REVIEW ARTICLE

Neuropathy target esterase

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Neuropathy target esterase (NTE) is an integral membrane protein present in all neurons and in some non-neural-cell types of vertebrates. Recent data indicate that NTE is involved in a cell-signalling pathway controlling interactions between neurons and accessory glial cells in the developing nervous system. NTE has serine esterase activity and efficiently catalyses the hydrolysis of phenyl valerate (PV) *in vitro*, but its physiological substrate is unknown. By sequence analysis NTE has been found to be related neither to the major serine esterase family, which includes acetylcholinesterase, nor to any other known serine hydrolases. NTE comprises at least two functional domains: an N-terminal putative regulatory domain and a C-terminal effector domain which contains the esterase activity and is, in part, conserved in proteins found in bacteria, yeast, nematodes and insects. NTE's effector domain contains three predicted transmembrane segments, and the active-site serine residue lies at the centre of one of these segments. The isolated recombinant domain shows PV hydrolase activity only when incorporated into phospholipid liposomes. NTE's esterase activity appears to be largely redundant in adult vertebrates, but organophosphates which react with NTE *in vivo* initiate unknown events which lead, after a delay of 1–3 weeks, to a neuropathy with degeneration of long axons. These neuropathic organophosphates leave a negatively charged group covalently attached to the active-site serine residue, and it is suggested that this may cause a toxic gain of function in NTE.

Key words: active site, gain of function, neural development, neurodegeneration, protein family.

INTRODUCTION

Organophosphosphorus esters (OP) can cause at least two neurotoxic syndromes in humans and other animals. Best known is the acute, sometimes fatal, neurotoxicity which results from covalent inhibition of acetylcholinesterase (AChE) at cholinergic synapses. Deliberate induction of this form of toxicity in insects is exploited in the use of OPs as pesticides. The second syndrome, a neuropathy characterized by flaccid paralysis of the lower limbs, becomes evident 1–3 weeks after exposure to those OPs which covalently modify a neuronal protein, now known as neuropathy target esterase (NTE). Although neuropathic OPs paralysed many thousands of people during the 20th century, this syndrome is now very rare. In part this is because, despite the worldwide use of very large quantities of OP pesticides, those with neuropathic potential have been screened out of the market.

Why, then, continue studies of NTE and OP-induced delayed neuropathy (OPIDN)? First, because it is now becoming apparent that the normal physiological function of NTE is in neural development and involves a novel cell-signalling pathway. Secondly, because elucidation of the molecular mechanisms of OPIDN may supply valuable clues to the working of the adult nervous system in health and during other (non-toxic) neurodegenerative conditions. Thirdly, because NTE is an interesting protein in its own right: it is a member of a newly discovered protein family, with a domain conserved through evolution which may have a second function in addition to its capacity to hydrolyse esters.

Serine esterases: reactions with carboxylate ester substrates and OP inhibitors

AChE is a member of a large family of serine esterases with primary sequence similarity. This family includes enzymes responsible for the hydrolysis of a wide range of carboxyl ester substrates ranging from acetylcholine, to long-chain fatty-acid esters of cholesterol or triacylglycerol and to the juvenile hormones of insects [1,2]. A particular serine residue at the active site of these enzymes is rendered reactive by the presence of a histidine and a glutamate (or aspartate) residue, the components of the catalytic triad. Serine esterases catalyse hydrolysis of carboxylate esters by the mechanism shown, in a highly simplified form, in Scheme 1(a). This involves formation of a covalent acyl-enzyme intermediate. Nucleophilic displacement of the acyl group by the hydroxy moiety of water liberates the free enzyme to complete the catalytic cycle (Scheme 1a). This mechanism is essentially the same in serine proteases such as chymotrypsin, which cleave peptide bonds with intermediate formation of a covalent acyl-enzyme [3].

OP compounds act as pseudo-substrates for a variety of serine esterases and proteases. As an example, the reaction of an organophosphate with a serine esterase is shown in simplified form in Scheme 1(b). The rate of hydrolysis of the phosphorylated enzyme is greatly (several orders of magnitude) decreased compared with that for the acyl-enzyme. Thus the enzyme becomes virtually permanently inhibited, although agents more nucleophilic than water, such as certain oximes, can effect a speedier dephosphorylation. However, the phosphorylated enzyme can

Abbreviations used: NTE, neuropathy target esterase; PV, phenyl valerate; OP, organophosphorus esters; AChE, acetylcholinesterase; OPIDN, OP-induced delayed neuropathy; TOCP, tri-*o*-cresyl phosphate; DFP, di-isopropyl fluorophosphate; PDPP, phenyl dipentyl phosphinate; EPN, ethyl 4-nitrophenyl phenyl phosphonate; S9B, 1-(saligenin cyclic phosphoro)-9-biotinyldiaminononane; NEST, **N**TE-**est**erase domain; TM, transmembrane; SWS, Swiss-cheese; JHE, juvenile-hormone esterase; ER, endoplasmic reticulum; BG, β-glucuronidase.

