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RESEARCH ARTICLE

Differentiated NSC-34 cells as an *in vitro* cell model for VX

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Abstract

The US military has placed major emphasis on developing therapeutics against nerve agents (NA). Current efforts are hindered by the lack of effective *in vitro* cellular models to aid in the preliminary screening of potential candidate drugs/antidotes. The development of an *in vitro* cellular model to aid in discovering new NA therapeutics would be highly beneficial. In this regard, we have examined the response of a differentiated hybrid neuronal cell line, NSC-34, to the NA VX. VX-induced apoptosis of differentiated NSC-34 cells was measured by monitoring the changes in caspase-3 and caspase-9 activity post-exposure. Differentiated NSC-34 cells showed an increase in caspase-3 activity in a manner dependent on both time (17–23 h post-exposure) and dose (10–100 nM). The maximal increase in caspase-3 activity was found to be at 20-h post-exposure. Caspase-9 activity was also measured in response to VX and was found to be elevated at all concentrations (10–100 nM) tested. VX-induced cell death was also observed by utilizing annexin V/propidium iodide flow cytometry. Finally, VX-induced caspase-3 or -9 activities were reduced with the addition of pralidoxime (2-PAM), one of the current therapeutics used against NA toxicity, and dizocilpine (MK-801). Overall the data presented here show that differentiated NSC-34 cells are sensitive to VX-induced cell death and could be a viable *in vitro* cell model for screening NA candidate therapeutics.

Keywords

Cell death, nerve agent, NSC-34 cells, VX

History

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Introduction

The organophosphate (OP) nerve agents (NA), including soman, sarin, tabun and VX, act principally as potent cholinesterase inhibitors. The toxicity of these compounds and their mode of action are attributed to the inhibition of the enzyme acetylcholinesterase (AChE), which leads to an excess of acetylcholine (ACh) at both peripheral and central nervous system (CNS) synapses (Bajgar et al., 2012; Upadhyay et al., 2009). This accumulation triggers a series of events resulting from its action on muscarinic- and nicotinic-mediated events, known as a cholinergic crisis. They include constriction of the pupil (miosis), increased production of saliva, runny nose, increased perspiration, urination, defecation, bronchosecretion, bronchoconstriction, decreased heart rate and blood pressure, muscular twitches and cramps, cardiac arrhythmias, tremors and convulsions. The most life-threatening effects are paralysis of the respiratory muscles and inhibition of the respiratory center resulting in death from respiratory paralysis.

In the CNS, this cholinergic abnormality is believed to trigger a glutamatergic response, which contributes to convulsions and subsequent damage to post-synaptic neurons (McDonough & Shih, 1997). A limitation of the current therapeutic regimen, i.e. atropine, 2-PAM (2-pyridine aldoxime methyl chloride) and an anti-convulsant compound (diazepam), is the small window of time that exists for maximum therapeutic effect (McDonough & Shih, 1997). One of the obstacles in developing new therapeutics is the lack of an *in vitro* model that can be used to investigate the possible mechanisms of toxicity and the prospective therapeutic interventions prior to their validation in a suitable animal model. The development of an *in vitro* screening model would be more cost effective and would also reduce animal usage of drug discovery (Sundstrom et al., 2005). Additionally, the use of an *in vitro* cell model would allow a fast, high throughput screening of drugs that are approved for other indications to investigate their suitability as a NA therapeutic.

The neural stem cell line used and investigated in this article is the NSC-34 cell line, which is a fusion of motor neuron-enriched, embryonic mouse spinal cord cells with mouse neuroblastoma as a potential neuronal model (Durham et al., 1993). This cell line has been previously explored as a tool for

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toxicological assessments but was found to have limitations (Durham et al., 1993). To address the limitations discovered (e.g. lack of glutamate toxicity), Eggett et al. (2000) were able to differentiate NSC-34 cells into a glutamate-sensitive neuronal cell population. Further studies with differentiated NSC-34 cells showed that these cells express a high degree of morphological and physiological properties of motor neurons, including extension of processes, formation of contacts with cultured myotubes, synthesis and storage of ACh, expression of choline acetyltransferase synthesis and storage of acetylcholine, the release of neurotransmitters (Cashman et al., 1992; Matusica et al., 2008). Moreover, differentiating these cells by modifying growth conditions via serum deprivation or by altering medium composition causes these cells to express different glutamate receptor proteins, thereby making the cells sensitive to glutamate stimulation. These properties give them the potential to be used as a model cell line to investigate cellular toxicity after insult/injury (e.g. NA exposure; Eggett et al., 2000).

