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Constructs of human neuropathy target esterase catalytic domain containing mutations related to motor neuron disease have altered enzymatic properties

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Abstract

Neuropathy target esterase (NTE) is a phospholipase/lysophospholipase associated with organophosphorus (OP) compound-induced delayed neurotoxicity (OPIDN). Distal degeneration of motor axons occurs in both OPIDN and the hereditary spastic paraplegias (HSPs). Recently, mutations within the esterase domain of NTE were identified in patients with a novel type of HSP (SPG39) designated NTE-related motor neuron disease (NTE-MND). Two of these mutations, arginine 890 to histidine (R890H) and methionine 1012 to valine (M1012V), were created in human recombinant NTE catalytic domain (NEST) to measure possible changes in catalytic properties. These mutated enzymes had decreased specific activities for hydrolysis of the artificial substrate, phenyl valerate. In addition, the M1012V mutant exhibited a reduced bimolecular rate constant of inhibition (k_i) for all three inhibitors tested: mipafox, diisopropylphosphorofluoridate, and chlorpyrifos oxon. Finally, while both mutated enzymes inhibited by OP compounds exhibited altered time-dependent loss of their ability to be reactivated by nucleophiles (aging), more pronounced effects were seen with the M1012V mutant. Taken together, the results from specific activity, inhibition, and aging experiments suggest that the mutations found in association with NTE-MND have functional correlates in altered enzymological properties of NTE.

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Conflict of Interest: JKF and SRR jointly hold patent #7384748 B2 that applies to the use of neuropathy target esterase (NTE) gene analysis for diagnosis of neurologic disease; however, the patent has not been licensed and no royalties have been obtained. RJR is a co-inventor on a patent application #12/121,389 on a nanostructured biosensor containing neuropathy target esterase activity that has potential applications for detection of neuropathic organophosphorus compounds; however, the patent has not been issued and no income has been received. NDH received partial support from a research grant from Dow Chemical Company, and RJR had a research grant and consultancy from Dow, which manufactures chlorpyrifos. The active metabolite, chlorpyrifos oxon, is a compound discussed in the manuscript; however, Dow had no involvement in the study design; collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

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Keywords

Hereditary spastic paraplegia (HSP); motor neuron disease (MND); neuropathy target esterase (NTE); organophosphorus compound-induced delayed neuropathy (OPIDN)

1. Introduction

Neuropathy target esterase (NTE) was first recognized as the site of initiation of organophosphorus (OP) compound-induced delayed neurotoxicity (OPIDN), a sensorimotor distal degeneration of long large-diameter axons in peripheral nerves and spinal cord (Davis and Richardson, 1980; Johnson, 1969). The pathogenesis of OPIDN is consistent with a transection-induced Wallerian-type distal axonopathy (Bouldin and Cavanagh, 1979), an active and wave-like pattern of degeneration that progresses in a proximal-to-distal direction (Beirowski et al., 2005). Clinical manifestations of OPIDN become apparent 1-4 weeks after exposure to a neuropathic OP compound and include paresthesias in the distal extremities, sensory loss, ataxia, and flaccid paralysis. Eventual reinnervation of muscles with persistent lesions in descending upper motor neuron axons leads to spastic paralysis (Lotti and Moretto, 2005; Richardson, 2005). OPIDN is associated with the inhibition of >70% of NTE by a neuropathic compound and aging of the NTE-OP conjugate, processes that can be measured using the artificial substrate phenyl valerate (PV) (Johnson, 1977).

Although the precise physiological function of NTE and its pathogenic role in OPIDN are unknown, it has been proposed that disruption of NTE activity by OP inhibition and aging could produce a perturbation in the metabolism of important membrane phospholipids, potentially leading to axonopathy (Quistad et al., 2003; Read et al., 2009; Vose et al., 2008). Indeed, given that NTE hydrolyzes membrane phospholipids with a preference *in vitro* for lysophosphatidylcholine (LPC) (van Tienhoven, et al., 2002), the enzyme has been functionally classified as a lysophospholipase (EC 3.1.1.5). However, there is also evidence from studies in yeast and mammalian cells that NTE acts as a phospholipase B, hydrolyzing phosphatidylcholine (PC) to glycerophosphatidylcholine (GPC) (Glynn, 2006; Zaccheo et al., 2004). Based on the homology of its catalytic domain to patatin, it has been named patatin-like phospholipase domain-containing protein-6, whose corresponding gene name is PNPLA6 (gene accession number AJ004832) (Lush et al., 1998; Uniprot, 2009; Wijeyesakere and Richardson, 2010).

