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The Effects of Repeated Sub-Toxic Sarin Exposure on Behavior, EEG and Blood and Brain AChE Activity

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14. ABSTRACT
Guinea pigs were exposed 5 days/week/two weeks to saline or one of two sarin doses (0.2 or 0.4 x LD_{50}). Animals were assessed for signs of toxicity, changes in body weight and temperature, EEG activity, behavioral and biochemical parameters during exposure (E1-10) and up to 100 days post-exposure (P100). Red blood cell AChE was depressed 60% and 80% respectively in the 0.2 and 0.4 x LD_{50} groups following E10, returning to baseline by P100. Brain AChE was significantly reduced in the 0.4 x LD_{50} animals only following E10, returning to baseline by P100. The 0.4 x LD_{50} animals exhibited a significant increase in $beta_2$ EEG activity across exposure days, but returned to control levels by P3. No differences were noted among groups on toxicity signs, weight or temperature. A significant increase in flinch threshold in both sarin groups was found only on P3, P10 and P30. There were significant increases in vertical movement and activity-chamber center time in the 0.4 xLD_{50} animals at P100; due to high variability, a supplementary cohort was exposed and tested; no significant changes were found. These results suggest that the initial depression of AChE does not result in persistent changes that influence physiology or behavior.

15. SUBJECT TERMS
Acetylcholinesterase; Activity Monitoring; Behavior; Body Temperature; Body Weight; Brain Regions; EEG; Electroencephalographic; Flinch Threshold; Nerve agents; Neurobehavioral; Nociception; Organophosphorus; Sarin

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Abstract

Adult male guinea pigs were exposed 5 days/week/two weeks to saline or to the nerve agent sarin (8.4 or 16.8 μg/kg, equivalent to 0.2 or 0.4 x LD_{50}). Animals were assessed for signs of toxicity and for changes in body weight and temperature, electroencephalographic (EEG) activity, behavioral and biochemical parameters during exposure and up to 100 days post-exposure (P100).

Red blood cell AChE activity was depressed 60% and 80% respectively by the 0.2 and 0.4 x LD_{50} sarin doses following the tenth exposure (P10) with a return to baseline by P100. Brain AChE was significantly reduced ($P<0.05$) in the 0.4 x LD_{50} sarin group following P10 with a return to baseline by P100. The 0.4 x LD_{50} animals exhibited a significant increase in $beta_2$ EEG activity across exposure days when compared with the 0.2 x LD_{50} and saline animals, but returned to control levels by 3 days post-exposure (P3). No differences were noted between saline and sarin groups on toxicity signs, body weight or temperature. There was a significant increase in flinch threshold in both sarin groups at P3, P10 and P30, but not at P100. There was a significant increase in vertical movement/rearing and in activity chamber center time in the 0.4 x LD_{50} animals at P100; however, since the variability was high, a supplementary cohort was exposed to sarin and tested. There were no significant changes in activity and the variability was low. Taken together, these results suggest that the initial depression of AChE does not result in persistent changes that influence behavior or EEG activity.

1. Introduction

Much remains to be learned about the effects of repeated low-level exposure to organophosphorus (OP) nerve agents, such as sarin (isopropyl methylphosphonofluoridate) or soman (pinacolylmethyl phosphonofluoridate), and there has been concern that this type of exposure may cause persistent adverse health effects. Consequently, there is a need to better understand the potential health consequences of repeated low-level exposure and to determine what level of exposure may produce adverse effects. One difficulty in evaluating the previous low-level exposure studies is the confusion created by the use of various definitions of "low-level exposure" and, indeed, by the many labels applied to the concept. This type of exposure, which often involves multiple (also called repeated or chronic) exposures, is sometimes called low-dose, clinically asymptomatic, subseizure, subtoxic or sublethal. This is in contrast to high-level exposures, which are variously referred to as acute, symptomatic, seizure-eliciting, toxic or lethal. When mentioning specific studies in this report, a study's unique terminology is used.

One obstacle in studying either low-level or acute exposure to nerve agents has been the difficulty of obtaining reliable data on accidental or wartime exposures (Brown and Brix, 1998). Animal studies have thus been employed to elucidate the neurochemical and pathological effects of acute, seizure-eliciting doses of the potent OP nerve agents (sarin, soman, VX), and the neurotoxic effects are now relatively well understood (for review see Somani et al., 1992; McDonough and Shih, 1993, 1997; McDonough et al., 1999, 2000; Kadar et al., 1995; Taylor, 1996). With regard to repeated low-level OP exposure, however, many questions remain. Some of the low-level studies have examined the neurobehavioral effects of a variety of OP agents of lesser potency, such as the pesticides diisopropyl fluorophosphate (DFP) or parathion (Russell and Overstreet, 1987; Prendergast et al., 1997; Olson et al., 1998; Deurveilher et al., 1999-a & b), while others have examined the effects of the chemical warfare nerve agents, such as sarin and soman (Burchfiel et al., 1976; Churchill et al., 1984; Russell et al., 1986; Modrow and McDonough, 1986; Nieminen et al., 1990; Sirkka et al., 1990; Olson et al., 1998; Pearce et al., 1999; Kassa et al., 2001-a & b; Conn et al., 2002).

Along with the range of terminology, these studies have employed a variety of definitions of so called "repeated low-dose exposure," including the use of different durations of exposure and varying delivery methods, such as inhalation or injection including subcutaneous (s.c.), intraperitoneal (i.p.) or intramuscular (i.m.)(Burchfiel et al., 1976; Sterri et al., 1980, 1981; Churchill et al., 1984; Russell et al., 1986; Modrow and McDonough, 1986; Hartgraves and Murphy, 1992; Olson et al., 1998; Kassa et al., 2001-a & b). Further, previous studies have used a number of animal species and have investigated the effects of low-level OP exposure on varied behavioral and neurochemical parameters.

Some previous studies found reversible changes in regional brain cholinesterase activity and muscarinic receptor density in rats following repeated sublethal soman injections (s.c.) (Churchill et al., 1984, 1985), as well as altered behavior as exposures continued (s.c. and i.p. injections; Hymowitz et al., 1985, 1990; Russell et al., 1986; Modrow and McDonough, 1986). It has also been observed in rats that i.p. injections of subtoxic doses of sarin or soman decreased locomotor activity and altered behavior on the plus-maze and elevated horizontal bridge tests (Sirkka et al., 1990; Nieminen et al., 1990). In studying the effects of repeated low-level sarin inhalation in rats, Kassa et al. (2001-a & b) concluded that clinically asymptomatic doses were disruptive to neurophysiological function and caused long-term memory impairments. Two related studies reported long-term alterations (up to 12 months) in immune function and liver nucleic acid metabolism (Kassa et al., 2000-a & b). In non-human primates, repeated low-level

exposure (1 μg/kg, i.m.,1/week x 10 weeks) to sarin has been reported to produce alterations in the encephalographic spectrum that far outlasted the period of exposure (Burchfiel et al., 1976).

Ostensibly conflicting results have been observed as well, such as a decrease (Nieminen et al., 1990; Koupilova et al., 1993; Kassa et al., 2001), an increase (Kassa et al., 2001), or no overall change (Russell et al., 1986) in locomotor activity following low-level exposure to nerve agents. Although Kassa and colleagues found an initial depression in activity and mobility in rats 3 months after low-level sarin exposure, an increase in exploratory activity was observed at 6 and 12 months post-exposure, suggesting variable and time-dependent behavioral changes. In another study that looked at s.c. as well as i.p. injections of soman, injection method-dependent differences in levels of brain and gut AChE activity were seen (Brezenoff et al., 1985), suggesting different underlying mechanisms for the behavioral changes observed following soman exposure via the two injection routes.

