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Research Report

Reduction of neuropathy target esterase does not affect neuronal differentiation, but moderate expression induces neuronal differentiation in human neuroblastoma (SK-N-SH) cell line

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Abstract

Neuropathy target esterase (NTE) is inhibited and aged by organophosphorus compounds that induce delayed neuropathy in human and some sensitive animals. NTE has been proposed to play a role in neurite outgrowth and process elongation during neurodifferentiation. However, to date, there is no direct evidence of the relevance of NTE in neurodifferentiation under physiological conditions. In this study, we have investigated a possible role for NTE in the all-*trans* retinoic acid-induced differentiation of neuroblastoma cells. The functional inactivation of NTE by RNA interference indicated that reduction of NTE does not affect process outgrowth or differentiation of the cells, although moderate expression of NTE by expression of the NTE esterase domain accelerates the elongation of neurite processes. Mipafox, a neurotoxic organophosphate, was shown to block process outgrowth and differentiation in cells that have lowered NTE activity due to RNA interference, suggesting that mipafox may interact with other molecules to exert its effect in this context.

Theme: Disorders of the nervous system
Topic: Neurotoxicity, cell differentiation and migration

Keywords: Neuropathy target esterase; Neural differentiation; Human neuroblastoma cell; RNA interference; Organophosphate

1. Introduction

Neuropathy target esterase (NTE) was identified over 30 years ago as the primary site of action of organophosphorus compounds (OP) that cause a delayed paralyzing syndrome with degeneration of nerve axons [14]. Although the inhibition and subsequent aging of NTE has been proposed to be an initiating event in OP-induced delayed neuropathy (OPIDN), the events that occur between NTE inhibition and the appearance of clinical effects are not completely understood [10]. Elucidation of the molecular and cellular functions of NTE is a priority in understanding the pathogenesis of OPIDN.

NTE is a polypeptide of 1327 amino acids and is anchored to the cytoplasmic face of endoplasmic reticulum by an amino terminal transmembrane segment in mammalian cells and neurons [1,18]. The NTE esterase domain (NEST), which comprises residues 727–1216 of human NTE, reacts with an ester substrate and covalent inhibitors in a manner very similar to NTE [2]. NTE is a novel serine esterase protein that is highly conserved among various species including insects, nematodes, yeast and bacteria [20]. Recently, NTE was identified in mammalian cells as a novel phospholipase B responsible for converting phosphatidylcholine to glycerophosphocholine and also has potent lysophospholipase activity in mouse [26,33]. In mice, complete inactivation of the NTE gene resulted in embryonic lethality due to placental failure and impaired vasculogenesis [21,31]. Additionally, brain-specific deletion of

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NTE resulted in neurodegeneration, thus indicating that NTE is essential for embryonic and nervous development [1].

Cultured cells with neuronal properties and stable cell lines are potential models for studying morphological and biochemical damage from neurotoxic OPs that induce delayed neurotoxicity in animal models can inhibit outgrowth of axon-like processes in several differentiated cell lines, such as human neuroblastoma SH-SY5Y cells [13], mouse neuroblastoma N2a cells, rat adrenal pheochromocytoma PC-12 cells and brain glial tumor C6 cells [6–8,12,17,28,29]. As such, the inhibitory effect of neuropathic OP on neurite outgrowth in cell cultures has provided a standard in the screening for delayed neurotoxicity in vitro [12]. NTE is a preferred candidate target since two direct-acting NTE inhibitors, mipafox and 2-octyl-4*H*-1,3,2-benzodioxophosphorin 2-oxide, selectively inhibit cell process outgrowth and NTE is more sensitive than acetylcholinesterase (AChE) to the active agents [17]. Therefore, it has been proposed that NTE's role in cytotoxicity is both in neural function and the maintenance of neural integrity. As such, noncytotoxic biochemical disruption of NTE phosphorylation and aging may lead to inhibition of neurite and process outgrowth [17]. Chlorpyrifos, a metabolic precursor of NTE inhibitor, inhibits outgrowth of axons and also decreases the activity of NTE in co-differentiation mouse neuroblastoma N2a cells [28]. Human neuroblastoma (SH-SY5Y) cells have been widely used as an in vitro cell model to test the inhibitory effects of different OPs on neural differentiation and previous reports have shown that the NTE activity of SH-SY5Y cells is higher in differentiated cells than in undifferentiated cells [13,23]. These results suggest that NTE may play a certain role in differentiation of neuroblastoma cells.