A recent neurogenetic evaluation of two non-related families discovered three separate mutations within the esterase domain of NTE (NEST) in persons affected by a form of hereditary spastic paraplegia (SPG39 HSP) that was named NTE-motor neuron disease (NTE-MND) (Rainier et al., 2008). The first point mutation was discovered in a consanguineous kindred homozygous for methionine 1012 to valine (M1012V), an interspecies conserved residue. A genetically unrelated second subject had non-related compound heterozygous mutations; one was a point mutation resulting in an arginine 890 to histidine (R890H), and the other was an insertion that led to a frameshift mutation and a premature truncation. These individuals had developed axonopathies of distal central and peripheral motor neurons resulting in progressive spastic weakness and muscular atrophy, clinical features that are similar to OPIDN.

NEST is a highly conserved region within NTE found in bacteria, yeast, nematodes, *Drosophila*, and mice, which further suggests that mutations within the region could have severe neurological implications (Lush et al., 1998). Site-directed mutagenesis studies of human recombinant NEST have confirmed serine 966 as the active site serine and shown that two aspartate residues (aspartate 1086 and aspartate 960) appear to be essential for enzymatic activity (Atkins and Glynn, 2000). A homology model of the NTE catalytic domain indicates that serine 966 and aspartate 1086 form the catalytic dyad, and that aspartate 960 is predicted to lie on the surface of the molecule (Wijeyesakere et al., 2007). The use of recombinant NEST to study the effect of site-directed mutagenesis on NTE catalysis has proved to be a useful tool. Wild-type NEST (*wt*NEST) has been well characterized: it retains the catalytic properties of full length NTE; it is more easily solubilized for *in vitro* investigations; and it permits facile introduction of mutations via site-directed mutagenesis (Atkins and Glynn, 2000; Kohli et al., 2007). The present study was carried out to test the hypothesis that mutations in the sequence of human recombinant NEST found in association with NTE-MND will have functional consequences detectable as changes in the kinetics of substrate hydrolysis, inhibition, and aging.

2. Materials and Methods

2.1. Chemicals

N,N'-diisopropylphosphorodiamidofluoridate (mipafox, MIP) was synthesized by ChemSyn, and purchased from the Midwest Research Institute (Kansas City, MO) (99% pure by HPLC). Diisopropylphosphorofluoridate (DFP) was purchased from Sigma Aldrich (St. Louis, MO) (98% pure by GC). *O,O*-diethyl-3,4,5-trichloro-2-pyridyl phosphate (chlorpyrifos oxon, CPO) was furnished by Dow AgroSciences (Indianapolis, IN) (purity 96% by GC). Dioleoylphosphatidylcholine (DOPC) was purchased from Avanti (Alabaster, AL) (purity > 98% by GC). All other chemicals were reagent grade or the highest grade commercially available. Aqueous solutions were prepared in deionized distilled water. CPO, DFP, and MIP present a potential neurotoxic hazard. Therefore, these chemicals were handled in a chemical fume hood and all items that contacted them were decontaminated by soaking in 1 M NaOH overnight.

2.2. Site-Directed Mutagenesis

Site-directed mutagenesis was performed using the QuikChange II Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA) to create the NTE-MND mutations R890H and M1012V with primers purchased from Invitrogen. The primers used for the R890H mutation were forward 5'-GCACCTGCGCTGTCCGCACCGCCTCTTTTCGCGC-3', and reverse 5'-GCGCGAAAAGAGGCGGTGCGGACAGCGCAGGTGC-3'. The primers used for the M1012V mutation were forward 5'-CGTACCCAGTCACCTCCGTGTTCACTGGGTCTGCC-3', and reverse 5'-GGCAGACCCAGTGAACACGGAGGTGACTGGGTACG-3'. A polymerase chain reaction was set up using *Pfu* DNA polymerase (Stratagene). The plasmids were then replicated in competent DH5α *E. coli* cells, and isolated using QIAprep from QIAGEN. The mutations were verified by DNA sequencing at the University of Michigan Core Facility (Ann Arbor, MI).

2.3. Protein Expression and Purification

*wt*NEST and its mutant forms were expressed and purified using the protocol published by Atkins and Glynn (2000) with some modifications. The original *wt*NEST construct subcloned into pET21b(+) vector was kindly provided by Dr. Paul Glynn (MRC Toxicology Unit, University of Leicester, UK). Cultures of CD41 cells containing NEST plasmids were grown in terrific broth media at 37 °C until $OD_{600} = 1.0$, when the temperature was reduced to 20 °C and the cells were induced with 0.4 mM isopropyl- β -D-thiogalactopyranoside for 16 h (overnight). The cells were centrifuged for 12 min at $12,000 \times g$ at 4 °C and the pellets were stored at -80 °C.

