

REPORT DOCUMENTATION PAGE			<i>Form Approved</i> OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.				
1. REPORT DATE (DD-MM-YYYY) 2010		2. REPORT TYPE Open Literature		3. DATES COVERED (From - To)
4. TITLE AND SUBTITLE Changes in extracellular striatal acetylcholine and brain seizure activity following acute exposure to nerve agents in freely moving guinea pigs		5a. CONTRACT NUMBER		
		5b. GRANT NUMBER		
		5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) O'Donnell, JC., McDonough, JH, Shih, T-M		5d. PROJECT NUMBER		
		5e. TASK NUMBER		
		5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) US Army Medical Research Institute of Chemical Defense ATTN: MCMR-CDR-P 3100 Ricketts Point Road		8. PERFORMING ORGANIZATION REPORT NUMBER USAMRICD-P09-037		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) US Army Medical Research Institute of Chemical Defense ATTN: MCMR-CDZ-I 3100 Ricketts Point Road		10. SPONSOR/MONITOR'S ACRONYM(S)		
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				
13. SUPPLEMENTARY NOTES Published in Toxicology Mechanisms and Methods, 20(3), 143-52, 2010. This research was supported by the Defense Threat Reduction Agency - Joint Service and Technology Office, Medical Science and Technology Division.				
14. ABSTRACT See reprint.				
15. SUBJECT TERMS Acetylcholine, acetylcholinesterase, choline, guinea pig, in vivo microdialysis, nerve agents, organophosphorus compounds, sarin, seizure activity, soman, VR, VX				
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UNLIMITED	18. NUMBER OF PAGES 10
a. REPORT UNCLASSIFIED	b. ABSTRACT UNCLASSIFIED	c. THIS PAGE UNCLASSIFIED		
			19a. NAME OF RESPONSIBLE PERSON Tsung-Ming Shih	
			19b. TELEPHONE NUMBER (include area code) 410-436-3414	

RESEARCH ARTICLE

Changes in extracellular striatal acetylcholine and brain seizure activity following acute exposure to nerve agents in freely moving guinea pigs

John C. O'Donnell, John H. McDonough, and Tsung-Ming Shih

Pharmacology Branch, Research Division, US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD 21010-5400, USA

Abstract

Organophosphorus nerve agents irreversibly inhibit acetylcholinesterase (AChE) in the peripheral and central nervous systems, causing an increase in the concentration of acetylcholine (ACh) in the synapse or neuromuscular junction and subsequent adverse effects. In this study, *in vivo* microdialysis was utilized to collect samples from the striatum for monitoring changes in extracellular ACh levels along with cortical electroencephalographic (EEG) recordings for identifying seizure activity after acute subcutaneous (s.c.) exposure to $1.0 \times LD_{50}$ of the nerve agents sarin, soman, or one of two V-type agents (VX, or a Russian V-agent, designated VR) in unanesthetized freely moving guinea pigs. Based on EEG recordings, these animals were subsequently divided into groups that developed seizures (S) and those that did not develop seizures (NS). Maximum ACh levels in the striatum were observed at 60–70 min for sarin and soman S groups and 105 min for VX and VR S groups. In all NS groups the greatest increase in extracellular ACh occurred within 30 min after exposure, although in the sarin NS group a few sporadic increases of ACh from control occurred. Animals that developed seizures, regardless of the nerve agent, had significantly higher extracellular striatal ACh levels compared to the controls or those animals that did not develop seizures, yet both S and NS groups displayed similar levels of blood AChE inhibition. Regardless of the agent, all animals in the non-seizure groups survived 24 h, while lethality (25–42%) was observed only in animals that experienced seizure activity.

Keywords: Acetylcholine; acetylcholinesterase; choline; guinea pig; *in vivo* microdialysis; nerve agents; organophosphorus compounds; sarin; seizure activity; soman; VR; VX

Introduction

Organophosphorus (OP) nerve agents are potent inhibitors of cholinesterase (ChE) enzymes, in particular acetylcholinesterase (AChE), the enzyme responsible for hydrolyzing acetylcholine (ACh) in the synapse and neuromuscular junctions (Taylor 2001). Inhibition of AChE leads to an accumulation of extracellular ACh within the synapses of both the central (CNS) and peripheral (PNS) nervous systems (Taylor 2001). Accumulation of ACh results in hyperactivity of the cholinergic system that can produce a sequence of toxic signs such as hypersecretion, bronchoconstriction, miosis, muscular twitching, mental confusion, convulsive seizures, flaccid paralysis, respiratory distress, and death (McDonough and Shih 1997; Ecobichon 2001; Taylor 2001;

Eddleston et al. 2004b). The current US military treatment regimen for OP poisoning consists of administration of atropine sulfate, a muscarinic ACh receptor antagonist, and the oxime 2-PAM (pralidoxime; pyridine-2-aldoxime methylchloride), an AChE reactivator. To curb brain seizures and motor convulsions the benzodiazepine drug diazepam is also used (Moore et al. 1995; Lallement et al. 1997; Taylor 2001; Eddleston et al. 2004a).

The progression of neurochemical events following nerve agent poisoning at doses sufficient to elicit seizures can be divided into three phases: an early cholinergic phase, a transitional phase of progressively mixed cholinergic/non-cholinergic modulation, and finally a predominately non-cholinergic phase (McDonough and Shih 1997; Shih

Address for Correspondence: Tsung-Ming Shih, Commander, US Army Medical Research Institute of Chemical Defense, 3100 Ricketts Point Road, Aberdeen Proving Ground, MD 21010-5400, USA. Tel: 410-436-3414. Fax: 410-436-2072. E-mail: tsungming.a.shih@us.army.mil

(Received 04 December 2009; revised 15 January 2010; accepted 26 January 2010)

ISSN 1537-6516 print/ISSN 1537-6524 online © 2010 Informa UK Ltd
DOI: 10.3109/15376511003657439

<http://www.informahealthcare.com/txm>

and McDonough 1997). Changes in neurotransmitter and metabolite levels have been measured in brain sub-region homogenates of rodents following intoxication with the nerve agent soman (Shih 1982; Fosbraey et al. 1990; Shih and McDonough 1997). These changes, however, did not discriminate between extra- and intra-cellular pools of ACh, which reflect, respectively, the synaptic cholinergic transmission and the storage of the transmitter (Lallement et al. 1992). The measurement of the extracellular ACh release provides more precise information on the involvement of the cholinergic system in the generation of seizures or other toxic signs. In previously reported research, the rat model has been used for intracranial microdialysis studies of extracellular ACh levels in soman-exposed animals (Lallement et al. 1992; Tonduli et al. 1999). In rats, soman intoxication caused a two-phase pattern of ACh release in the medial septum and hippocampus, while in the amygdala there was a steady rise to a maximum ACh release after 50 min (Lallement et al. 1992). These earlier studies focused only on the effects of soman. However, other nerve agents, such as sarin and VX, that had been reported to be employed during war conflicts and terrorist attacks (Malloy 2000; Szinicz 2005; Smart et al. 2008), were not investigated.

