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Repeated Exposure to Sublethal Doses of the Organophosphorus Compound VX Activates BDNF Expression in Mouse Brain

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The highly toxic organophosphorus compound VX [*O*-ethyl *S*-[2-(diisopropylamino)ethyl]methylphosphonate] is an irreversible inhibitor of the enzyme acetylcholinesterase (AChE). Prolonged inhibition of AChE increases endogenous levels of acetylcholine and is toxic at nerve synapses and neuromuscular junctions. We hypothesized that repeated exposure to sublethal doses of VX would affect genes associated with cell survival, neuronal plasticity, and neuronal remodeling, including brain-derived neurotrophic factor (BDNF). We examined the time course of BDNF expression in C57BL/6 mouse brain following repeated exposure (1/day × 5 days/week × 2 weeks) to sublethal doses of VX (0.2 LD₅₀ and 0.4 LD₅₀). BDNF messenger RNA expression was significantly ($p < 0.05$) elevated in multiple brain regions, including the dentate gyrus, CA3, and CA1 regions of the hippocampal formation, as well as the piriform cortex, hypothalamus, amygdala, and thalamus, 72 h after the last 0.4 LD₅₀ VX exposure. BDNF protein expression, however, was only increased in the CA3 region of the hippocampus. Whether increased BDNF in response to sublethal doses of VX exposure is an adaptive response to prevent cellular damage or a precursor to impending brain damage remains to be determined. If elevated BDNF is an adaptive response, exogenous BDNF may be a potential therapeutic target to reduce the toxic effects of nerve agent exposure.

Key Words: neurotrophins; BDNF; organophosphorus compounds; chemical warfare nerve agents; VX; mice.

VX (*O*-ethyl *S*-[2-(diisopropylamino)ethyl]methylphosphonate) is a chemical warfare nerve agent (CWNA) that irreversibly inhibits serine esterases, including acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Prolonged inhibition of

AChE leads to increased levels of acetylcholine (ACh) at neuronal synapses and neuromuscular junctions, which results in symptoms of acute toxicity, including convulsions, tremors, hypothermia, urinary and fecal incontinence, and bronchial constriction (reviewed in Russell and Overstreet, 1987). Acute toxic levels of CWNA, particularly at doses producing seizures, induce widespread neuropathological damage in several areas, including the piriform cortex, thalamus, amygdala, and hippocampus (reviewed in Petras, 1994). In addition, CWNA-induced seizures alter inflammatory responses and signaling pathways associated with inflammation in these brain regions (Spradling *et al.*, 2011).

Although the seizurogenic and neuropathological effects of acute exposure to toxic levels of CWNA have been well characterized, less knowledge exists about the effects of repeated exposure to sublethal doses of CWNA. Incidents such as the release of sarin in Tokyo subways (Ohbu *et al.*, 1997) and the destruction of an ammunition depot that contained both sarin and cyclosarin during the Persian Gulf War (reviewed in McCauley *et al.*, 2002), as well as concerns about the persistence of VX in the environment due to its low volatility and high stability in surfaces such as asphalt (Gura *et al.*, 2006), have increased awareness of the need to understand the short- and long-term effects of exposure to sublethal doses of CWNA. Bloch-Shilderman *et al.* (2008) have reported that rats exposed to 0.05 LD₅₀ VX for 3 months via an osmotic minipump show an impairment in the open field test as well as reduction in the expression of vesicle-associated membrane protein in hippocampal neurons. However, CWNA can also be administered repeatedly with minimal overt neurobehavioral effects, suggesting the development of tolerance to the disruptive effects of exposure (Russell *et al.*, 1986). Blood and brain AChE levels can be reduced with appropriate dosing schedules to > 20% of normal with no observable signs of toxicity (Sterri *et al.*, 1980). Unfortunately,

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there has been minimal research into the molecular mechanisms of exposure to sublethal doses of CWNA, with the exception of Blanton *et al.* (2004), who evaluated the effects of repeated exposure to sublethal doses of VX on neuronal gene expression using microarrays.

Peptides with known trophic effects may be unique targets of intoxication and important factors in the recovery of surviving subjects. In addition, some latent effects of CWNA may be partially due to altered expression or action of neurotrophins. Neurotrophins, including brain-derived neurotrophic factor (BDNF), act through discrete tyrosine kinase (trk) receptors and are important for neuronal development, plasticity, cell survival, and remodeling following ischemia, trauma, and toxin exposure (reviewed in Mocchetti and Wrathall, 1995). BDNF plays a critical role in neuronal plasticity, including the morphology of dendritic spines, demonstrated by the fact that BDNF/TrkB signaling increased dendritic spine density in apical dendrites of hippocampal CA1 pyramidal neurons at 24 h (Alonso *et al.*, 2004). In rat hippocampus, BDNF is increased following traumatic brain injury (Grundy *et al.*, 2000) and ischemia (Tsukahara *et al.*, 1998), which may be neuroprotective. BDNF has also been shown to rescue motor neurons and substantia nigra dopaminergic cells from traumatic brain injury (Sendtner *et al.*, 1992) and reduce striatal damage and Parkinson's-like symptoms induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in monkeys (Tsukahara *et al.*, 1995).