Thawed cell pellets were resuspended in PEN buffer (50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl, and 0.1 mM EDTA) containing 3% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and sonicated at 30 sec intervals for 5 min with an output of 25 mA. The resulting mixture was centrifuged at $20,000 \times g$ for 60 min at 4 °C. The supernatant was absorbed onto nickel-nitrilotriacetic acid-agarose and washed three times with PEN buffer containing 0.3% (w/v) CHAPS and three times with wash buffer containing 10 mM imidazole. The protein was eluted with PEN buffer containing 0.3% (w/v) CHAPS and 300 mM imidazole. The protein was then diluted to 0.1 mg/mL, incorporated into DOPC liposomes by mixing at a 3:1 (w:w) ratio, and dialyzed 3 times in PEN buffer containing 1mM dithiothreitol at 4 °C (2h, 2h, overnight). The purification was monitored on SDS-PAGE, and protein concentration was determined using A_{280} on a NanoDrop ND-1000 UV/Vis spectrophotometer (Thermo Scientific, Wilmington, DE).

2.4. Determination of NEST Activity

The activity of NEST was determined by a colorimetric assay as described by Johnson (1977) and modified by Kayyali et al. (1991), without the use of inhibitors. All reactions were carried out at 37 °C for the entire assay. Aliquots (50 μ L; 25 - 2.5 ng) of NEST diluted in buffer containing 50 mM Tris-HCl and 0.1 mM EDTA, pH 8.0 at 25 °C, were added to 96-well plates. Substrate solution (100 μ L) of phenyl valerate (2.71 mM final) / *N,N'*-dimethylformamide diluted in 0.03% (w/v) Triton X-100 was added, and the reaction was allowed to proceed for 20 min. A stop solution of 100 μ L of 9.5 mg/mL sodium dodecyl sulfate / 11.2 mM 4-aminoantipyrine was added and the reaction with the phenol produced was allowed to proceed for 5 min. Finally, 50 μ L of 12.1 mM $K_3Fe(CN)_6$ was added and color was allowed to develop and stabilize for 10 min. The endpoint absorbance was measured at 486 nm using a SpectraMax 340 microplate reader (Molecular Devices, Sunnydale, CA).

2.5. Determination of NEST Inhibition and k_i

The apparent bimolecular rate constants of inhibition (k_i) of the OP inhibitors against the NEST enzymes were determined as previously described (Aldridge and Reiner, 1972; Kropp and Richardson, 2003; Kropp et al., 2004). The reaction with inhibitor (*I*) was carried out so that $[I] > 10$ [enzyme] and $[I] \ll K_d$. These conditions were used so that k_i could be measured using linear regression to model pseudo-first-order kinetics. The slopes of the primary linear kinetic plots of $\ln(\% \text{activity})$ vs time were the apparent first-order rate

constants of inhibition (k_i') for each $[I]$. The slope of the secondary linear kinetic plot of $-k_i'$ vs $[I]$ was the k_i for the inhibitor. The harmonic means (Raffa, 2002) of the 20-min IC_{50} values (inhibitor concentration required to produce 50% inhibition after incubation of inhibitor with enzyme for 20 min) were calculated from the equation, $IC_{50} = \ln(2)/(k_{i(m)} \times t)$, where $k_{i(m)} = \text{mean } k_i$ and time (t) = 20 min. The associated SEM values were calculated from the propagation of error for reciprocals (Harris, 2002).