To develop improved broad-spectrum therapeutic antidotes to treat nerve agent poisoning, more work is needed to understand the diverse effects of these nerve agents as they relate to differences in the agents' structures, physical properties, and metabolism. For example, sarin and soman are methylphosphonofluoridate anhydrides and are clear, colorless liquids that are volatile at room temperature and pose a vapor threat. In contrast, V-type nerve agents such as VX or the Russian V-type agent, designated VR, are phosphonic acid thioesters and are oily liquids with low volatility at ambient temperature and pose primarily a percutaneous threat (structures shown in Figure 1). Both VR and VX are charged, whereas the sarin or soman do not carry a charge. V-agents circulate in vivo as protonated amines and are metabolically more persistent than agents like sarin or soman. V-agents are hydrolyzed much more slowly than agents like sarin or soman, and some of the hydrolysis products of V-agents are presumably toxic. V-agents may be metabolized by routes that are not available for agents like sarin or soman such as oxidation reactions at nitrogen and/or sulfur moieties. V-agents react more slowly, if at all,

with carboxylesterases and phosphorylphosphatases than do agents like sarin or soman (Munro et al. 1999; van der Schans et al. 2003). Additionally, V-agents and agents like sarin or soman are brain region and tissue compartment specific in their ability to inhibit AChE activity (Shih et al. 2005). Clearly, the large body of pharmacological research that has focused on soman alone may not be sufficient to guide efforts in developing medical countermeasures for a broad spectrum of diverse OP nerve agents.

In this study we examine and compare the time-course effects of a single acute exposure to toxic doses of the nerve agents sarin and soman to the V-agents, VX or VR, on brain seizure activity and extracellular striatal levels of ACh simultaneously in unanesthetized, freely moving guinea pigs. In addition, we examine the relationship between ACh changes and CNS signs (e.g. seizures) of nerve agent poisoning, and between seizures and lethality of nerve agent toxicity.

Extracellular ACh measurements for evaluation of the aforementioned relationships were obtained by microdialysis collection in the striatum. The striatum is a relatively large structure that allows for consistent and accurate cannula implantation. Additionally, this brain region is composed of a relatively high number of cholinergic interneurons, providing a high density of AChE and easily detectable extracellular concentrations of ACh. Most importantly, it serves as an excellent indicator of events taking place throughout the cholinergic system following nerve agent exposure. The striatum accounts for most of the basal ganglion, which has been implicated in OP-induced seizure potentiation, and is extensively innervated by excitatory afferent neurons from the neocortex and substantia nigra, which have been respectively identified as a seizurogenic area and an area vital for seizure propagation during nerve agent poisoning (Glenn et al. 1987).

Materials and methods

Subjects

Male Hartley guinea pigs (CrI:(HA) BR COBS) weighing 250–300 g were purchased from Charles River Labs (Kingston, NY). They were individually housed in polycarbonate cages in temperature ($21 \pm 2^\circ\text{C}$) and humidity ($50 \pm 10\%$) controlled quarters that were maintained on a 12h light-dark schedule

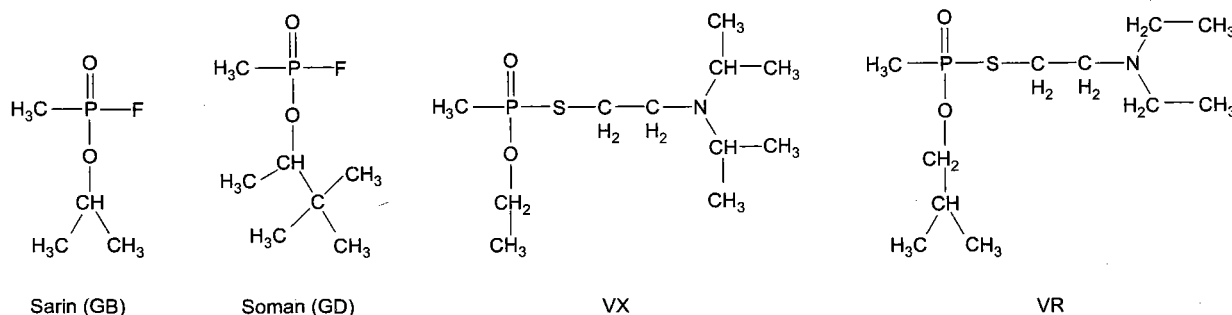


Figure 1. Chemical structures of the nerve agents sarin, soman, and the V-agents VR and VX.

(with lights on at 06:00 h). Laboratory chow and filtered tap water were freely available whenever the animals were in home cages. Animals were allowed to acclimate for 1 week prior to experimentation.

Although rat and mouse models have been developed for nerve agent studies, the guinea pig is considered to be a more suitable animal model (Inns and Leadbeater 1983). Unlike rats or mice, guinea pigs do not possess high levels of carboxylesterase, which non-specifically bind nerve agents such as sarin or soman and reduce their availability. In addition, guinea pigs are more similar to non-human primates in their response to pyridostigmine bromide pre-treatment for protection against nerve agents (Maxwell et al. 1987; 1988). Guinea pigs have been used previously to study protection against acute nerve agent toxicity, prophylactic and therapeutic treatment of nerve agent-induced seizures, and pathology following nerve agent exposure (Shih et al. 1996; 2003; 2007; 2009; McDonough and Shih 1997; Shih and McDonough 1999; Atchison et al. 2004).

Materials

Chemicals/drugs

Atropine methyl nitrate (AMN), neostigmine bromide (Br), ACh chloride, choline chloride, acetylthiocholine iodide, glucose, sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$), 1-octanesulfonic acid sodium salt, Triton-X100, and monobasic sodium phosphate (NaH_2PO_4) were purchased from Sigma-Aldrich (St. Louis, MO). Reagent MB was obtained from ESA Biosciences, Inc. (Chelmsford, MA). Phosphoric acid (HPLC Grade), dibasic sodium phosphate (Na_2HPO_4), and Tris (hydroxymethyl) amino methane were purchased from Fischer Scientific (Fair Lawn, NJ). Saline (U.S.P.) was purchased from Braun Medical, Inc. (Irvine, CA). Heparin sodium was purchased from U.S.P., Inc. (Rockville, MD). Pentobarbital sodium and buprenorphine HCl were purchased from Ovation Pharmaceuticals (Deerfield, IL) and Reckitt Benckiser Pharmaceuticals, Inc. (Richmond, VA), respectively. Isoflurane liquid for inhalation was purchased from MINRAD Inc. (Bethlehem, PA). Soman (pinacolyl methylphosphonofluoridate), sarin (isopropyl methylphosphonofluoridate), VX (O-ethyl S-(2-(diisopropylamino)ethyl) methylphosphonothioate), and the Russian V-agent VR (O-isobutyl S-(2-(diethylamino)ethyl)methylphosphonothioate) were obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). DTNB (5, 5'-dithiobis (2-nitrobenzoic acid), bicinchoninic acid (BCA) Protein Assay Reagent A (sodium carbonate, sodium bicarbonate, BCA⁻ detection reagent, and sodium tartrate in 0.1 N sodium hydroxide), and BCA Protein Assay Reagent B (4% cupric sulfate) were purchased from Pierce Biotechnology, Inc. (Rockford, IL). DTNB was prepared in Tris buffer (0.05M, pH 8.2) to a concentration of 0.424M. Nerve agents and AMN were diluted in normal saline to a concentration to inject 0.5 ml/kg.

