knockouts in general and the *nestin-cre/loxP* system in particular (Harno et al., 2013; Liang et al., 2012; Yang et al., 2009), it seems unlikely that the cKO NTE mice could have had a partially disrupted *PNPLA6* gene giving rise to truncated NTE proteins that escaped detection from the polyclonal antibody directed against the catalytic domain region of the enzyme (Akassoglou et al., 2004; Read et al., 2009). Therefore, assuming the cKO experiments resulted in a complete disruption of the *PNPLA6* gene, there would have been no expression of truncated or otherwise abnormal forms of NTE protein to serve as the counterpart to inhibited/aged NTE in OPIDN. However, given that the *nestin-cre/loxP* system initiates the cKO of NTE starting at embryonic day 11, the possibility remains that the resulting neurological abnormalities reflect neurodevelopmental sequelae from the absence of the NTE protein and its enzymatic activity. Although the neuropathological changes in the spinal cords of cKO mice had a similar appearance to those in the MOCP-dosed mice

(Read et al., 2009), axonal degeneration arising from a wide range of causes can have a similar appearance and distribution of lesions (Coleman, 2005).

Likewise, the knockdown experiments in zebrafish indicate that suppression of a critical amount of PNPLA6 is sufficient to cause motor neuron defects and other abnormalities, but here again, the knockdown was done during embryonic development (Song et al., 2013), so that the observed effects were the result of neurodevelopmental phenomena rather than an effect on adult axonal maintenance.

Moreover, when adult zebrafish brain NTE was inhibited over 70% following dosing with CBDP or DFP, no behavioral deficits or spinal cord histopathological lesions were detected. In addition, a thorough lipidomic analysis detected only a small increase in brain lysophospholipids 48 hours after DFP but not following CBDP, and no changes in brain PtdCho resulted from treatment with either inhibitor. The authors concluded that the zebrafish was not a good model of human OPIDN (Faria et al., 2018).

Johnson had noted that all neuropathic OP compounds had one thing in common—the ability to age after inhibiting NTE. Accordingly, he reasoned that the aged protein served as a signal to initiate axonal degeneration. Loss of NTE catalytic activity seemed irrelevant for the induction of lesions, because nonaging compounds inhibited NTE without producing OPIDN. Furthermore, nonaging inhibitors of NTE were not inert, because pretreating animals with nonaging inhibitors conferred protection against OPIDN from subsequent dosing with aging inhibitors, as long as the inhibition level from the pretreatment was ∼30% or more (Carrington, 1989; Johnson, 1970, 1974; Johnson and Read, 1993; Moretto et al., 2001).

However, it is also true that aging compounds necessarily result in longer-term inhibition than at least some nonaging inhibitors (e.g., carbamates) because aging inhibitors yield a highly stable organophosphylated NTE that cannot undergo spontaneous reactivation. Thus, recovery of NTE activity following inhibition by ageable inhibitors depends solely upon resynthesis of the enzyme rather than a combination of resynthesis and reactivation. Nevertheless, prolonged inhibition of NTE has also been produced by various dosing regimens with nonaging inhibitors, such as repeated dosing with carbamates, single or repeated dosing with phosphinates, and single or repeated dosing with sulfonyl fluorides, such as PMSF. NTE inhibited by phosphinates can be reactivated in vitro by KF or oximes, but NTE inhibited by PMSF apparently cannot be reactivated either spontaneously or by powerful nucleophiles. Yet, despite prolonged inhibition by these nonaging inhibitors of NTE, no axonal degeneration is produced (Meredith and Johnson, 1988; Richardson et al., 2020).

A possible resolution of the apparent paradox concerning nonaging NTE inhibitors is to propose that they are nonspecific and could theoretically inhibit other serine hydrolases whose inactivation would serve to negate the loss of NTE activity (Wijeyesakere and Richardson, 2010). After all, the human genome encodes >200 serine hydrolases, and at least half of these have not been functionally characterized (Lenfant et al., 2016; Simon and Cravatt, 2010). However, it would seem that the simpler explanation for the nonpathogenicity and protection afforded by nonaging NTE inhibitors is that these compounds lack the distinctive common feature of aging inhibitors—the ability to place a negatively charged phosphyl group in the active site of NTE (Johnson, 1990). This is especially so, considering that some of the nonaging phosphinate inhibitors exhibit high potency and selectivity for NTE. Thus, from a neurotoxicological perspective, we are left with two theoretical possibilities: (1) OPIDN results from the presence of aged NTE; or (2) OPIDN results from a combination of NTE inhibition plus the presence of the aged protein. As a practical matter, in order to produce aged NTE, it is necessary first to inhibit the enzyme with an ageable inhibitor; therefore, the toxicological data ultimately lead to the conclusion that OPIDN requires both inhibition and aging of NTE.