In the present study, we investigated VX-induced toxicity on differentiated NSC-34 cells as measured by caspase-3, -9 and flow cytometry. Our goal was to determine if differentiated NSC-34 cells are an appropriate *in vitro* cell model for VX. The results show that glutamate induced cell death by increasing caspase-3 activation in the differentiated NSC-34 cell line. Differentiated NSC-34 cells showed an increase in caspase-3 in a time- and dose-dependent manner. The maximal increase in caspase-3 was found to be at 20-h post-exposure. Caspase-9 was also measured in response to VX and was found to be elevated at all concentrations of VX tested. VX-induced cell death was also observed by utilizing annexin V/propidium iodide flow cytometry. Finally, VX-induced caspase-3 or -9 activities were reduced with the addition of pralidoxime (2-PAM) or dizocilpine (MK-801). Taken together, these results demonstrate that NSC-34 cells are sensitive to both VX and glutamate; however, whether glutamate is directly involved in the VX effect needs to be established.

Methods

Cell culture and chemicals

VX was obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). VX was supplied as a 3.5 mM solution diluted into PBS, which was stored at −80 °C until use. VX was diluted (1:1000) in cell media just prior to exposure. Stock NSC-34 cells were purchased from Cederlane (Burlington, NC), and growth media (DMEM+ Gluta MAX-1) was purchased from Invitrogen (Frederick, MD). DMEM+ Gluta MAX-1 was supplemented with 10% FBS (fetal bovine serum) purchased from ATCC (Manassas, VA). NSC-34 cells were maintained in DMEM Gluta-MAX 1 and cultured according to the company's instructions. All other chemicals were from Sigma (St. Louis, MO) and were purchased at the highest purity available.

Neuronal differentiation

Once NSC-34 cells reached 100% confluency, they were differentiated by following a previously published method (Eggett et al., 2000). Differentiation media used was 1:1 DMEM/F:12 media which was purchased from Invitrogen (Frederick, MD). Differentiation media was supplemented with 1% FBS (ATCC, Manassas, VA) and 1% MEM non-essential amino acids (Life Technologies, Grand Island, NY). Differentiation occurred for 2–4 weeks, and cells were allowed to be cultured up to three passages.

VX exposure of cells

Differentiated NSC-34 cells were exposed to 10–100 nM of VX. Control cells were treated in the exact same manner as exposed cells; however, they were exposed to vehicle instead of VX. Pralidoxime chloride (2-PAM) (Sigma, St. Louis, MO) or (+)-MK-801 hydrogen maleate (MK-801) (Sigma, St. Louis, MO) were applied immediately prior to exposure. Both of these compounds were not found to alter the baseline activity of either caspase-3 or -9 (data not shown). Media was not changed until the desired time period was met. Plates or flasks were washed 5 times with Hank's buffered saline solution (HBSS) (Life Technologies, Grand Island, NY) to remove VX prior to all assay preparation.

Caspase-3 and -9 activation

Caspase-3/-9 activation and sequential activity were measured by Caspase-Glo® 3/7 and 9 assay respectively, from Promega (Madison, WI). Caspase-Glo assay is a homogeneous, luminescent assay that measures caspase activity by measuring the luminescence generated from the cleavage of a luminogenic substrate containing an aspartic acid-glutamic acid-valine-aspartic acid (DEVD) sequence for caspase 3/7 and a leucine-glutamic acid-histidine-aspartic acid (LEHD) sequence for caspase-9. Following this cleavage, a substrate for luciferase (amino-luciferin) is released. Caspase-Glo assays were performed according to the manufacturer's instructions. Briefly, Caspase-Glo reagent mixture was added in a 1:1 ratio in cellular media. At 1 h prior to the time point, Caspase-Glo reagent was added to the cells, and caspase activity was measured approximately 1 hour later with a SpectraMax Gemini EM (MDS Analytical Technologies, Toronto, Canada).