Equipment

Microdialysis guide cannulae (15 mm; part No. MD2250) and probes (BAS BR-2; 0.34 mm OD, part no. MD-2200) were purchased from Bioanalytical Systems (BAS) (W. Lafayette, IN). QND software and NE-4 amplifier were purchased from Neurodata, Inc. (Pasadena, CA). Kopf dual arm stereotaxic frame and Kopf electrode angle calibrator (model 935) were purchased from David Kopf Instruments (Tujunga, CA). Cortical electroencephalographic (EEG) screws were purchased from Plastics One, Inc. (Roanoke, VA), stainless steel wire from A-M Systems, Inc. (Sequim, WA), connector from March Electronics, Inc. (Bohemia, NY), and dental acrylic from Lang Dental Mfg. Co., Inc. (Wheeling, IL). The high pressure liquid chromatography (HPLC) system consisted of a pump (Model 582), autosampler (Model 542), detector (Coulachem III), analytical column (ESA ACH-250 5- μ m particle size, 250 $\times$ 3.2 mm), post column reactor (ACH-SPR Part No. 70-0640), and analytical cell (Model 5040), all from ESA Biosciences, Inc. (Chelmsford, MA).

Surgery

Guinea pigs were anesthetized with isoflurane and surgically implanted with both cortical screw electrodes and a microdialysis guide cannula, using standard aseptic surgical techniques reported previously (Shih and McDonough 1999). Briefly, guinea pigs were placed in the stereotaxic frame and three cortical stainless-steel screw electrodes were implanted in the skull: two were placed bilaterally ~3.0 mm lateral from the midline and equidistant between bregma and lambda; the third was placed on the posterior calvaria as the reference electrode. Stainless-steel wires attached the screws to a miniature connector plug. The guide cannula was aimed at the striatum (+11.4 mm anterior, +3.6 mm lateral, -4.6 mm ventral to the skull surface) based on the atlas of Luparello (1967) using a zero coordinate. The screws, wires, connector, and guide cannula were then anchored to the skull with dental acrylic. The guinea pigs received 0.07 ml of 0.15 mg/ml buprenorphine HCl s.c. prior to and following surgery for pain control. The guinea pigs were allowed to recover for 6-10 days before the experiment began.

Experimental procedures

A schematic representation of the time-line of the experiment is presented in Figure 2. On the day of the experiment, blood (0.25-0.5 ml) was drawn using the toe nail clip method (Vallejo-Freire 1951) for determination of baseline AChE activity. The animal was then placed in an individual sample collection chamber (23 cm deep $\times$ 31 cm wide $\times$ 45 cm high). The cortical screw electrodes were connected to an amplifier. EEG recordings were captured using QND software on a Macintosh personal computer (low frequency filter = 0.3 Hz; high frequency filter = 40 Hz; sampling rate = 128 Hz) and displayed on a monitor, as reported elsewhere (Shih and McDonough 1999). The analog signal was amplified with an NE-4 amplifier before being processed on the Neurodata digital signal processor board. A brain microdialysis probe

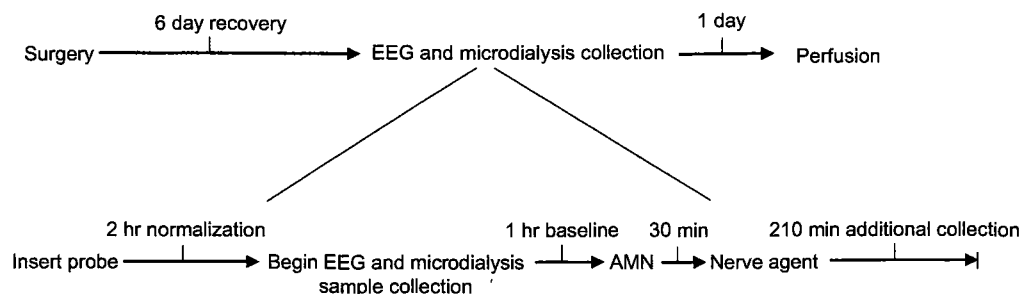


Figure 2. Schematic depiction of the time-line of the microdialysis experiment.

was inserted into the guide cannula, and perfused at a constant rate of 1.5 $\mu\text{L}/\text{min}$ with an artificial cerebrospinal fluid containing 140 mM NaCl, 3 mM KCl, 1 mM MgCl_2 , 1.2 mM CaCl_2 , 1.2 mM Na_2HPO_4 , 0.27 mM NaH_2PO_4 , 74 mM glucose, and 2 μM neostigmine Br (pH 7.4). Animals were allowed to move freely during the course of the experiment, as the EEG/microdialysis tether allowed them to reach any area of the collection chamber. A 2-h normalization period was allowed to pass before commencing the continuous collection of dialysate samples at 15-min intervals (volume per sample = 22.5 μL) in 250- μL microcentrifuge tubes. Baseline dialysate samples were taken for 60 min (four 15-min fractions) prior to administering AMN, 1.0 mg/kg, i.m. AMN limits secretions of mucus and saliva to keep the airways clear to enhance survival during the collection period, but it does not cross the blood-brain barrier to interfere with CNS events. AMN was administered 30 min before a $1.0 \times \text{LD}_{50}$ s.c. dose of a nerve agent (sarin, 42.0 $\mu\text{g}/\text{kg}$; soman, 28.0 $\mu\text{g}/\text{kg}$; VR, 11.3 $\mu\text{g}/\text{kg}$; or VX, 8.0 $\mu\text{g}/\text{kg}$). Agent vehicle control animals (AMN/SAL) were administered AMN i.m. followed 30 min later by saline s.c. instead of nerve agent. A separate group of animals was treated with saline i.m. in place of AMN and 30 min later with another dose of saline s.c. to serve as the saline/saline (SAL/SAL) control group. In total, there were six separate treatment groups: a SAL/SAL control group, an AMN/SAL control group, and four AMN/nerve agent groups (sarin, soman, VR, or VX). EEG recording and dialysate collection were continued for at least 210 min after nerve agent administration. The collected dialysate samples were stored at -20°C until analysis. At ~ 24 h post-exposure, a second blood sample was drawn for AChE analysis from the subjects that survived. Survivors were then deeply anesthetized with pentobarbital sodium (75–100 mg/kg, i.p.) and perfused with saline followed by 10% neutral buffered formalin for verification of probe location.

ACh and choline (Ch) analysis

ACh and Ch levels within dialysate samples were quantified using isocratic HPLC with electrochemical (EC) detection. The autosampler injected 10 μL of each sample and separation was achieved with the ESA analytical column at 27°C . The mobile phase (pH 8.0) was composed of anhydrous Na_2HPO_4 (100 mM), 1-octanesulfonic acid (2 mM), and an anti-microbial agent Reagent MB (50 $\mu\text{L}/\text{L}$ of deionized water) and was delivered at a constant rate of 0.350 mL/min.

To facilitate EC detection, a post column enzymatic reactor was utilized to convert ACh and Ch to detectable hydrogen peroxide (Lallement et al. 1992; Moor et al. 1998; Tonduli et al. 1999). The detector gain was set at 100 nA for Ch and 50 nA for ACh. The signal from the amperometric cell was delivered to a computer, and the digitized signal was then analyzed with EZchrom elite software (Scientific Software, Pleasanton, CA). The system was calibrated using a set of standards ranging from 0.63–10 pmoles of ACh and 1.07–25 pmoles of Ch. The typical retention time was 5.0 min for Ch and 7.6 min for ACh with excellent peak separation.