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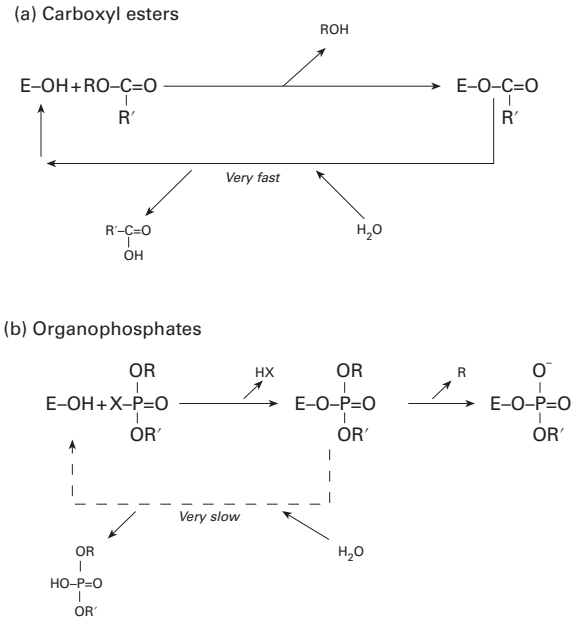

Scheme 1 Reaction of a serine esterase with carboxylate ester substrates and with organophosphate inhibitors

The reactive serine residue at the enzyme's (E) active site is represented by –OH. (**a**) The serine residue makes a nucleophilic attack on the acyl carbon of the ester and forms a tetrahedral hemiacetal intermediate (not shown). The alcohol moiety (ROH) is rapidly expelled from this intermediate to produce a covalent acyl-enzyme. Rapid aqueous hydrolysis of the acyl-enzyme liberates the carboxylic acid and regenerates free enzyme. (**b**) Part of the efficacy of OPs as serine-esterase inhibitors results from their structural resemblance to the tetrahedral hemiacetal intermediate formed between the enzyme and carboxylate ester substrates. The rate of hydrolysis of the organophosphorylated enzyme is much lower than that of the acyl-enzyme, resulting in essentially irreversible inhibition. In addition, organophosphates, but not organophosphinates, are able to undergo a second reaction, termed aging. This entails loss of one of the R groups from the organophosphorylated enzyme, leaving a negatively charged species attached to the active site.

subsequently undergo a second reaction, known as aging, which in the case shown of an organophosphate, results in the liberation of one of the bound OP's R groups (Scheme 1b). This leaves the active-site serine residue covalently attached to a negatively charged mono-organophosphoryl moiety which is significantly more resistant to removal by nucleophiles. Organophosphinates, in which both R groups are directly attached to the phosphorus atom, also covalently react with the active-site serine residue, but cannot undergo the aging reaction; consequently, esterases inhibited by organophosphinates do not carry a covalently bound negatively charged adduct and are more readily re-activated by oximes [3].

OPIDN, NTE and the mechanism of initiation

Early in 1930, thousands of people were paralysed in a poisoning epidemic in the Southern U.S.A. An OP, tri-*o*-cresyl phosphate (TOCP), was identified as the toxic agent and the adult chicken as the optimal animal model for studying this syndrome [4]. Leg weakness becomes apparent about 1 week after dosing chickens with TOCP and, by 2 weeks, birds are severely paralysed; histopathological examination reveals degeneration of long large-diameter axons in the spinal cord and peripheral nerves [5].

NTE was originally identified as a set of sites in chicken brain homogenates which reacted with radiolabelled di-isopropyl fluorophosphate (DFP) or hydrolysed a phenolic ester: the portion of the labelling or of the esterase activity which was resistant to a non-neuropathic OP but sensitive to a neuropathic one was defined as NTE [6–8]. Determination of the portion of phenyl valerate (PV) hydrolase activity in tissue homogenates which is resistant to paraoxon (a non-neuropathic OP) and sensitive to mipafox (a neuropathic OP) has become the internationally recognized assay for NTE [9].

Dosing chickens with various organophosphinates does not lead to OPIDN, even though NTE catalytic activity in brains of dosed birds was shown to be inhibited by $> 80\,\%$; furthermore, predosing with these phosphinates protects birds from OPIDN induced by a subsequent dose of DFP. These observations led Martin Johnson to postulate that only NTE inhibitors which can undergo the aging reaction (see Scheme 1b) are neuropathic and that generation of a negatively charged group at NTE's active site is essential for initiation of OPIDN [10]. Subsequently, much further evidence has accumulated to support this view for the initiation mechanism [11–14].