Clearly, no laboratory exposure method can mimic exactly the type(s) of exposure for which either military personnel or civilian populations are currently at risk. It is important, however, that a comprehensive series of animal studies on the effects of low-level exposure to the potent nerve agents be conducted. It is the intention that such a series of studies, of which the current report is a part, should compliment each other so that concentrations, duration and route of exposure, animal species and other parameters are kept constant across experimental protocols with the goal of producing a more comprehensive, comparable body of data. The study detailed in this report examined electrophysiological, behavioral, blood and neurochemical parameters in a guinea pig model that used repeated exposure to low levels of the nerve agent sarin. The doses of sarin (0.2 and 0.4 x LD_{50}) were selected to allow replication and expansion on the database of low-level work already begun (Atchison et al., 2003; Hulet et al., 2002; Sipos et al., 2001). The exposure schedule and doses were selected because while repeated exposures at these doses over a two-week period produce no observable symptoms, RBC AChE levels are driven to low, stable levels.

2. Materials and Methods

2.1 Animals

Male Hartley guinea pigs (Crl: (HA) BR; N = 180), obtained from Charles River Laboratories (Kingston, NY) with an average weight of 350g at baseline, were housed individually in hanging cages in a temperature (20 ± 2º) and humidity (50 ± 10%) maintained room, with an alternating 12-hour light/dark cycle with lights on at 0600 hours. Standard laboratory chow and water were available *ad libitum*, except during periods of (electroencephalographic) EEG recording and neurobehavioral testing.

Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals, and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals,* National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.2 Surgery

Guinea pigs were anesthetized with isoflurane and stereotaxically implanted with stainless steel cortical screw electrodes using standard rodent aseptic surgical techniques previously described (McDonough et al., 1995; Shih and McDonough, 1999). Following anesthesia, IPTT-200 temperature transponders (Bio Medic Data Systems Inc. (BMDS), Seaford, DE) were

inserted between the shoulder blades. Stainless steel cortical EEG screws were then placed approximately 3.0 mm lateral from midline and equidistant between bregma and lambda. The screws in the skull were attached to a miniature connector via stainless steel wires, and the assembly was then anchored to the skull with dental acrylic. The animals were allowed to recover for at least 5 days prior to experimentation.

2.3 Nerve Agent

Sarin was obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). The agent was diluted in sterile saline (0.9% NaCl, USP) in concentrations to deliver volumes equal to 0.5 ml/kg and maintained on ice. The most recently determined LD_{50} of 42 μg/kg in guinea pig (Hulet et al., 2002) was used in this study.

2.4 Experimental protocol

Animals (N = 180) were randomly assigned to saline (0.5 ml/kg, s.c.) or to one of two sarin doses, 8.4 μg/kg (0.2 x LD_{50}) or 16.8 μg/kg (0.4 x LD_{50}) (s.c.; LD_{50} = 42 μg/kg), and to one of five time points (2 hr and 3, 10, 30 and 100 days following the last exposure) for a total of 15 groups. Each group consisted of 12 animals, with 6 animals designated for post-sacrifice assessment of neuropathology and 6 designated for post-sacrifice neurochemical assays. Additionally, 6 animals from each group received activity testing, and 6 were assessed for nociceptive changes/flinch threshold.

Following the post-surgery recovery period, the guinea pigs were subjected to the three segments of the experimental schedule: the pre-exposure (1 week), the exposure (2 weeks), and the post-exposure (periodically up to 100 days) phases. In the pre-exposure phase, animals received 2 days (Tuesday, Wednesday) of handling and familiarization with the test chambers in order to minimize their initial stress. On the following 2 days (Thursday, Friday), baseline EEG (30 min/day), baseline behavioral data (flinch threshold and activity), and blood for baseline AChE assessment were collected. Animals were observed for normal behavior during the pre-exposure handling and baseline days (designated B1 and B2). Exposure days were designated E1 through E10; animals were injected daily, s.c., 5 days/week for 2 weeks (Monday-Friday) with saline or sarin, and body weight and pre- and post-exposure body temperature (temperature measured with BMDS Notebook and IPTT-200 transponders, Bio Medic Data Systems Inc., Seaford, DE) were measured daily. Prior to the daily injections, animals were monitored for EEG activity for 15 min to establish a pre-exposure baseline. Post-injection EEG monitoring continued for 1 hr, during which time the animals were assessed for signs of sarin intoxication, including eyelid closure, facial tremor, fasciculation, writhing, vocalization, circling, ease of handling, lacrimation and salivation. Following the daily EEG recording, animals were assessed for change on measures of general activity or nociception (flinch threshold/foot shock). After the termination of the exposure phase, separate groups of animals were evaluated for EEG and behavioral changes at post-exposure days 3, 10, 30 and 100, designated P3, P10, P30 and P100. In addition, at each of these times (plus at 2 hr post-exposure), groups of animals were euthanized (75 mg/kg, i.p., pentobarbital) and transcardially perfused. Animals designated for histopathology were perfused with a 0.9% saline flush followed by 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA). Animals designated for neurochemical assays were perfused with saline only and the brains dissected into six regions (cortex, hippocampus, striatum, midbrain, brainstem and cerebellum). Individual regions were weighed, immediately

frozen on dry ice and then stored at -70ºC for subsequent AChE assay. Blood was drawn on selected days throughout the experiment for analysis of red blood cell (RBC) AChE activity.

2.5 RBC and brain AChE assays

Blood collection was always performed after all other testing was completed on any given day. RBC AChE activity was assessed by extracting approximately 0.3 ml of blood via toenail clip (Vallejo-Freire, 1951) collected in microfuge tubes containing 16 µl EDTA. Whole blood was separated into plasma and RBC by centrifugation (11 min at 14,000 x g). RBC AChE activity was determined using an automated COBAS/FARA clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IL), with a method based on that of Ellman et al. (1961) and modified for COBAS/FARA by Hobson et al. (1988). RBC AChE activity is expressed as a percent of baseline values. Individual brain regions designated for brain AChE analysis were homogenized (1:20 w:v) in 50 mM sodium phosphate buffer, and an aliquot of homogenate was further diluted (4:1) with 5% Triton-X 100 (Bio-Rad Laboratories, Hercules, CA) in saline to achieve a final concentration of 1% Triton-X. The brain AChE assay was performed according to the Ellman method modified for microplate reader (SpectraMax plate reader and SoftMax software, Molecular Devices Corporation, Sunnyvale, CA) using acetylthiocholine iodide (Sigma-Aldrich, St. Louis, MO) as the substrate. The BCA protein assay (Pierce Biotechnology, Rockford, IL) was performed in conjunction with the brain AChE assay, and brain AChE activity is expressed as pmol activity/g protein.

2.6 General locomotor activity

Activity was measured in a clear Plexiglas chamber (40 cm L x 40 cm W x 30 cm H) utilizing a grid of horizontal and vertical photo beams (AccuScan Instruments, Inc., Columbus, OH). Horizontal and vertical movement (number of beam breaks) was measured during 30-min sessions, and four activity variables were selected for analysis: center time, total distance traveled, vertical movement and stereotypic movement (Versamax Analyzer and software, AccuScan Instruments, Columbus, OH). Each session was divided into three 10-min segments to assess habituation along with total activity. Total distance traveled (cm) is a function of horizontal beam breaks in the activity chamber, while vertical movement (rearing) is a function of vertical beam breaks. Center time is defined as the amount of time spent away from the perimeter of the chamber. Stereotypic movement is defined as a break in the same beam or set of beams repeatedly (with a 1-sec interval separating episodes), and typical behaviors include grooming and head bobbing.

2.7 EEG Activity

Animals were placed in single EEG recording chambers (45 cm H x 30 cm W x 25 cm D) with clear Plexiglas front panels. Individual animal headpieces were connected to the EEG recording apparatus, and QND Software and amplifiers (NeuroData, Inc., Pasadena, CA) were used to record activity (low frequency filter = 0.3 Hz; high frequency filter = 40 Hz; sampling rate = 128 Hz). Both total power and individual band power (spectral analysis: delta = 1-3.5 Hz; theta = 4-7.5 Hz; alpha = 8-12 Hz; $beta_1$ = 21-31.5 Hz; $beta_2$ = 21-31.5 Hz) were analyzed following data collection, and only data from fully awake periods was analyzed. A two-min [total] segment(s) of EEG recording from awake, resting animals was randomly selected for analysis. One segment was selected for analysis on each pre-exposure baseline and post-exposure day. Two (two-min) segments were analyzed on exposure days with one segment

being selected from the 15-min pre-injection recording period, and the second selection being made at/near the 45-min post-injection time point.

2.8 Nociceptive (flinch) thresholds

The determination of flinch thresholds was conducted using an adaptation of the up-and-down procedure (Crocker and Russell, 1984). Thresholds were measured by placing the animals in a test chamber (16 cm L x 11 cm W x 13 cm H) with a stainless steel grid floor, through which varying intensities of electric shock could be delivered (shocker, MED Associates, Inc., St. Albans, VT). After a 1-min habituation period, shock pulses (0.5 sec) were delivered at 15-sec intervals, and shock intensities were available from 0.05 to 4.0 mA in twenty 0.1 log unit steps. The procedure was as follows: a series of trials are carried out in accordance with the rules that a flinch response is followed by a step decrease in shock intensity, and that a nonresponse (no flinch) is followed by a similar step increase in shock intensity; trials are continued in each series until a change in behavior (called the first reversal, "flinch to no flinch" or "no flinch to flinch") occurs; the session is terminated at 4 trials following the first reversal. On each of the 2 baseline days, the session began with a presentation of a 0.55 mA shock, an intensity found to be an appropriate starting point for most guinea pigs; the ending values for the 2 baseline sessions were averaged, and the first exposure-day series began with a shock intensity as close as possible to the averaged baseline flinch threshold. Flinch was defined as any visually recognizable withdrawal reaction in response to shock presentation, and consensus between 2 trained observers was required. Flinch thresholds were determined using the up-down method for small samples (Dixon and Massey, 1981). Testing was always conducted after all other behavioral procedures had been completed on any given day, but before scheduled blood collection.

2.9 Data analysis

Two-way (dose, days) repeated measures analysis of variance (ANOVA) was used to determine the effect of experimental manipulations on body weight, body temperature, activity levels and nociceptive thresholds from the baseline measurements through the post-exposure determination performed 2 hr after the last exposure. For the evaluations of post-exposure days (P3, P10, P30 and P100), independent one-way ANOVA was performed at each time point since group size changed after each post-exposure determination. If overall main effects were significant, all possible comparisons were made using the Neuman-Keuls test. EEG data was evaluated using a three-factor (dose, EEG band, time) ANOVA with repeated measures on 2 factors (EEG band, time). Statistical significance was defined as $P<0.05$.

3. Results

3.1 Behavioral signs

Animals were observed for normal behavior during the pre-exposure handling and baseline days and for signs of sarin intoxication during exposure and post-exposure phases of the study. No observable signs of intoxication were seen at any time in either the 0.2 or 0.4 x LD_{50} sarin groups.

Figure 1: Red Blood Cell Acetylcholinesterase Activity

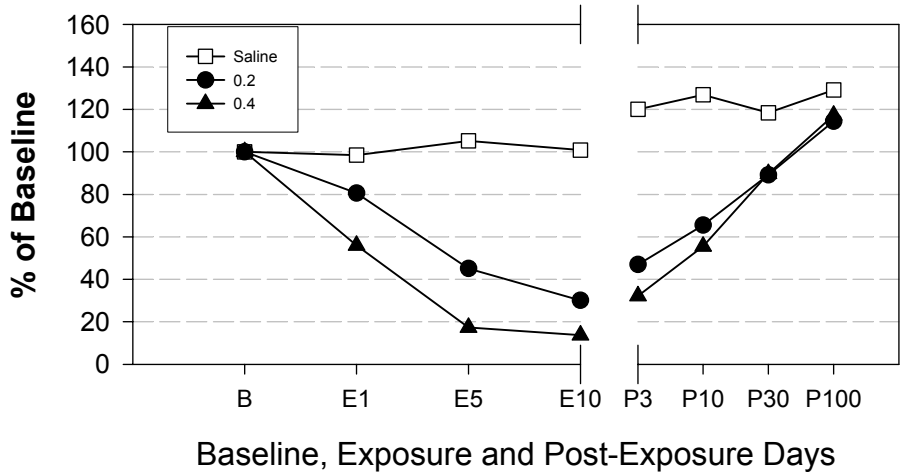

Figure 1: Red blood cell (RBC) acetylcholinesterase (AChE) activity was depressed 60% and 80% by the 0.2 and 0.4 x LD_{50} sarin treatments, respectively, following the tenth exposure, with a return to baseline levels by 100 days.

3.2 RBC AChE level

Three hours following administration of the first sarin injection, RBC AChE activity fell to 80% and less than 60% of control values in the 0.2 and 0.4 x LD_{50} sarin animals, respectively (Figure 1). RBC AChE activity continued to drop, and following the tenth and last injection, AChE levels had decreased to less than 40% of baseline in the 0.2 x LD_{50} sarin animals and to less than 20% of baseline in the 0.4 x LD_{50} sarin animals. Both agent groups showed a progressive increase in AChE activity following the exposure period, with all animals returning to greater than their own pre-exposure baseline level at 100 days. There was an expected significant effect of group ($F_{2,407}$=355.7; P<0.001) and day ($F_{3,407}$=557.9; P<0.001), and a significant group x day interaction ($F_{6,407}$=179.0; P<0.001). All three groups were statistically similar at baseline, but a significant dose-dependent separation was apparent by three hours following the first exposure (saline > 0.2 > 0.4 x LD_{50} sarin; P<0.05); the separation continued through P10 (saline > 0.2 > 0.4 x LD_{50} sarin; P<0.001). By P30, the sarin groups were no longer significantly different from each other, but AChE values remained significantly lower than in saline groups (P<0.05).

Brain Acetylcholinesterase Activity (pmol activity/g protein)

		Time Point				
REGION	**DOSE**	**E10**	**P3**	**P10**	**P30**	**P100**
Striatum	Saline	326.3 (±6.7)	352.4 (±10.9)	373.6 (±14.2)	356.0 (±13.1)	378.1 (±7.7)
	0.2 LD50	303.3(±11.2)	323.5 (±9.2)	319.1 (±11.4)	344.9 (±10.2)	370.9 (±8.7)
	0.4 LD50	*214.9 (±26.5)	*221.1 (±20.1)	*235.6 (±22.3)	308.9 (±9.8)	414.2 (±21.7)
Cerebellum	Saline	223.56 (±7.3)	230.5 (±10.5)	223.7 (±4.2)	230.9 (±16.3)	250.3 (±5.9)
	0.2 LD50	226.99 (±9.0)	230.7 (±3.5)	227.3 (±9.6)	226.8 (±8.8)	262.4 (±4.5)
	0.4 LD50	*154.3 (±14.8)	182.72 (±8.8)	*176.1 (±6.8)	220.4 (±8.6)	226.7 (±6.1)
Brainstem	Saline	169.6 (±11.0)	176.7 (±7.0)	178.0 (±9.4)	186.9 (±12.7)	166.4 (±4.6)
	0.2 LD50	188.1 (±9.8)	182.63 (±6.5)	177.6 (±21.0)	184.4 (±10.0)	186.2 (±5.4)
	0.4 LD50	*146.5 (±19.0)	144.2 (±12.4)	152.5 (±14.9)	169.2 (±9.4)	182.6 (±10.1)
Midbrain	Saline	147.2 (±61.5)	138.1 (±52.7)	137.1 (±20.0)	147.9 (±25.7)	131.1 (±21.0)
	0.2 LD50	149.38 (±36.3)	141.8 (±31.1)	147.7 (±40.5)	137.4 (±24.8)	145.4 (±14.8)
	0.4 LD50	*83.9 (±59.0)	116.8 (±43.3)	*105.2 (±36.9)	130.0 (±44.8)	133.6 (±12.1)
Cortex	Saline	105.7 (±6.2)	96.7 (±6.2)	98.2 (±8.5)	99.2 (±7.7)	93.6 (±3.6)
	0.2 LD50	84.4 (±10.9)	92.4 (±5.4)	100.0 (±8.1)	108.4 (±8.4)	105.0 (±5.7)
	0.4 LD50	*62.3 (±14.3)	83.3 (±4.4)	64.6 (±11.5)	91.4 (±3.9)	100.4 (±6.4)
Hippocampus	Saline	100.4 (±5.1)	107.8 (±5.3)	107.4 (±3.5)	98.7 (±3.5)	101.9 (±1.6)
	0.2 LD50	103.2 (±4.1)	97.5 (±9.6)	101.84 (±1.9)	98.2 (±6.5)	104.6 (±6.2)
	0.4 LD50	*68.78 (±14.0)	*76.7 (±5.5)	*73.65 (±12.8)	90.8 (±3.9)	103.6 (±21.7)

Table 1: Brain regions are arranged in order of AChE activity level, with striatum containing the highest level, and cortex and hippocampus the lowest levels. Significant reductions (*) in AChE activity occurred primarily at E10 (day of tenth injection) in the 0.4 x LD50 sarin group, with a steady increase back to control levels by P100.

3.4 General activity

There was no significant difference between groups at baseline on any of the 4 activity variables analyzed (total distance traveled, vertical movement, stereotypic movement or center time) (Figure 2, Panels A-D) whether considering all animals from the five time point designations of the original study or animals from the 100-day time point only. Because supplemental groups of 100-day animals were sarin-exposed and activity tested to confirm the results (See Original Study/100-day Animals Alone and Confirmation Group/100-day Animals Only), only original animals participating in the study from baseline through P100 were considered in the following analyses.

Original Study/100-Day Animals: There was no significant effect of treatment group on total distance traveled (Panel A) or stereotypic movement (Panel C) from E1 to E10, or at P3, P10, P30 or P100 (total: $F_{2,269}$=1.73 ; P=0.21; stereotypic: $F_{2,269}$=1.77; P=0.2), and no significant group x day interaction (total: $F_{28,269}$=0.96; P=0.53; stereotypic: $F_{28,269}$= 0.78; P=0.78). Although there was a significant effect of day on both of these activity variables (total: $F_{14,269}$= 8.2; P<0.001; stereotypic: $F_{14,269}$=0.78; P<0.001), the effect is not sarin-dependent. There were significant effects of group ($F_{2,269}$=3.7; P=0.05) and day ($F_{14,269}$=12.37; P<0.001) on vertical movement (Panel B), and a significant group x day interaction ($F_{28,269}$=3.7; P<0.001) as well. A significant increase in vertical movement (Panel B) was observed in the 0.4 x LD_{50} sarin animals when compared with the saline animals at P10 (P=0.03) and P30 (P=0.009), and by P100 both

the 0.2 and 0.4 x LD_{50} sarin animals exhibited significantly more vertical or rearing activity when compared with the saline group (P<0.001). Only the 0.4 x LD_{50} sarin animals exhibited significantly more vertical movement when compared with their own pre-exposure baseline levels (P<0.001); however, both the 0.2 and 0.4 x LD_{50} sarin animals spent more time in the center of the activity chamber when compared with their own baseline (Panel D; P<0.001). On center time there was a significant effect of day ($F_{14,268}$=13.87; P<0.001) and a group x day interaction ($F_{28,268}$=3.39; P<0.001); however, there was no significant effect of sarin exposure (group: $F_{2,268}$=3.57; P=0.054).

Confirmation Group: In spite of the significance, there was a large standard error (SEM) on all four variables in both the 0.2 and 0.4 x LD_{50} sarin groups at the post-exposure time points (Panels A-D). Consequently, activity testing in additional animals not included in the original 180 (N = 30; 10 animals/group; saline, 0.2 and 0.4 x LD_{50} sarin) was performed to confirm the initial activity findings. The results of the supplemental testing failed to confirm the initial observed increases in activity at the later post-exposure time points (Panels E-H); further, the SEM was quite low in the confirmation study, compared with that of the original study. Again, the significant effect of day, seen on all four variables, was not sarin-dependent, and the large increase in activity originally seen at P3 occurred in the confirmation study as well. At P3 and P10, the 0.2 LD_{50} animals exhibited a significant increase in vertical movement ($F_{2,431}$=3.52; P=0.04) and near-significance on total distance ($F_{2,431}$=3.2; P=0.055); however, this behavior returned to control level by P30.

Figure 2: There was a significant increase in locomotor activity in both sarin groups (vertical movement or rearing; Panel B), and an increase in time spent in the center of the activity chamber (Panel D) in the 0.4 x LD_{50} sarin animals only at 100 days post-exposure. Since there was high variability in the activity scores of the exposed animals at the post-exposure time-points, a supplementary cohort of 100-day animals was exposed and tested (Panels E-H). No significant dose- or sarin-related changes in activity were observed in these animals, and there was low variability as well. (Panels A-D/Original Study: N = 6 animals/group; only 100-day animals shown) (Panels E-H/Confirmation Study: N = 10 animals/groups; 100-day animals only.)

Figure on following page.

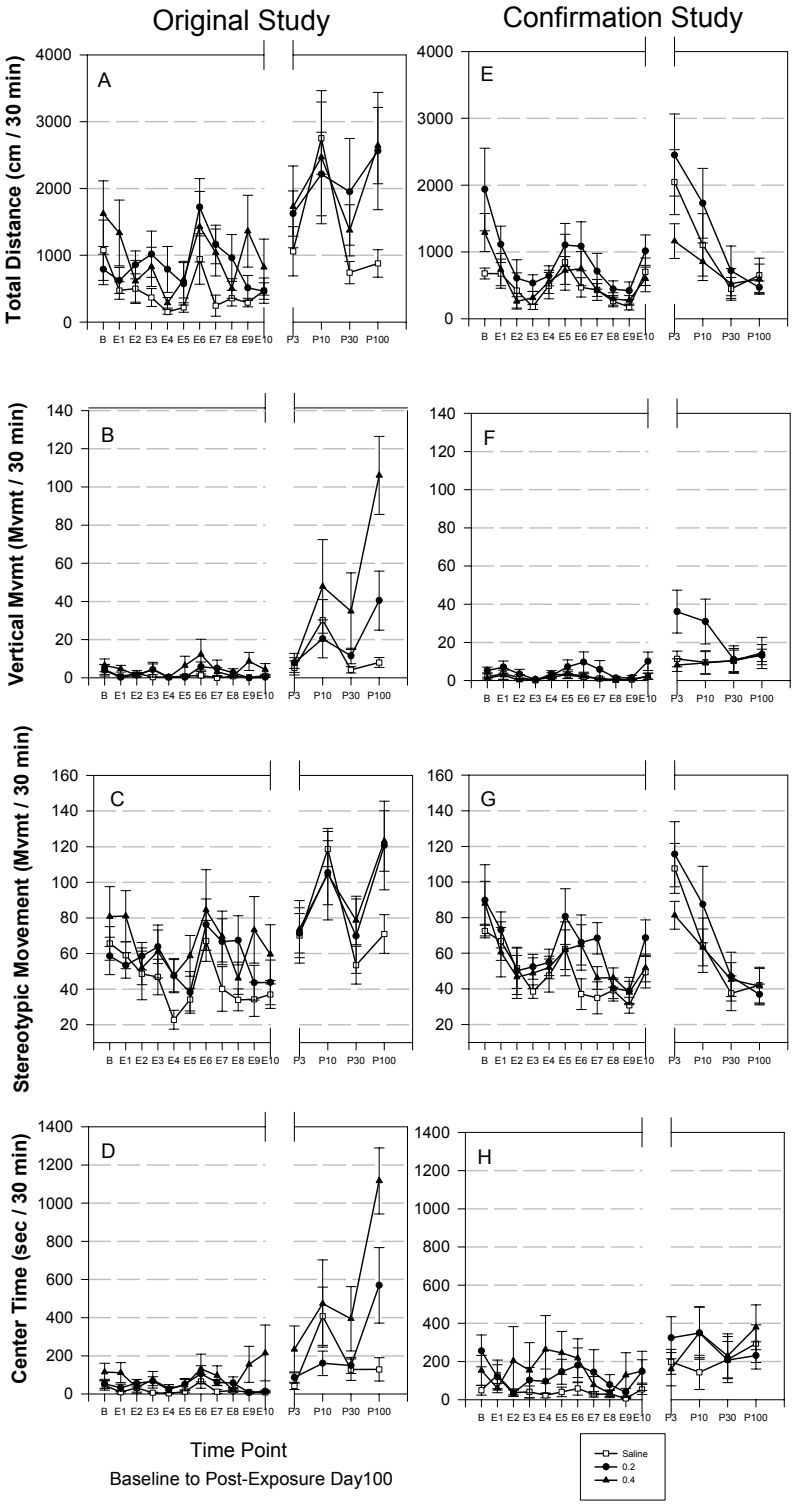

Figure 2: Spontaneous Locomotor Activity

3.5 Body weight and temperature

When examining raw body weight from baseline through exposure ten, there was an expected significant effect of day ($F_{10,2034}$=1855.9; P=0.594) and an expected significant group x day interaction ($F_{20,2034}$=1.62; P<0.001), indicating that all animals gained weight, whether considered altogether or in designated groups. However, there was no significant group effect (sarin dose) ($F_{2,2034}$=0.52; P=0.594), demonstrating that animals exposed to either 0.2 or 0.4 x LD_{50} sarin did not show any difference in body weight gain during the exposure period when compared with the saline controls (Figure 3, Panel A; shown is body weight gain). This is consistent with other recent findings (Hulet et al., 2002) that the two-week schedule of 0.4 x LD_{50} sarin administration (s.c.) used in this study does not cause a change in weight gain during the exposure period. Likewise, there was no significant effect of sarin treatment on body weight at any of the post-exposure time points, P3 ($F_{2,149}$=0.76; P=0.469), P10 ($F_{2,109}$=0.198; P=0.821), P30 ($F_{2,73}$=0.705; P=0.498) or P100($F_{2,39}$=0.187; P=0.830).

There were no sarin-related differences in body temperature (Figure 3, Panel B) between exposed animals and controls on temperature readings taken prior to injections during the exposure phase or at the post-exposure time points. The one exception was day E6 where the 0.2 x LD_{50} sarin animals exhibited temperatures slightly higher by 0.26°C than either the 0.4 x LD_{50} sarin or saline animals ($F_{2,1378}$=5.45; P=0.004). This is likely related to the normal daily fluctuation in guinea pig body temperature and not sarin treatment, since the readings of the 0.4 x LD_{50} sarin and saline animals were similar. There was a significant effect of treatment day ($F_{9,1378}$=9.27; P<0.001), with daily temperatures of all groups fluctuating slightly over the course of the study, but remaining within the normal range for guinea pigs. At P100, there was a significant (P<0.001) drop in temperature across all groups, but again, there was no significant effect of sarin treatment (P>0.25; see Discussion). When the exposure period post-injection temperature readings (measured immediately after removal from the EEG recording chambers) were compared (Figure 3, Panel C), there were no sarin or dose-related differences ($F_{2,1037}$=1.27; P=0.28), although some normal fluctuation was seen. A small post-injection decrease (~0.5°) in body temperature was observed; however, the difference was observed across all groups including saline controls and is likely a function of resting state during the hour-long EEG recording session. Day E1 post-injection readings were significantly higher and day E2 readings were slightly higher than all other days (P<0.004), suggesting a temperature-sensitive habituation to the treatment handling over time. The same phenomenon was observed in the activity data with a gradual decrease in activity across groups from baseline and over the first several exposures (Figure 2).

Figure 3: There was no difference among groups on body weight change (Panel A). On body temperature, there were no differences among groups (Panel B) except at Exposure 6, and the 0.2 x LD_{50} animals (a) exhibited significantly higher temperature than both the 0.4 x LD_{50} and the control animals. At P100, the temperature of all groups decreased and is likely a function of the peripherally implanted transponders being displaced away from the body core by the large weight-gain from P30 to P100. Panel C illustrates the post-injection drop in temperature observed in all three groups.

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Figure 3-A: Body Weight Change

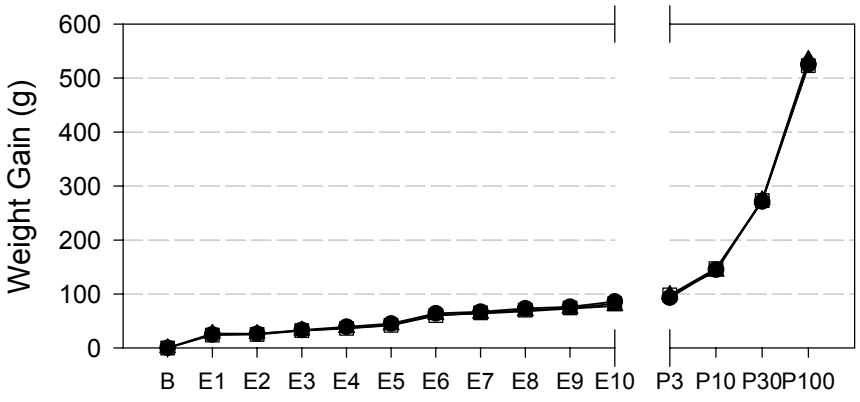


Figure 3-B: Average Daily Body Temperatures

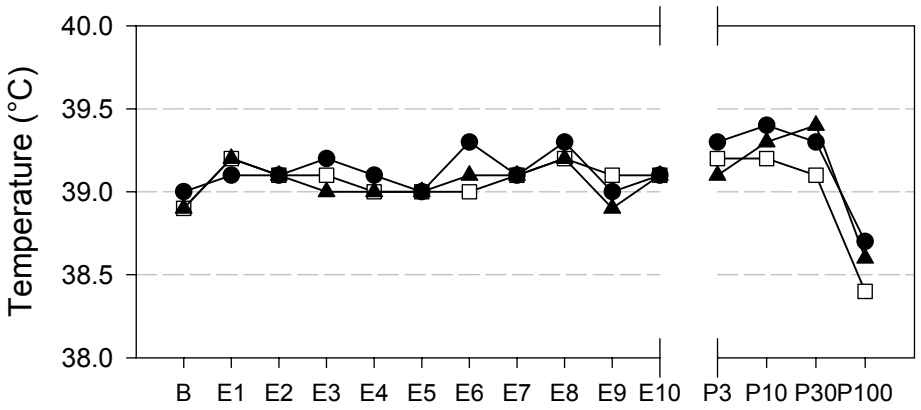
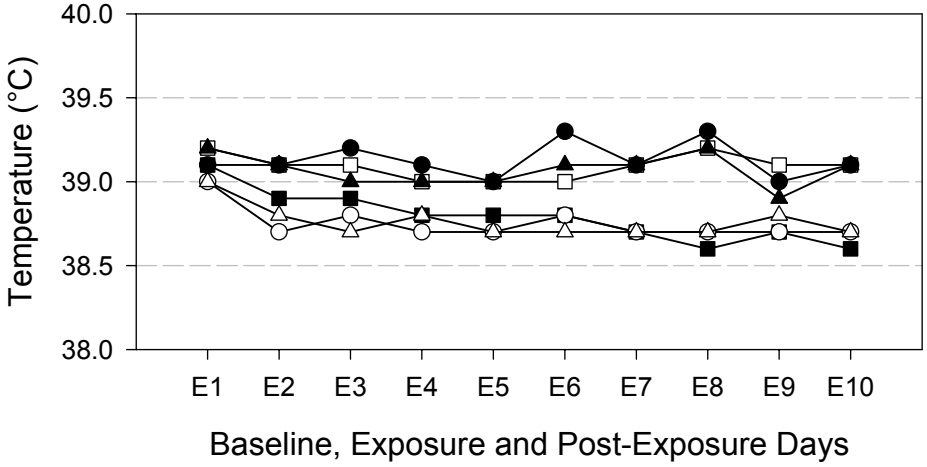


Figure 3-C: Daily Pre- and Post-Exposure Temperatures



3.6 EEG recording

EEG activity (total and individual band power in Hz) was analyzed as follows: baseline and post-exposure time points were analyzed separately, while samples from the daily 15-min pre-injection EEG recordings were compared with each other, as were samples from the daily post-injection EEG recordings (Table 2).

Total Power: There was no difference at baseline among groups on total power ($F_{2,12}$=0.26; P=0.77), or at P3 ($F_{2,95}$=0.15; P=0.86), P10 ($F_{2,83}$=0.23; P=0.79), P30 ($F_{2,55}$=2.05; P=0.14) or P100 ($F_{2,28}$=0.85; P=0.44). When samples from the daily 15-minute pre-injection EEG recordings were compared with each other, there was no effect of group ($F_{2,758}$=2.19; P=0.11) or day ($F_{5,758}$=1.36; P=0.24) on total power, and there was no group x day interaction ($F_{10,758}$=0.59; P=0.82).

Individual Band Power: Upon spectral analysis (five bands: delta, theta, alpha, beta$_1$, beta$_2$), the three groups of animals were equivalent at baseline ($F_{2, 127}$ (delta=0.31; theta=0.016, alpha=0.19, beta$_1$=0.63, beta$_2$=2.04); P>0.05). Likewise, there was no difference among groups in any band at any post-exposure time point (P3, P10, P30 and P100; P>0.05). When samples from the daily 15-minute pre-injection EEG recordings were compared with each other, there was, however, a significant increase in high frequency band (beta$_2$) power in the 0.4 x LD_{50} sarin animals (group: $F_{2,758}$ =11.65; P<0.001), when compared with both the 0.2 x LD_{50} (P<0.001) and saline (P<0.001) animals. There was no significant effect of day on beta$_2$ power ($F_{5,758}$=1.09; P=0.36), and there was no significant group x day interaction ($F_{10,758}$=0.391; P=0.95). Likewise, there was a significant increase in high frequency band (beta$_2$) power in the 0.4 x LD_{50} sarin animals, as compared with both the 0.2 x LD_{50} (P=0.003) and saline (P<0.001) animals, when samples from the daily post-injection EEG recordings were examined (group: $F_{2,755}$=9.23; P<0.001); however, there was no significant effect of day ($F_{5,755}$=0.85; P=0.51) and no group x day interaction ($F_{10,755}$=0.16.; P=0.99).

EEG Activity: Total and Individual Band Power (Hz)

Total Power			**delta**	**theta**	**alpha**	**beta1**	**beta2**
GROUP		**0.2 / 0.4**	**0.2 / 0.4**	**0.2 / 0.4**	**0.2 / 0.4**	**0.2 / 0.4**	**0.2 / 0.4**
Baseline		NS / NS	NS / NS	NS / NS	NS / NS	NS / NS	NS / NS
Pre-injection	Group:	NS / NS	NS / NS	NS / NS	NS / NS	NS / NS	NS/*P*<0.001
	Day:	NS / NS	NS / NS	NS / NS	NS / NS	NS/*P*=0.03	NS / NS
	G X D:	NS / NS	NS / NS	NS / NS	NS / NS	NS / NS	NS / NS
Post-injection	Group:	NS / NS	NS / NS	NS / NS	NS / NS	NS / NS	NS/*P*<0.001
	Day:	NS / NS	NS / NS	NS/*P*=0.04	NS/*P*=0.02	NS/*P*=0.01	NS / NS
	G X D:	NS / NS	NS / NS	NS / NS	NS / NS	NS / NS	NS / NS
P3		NS / NS	NS / NS	NS / NS	NS / NS	NS / NS	NS / NS
P10		NS / NS	NS / NS	NS / NS	NS / NS	NS / NS	NS / NS
P30		NS / NS	NS / NS	NS / NS	NS / NS	NS / NS	NS / NS
P100		NS / NS	NS / NS	NS / NS	NS / NS	NS / NS	NS / NS

Table 2: NS indicates non-significance. *P* values are present when significance occurs, but only the EEG activity (beta 2 band) of the 0.4 x LD50 animals was significantly different from controls. The significant effects of day were not sarin- or dose-dependent.

3.7 Nociceptive (flinch) thresholds

When flinch thresholds from baseline through P100 were assessed (Figure 4), there were significant effects of group ($F_{2,1185}$=20.93; P<0.001) and day ($F_{14,1185}$=2.00; P=0.015) on this measure of nociceptive threshold, with the 0.2 and 0.4 x LD_{50} sarin animals exhibiting higher flinch thresholds than saline controls only at P3 (P<0.02), P10 (P<0.02) and P30 (P<0.03). There was, however, no significant group x day interaction ($F_{28,1185}$ = 0.83; P=0.72).

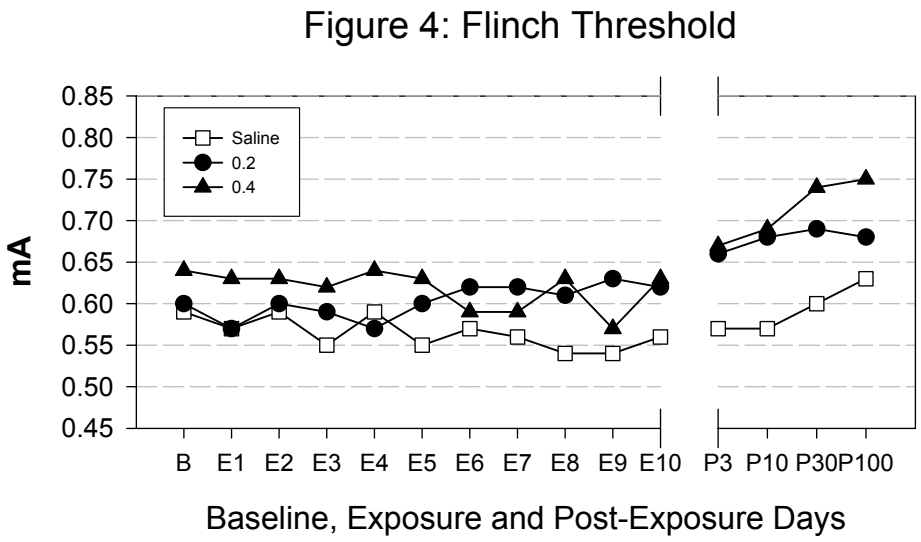


Figure 4: There was a significant increase in flinch threshold in both sarin groups at 3, 10 and 30 days post-exposure; however, there was no significant difference among groups by 100 days post-exposure.

4. Discussion

The objective of this study was to determine whether there are significant changes in a number of parameters, including neurobehavioral and biochemical, following repeated exposure to low-level doses of sarin (0.2 or 0.4 x LD_{50}, s.c.). Additionally, animals were followed to 3, 10, 30 and 100 days after the last exposure to determine whether either persistent or delayed effects of low-level sarin exposure were present. As has been noted, 0.4 x LD_{50} was selected because it is the highest dose that can be given repeatedly (5 days/week for two weeks, s.c.) without eliciting signs of nerve agent intoxication (Hulet et al., 2002). When Hulet et al. (2002) assessed the 0.5 x LD_{50} sarin dose in guinea pigs, based on the same LD_{50} and using the same dosing regimen as the current study, cholinergic signs were elicited although no epileptiform seizures were observed.

The current study confirms the previous results of Hulet et al. (2002) that this exposure and dosing regimen is clinically asymptomatic; that is, no physical signs of sarin intoxication were observed in exposed animals. The most dramatic effect of this dosing regimen was found on RBC AChE activity during the exposure phase (Figure 1). Although animals remained clinically asymptomatic, RBC AChE levels had decreased by 65% and 85% following the last exposure in the 0.2 and 0.4 x LD_{50} sarin animals, respectively, with a progressive return to baseline levels by P100 (Figure 1). Brain AChE activity was less severely altered by sarin (Table 1). There was a

significant reduction in brain AChE activity in all 6 brain regions tested at day E10, but only in the 0.4 (and not in the 0.2) x LD_{50} sarin animals, with a return to control levels by P100.

Interestingly, while the greatest inhibition of both RBC and brain AChE appears at the end of the exposure period, it is at P100--when AChE activity has returned to near-control levels--that the initial behavioral differences were observed (Figure 2). Both sarin groups exhibited an increase in rearing activity, but only the 0.4 LD_{50} group showed a significant increase, albeit with high variability, in time spent in the center of the activity chamber. There was no significant effect of sarin on total distance traveled or stereotypical behavior during the exposure period or at P3, P10, P30 or P100 (Figure 2, Panels A-D). The small increase seen in all three groups on day E6 (a Monday) is likely due to the absence of handling during the preceding weekend. The large increase in activity level observed in all three groups at P3 (again, a Monday) is also likely due to the absence of handling. Also at P3, animals were present in the EEG chamber for only 15 minutes prior to activity testing, compared with 1 hour and 15 minutes on injection days. This effect occurred in both the original and confirmation studies.

The reason for the significant increase in rearing in the 0.2 x LD_{50} group is unclear (Figure 2, Panel B); however, Kassa et al. (2001) reported subtle behavioral and physical changes in rats following low-level sarin inhalation that produced a far lower reduction in RBC AChE activity (20%) than was observed in this study. As noted in the Introduction, increases and decreases as well as minimal impact on locomotor or exploratory activity have been reported previously following sub-toxic OP exposure (Nieminen et al., 1990; Koupilova et al., 1993; Kassa et al., 2001; Conn et al., 2002; Scremin et al., 2003). Further, Kassa et al. (2001) reported a decrease, rather than an increase, in activity and mobility at 3 months following exposure, a time point similar to the P100 used in this study. The decrease in exploratory activity was observed only at their Level 2-S (asymptomatic single inhalation exposure with RBC AChE inhibition of 20%) and the decrease was not significant, although a significant decrease in mobility was reported in animals receiving repeated asymptomatic inhalation exposures (the degree of RBC AChE inhibition was not provided). Nieminen et al. (1990) also observed a decrease in locomotor activity following a single subtoxic dose of sarin or soman, with one sarin dose (12.5 μg/kg) falling roughly in between the 0.2 and 0.4 x LD_{50} doses of the current study (see 2.4 Experimental Protocol). However, the route of administration was i.p., and Brezenoff et al. (1985) suggest that the inhibition of AChE in the gastrointestinal tract following i.p. injection of a nerve agent may be involved in the behavioral effects attributed to these drugs. Further, the rodent species used in the former studies was the rat, rather than guinea pig, further confounding comparison with the current study.

Because of the high variability observed in the initial study at the post-exposure time points (see Figure 2, Panels A-D), and because of the lack of corroborating evidence (no sarin-related effect on body weight, temperature, total EEG power or general behavioral signs), it was necessary to confirm the initial findings. Although there was a significant depression of brain AChE activity in the 0.4 x LD_{50} animals following day E10 in the original study, it is not clear that this early decrease was sufficient to lead to persistent neurochemical or pathological changes that could affect long-term behavior. This, combined with the confirmation study data where the P100 values are tightly clustered with low within-group variability (see Figure 2, Panels E-H), suggests that the decrease in AChE observed under our conditions does not lead to alterations in behavior. Additionally, the conditions of the confirmation study were essentially the same as those of the initial study, with the daily schedule remaining constant. Although animals were not operated to implant EEG electrodes, they remained in the EEG chambers for the same length of

time and were exposed and handled identically to the previous animals, including blood draws for measurement of RBC AChE on selected days. Further, body weight change, body temperature and the depression of RBC AChE at E10 were equivalent.

EEG activity was recorded for one hour following each daily sarin injection; were changes in EEG to occur in response to repeated low-level exposure, it is thought they would be apparent during the first hour following exposure. No significant effect of repeated low-level exposure was seen on total EEG activity at any time point in this study (see Table 2); this includes daily pre- and post-injection total EEG power. A significant increase in high frequency band ($beta_2$) power was seen in the 0.4 x LD_{50} sarin animals across the 10-day exposure period; however, this increase had returned to normal by three days after the last exposure and was not evident during the P10, P30 or P100 testing sessions. Unlike the increase in activity (rearing and center time) observed at P100, the increase in EEG $beta_2$ power coincided with the exposure period reduction of RBC and brain AChE activity, and like the reduction in brain AChE activity, the $beta_2$ power increase was seen only in the 0.4 x LD_{50} sarin animals. Long-lasting effects on the cortical EEG spectrum following repeated asymptomatic sarin exposure have been reported in non-human primates (1 μg/kg once a week/10 weeks; Burchfiel et al., 1976). However, Hulet et al. (2002) report that repeated subcutaneous injections (daily; Mon-Fri/two weeks) of 0.3, 0.4 or 0.5 x LD_{50} sarin in guinea pigs failed to elicit seizures or cause changes in the EEG spectrum in the lower two doses, and Pearce et al. (1999) found no significant changes in EEG patterns in the common marmoset when followed for up to 15 months (one dose, 2.5-3.0 μg/kg sarin, i.m.). Interestingly, as with the current study, Burchfiel at al. (1976) observed changes in the beta frequency bands; however, a persistent increase in the relative amount of voltage that was present at 24 hr was also present one year post-exposure. Pearce et al. (1999) also found increases in $beta_2$ amplitude that approached significance (P=0.07), and also cited an unpublished study from the Chemical Biological Defence Establishment (CBDE) Porton Down that showed small changes in the $beta_2$ band in a small number of rhesus monkeys 7-9 months following a similar dose of sarin. The Burchfiel study, however, found the significant increase in beta activity during the states of drowsiness and awake in darkness; both the Burchfiel and Pearce studies used far fewer animals than the current study (average N for pre- and post-injection EEG data across exposure days = 127 animals/day, groups combined). While the current study defined the $beta_2$ band as 21-31.5 Hz, it should be noted that the Burchfiel study designated $beta_2$ as 22-50 Hz and the Pearce study identified $beta_2$ as 22-40 Hz.

The lack of significant effect on flinch threshold (Figure 4), a measure of nociception, during the exposure period was surprising, because it has been reported that both soman and sarin induce an analgesic effect in rats and mice (Clement and Copeman, 1984; Shih and Romano, 1988). These studies used acute toxic administration of soman (50-80 μg/kg) and sarin (120 μg/kg); however, Russell et al. (1986) observed elevated nociceptive thresholds, which remained so throughout treatment, with subsymtomatic exposures of soman (35 μg/kg, s.c., 11 injections). One study that examined nociception using the hot-plate test in rats following single subtoxic doses of soman (4 and 20 μg/kg) or sarin (12.5 and 50 μg/kg) found that these doses did not impair nociception (Sirkka et al., 1990), and Scremin et al. (2003) did not observe any effect of sarin (62.5 μg/kg; 0.5 x LD_{50}, s.c.; 3 days/week for 3 weeks) on nociceptive threshold in rats when observed up to 16 weeks post-exposure. In the current study, there was no sarin-related change in flinch threshold during the exposure period, although a small but significant increase in flinch threshold was seen in both the 0.2 and 0.4 x LD_{50} sarin animals at P3, P10 and P30 when compared with controls. However, no significant difference was observed among groups

at P100. Thus, under the conditions used, repeated low-level exposure to sarin appears to mediate a small, transient elevation but does not result in persistent changes in flinch threshold. A slight increase was observed across all groups over time, but with no significant differences (P>0.06) within groups when P100 flinch thresholds were compared with the respective group measurements at baseline. The increase is likely a function of increasing age and size.

The repeated low-level exposure to sarin used in this study does not appear to result in changes in either body temperature or body weight gain (Figure 3, Panels A-C). It has been reported, however, that sublethal doses of sarin or soman cause a severe, transient hypothermia in rats and mice (Meeter et al., 1969, 1971; Coudray-Lucas et al., 1983; Clement, 1991, 1993). Clement (1993) injected mice with sublethal soman (70 μg/kg, s.c.; LD_{50} = 130-140 μg/kg, s.c.) and observed a maximum decrease in temperature of approximately 6˚C at 90 minutes with a return to normal by 400 minutes. In the current study, not only was there no effect of 0.2 or 0.4 x LD_{50} sarin on daily pre-injection body temperature over the course of the exposure phase, but also there was no sarin- or dose-related effect when temperature was measured one hour post-injection. The slight (less than 0.5˚ C) drop in body temperature seen across all groups, including saline controls, at this time is likely the result of resting state during the hour-long post-injection EEG recording session, since the animals often fall asleep during this period. Clement (1993) described a drop in core temperature over the observation period in control mice, and that post-injection temperature decrease observed in the current study could also be the result of a diurnal rhythm as the Clement study suggested. There was an approximate 1˚ C drop in temperature seen at P100 across all groups, and this could be a function of the peripheral implantation of the temperature transponders. Since the animals increase in size by an average of 254g from P30 to P100, it is possible that at P100, the transponders have become located too far from the body core to register core temperature accurately.

There was no difference among groups in body weight gain during the pre-exposure, exposure or post-exposure phases of the study. This is in concurrence with the finding of Hulet et al. (2002) that there was no significant difference between the weight gains of the 0.4 x LD_{50} sarin and saline control animals, with a weight gain difference being observed only with the 0.5 LD_{50} sarin dose. The authors did not report weight gain past the exposure period, but there is no reason to suspect that the 0.4 x LD_{50} animals in that study would not have continued to gain weight normally.

5. Conclusions

The many variations of experimental factors (including nerve agents, doses, animal species, dosing regimen, route of administration and behavioral test parameters) mentioned in this article underscore the difficulty of reconciling previous data to form a coherent hypothesis of the effects of low-level nerve agent exposure. Even though the doses of 0.2 and 0.4 x LD_{50} sarin used in this study produce no cholinergic signs (i.e., the animals are clinically asymptomatic), there is a dramatic reduction of RBC AChE in both agent groups and a significant reduction in brain AChE in the 0.4 x LD_{50} animals. Additionally, a significant, but temporary, increase in high frequency ($beta_2$) EEG power was seen in the 0.4 x LD_{50} sarin animals across the 10-day exposure period. While the greatest RBC and brain AChE inhibition occurs following exposure E10 and the increase in $beta_2$ EEG power appears during the exposure period, it is at the last time point examined (P100), when AChE levels and EEG power are at or near control levels, that the behavioral differences (activity and flinch threshold) occurred in the original study. A supplementary cohort of 100-day animals was exposed and tested to confirm the activity

findings; however, the supplementary study failed to confirm the increases observed in either rearing or time spent in the center of the activity chamber. Regarding locomotor activity, the studies mentioned earlier report both increases and decreases in activity following low-level nerve agent exposure, although the results were not significant in some cases. Likewise, varying effects of low-level nerve agent exposure have been observed on flinch threshold, with elevated thresholds during the exposure period and no effect up to 16 weeks post-exposure. Other EEG studies observed either a significant or marginally significant increase in $beta_2$ power or amplitude following low-level sarin exposure. However, Burchfiel et al. (1976) warn that it is not necessarily valid to suggest that long-term beta activity increases support the idea of chronic behavioral disorders, and it has been reported that neural lesions detected following soman-induced seizures in the rat are correlated with increases in delta, not beta, band activity (McDonough et al., 1998).

The results of the current study, combined with previous low-level nerve agent work, suggest that possible changes in locomotor activity due to low-level nerve agent exposure may occur under some conditions, but the effects are variable and may be difficult to quantify. Indeed, no significant increases in total distance traveled or stereotypic behavior were found in the current study. Additionally, the observed post-exposure period differences in flinch threshold were not present at the longest time point examined (P100), and EEG activity changes were apparent only during the exposure period and disappeared rapidly. Further, no sarin-related changes were found in body weight gain, body temperature or on general signs of nerve agent intoxication. The initial depression of both brain and RBC AChE activity returned to baseline levels by P100; these results suggest that this initial depression does not result in persistent changes that influence behavior.

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