Blood collection and processing

Approximately 0.25–0.5 mL of blood was collected into a tube with 50 μL of heparin sodium (15 units/mL) prior to exposure, and then again from 24-h survivors. For whole blood (WB) preparation, 20 μL of collected blood was diluted (1:25) in 1% Triton-X100 (in saline) and mixed briefly. For red blood cell (RBC) samples, the remaining blood was centrifuged (5 min; 16,000 $\times g$), the plasma removed, and 10 μL of packed RBC were diluted (1:50) in 1% Triton-X100 (in saline) and mixed briefly. The diluted WB and RBC samples were frozen at -80°C until analyzed for AChE activity.

AChE activity

AChE activity was measured in a spectrophotometer using the colorimetric method of Ellman et al. (1961) modified for use with a microplate reader (Shih et al. 2005; 2009). The WB and RBC AChE activities were determined in triplicate using DTNB (0.424 M) as the chromophore and a supersaturated concentration of acetylthiocholine (17.1 mM) as the substrate. A sample volume of 10 μL was added to each well to place results within the optimal detectable range of the assay. The plate was read at 410 nm at 20-s intervals for 3.5 min using a Spectramax Plus 384 microplate reader (Molecular Devices Corporation, Sunnyvale, CA), and the activity ($\mu\text{mol}/\text{min}/\text{mL}$) was determined using Softmax Plus 4.3 LS software (Molecular Devices Corporation).

Statistical analysis

All results are expressed as mean values $\pm$ SEM. Blood AChE data were analyzed via a univariate general linear model with a type I sum of squares (fixed factor-group; covariant-baseline; dependent-exposure). Comparison of groups according to seizure occurrence and mortality within seizure groups

was made using a cross-tabulated Chi-square test followed by pair-wise comparisons with Fisher's exact test. Seizure onset times were compared using a one-factor ANOVA. Raw baseline ACh and Ch concentrations (first four 15-min samples before AMN administration) for the control and experimental groups were subjected to ANOVA with repeated measures. The post-exposure values of each subject were taken as a percentage of that subject's average baseline concentration, and these values were used for statistical analyses to reduce error and focus on time-course changes. It was necessary to perform a natural log transform of the data to meet the homogeneity of variance assumption. First, SAL/SAL and AMN/SAL groups were compared to examine the effect of AMN, and then seizure (S) and non-seizure (NS) groups were compared to the AMN/SAL group separately for each individual agent. Analyses were performed with repeated measure ANOVA followed by one-way ANOVA to compare nerve agents at each observation time. Post-hoc analysis was done using Tukey's Multiple Comparison test. In all cases, significance was set at $p \leq 0.05$. All tests were run using SPSS (PASW) 17 for windows.

Blood could not be drawn from subjects that did not survive or were in a near death state at 24h, and was not collected from a pair of VX NS animals at time of perfusion. Therefore, blood AChE data from animals with poor microdialysis collection volumes and unusable HPLC results were included out of necessity to obtain enough samples for statistical analysis. Animals with dialysate volume insufficient for HPLC analysis were, however, not included in seizure occurrence and mortality figures so that comparison of the ACh and seizure/mortality results could be consistent within the same group.

Results

Seizure occurrence and lethality

EEG recordings were used to identify whether or not seizure activity occurred. Seizure onset was operationally defined as the appearance of ≥ 10 s of rhythmic high amplitude spikes or sharp wave activity in the EEG. Once developed, seizures continued throughout the rest of the day during the period of dialysis collection. Typical examples of cortical EEG recordings of baseline, non-seizure, and seizure activity are displayed in Figure 3. The toxic response to a $1.0 \times \text{LD}_{50}$ nerve agent dose was highly variable; in some animals the toxic signs progressively developed into seizures, whereas in others, they did not. Figures 3(A) and (B) show baseline EEG activity and non-seizure EEG activity, respectively. When seizures did develop in an animal, seizure activity was typically maintained for the duration of collection (~ 4 h). Figure 3(C) shows representative EEG seizure activity or *status epilepticus* elicited by the agents. Seizure occurrence rates were 42% (8/19) for sarin, 68% (19/28) for soman, 84% (16/19) for VX, and 80% (12/15) for VR. Seizure occurrence in VX and VR groups was significantly higher than that of the sarin group. Twenty-four hour mortality, 26% (5/19) for sarin, 25% (7/28) for soman, 42% (8/19) for VX, and 27% (4/15) for VR, did

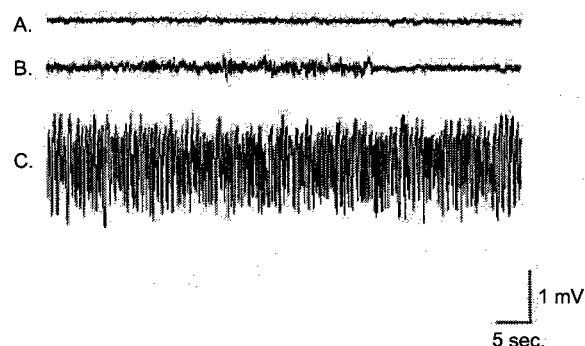


Figure 3. Typical EEG recordings before and after nerve agent exposure. (A) Baseline EEG activity. (B) EEG in non-seizure group. There were small EEG disturbances; however, no seizure activity followed. (C) *Status epilepticus* in nerve agent-treated seizure animals.

not differ between agents and, most notably, mortality was only observed in animals that developed seizures. All of the animals that failed to develop seizures survived for 24h after exposure. This difference in mortality between seizure and non-seizure groups was highly significant (Chi-square = 21.41, $df = 1$, $p < 0.001$).

The mean time for seizure onset observed for sarin and soman was 18 ± 4.8 ($n = 8$) and 23 ± 2.0 ($n = 19$) min, respectively. VR followed next with a mean seizure onset time of 33 ± 3.0 ($n = 12$) min, while VX showed the longest time from exposure to seizure onset with a mean time of 41 ± 2.8 ($n = 16$) min. Sarin-induced seizures had significantly shorter latencies than VR- or VX-induced seizures, while soman-induced seizures had significantly shorter latencies than VX-induced seizures.

Blood AChE activity

Average baseline blood AChE activities for the treatment groups ranged from 2.1–2.7 $\mu\text{mol/min/ml}$ for RBC and WB (Table 1). The SAL/SAL and AMN/SAL controls showed no effects on AChE activity. As would be expected, WB and RBC AChE activity was significantly inhibited in the nerve agent exposed groups relative to their baseline values or the SAL/SAL and AMN/SAL groups when measured 24h after agent exposure. Notably, there were no differences in either RBC or WB enzyme activity between S and NS groups within the four nerve agent groups 24h after exposure.

Striatal ACh and Ch levels

Basal extracellular striatal ACh ranged between 0.27–7.10 pmol/10 μl sample. No significant differences were found between the basal levels of the individual treatment groups. The SAL/SAL control group showed no significant change in ACh during the entire experimental period. Figure 4 shows the time-course changes of ACh in S and NS groups after exposure to $1.0 \times \text{LD}_{50}$ of sarin (Figure 4A), soman (Figure 4B), VX (Figure 4C) or VR (Figure 4D).

