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Effects of Repeated Sublethal VX Exposure on Operant Time Estimation in Rats

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ABSTRACT

Thirty-two rats were trained to stability under a differential-reinforcement-of-low rates 20-s schedule with a sucrose pellet as the reinforcer and a lever press as the operant response. The exposure groups were saline, 0.3, 0.4, and 0.5LD50 VX delivered subcutaneously. Injections were given for three consecutive days, and neurobehavioral evaluations were conducted throughout the week of exposure and for two weeks following. Toxic signs scores were significantly higher in the 0.4 and 0.5LD50 groups. Additionally, body weight, number of responses, and reinforcers earned were significantly lower in the 0.4 and 0.5LD50 groups. Interresponse times changed in the VX-exposed groups. Operant performance recovered to pre-exposure levels by the first day of the following week and remained stable. No persistent or delayed-onset neurobehavioral effects were observed following discontinuation of VX injections. Repeated sublethal exposure to VX produced robust but transient response suppression in the 0.4LD50 and 0.5LD50 groups that recovered within two days of terminating the VX exposures. Toxic signs and body weight corroborated the intoxication observed, but these measures were less sensitive than the operant performance measures. Reinforcer-retrieval latency was a sensitive measure of impairment, revealing a deficit in the 0.3LD50 group as early as the second day of exposure.

INTRODUCTION

Studies of humans exposed to nerve agents (such as soman, sarin, VX, VR, tabun, and cyclosarin) or other organophosphorus compounds that inhibit acetylcholinesterase have shown disturbances in electroencephalogram (EEG), emotion, memory, focus, alertness, and/or motor performance (Bowers et al., 1964; Duffy et al., 1979; Holmes & Gaon, 1956; Tabershaw & Cooper, 1966; Yokoyama et al., 1998; Rosenstock et al., 1991; Savage et al., 1988; Steenland et al., 1994). Experimentally, the neurobehavioral effects of nerve agent exposure have not been adequately characterized. This is true of both acute, high-dose exposure as well as repeated exposure to low doses. A limited set of studies has examined the behavioral deficits induced by nerve agent poisoning. Geller et al. (1985) evaluated acute low-dose soman exposure on the visual discrimination and memory performance of baboons using a match-to-sample task. At doses sufficient to greatly reduce blood acetylcholinesterase, temporary response suppression was observed. Castro et al. (1992) trained rhesus monkeys to criterion levels of performance on a serial-probe recognition (SPR) task prior to exposing them to 38 ug/kg (5LD50) soman IM. Because the animals had received pyridostigmine pretreatment and virtually immediate atropine and 2-PAM therapy, all survived. The average duration of convulsions was 9 minutes, and within 1 hour convulsions ceased for all animals. SPR testing occurred on the day of exposure and for 20 consecutive daily sessions thereafter. On average, recovery of SPR performance to pre-exposure levels took 15 daily sessions. A second cohort of rhesus monkeys, treated identically except for the administration of diazepam (0.214 mg/kg, IM) one minute after exposure, exhibited virtually no convulsions, and SPR performance recovered on the sixth day after exposure. Thus, recovery of neurobehavioral function as indexed by the SPR task was faster in those animals exhibiting fewer convulsions, presumably because brain damage was less extensive in those subjects (c.f., McDonough et al., 1995; McDonough & Shih, 1997).

Using rats, McDonough and colleagues (1986) demonstrated that survivors of acute high-dose soman poisoning (100 ug/kg, SC) exhibited deficits in the acquisition of a differential-reinforcement-of-low-rates (DRL) schedule beginning 3 weeks following recovery and continuing for 45 sessions thereafter. Neuropathology, toxic signs scores, and neurobehavioral deficits were correlated in soman-exposed animals. More recently, rhesus monkeys surviving acute high-dose soman poisoning were allowed 7 weeks of recovery before being tested on acquisition of a DRL task for 20 daily sessions to assess neurobehavioral pathology relative to unexposed controls (Myers et al., 2006). Soman survivors exhibited a significantly lower rate of acquisition and attained a lower level of asymptotic performance. Timing accuracy and precision (as indexed by the distribution of response latencies on the DRL schedule) were poorer in the soman survivors. Bizot (1998) extended the utility of the DRL schedule to other compounds of military relevance, including the reversible acetylcholinesterase inhibitor physostigmine. In his study, acute physostigmine produced transient but marked response suppression and reinforcer loss in the DRL paradigm. The current study used a comparable DRL schedule in rats to evaluate neurobehavioral deficits following three consecutive days of sublethal exposure to VX and subsequent recovery.

METHOD

Subjects

Thirty-two male Sprague-Dawley rats (Crl:CD(SD)) were obtained from Charles River Laboratories (Wilmington, MA, USA). Rats weighed between 250-300 g at the time of shipping and were allowed two weeks to acclimate to our facility prior to initiating the study. Rats were fed *ad libitum* and weighed daily (M-F) for two weeks. Food restriction was then implemented in which each rat was given 90 minutes of access to rodent chow each afternoon (from about 1430-1600) to maintain food-motivated responding and control growth. After 1 week of food restriction, preliminary behavioral training (autoshaping) began.