2.6. Determination of Inhibited NEST Aging and k_4

Aging experiments and determination of the first-order rate constant of aging (k_4) were carried out similarly to previous methods for NEST and other esterases (Clothier et al., 1981; Jiangmongkol et al., 1999; Kropp et al., 2004). Aliquots of NEST were diluted using the same conditions as described for the activity determination, and all assays were performed at 37 °C. The aliquots were inhibited for 2 min with OP compounds at concentrations required to yield 90% inhibition using the equation ($IC_{90} = \ln(10)/k_i \times t$). The concentrations used were specific for inhibitor and enzyme. The enzyme solution was diluted 1:300 (v/v) with Tris-HCl buffer, pH 8.0 to stop inhibition and the aging reaction was allowed to proceed for timed intervals from 0 to 15 min. At each time point, 25 μ L of KF was added to 25 μ L of enzyme solution to yield a final KF concentration of 200 mM and enzyme was allowed to reactivate for 20 min. In parallel, 25 μ L KCl was added to 25 μ L aliquots of enzyme solution at each time point to serve as the inhibited nonreactivated control (final KCl concentration = 200 mM). An uninhibited KCl-treated NEST control was also measured. Enzyme activity was measured as described above. The first-order rate constant of aging (k_4) was determined by the slope of the linear plot of $\ln(100/\%$ reactivation) against time. The % reactivation was determined by the expression $[(AR_t) - (AI_t)]/[(AR_0) - (AI_0)] \times 100$, where AR_t is the activity of reactivated enzyme at t_{aging} , AI_t is the activity of inhibited enzyme without oxime or KF at t_{aging} , AR_0 is the activity of the reactivated enzyme at t_0 , and AI_0 is the activity of inhibited enzyme without oxime or KF at t_0 . Harmonic means (Raffa, 2002) of half-life ($t_{1/2}$) values were calculated from the equation $t_{1/2} = \ln(2)/k_{4(m)}$, where $k_{4(m)} = \text{mean } k_4$; associated SEM values were calculated from propagation of error for reciprocals (Harris, 2002).

2.7. Statistical Analyses

Except as otherwise specified, data are presented as means $\pm$ SEM, with $n = 3$ or 4 as noted. The specific activity for each NEST enzyme was calculated by three paired determinations of protein concentration and phenol produced. Comparisons of more than two means with a single factor were done with one-way analysis of variance (ANOVA) and the Tukey post hoc test for multiple comparisons. Comparisons of more than two means with two factors were done by two-way ANOVA and the Bonferroni post hoc test for multiple comparisons. The maximum p -value for statistical significance was $p < 0.05$ with lower p -values as indicated. Linear regressions and statistical analyses were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. Specific Activity

Human recombinant NEST was used to investigate the enzymological properties of NTE mutations found in patients with NTE-MND. The PV hydrolase specific activity, a measurement of esterase activity, of *wt*NEST was found to be 2.43 mmol phenol produced/mg protein/min (Table 1). The specific activities for the R890H and M1012V mutant forms of NEST were 71.6% and 63.9% of that for *wt*NEST, respectively. The specific activities of both mutant enzymes were reduced statistically from that of the wild type, but they were not significantly different from each other.

3.2. Kinetics of Inhibition

Because the PV hydrolase specific activities were significantly reduced in the two mutant forms of NEST compared to that of the wild-type enzyme, further characterization of the enzymological characteristics of these proteins were carried out, starting with an examination of the inhibitory potencies of three OP compounds: MIP, DFP, and CPO (Fig. 1) (Table 2). These OP compounds were chosen because they represent a variety of structures whose inhibitory potencies against NTE or NEST encompass a 100-fold range, and their interactions with these enzymes have been thoroughly established in the literature (Table 3).

Inhibitory constants for the R890H mutant were not significantly different from those for *wt*NEST (Table 2). In contrast, the inhibitor sensitivity of the M1012V mutant was significantly reduced for all three inhibitors relative to both *wt*NEST and the R890H mutant. The altered inhibitory potency against the M1012V mutant was especially pronounced for CPO, where the difference between *wt*NEST was approximately 12-fold.

3.3. Kinetics of Aging

The changes in kinetics of inhibition led us to investigate possible changes in post-inhibitory kinetics—specifically, the kinetics of aging. OP-inhibited *wt*NEST aged rapidly; MIP-inhibited enzyme could not be reactivated at pH 8.0 even at the shortest time point of 1.5 min, and the $t_{1/2}$ values for aging of DFP- and CPO-inhibited *wt*NEST, were 6.19 and 4.30 min, respectively (Table 4). The *wt*NEST activity for the KCl control was approximately 10% of the noninhibited activity; this was expected, because this control enzyme was inhibited by 90% and activity would normally not be restored in the absence of a strong nucleophile, such as KF (Fig. 2).

Surprisingly, the results of aging experiments did not permit calculation of a rate constant of aging for the M1012V mutant of NEST inhibited by any of the three inhibitors (Fig. 2). Activities were higher at all time points for samples treated with KCl than for those treated with the presumed nucleophilic reactivator, KF. In order to calculate a k_4 , the difference between the reactivated and non-reactivated enzymes must be positive. Because the activities were reversed, the kinetic constants were indeterminate (Table 4). Thus, these data reveal that the M1012V mutation exerts a drastic and unexpected effect on this aspect of NEST postinhibitory kinetics.