The ACh levels of the AMN/SAL group were significantly elevated relative to the SAL/SAL group for a 1h period from 45–90 min after the sham agent injection, as indicated in Figure 4(A). In contrast, the SAL/SAL group showed no

Table 1. AChE activity in RBC and WB (mean activity $\pm$ SEM).

Treatment group	n	RBC			WB		
		avg baseline activity (μ moles/ml/min)	avg 24 h post-exposure (μ moles/ml/min)	avg % baseline	avg baseline activity (μ moles/ml/min)	avg 24 h post-exposure (μ moles/ml/min)	avg % baseline
Saline/saline	11	2.412 $\pm$ 0.065	2.328 $\pm$ 0.102	96.8% $\pm$ 4.232	2.415 $\pm$ 0.123	2.330 $\pm$ 0.114	97.2% $\pm$ 3.637
AMN/saline	6	2.220 $\pm$ 0.208	2.382 $\pm$ 0.208	107.7% $\pm$ 4.882	2.245 $\pm$ 0.141	2.273 $\pm$ 0.164	101.5% $\pm$ 3.724
Sarin seizure	5	2.346 $\pm$ 0.160	0.122 $\pm$ 0.017	5.3% $\pm$ 0.798	2.472 $\pm$ 0.033	0.516 $\pm$ 0.058	21.1% $\pm$ 2.536
Sarin non-seizure	9	2.562 $\pm$ 0.245	0.171 $\pm$ 0.020	6.9% $\pm$ 0.751	2.231 $\pm$ 0.148	0.651 $\pm$ 0.037	29.6% $\pm$ 1.278
Soman seizure	15	2.489 $\pm$ 0.081	0.161 $\pm$ 0.029	6.5% $\pm$ 1.093	2.133 $\pm$ 0.139	0.375 $\pm$ 0.033	18.3% $\pm$ 1.654
Soman non-seizure	10	2.417 $\pm$ 0.150	0.210 $\pm$ 0.046	8.9% $\pm$ 1.688	2.105 $\pm$ 0.252	0.366 $\pm$ 0.030	16.5% $\pm$ 1.353
VX seizure	5	2.444 $\pm$ 0.122	0.644 $\pm$ 0.100	26.2% $\pm$ 3.395	2.666 $\pm$ 0.144	1.100 $\pm$ 0.057	42.0% $\pm$ 4.130
VX non-seizure	1	2.731 $\pm$ n/a	0.220 $\pm$ n/a	8.1% $\pm$ n/a	2.576 $\pm$ n/a	0.805 $\pm$ n/a	23.5% $\pm$ n/a
VR seizure	10	2.448 $\pm$ 0.074	0.568 $\pm$ 0.132	22.8% $\pm$ 4.659	2.427 $\pm$ 0.078	1.513 $\pm$ 0.132	62.9% $\pm$ 5.950
VR non-seizure	3	2.503 $\pm$ 0.090	0.437 $\pm$ 0.048	17.3% $\pm$ 1.347	2.740 $\pm$ 0.107	1.327 $\pm$ 0.122	48.3% $\pm$ 2.992

changes in ACh levels throughout the different collection periods. In subsequent analysis, all agent exposed groups were compared to the AMN/SAL group. Figure 5.

Both the sarin S and sarin NS groups (Figure 4A) had ACh levels that were significantly elevated over those of the AMN/SAL group. The sarin S group showed a significant ACh increase ($354.3 \pm 84.8\%$) 45 min after the nerve agent injection and a maximal increase ($411.1 \pm 84.8\%$) at the 90 min post-exposure sample time. The sarin S group also had significantly higher ACh levels than the sarin NS group 90 min after exposure (431.36 ± 68.2 and $245.41 \pm 27.9\%$, respectively). Both the sarin S and sarin NS groups had significantly elevated ACh levels relative to the AMN/SAL group starting ~45 min after sarin administration and lasting for almost 2 h after that.

The soman S group (Figure 4B) showed a significant rise in ACh ($258.3 \pm 17.1\%$) relative to the AMN/SAL group at starting 60 min after exposure, with a maximal increase in ACh occurring at 75 min after exposure. ACh levels remained significantly above AMN/SAL control levels and the soman NS group at virtually all subsequent time points.

The VX S group (Figure 4C) showed a significant increase in ACh ($261.4 \pm 27.6\%$) levels relative to AMN/SAL group starting at 60 min after exposure, and experienced its greatest increase in extracellular ACh levels ($415.9 \pm 33.7\%$) within the first 105 min following exposure. The ACh increase remained above control levels until 195 min after VX exposure. The VX NS group was similar to the AMN/SAL group through all but one collection period. The VX S and VX NS groups were not significantly different, although the VX S group had higher ACh levels than the VX NS group in all but three of the 14 post-exposure collection periods.

The VR S group (Figure 4D) showed a significant increase in ACh levels starting at 60 min after exposure compared to the AMN/SAL group. These ACh levels remained 300–400% above control levels throughout the duration of the collection, and were significantly greater than VR NS levels from 60–90 min post-exposure.

For all experimental groups, baseline Ch levels ranged between 2.01–5.20 pmol/10 μ l of sample. When compared with ACh levels, Ch showed a larger variability among

animals. Basal Ch levels were not significantly different between treatment groups. Additionally, nerve agent treatment did not significantly alter Ch levels, and no general trends were evident (data not shown).

Discussion

In this study intracranial microdialysis was used to monitor changes in extracellular ACh levels in the striatum during cortical EEG recording following acute exposure to $1.0 \times LD_{50}$ of the four nerve agents sarin, soman, VX, or VR in unanesthetized freely moving guinea pigs. This exposure procedure resulted in two populations of animals, those that developed seizures following agent exposure and those that did not seize. Across all agents, those animals that developed seizures displayed higher levels of striatal ACh following seizure onset than those animals that did not seize or the AMN/SAL controls. While the elevations in ACh levels in the seizure animals relative to the non-seizure animals were not always statistically significant at many time points (sarin) or with all agents (VX), there was a clear trend across all the agents that seizure activity resulted in significant and sustained elevations in ACh compared to the animals that did not develop seizures. There were no differences in the degree of AChE inhibition in blood produced by the different agents between the animals that developed seizures and those that did not. Also, 24-h lethality was observed across all the agents, but only in animals that developed sustained seizure activity.