Apparatus

Eight commercially available operant chambers for rodents were used (Model ENV-009, extra wide 5-bay rodent chamber, MED Associates, St. Albans, VT). Each contained a retractable response lever (ENV-112CM) on the far left of the front wall and a houselight mounted centrally on the upper portion of the rear wall. Above each response lever was a triple stimulus display ("cue light"; ENV-222M) comprised of three LEDs, red, yellow, and green. Whitenoise (approximately 80 dB) was presented by a speaker mounted behind the back wall. A pellet dispenser (ENV-203M) could deliver 45-mg sucrose pellets (Bio-Serv, Frenchtown, NJ; F0042) to a food trough (ENV-200R2M) located centrally near the bottom of the front wall, approximately 3 cm above the grid floor. The food trough contained a photobeam (ENV-254) for detecting pellet retrieval by the subject. A response feedback clicker module (ENV-135M) was mounted above the lever behind the front wall and a click accompanied each pellet delivery. A PC running MED-PC IV software was used to present stimuli and record events with 0.01-s resolution.

Behavioral Procedure

Sessions began with onset of the houselight and whitenoise, and these stimuli were terminated at the end of the session. Autoshaping was used to produce acquisition of lever pressing. Every 50 seconds (range = 10-90 s) on average, the cue light was illuminated and the lever inserted. After three sessions with little or no pressing, manual shaping was used in three cases. All rats acquired the leverpress response within five sessions, and a fixed-ratio 1 schedule was implemented for two sessions. Under this schedule, the lever remained inserted and the houselight and whitenoise remained on throughout the session. Each lever press produced a pellet and the session ended after 30 minutes or 60 reinforcers, whichever occurred first. The DRL schedule was implemented next and remained in effect for the remainder of the study (approximately 20 weeks; 15 weeks to obtain stable performance, 2 weeks of vehicle injections, exposure week, and 2 weeks of post-exposure evaluation). Each session ended at 30 minutes. A response was reinforced if and only if the previous response (or the start of the session) occurred greater than 20 s prior to the current response. Thus, by spacing responses appropriately, each rat could earn about 3 reinforcers a minute, or 90 pellets in the 30-min session.

Pharmacological Procedure

VX was obtained from the Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, and diluted to 19.2 ug/mL in physiologic saline and frozen until subsequent use. Each rat was injected with saline or VX at a maximum volume of 0.42 mL/Kg (actual volume varied with dose from 0.09 – 0.20 mL; the 1LD50 estimate equaled 16 ug/Kg). Each injection was given subcutaneously into the right flank while the animal was restrained with a towel in the chemical fume hood 30 minutes prior to the start of the behavioral test session. Each rat was injected with saline on Monday, Tuesday, and Wednesday the week before VX injections to habituate it to the process of receiving injections and to provide vehicle injection control data. Similarly, on the week of VX injections, injections occurred on Monday, Tuesday, and Wednesday.

Group Assignment

On the Friday before the week of vehicle injections, the number of reinforcers earned for each animal was recorded. The animals were ranked in ascending order based on this performance measure and sequentially divided into four equal groups of eight subjects each (i.e., subjects with rankings 1, 5, 9, 13, 17, 21, 25, and 29 constituted the saline group). The exposure groups were saline, 0.3, 0.4, and 0.5LD50 VX.

Toxic Signs

During the week of exposures, toxic signs were rated for each animal prior to the start of the daily behavioral test session. This occurred about 5 minutes prior to placing the rat in the operant chamber and, on injection days, occurred between 20-25 minutes following injection. The toxic signs score sheet is shown as Appendix A and includes measures of posture, movement, and cholinergic overstimulation.

Body Weight

Body weights were recorded daily from each subject throughout training, during exposure, and in the weeks following exposure. Weights were taken approximately 10-30 minutes prior to injection or (on non-injection days) the start of the behavioral test session.

Data Analysis

A between-groups repeated-measures ANOVA (with Dose as the between-subjects factor and Day as the within-subjects factor) was performed separately for each of the following dependent measures for each subject on each day: toxic signs, body weight, total responses, and total reinforcers. Following a significant main effect or interaction, post-hoc comparisons were made using Fisher's least-significant-difference test. For the 5^{th}, 10^{th}, 25^{th}, 50^{th}, 75^{th}, 90^{th}, and 95^{th} percentiles of the interresponse time distribution and for the median of the reinforcer-retrieval latencies, separate one-way (Dose) ANOVAs or t-tests were conducted for each exposure day because there were often too few responders in the highest dose groups to conduct a repeated-measures analysis across days. An alpha level of .05 was used for all statistical tests.