We hypothesized that repeated exposure to low levels of VX would affect neurotrophin expression in brain regions previously shown to be affected by CWNA. C57BL/6 mice were exposed to low doses (0.2 or 0.4 LD₅₀) of VX five times per week (Monday to Friday) for 2 weeks. Expression of BDNF in mouse brain was measured at 2 or 72 h after the last VX exposure, similar to time points measured by Blanton *et al.* (2004) using microarrays, in which they observed a transient increase in gene clusters following repeated exposure to sublethal doses of VX. In our study, we hypothesized that BDNF levels would be affected by 2 h post-exposure and return to baseline by 72 h post-exposure.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (25–30 g, 10–16 weeks old; Jackson Laboratories, Bar Harbor, ME) were group-housed (six per cage) upon arrival at the US Army Medical Research Institute of Chemical Defense (USAMRICD; Aberdeen Proving Ground, MD) under a reverse light-dark cycle (lights off at 1100), with food and water available *ad libitum*. Mice were sc implanted with identification chips (BioMedic Data Systems Inc., Seaford, DE) 1 week before the start of VX exposures.

Drug Exposure

VX, diluted in saline (Phoenix Scientific Inc., Ft Dodge, IA), was obtained from the Surety Issue Laboratory at USAMRICD, aliquoted into serum vials, sealed with Teflon septa, and stored at -80°C to ensure that mice received the same dilution and lot over the course of the exposures. Mice received saline ($n = 11$),

0.2 LD₅₀ VX (4.2 $\mu\text{g}/\text{kg}$; $n = 11$), or 0.4 LD₅₀ VX (8.4 $\mu\text{g}/\text{kg}$; $n = 9$) once per day Monday through Friday at 0830 ± 1 h for 2 weeks, totaling 10 exposures. VX was sc injected between the shoulder blades in a volume of 5 ml/kg, and the LD₅₀ value for VX given sc in mice is 21 $\mu\text{g}/\text{kg}$ (Boskovic, 1979).

Measurement of AChE and BuChE

Tail blood (14 μl) was collected by tail nick and pipetted, using a heparin-dipped pipette tip, into centrifuge tubes that contained 186 ml sterile water. Samples were collected 2 or 72 h following the last VX exposure and stored at -80°C until assays were performed. Whole blood concentrations of AChE and BuChE were determined using methods developed at the Walter Reed Army Institute of Research (Haigh *et al.*, 2008).

Euthanasia

Mice were euthanized by decapitation either 2 or 72 h after their last exposure, and brains were removed and stored in isopentane (-80°C) until sectioned. Coronal sections (14 μm) were cut using a cryostat, placed onto poly-L-lysine precoated slides (Cell Associates, Houston, TX), and kept frozen until processed.

Measurement of BDNF Messenger RNA Expression

BDNF probe preparation. RNA probes for BDNF were generated by *in vitro* transcription reactions according to the instructions provided by the manufacturer (Strip-EZ RNA; Ambion, Austin, TX). For the production of antisense BDNF RNA probes, a plasmid containing a 460-bp BDNF insert (Smith and Cizza, 1996) was first linearized with EcoRI and transcribed with T7 polymerase. For sense probes, this plasmid was linearized with SalI and transcribed with SP6 polymerase. All complementary RNA probes were synthesized using ^{33}P -labeled uridine triphosphate (UTP). For the *in vitro* synthesis of RNA transcripts from DNA templates, a cocktail consisting of 0.5 μg DNA template, 5 μl ribonucleotide triphosphates (1 μl of each 10mM solution of adenosine triphosphate, guanosine triphosphate and UTP, and 2 μl modified cytidine triphosphate solution [2mM]), ^{33}P -UTP (specific activity of 5×10^9 cpm/ μg), 2 μl RNA polymerase, 2 μl of $10\times$ transcription buffer and nuclease-free H₂O needed to bring the final volume to 20 μl . The reaction mixture was incubated for 1 h at 37°C .

In situ hybridization. Slides were allowed to warm up to room temperature and fixed in 4% paraformaldehyde-EM grade (Electron Microscopy Sciences, Hatfield, PA) in $1\times$ phosphate-buffered saline (PBS) (0.01M KH₂PO₄, 0.1M Na₂HPO₄, 1.37M NaCl, 0.027M KCl, pH 7.2) for 20 min. Sections were washed with $1\times$ PBS for 5 min and then washed with a solution containing 20 $\mu\text{g}/\text{ml}$ proteinase K in 50mM Tris-HCl, pH 8.0, and 5mM ethylenediaminetetraacetic acid (EDTA) for 5 min. Slides were then washed with $1\times$ PBS for 5 min and refixed in 4% paraformaldehyde-EM grade in $1\times$ PBS for 20 min. To diminish probe binding to protein moieties and thus reduce background hybridization, tissue sections were acetylated in 300 ml 0.1M triethanolamine, pH 8.0, containing 0.75 ml acetic anhydride and incubated for 10 min. Slides were then washed in PBS and in diethylpyrocarbonate-treated water (DEPC-H₂O) for 5 min each. Next, the slides were dehydrated by washing in an ascending ethyl alcohol (EtOH) concentration series: 60% EtOH (1 min), 80% EtOH (1 min), 95% EtOH (2 min), 99% EtOH (1 min), chloroform (5 min), 99% EtOH (1 min), and 95% EtOH (1 min). Sections were allowed to air dry and used immediately for *in situ* hybridization according to the method of Wisden *et al.* (1991). Hybridization solution (50% formamide, 0.6M NaCl, 10mM Tris-HCl pH 7.5, 1mM EDTA, 50 μg heparin, 10mM dithiothreitol, 0.5 mg/ml single-stranded DNA, 0.5 mg/ml transfer RNA, 10% polyethylene glycol 8000, and $1\times$ Denhardt's solution: Novagen), containing 4×10^6 cpm/ μl of either sense or antisense ^{33}P -labeled UTP RNA probes (specific activity of $> 1.1 \times 10^8$ cpm/ μg), was heated to 80°C , placed on ice for 1 min, and pipetted onto the sections, which were then covered with cover slips. Slides were placed horizontally in a slide box containing tissue paper soaked with 5 ml PBS. Slides were then incubated in a hybridization oven at 56°C for 16–24 h. Next, cover slips were removed from brain sections by placing slides vertically and then washed twice in $2\times$ saline-sodium citrate (SSC) (3.0M NaCl and 0.3M sodium citrate) at 50°C for 30 min. This was followed by

