Repeated exposure to sublethal doses of the organophosphorus compound VX activates BDNF expression in mouse brain


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Short Title: VX Increases CNS BDNF

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Abstract:

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 x 5 days/week x2 weeks) to sublethal doses of VX (0.2 LD<sub>50</sub> and 0.4 LD<sub>50</sub>). BDNF mRNA 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 hr after the last 0.4 LD<sub>50</sub> 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.

Keywords: neurotrophins, BDNF, organophosphorus compounds, chemical warfare nerve agents, VX, mice
1. Introduction

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 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).

While 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 LD_{50} VX for three 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 (VAMP) 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, there has been minimal research
into the molecular mechanisms of exposure to sub-lethal 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 hr (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 MPTP 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 LD_{50}) of VX five times per week (Monday-Friday) for two weeks. Expression of BDNF in mouse brain was measured at 2 or 72 hr 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 hr post-exposure, and return to baseline by 72 hr post-exposure.

2. Materials and Methods

2.1 Animals

Male C57BL/6 mice (25-30 g, 10-16 weeks old; Jackson Laboratories, Bar Harbor, ME) were group-housed (6/cage) upon arrival at the U.S. 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 subcutaneously (sc) implanted with identification chips (BioMedic Data Systems Inc., Seaford, DE) one week before the start of VX exposures.

2.2 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 LD_{50} VX (4.2 μg/kg; n=11) or 0.4 LD_{50} VX (8.4 μg/kg; n=9) once per day Monday through Friday at 0830 ± 1 hr for 2 weeks, totaling 10
exposures. VX was sc injected between the shoulder blades in a volume of 5 ml/kg, and the
LD_{50} value for VX given sc in mice is 21 µg/kg (Boskovic, 1979).

2.3 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 hr
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).

2.4 Euthanasia

Mice were euthanized by decapitation either 2 or 72 hr 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.

2.5 Measurement of BDNF mRNA Expression

2.5.1 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 cRNA
probes were synthesized using $^{33}$P-labeled UTP. For the in vitro synthesis of RNA transcripts from DNA templates, a cocktail consisting of 0.5 µg DNA template, 5 µl rNTPs (1 µl of each 10 mM solution of ATP, GTP and UTP and 2 µl Modified CTP Solution [2 mM]), $^{33}$P-UTP (specific activity of $5 \times 10^9$ cp/m/µg), 2 µl RNA polymerase, 2 µl of 10X transcription buffer and nuclease-free H$_2$O needed to bring the final volume to 20 µl. The reaction mixture was incubated for 1 hr at 37°C.

2.5.2 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 1X phosphate buffered saline (PBS: 0.01 M KH$_2$PO$_4$, 0.1 M Na$_2$HPO$_4$, 1.37 M NaCl, 0.027 M KCl, pH 7.2) for 20 min. Sections were washed with 1X PBS for 5 min and then washed with a solution containing 20 µg/ml proteinase K in 50 mM Tris-HCl, pH 8.0 and 5 mM EDTA for 5 min. Slides were then washed with 1X PBS for 5 min and refixed in 4% paraformaldehyde-EM grade in 1X PBS for 20 min. To diminish probe binding to protein moieties, and thus reduce background hybridization, tissue sections were acetylated in 300 ml 0.1 M triethanolamine, pH 8.0 containing 0.75 ml acetic anhydride and incubated for 10 min. Slides were then washed in PBS and in DEPC-H$_2$O for 5 min each. Next, 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, 0.6 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 50 µg heparin, 10 mM DTT, 0.5 mg/ml ssDNA, 0.5 mg/ml tRNA, 10% PEG 8000, 1X
Denhardt’s solution: Novagen®), containing 4 x 10^6 cpm/µl of either sense or antisense 33P-labeled UTP RNA probes (specific activity of > 1.1 x 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 hr. Next, cover slips were removed from brain sections by placing slides vertically and then washed twice in 2X SSC (SSC: 3.0 M NaCl and 0.3 M sodium citrate) at 50°C for 30 min. This was followed by a 30-min wash using slow agitation in a solution containing 2X SSC and 14 M 2-β-mercaptoethanol (BME) at 50°C, one 30-min wash in a RNase solution containing 10 µl of 5-10U/µl RNase One (Promega, Madison, WI), 2X SSC and 14 M BME at 37°C, a 30-min wash in a solution containing 2X SSC, 14 M BME, and dI formamide at 50°C, and then a 30-min wash in a solution containing 2X SSC, 14 M BME, and 1% Na pyrophosphate. Finally, 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 ribonuclease A (RNase A) treated sections. For RNase controls, brain sections were fixed as described above, and then pre-treated with RNase A (20 µg/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 hr with 10X unlabeled-antisense before being treated with 33P-labeled RNA probes as described above. Control brain sections did not
have any bound $^{33}$P-labeled oligonucleotide probe. In situ hybridization autoradiographs of controls are shown elsewhere (Pizarro et al., 2004).