Kinetics results for aging of inhibited NEST were not as markedly altered by the R890H mutation as by the M1012V mutation (Fig. 2 and Table 4). Nevertheless, aging of MIP-inhibited R890H was indeterminate, because the KCl control activities were higher than the KF-treated samples for all time points. Moreover, although it was possible to calculate k_4 values for DFP and CPO based on the first 2-3 time points, activities for both inhibitors were higher in the KCl controls than in the KF-treated samples after approximately 6 min.

4. Discussion

Mutations of arginine (R890H) and methionine (M1012V) residues within NEST were discovered in patients with NTE-MND (Rainier et al., 2008). Although not previously the focus of homology studies of NTE, arginine 890 is conserved within the patatin domain of NTE in humans and mice; however, SWS of *Drosophila* and YOL4 in *C. elegans* contain a lysine at this residue. In contrast, the methionine 1012 residue is conserved across mouse, *Drosophila*, and *C. elegans*. Although patients with either mutation exhibited the NTE-MND phenotype, disruption of the more conserved methionine residue had a greater overall effect on the enzymological properties of NEST in terms of kinetics of inhibition and aging. However, in assessing the potential relative impact of the two mutations in these particular patients, it is important to consider gene dosage. Thus, the NTE-MND patient with the M1012V mutation was homozygous for this point defect; therefore, all of the expressed NTE would have this mutation. On the other hand, the NTE-MND patient with the R890H mutation had a compound heterozygous mutation paired with an insertion causing a frameshift and premature stop. The truncated protein is predicted to lack the last 235 residues of its catalytic domain and to be devoid of enzymatic activity. Consequently, the R890H form of NTE would be the only remaining enzymologically functioning protein from the two alleles. Therefore, the patient with the R890H mutation on one allele and the truncation mutation on the other allele would be expected to have half the amount of less severely affected NTE, whereas the patient with the M1012V mutation on both alleles would be expected to have the full complement of the more severely affected form of NTE. In order to test these predictions, it will be necessary to measure NTE activity in cells expressing the enzyme from the NTE-MND patients.

The *wt*NEST PV hydrolase specific activity of 2.43 mmol/min/mg obtained in this study (Table 1) is similar to the approximately 3 mmol/min/mg that has been previously reported (van Tienhoven et al., 2002). It is important to note that the data from the present study show that both of the NTE-MND mutations in the NEST domain produce a significant reduction of PV hydrolase specific activity in the mutant NEST constructs without abolishing activity altogether. Thus, conventional knockout of NTE is known to be embryonic lethal (Moser et al., 2004; Winrow et al., 2003), whereas conditional knockout permits survival but results in neurodegeneration (Akassoglou et al., 2004; Read et al., 2009). Clearly, certain other point mutations in NEST are known to eliminate PV hydrolase activity (Atkins and Glynn, 2000), but the NTE-MND mutations were not one of these.

Along with reduced specific activity, one of the NTE-MND mutations in NEST (M1012V) also produced changes in inhibition kinetics. MIP, DFP, and CPO were chosen as inhibitors because they are structurally different from one another and their reported inhibitory

potencies against NEST and NTE are spread over a 100-fold range. Fixed-time IC_{50} values for *wt*NEST calculated from the k_i values obtained in the present study (Table 2) align well with the medians of the ranges of published values (Table 3). Relative to *wt*NEST, the k_i values for R890H NEST were not significantly different. Accordingly, R890H NEST had IC_{50} values within the interquartile range of published values for the three inhibitors tested. In contrast, M1012V NEST had significantly lower k_i values for all three inhibitors compared to *wt*NEST. Moreover, the k_i reductions between M1012V NEST and *wt*NEST were substantial: 2.1-, 3.4-, and 12-fold for MIP, DFP, and CPO, respectively. Consequently, the corresponding IC_{50} values for all three inhibitors against M1012V NEST were above the ranges for all but one of the values previously reported for hen or human NTE or NEST. The lone exception was the calculated IC_{50} value for MIP-inhibited NEST gathered at 25 °C (Kropp et al., 2004); however, recalculating this reported value to account for the temperature difference (Aldridge and Reiner, 1972) would result in an adjusted IC_{50} of 7.5 μ M, which is closer to the median value of other published data. Overall, the results show that the R890H mutation had no significant influence on the inhibitory response of NEST to the compounds used in this study, whereas the M1012V mutation had a marked effect.