a 30-min wash using slow agitation in a solution containing $2\times$ SSC and 14M 2- β -mercaptoethanol (BME) at 50°C, one 30-min wash in a ribonuclease (RNase) solution containing 10 μ l of 5–10 U/ μ l RNase One (Promega, Madison, WI), $2\times$ SSC and, 14M BME at 37°C, a 30-min wash in a solution containing $2\times$ SSC, 14M BME, and deionized formamide at 50°C, and then a 30-min wash in a solution containing $2\times$ SSC, 14M BME, and 1% Na pyrophosphate. Finally, the slides were washed in DEPC-H₂O for 5 min and then dehydrated in 60% EtOH for 1 min and then in 95% EtOH for 5 min. Sections were allowed to air dry and were autoradiographed on Kodak BioMax maximum sensitivity (Eastman Kodak Co, Rochester, NY) film at 80°C for 3 days. The film was developed and scanned for analysis. Controls for the *in situ* hybridization included brain sections hybridized with both sense or unlabeled antisense oligonucleotide probes and RNase A-treated sections. For RNase controls, brain sections were fixed as described above and then pretreated with RNase A (20 μ g/ml) overnight at 37°C. Next, sections were treated with ³³P-labeled RNA probes as described above. For unlabeled-antisense controls, brain sections were fixed as described above and then incubated at 42°C for 16–24 h with $10\times$ unlabeled antisense before being treated with ³³P-labeled RNA probes as described above. Control brain sections did not have any bound ³³P-labeled oligonucleotide probe. *In situ* hybridization autoradiographs of controls are shown elsewhere (Pizarro *et al.*, 2004).

Quantification of BDNF messenger RNA expression. Image analysis of the *in situ* hybridization was done in a blind fashion (groups were unknown to the data analyst). Films exposed to the hybridized sections were used for densitometric analysis to measure changes in BDNF messenger RNA (mRNA) levels in the whole brain and the regions of the hippocampal formation. For *in situ* hybridization analysis of the dentate gyrus (DG), CA3, and CA1 regions of the hippocampal formation, optical density measurements were obtained by tracing each region. The same procedure was used to measure BDNF mRNA in regions of the thalamus, hypothalamus, amygdala, piriform cortex, and other cortical areas. Films were captured electronically for densitometric analysis using the Model GS-800 Calibrated Imaging Densitometer and the Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Measurement of BDNF Protein Expression

Immunohistochemistry. Slides were thawed at room temperature for 15–30 min and then fixed in cold acetone for 10 min. Slides were blocked with 3% donkey serum in PBS with mild agitation for 1 h, and only the BDNF slides were incubated with 0.5% Triton X-100 in PBS with mild agitation for 30 min. The slides were incubated with primary antibody or antiBDNF (SC-546 at 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), with mild agitation for 2 h. They were incubated with a secondary antibody, Alexa Fluor 555-conjugated donkey anti-rabbit IgG (A31572 at 1:1000; Molecular Probes, Inc., Eugene, OR), with mild agitation for 50 min. To ensure that there was no nonspecific binding from the antibodies themselves to the tissues, three types of negative controls were examined. For the first control, there was no primary antibody applied to the tissue sections. For the second control, the normal rabbit IgG was used instead of the primary antibody. For the third control, the primary antibody is mixed with a blocking peptide (Ab:blocking peptide 1:30) and incubated for 1 h before adding the secondary antibody. The images of the best two sections on each slide were taken with a charge-coupled device camera in an Olympus BX51 microscope controlled by the OASIS Turboscan (Objective Imaging, Cambridge, U.K.) on the second and third day after each assay. Turboscan is a high-speed mosaic imaging system adapted in Image-Pro Plus 6.0 (Media Cybernetics, Inc., Bethesda, MA) software. The images of each section were aligned and put together automatically using Turboscan module and saved as a mosaic image. The predictive focus setting was used to adjust the focus for three corners (upper right, bottom right, and upper left). The exposure time was 3 s for scanning sections using a $\times 4$ objective, 900 ms using a $\times 10$ objective, 300 ms using a $\times 20$ objective, and 600 ms using a $\times 40$ objective. The intensities of mouse hippocampus region and background (nontissue area) were measured using the Quantity One software (Bio-Rad Laboratories).

Quantification of BDNF protein expression. The mouse hippocampus region was identified and traced for each image collected in Quantity One software. The background region was also traced for each image. The intensities of each traced area and background region were measured. The protein expression was defined by the ratio of hippocampus intensity versus background for each image, and it was globally normalized against each collection day.

Data Analysis

Results are presented as mean \pm SEM. One-way ANOVA with a factor of drug dose was used to compare significant changes in BDNF expression (mRNA or protein) and to compare AChE and BuChE levels following VX exposure at each time point. *Post hoc* tests were performed using least significant difference test.

RESULTS

Spatiotemporal Distribution of BDNF

We examined the temporal profile of BDNF mRNA expression at 2 and 72 h after repeated injections of saline and 0.2 or 0.4 LD₅₀ VX, using *in situ* hybridization analysis (Fig. 1). Mice that were injected with 0.4 LD₅₀ VX showed elevated levels of BDNF mRNA expression at 2 and 72 h following the final exposure in areas of the hippocampus, lateral amygdala, cortex, hypothalamus, and thalamus. In some brain regions, 0.2 LD₅₀ VX also increased BDNF mRNA expression at 2 h, but not at 72 h, post-exposure.