SK-N-SH cells are a good in vitro cell model for studying the mechanism of neurotoxicity since they extend processes following retinoic acid treatment, maintain the properties of neuroblastoma cells and have higher NTE activity than PC-12 cells [22]. Therefore, SK-N-SH was chosen as cell model in this study. RNA interference (RNAi) has been a new effective way to inhibit the expression of gene in mammalian cells; pSUPER vector was used as a stable system to produce short interfering RNAs (siRNA) against NTE in mammalian cells [4,30,32]. The present investigation was designed to study the role of NTE in all-*trans* retinoic acid-induced neurodifferentiation by genetic methods. In addition, the relation between OP and its target was verified in this paper.

2. Materials and methods

2.1. Materials

The NTE cDNA clone D16 and the construct pNTE-GFP were kindly provided by Dr. Paul Glynn and Dr. Yong Li (The MRC Toxicology Unit, University of Leicester, UK). pSUPER and pcDNA3.1(+) vectors were purchased from Oligoengine (Seattle, Washington) and Invitrogen (Groningen, The Netherlands), respectively. The human neuroblastoma SK-N-SH cell line was purchased from the Cell Center of Chinese Academy of Medical Sciences. Cell culture reagents were obtained from Gibco (Grand Island, NY, USA). Human NTE-specific antibodies against a peptide corresponding to amino acids 37–48 (LPQEPPGSATDA) were generated by Shanghai Casarray Co. LTD (Shanghai, China). Paraoxon, all-*trans* retinoic acid (ATRA), monoclonal anti-HA antibody (clone HA-7), monoclonal anti-neurofilament 200 antibody (clone N52), anti-mouse IgG (Fc specific) peroxidase conjugate antibody and propidium iodide (PI) were obtained from Sigma (St. Louis, MO, USA). Horse fluorescein anti-mouse IgG (H + L) was purchased from Vector laboratories (Burlingame, CA, USA). Mipafox and phenyl valerate were synthesized in our laboratory. Transfection reagent lipofectamine 2000 was purchased from Invitrogen (Groningen, The Netherlands).

2.2. Cell culture

SK-N-SH cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and streptomycin. Incubations were carried out at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were maintained in the logarithmic phase of growth and subcultured at 3- to 4-day intervals.

2.3. DNA constructs

To construct a stable RNA interference expression vector of human NTE to be expressed in mammalian cells, a pSUPER neo vector was generated by cloning a *Spe*I- and *Xho*I-digested fragment from pSUPER, which contains the H1 RNA Polymerase III promoter and the multiple cloning site, into the compatible in the pcDNA3.1(+) vector. Next, NTEiF 5′-GATCCCC**CGTCCGGGTGCTGGGCCAC**TTCAAGAGA**GTGGCCCAGCACCCGGACG**TTTTTGGAAA-3′ and NTEiR 3′-GGG**GCAGGCCCACGACCCGGTG**AAGTTCTCT-**CACCGGGTCGTGGGCCTGC**AAAAACCTTTTCGA-5′ were annealed to form a double-strand DNA (NTEi) with *Hin*dIII and *Bgl*II sites at the 5′ and 3′ ends, respectively. NTEi was ligated into the *Hin*dIII and *Bgl*II sites of the pSUPER neo vector. The construct was verified by DNA sequencing and was shown to produce siRNA targeted against nucleotides 430–448 of NTE mRNA in mammalian cells.

To construct an expression vector for human NEST, PCR was used to add a *Bam*HI site and the influenza hemagglutinin (HA) tag in frame at the 5′ end with the following primers: NESTF 5′-TTGGATCCGCCATGGGCTACCCA-TACGATGTTCCAGATTACGCTACCCGCCTTATC-CACCTACTG-3′ and NESTR 5′-GCGAATTCTTACC-

GCCGGTCTGTGAGCATT-3′. This product was then cloned into the *Bam*HI and *Eco*RI sites of the vector pcDNA3.1(+) to make pcDNA-NEST. The construct, verified by DNA sequencing, contained the protein-coding sequence from 2331 to 3826 bp of NTE cDNA tagged with HA.

2.4. Stable expression cell clone

The two constructs (pSUPER-neo-NTE and pcDNA-NEST) and vectors (pSUPER-neo and pcDNA3.1(+)) were transfected into human neuroblastoma SK-N-SH cells with lipofectamine 2000 and selected with 500 μg/ml G418 for 3 weeks. Positive clones (SH/dsRNA-NTE cell) were confirmed by Western blotting and NTE activity assay. Stable clones were maintained in DMEM medium containing 200 μg/ml G418.