Acetylcholine/acetylcholinesterase assay

AChE activity and ACh were measured by Amplex® Acetylcholine/Acetylcholinesterase Assay Kit which was purchased from Invitrogen. Acetylcholinesterase is converted to choline, which is then oxidized by choline oxidases to H_2O_2 and betaine. The H_2O_2 reacts with the Amplex Red in the presence of horseradish peroxidase to produce a fluorescent product. This assay was conducted in 96-well microplates, and cells were washed with HBSS up to 2 h before exposure with VX. Exposure was completed using HBSS instead of supplemented media. Control cells were treated in the exact same manner as exposed cells; however, they were exposed to vehicle instead of VX. The assay kit was preformed according to the manufacturer's instructions. Fluorescence was measured a half hour later using a SpectraMax Gemini EM (MDS Analytical Technologies, Toronto, Canada) with excitation range of 530–560 nm and emission detection at ~590 nm.

Flow cytometry

Cells were stained with annexin V Alexa Fluor® 488 and propidium iodide utilizing the dead cell apoptosis kit (Cat# V23200) from Invitrogen (Carlsbad, CA). After the final incubation, step cells were analyzed by flow cytometry. A minimum of 10 000 cells were analyzed using a Becton Dickinson (San Jose, CA) FACSAria II flow cytometer. Data were analyzed using FlowJo (Tree Star, Inc., Ashland, OR) flow cytometry analysis software.

Data analysis

Data are shown as mean ± standard deviation. Group comparisons were conducted using one-way analysis of variance followed by Tukey's *post-hoc* multiple comparison test. Significant results were identified when $p < 0.05$ or smaller.

Results

Differentiated NSC-34 cells are sensitive to glutamate and VX

The first set of experiments examined the dose-dependent sensitivity of differentiated NSC-34 cells to glutamate; these experiments showed that these cells undergo a significant increase in caspase-3 activation 24 h after exposure to glutamate. The increase in caspase-3 activation was seen at 0.1–5 mM glutamate with the maximal increase occurring at 5 mM glutamate ($p < 0.01$) compared with no increase in controls evaluated at the same time point (Figure 1A). Higher concentrations of glutamate (10–20 mM) did not increase caspase-3 activation, but lowered caspase-3 activation, suggesting a desensitization to the glutamate. This result is not unexpected since stimulation of glutamate has been shown to evoke a dose-dependent early (~0.5–2 h post-exposure) phase of necrosis with a late (12–24 h post-exposure) phase of apoptosis (Ankarcrona et al., 1995). High concentrations (>10 mM) of glutamate have been previously shown to cause >80% cell death and low caspase-3 activation at 24-h post-exposure (Hirata et al., 2011; Moroni et al., 2001).

Time- and dose-dependence of caspase-3 activation was examined in differentiated NSC-34 cells following VX exposure. In these experiments, differentiated NSC-34 cells were exposed to VX (10–100 nM), and caspase-3 activation was examined 17 (Figure 1B), 20 (Figure 1C) and 23 h (Figure 1D) later. At each of these time points, caspase-3 activity significantly ($p < 0.01$) increased in response to VX. At least two concentrations of VX were significantly ($p < 0.05$) increased at all time points; however, only the 75 nM concentration of VX was seen to be significantly ($p < 0.01$) increased at all time points. The time point that saw the most significant ($p < 0.05$) elevation in VX-induced caspase-3 activation was 20 h (25–100 nM) and was used for all sequential experiments.

Since cell death from exposure to NA has been proposed due to intracellular calcium overload, we studied caspase-9 activation in addition to caspase-3 activation following VX exposure. Caspase-9 activation was examined 20 h after VX (10–100 nM) exposure in differentiated NSC-34 cells. VX-induced caspase-9 activation was significantly ($p < 0.05$) increased at all the concentrations examined, which varied from 119 ± 5% (10 nM) to 124 ± 4% (75 nM) of control (Figure 1E). Activation of caspase-9 was not dose-dependent at the doses tested but was consistently ~120% of control, unlike VX-induced caspase-3 activation.

Flow cytometry of NSC-34 cells

Cell death can be divided into two different processes, apoptosis and necrosis. To determine the total cell death from VX, however, another way of assessing cell death was used since necrosis and a subtype of apoptosis are not caspase-dependent. Flow cytometry using annexin V (apoptosis marker) and propidium iodide (PI) (necrosis marker) staining was employed. The results showed proportions of cells as live and dead (via both apoptosis and necrosis) (Figure 2A–E). Differentiated NSC-34 cells were exposed to VX for 20 h followed by annexin V/PI staining. Cell death (apoptosis/necrosis) from VX (Figure 2D) was 20–29% with the peak occurring from the 75-nM dose. In the lower right quadrant (annexin V staining only, indicator of early apoptosis), the percentage of staining increases from 7% to a peak of 14% (at 75 nM); this mirrors the total amount of cell death. This trend is not evident in the upper right quadrant (annexin V and propidium iodide, indicator of late apoptosis/early necrosis) or the upper left quadrant (propidium iodide staining only, indicator of necrosis). The reasons for this are unknown and remain to be studied. These results indicate that VX causes cell death both via apoptosis and necrosis, with more cells undergoing apoptosis.