BIOCHEMICAL STUDIES ON NTE

Until quite recently all studies on NTE relied on detection of its PV hydrolase activity or labelling by [^{3}H]DFP. Using these methods it was shown that, in the adult chicken, the highest specific activities of NTE are found in brain, while spinal cord

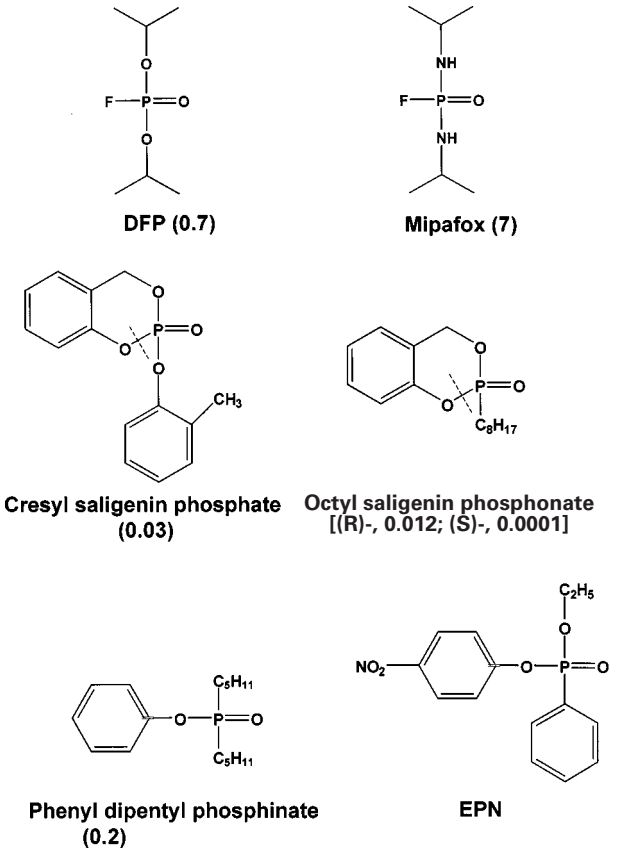

Figure 1 Some OPs used in studies of NTE

All OPs are shown with the leaving group X (see Scheme 1b) to the left-hand side of the phosphorus atom. The I_{50} values shown in parentheses (in μM) represent the concentration required in a 20 min preincubation with chicken brain homogenates to produce a 50% inhibition of NTE's PV hydrolase activity [8,23–26]. The broken line indicates the bond that is broken when saligenin cyclic OPs react with NTE.

and sciatic nerve contain substantially less. Relatively high levels of NTE are present in several non-neural chicken tissues, including intestine, spleen and thymus [11]. NTE is enriched in microsomal membrane fractions of chicken brain homogenates, yet comprises less than 0.1 % of total microsomal protein [15,16]. The catalytic-centre activity of NTE for PV is about 4000 s^{-1}, a rate approaching that of a diffusion-limited reaction [16].

NTE requires detergent for solubilization, and its phase-partioning characteristics indicate that it is an integral membrane protein [17]. The type and concentration of detergent used in the solubilization are important for the maintenance of NTE activity [18]. Fractionation of the solubilized material generally leads to substantial loss of NTE activity, which can be partially ameliorated by addition of phospholipids [19]. On SDS/PAGE, [^{3}H]DFP-labelled NTE runs as a 155 kDa polypeptide [16], while on gel filtration, detergent-solubilized NTE migrates as a complex with an apparent molecular mass of more than 850 kDa [20,21]. Radiation-inactivation experiments on chicken brain membranes indicate a target size of about 105 kDa for the catalytically active portion of NTE [22].

Some of the OP inhibitors frequently used in NTE studies are shown in Figure 1, together with an index of their *in vitro* potency, the I_{50} value determined with a standardized 20 min (37 °C) preincubation. The saligenin cyclic phosphorus esters are among the most potent OP inhibitors of NTE [25]. Cresyl cyclic saligenin phosphate is the active metabolite of TOCP, the agent which caused the 1930 poisoning epidemic in the U.S.A. [27]. The reaction of the saligenin cyclic phosphates with NTE is special in that there is no independent leaving group X (cf. Scheme 1b), but the P—O bond indicated in Figure 1 is broken to allow formation of the phosphorylated enzyme; the saligenin moiety is then shed during the aging reaction [26]. For chiral compounds like the cyclic saligenin OPs, NTE's active site shows a marked preference for one of the two stereoisomers, as has been shown for octyl cyclic saligenin phosphonate (Figure 1).

Phenyl dipentyl phosphinate (PDPP; Figure 1) and related phosphinates produce no obvious adverse effects in adult chickens, even when NTE activity is inhibited for prolonged periods by repeated dosing; these observations were important in formulating the proposal that the aging reaction is essential for initiation of OPIDN [10,24]. The importance of stereochemistry in the OP aging reaction with NTE has been demonstrated dramatically in the case of ethyl 4-nitrophenyl phenyl phosphonate (EPN) (Figure 1). The L-(−) isomer of EPN causes OPIDN, but the D-(+) isomer is not neuropathic, and, in fact, when dosed prophylactically, protects against a subsequent dose of the L-(−) isomer [28].