RESULTS

Figure 1 shows clinical signs of nerve agent toxicity across exposure week. The mean of the total toxic signs score for each dose group (indicated in the legend) is shown. Significant effects of Dose ($F_{3,112}$=14.0, p< .001), Day ($F_{4, 112}$=31.6, p< .001), and a Dose X Day interaction ($F_{12, 112}$=9.3, p< .001) were observed. Follow-up tests revealed that the toxic signs in the 0.4 and 0.5LD50 groups on Days 2 and 3 of VX injection were significantly higher than in the other two groups.

Figure 2 shows mean body weight before each session during exposure week. A significant effect of Day ($F_{4, 112}$=20.6, p< .001) and a Dose X Day interaction ($F_{12, 112}$= 9.5, p< .001) were observed. Follow-up tests revealed that, relative to Day 1, body weights were significantly reduced on Days 3-5 within the 0.4 and 0.5LD50 groups.

Figure 3 shows the average total number of responses for each DRL session during exposure week. Significant effects of Dose ($F_{3,112}$=18.4, p< .001), Day ($F_{4, 112}$=16.0, p< .001), and a Dose X Day interaction ($F_{12, 112}$=3.4, p< .001) were observed. Follow-up tests revealed that, relative to the saline group, responding was significantly suppressed in the 0.5LD50 group on Days 1-4 and in the 0.4LD50 group on Days 2 and 3.

Figure 4 shows the average total number of reinforcers earned for each DRL session during exposure week. Significant effects of Dose ($F_{3,112}$=7.7, p< .001), Day ($F_{4, 112}$ = 15.6, p< .001), and a Dose X Day interaction ($F_{12, 112}$=4.7, p< .001) were observed. Follow-up tests revealed that, relative to the saline group, the number of reinforcers earned was significantly lower in the 0.4 and 0.5LD50 groups on Days 2 and 3.

Because results indicated that responding changed as a function of exposure, we conducted an interresponse time (IRT) analysis to better characterize the nature of the changed responding. For each subject for each session, we calculated the following percentiles: 5, 10, 25, 50, 75, 90, and 95. However, in several cases, these percentiles could not be accurately calculated because fewer than 5 responses were emitted in a given session. For such sessions, these "non-responders" were excluded from analysis of IRT percentiles. The proportion of "responders" as a function of dose group is shown in Figure 5 for each day of exposure week. Significant effects of Dose ($F_{3,112}$=64.6, p< .001), Day ($F_{4, 112}$=12.3, p< .001), and a Dose X Day interaction ($F_{12, 112}$=4.6, p< .001) were observed. Follow-up tests revealed that, relative to the saline group, the proportion of responders was significantly lower in the 0.5LD50 group on Days 1-3 and in the 0.4LD50 group on Days 2 and 3. The IRT results were analyzed by conducting a separate one-way (Dose) ANOVA for each exposure day. On Day 1, the 90^{th} and 95^{th} percentiles were significantly greater in the 0.4LD50 group and the 95^{th} percentile was significantly greater in the 0.5LD50 group, relative to the saline group. On Days 2 and 3, there was only one responder in each of the two highest dose groups, too few to conduct a statistical analysis of the IRTs in those two groups. On Day 2, the 90^{th} and 95^{th} percentiles were significantly greater in the 0.3LD50 group than in the saline group. On Day 3, no statistically significant differences were observed between the 0.3LD50 and saline groups. On Day 4, the 75^{th}, 90^{th}, and 95^{th} percentiles were significantly

greater in the 0.5LD50 group than in the saline group. On Day 5, the 10^{th} and 25^{th} percentiles in the 0.5LD50 group were significantly *lower* than in the saline and 0.3LD50 groups. Similarly, the 5^{th} and 10^{th} percentiles in the 0.4 and 0.5LD50 groups were significantly lower than in the 0.3LD50 group on Day 5.

The median latency to retrieve the pellet (i.e., break the photobeam in the food trough after pellet delivery) was analyzed for each subject for each day of exposure week, but (similar to the IRT analysis above) only for those subjects that earned at least five reinforcers. The median was selected (rather than the mean) as the measure of central tendency because latency distributions are often positively skewed and medians are less affected by extreme scores. For the saline group, the average median reinforcer-retrieval latency was relatively invariant and ranged from 0.93 -1.0 seconds across the five exposure days. No significant main effect of Dose was observed on Day 1 for reinforcer-retrieval latency. On Days 2 and 3, only the saline and 0.3LD50 groups could be compared. The 0.3LD50 group exhibited significantly longer reinforcer-retrieval latencies than the saline group on Day 2 (t_{14}=3.55, p<.005) and on Day 3 (t_{14}=2.90, p<.02). On Days 4 and 5, a main effect of Dose was observed ($F_{3,25}$=5.15, p<.02 and $F_{3,28}$=4.33, p<.02, respectively). On Days 4 and 5, the 0.5LD50 group had significantly longer reinforcer-retrieval latencies than the saline and 0.3LD50 groups.