Acetylcholinesterase inhibition by VX

Since VX is an AChE inhibitor, the amount of VX-induced AChE inhibition was measured to demonstrate the effect of VX on NSC-34 AChE. In this set of experiments, the cellular media was removed and replaced with Hank's buffered salt solution (HBSS) for 2 h prior to VX or vehicle addition. The use of HBSS was necessary since the acetylcholinesterase inhibition kit uses Amplex Red as the substrate for detection of acetylcholinesterase activity. In this series of reactions, acetylcholinesterase converts acetylcholine into choline. The choline reacts with choline oxidase to H_2O_2, which reacts with Amplex Red to produce fluorescence (Mohanty et al., 1997; Zhou et al., 1997). The cellular media contains choline, and its use caused no inhibition to be seen (results not shown), hence, the use of HBSS instead of the cellular media for these experiments. AChE inhibition was measured 2 h after VX exposure; a highly significant ($p < 0.001$) reduction in AChE activity was seen for all concentrations of VX with peak inhibition (70%) occurring at 100 nM (Figure 3).

Effect of MK-801 and 2-PAM on VX-induced caspase activation

In Figure 1, we show that VX increased both caspase-3 and -9 significantly over control. To provide evidence that the increase in caspase-3 and -9 was due to VX, two compounds used extensively to reduce the effect of NA toxicity (MK-801 and 2-PAM) were administered to see whether this VX-induced caspase-3 and -9 activation was reversible. MK-801, an NMDA antagonist, has been used in differentiated NSC-34 cells previously, dose-dependently with 1 μM