Referring back to the neurogenetics findings summarized above, it would appear that disease-causing NTE mutations could produce neurodegeneration via loss of NTE activity and/or through the presence of abnormal forms of NTE protein. On the other hand, knockdown or cKO of NTE most likely produces neurodegeneration from loss of NTE protein and concomitant absence of its enzymatic activity and/or deficiency of unknown functions of its other domains. In any event, it is important to bear in mind that thus far, the neurogenetics findings do not distinguish between developmental effects versus effects on maintenance of a healthy nervous system in the adult.
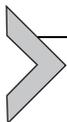
10. Suggested future research on elucidating the role of NTE in OPIDN

In consideration of the toxicological and neurogenetics findings reviewed above, we propose two hypothetical models to help guide future research on elucidating the role of NTE in OPIDN: (1) phospholipid homeostasis disruption (PHD); and (2) OP-induced Wallerian degeneration (OPIWAD). These are not necessarily mutually exclusive models.

Indeed, they could very well be viewed as sequential, whereby disruption of axonal transport in the PHD model could serve as a trigger for axonal degeneration via the OPIWAD model.

10.1 Phospholipid homeostasis disruption (PHD)

Fig. 14 is a depiction of a hypothetical phospholipid homeostasis disruption (PHD) model, showing some of the partial pathways of phospholipid metabolism in a neuron. This model represents a synthesis of the "lipid hypothesis" (Wijeyesakere and Richardson, 2010) together with an extension to mammalian systems of a model based on studies in yeast (Glynn, 2005, 2013). In this model, the final stage of PtdCho synthesis takes place in the Golgi, and the product is retrogradely transported to the ER by a PtdCho/PtdIns transport protein (PITP), a mammalian homologue of the yeast transport protein, sec14p (Wyckoff et al., 2010). Despite being named for their originally discovered function of shuttling PtdIns between membrane compartments, PITPs can also accommodate PtdCho as cargo (Carvou et al., 2010; Grabon et al., 2015).

In Fig. 14, *Pathway (1)*, we hypothesize that some fraction of the PtdCho transported to the ER in complex with a PITP is degraded by NTE, liberating PITP in the process (Glynn, 2005, 2013). If this function of NTE were deficient, then an increased fraction of the available PITP pool would remain associated with PtdCho rather than becoming available for transporting PtdIns to the Golgi. The resulting deficiency of PtdIns and concomitantly phosphorylated PtdIns in the Golgi would compromise the constitutive secretory pathway and axonal transport, thus precipitating axonal degeneration (Bartlett et al., 2002; Lorenzo et al., 2014). In this connection, it is noteworthy that disruptions in axonal transport have been shown to precede clinical signs of OPIDN in hens treated with neuropathic OP compounds (Gupta et al., 1997; Moretto et al., 1987; Song et al., 2012). In addition, NTE has been shown to undergo fast axonal transport in hen sciatic nerve (Carrington and Abou-Donia, 1985).