For all measures of operant performance, the total number of responses and reinforcers, IRTs, and reinforcer-retrieval latencies recovered by the first day of the following week (data not shown) and remained stable for two weeks; thus, no persistent or delayed-onset neurobehavioral effects were observed.

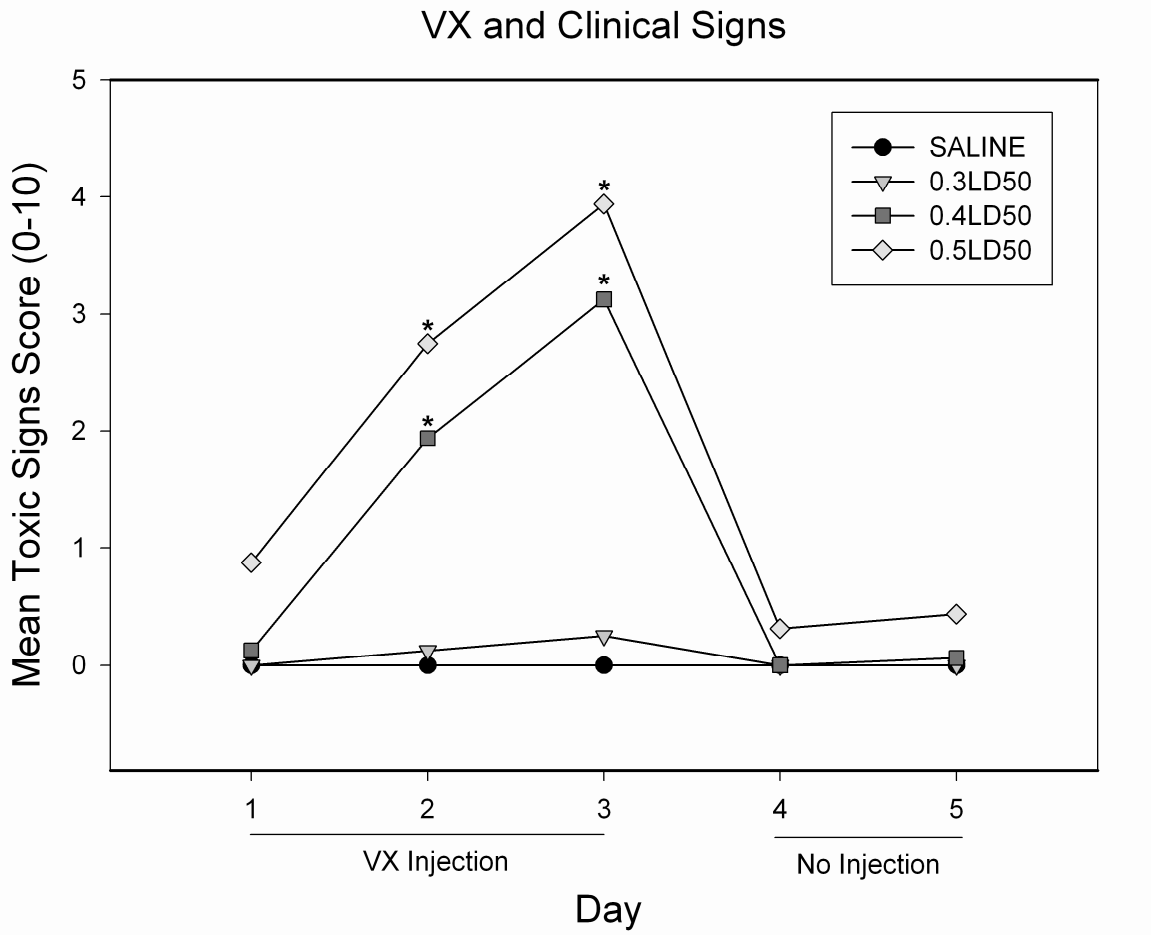

Figure 1. Relative to the saline control, toxic signs were significantly higher in the 0.4LD50 and 0.5LD50 groups on Days 2 and 3 of VX injection. Asterisks indicate a statistically significant difference from the saline group.