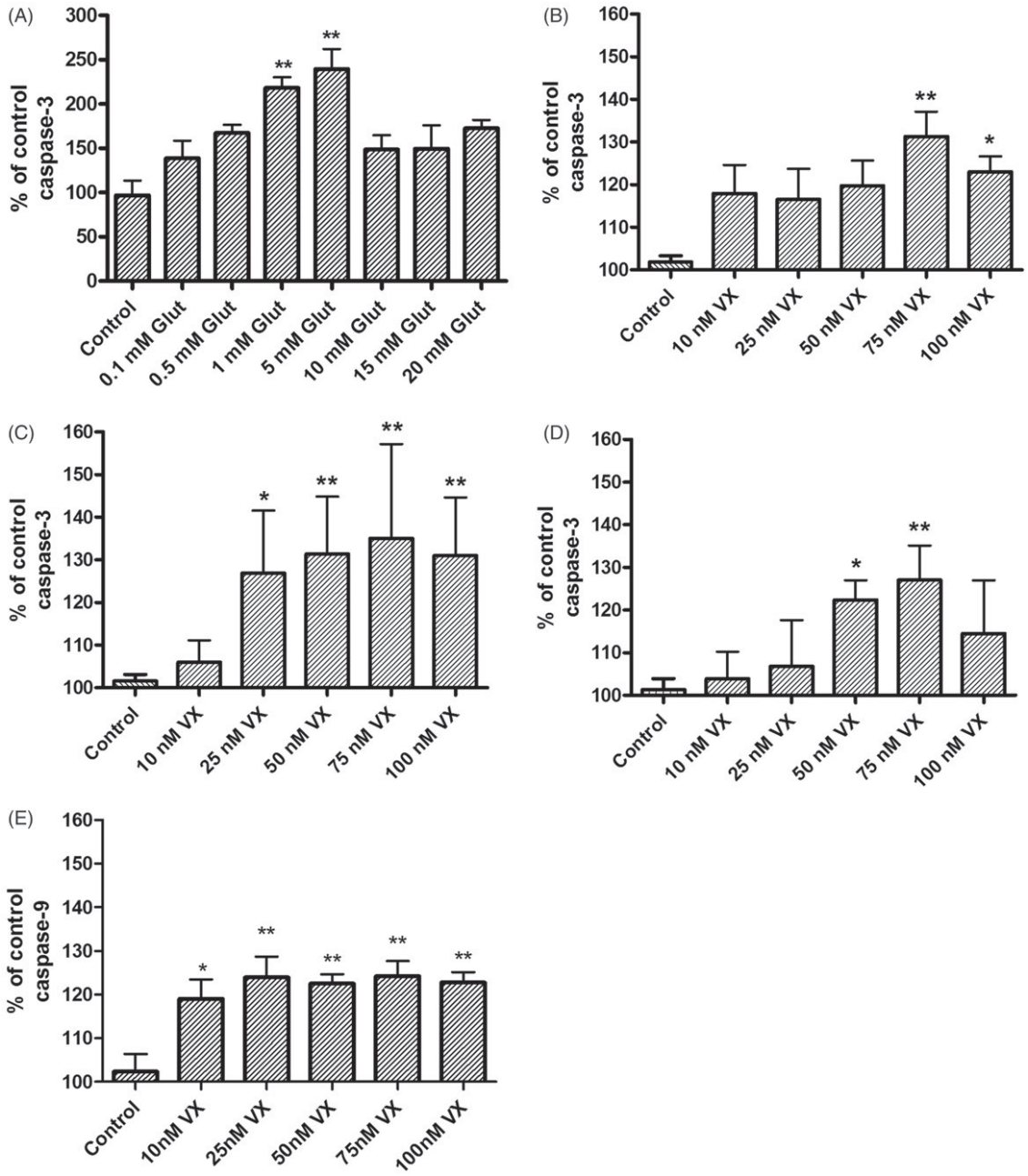

Figure 1. Glutamate- and VX-induced activation of caspase-3 in differentiated NSC-34 cells. Differentiated NSC-34 cells were exposed to glutamate for 24 h (A) or to 10–100 nM VX for 17 h (B), 20 h (C) and 23 h (D). Caspase-3 activation was measured at the designated time points. Capase-9 activation was measured at 20 h post-exposure to 10–100 nM VX (E). Data points are mean ± SD of four replicate determinations, $*p < 0.05$, $**p < 0.01$ when compared to control. Black boxes indicate control which was caspase substrate added to untreated cells to indicate basal apoptosis. Glut = glutamate.

being the optimal dose (Chen et al., 2011). The activation of N-methyl-D-aspartate (NMDA) receptors resulting from NA exposure has been documented in the past (for review, see McDonough & Shih, 1997). In Figure 4(A) and (C), 1 μM MK-801 was added to differentiated NSC-34 cells just prior to VX exposure, and caspase-3 and -9 activities were examined 20 h after VX exposure. MK-801 was able to reduce VX-induced caspase-3 activity to near baseline levels; however, this was not the case for caspase-9. The reduction of VX-induced caspase-9 activity via MK-801 was only 5–10% (Figure 4C).

Additionally, we used 2-PAM, an AChE reactivator, at a concentration of 600 μM, which has been shown to be an effective dose that reactivates AChE (Kuca et al., 2005). The addition of 2-PAM was also just prior to VX exposure, and caspase-3 and -9 activities were examined 20 h post-exposure. Neither of these two compounds, MK-801 nor 2-PAM, caused a significant increase in caspase-3 or -9 activity when added alone (data not shown). The results of 2-PAM mimic those of MK-801, with a reduction of caspase-3 to baseline but a less pronounced effect on caspase-9 (5–10% reduction).

Discussion

The results in this study demonstrate that a chemical warfare agent, VX, was able to induce dose-dependent cell death in a