The aging reaction (cf. Scheme 1b) of [^{3}H]DFP-inhibited NTE is very rapid, with a half-life of a few minutes, and the aged isopropyl group is quantitatively transferred to a covalent acceptor site, dubbed 'site Z', within NTE itself This contrasts with a much lower rate of aging for DFP-inhibited cholinesterases, in which the aged isopropyl group is liberated into free solution [29]. More recently, Yoshida et al. [26] have investigated the reaction of chicken brain NTE with ^{3}H-labelled octyl cyclic saligenin phosphonate (cf. Figure 1); those authors reported that only about 15 % of the aged saligenin group was transferred to site Z.

MOLECULAR STUDIES ON NTE

Isolation and immunohistochemical localization

The low abundance and apparent requirement for membrane lipid to maintain NTE activity impeded its isolation for several years. A fraction substantially enriched in [^{3}H]DFP-labelled NTE, but far from homogeneous, was isolated from chicken brain [17]. An apparently homogenous NTE preparation from phospholipase A_2-solubilized embryonic chicken brain had a specific activity only about half that in the initial crude solubilized extract [30]. A breakthrough was finally achieved by the synthesis of a novel reagent, S9B [1-(saligenin cyclic phosphoro)-9-biotinyl-diaminononane], for affinity purification of NTE [31]. S9B reacted rapidly and specifically with NTE in brain microsomes, and this resulted in the covalent attachment, via a long alkyl spacer, of a biotin molecule to the active-site serine residue. Microsomal proteins, quantitatively solubilized by boiling in dilute SDS, were then subjected to affinity chromatography with avidin–Sepharose, which binds biotinylated polypeptides. S9B-labelled NTE was eluted from the avidin by boiling in SDS. Two polypeptides (carboxylases) with endogenous covalent biotin prosthetic groups which were co-eluted from avidin–Sepharose with S9B-labelled NTE were removed by subsequent preparative electrophoresis [31].

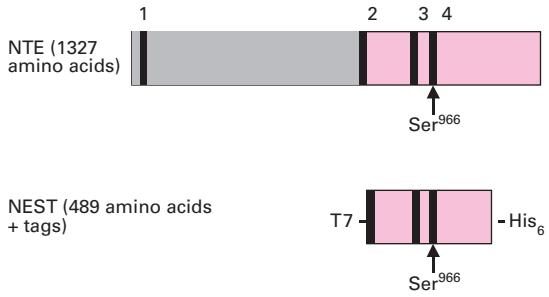

Figure 2 Predicted secondary structure of NTE and NEST

NTE is shown as a linear polypeptide of 1327 amino acids with two major functional domains: an N-terminal regulatory domain (grey), which contains regions with some similarity to cyclic AMP-binding proteins, and a C-terminal effector domain (pink) which contains the esterase activity. Four TM segments predicted by TM-pred analysis are shown as black vertical bars. The active-site serine residue (Ser^{966}) lies at the centre of putative TM segment number 4. NTE residues 727–1216 were cloned into a pET vector and expressed in *E. coli* with a short N-terminal (T7) tag sequence and a C-terminal His_6 tag: this construct, dubbed NEST, has all the OP-sensitive PV hydrolase activity of full-length NTE.

Isolated chicken NTE was digested with endoproteinase Glu-C and the resulting peptide fragments resolved by SDS/PAGE. The N-terminal amino acid sequence of one of these fragments provided sufficient information to synthesize an 11-residue peptide which was used to raise a rabbit antiserum to NTE [32]. An immunohistochemical survey of the chicken nervous system using this antiserum showed that NTE is present in essentially all neurons, but is absent from glia. NTE immunostaining could not be detected in normal sciatic nerve, but accumulated at the constriction site 8 h after nerve ligation, indicating that NTE undergoes fast axonal transport. NTE immunostaining filled neuronal cell bodies (except the nucleus) and sometimes extended into the proximal axon; this pattern, taken together with the biochemical data on NTE in microsomal fractions, indicated that NTE is probably associated with the endoplasmic reticulum (ER). These immunostaining characteristics were not detectably altered in chickens 1 or 3 days after treatment with a neuropathic OP, suggesting that OP-modified NTE is neither grossly redistributed nor degraded faster than native NTE [32].