To explore further the deviations in catalytic properties of mutant forms of NEST from *wt*NEST, the aging of the OP-inhibited enzymes was investigated. The calculated $t_{1/2}$ of aging for *wt*NEST treated with DFP (6.19 min) (Table 4) is comparable to a previously reported literature value for NEST, which was 6.41 minutes (Kropp et al., 2004), as well as values reported for NTE, which were 3.25 and 7.4 minutes (Clothier and Johnson, 1979; Jokanovic et al., 1998). The rapid and complete aging of MIP-inhibited NEST at pH 8.0 was also previously reported (Kropp et al., 2004).

The rate constant of aging, k_4 , could be calculated from early time points for R890H NEST inhibited with DFP or CPO (Table 4); however, this value could not be calculated for MIP-inhibited R890H NEST or M1012V inhibited with any of the three inhibitors. This was because the KCl-treated enzyme, which was intended to serve as the nonreactivated species, was apparently being reactivated, at times greater than the KF-treated enzyme. The most notable deviation was seen with the MIP-inhibited enzymes, where *wt*NEST was clearly nonreactivable; the mutants were not only reactivated by KF, but the reactivation was higher by KCl (Fig.2). This unexpected behavior of the mutant enzymes could be due to a weakening of the OP-esterase bond, such that it could be hydrolyzed under simple aqueous dilution. We also investigated the possibility that 90% inhibition was not being reached at 2 min with the inhibitor concentrations used; however, a 2-min inhibition curve was performed to confirm that 90% inhibition was indeed being reached (data not shown). Therefore, it seems that the covalent bond between the serine and OP moiety in the inhibited NEST containing the mutations is more vulnerable to hydrolysis than it is in inhibited *wt*NEST. Further investigations into the reactivation of the NTE-MND mutant forms of inhibited NEST will be needed to test this hypothesis. In any event, it is apparent that both NTE-MND mutations have produced a marked change in the reactivation characteristics of inhibited NEST that can be readily seen following inhibition with the phosphoramidate, MIP.

Changes within conserved residues of enzymes can cause altered inhibitor and substrate binding, leading to disease, drug resistance, or drug sensitivity (Dussault et al., 2008; Hersey et al., 2009). Although the type of inhibitor profiling carried out in the present study has not previously been performed on NEST mutations, it has been done with another serine esterase, acetylcholinesterase, where mutations made within the acyl pocket surrounding the active-site gorge altered the inhibitor specificity, ultimately causing the enzyme to conform to the inhibitor profile of butyrylcholinesterase (Kovarik et al., 2003). The changes in specific activity along with inhibitory and postinhibitory properties observed with NEST mutants indicate that the NTE-MND mutations confer altered enzymological characteristics on the active center of NEST. Given that NEST is the catalytic domain of NTE, it is likely that the NTE-MND mutations would produce similar alterations in the enzymological properties of NTE. Such alterations have obvious toxicological implications, particularly for the M1012V mutant with its more pronounced effects. Thus, the predicted decreased sensitivity of mutated NTE to neuropathic inhibitors and its increased rate of reactivation suggest that affected individuals might be less susceptible to OPIDN, although this would be difficult to assess because they would be developing NTE-MND due to their already-altered NTE. In any event, these contentions will require studies of NTE activity in cells obtained from NTE-MND patients, investigations of full-length mutated protein, and examinations of animal models with NTE-MND mutations.

Although the physiological functions and pathogenic roles of NTE have not yet been definitively elucidated, it is clear from conventional and conditional knockout experiments that the protein is essential for survival of the developing embryo and maintenance of axons in adults (Akassoglou, 2004; Moser et al., 2004; Read et al., 2009). It is also becoming apparent that NTE participates in the regulation of phospholipid homeostasis necessary for maintaining cellular membrane integrity. For example, PC and LPC are both deacylated to GPC by NTE and NEST *in vitro*; and overexpression of NTE is protective against LPC toxicity, while decreased levels of NTE result in increased levels of PC and neurodegeneration (Mühlig-Versen et al., 2005; Read et al., 2009; Vose et al., 2008). In the present study, we have shown that NTE-MND mutations in NEST produce alterations in enzymological characteristics such as specific activity toward an artificial substrate as well as interactions with OP inhibitors. It will be of interest in future work to determine if the NTE-MND mutations also produce changes in the processing of PC and LPC by NTE, and to determine how such perturbations in lipid membrane metabolism lead to neurodegenerative disease.

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Fig 1.

Chemical structures of inhibitors. *O,O*-diethyl-3,4,5-trichloro-2-pyridyl phosphate (chlorpyrifos oxon, CPO), diisopropylphosphorofluoridate (DFP), and *N,N'*-diisopropylphosphorodiamidofluoridate (mipafos, MIP).

Fig 2.

Time course of residual activity of inhibited NEST in aging experiments. Enzymes are shown as column headings: *wt*NEST = wild type neuropathy target esterase catalytic domain (NEST); R890H = R890H mutant NEST; M1012V = M1012V mutant NEST. Inhibitors are shown as row titles: MIP = mipafox; DFP = diisopropylphosphorofluoridate; CPO = chlorpyrifos oxon. Values for each form of inhibited NEST are expressed as percent uninhibited control. Filled symbols (●) = KF-treated; open symbols (○) = KCl-treated. Data are means $\pm$ SEM, $n = 3$.

Table 1

Phenyl valerate (PV) hydrolase specific activity for wild-type neuropathy target esterase (NTE) catalytic domain (*wt*NEST) and the NTE-related motor neuron disease mutations, R890H and M1012V^a

	<i>wt</i> NEST	R890H	M1012V
PV specific activity ^b	2.43 ± 0.08	1.74 ± 0.02 ***	1.55 ± 0.05 ***
% <i>wt</i> NEST ^c	100.0 ± 4.9	71.6 ± 2.7 **	63.9 ± 3.0 **

^aData are means ± SEM, *n* = 3

^bPV specific activity is expressed as mmol phenol produced/mg protein/min.

^c% *wt*NEST specific activity calculated as the fraction of PV specific activity × 100. The associated SEM was calculated by propagation of error for quotients (Harris, 2002); the calculation included the SEM of the control (*wt*NEST).

*** R890H and M1012V specific activity significantly different from *wt*NEST (*p* < 0.001), but not significantly different from each other (*p* > 0.05).

** R890H and M1012V % *wt*NEST significantly different from *wt*NEST (*p* < 0.01), but not significantly different from each other (*p* > 0.05).

Table 2

Bimolecular rate constants of inhibition (k_i) and calculated 20-min IC_{50} values for MIP, DPF, and CPO against wild-type neuropathy target esterase (NTE) catalytic domain (wNTE) and its two NTE-related motor neuron disease mutant forms, R890H and M1012V^a

Inhibitor	Inhibitor Constant	Enzyme	
		wNTE	M1012V
MIP	k_i ($M^{-1}min^{-1}$)	$(4.36 \pm 0.13) \times 10^3$	$(2.09 \pm 0.14) \times 10^3^{***\dagger}$
	IC_{50} (μM)	7.95 ± 0.24	$16.6 \pm 1.11^{***\dagger}$
DFP	k_i ($M^{-1}min^{-1}$)	$(3.75 \pm 0.24) \times 10^4$	$(1.10 \pm 0.11) \times 10^4^{***\dagger}$
	IC_{50} (μM)	0.92 ± 0.06	$3.15 \pm 0.32^{**\#}$
CPO	k_i ($M^{-1}min^{-1}$)	$(1.54 \pm 0.34) \times 10^5$	$(1.28 \pm 0.05) \times 10^4^{***\dagger}$
	IC_{50} (μM)	0.23 ± 0.05	$2.71 \pm 0.11^{***\dagger}$

^aData are means $\pm$ SEM, $n = 4$ (wNTE) or 3 (R890H and M1012V). IC_{50} harmonic mean (Raffa, 2002) values were calculated using the equation, $IC_{50} = \ln(2)/(k_i(m) \times t)$, where $k_i(m)$ = mean k_i and time (t) = 20 min; IC_{50} SEM values were calculated by propagation of error for reciprocals (Harris, 2002). Two-way ANOVA for k_i was performed on log-transformed data. MIP = mipafox; DFP = diisopropylphosphorofluoridate; CPO = chlorpyrifos oxon.

*** Significantly different from wNTE ($p < 0.001$).

** Significantly different from wNTE ($p < 0.01$).

[†] Significantly different from R890H ($p < 0.001$).

[‡] Significantly different from R890H ($p < 0.01$).

[#] Significantly different from R890H ($p < 0.05$).