Tonduli et al. (1999) administered a seizurogenic ED_{50} of soman to rats and recorded EEG for power spectrum analysis as well as assaying for cortical AChE and extracellular ACh concentrations. Much like the current data, they found seizures associated with AChE inhibition $> 65\%$ and an ACh elevation $> 200\%$ of baseline. They found that only rats that lacked an increase in the gamma band developed seizures. They concluded that seizure occurrence was determined not only by ACh increase, but also by differences in stress response, because the gamma band is associated with noradrenergic pathway activation and the sympathetic response to stress. Our non-seizure ACh

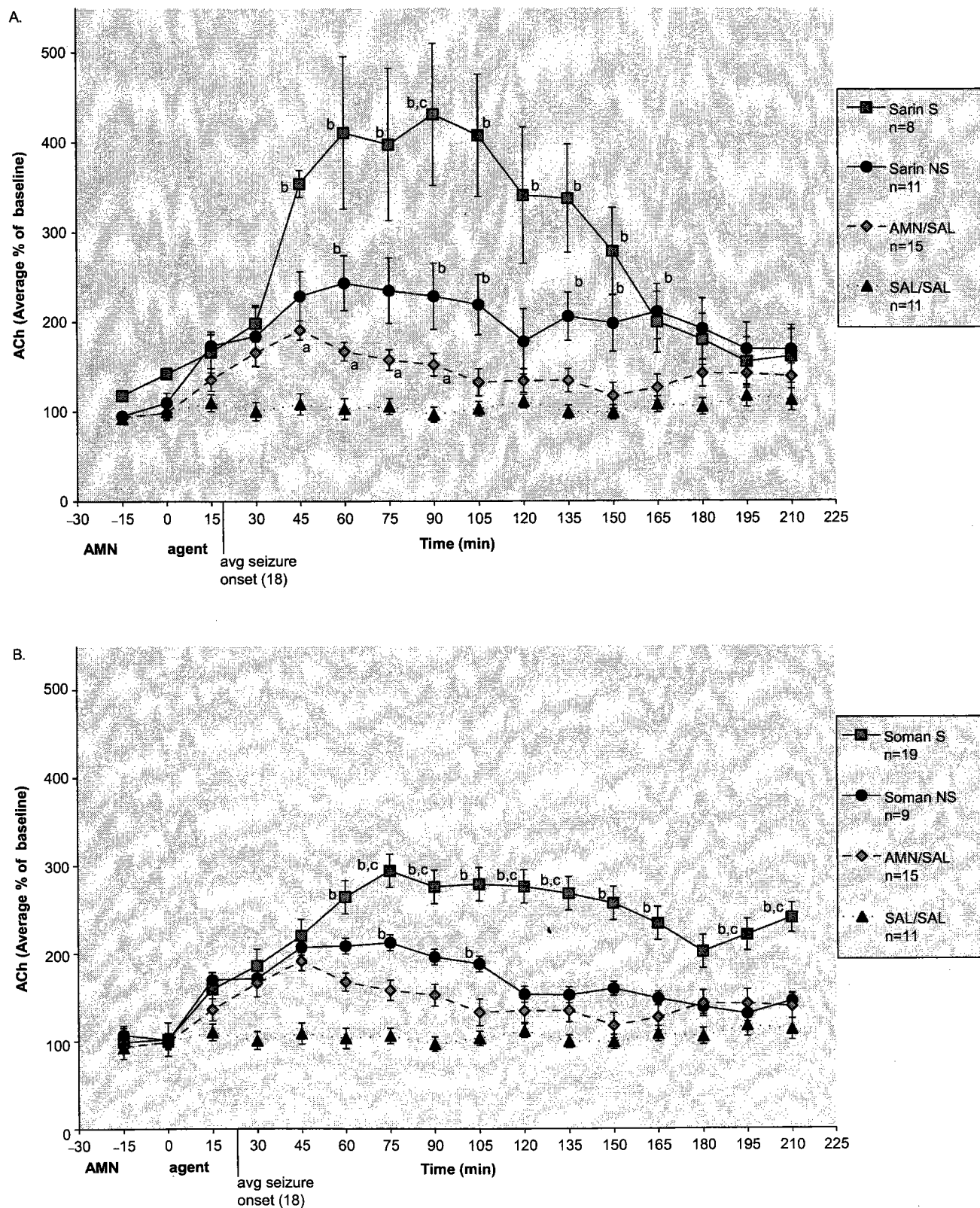
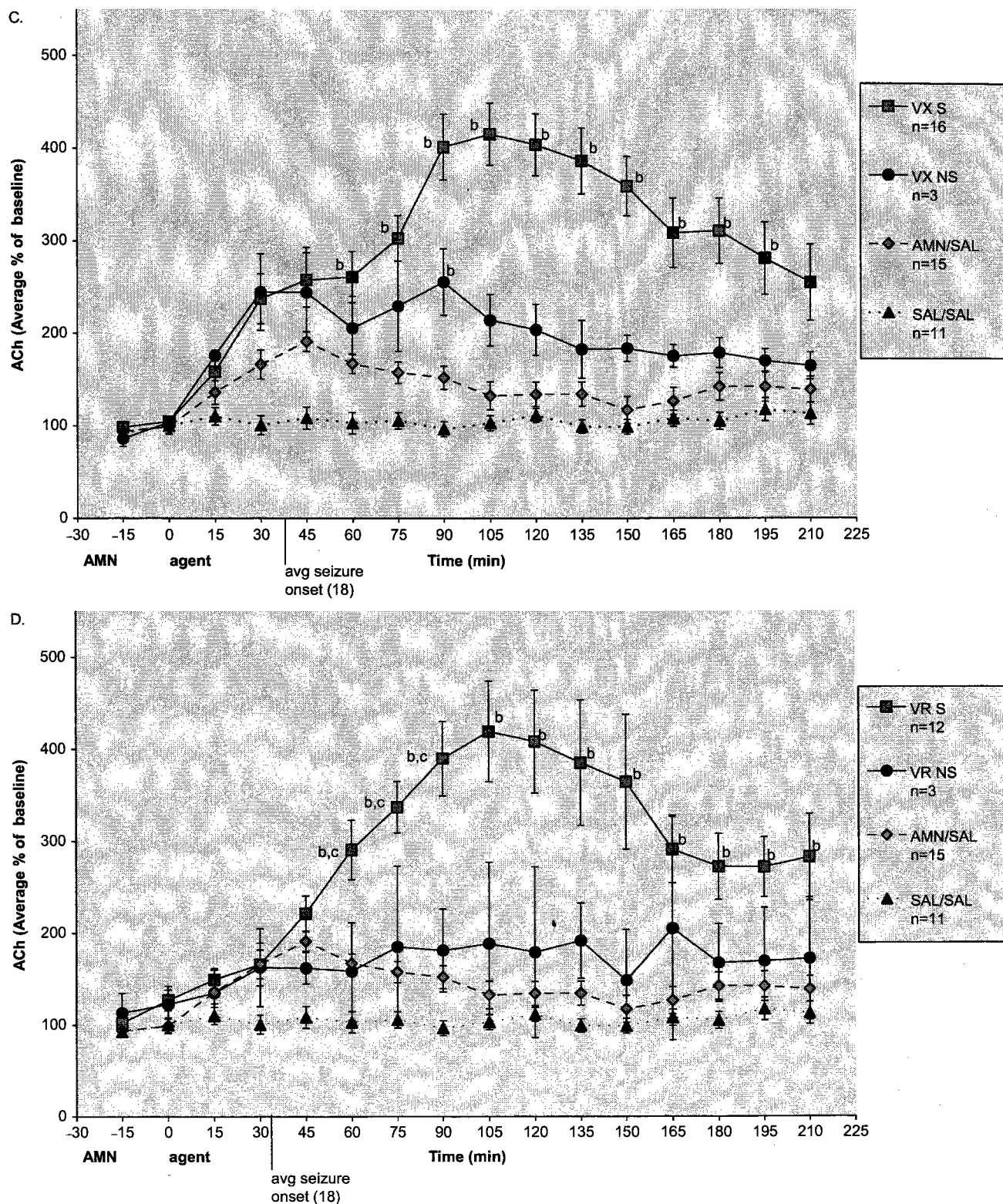