Molecular cloning of human NTE: implications for structure and function

The N-terminal sequence of an endoproteinase Glu-C fragment of S9B-labelled pig brain NTE was found to be very similar to

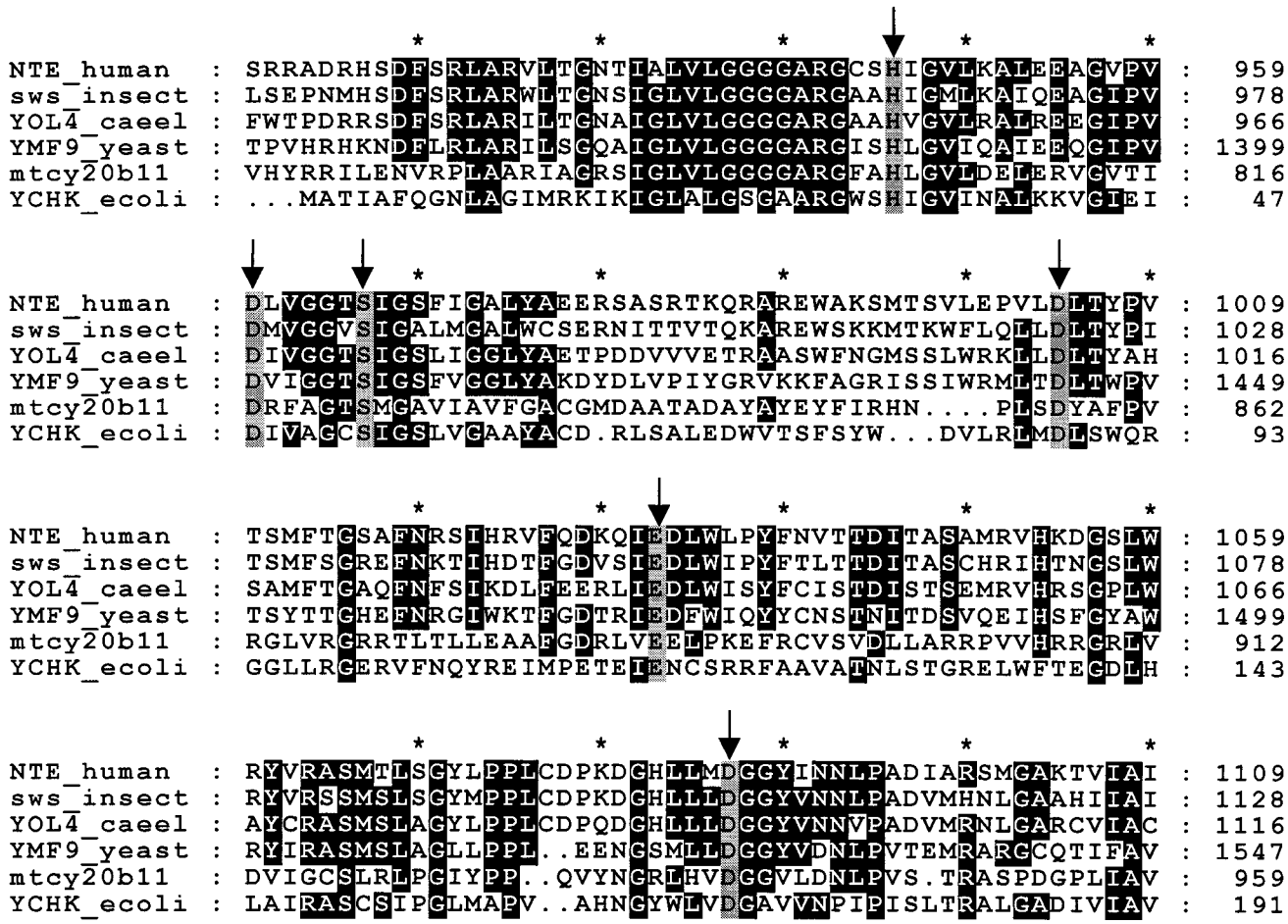

Figure 3 Alignment of amino acid sequences in the highly conserved C-terminal region of NTE with homologues from various species

Amino acids 910–1109 of NTE (complete sequence contains 1327 amino acids) are aligned with homologous regions from: SWS (*Drosophila*; 1425 amino acids), YOL4 (*C. elegans*; 1351 amino acids), YMF9 [*Saccharomyces cerevisiae* (baker's yeast); 1679 amino acids], MTCY20B11.14c (*Mycobacterium tuberculosis*; 1048 amino acids) and YCHK (*E. coli*; 314 amino acids). Residues identical in at least four of the proteins are shown white letters on a black background. The positions of the active-site serine (S) residue (Ser^{966}) and of conserved histidine (H), aspartic acid (D) and glutamic acid (E) residues are shown as black letters on a grey background and are also indicated by arrows.

the translated sequence of a human expressed-sequence-tag cDNA; the latter was used to initiate screening of human brain cDNA libraries, from which a full-length NTE cDNA clone was finally isolated [33]. The NTE cDNA clone D16 encoded a polypeptide of 1327 amino acids, and analysis of this sequence with the TM-pred program [34] indicated the presence of four potential transmembrane (TM) segments (see Figure 2). Biochemical experiments indicated that the active-site serine residue labelled by S9B lay between residues 955 and 1033, and attention was drawn to Ser^{966}, which lies in the motif G-X-S-X-G common to most known serine hydrolases [33].