2.5.3 Quantification of BDNF mRNA 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 mRNA levels in the whole brain and the regions of the hippocampal formation. For in situ hybridization analysis of the dentate gyrus, 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).

2.6 Measurement of BDNF Protein Expression

2.6.1 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 hr, 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 anti-BDNF (SC-546 at 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), with mild agitation for 2 hr. They were incubated with a secondary antibody, Alexa Fluro 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 non-specific 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 hr before adding the secondary antibody. The images of the best two sections on each slide were taken with a CCD camera in an Olympus BX51 microscope controlled by the OASIS Turboscan (Objective Imaging, Cambridge, UK) 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 4x objective, 900 ms using a 10x objective, 300 ms using a 20x objective and 600 ms using a 40x objective. The intensities of mouse hippocampus region and background (non-tissue area) were measured using the Quantity One software (Bio-Rad Laboratories).

2.6.2 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.
2.7 Data Analysis

Results are presented as mean ± standard error of the mean (SEM). One-way analysis of variance (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.

3. Results

3.1 Spatio-Temporal Distribution of BDNF

We examined the temporal profile of BDNF mRNA expression at 2 and 72 hr after repeated injections of saline, 0.2 or 0.4 LD$_{50}$ VX, using in situ hybridization analysis (Figure 1). Mice that were injected with 0.4 LD$_{50}$ VX showed elevated levels of BDNF mRNA expression at 2 and 72 hr following the final exposure, in areas of the hippocampus, lateral amygdala, cortex, hypothalamus, and thalamus. In some brain regions, 0.2 LD$_{50}$ VX also increased BDNF mRNA expression at 2 hr, but not at 72 hr, post-exposure.

Quantitative measures of the effects of VX on BDNF mRNA levels in the piriform cortex (Pir), amygdala (anterior basolateral amygdala [BLA], posterior basolateral amygdala [BLP], and posterior basomedial amygdala [BMP]), ventromedial hypothalamic nucleus (VMH), dorsomedial hypothalamic nucleus (DM), parafascicular thalamic nucleus (PF), intermediodorsal thalamic nucleus (IMD), paraventricular thalamic nucleus (PV), medial habenular nucleus (MHb), retrosplenial granular cortex (RSG), retrosplenial agranular cortex (RSA), dentate gyrus (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 Frankin, 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.

3.2 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]; Figure 2). Both 0.2 LD$_{50}$ and 0.4 LD$_{50}$ VX-treated mice had increased BDNF mRNA expression in the DG at 2 hr after the last VX exposure, while only 0.4 LD$_{50}$ VX-treated mice had significant increased expression in CA1 and CA3, relative to saline-treated mice. BDNF mRNA expression was also increased in regions of the hippocampus 72 hr after the last 0.4 LD$_{50}$ 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 hr after the last 0.4 LD$_{50}$ VX exposure ([F (2, 28) = 4.01; p=0.030]; Figure 3).

In the medial habenular nucleus, BDNF mRNA expression was increased by 0.4 LD$_{50}$ VX at both 2 hr [F (2, 13) = 5.47; p=0.02] and 72 hr [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 hr after the last 0.4 LD$_{50}$ 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 hr after the last 0.4 LD$_{50}$ 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 LD$_{50}$ VX increased BDNF mRNA expression 2 hr 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 hr 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 LD_{50} and 0.4 LD_{50} VX increased BDNF mRNA expression 2 hr post-exposure [F (2, 13) = 6.26; p<0.02], and BDNF remained increased 72 hr after the last 0.4 LD_{50} VX exposure [F (2, 16) = 10.98; p<0.02].

In areas of the hypothalamus, 0.4 LD_{50} VX increased BDNF mRNA expression 2 hr (DMH [F (2, 13) = 9.74 p<0.01] and VMH [F (2, 13) = 9.74; p<0.01]) and 72 hr (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 LD_{50} VX at both 2 hr [F (2, 13) = 4.78; p<0.04] and 72 hr [F (2, 16) = 8.90; p=0.003] following the last VX exposure.