2.5. Immunoblotting

NTE expression levels in pSUPER-neo-NTE-transfected cell lines were evaluated by Western blotting. Cell extracts were prepared by washing cells twice with cold PBS, harvested in lysis buffer (50 mM Tris pH 7.5, 0.3 M NaCl, 5 mM EGTA, 1 mM EDTA, 0.5% Triton X-100, 0.5% NP40, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and a 10 μg/ml final concentration of each of aprotinin, leupeptin and pepstatin), and then sonicated on ice. Lysates were clarified, and the protein concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). For cells transfected with pcDNA-NEST, cells were harvested by trypsinization. Cell pellets were resuspended in TE buffer (50 mM Tris–HCl, 0.2 mM EDTA, pH 8.0), homogenized with 10 passages through a 25-gauge hypodermic needle and centrifuged at 100 × *g* at 4 °C for 2 min. The supernatant fraction was further centrifuged at 100,000 × *g* at 4 °C for 1 h. After removing the soluble cytosolic fraction, the particulate fraction was washed 1×, centrifuged and finally resuspended in the original volume of TE buffer. The protein concentrations were determined using the BCA assay. Lysates (40 μg) were electrophoresed on 10% SDS–polyacrylamide gels and then transferred to nitrocellulose and probed with primary antibody. Blots were washed and then incubated with horseradish peroxidase-conjugated secondary antibody. The Western blots were developed with using standard enhanced chemiluminescence (ECL) (Pierce, Rockford, IL, USA).

2.6. Northern blot

Total RNA was obtained from PBS-washed cell monolayers with Trizol. Total RNA (15–20 μg/lane) was denatured, electrophoresed through 1% agarose–formaldehyde gels and transferred to nylon membranes (Amersham Biosciences) following standard techniques. DNA probes were labeled with (α-^{32}P) dCTP (Amersham Biosciences, specific activity 3000 Ci/mmol) by random prime labeling (Prime-a-Gene Labeling System, Promega) (Madison, WI, USA). Hybridization was carried out overnight at 65 °C according to Church and Gilbert [5]. The filters were washed with 0.5× SSC, 0.1% SDS, first at room temperature for 10 min and then for 30 min at 65 °C. Membranes were exposed to X-ray films at −70 °C with intensifying screens.

2.7. NTE and AChE assay

Cellular NTE activity was determined in cellular homogenates as the difference between the colorimetric determination of the phenol liberated from the paraoxon-resistant (40 μM) and mipafox-sensitive (50 μM) hydrolysis of phenyl valerate, as previously described by Johnson [15], and expressed as nanomoles of phenyl valerate hydrolyzed per minute per milligram of protein with phenol as the standard. AChE activity was assayed as described by Gorun et al. [11]. Concentration of protein was measured by the method of Lowery et al. using bovine serum albumin as the standard [19].

2.8. Differentiation of SK-N-SH clones

To initiate ATRA-mediated differentiation, cells were grown in the DMEM medium containing 20 μM ATRA for 1 week in the dark with replacement of the conditioned media every 48 h. To assess the effect of lowered expression of NTE, cells were photographed in an inverted phase contrast microscope (DMRBE, Leica). Differentiated cells were quantified by counting at least 500 cells in randomly chosen fields. Cells were considered to be differentiated if they had at least one process longer than the cell body, which could be regarded as a neurite [27]. The results are expressed as percent of cells differentiated. The length of the longest neurite was measured in at least 100 cells in randomly chosen fields (40× magnification). At least three independent experiments were conducted with each clone and the results are expressed as mean ± SE.

2.9. Immunofluorescence analysis

Cells were initially seeded in Petri dishes containing 3-aminotriethoxysilane-coated glass slides and were induced to differentiate as described above. Slides containing the attached cells were initially fixed in 90% (v/v) methanol in TBS at −20 °C for 20 min. Cells were permeabilized for 10 min using 1% (v/v) Triton X-100 in TBS at room temperature. Slides were then washed in TBS and incubated in blocking solution [3% (w/v) bovine serum albumin (BSA) in TBS, BSA/TBS] for 1 h at room temperature. Cells were incubated with monoclonal NF-H antibody N52 (diluted as 1:100 in BSA/TBS) overnight in a humidified atmosphere. Following extensive washing in TBS/Tween-20 for a total of 1 h, cells were incubated with horse fluorescein anti-mouse IgG (H + L) (100 μ g/ml in

BSA/TBS) for 2 h at room temperature. Slides were again washed in TBS/Tween for 1 h before staining with 10 μ g/ml PI and mounting with anti-fade mountant. Cells were viewed using a TCS-4D laser scanning confocal microscope (Leica Microsystems).

2.10. Effect of OPs on SH/dsRNA-NTE cell clone

The SK-N-SH cells and the cell clone SH/dsRNA-NTE, which has lowered expression of NTE, were treated with 50 μM paraoxon or mipafox in 0.1% DMSO and induced to differentiate with 20 μM ATRA for 1 week in the dark with replacement of the conditioned media containing corresponding concentration OPs and ATRA every 48 h. SK-N-SH cells were treated with 0.1% DMSO and 20 μM ATRA as the control. After 1 week of treatment, cells were washed with cold phosphate-buffered saline (PBS) and fixed with 90% (v/v) methanol in PBS at −20 °C for 20 min and stained with Coomassie brilliant blue R-250 [1.25% (w/v) in 40% (v/v) methanol and 20% (v/v) glacial acetic acid in distilled water] for 1 min at room temperature. Stained cells were washed and left to air-dry for 24 h. The effect of OP on the neuronal differentiation was monitored as described above. The activity of NTE in cells was assayed as described above after treatment and washing 2× with cold PBS.