Subsequently we have cloned portions of NTE cDNA into a pET vector and expressed the recombinant polypeptides in *Escherichia coli*. The minimum sequence expressing full OP-sensitive PV hydrolase activity corresponds to NTE residues 727–1216, and this construct has been dubbed 'NEST', for **NTE-est**erase domain (Figure 2). Ser^{966} has been confirmed as the active-site residue by [^{3}H]DFP labelling and protease digestion of NEST (J. Atkins and P. Glynn, unpublished work).

To the N-terminal side of NEST, NTE displays moderate to weak sequence similarity to cyclic nucleotide-binding proteins (protein kinase A regulatory subunit and cyclic nucleotide-gated ion channels). Provisionally it is suggested that NTE comprises at least two functional domains (Figure 2): an N-terminal regulatory domain and a C-terminal effector domain. The N-terminal domain may bind a cyclic nucleotide or similar molecule and thereby modulate the activity of the C-terminal effector domain. The most obvious potential function of the effector domain may be hydrolysis of a carboxyl ester, but other possibilities are discussed below.

Interestingly, Ser^{966} is at the centre of the fourth predicted TM segment in NTE [33]. Whether the segment of NTE containing the active-site serine residue is actually located within a membrane lipid bilayer is currently under investigation. If this proves to be the case, then, in order to achieve hydrolysis of the acyl-enzyme intermediate (Scheme 1a), the active-site serine residue of NTE would have to lie in an aqueous transmembrane pore. In turn, this structure would suggest that NTE's physiological function may not be due simply to its esterase activity. Recombinant NEST (which contains predicted transmembrane segments 2, 3 and 4; see Figure 2) is firmly associated with *E. coli* membranes, requires detergent for extraction, is insoluble in aqueous media lacking detergent, and the purified protein must be reconstituted into phospholipid vesicles to allow expression of PV hydrolase

activity (J. Atkins and P. Glynn, unpublished work). Thus at least one of the three predicted TM segments in NEST appears functional.

Members of the acetylcholinesterase family share a tertiary structural feature – the α/β hydrolase fold – with a variety of other proteins which hydrolyse a wide range of substrates; a common feature of these enzymes is that the nucleophilic catalytic residue (serine in AChE and carboxypeptidase II, cysteine in diene lactone hydrolase and aspartate in haloalkane dehalogenase) is situated at a point of a sharp elbow in the protein backbone between an α-helix and a β-sheet [35]. The crystal structures of a human triacylglycerol lipase and of a lipase from the yeast *Geotrichum* (both in the AChE family) have been solved: the catalytic serine residue in these lipases also is located at this characteristic elbow [36], and yet secondary-structure analysis by the TM-pred program [34] predicts that this serine residue lies within a TM segment. Clearly, great caution must be exercised in intrepreting such predictions of secondary structure. However, it is notable that while the human hepatic and *Geotrichum* lipases are soluble proteins which hydrolyse aqueous-insoluble substrates at a lipid/water interface, NEST (see above) appears to be an integral membrane polypeptide. Ongoing patch-clamp experiments aim to determine whether NEST does form a TM pore in phospholipid liposomes.

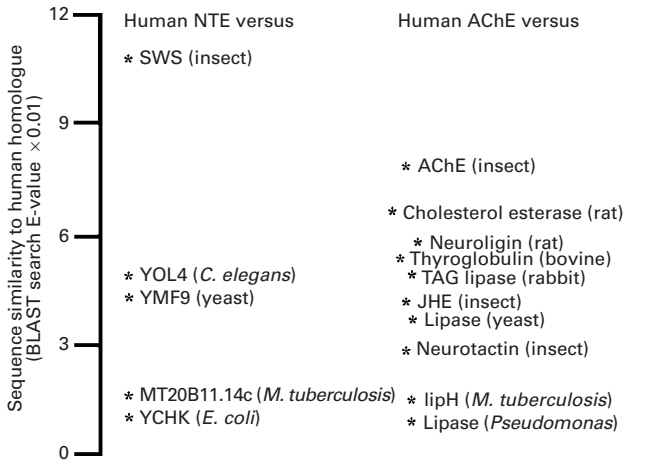

Figure 4 Comparison of the degree of similarity between human NTE (left) and human AChE (right) with homologues in their respective protein families

The vertical axis shows the E-values for each protein returned by a BLAST search [39] using either human NTE or human AChE as the input sequences; higher E-values reflect a greater degree of sequence similarity. Abbreviations: TAG lipase, triacylglycerol lipase; lipH, possible lipase (MTCY21B4.16c).