Figure 4. Sustained significant ACh elevation distinguishes S from NS groups, and earlier ACh increases distinguish sarin and soman from the V-agent groups. Changes in extracellular striatal ACh concentrations after exposure to $1 \times LD_{50}$ of sarin (A), soman (B), VX (C), or VR (D). Points and error bars represent the mean of ACh concentrations taken as percentages of the baseline of each individual subject $\pm$ SEM. Times of AMN and nerve agent injections are indicated on the x-axis, as well as average seizure onset times. a. $p < 0.05$ vs SAL/SAL (SAL = saline). b. $p < 0.05$ vs AMN/Nerve agent NS (non-seizure). c. $p < 0.05$ vs AMN/

Figure 4. continued on next page

Figure 4. Continued.



levels were nearly identical to their seizure counterparts prior to seizure initiation. It was only after seizure onset that the two groups started to separate. Therefore, while ACh elevations and subsequent enhanced neuronal

activity may be a major driving force behind seizure initiation, there are likely to be other factors that trigger seizure activity and the subsequent dramatic increases in extracellular ACh.

The presence and sustained duration of elevated extracellular ACh levels can be used to make clear distinctions between seizure and non-seizure groups within each nerve agent exposure group. Elevated extracellular ACh levels in the sarin and VX seizure groups returned to levels that were not significantly different from controls within 4 h after nerve agent administration, even while seizure activity persisted. In contrast, concentrations of ACh in the soman and VR seizure groups were still elevated above control levels at the end of the experimental collection period, indicating a more prolonged effect of these agents on this measure. It is clear from the data that the seizure activity per se played a significant role in increasing striatal ACh levels over and above that produced by nerve agent intoxication alone. This demonstrates that in animals experiencing cholinergic intoxication sufficient for seizure to develop, the sustained elevation of striatal ACh greater than the animals that did not develop seizures results from a cascade effect of additional ACh release brought on by, and in turn possibly contributing to, the seizure itself. The exact mechanism(s) by which the seizures increase ACh may not be specific to nerve agent-induced seizures, and may be a general feature of prolonged experimental seizures. This is a potential area for future research.

Agent groups were compared with the AMN/SAL group and not the SAL/SAL group because all agent groups received AMN pre-treatment. AMN has a predominately peripheral effect and does not readily cross the blood-brain barrier (Chambers and Chambers 1989; Shih 1991; Maalouf et al. 1998). AMN was administered to reduce the peripheral toxic effects, in particular salivation and mucus secretion, in the nerve agent-exposed subjects, and was not expected to interfere with seizure occurrence or the extracellular striatal ACh levels. However, the AMN/SAL group showed a rise in ACh levels compared with the SAL/SAL group that was significant when the normalized percent of baseline values were tested, but not significant when testing the raw concentrations. A similar effect was observed when the combination of neostigmine and atropine led to a significant rise in hippocampal ACh levels (Moor et al. 1998). There is some additional evidence that the microdialysis guide cannula and probe may disrupt the blood-brain barrier, allowing drugs to cross into the area around the probe insertion site, which is why a sufficient recovery period must precede collection (Allen et al. 1992; Westergren et al. 1995; Groothuis et al. 1998). Thus, the ACh increases seen in the AMN/SAL group, as well as in the agent non-seizure groups, may be partially attributed to AMN acting on cholinergic autoreceptors.

Similar degrees of AChE inhibition in both WB and RBC were observed in the animals that both did and did not develop seizures. Thus, blood AChE concentration may not reliably predict between seizure and non-seizure outcomes. The two V-agents exhibited slower seizure onset and striatal ACh elevations when compared to sarin or soman. Such a difference in delay was also observed in a previous study (Shih et al. 2005), where inhibition of striatal AChE after

sarin or soman exposure ($1.0 \times \text{LD}_{50}$) was both more rapid and of greater magnitude than seen in VX or VR exposed animals. In that study, there were no differences in the onset or rate of AChE inhibition in blood or peripheral muscle tissues. Thus, the differences in the rate of AChE inhibition between peripheral tissue and brain have been attributed to the charge in structures and other physical properties associated with VX and VR molecules, which delay passage across the blood-brain barrier (Shih et al. 2005).

One of the more interesting results of this study was the observation that only animals that experienced seizures were at risk of 24-h mortality, the traditional measure of nerve agent toxicity and the metric used to evaluate the success or failure of medical countermeasures. None of the animals that failed to develop seizure activity succumbed to the acute toxic effects of any of these four nerve agents. This observation reinforces previous data demonstrating that prevention or rapid control of seizure activity is critical for survival of nerve agent intoxication and that seizure activity is a significant contributor to the lethal effects of these agents (Shih et al. 2003; 2007).

In summary, following exposure to an LD_{50} dose of the nerve agents sarin, soman, VX, or VR, striatal ACh levels increased and animals that developed seizures, regardless of the nerve agent, had significantly greater elevations in extracellular striatal ACh levels compared to those animals that did not develop seizures or the controls. This was taken to indicate that the seizures per se contributed to this greater ACh increase, the mechanism by which this occurs is unknown. While AChE inhibition in blood was greater with sarin and soman than with either of the V-agents, there was no difference in levels of blood AChE inhibition between animals that developed seizures and those that did not. Thus, blood AChE inhibition levels produced by the agents were not reliable indicators of seizure development. Regardless of the agent, all animals that did not develop seizures survived 24 h, while lethality was observed only in animals that experienced seizure activity.

Acknowledgements

The authors express their appreciation for the excellent technical assistance of Steven Raiker, Kathleen McAvoy, Cindy Acon-Chen, Jeffrey Koenig, Teresa Ferrara, and Dr Bruce J. Jung. This research was supported by the Defense Threat Reduction Agency-Joint Service and Technology Office, Medical Science and Technology Division.

Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adhered to the *Guide for the Care and Use of Laboratory Animals*, by the Institute of Laboratory Animal Resources, National Research Council (National Research Council Publication No. 85-23, 1996). The research environment and protocols for animal experimentation were approved by the Institutional Animal Care and Use Committee (IACUC) of the US Army Medical Research Institute of Chemical

Defense. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The opinions or assertions contained herein are the private views of the authors, and are not to be construed as reflecting the views of the Department of the Army or the Department of Defense.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

- Allen DD, Crooks PA, Yokel RA. 1992. 4-Trimethylammonium antipyrine: a quaternary ammonium nonradionuclide marker for blood-brain barrier integrity during in vivo microdialysis. *J Pharmacol Toxicol Methods* 28:129-135.
- Atchison CR, Sheridan RE, Duniho SM, Shih T-M. 2004. Development of a guinea pig model for low-dose long-term exposure to organophosphorus nerve agents. *Toxicol Mech Methods* 14:183-194.
- Chambers JE, Chambers HW. 1989. Short-term effects of paraoxon and atropine on schedule-controlled behavior in rats. *Neurotoxicol Teratol* 11:427-432.
- Ecobichon DJ. 2001. Toxic effects of pesticides. In: Klaassen CD, editor. *Casarett and Doull's toxicology: The basic science of poisons*. 6th ed. New York: McGraw-Hill. pp 763-810.
- Eddleston M, Dawson A, Karalliedde L, Dissanayake W, Hittarage A, Azher S, Buckley N. 2004a. Early management after self-poisoning with an organophosphorus or carbamate pesticide—a treatment protocol for junior doctors. *Crit Care* 8:R391-R397.
- Eddleston M, Singh S, Buckley N. 2004b. Organophosphorus poisoning (acute). *Clin Evid* 12:1941-1953.
- Ellman GL, Courtney KD, Andres V, Featherstone RM. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7:88-95.
- Fosbraey P, Wetherell JR, French MC. 1990. Neurotransmitter changes in guinea-pig brain regions following soman intoxication. *J Neurochem* 54:72-79.
- Glenn JE, Hinman DJ, McMaster SB. 1987. Electroencephalographic correlates of nerve agent poisoning. In: Dun NJ, Perlman RL, editors. *Neurobiology of acetylcholine*. New York and London: Plenum Press. pp 503-534.
- Groothuis DR, Ward S, Schlageter KE, Itskovich AC, Schwerin SC, Allen CV, Dills C, Levy RM. 1998. Changes in blood-brain barrier permeability associated with insertion of brain cannulas and microdialysis probes. *Brain Res* 803:218-230.
- Inns RH, Leadbeater L. 1983. The efficacy of bispyridinium derivatives in the treatment of organophosphonate poisoning in the guinea pig. *J Pharm Pharmacol* 35:427-433.
- Lallement G, Carpentier P, Collet A, Baubichon D, Pernot-Marino I, Blanchet G. 1992. Extracellular acetylcholine changes in rat limbic structures during soman-induced seizures. *Neurotoxicology* 13:557-13567.
- Lallement G, Clarencon D, Brochier G, Baubichon D, Galonnier M, Blanchet G, Mestries J-C. 1997. Efficacy of atropine/pralidoxime/diazepam or atropine/HI-6/prodiazepam in primates intoxicated by soman. *Pharmacol Biochem Behav* 56:325-332.
- Luparello TJ. 1967. *Stereotaxic atlas of the forebrain of the guinea pig*. Baltimore: Williams & Wilkins. p 36.
- Maalouf M, Miasnikov AA, Dykes RW. 1998. Blockade of cholinergic receptors in rat barrel cortex prevents long-term changes in the evoked potential during sensory preconditioning. *J Neurophysiol* 80:529-545.
- Malloy CD. 2000. A history of biological and chemical warfare and terrorism. *J Public Health Manage Pract* 6:30-37.
- Maxwell DM, Brecht KM, Lenz DE, O'Neill BL. 1988. Effect of carboxylesterase inhibition on carbamate protection against soman toxicity. *J Pharmacol Exp Ther* 246:986-991.
- Maxwell DM, Brecht KM, O'Neill BL. 1987. The effect of carboxylesterase inhibition on interspecies differences in soman toxicity. *Toxicol Lett* 39:35-42.
- McDonough JH, Shih T-M. 1997. Neuropharmacological mechanisms of nerve agent-induced seizure and neuropathology. *Neurosci Biobehav Rev* 21:559-579.
- Moor E, Schirm E, Jacso J, Westerink BH. 1998. Effects of neostigmine and atropine on basal and handling-induced acetylcholine output from ventral hippocampus. *Neuroscience* 82:819-825.
- Moore DH, Clifford CB, Crawford IT, Cole GM, Baggett JM. 1995. Review of nerve agent inhibitors and reactivators of acetylcholinesterase. In: Quinn DM, Balasubramanian AS, Doctor BP, Taylor P, editors. *Enzymes of the cholinesterase family*. New York: Plenum Press. pp 297-304.
- Munro NB, Talmage SS, Griffin GD, Waters LC, Watson AP, King JF, Hauschild V. 1999. The sources, fate, and toxicity of chemical warfare agent degradation products. *Environ Health Perspect* 107:933-974.
- Shih T-M. 1982. Time course effects of soman on acetylcholine and choline levels in six discrete areas of the rat brain. *Psychopharmacology (Berl)* 78:170-175.
- Shih T-M. 1991. Cholinergic actions of diazepam and atropine sulfate in soman poisoning. *Brain Res Bull* 26:565-73.
- Shih T-M, Duniho SM, McDonough JH. 2003. Control of nerve agents induced seizures is critical for neuroprotection and survival. *Toxicol Appl Pharmacol* 188:69-80.
- Shih T-M, Kan RK, McDonough JH. 2005. In vivo cholinesterase inhibitory specificity of organophosphorus nerve agents. *Chem-Biol Interact* 157-158:293-303.
- Shih T-M, Koplovitz I, McDonough JH. 1996. Evaluation of anticonvulsant drugs for soman-induced seizure activity. *J Am Coll Toxicol* 15(Suppl 2):S43-S60.
- Shih T-M, McDonough JH. 1997. Neurochemical mechanisms in soman-induced seizures. *J Appl Toxicol* 17:255-264.
- Shih T-M, McDonough JH. 1999. Organophosphorus nerve agents-induced seizures and efficacy of atropine sulfate as anticonvulsant treatment. *Pharmacol Biochem Behav* 64:147-153.
- Shih T-M, Rowland TC, McDonough JH. 2007. Anticonvulsants for nerve agent-induced seizures: the influence of the therapeutic dose of atropine. *J Pharmacol Exp Ther* 320:154-161.
- Shih T-M, Skovira JW, O'Donnell JC, McDonough JH. 2009. Central acetylcholinesterase reactivation by oximes improves survival and terminates seizures following nerve agent intoxication. *Adv Stud Biol* 1:155-196.
- Smart JK, Mauroni A, Hill BA, Kok AB. 2008. History of the chemical threat, chemical terrorism, and its implications for military medicine. In: Tuorinsky SD, editor. *Medical aspects of chemical warfare, textbooks of military medicine*. Washington, DC: The Office of the Surgeon General at TMM Publications, Borden Institute. Chapter 4, pp 115-153.
- Szinicz L. 2005. History of chemical and biological warfare agents. *Toxicology* 214:167-181.
- Taylor P. 2001. Anticholinesterase agents. In: Hardman JG, Limbird LE, Gilman AG, editors. *Goodman and Gilman's The pharmacological basis of therapeutics*. 10th ed. New York: McGraw-Hill. pp 175-191.
- Tonduli LS, Testylier G, Pernot-Marino I, Lallement G. 1999. Triggering of soman induced seizures in rats: multiparametric analysis with special correlation between enzymatic, neurochemical and electrophysiological data. *J Neurosci Res* 58:464-473.
- Vallejo-Freire AA. 1951. A simple technique for repeated collection of blood samples from guinea pigs. *Science* 114:524-525.
- Van der Schans MJ, Lander BJ, Van der Wiel H, Langenberg JP, Benschop HP. 2003. Toxicokinetics of the nerve agent (±)-VX in anesthetized and atropinized hairless guinea pigs and marmosets after intravenous and percutaneous administration. *Toxicol Appl Pharmacol* 191:48-62.
- Westergren I, Nystrom B, Hamberger A, Johansson BB. 1995. Intracerebral dialysis and the blood-brain barrier. *J Neurochem* 64:229-234.