3. Results

3.1. Silencing of NTE in SK-N-SH cells

To generate stable cell lines that express siRNA constructs, pSUPER-neo, a derivative of the pSUPER vector, was constructed in order to allow selection by antibiotics in mammalian cells. In order to evaluate the role of NTE in neurodifferentiation, human neuroblastoma SK-N-SH cells were stably transfected with pSUPER-neo-NTE and named SH/dsRNA-NTE. As shown in Fig. 1A, the expression of NTE was virtually eliminated in SH/dsRNA-NTE cells since no NTE proteins could be detected by immunoblotting. The levels of NTE protein remained undetectable in SH/dsRNA-NTE cells upon longer exposure (data not shown). To measure the basal in situ NTE activity, we assayed the absorbance difference for phenyl valerate hydrolysis between samples exposed to paraoxon and paraoxon plus mipafox. In wild-type cells, the basal NTE activity was 15.92 ± 1.39 nmol phenol/min/mg

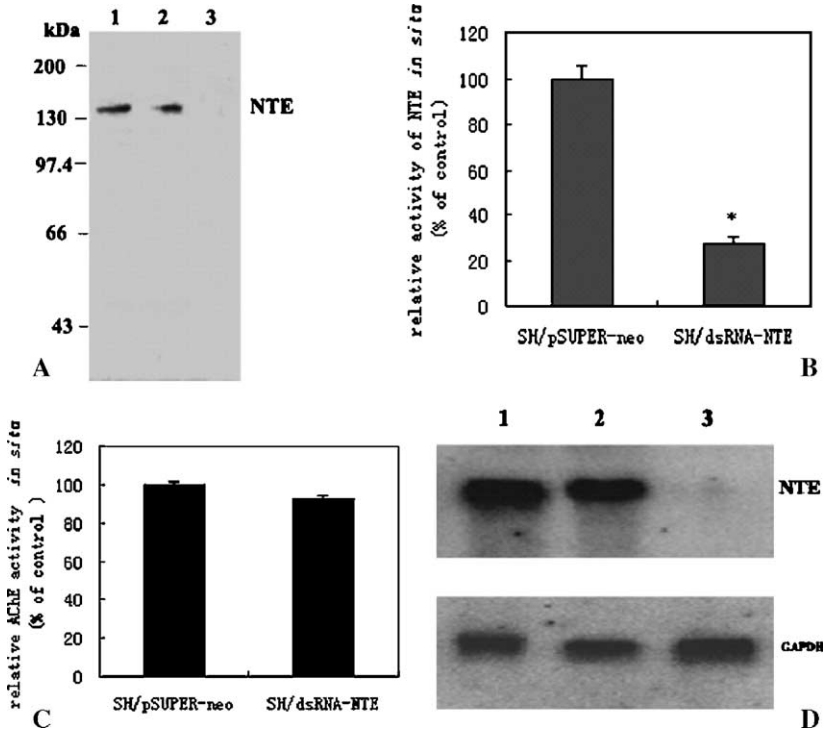

Fig. 1. Silencing of NTE in human neuroblastoma SK-N-SH cells. (A) Representative immunoblots of NTE expression levels in cells stably transfected with wild-type NTE (lane 1), pSUPER/neo (vector control) (lane 2), and pSUPER/neo-NTE (lane 3). (B) Quantitative analysis of basal in situ NTE activity. Data are presented as a percentage of in situ NTE activity. In wild-type cells, NTE activity was defined as 15.92 ± 1.39 nmol phenol/min/mg protein. The basal in situ NTE activity was significantly decreased in SH/dsRNA-NTE cells ($n = 3$, $^*P < 0.05$). (C) Quantitative analysis of basal in situ AChE activity. Data are presented as a percentage of in situ AChE activity. In wild-type cells, basal AChE activity was defined as 22.75 ± 1.79 nmol acetylthiocholine hydrolyzed/min/mg protein. Basal in situ AChE activity was not significantly decreased in SH/dsRNA-NTE cells ($n = 3$, $P > 0.05$). (D) Representative Northern blots of NTE and GAPDH mRNA expression levels in mock-transfected human neuroblastoma SK-N-SH cells (wild-type NTE) (lane 1), SK-N-SH cells stably transfected with pSUPER-neo (vector control) (lane 2), or pSUPER-neo-NTE (lane 3). The expression levels of GAPDH mRNA was used as a loading control.