3.3 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 LD_{50} VX and 0.4 LD_{50} VX, 2 hr after the last exposure, relative to control (Figure 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 hr post-exposure. 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 hr after the last exposure.

4. Discussion
Repeated exposure to 0.4 LD$_{50}$ 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 hr following the last exposure to 0.4 LD$_{50}$ VX. In two brain regions, the piriform cortex and the dentate gyrus of the hippocampus, BDNF mRNA expression was also increased at 2 hr, but not 72 hr, after the last exposure to 0.2 LD$_{50}$ VX. BDNF protein expression, on the other hand, was only increased in the CA3 region of the hippocampus at 72 hr following the last exposure to 0.4 LD$_{50}$ 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 LD$_{50}$ VX is of interest since these two limbic regions are important for the generation and propagation of seizures. In addition, the piriform cortex and 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). Since 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 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), while training in a radial arm spatial memory 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 central nervous system (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 hr after the last VX exposure and approached that of controls by 72 hr post-exposure. The current findings indicate that although blood levels of AChE return to that of control by 72 hr 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 than 2-fold increase in incidence of ALS (Haley, 2003). More research is needed to extend the time course of the up-regulation 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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Medical Research Institute of Chemical Defense and all procedures were conducted in
accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals
References:


Figure Legends:

Figure 1. Differential spatio-temporal 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 spatio-temporal binding of the $^{33}$P-labeled probe against BDNF mRNA in mouse brains after exposure to saline control (A, B), 0.2 LD$_{50}$ dose of VX (C, D), and 0.4 LD$_{50}$ dose of VX (E, F). A significant increase in BDNF mRNA expression in the brain is observed 2 hr and 72 hr following the last 0.4 LD$_{50}$ exposure compared to controls.

Figure 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 Frankin, 2001). A significant increase in BDNF expression was observed 2 hr and 72 hr after 0.4 LD$_{50}$ exposure, compared to controls in the CA1 (B), dentate gyrus (C) and CA3 (D). In addition, a significant increase in BDNF expression was observed in the dentate gyrus 2 hr, but not 72 hr, after the last exposure to 0.2 LD$_{50}$ VX. *p<0.05; **p<0.01; ***p<0.001

Figure 3. Immunohistochemistry analysis of BDNF protein expression in hippocampal regions following repeated exposure to low levels of VX. Regions analyzed included the CA1 (A), dentate gyrus (B) and CA3 (C). A significant increase in BDNF expression was observed 72 hr after 0.4 LD$_{50}$ exposure in CA3 compared to controls. **p<0.01
Figure 4. Mice that received repeated exposure to low levels of VX (0.2 LD$_{50}$ and 0.4 LD$_{50}$) had significantly lower blood levels of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) 2 hr after the last exposure, relative to control (saline-treated mice). Mice evaluated 72 hr 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 hr after the last exposure. ***p<0.001
Table 1. *In Situ* hybridization analysis of BDNF mRNA expression (mean optical density ± SEM) in other brain regions following repeated exposure to low levels of VX