Quantitative measures of the effects of VX on BDNF mRNA levels in the piriform cortex (Pir), amygdala (anterior basolateral amygdala, posterior basolateral amygdala, and posterior basomedial amygdala), ventromedial hypothalamic nucleus (VMH), dorsomedial hypothalamic nucleus (DMH), parafascicular thalamic nucleus (PF), intermediodorsal thalamic nucleus (IMD), paraventricular thalamic nucleus (PVT), medial habenular nucleus (MHb), retrosplenial granular cortex (RSG), retrosplenial agranular cortex (RSA), DG, hippocampus CA1 field, hippocampus CA3 field, and posterior parietal association area (PPtA) were assessed by densitometric analysis. As illustrated in Figure 2A, these brain regions were identified using *The Mouse Brain in Stereotaxic Coordinates* (Paxinos and Franklin, 2001). The most intensely expressed regions in the brain include the cell granule layer of the DG, CA3, and CA1 subregions of the hippocampus, amygdala, frontal cortex, and the piriform cortex.

VX Effects on BDNF

Two hours following the last VX exposure, BDNF mRNA expression was increased in regions of the hippocampal formation (DG [$F_{2,13} = 9.74$; $p < 0.01$], CA1 [$F_{2,13} = 6.15$; $p < 0.02$], and CA3 [$F_{2,13} = 6.70$; $p < 0.02$]; Fig. 2). Both 0.2 LD₅₀ and 0.4 LD₅₀ VX-treated mice had increased BDNF mRNA expression in the DG at 2 h after the last VX exposure, whereas only 0.4 LD₅₀ VX-treated mice had significant increased expression in CA1 and CA3, relative to saline-treated mice. BDNF mRNA expression was also increased in

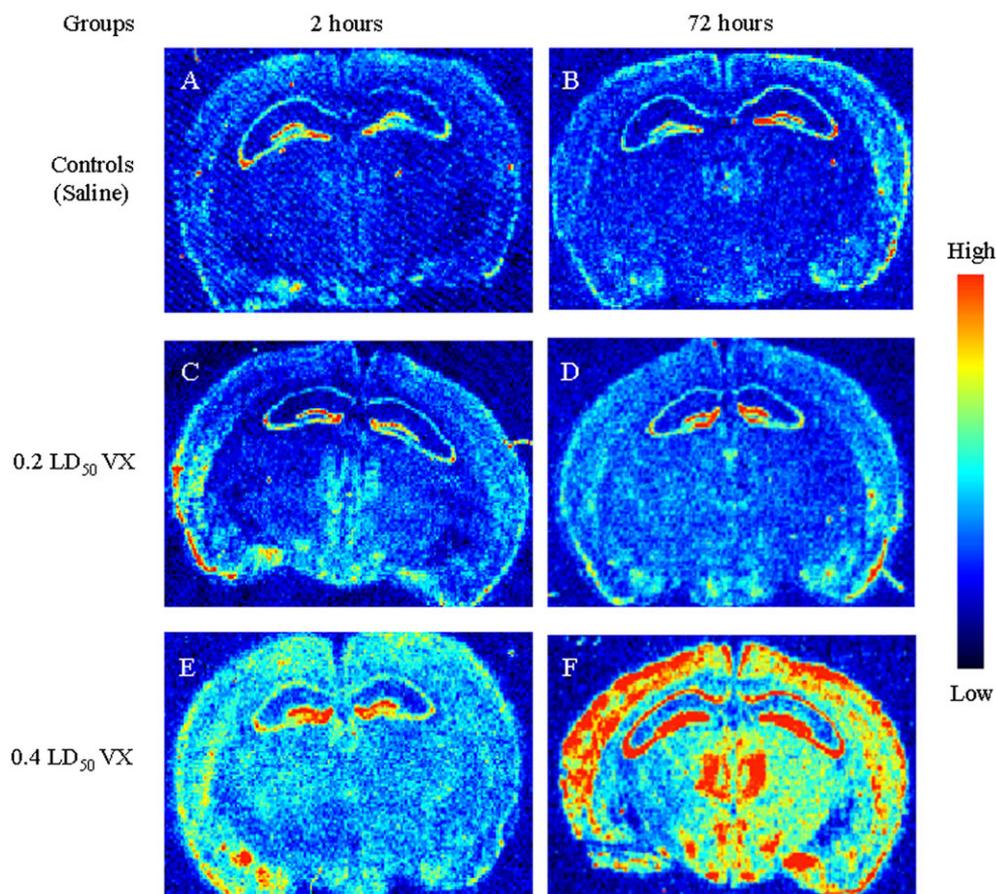


FIG. 1. Differential spatiotemporal distribution of BDNF mRNA in the brain after repeated exposure to low levels of the chemical warfare agent VX. Graded densitometric display of autoradiographs of *in situ* hybridizations showed spatiotemporal binding of the ^{33}P -labeled probe against BDNF mRNA in mouse brains after exposure to saline control (A and B), 0.2 LD₅₀ dose of VX (C and D), and 0.4 LD₅₀ dose of VX (E and F). A significant increase in BDNF mRNA expression in the brain is observed 2 h and 72 h following the last 0.4 LD₅₀ exposure compared with controls.

regions of the hippocampus 72 h after the last 0.4 LD₅₀ VX exposure (DG [$F_{2,16} = 9.74$; $p < 0.01$], CA1 [$F_{2,16} = 11.75$; $p = 0.001$], and CA3 [$F_{2,16} = 10.78$; $p = 0.001$]). BDNF protein expression, on the other hand, was only increased in the CA3 region of the hippocampus at 72 h after the last 0.4 LD₅₀ VX exposure ($F_{2,28} = 4.01$; $p = 0.030$; Fig. 3).