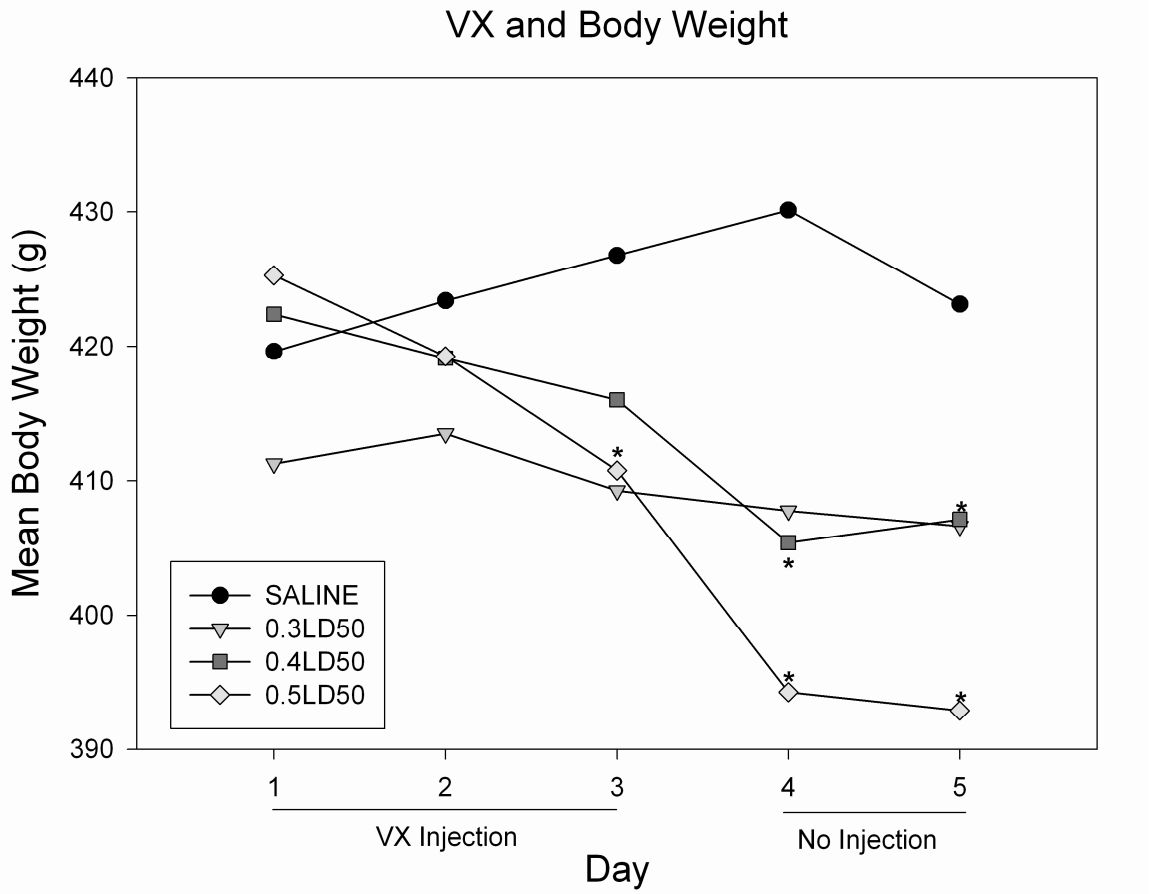

Figure 2. Relative to Day 1, body weights were significantly reduced on Days 3-5 for the 0.5LD50 group and on Days 3 and 4 for the 0.4LD50 group. Asterisks indicate a statistically significant difference from Day 1.