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Saline</th>
<th>0.2 LD₅₀ VX</th>
<th>0.4 LD₅₀ VX</th>
<th>Saline</th>
<th>0.2 LD₅₀ VX</th>
<th>0.4 LD₅₀ VX</th>
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<tr>
<td>Amygdala</td>
<td>0.0528 ± 0.0005</td>
<td>0.0618 ± 0.0018</td>
<td>0.0782 ± 0.0116</td>
<td>0.0693 ± 0.0066</td>
<td>0.0692 ± 0.0034</td>
<td>0.0970 ± 0.0049</td>
</tr>
<tr>
<td>Dorsomedial Hypothalamic Nucleus (DM)</td>
<td>0.0533 ± 0.0006</td>
<td>0.0639 ± 0.0026</td>
<td>0.0775 ± 0.0114*</td>
<td>0.0711 ± 0.0079</td>
<td>0.0706 ± 0.0047</td>
<td>0.0976 ± 0.0072*</td>
</tr>
<tr>
<td>Intermediodorsal Thalamic Nucleus (IMD)</td>
<td>0.0532 ± 0.0005</td>
<td>0.0639 ± 0.0037</td>
<td>0.0758 ± 0.0064**</td>
<td>0.0731 ± 0.0095</td>
<td>0.0722 ± 0.0055</td>
<td>0.1090 ± 0.0093**</td>
</tr>
<tr>
<td>Medial Habenular Nucleus (MHb)</td>
<td>0.0529 ± 0.0005</td>
<td>0.0616 ± 0.0020</td>
<td>0.0741 ± 0.0087**</td>
<td>0.0715 ± 0.0083</td>
<td>0.0669 ± 0.0038</td>
<td>0.0945 ± 0.0047*</td>
</tr>
<tr>
<td>Parafascicular Thalamic Nucleus (PF)</td>
<td>0.0537 ± 0.0007</td>
<td>0.0619 ± 0.0025</td>
<td>0.0723 ± 0.0068**</td>
<td>0.0734 ± 0.0090</td>
<td>0.0675 ± 0.0043</td>
<td>0.0959 ± 0.0046*</td>
</tr>
<tr>
<td>Paraventricular Thalamic Nucleus (PV)</td>
<td>0.0530 ± 0.0007</td>
<td>0.0616 ± 0.0021</td>
<td>0.0770 ± 0.0081**</td>
<td>0.0728 ± 0.0098</td>
<td>0.0703 ± 0.0043</td>
<td>0.0987 ± 0.0044*</td>
</tr>
<tr>
<td>Piriform Cortex (Pir)</td>
<td>0.0582 ± 0.0010</td>
<td>0.0850 ± 0.0050*</td>
<td>0.1020 ± 0.0170**</td>
<td>0.0918 ± 0.0133</td>
<td>0.0948 ± 0.0060</td>
<td>0.1510 ± 0.0072***</td>
</tr>
<tr>
<td>Posterior Parietal Association Area (PPtA)</td>
<td>0.0547 ± 0.0005</td>
<td>0.0614 ± 0.0023</td>
<td>0.0804 ± 0.0106**</td>
<td>0.0714 ± 0.0071</td>
<td>0.0687 ± 0.0046</td>
<td>0.1080 ± 0.0076**</td>
</tr>
<tr>
<td>Retrosplenic Angular Cortex (RSA)</td>
<td>0.0544 ± 0.0008</td>
<td>0.0622 ± 0.0023</td>
<td>0.0791 ± 0.0095**</td>
<td>0.0699 ± 0.0065</td>
<td>0.0684 ± 0.0039</td>
<td>0.1040 ± 0.0061***</td>
</tr>
<tr>
<td>Retrosplenic Granular Cortex (RSG)</td>
<td>0.0530 ± 0.0006</td>
<td>0.0584 ± 0.0018</td>
<td>0.0738 ± 0.0087**</td>
<td>0.0666 ± 0.0060</td>
<td>0.0647 ± 0.0036</td>
<td>0.0900 ± 0.0045**</td>
</tr>
<tr>
<td>Ventromedial Hypothalamic Nucleus (VMH)</td>
<td>0.0542 ± 0.0008</td>
<td>0.0675 ± 0.0025</td>
<td>0.0813 ± 0.0116**</td>
<td>0.0752 ± 0.0088</td>
<td>0.0725 ± 0.0038</td>
<td>0.1080 ± 0.0071**</td>
</tr>
</tbody>
</table>

* p<0.05; ** p<0.01; *** p<0.001
Figure 1. Differential spatio-temporal 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 spatio-temporal binding of the 33P-labeled probe against BDNF mRNA in mouse brains after exposure to saline control (A, B), 0.2 LD50 dose of VX (C, D), and 0.4 LD50 dose of VX (E, F). A significant increase in BDNF mRNA expression in the brain is observed 2 hr and 72 hr following the last 0.4 LD50 exposure compared to controls.

180x173mm (300 x 300 DPI)
Figure 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 hr and 72 hr after 0.4 LD50 exposure, compared to controls in the CA1 (B), dentate gyrus (C) and CA3 (D). In addition, a significant increase in BDNF expression was observed in the dentate gyrus 2 hr, but not 72 hr, after the last exposure to 0.2 LD50 VX. *p<0.05; **p<0.01; ***p<0.001

188x159mm (300 x 300 DPI)
Figure 3. Immunohistochemistry analysis of BDNF protein expression in hippocampal regions following repeated exposure to low levels of VX. Regions analyzed included the CA1 (A), dentate gyrus (B) and CA3 (C). A significant increase in BDNF expression was observed 72 hr after 0.4 LD50 exposure in CA3 compared to controls. **p<0.01

152x106mm (300 x 300 DPI)
Figure 4. Mice that received repeated exposure to low levels of VX (0.2 LD50 and 0.4 LD50) had significantly lower blood levels of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) 2 hr after the last exposure, relative to control (saline-treated mice). Mice evaluated 72 hr 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 hr after the last exposure. ***p<0.001
139x68mm (300 x 300 DPI)