In contrast, *Pathway (2)* in Fig. 14 involves direct deacylation of free PtdCho by NTE. Compromising this function of NTE would not affect PITP levels, so that PtdIns could function normally to support the constitutive secretory pathway and axonal transport. Thus, a neuropathic OP compound would be expected preferentially to inhibit *Pathway (1)*, whereas a nonneuropathic protective NTE inhibitor would be expected preferentially to inhibit *Pathway (2)*.
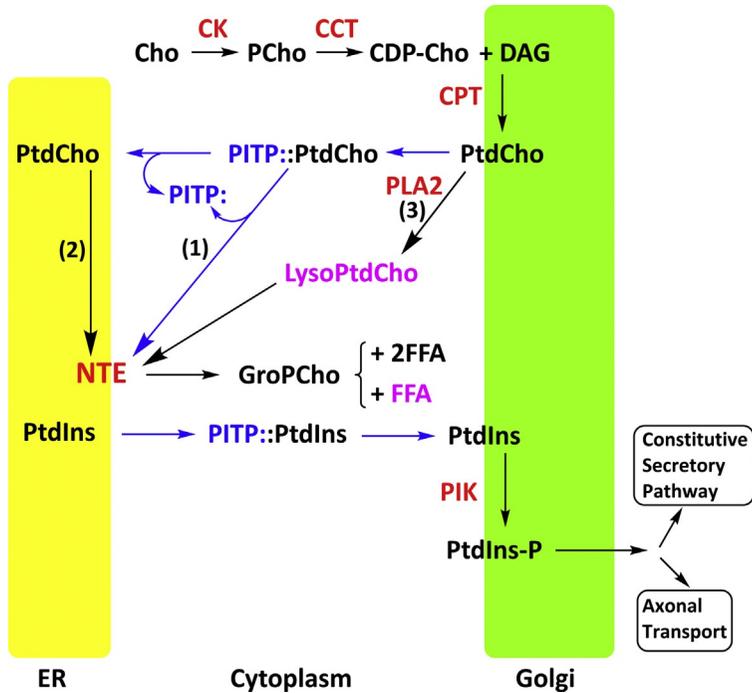
Fig. 14 Phospholipid homeostasis disruption (PHD) model of the role of NTE in OPIDN. Partial pathways of phospholipid metabolism in a neuron are shown. Choline (Cho) taken up by the cell is phosphorylated by choline kinase (CK) to phosphocholine (PCho), which is then converted to cytidine diphosphate-choline (CDP-Cho) via cytidine triphosphate phosphocholine cytidyltransferase (CCT) catalysis. The final step carried out in the Golgi involves addition of a diacylglycerol (DAG) to CDP-Cho catalyzed by choline phosphotransferase (CPT) to form phosphatidylcholine (PtdCho). The PtdCho product forms a complex with a phosphatidylcholine/phosphatidylinositol transport protein (PITP), which conveys PtdCho to the endoplasmic reticulum (ER), releasing uncomplexed PITP back into the cytoplasm. In *Pathway (1)*, some fraction of the PtdCho being transported to the ER is deacylated by NTE to glycerophosphocholine (GroPCho) and two free fatty acids (FFA), releasing free PITP in the process. *Pathway (2)* involves deacylation of PtdCho in the ER to GroPCho +2 FFA. *Pathway (3)* depicts hydrolysis of PtdCho by a phospholipase A2 (PLA2) to lysophosphatidylcholine (LysoPtdCho), which is then further degraded by NTE to GroPCho+FFA. Phosphatidylinositol (PtdIns) in the ER is transported via a PITP to the Golgi, where it is phosphorylated by phosphatidylinositol kinases (PIK) to various phosphorylated PtdIns species (PtdIns-P), which help regulate the constitutive secretory pathway and axonal transport. *Colors*: blue, PITPs and their pathways; green, Golgi; magenta, LysoPtdCho and the FFA formed from its hydrolysis; red, enzymes; yellow, ER. See text for explanation of how NTE inactivation in this model could precipitate OPIDN (Carvou et al., 2010; Glynn, 2005, 2013; Marcucci et al., 2010; Ong et al., 2015).

Pathway (3) in Fig. 14 involves one or more non–NTE phospholipase A2 (PLA2) enzymes hydrolyzing PtdCho directly to LysoPtdCho, which would then be expected to be rapidly degraded by NTE to yield GroPCho and an FFA. However, if NTE were preferentially inhibited, LysoPtdCho could increase to injurious levels, leading to axonal degeneration (De et al., 2003). On the other hand, preferential inhibition of PLA2 activity would block LysoPtdCho formation, thus conferring protection against NTE inhibition (Wijeyesakere and Richardson, 2010). Indeed, inhibitors of various brain PLA2 enzymes are considered an important neuropharmacological class of agents for the treatment of a multiplicity of neurological disorders (Dennis et al., 2011; Farooqui et al., 2006; Ong et al., 2015).

10.2 OP-induced Wallerian degeneration (OPIWAD)

Fig. 15 depicts a simplified model of OP-induced Wallerian degeneration (OPIWAD). This model provides a conceptual framework for understanding potential actions of neuropathic and nonneuropathic NTE inhibitors in initiating or preventing axonal degeneration. Wallerian degeneration and its relationship to OPIDN are discussed below.

In his insightful histopathological observations of OPIDN histopathology produced by TOCP in hens, Cavanagh (1954) described the spatial-temporal evolution of lesions in the spinal cord and peripheral nerves as a process of primary axonal degeneration followed by demyelination that closely resembled Wallerian degeneration induced by cutting or crushing spinal cord tracts or peripheral nerves. He also noted that the lesions were similar to those seen in thiamine deficiency, which results in a slowing of energy metabolism that leads to degeneration of distal axons—the nerve cell components farthest from the cell body. In a follow-up study of OPIDN produced by DFP in cats, Bouldin and Cavanagh (1979a) described the lesions as a "chemical transection" of axons that precipitated Wallerian degeneration.

Originally described by Waller in 1850, Wallerian degeneration was considered for the next 139 years to be a passive degenerative process—a consequence of separating the distal axon from vital substances supplied to it by the cell body (Waller, 1850). Then, in 1989, investigators in Oxford University made the astounding discovery of a strain of mouse whose distal axons remained intact for relatively long periods following transection of a peripheral nerve (Lunn et al., 1989). Ultimately, the cause of this
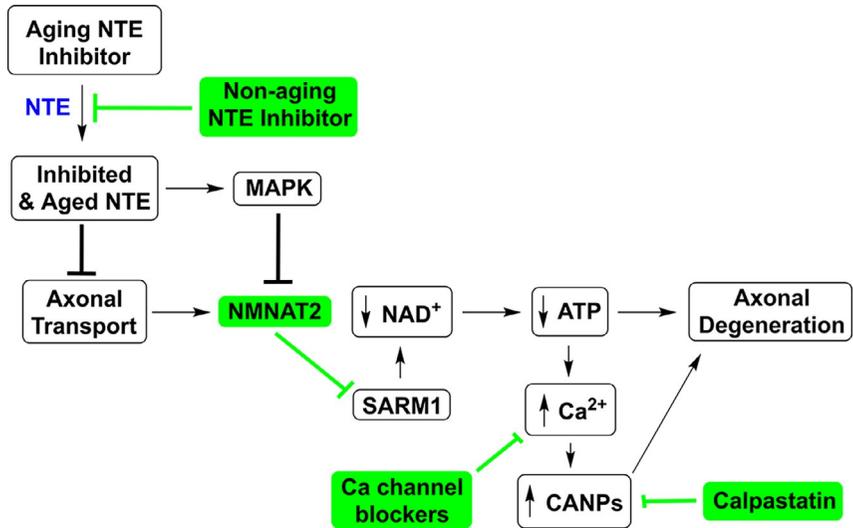
Fig. 15 Simplified model of OP-induced Wallerian degeneration (OPIWAD). Exposure to a neuropathic (aging) OP NTE inhibitor produces inhibited and aged NTE, which triggers Wallerian degeneration via inhibition of axonal transport and/or stimulation of mitogen-activated protein kinase (MAPK) and/or associated kinase cascade pathways. Nicotinamide mononucleotide adenylyltransferase 2 (NMNAT2) is a prosurvival factor continuously supplied to the axon via axonal transport; it blocks sterile alpha and toll-interleukin receptor (TIR) motif containing 1 (SARM1) from depleting nicotinamide adenine dinucleotide (NAD^+) levels. Activation of kinase cascades lowers NMNAT2 levels enabling SARM1 to deplete NAD^+, leading to ATP depletion. The resulting energy crisis increases Ca^{2+} concentrations from external and internal (ER and mitochondrial) sources, thereby activating calcium-activated neutral proteases (CANPs; calpains), which degrade axonal proteins resulting in axonal degeneration. Nonaging NTE inhibitors block formation of aged NTE, conferring protection against OPIDN by blocking initiation. In the execution phase of Wallerian degeneration, axonopathy can be ameliorated by Ca^{2+}-channel blockers and/or CANP inhibitors such as calpastatin. Components of the model that block axonopathy are shown in green; NTE is colored blue (Conforti et al., 2014; Rosell and Neukomm, 2019; Walker et al., 2017).

axonal resiliency was found to be a mutation, aptly named Wallerian degeneration slow (Wld^S). The discovery was serendipitous, as the mutation arose spontaneously and permitted detection coincidentally only after becoming homozygous. The revelation of Wld^S fundamentally changed our concept of Wallerian degeneration from a passive process to an active program of axonal self-destruction—a kind of local apoptosis confined to the axon (Gerdts et al., 2016).

Ultimately, it was established that Wld^S protects axons from degeneration in a gain of function manner by coding for a stable fusion protein that substitutes for the labile cytosolic nicotinamide mononucleotide adenyltransferase 2 (NMNAT2). This is an enzyme that catalyzes the formation of nicotinamide adenine dinucleotide (NAD$^+$), an essential cofactor for numerous enzymatic pathways, including those involved in redox reactions of energy metabolism and the production of the "energy currency" of the cell, adenosine triphosphate (ATP) (Coleman and Freeman, 2010). In a normal axon, NMNAT2 is degraded rapidly, but it is constantly resupplied from the cell body by axonal transport. Disruption of axonal transport by injury, disease, or certain neurotoxic agents interrupts the supply of NMNAT2, which quickly leads to NAD$^+$ depletion, followed closely by loss of ATP. The resulting local energy crisis permits intra-axonal calcium levels to rise sharply, thereby activating calcium–activated neutral proteases (CANPs; calpains), which mediate destruction of the axon. Given its central role as a prosurvival factor that helps maintain axons in a healthy state (Gilley and Coleman, 2010), NMNAT2 is tightly regulated by multiple pathways and processes including cAMP signaling, protein phosphorylation cascades, palmitoylation, and ubiquitin ligases (Geden and Deshmukh, 2016; Rosell and Neukomm, 2019).

Complementary studies have identified prodegenerative factors and pathways, including sterile alpha and Toll/interleukin-1 receptor motif-containing 1 (SARM1) and the p38–mitogen-activated protein kinase (MAPK) cascade. Loss of function mutations in SARM1 or blockades of the MAPK pathway confer protection against axonal degeneration induced by various triggers, including physical or chemical agents. Conversely, activation of SARM1 and/or the MAPK pathway triggers axonal degeneration, possibly by decreasing the stability of NMNAT2, thus leading to depletion of NAD$^+$ and ATP (Loring and Thompson, 2020).

Wallerian degeneration appears to be a biphasic process consisting of an initiation phase of early events contributing to NAD$^+$ depletion followed after a latent period by an execution phase (Chang et al., 2016; Coleman and Freeman, 2010). The precise events that mark the transition from one stage to another are still being investigated, but it appears that the execution interval entails increased intra-axonal Ca^{2+} concentrations and activation of CANPs that contribute to the dissolution of the axon (Conforti et al., 2014; Song and Xie, 2012). However, calcium entry and/or release of calcium from internal storage sites such as the ER and mitochondria could also occur during the initiation phase, even in toxic neuropathies

that do not involve physical trauma to axons. For example, at 24 hours after a neuropathic dose of TOCP in hens, CANP activity was significantly elevated in sciatic nerve, and administration of the calcium-channel blocker, nimodipine, within 24 hours after TOCP was effective at normalizing CANP activity measured at 24 hours and 28 days post-TOCP. This treatment was partially effective at ameliorating clinical signs of OPIDN assessed 28 days post-TOCP (Emerick et al., 2010).

Additional pathways and targets that modulate initiation and/or execution of Wallerian degeneration are being continually discovered. Among these are as-yet unidentified serine proteases whose inhibition delays neurite degeneration in primary neuronal cultures (Conforti et al., 2014). Not surprisingly, mitochondria have been implicated in Wallerian degeneration (Barrientos et al., 2011), and studies are ongoing to determine whether mitochondrial perturbations correspond to cause or effect as well as to initiation, execution, or both phases of axonal degeneration (Conforti et al., 2014). However, recent work indicates that inhibition of mitochondrial respiration is a downstream event in axonal degeneration triggered by activation of SARM1 via c-Jun N-terminal kinase (JNK)-mediated phosphorylation (Murata et al., 2018).

Of particular relevance to OPIDN is the fact that many of the regulatory pathways in Wallerian degeneration involve a balance between the actions of protein kinases and phosphoprotein phosphatases to phosphorylate and dephosphorylate proteins (Ali et al., 2017; Araki and Wakatsuki, 2019; Murata et al., 2018). These processes can operate on both sides of the opposing forces of prosurvival and prodegenerative pathways. For example, it is conceivable that organophosphylation and aging of NTE could serve as a signal to initiate Wallerian degeneration, as proposed previously, but not yet rigorously tested (Richardson, 1984). Furthermore, chemical modification of NTE by nonaging adduction might confer protection against axonopathy by maintaining NMNAT2 levels or blocking activation of SARM1 or MAPK pathways (Walker et al., 2017). Apropos of these concepts, studies of organophosphylation of proteins by in vitro or in vivo exposures to OP insecticides or nerve agents have of course found OP adducts on esteratic Ser residues (Black and Read, 2013; Graham et al., 2016). In addition, these investigations have detected OP adducts on nonesteratic Thy, Tyr, and Lys residues of multiple proteins, including α- and β-tubulin and ubiquitin (John et al., 2010, 2018; Onder et al., 2018; Schmidt et al., 2014; Schopfer and Lockridge, 2018). Moreover, many of the OP-adducted residues are phosphorylation sites for protein kinases.

Other studies have shown that OP compounds can disrupt kinase-mediated phosphorylation in *Drosophila*, rat H9c2 cells, and mouse brain, resulting in hypo- or hyperphosphorylation of specific regulatory sites (Batista et al., 2016; Felemban et al., 2015; Zhu et al., 2010). In addition, OP compounds reacting directly with Lys residues in proteins can form isopeptide crosslinks with Glu or Asp residues, and tubulin modified in this manner forms high molecular weight intermolecular aggregates (Schmidt et al., 2014; Schopfer and Lockridge, 2018). Taken together, these findings clearly demonstrate the possibility that exposure to OP compounds could disrupt physiological or pathogenic processes that are regulated by protein phosphorylation and dephosphorylation events normally mediated by protein kinases and phosphoprotein phosphatases. Such events are now known to include the induction or prevention of Wallerian degeneration. As noted previously, NTE has at least six nonesteratic phosphorylation sites (UniProt, 2019a), and these could be candidates for disruption by organophosphylation.
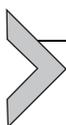
11. Conclusion

Despite the considerable advances that have been made in understanding the physiological and pathogenic roles of NTE since its discovery, there remains a major obstacle for making further progress—the lack of a 3D structure of the protein. The fact that NTE is a large membrane-bound and multi-domain molecule hinders structural determination by X-ray crystallography. Nevertheless, solving its individual domains can be undertaken using X-ray and NMR techniques (Puthenveetil and Vinogradova, 2019), and determining how its domains interact with each other can be approached by combining site-directed fluorescence, chemical crosslinking, isotope exchange, and mass spectrometry (Hodge et al., 2019; Raghuraman et al., 2019; Xiang et al., 2020). In addition, cryogenic electron microscopy has recently emerged as an ideal technique for solving the structures of "difficult" proteins, and its resolution is now approaching that of X-ray methods (Nwanochie and Uversky, 2019; Schur, 2019). In the meantime, our work on the structure and dynamics of NTE is in progress using a coordinated suite of computational molecular modeling approaches, including iterative threading, global exploration with loop perturbation, and coarse-grained molecular dynamics simulations (Liu et al., 2019; Singh and Lee, 2019). Subsequently, in silico mutagenesis and covalent docking will be used

to study alterations in structure, flexibility, and stability of NTE induced by mutations and chemical modification with aging vs. nonaging inhibitors (Doering et al., 2018; Scarpino et al., 2018).

Our current computational work on the NTE catalytic domain has shown that a patatin-like protein, PlpD, is a better template than patatin itself for creating a homology model (RJR, unpublished observations). PlpD is a virulence factor with phospholipase A1 activity secreted by *Pseudomonas aeroginosa,* a bacterium commonly associated with opportunistic and hospital-acquired infections (da Mata Madeira et al., 2016; Foulkes et al., 2019). Similarly, replication of coronaviruses and flaviviruses have been shown to involve manipulation of host phospholipid homeostasis via activation of phospholipase A2 enzymes, and inhibition of these activities compromises viral replication (Liebscher et al., 2018; Müller et al., 2018). These findings have suggested an entirely new direction for future work on NTE that should help inform studies of important human pathogens. Thirty years ago, M.K. Johnson published a "Contemporary Issues in Toxicology" article titled, "Organophosphates and Delayed Neuropathy— Is NTE Alive and Well?" This was a report on the status of our understanding of NTE and the mechanism of OPIDN along with an outline of how to use this knowledge for the intelligent and economic assessment of the relative neurotoxicological risks of exposures to OP compounds. He ended the article with words of encouragement that also served as a call to action for further work leading to ever-greater understanding (Johnson, 1990). It is fitting to requote his words here:

"The saying, 'If you do not use your lamp in the dark, whose fault is it that you stumble?' has been attributed to Gilbert of Newminster (ca. 1200–1250), and Winston S. Churchill in 1941 said, 'Give us the tools and we'll finish the job.' We have the 'lamp'. We have the 'tools'."

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