Table 3

Reported or calculated fixed-time IC₅₀ values for MIP, DFP, and CPO against neuropathy target esterase (NTE) or NTE catalytic domain (NEST)^a

Inhibitor	Reported or calculated IC ₅₀ (μM)	Median IC ₅₀ (μM); 25 th -75 th percentile
MIP	1.9 ^b , 6.1 ^c , 7.0 ^d , 7.0 ^e , 7.3 ^f 8.7 ^g , 9.6 ^h , 12 ⁱ , 14 ^j , 18 ^k ,	7.30; 4.45 – 10.8
DFP	0.56 ^c , 0.7 ^d , 0.96 ^g , 1.0 ⁱ , 1.2 ⁱ , 2.0 ^f	0.98; 0.66 – 1.40
CPO	0.069 ^l , 0.15 ^l , 0.18 ^m , 0.21 ⁿ , 0.24 ^o , 0.35 ^e	0.20; 0.13 – 0.27

^aIC₅₀ values reported from the literature or calculated from reported bimolecular rates of inhibition (*k_i*) using the equation $IC_{50} = \ln(2)/(k_i \times t)$ where time (*t*) is 20 min. All values were determined at 37 °C unless noted otherwise. MIP = mipafox; DFP = diisopropylphosphorofluoridate; CPO = chlorpyrifos oxon.

^bMacKay et al. (1996); 30-min IC₅₀ value for the solubilized microsomal fraction of embryonic chicken brain homogenate NTE.

^cAtkins and Glynn (2000); 20-min IC₅₀ values for hen brain homogenate NTE.

^dLotti and Johnson (1978); 20-min IC₅₀ values for hen brain homogenate NTE.

^eKropp and Richardson (2003); derived from *k_i* values for hen brain microsomal NTE.

^fNovak and Padilla (1986); 20-min IC₅₀ value for chicken brain homogenate NTE.

^gAtkins and Glynn (2000); 20-min IC₅₀ values for human recombinant NEST.

^hCorrell and Ehrich (1987); IC₅₀ value for chicken brain homogenate NTE; neither time nor temperature of incubation were indicated.

ⁱLotti and Johnson (1978); 20-min IC₅₀ values for human brain homogenate NTE.

^jEhrich et al. (1997); 60-min IC₅₀ values for differentiated human SH-SY5Y neuroblastoma cell NTE.

^kKropp et al. (2004); derived from *k_i* values for human recombinant NEST at 25 °C.

^lCapodicasa et al. (1991); 20-min IC₅₀ value for hen brain homogenate NTE.

^mCapodicasa et al. (1991); 20-min IC₅₀ value for human brain homogenate NTE.

ⁿRichardson et al. (1993); 20-min IC₅₀ value for hen brain homogenate NTE.

^oRichardson et al. (1993); derived from *k_i* value for hen brain homogenate NTE.

Table 4

Kinetic constants of aging for wild-type neuropathy target esterase catalytic domain (wtNEST) and its two motor neuron disease mutant forms, R890H and M1012V^a

Inhibitor ^b	Inhibitor Constant	Enzyme		
		wtNEST	R890H	M1012V
MIP	k_4 (min ⁻¹)	No reactivation ^c	Indeterminate ^d	Indeterminate ^d
	$t_{1/2}$ (min)	Indeterminate ^c	Indeterminate ^d	Indeterminate ^d
DFP	k_4 (min ⁻¹)	0.112 ± 0.037	0.408 ± 0.025 ^{***}	Indeterminate ^d
	$t_{1/2}$ (min)	6.19 ± 2.04	1.70 ± 0.10 [*]	Indeterminate ^d
CPO	k_4 (min ⁻¹)	0.161 ± 0.044	0.141 ± 0.028 ^e	Indeterminate ^d
	$t_{1/2}$ (min)	4.30 ± 1.18	4.91 ± 0.98 ^e	Indeterminate ^d

^aData are means ± SEM; except as noted below, $n = 3$. Harmonic mean (Raffa, 2002) of the half-life of aging was calculated from the equation $(t_{1/2}) = \ln(2)/k_4(m)$, where $k_4(m) = \text{mean } k_4$; associated SEM calculated by propagation of error for reciprocals (Harris, 2002).

^bMIP = mipatof; DFP = diisopropylphosphorofluoridate; CPO = chlorpyrifos oxon.

^cThere was no reactivation for MIP-inhibited NEST at any time point; therefore, $t_{1/2}$ could not be determined.

^dKCl control activities were higher than KF-treated activities; therefore, k_4 and $t_{1/2}$ were indeterminate (see Fig. 2 and text).

^e $n = 2$; indeterminate for the third pair of data points.

^{***}Significantly different from wtNEST ($p < 0.01$).

^{*}Significantly different from wtNEST ($p < 0.05$).