protein. However, in SH/dsRNA-NTE cells, the basal NTE activity was significantly decreased in situ compared to the vector control cells, such that SH/dsRNA-NTE abolished 70% of NTE activity (Fig. 1B). As a control, AChE activity was assayed. In wild-type cells, the basal activity of AChE was 22.75 ± 1.79 nmol acetylthiocholine hydrolyzed/min/mg protein. As shown in Fig. 1C, AChE activity did not show a significant reduction between vector control cells and SH/dsRNA-NTE cells. In addition, the mRNA level of NTE in SH/dsRNA-NTE was monitored with Northern blots. The results showed that NTE mRNA in SH/dsRNA-NTE was almost eliminated compared to the vector control and wild-type cells (Fig. 1D). These results demonstrate that the construct pSUPER-neo-NTE efficiently and specifically inhibited the expression of NTE at a post-transcription level.

3.2. Reduction of NTE does not affect neural differentiation in human neuroblastoma

To determine whether NTE is required for neural differentiation, we transfected cells with either pSUPER vector or pSUPER-neo-NTE vector. Transfection with either the empty pSUPER vector or the pSUPER-neo-NTE vector did not result in any morphological alterations. Furthermore, we observed no detectable differences in the growth rates of SH/dsRNA-NTE and control cells (data not shown). In order to evaluate a role for NTE in ATRA-mediated neurodifferentiation of SK-N-SH cells, these cell lines were treated with ATRA for 1 week and their morphology was evaluated and the cell marker protein was detected. ATRA significantly increased neurite outgrowth in the vector control-transfected cells (compare Figs. 2A with B). Similarly, ATRA treatment resulted in the same effect in SH/dsRNA-NTE cells (compare Figs. 2C with D). Both the control cells and the SH/dsRNA-NTE cells after ATRA treatment expressed neuronal marker protein stained by NF-H antibody N52 in their initiated neurite outgrowth (Supplementary Fig. 1). The effect of ATRA on neuronal differentiation of SH/dsRNA-NTE was quantified. As shown in Table 1, the percentage and average length of differentiated cells in SH/dsNRA-NTE were not significantly different from those of vector control cells. These findings indicate that reduction of NTE does not affect ATRA-induced neuronal differentiation in SK-N-SH cells.

Table 1
NTE lowered expression does not affect ATRA-induced neuronal differentiation in vector control and SH/dsRNA-NTE cells

	Average neurite length (μm)	Differential cells (% of total cells)
Control	114.4 ± 7.0	33.9 ± 1.3
SH/dsRNA-NTE	112.8 ± 3.5	31.2 ± 0.8

Percentage of the cells differentiated was counted in five randomly chosen fields (20× magnification) from each dish. The data represent mean ± SE from three independent experiments. The length of neurite on each cell (40× magnification) was measured in at least 100 cells per treatment. Results given are the mean ± SE from three independent experiments.

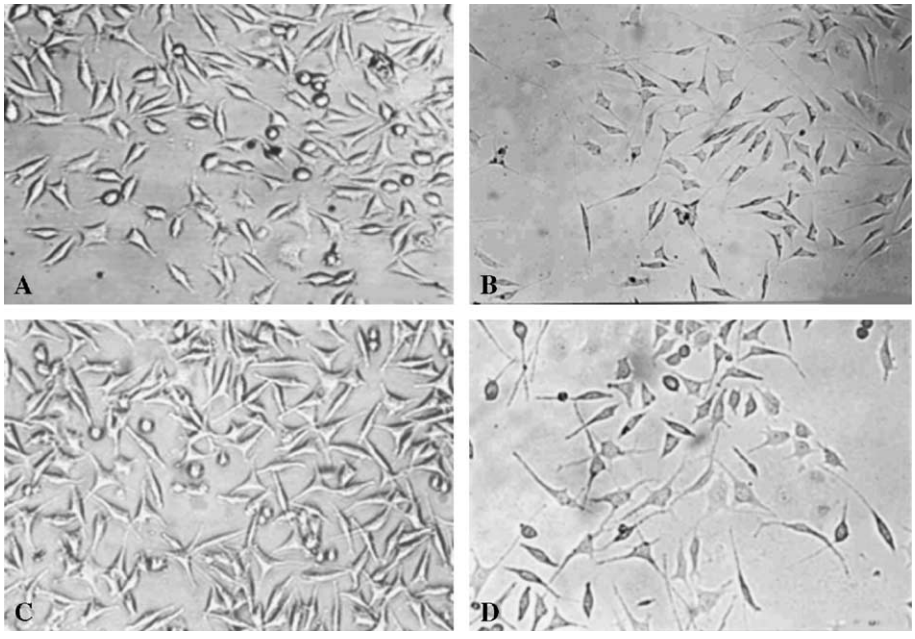

Fig. 2. ATRA-induced neuronal differentiation in SH/dsRNA-NTE cells. To induce ATRA-mediated differentiation, cells were grown in DMEM medium containing 20 μM ATRA. The medium was replaced with fresh conditioned medium once every 48 h. The cells were photographed on day 7 using an inverted phase contrast microscope. At least three independent experiments were conducted with each clone and the photographs shown are representative of these experiments. Undifferentiated control cells (A), differentiated control cells (B), undifferentiated SH/dsRNA-NTE cells (C), and differentiated SH/dsRNA-NTE cells (D).