In the MHb, BDNF mRNA expression was increased by 0.4 LD₅₀ VX at both 2 h [$F_{2,13} = 5.47$; $p = 0.02$] and 72 h [$F_{2,16} = 5.51$; $p = 0.02$] after the last VX exposure, relative to saline-treated mice (Table 1). In thalamic nuclei, BDNF mRNA expression was also increased 2 h after the last 0.4 LD₅₀ VX exposure (PVT [$F_{2,13} = 8.00$; $p < 0.01$], PF [$F_{2,13} = 6.18$; $p < 0.02$], and IMD [$F_{2,13} = 8.22$; $p < 0.01$]) and 72 h after the last 0.4 LD₅₀ VX exposure (PVT [$F_{2,16} = 4.81$; $p < 0.03$], PF [$F_{2,16} = 4.94$; $p < 0.03$], and IMD [$F_{2,16} = 6.28$; $p < 0.02$]).

In cortical areas, including the PPTA, RSA, and RSG, 0.4 LD₅₀ VX increased BDNF mRNA expression 2 h after the last exposure (PPTA [$F_{2,13} = 5.81$; $p < 0.02$], RSA [$F_{2,13} = 6.30$; $p < 0.02$], and RSG [$F_{2,13} = 5.74$; $p = 0.02$]). BDNF mRNA expression remained increased 72 h after the last VX exposure (PPTA [$F_{2,16} = 10.71$; $p = 0.002$], RSA [$F_{2,16} = 12.52$; $p =$

0.001], and RSG [$F_{2,16} = 7.85$; $p = 0.005$]). In piriform cortex, both 0.2 LD₅₀ and 0.4 LD₅₀ VX increased BDNF mRNA expression 2 h post-exposure [$F_{2,13} = 6.26$; $p < 0.02$], and BDNF remained increased 72 h after the last 0.4 LD₅₀ VX exposure [$F_{2,16} = 10.98$; $p < 0.02$].

In areas of the hypothalamus, 0.4 LD₅₀ VX increased BDNF mRNA expression 2 h (DMH [$F_{2,13} = 9.74$; $p < 0.01$] and VMH [$F_{2,13} = 9.74$; $p < 0.01$]) and 72 h (DMH [$F_{2,16} = 4.91$; $p < 0.03$] and VMH [$F_{2,16} = 7.82$; $p = 0.005$]) after the last VX exposure, relative to saline-treated mice. In the lateral amygdala, BDNF mRNA expression was also increased by 0.4 LD₅₀ VX at both 2 h [$F_{2,13} = 4.78$; $p < 0.04$] and 72 h [$F_{2,16} = 8.90$; $p = 0.003$] following the last VX exposure.

VX Effects on Blood Enzymes

Whole blood levels of AChE [$F_{2,13} = 90.10$; $p < 0.001$] and BuChE [$F_{2,13} = 981$; $p < 0.001$] were significantly less in mice repeatedly exposed to 0.2 LD₅₀ VX and 0.4 LD₅₀ VX, 2 h after the last exposure, relative to control (Fig. 4). Levels of AChE [$F_{2,16} = 2.73$; $p = 0.1$] in VX-treated mice returned to control levels and were not significantly different 72 h post-exposure.

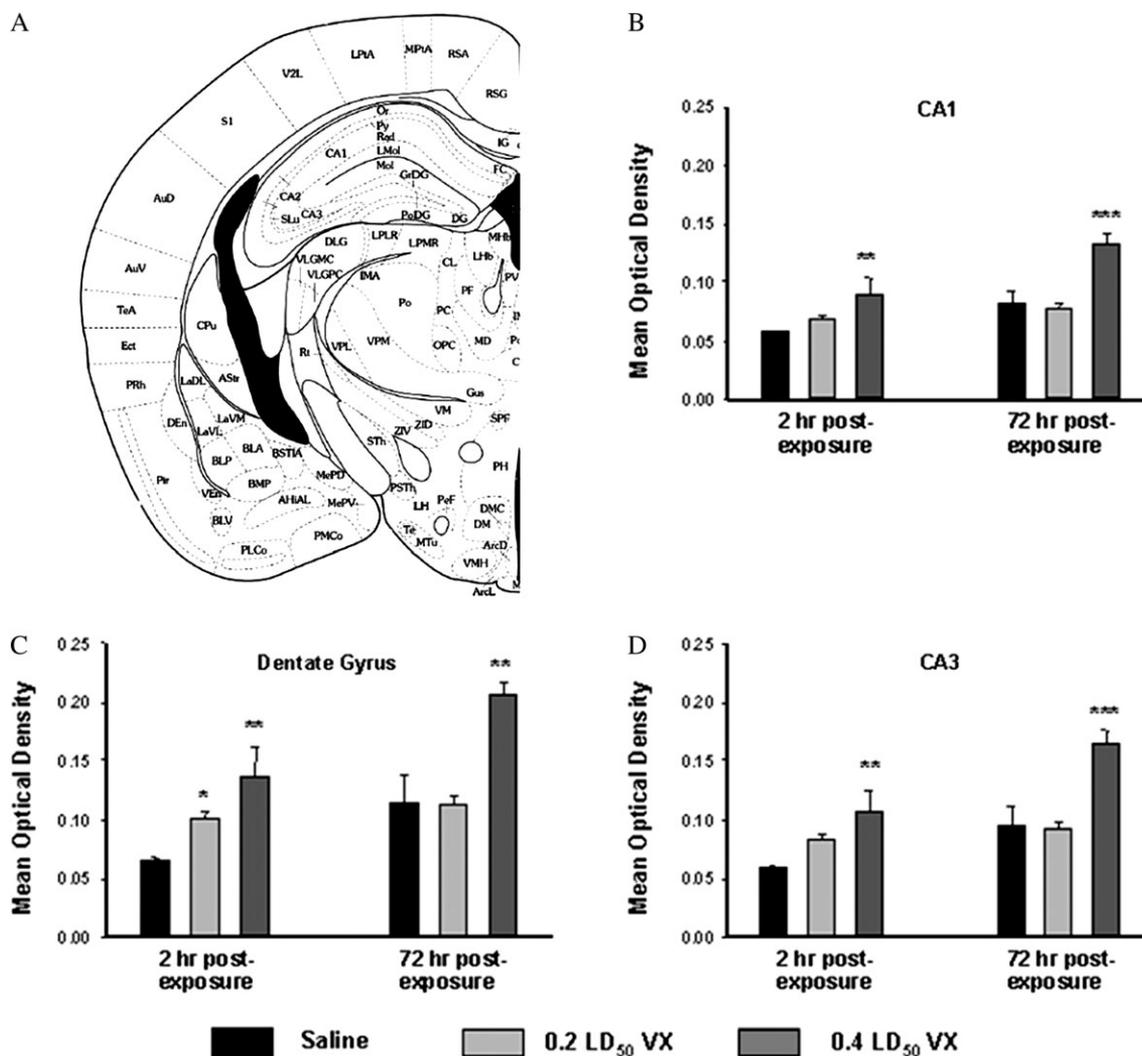


FIG. 2. *In situ* hybridization analysis of BDNF mRNA expression in hippocampal regions following repeated exposure to low levels of VX. (A) Regions analyzed were identified using *The Mouse Brain in Stereotaxic Coordinates* (Paxinos and Franklin, 2001). A significant increase in BDNF expression was observed 2 h and 72 h after 0.4 LD₅₀ exposure compared with controls in the CA1 (B), DG (C), and CA3 (D). In addition, a significant increase in BDNF expression was observed in the DG 2 h, but not 72 h, after the last exposure to 0.2 LD₅₀ VX. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Levels of BuChE [$F_{2,16} = 20.53$; $p < 0.001$] increased with time following VX exposure but were still significantly lower than that of control mice 72 h after the last exposure.

DISCUSSION

Repeated exposure to 0.4 LD₅₀ VX increased BDNF mRNA expression in multiple brain regions, including areas of the hippocampus, amygdala, hypothalamus, thalamus, and cortex. BDNF mRNA expression was increased in these regions at both 2 and 72 h following the last exposure to 0.4 LD₅₀ VX. In two brain regions, the piriform cortex and the DG of the hippocampus, BDNF mRNA expression was also increased at 2 h, but not 72 h, after the last exposure to 0.2 LD₅₀ VX. BDNF

protein expression, on the other hand, was only increased in the CA3 region of the hippocampus at 72 h following the last exposure to 0.4 LD₅₀ VX. The lack of change in BDNF protein expression in the other brain regions may reflect a deficiency in the translation of the newly synthesized BDNF mRNA, indicating that the BDNF gene is regulated at the level of transcription and translation or an increased turnover rate of BDNF protein. Several other studies have also shown that changes in BDNF mRNA and protein are not always correlated (Nanda and Mack, 2000; Pollock *et al.*, 2001).

The greater susceptibility of the piriform cortex and hippocampus to changes in BDNF mRNA expression following repeated exposure to 0.2 LD₅₀ VX is of interest because these two limbic regions are important for the generation and propagation of seizures. In addition, the piriform cortex and

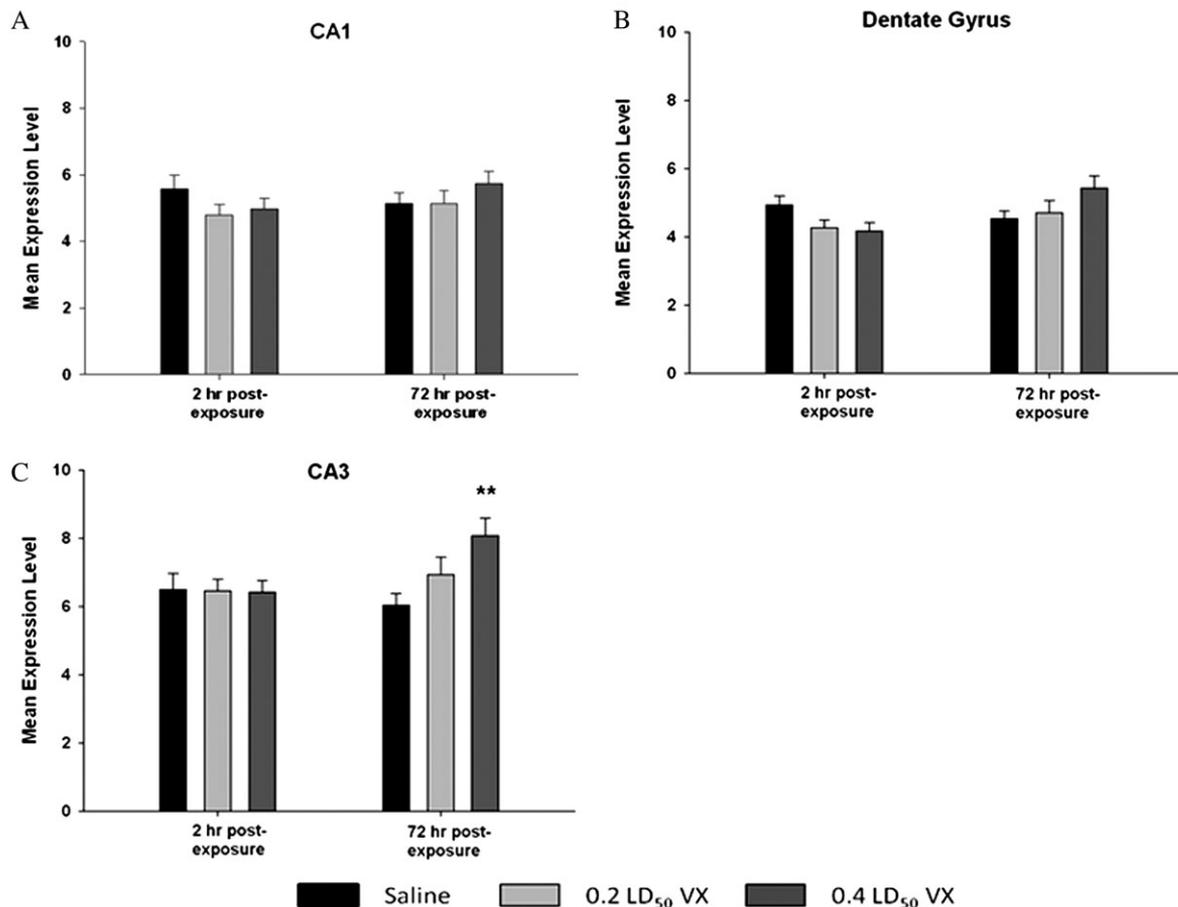


FIG. 3. Immunohistochemistry analysis of BDNF protein expression in hippocampal regions following repeated exposure to low levels of VX. Regions analyzed included the CA1 (A), DG (B), and CA3 (C). A significant increase in BDNF expression was observed 72 h after 0.4 LD₅₀ exposure in CA3 compared with controls. ** $p < 0.01$.

hippocampus are particularly sensitive to showing prolonged cholinesterase inhibition following repeated low-dose soman exposure; other regions, including the thalamus and hypothalamus, are also affected but to a lesser extent (Howerton *et al.*, 1991). Although we did not observe convulsions in mice exposed to low levels of VX, we did see transient tremors following the last VX exposures. Evaluation of seizure activity was not recorded in this study but may provide useful information in future studies.

Seizures increase BDNF mRNA expression in the hippocampal formation, cerebral cortex, and amygdaloid complex of rodents (reviewed in Murer *et al.*, 2001). Because chronic infusion of BDNF into the hippocampus inhibits kindling (Larmet *et al.*, 1995), it has been suggested that upregulation of BDNF by seizures may in turn limit epileptogenesis, possibly through upregulation of neuropeptide Y, which has anticonvulsant properties (Reibel *et al.*, 2001). Our findings that repeated exposures to low doses of VX increase BDNF mRNA expression in brain regions important for seizure initiation and propagation may have implications for failure of these mice to develop convulsions.

Increased BDNF expression following hypoxia-ischemia, hypoglycemic coma, and traumatic brain injury has been well documented (reviewed in Murer *et al.*, 2001). Many studies suggest that the functional effects of insult-induced neurotrophin expression in the central nervous system (CNS) result in neuronal protection (reviewed in Lindvall *et al.*, 1994). One suggested mechanism by which BDNF may be neuroprotective in stroke models is through the prevention of apoptotic cell death (Schabitz *et al.*, 2000). Other suggested mechanisms include the prevention of glutamate-induced excitotoxicity and cytoskeletal protein degradation. Exogenous BDNF treatment protects and increases survival of basal forebrain and hippocampal/cortical neuron cultures after insults, such as excitotoxins, calcium overload, and elevated concentrations of free radicals (Lowenstein and Arsenault, 1996).

Neurotrophins in the hippocampus also contribute to long-term potentiation (Messaoudi *et al.*, 1998) and learning and memory. In rats with hippocampal damage and spatial memory impairment, BDNF levels are decreased (reviewed in van Praag *et al.*, 1998), whereas training in a radial arm spatial memory

TABLE 1
In Situ Hybridization Analysis of BDNF mRNA Expression (Mean Optical Density \pm SEM) in Other Brain Regions Following Repeated Exposure to Low Levels of VX

Brain region	2 h post-exposure			72 h post-exposure		
	Saline	0.2 LD ₅₀ VX	0.4 LD ₅₀ VX	Saline	0.2 LD ₅₀ VX	0.4 LD ₅₀ VX
Amygdala	0.0528 \pm 0.0005	0.0618 \pm 0.0018	0.0782 \pm 0.0116*	0.0693 \pm 0.0066	0.0692 \pm 0.0034	0.0970 \pm 0.0049**
DMH	0.0533 \pm 0.0006	0.0639 \pm 0.0026	0.0775 \pm 0.0114*	0.0711 \pm 0.0079	0.0706 \pm 0.0047	0.0976 \pm 0.0072*
IMD	0.0532 \pm 0.0005	0.0639 \pm 0.0037	0.0758 \pm 0.0064**	0.0731 \pm 0.0095	0.0722 \pm 0.0055	0.1090 \pm 0.0093**
MHb	0.0529 \pm 0.0005	0.0616 \pm 0.0020	0.0741 \pm 0.0087**	0.0715 \pm 0.0083	0.0669 \pm 0.0038	0.0945 \pm 0.0047*
PF	0.0537 \pm 0.0007	0.0619 \pm 0.0025	0.0723 \pm 0.0068**	0.0734 \pm 0.0090	0.0675 \pm 0.0043	0.0959 \pm 0.0046*
PVT	0.0530 \pm 0.0007	0.0616 \pm 0.0021	0.0770 \pm 0.0081**	0.0728 \pm 0.0098	0.0703 \pm 0.0043	0.0987 \pm 0.0044*
Pir	0.0582 \pm 0.0010	0.0850 \pm 0.0050*	0.1020 \pm 0.0170**	0.0918 \pm 0.0133	0.0948 \pm 0.0060	0.1510 \pm 0.0072***
PPtA	0.0547 \pm 0.0005	0.0614 \pm 0.0023	0.0804 \pm 0.0106**	0.0714 \pm 0.0071	0.0687 \pm 0.0046	0.1080 \pm 0.0076**
RSA	0.0544 \pm 0.0008	0.0622 \pm 0.0023	0.0791 \pm 0.0095**	0.0699 \pm 0.0065	0.0684 \pm 0.0039	0.1040 \pm 0.0061***
RSG	0.0530 \pm 0.0006	0.0584 \pm 0.0018	0.0738 \pm 0.0087**	0.0666 \pm 0.0060	0.0647 \pm 0.0036	0.0900 \pm 0.0045**
VMH	0.0542 \pm 0.0008	0.0675 \pm 0.0025	0.0813 \pm 0.0116**	0.0752 \pm 0.0088	0.0725 \pm 0.0038	0.1080 \pm 0.0071**

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

test increases hippocampal BDNF in rats (Mizuno *et al.*, 2000). Administration of antisense BDNF (i.c.v.) prevents acquisition and blocks retention in trained rats. In humans, a deficit in neurotrophins was found in areas with Alzheimer's plaques (Soontornniyomkij *et al.*, 1999).

Given that the administration of ACh agonists into the hippocampus increases expression of BDNF and trkB mRNA (French *et al.*, 1999), ACh is a mediator of BDNF expression in the CNS. This is in agreement with our findings that inhibiting AChE and thereby increasing ACh levels increases BDNF expression. As expected, levels of blood AChE and BuChE were significantly reduced 2 h after the last VX exposure and approached that of controls by 72 h post-exposure. The current findings indicate that although blood levels of AChE return to that of control by 72 h

post-exposure, changes in gene expression in regions of the CNS are still present.

Whether BDNF might be a therapeutic target for neurobehavioral deficits following repeated exposure to low-dose VX remains to be determined. The safety of using recombinant methionyl human BDNF administered intrathecally was tested in phase I/II clinical trials as a potential treatment for amyotrophic lateral sclerosis (ALS) (Ochs *et al.*, 2000), and in phase III trials for ALS (Kalra *et al.*, 2003). Unidirectional transport of BDNF from the periphery into the CNS has been shown in mice, indicating that pharmacokinetically this neurotrophin is a potential therapeutic candidate (Pan *et al.*, 1998). Of potential interest, soldiers in the Gulf War, some of whom may have been exposed to a combination of organophosphates or other environmental insults, have a greater

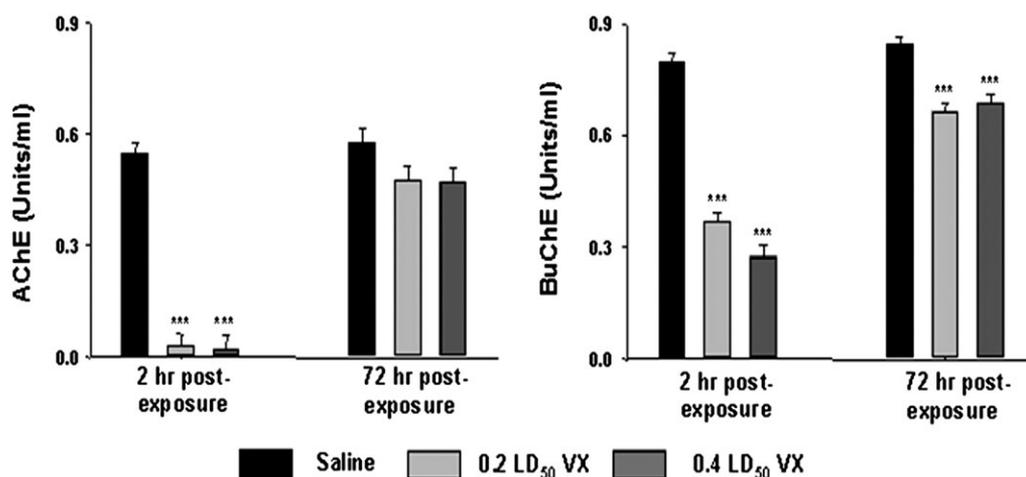


FIG. 4. Mice that received repeated exposure to low levels of VX (0.2 LD₅₀ and 0.4 LD₅₀) had significantly lower blood levels of AChE and BuChE 2 h after the last exposure, relative to control (saline-treated mice). Mice evaluated 72 h after their last VX exposure had similar levels of AChE to control. Although levels of BuChE began to return to that of control, levels in VX-treated mice were significantly less than control 72 h after the last exposure. *** $p < 0.001$.

than twofold increase in incidence of ALS (Haley, 2003). More research is needed to extend the time course of the upregulation of BDNF following repeated low-dose exposure to VX and to determine how these molecular changes may relate to neurobehavioral deficits and neuropathology. Although exposure to organophosphates such as pesticides has been shown to affect the expression of many neurotrophic factors (Slotkin and Seidler, 2007), it must be determined whether VX also affects neurotrophin expression, and whether there are sex differences in these effects. The current findings have important implications for a potential therapeutic target for functional deficits that may follow CWNA exposure.

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