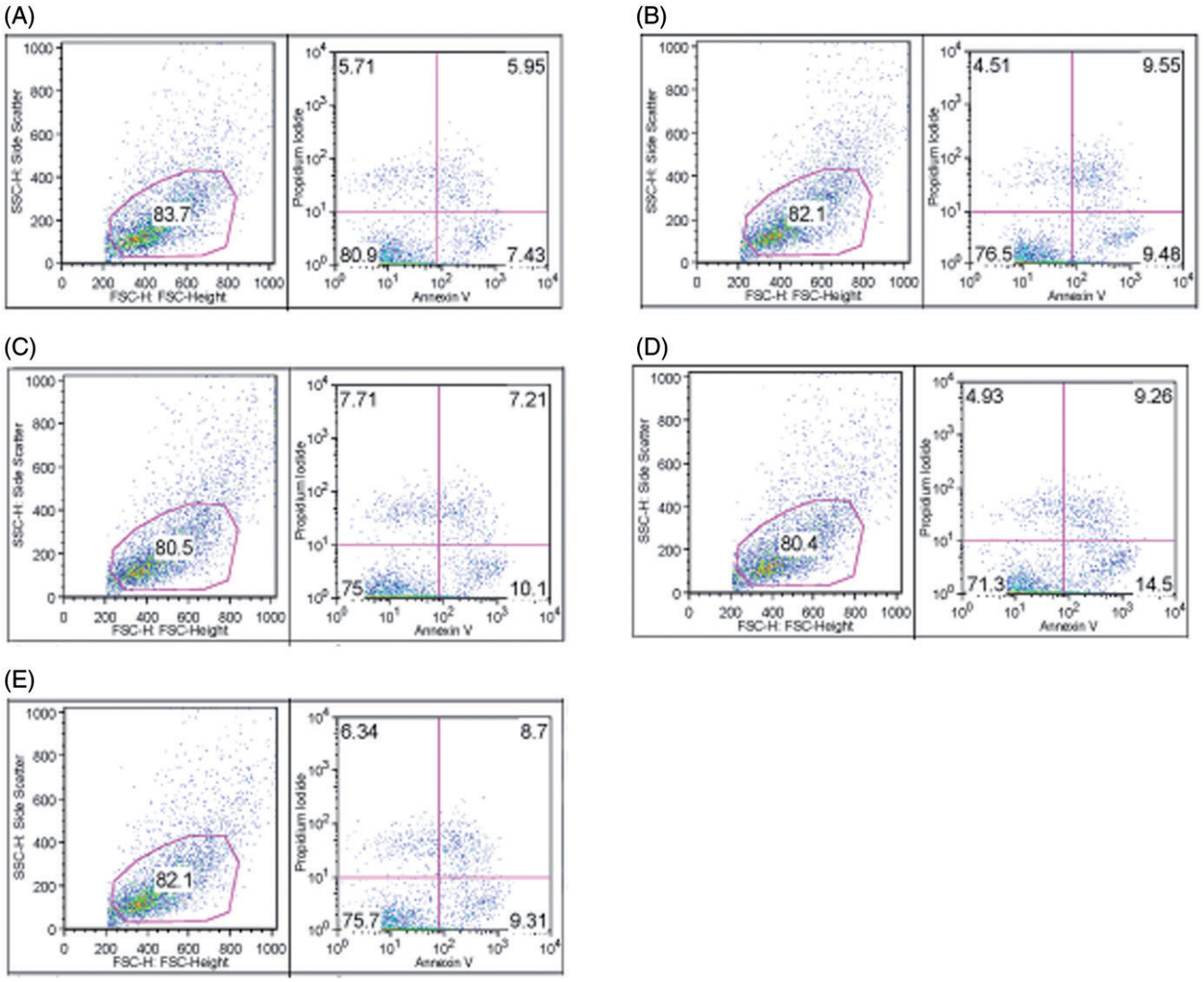

Figure 2. Annexin V/PI staining of differentiated NSC-34 cells exposed to VX. Differentiated NSC-34 cells were exposed to 10 nM (A), 25 nM (B), 50 nM (C), 75 nM (D) or 100 nM (E) VX for 20 h and stained for annexin V/PI flow cytometry. Lower left quadrant (unstained), no cell death is apparent; lower right quadrant (annexin V only), cells are in early apoptosis; upper right quadrant (annexin V and PI), cells are in late apoptosis; upper left quadrant (PI only), cells are in necrosis.

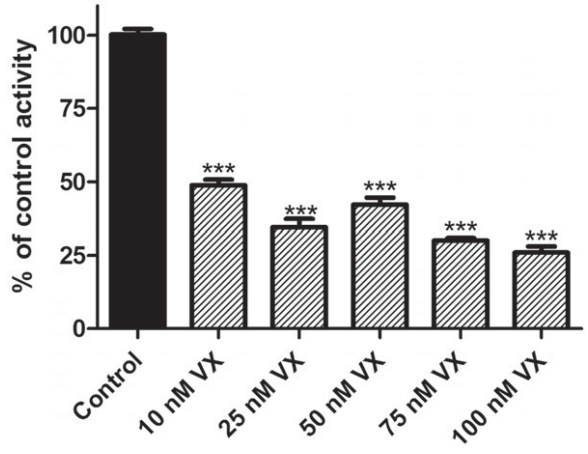

Figure 3. VX-induced inhibition of AChE from differentiated NSC-34 cells. Differentiated NSC-34 cells were exposed to VX and AChE activity was measured. AChE activity was normalized to the control (black box, untreated) from each experiment. ***$p < 0.001$.

differentiated neuronal cell line, NSC-34. This cell line was originally derived by fusing motor neuron-enriched embryonic mouse spinal cord cells with mouse neuroblastoma cells (Cashman et al., 1992). This cell line has been previously explored as a tool for toxicological assessments but was found to have limitations (Durham et al., 1993). To address the limitations discovered (e.g. lack of glutamate toxicity), Eggett et al. (2000) were able to differentiate NSC-34 cells into a glutamate-sensitive neuronal cell population. Following this, systematic studies have been undertaken to compare the expression of various neuronal proteins in non-differentiated NSC-34 with that in differentiated NSC-34 cells (for review, see Matusica et al., 2008). These studies laid the groundwork for the use of differentiated NSC-34 cells as an *in vitro* cell line for toxicity studies. Differentiated NSC-34 or any cell line used as neurotoxicity model would only be effective if used as a preliminary screening tool, since it cannot replicate the complex interaction between the numerous cell types in the brain that is seen *in vivo*. Another issue that arises with the use of *in vitro* cell lines in toxicology studies is determining

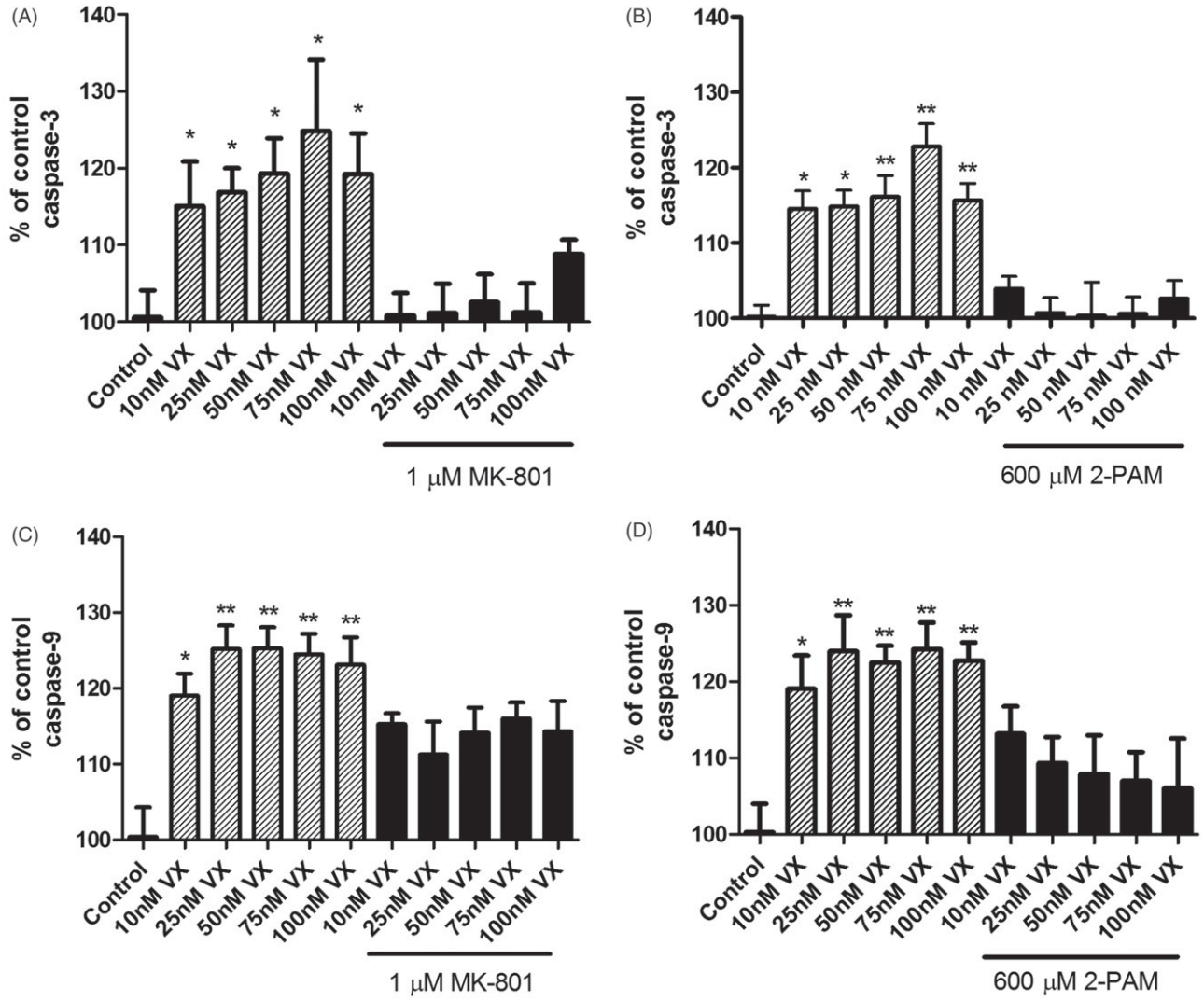

Figure 4. Effect of MK-801 and 2-PAM on VX-induced activation of caspase-3 and -9. Differentiated NSC-34 cells were incubated with either 1 μM MK-801 or 600 μM 2-PAM 1 min prior to exposure with VX. VX-induced caspase-3 (A and B) or caspase-9 (C and D) was measured 20 h after exposure. Black boxes indicate the addition of MK-801 or 2-PAM. $*p < 0.05$, $**p < 0.01$ when compared to control.

an appropriate dose range to test. Using microdialysis experiments with 267.4 μg/kg VX percutaneously applied to clipped haired guinea pigs, it was found that the concentration of VX in the brain was ~270 pM; this dose resulted in a maximal inhibition of plasma butyrylcholinesterase and erythrocyte AChE of 65.5% and 70.2%, respectively (Mumford et al., 2013). Since the dose range for the experiments presented here was 10–100 nM (i.e. 35- to 370-fold higher), it is reasonable to believe these concentrations would be toxic *in vivo*.

In the first few experiments, we tried to reproduce the sensitivity of differentiated NSC-34 cell lines to glutamate, as previously reported, prior to exploring the toxicity of VX on these cells (Figure 1). The results seen agree with previous reports. The maximal increase of caspase-3 from glutamate was seen at 5 mM with higher concentrations activating caspase-3 to a lesser degree. This effect could be due to an increase in other types of cell death (i.e. necrosis, caspase-independent cell death) resulting in a lower activation of caspase-3. Both necrosis and caspase-independent cell death have been reported to be caused by glutamate incubation (Ankarcrona et al., 1995; Landshamer et al., 2008; Zhang & Bhavnani, 2006). The dose- and time-dependent activation of caspase-3 by VX revealed a significant increase with the 75 nM concentration for all the time points examined. The rise in caspase-3 activation was dose-dependent at all of the time points with the highest activation seen with 75 nM. Since caspase-3 activation only measures apoptosis, we also used flow cytometry with cells stained with annexin V and PI for a clearer picture of the types and amount of total cell death following exposure to VX. The results of the flow cytometry experiments, shown in Figure 2, complement the results seen in Figure 1. The amount of apoptosis (annexin V only and annexin V & PI staining) seen is similar at all concentrations of VX assayed, which is also seen in Figure 1(C). At all concentrations of VX, an appreciable amount of necrosis (PI only staining) is seen; this shows that VX induces not only apoptotic cell death but necrosis as well.

The amount of VX-induced AChE inhibition was found to range from 50 to 80% was in correlation with total cell death from Figures 1 and 2. The maximal inhibition of AChE with the concentrations tested appears to be with exposure to 100 nM, whereas the most cell death was seen with 75 nM. A closer examination of the data shows that the VX-induced

AChE inhibition with 100 nM is not significantly different from that with 75 nM. These data show that VX can induce the inhibition of AChE in differentiated NSC-34 cells. The reversal of VX-induced activation of caspase-3 and -9 with both MK-801 and 2-PAM indicates that the activation of these caspases is due to VX. This reversal was such that the activation of both of these caspases was not significantly elevated over control. Overall, these results indicate that apoptotic cell death of differentiated NSC-34 cells following exposure to VX was a direct effect of this specific NA toxicity as the effect was reversed by 2-PAM and MK-801, common therapeutic agents used to alleviate NA toxicity.

Conclusions

The data presented here show that differentiated NSC-34 cells are sensitive to VX-induced cell death and could be a viable *in vitro* cell model for neuroprotective screening studies; however, more studies need to be completed to fully validate this cell model for other NA toxicity studies.

Declaration of interest

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