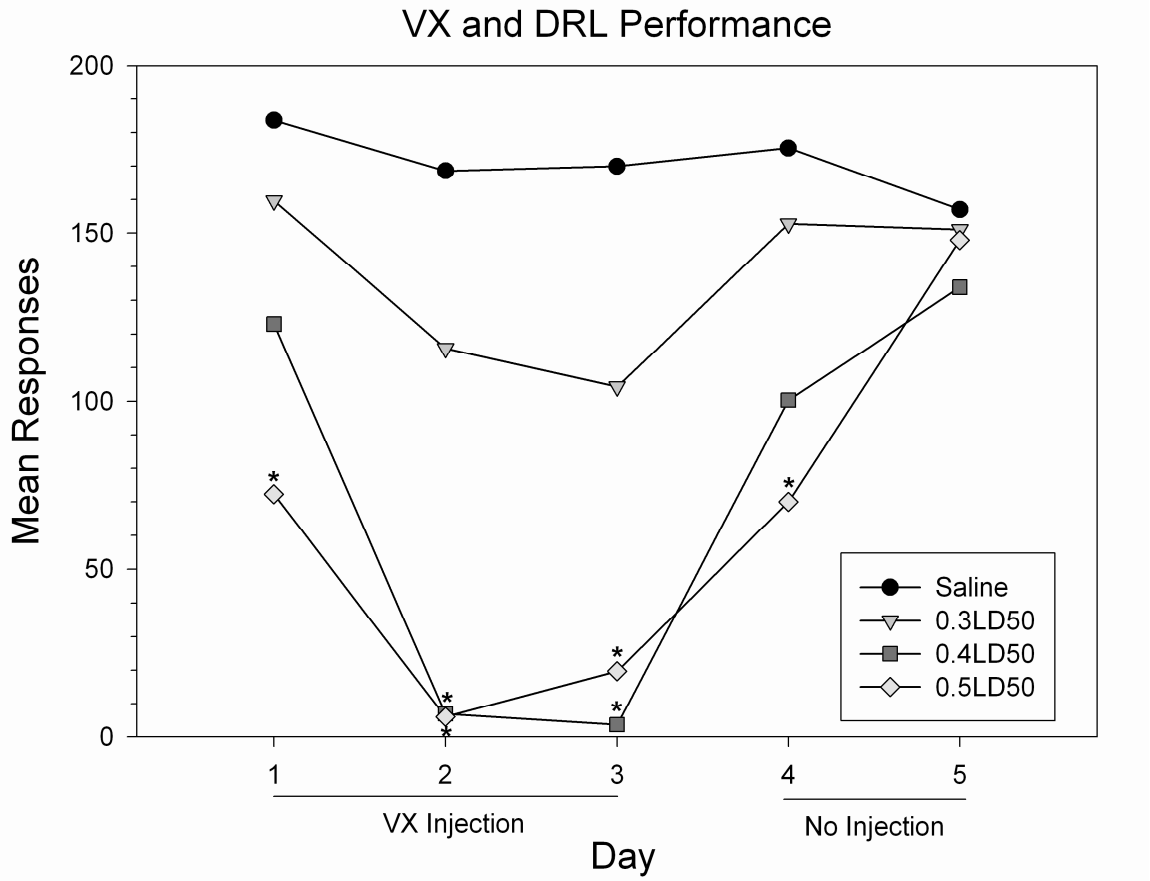

Figure 3. Relative to the saline control, responses were significantly lower in the 0.4LD50 group on Days 2 and 3 of VX injection and in the 0.5LD50 group on Days 1, 2, 3, and 4. Asterisks indicate a statistically significant difference from the saline group.

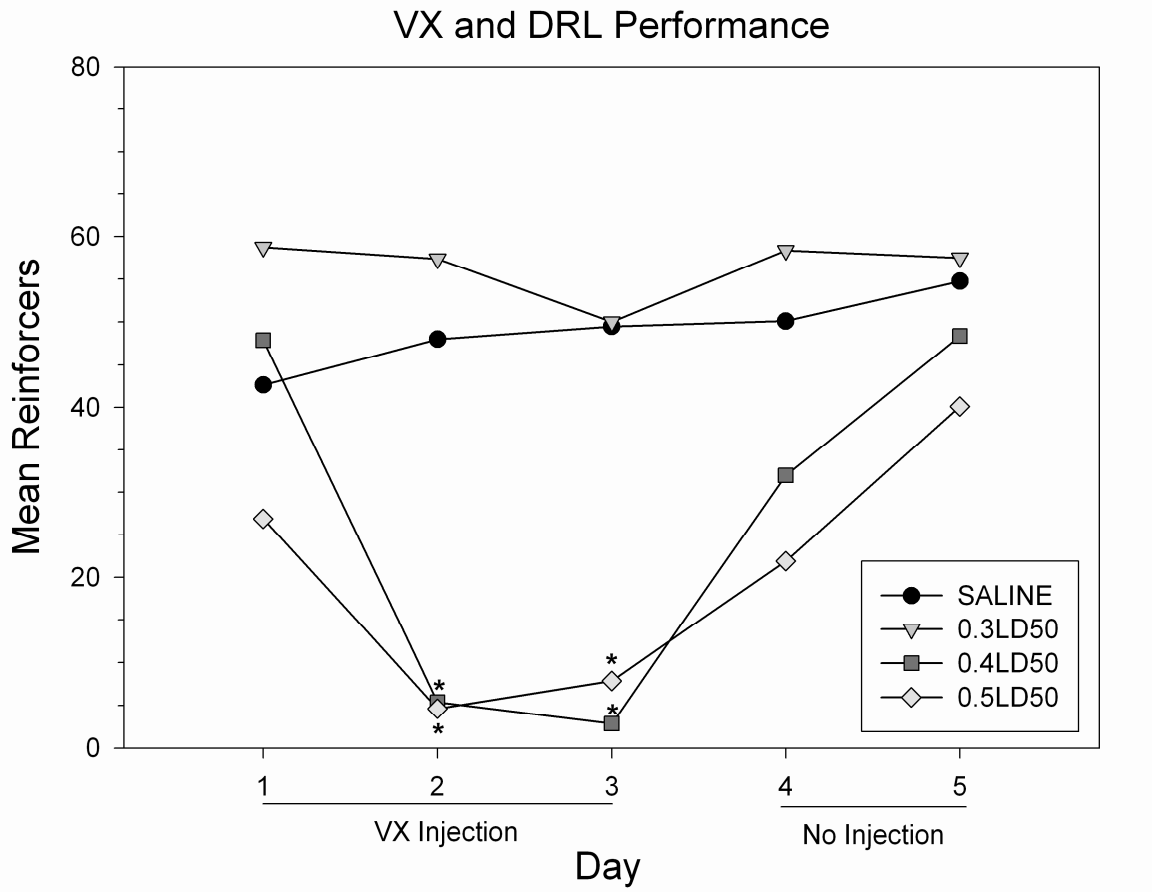

Figure 4. Relative to the saline control, reinforcers were significantly lower in the 0.4LD50 and 0.5LD50 groups on Days 2 and 3 of VX injection. Asterisks indicate a statistically significant difference from the saline group.