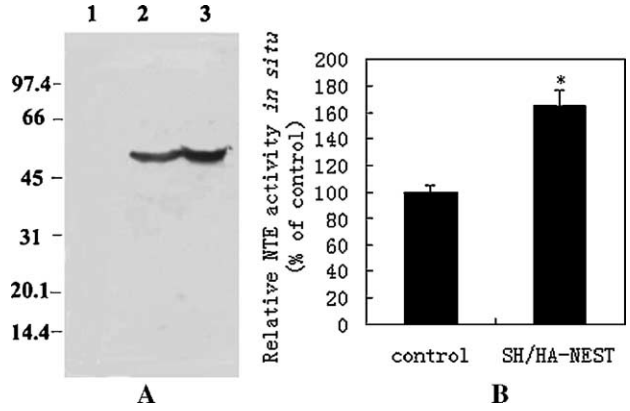

Fig. 3. Stable expression of NEST in SK-N-SH cells. (A) Soluble/particulate distribution of HA-tagged NEST construction stably transfected SK-N-SH cells. Whole cell extraction of SH/pcDNA cells (lane 1), particulate (lane 2), and soluble fractions (lane 3) of SH/HA-NEST cells were subjected to Western blotting with an antibody to HA. (B) Quantitative analysis of basal in situ NTE activity. Data are presented as a percentage of in situ NTE activity. In wild-type control cells, the basal in situ NTE activity was defined as 15.92 ± 1.39 nmol phenol/min/mg protein. The basal in situ NTE activity was significantly elevated in SH/HA-NEST cells ($n = 3$, $^*P < 0.01$).

3.3. Moderate expression of NTE in SK-N-SH

To investigate the effect of higher expression levels of NTE on neural differentiation compared to the effects seen with lower NTE levels, we designed an experiment to express full-length NTE protein in SK-N-SH cells. However, we found that the expression of the full-length NTE protein inhibited cell cycle and therefore we could not obtain stable clones expressing full-length NTE protein (unpublished data). Because the NTE esterase domain, comprising residues 727–1216 of human NTE, reacts with an ester substrate and covalent inhibitors in a manner very similar to NTE [2], we generated a construct pcDNA-NEST which encodes amino acid residues 725–1223 of human NTE and stably transfected it into SK-N-SH cells (SH/HA-NEST). HA-NEST is expressed as a protein of 53 kDa. Upon comparison, the NTE activity of cells expressing NEST was approximately one-third the activity of cells expressing full-length NTE (data not shown). As such, we defined the NTE activity of cells expressing NEST as moderate NTE activity [18]. To detect NEST expression, NEST was tagged with the HA epitope and identified with an HA antibody. As indicated in Fig. 3A, NEST is expressed in the soluble cytosolic fraction and in the particulate fraction, although it does not contain the transmembrane sequence. Furthermore, our results showed that the level of NTE activity in SH/HA-NEST cells is significantly elevated compared to that in the cells transfected with control vector (Fig. 3B).

3.4. NTE moderate expression induces neuronal differentiation in SK-N-SH cells

Because NTE activity was increased in the differentiated cells, we sought to determine if forced expression of NTE induces differentiation in the human neuroblastoma SK-N-SH cells. To quantitate differentiation, we counted the number of cells showing significant neurite outgrowth and measured the length of neurites in control cells and SH/HA-NEST clones. At low density, cells transfected with the control vector pcDNA displayed cell bodies with multiple protrusions and some minor neurites (Fig. 4A). In contrast, SH/HA-NEST cells displayed a distinct neuronal morphology defined by characteristic neurite outgrowth and exhibited extensive neurites with multiple branches (Fig. 4B). Correspondingly, SH/HA-NEST cells displayed more extensive neuritis with multiple branches stained with NF-H (neuronal marker protein) antibody defined by fluorescence intensity (Supplementary Fig. 2). There was a significant increase both in the percent of the cells differentiated and the length of the neurites in SH/HA-NEST cells (Table 2).

3.5. OP may interact with other molecules than NTE to exert its effect on neuronal differentiation

NTE inhibitors selectively block neurite-like outgrowth and cell processes and NTE is considered the preferred candidate reacting with OPs. To determine whether OP inhibit neuronal differentiation by targeting NTE, control and SH/dsRNA-NTE cells were treated with 50 μM paraoxon, an organophosphate that does not inhibit NTE activity or neuronal differentiation, or mipafox, an organo-

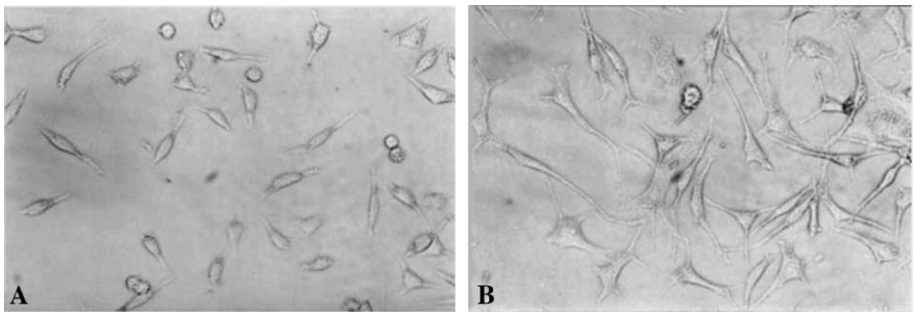

Fig. 4. Phase contrast photomicrographs of SH/HA-NEST cells. SH/pcDNA cells exhibit phase bright cell bodies with multiple protrusions and some minor neuritis (A). In contrast, SH/HA-NEST cells exhibit extensive neurites with multiple branches (B).

Table 2
Stable SH/HA-NEST cell undergoes neuronal differentiation

	Average neurite length (μm)	Differential cells (% of total cells)
Control	27.6 ± 0.4	11.3 ± 0.5
SH/NEST	53.1 ± 2.6*	21.3 ± 1.0*

Differentiated cells were counted in five randomly chosen fields (20× magnification) from each dish and expressed as percent of cells counted. In the three independent experiments, at least 500 cells were counted for each treatment and the data represent mean ± SE from three experiments. The length of the processes/neurites on each cell (40× magnification) was measured for at least 100 cells per treatment in three independent experiments. Results are expressed as mean ± SE of neurite length from three experiments. Differences between the control cells and SH/HA-NEST clones were subjected to statistical analysis by Student's *t* test: $*P < 0.05$.

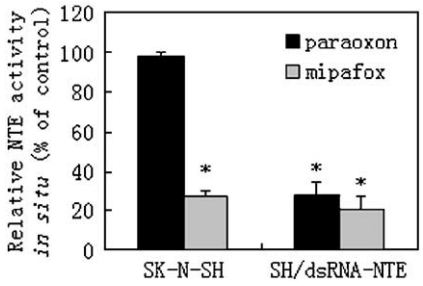

Fig. 6. Quantitative analysis of basal in situ NTE activity treated with paraoxon and mipafox. Data are presented as a percentage of in situ NTE activity in DMSO-treated SK-N-SH cells. Basal in situ NTE activity was significantly decreased in SH/dsRNA-NTE and mipafox-treated SK-N-SH cells, $n = 3$, $*P < 0.05$. The basal NTE activity in DMSO-treated SK-N-SH cells was defined as 15.92 ± 1.39 nmol phenol/min/mg protein.

phosphate that does inhibit both NTE activity and neuronal differentiation. Simultaneously, cells were also induced to differentiation with ATRA. After 7 days, the effect of mipafox and paraoxon on the neuronal differentiation and NTE activity was analyzed. As shown in Fig. 5, mipafox significantly decreased the average length of neurite in both differentiating control cells and differentiating SH/dsRNA-NTE cells, whereas paraoxon did not inhibit these processes in either cell line. In control cells, mipafox significantly decreased the level of NTE activity while paraoxon had no effect on NTE activity in these cells. In SH/dsRNA-NTE cells, the levels of NTE activity were not significantly different between paraoxon and mipafox treatment. However, the NTE activity was significantly suppressed by paraoxon or mipafox compared with that in differentiated control cells (Fig. 6). As such, these data suggest that NTE may contribute to but is not solely responsible for neural differentiation in human neuroblastoma cell.

4. Discussion

NTE is concentrated in the nervous system in mammalian animals and anchored to the cytoplasmic face of endoplasmic reticulum [1,18]. As such, it may play an important role in the nerve development. Indeed, mutation of Swiss cheese protein, its homologous protein in *Drosophila*, resulted in an age-dependent neurodegeneration in flies [9,16]. Moreover, brain-specific deletion of NTE led to neurodegeneration in mouse and the absence of NTE resulted in disruption of the endoplasmic reticulum, vacuolation of nerve cell bodies and abnormal reticular aggregates [1].

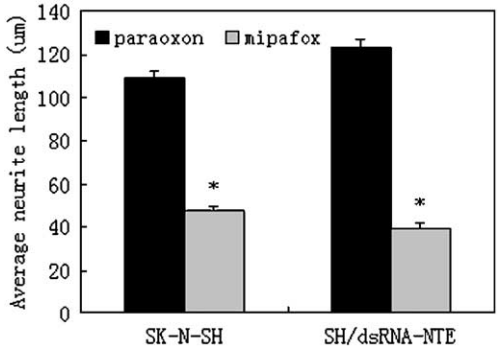

Fig. 5. Effects of paraoxon and mipafox on the neurite length of differentiating human neuroblastoma SK-N-SH cells and SH/dsRNA-NTE cells induced by ATRA. The neurite length was measured as described in Materials and methods. Mipafox significantly blocked the neurite length in both cells, $P < 0.05$.

SK-N-SH cells, a human neuroblastoma cell line, withdraws from the cell cycle and expresses a distinct neuronal phenotype when treated with ATRA [22]. SK-N-SH cells are used extensively as a cell culture model of neurodifferentiation. Therefore, SK-N-SH cells provide a useful model for studying changes in signal transducing proteins, cytoskeletal proteins and neural proteins as well as the expression and activity of neural proteins during the process of differentiation into neuronal phenotype. NTE, the main target protein in OPIDN, was also considered to be the preferred candidate target for inhibitory neuronal differentiation by OP [17]. Therefore, we studied the role of NTE in the neural differentiation in SK-N-SH cells.

Deletion of NTE in SK-N-SH with small interfering RNA did not alter the morphology nor affected the neural differentiation of SK-N-SH cells treated with ATRA. However, moderate expression of NTE by expression of NEST induced neural differentiation. Although deletion of NTE in mouse resulted in embryonic lethality at embryonic day 9 postcoitum (E9), E8 embryo cells survived after 1 week of culture in vitro and gave rise to different cell types with different cell morphologies including some highly differentiated cells, such as beating cardiac myocytes and cells resembling neurons with long branched neurite-like processes [21]. Taken together with our results, a number of different cells can survive and differentiate in the absence of NTE activity.

Using cells that express HA-NEST, we showed that moderate expression of NTE induced neural differentiation and partly inhibited cell division, although the growth rate of cells transfected with either control vector or HA-NEST

was similar. SK-N-SH cells expressing high levels of NTE transfected with GFP-tagged NTE construct pNTE-GFP showed inhibition of cell division (our unpublished observation). NTE, as a new phospholipase B in mammalian cells, is responsible for converting phosphatidylcholine to glycerophosphocholine and overexpression of NTE resulted in increasing level of glycerophosphocholine [33]. We speculate that in SH/dsRNA-NTE cells, which have lowered levels of NTE activity, the turnover rate of phosphatidylcholine to glycerophosphocholine is likely reduced and leads to increasing levels of phosphatidylcholine. In mammalian cells, the increased levels of phosphatidylcholine may be reduced through the activity of phospholipase D. Alternatively, increased phosphatidylcholine levels may inhibit the synthesis of new phosphatidylcholine by decreasing the activity of CTP-phosphocholine cytidylyltransferase, the rate-limiting step in phosphatidylcholine synthesis [3]. In yeast lacking YML059c, a protein with sequence homology to NTE, the rate of phosphatidylcholine synthesis is determined by the rate-limiting step catalyzed by CTP-phosphocholine cytidylyltransferase [33]. As such, the levels of phosphatidylcholine and glycerophosphocholine in SH/HA-NEST and SH/dsRNA-NTE cells need to be assayed further.

SH/dsRNA-NTE cells treated with mipafox showed a decrease in the average length of neurites. Although the level of NTE activity in the cells was not significantly decreased by the neurotoxicant, targets other than NTE may react with mipafox. Indeed, the outgrowth of axons by N2a cells after exposure to chlorpyrifos, an OP insecticide, is affected by several different mechanisms not including the inhibition of NTE [28]. There are several potential targets for OP in the cells [24]. For example, OP has been to shown to inhibit lysophospholipase. Although NTE also has potent lysophospholipase activity, NTE only constitutes 29% of the total lysophospholipase in mouse brain [25]. The target and mechanism of inhibitory neural differentiation by OP remains to be studied.

In summary, noncytotoxic biochemical disruptions through NTE inhibition with genetic methods does not lead to inhibition of neurite and process outgrowth. However, moderate increase in the levels of NTE induces neural differentiation. As such, OP may interact with certain molecules, other than NTE, to exert its inhibitory effect on neural differentiation in neuroblastoma cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbrainres.2005.07.012.

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