Clues to NTE's physiological function: the Swiss-cheese connection

Human NTE is very similar (41 % identical) to a *Drosophila* neuronal protein called Swiss cheese (SWS; [33]). In wild-type flies, developing neurons are ensheathed by a single layer of the plasma membrane of accessory glial cells. Mutations in the SWS protein cause neurons to become loosely wrapped with several layers of glial-cell membrane. This breakdown in the normal interaction between neurons and glia leads to apoptosis of both cell types. The name 'Swiss cheese' derives from the vacuolated appearance of the mutant brains [37]. It has been suggested that the SWS protein is involved in a cell-signalling pathway between developing neurons and glia. Attention has been drawn to an N-terminal domain of SWS (also present in NTE; see above) with some resemblance to the cyclic AMP-binding regulatory subunit of protein kinase A [37]. In addition to sharing sequence similarity, biochemical assays have shown that NTE-like PV hydrolase activity is present in wild-type *Drosophila*, but absent from *sws* mutants (P. Glynn and D. Kretzschmar, unpublished work).

From the above it seems likely that SWS is the *Drosophila* homologue of NTE and, by analogy, NTE may mediate cell signalling in the developing vertebrate brain. *In situ*-hybridization experiments on mouse embryos show that NTE mRNA is expressed in neurons from their earliest appearance in the developing nervous system [38]. Attempts are now underway to create NTE knock-out mice to determine whether these animals will show a neurodegenerative phenotype similar to that of *sws* mutant flies.

NTE is a member of a novel protein family

Sequence database searches revealed that NTE is not related to any known serine esterases or proteases, but, in addition to its close similarity with *Drosophila* SWS, it shares similarity to a number of polypeptides predicted from the sequencing of genomes of bacteria, yeast and nematodes [33]. In particular, a 200-amino-acid domain, corresponding to NTE residues 910–1109, is highly conserved (29 % identity between human NTE and the *E. coli* homologue YCHK), and, notably, all the homologues contain a serine residue in the same position as Ser^{966} of NTE. In addition, a completely conserved histidine and several acidic (aspartic acid and glutamic acid) residues are found within this domain, which, together with the serine residue, could comprise a catalytic triad of the kind found in conventional serine hydrolases (Figure 3; [33]).

Thus NTE is a member of a novel protein family which appear to be potential serine hydrolases. However, while recombinant fragments of both NTE (residues 727–1216) and SWS (residues 746–1235) show substantial PV hydrolase activity when expressed in the pET vector/*E. coli* system, we have been unable to detect PV hydrolase activity for analogous portions of the bacterial or yeast homologues expressed in the same system (Figure 3; J. Atkins, Y. Li and P. Glynn, unpublished work). The degree of sequence similarity between members of the NTE protein family is compared with that between members of the AChE family in Figure 4. *Drosophila* SWS appears to be as closely related to human NTE as *Drosophila* AChE is to human AChE – supporting the contention that SWS is the insect homologue of NTE; as enzymes, both NTE and SWS can hydrolyse PV and react with DFP. On the other hand, YOL4 (from the nematode worm *Caenorhabditis elegans*) and YMF9 (yeast) are no more related to human NTE than insect juvenile-hormone esterase (JHE) is to human AChE (Figure 4). Although both are clearly esterases, JHE and AChE have mutually exclusive ester substrates and widely differing reactivity with DFP. By analogy, it is possible that YOL4 and YMF9 have esterase activity, but their substrate and OP specificities remain to be determined.

At the lower end of the similarity scales in Figure 4, both NTE and AChE have related proteins in bacteria. The NTE-related protein in *E. coli*, YCHK (also known as RssA) is transcribed in an operon which also encodes a protein RssB [40]. RssB is involved in promoting the post-translational proteolytic turnover of the sigma-S subunit of RNA polymerase [41]. In actively growing *E. coli*, sigma-S turns over extremely rapidly ($t_{\frac{1}{2}} =$ 2 min), but under certain adverse conditions, such as sudden starvation due to exhaustion of glucose in the medium, it is

substantially stabilized [42]. The mechanisms whereby RssB activity is modulated by glucose starvation and whereby RssB promotes sigma-S proteolysis are unknown, but there is evidence that YCHK acts in the same pathway as RssB [41]. In broad terms, YCHK plays a role in a signalling pathway which regulates cellular responses to environmental stimuli.

THE ROLE OF NTE IN OPIDN: LOSS OF AN ESSENTIAL NON-ESTERASE FUNCTION OR A TOXIC GAIN OF FUNCTION?

The close similarity between NTE and *Drosophila* SWS suggests that NTE may have an important function during brain development, through involvement in a cell-signalling pathway. The role of NTE in OPIDN is a rather different issue. It has long been clear that prolonged simple inhibition of NTE's esterase activity by repeated dosing with non-aging organophosphinates over periods of 2 weeks has no obvious adverse effects in the adult chicken [12]. Thus it may be that, while NTE's esterase activity is required during development, this function is largely redundant in the adult animal.

As discussed above, the essential event in initiation of OPIDN by neuropathic OPs is the generation of a negative charge at the active site of NTE (cf. Scheme 1b). This suggests that either (1) neuropathic OPs cause loss of a non-esterase function of NTE which is required by neurons and/or their axons, or (2) NTE has no essential role in the adult animal, but placing a negative charge at its active site induces a toxic gain of function which initiates events culminating in neuropathy.

What could be a putative non-esterase function of NTE? At this point we can only speculate from analogy. It is interesting that several members of the AChE protein family (see Figure 4) lack the catalytic serine residue and so are not functional esterases [1,2]: thyroglobulin is a polypeptide hormone, neuroligins are a cell-surface proteins which bind neurexins and are involved in nerve-synapse formation in mammalian brain [43], and neurotactin [44,45] is a TM protein which mediates cell–cell interactions in the developing nervous system of *Drosophila*. Thus, in some members of the AChE protein family, the esterase domain (of about 400 amino acids; [1]) has evolved for a protein-binding, rather than a catalytic, function.

While their substrates have not yet been identified, all the known members of the NTE protein family do have a conserved serine residue in the same position as Ser^{966} of NTE (Figure 3) and so are at least potential esterases. However, even AChE itself appears to have neuromodulatory functions independent of its catalytic site [46], and it seems likely that these would be mediated by protein-binding interactions. An interesting example of the modulation by OPs of a protein-binding function of a serine esterase is that of egasyn, a carboxyl esterase present in the lumen of the ER of mouse liver. About 10 % of the egasyn is present as a non-covalent complex with β-glucuronidase (BG), so that about 30–50 % of cellular BG is retained in the ER while the remainder reaches the lysosomes. Reaction of the catalytic serine of egasyn with OPs, including paraoxon and DFP, releases BG from the complex so that it is secreted from the liver into the blood [47,48].

Whether NTE has any protein-binding capability which is inhibited by neuropathic (but not by non-neuropathic) OPs is under investigation; for the present, it is noteworthy that all the OPs shown to disrupt the egasyn–BG interaction are those which can undergo the aging reaction (Scheme 1b). However, the converse of this situation, that is, that reaction with a neuropathic OP could lead NTE to acquire a protein-binding function that it normally lacked, also merits consideration. The interaction of the cellular protein cyclophilin with the fungal metabolite cyclosporin A (CsA) provides a classic example of a gain of protein-binding function. In this case the cyclophilin–cyclosporin A complex binds to calcineurin (a calmodulin-dependent phosphatase), inhibiting its activity and, in the T-lymphocyte at least, disrupts the calcineurin/NF-AT (nuclear factor of activator T-cells) signal-transduction pathway [49,50].

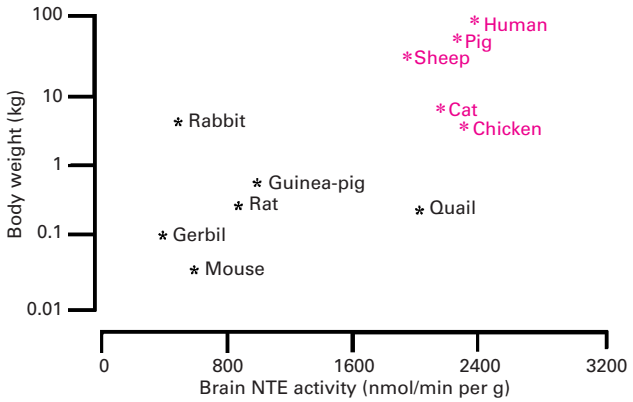

Figure 5 Species variation in body weight, brain NTE activity and susceptibility to OPIDN

Species susceptible to induction of OPIDN by a single dose of a neuropathic OP are shown in red. Data are from [11] and D. J. Read and P. Glynn (unpublished work).

Thus loss of an essential protein-binding function, or gain of a novel (and ultimately toxic) protein-binding function, are two theoretical consequences of the generation of a negative charge at NTE's active site by neuropathic OPs. An alternative function which might be lost or gained (or amplified) is that of a TM pore in the neuronal/axonal ER.

Consideration of the variation in susceptibility to OPIDN between different animal species (Figure 5) may also provide clues as to whether the initiation process involves a loss or a gain of function in NTE. Susceptible species are generally larger, and hence have longer axons, than resistant species. However, an additional consistent observation is that animals with relatively low levels of NTE (< 1000 nmol/min per g) are relatively resistant to OPIDN. Furthermore, in mice (a particularly resistant species), NTE appears to turn over faster ($t_{\frac{1}{2}} = 2$ days; D. J. Read and P. Glynn, unpublished work) than in chickens ($t_{\frac{1}{2}} = 4$–5 days; [12,24]). This would be consistent with a mechanism whereby a certain absolute level of OP-modified NTE must be achieved and then maintained for a finite period in order to initiate OPIDN. At the cellular level, neurons susceptible to OPIDN may be those with both high levels of NTE and the high energy demand required to maintain axons with volumes many times times greater than their own cell bodies. We are now attempting to generate transgenic mice expressing very high levels of NTE to determine whether these animals show a heightened susceptibility to OPIDN.

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