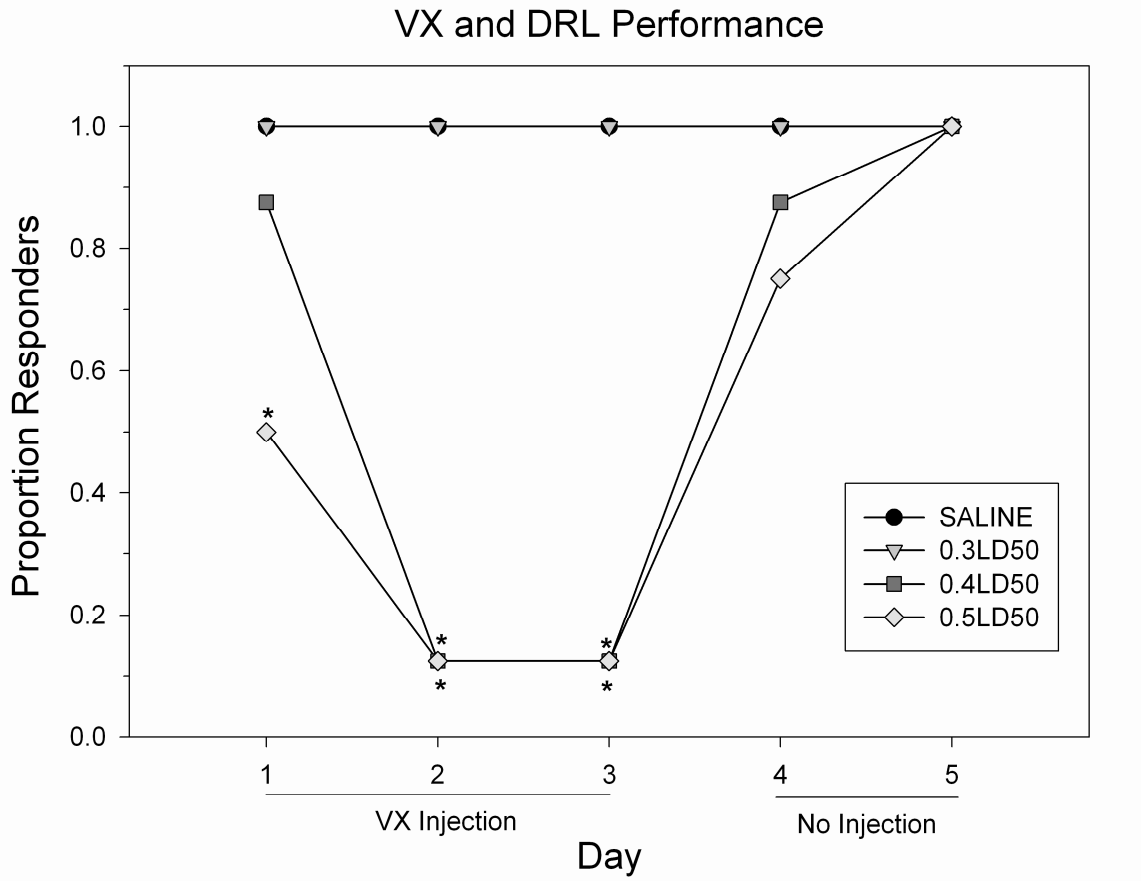

Figure 5. Relative to the saline control, the proportion of animals emitting at least 5 responses per session (i.e., "responders") was significantly lower in the 0.5LD50 group on Days 1, 2, and 3 of VX injection and on Days 2 and 3 of VX injection in the 0.4LD50 group. Asterisks indicate a statistically significant difference from the saline group.

DISCUSSION

Relative to saline control animals, toxic signs of nerve agent intoxication were significantly greater for the 0.4LD50 and 0.5LD50 groups on the second and third exposure days only. Body weight decreased significantly on the last 2 or 3 days of exposure week for the 0.4LD50 and 0.5LD50 groups, respectively. Operant responding was substantially decreased on the first day of VX injection for the 0.5LD50 group and remained suppressed through Day 4, but recovered by Day 5 (two days after ceasing VX injections). The 0.4LD50 group showed a similar but attenuated effect, with significant response suppression on Days 2 and 3 only. The number of reinforcers earned was substantially reduced on Days 2 and 3 for the 0.4 and 0.5LD50 groups. The proportion of animals responding on the DRL schedule was significantly reduced on Days 1-3 for the 0.5LD50 group and on Days 2 and 3 for the 0.4LD50 group. An analysis of IRTs indicated that the long IRTs significantly increased in all three VX groups on Day 1 or 2. On Day 4, IRTs were still significantly longer in the 0.5LD50 group relative to saline. On Day 5, the two highest dose groups exhibited significantly *shorter* interresponse times than the saline and/or 0.3LD50 group. This latter finding may be interpreted as a reduction in temporal control, increased appetitive motivation, or a failure to inhibit responding.

Additional operant tests could be used to better characterize the nature of this deficit. For example, adjusting-delay choice procedures may be particularly useful for assessing "self control" during nerve agent exposure and recovery. Temporal control could be further evaluated by a temporal-bisection or duration-discrimination procedure. A variable- or progressive-ratio schedule could be used to assess motivation. On this latter point, the results of Langston et al. (2005) are relevant. In their study, guinea pigs were pretrained to stable performance on a progressive-ratio schedule and then exposed to 0.1, 0.2, or 0.4LD50 sarin for 5 consecutive days (M-F) for two consecutive weeks. Those guinea pigs in the 0.4LD50 group exhibited the greatest weight loss and, during post-exposure recovery, exhibited higher response rates consistent with increased appetitive motivation on the progressive-ratio schedule. Thus, in the present study, it is possible that the shorter IRTs observed on Day 5 in the two highest dose groups were due to increased appetitive motivation as a result of weight loss during exposure. Indeed, increased motivation appears to produce less efficient DRL performance (c.f. Doughty & Richards, 2002; Lewis & Dougherty, 1992).

The present results are consistent with the body of research on cholinesterase inhibitors in general and nerve agents in particular. For example, Clark et al. (2005) and Mach et al. (2004) demonstrated that physostigmine reduced acoustic startle amplitude in mice. Philippens and colleagues (1992, 1996, & 1997) showed disruptions in acoustic startle and shuttle-box avoidance performance in guinea pigs following acute physostigmine administration. With specific regard to the repeated sublethal exposure to nerve agents, studies have shown behavioral alterations as well as reduced weight gain and clinical signs of toxicity at doses comparable to those producing effects in the present study. For example, Hulet et al. (2002) and Shih et al. (2006) exposed guinea pigs to 0.3, 0.4, 0.5, or 0.6LD50 sarin for five consecutive days for two consecutive weeks (i.e., 10

injections in 12 days). Both studies produced comparable results. Overt signs of toxicity (as indexed by the functional observational battery) and reduced weight gain were reliable only in the 0.5 LD50 group. Likewise, Atchison et al. (2004), using equivalent doses of sarin, soman, and VX, observed no signs of toxicity with a dose 0.4LD50 administered similarly. However, using an identical dosing procedure, Langston et al. (2005) showed weight loss and reduced responding on a progressive-ratio schedule of food reinforcement during the first week of exposure to 0.4LD50 (the day on which differences emerged was not specified). The reason for the apparently greater sensitivity of the guinea pigs from the Langston et al. study is not clear, but may be due to differences in toxicity resulting from age and/or dietary variables (those guinea pigs were older at the time of injection and maintained under both short-term and long-term dietary restriction; for a brief discussion, see Langston et al., 2005). This agrees well with the present study in that our rats were similarly diet restricted and, due to extensive training prior to exposure, older than those commonly employed in similar toxicity studies. Indeed, Shih et al. (1990) previously demonstrated that age and soman toxicity are positively related in rats. An alternative explanation focuses on the evaluative techniques themselves. That is, automated tests of operant responding using food reinforcement may be more sensitive than subjective measures of untrained performances (e.g., the functional observational battery). This does not account, however, for the greater sensitivity of the body weight data in the Langston study and the present one relative to the aforementioned (Hulet et al., Shih et al., and Atchison et al.) studies.

It does appear that operant procedures are particularly well suited for the evaluation of sublethal nerve agent intoxication. The present study was capable of detecting decrements in performance even in the 0.3LD50 group as early as Day 2 using IRT and reinforcer-retrieval analyses. It would be useful to utilize complementary *negative* reinforcement procedures to establish that such deficits are not limited to appetitively motivated behavior.

In summary, repeated sublethal exposure to VX produced robust but transient response suppression and reinforcer loss in the 0.4LD50 and 0.5LD50 groups that recovered within two days of completing the VX exposures. Toxic signs and body weight corroborated the intoxication observed in the 0.4LD50 and 0.5LD50 groups, but these measures were less sensitive than the operant performance measures.

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Appendix A.
Toxic Signs Score Sheet for Rat

RL _____ Date _____ Time _____
Post-Exposure Time (*circle one*) ~25 minutes, 24 h, 48 h, 72 h, 96 h

0 Normal
1-3 Mild Intoxication
4-6 Moderate Intoxication
7-10 Severe Intoxication

1. *General Motor Signs*
   0----------1----------2----------3
   Normal | Fasciculations | Tremors | Convulsions, Whole body

2. *Mastication*
   0----------1
   Normal | Abnormal, Excessive

3. *Salivation*
   0----------1
   Absent (Normal) | Present

4. *Lacrimation*
   0----------1
   Absent (Normal) | Present

5. *Piloerection*
   0----------1
   Absent (Normal) | Present

6. *General State*
   0----------1----------2----------3
   Normal, Coordination | Mildly Uncoordinated | Impaired movement | Prostrated

   Found DEAD at _____ (Date and Time)

   Comments: