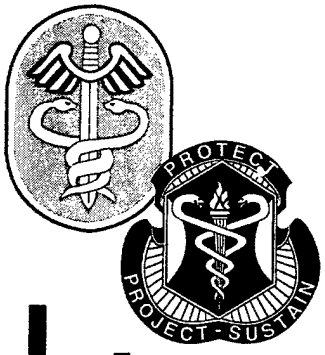

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Control of Nerve Agent-induced Seizures is Critical for Neuroprotection and Survival

Tsung-Ming A. Shih
Steven M. Duniho
John H. McDonough

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U.S. Army Medical Research
Institute of Chemical Defense
Aberdeen Proving Ground, MD 21010-5400

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14. ABSTRACT

The effectiveness of some anticholinergics (atropine, biperiden or trihexyphenidyl) and benzodiazepines (diazepam or midazolam) as an anticonvulsant treatment against seizures induced by six nerve agents (tabun, sarin, soman, cyclosarin, VR and VX) was studied in a guinea pig model that closely approximates the use of pretreatment and therapy drugs as medical countermeasures for nerve agent exposure. The time to seizure onset, the time to seizure termination, and 24-h lethality were recorded. The anticonvulsant ED50 of each drug for termination of seizures induced by each agent was calculated and compared. Brain tissue from animals that survived 24 h was examined for pathology. Results suggest that all nerve agents produce EEG seizures via a common mechanism and can be controlled by anticholinergic or benzodiazepine anticonvulsant treatment. While control of seizures was known to protect against brain pathology, the relationship between seizure control and the acute lethal effects of these agents appears to have been previously under-appreciated. Effective anticonvulsant treatment of a nerve agent casualty is, therefore, critical to immediate and long-term recovery.

15. SUBJECT TERMS

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Summary

This study evaluated the potency and rapidity of some anticholinergics (atropine, biperiden or trihexyphenidyl) and benzodiazepines (diazepam or midazolam) as an anticonvulsant treatment against seizures induced by six nerve agents (tabun, sarin, soman, cyclosarin, VR and VX) and summarized the relationship between anticonvulsant activity and nerve agent-induced lethality and neuropathology. Guinea pigs, previously implanted with cortical electrodes for electroencephalographic (EEG) recording, were pretreated with pyridostigmine bromide (0.026 mg/kg, im) 30 min prior to challenge with 2 x LD_{50} dose (sc) of a given nerve agent; in a separate experiment, animals were challenged with 5 x LD_{50}, sc, of soman. One min after agent challenge the animals were treated im with 2 mg/kg atropine SO_4 admixed with 25 mg/kg 2-PAM Cl. Five min after the start of EEG seizures, animals were treated im with different doses of anticholinergics or benzodiazepines and observed for seizure termination. The time to seizure onset, the time to seizure termination, and 24-h lethality were recorded. The anticonvulsant ED_{50} of each drug for termination of seizures induced by each agent was calculated and compared. Brain tissue from animals that survived 24 h was examined for pathology. All drugs were capable of terminating seizure activity with midazolam and trihexyphenidyl being significantly more potent than the other drugs, and midazolam being more rapid in controlling seizure than atropine, trihexyphenidyl or diazepam against each agent. Seizures induced by sarin or VX required lower doses of all the test anticonvulsants. The dose of a given drug that was an effective anticonvulsant against a 2 x LD_{50} challenge of soman was equally effective against seizures induced by a 5 x LD_{50} challenge. All nerve agents were capable of producing neuropathology. Seizure control was strongly associated with protection against acute lethality and brain pathology.

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INTRODUCTION

The potential for exposure to organophosphorus (OP) chemical warfare nerve agents exists on the battlefield and as a terrorist threat to civilian populations (e.g., Tokyo subway incident). Nerve agents are irreversible inhibitors of the cholinesterase (ChE) enzyme. Their toxic effects are due to hyperactivity of the cholinergic system as a result of inhibition of ChE, in particular, acetylcholinesterase (AChE), and the subsequent increase in the concentration of the neurotransmitter acetylcholine (ACh) in the brain and periphery (Taylor, 1995). Exposure causes a progression of toxic signs, to include hypersecretions, fasciculations, tremor, convulsions, respiratory distress and death. A combined regimen of prophylaxis and therapy is the most effective medical countermeasure for dealing with the threat of nerve agent poisoning to military personnel (Dunn and Sidell, 1989; Sidell, 1997). Pretreatment with carbamate ChE inhibitors, such as pyridostigmine, shields a fraction of ChE in the periphery from irreversible inhibition by the nerve agents. In the event of poisoning, immediate therapeutic treatment with an anticholinergic drug, such as atropine sulfate, antagonizes the effects of excess ACh at muscarinic receptor sites, and an oxime, such as pyridine-2-aldoxime methylchloride (2-PAM), is used to reactivate any unaged inhibited enzyme.

This combined prophylaxis and therapy regimen, however, does not prevent nerve agent-induced seizures. Prolonged epileptic seizures in a nerve agent casualty will produce profound, irreversible, brain damage that, in turn, will result in long-term deficits in cognitive function and behavior (Petras, 1981, 1994; Lemercier et al., 1983; McLeod et al., 1984; Kadar et al., 1995; Raffaele et al., 1987; Philippens et al., 1992; Filliat et al., 1999). Concomitant administration of an anticonvulsant drug such as diazepam (Valium®) is now considered essential to optimize the currently utilized regimen of carbamate pretreatment plus atropine and oxime therapy for severely exposed nerve agent casualties (Dunn and Sidell, 1989; Moore et al., 1995). Diazepam was provided to U.S. military forces in the early 1990s. However, experimental studies have shown diazepam to provide less than total protection against the neuropathological consequences of nerve agent exposure (Hayward et al., 1990; McDonough et al., 1995), and it has been hypothesized that low drug potency and/or slow speed of anticonvulsant activity are the key features that contribute to this situation (McDonough et al., 1999; Capacio et al., 2001). Thus, there has been an active research effort to find better drugs to treat seizures elicited by nerve agents.

However, drug potency and speed of activity are only two factors to be considered when evaluating a potential replacement anticonvulsant drug. An improved anticonvulsant treatment needs to protect against seizures elicited by all militarily relevant threat nerve agents, yet most previous anticonvulsant studies focused exclusively on seizures elicited by the nerve agent soman. Likewise, the dose of drug chosen should be sufficient to stop seizures elicited by all agents rapidly and should show efficacy across a wide range of agent exposure levels.

Studies from our institute over the past decade have identified a number of drugs that have greater anticonvulsant activity against nerve agent seizures than is now provided by diazepam. Among the benzodiazepine-type drugs, midazolam is more potent and rapidly acting as an anticonvulsant than diazepam (Anderson et al., 1997; McDonough et al., 1999). Additionally, several potent centrally acting anticholinergic drugs (such as biperiden and trihexyphenidyl) show robust anticonvulsant activity especially when given shortly after seizure onset (Capacio and Shih, 1991; McDonough and Shih, 1993; McDonough et al., 2000).

Therefore, the present study was performed to assess and compare the anticonvulsant potency and speed of action of three anticholinergic drugs (atropine sulfate, biperiden HCl, trihexyphenidyl HCl) and two benzodiazepines (diazepam and midazolam) against electroencephalographic (EEG) seizures produced by six chemical warfare nerve agents, and to analyze fully the relationship between anticonvulsant efficacy and nerve agent-induced neuropathology or lethality. The six nerve agents studied were tabun (GA; ethyl N,N-dimethyl phosphoramidocyanidate), sarin (GB; isopropyl methylphosphonofluoridate), soman (GD; pinacolyl methylphosphonofluoridate), cyclosarin (GF; cyclohexylmethylphosphonofluoridate), VX (0-ethyl S-(2-(diisopropylamino)ethyl)methylphosphonothioate), and a Russian V-type agent designated VR (0-isobutyl S-(2-diethylamino)ethyl)methylphosphonothioate). Their chemical structures are shown in Figure 1. In addition, the anticonvulsant efficacy of these drugs against seizures produced by a higher challenge dose of soman was assessed. These experiments were conducted in a guinea pig model that closely simulates the U.S. military doctrine of pyridostigmine pretreatment and atropine sulfate plus 2-PAM therapy after exposure (Keeler et al. 1991; Moore et al. 1995).

MATERIALS AND METHODS

Subjects. Male Hartley guinea pigs (Crl: (HA) BR COBS; Charles River Labs, Kingston, NY, USA), weighing 250-300 g before surgery, served as subjects. They were housed in individual cages in temperature ($21 \pm 2^{\circ}$ C) and humidity ($50\% \pm 10\%$) controlled quarters that were maintained on a 12-h light-dark cycle (lights on at 0600 h), and received food and water *ad libitum* except during experimental periods. The animal care program at USAMRICD is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Materials. Atropine sulfate, biperiden HCl, trihexyphenidyl HCl and sodium pentobarbital were purchased from Sigma Chemical Co. (St. Louis, MO). Pyridostigmine bromide, diazepam and midazolam were obtained from Hoffmann-La Roche Inc. (Nutley, NJ), and 2-PAM from Ayerst Labs, Inc. (New York City, NY). Tabun, sarin, soman, cyclosarin, VR and VX were obtained from the U. S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). Nerve agents were diluted in ice-cold saline prior to injection. Atropine sulfate, midazolam and 2-PAM were prepared in saline. Biperiden HCl, trihexyphenidyl HCl and diazepam and pentobarbital were prepared in a vehicle containing 40% propylene glycol, 10% ethanol, 1.5% benzyl alcohol and 48.5% distilled water. Atropine sulfate (2 mg/kg) and 2-PAM (25 mg/kg) therapies were admixed; all other solutions were prepared and injected separately. Injection volume was 0.5 ml/kg for all nerve agents and treatment drugs.

Surgery. Each guinea pig was anesthetized with isoflurane (3% induction, 1.5 – 2% maintenance; with oxygen) and set in a Kopf stereotaxic frame. Three cortical stainless steel screw electrodes were implanted in the skull. Two electrodes were placed bilaterally approximately 3.0 mm lateral to the midline and equidistant between bregma and lambda. A third was placed on the posterior calvaria as a ground. Stainless steel wires, previously soldered to these electrodes, were secured to a connector plug. Both the electrodes and plug were encased in cranioplastic cement. The incision site was sutured; the guinea pig was removed from the frame, and given buprenorphine HCl (0.03 mg/kg, sc) for post-operative analgesia. The animals were given seven to ten days rest prior to experimentation.

Experimental Procedure. EEG recordings were made using amplifiers and QND software supplied by Neurodata Inc. (Pasadena, CA) (low frequency filter = 0.3 Hz; high frequency filter = 40 Hz; sampling rate = 128 Hz) and displayed on a computer monitor. On the day of the experiment, guinea pigs were placed in individual recording chambers and continuously monitored for EEG activity. After a 15-min recording of baseline EEG, animals received a dose of pyridostigmine (0.026 mg/kg, im) determined to produce ~30% whole blood ChE inhibition (Lennox et al., 1985). Thirty min later, animals were challenged with 2 x LD_{50}, sc, dose of one of the different nerve agents: tabun (240 ug/kg), sarin (84 ug/kg), soman (56 ug/kg), cyclosarin (114 ug/kg), VX (16 ug/kg), or VR (22 ug/kg). In a separate experiment, animals were challenged with 5 x LD_{50} (140 ug/kg, sc) of soman. In all cases, one min after nerve agent challenge, the animals were treated with atropine sulfate (2 mg/kg, im) plus 2-PAM (25 mg/kg, im). This dose of 2-PAM approximates the total dose of 2-PAM in 3 autoinjectors given to a 70-75 kg human. The 2 mg/kg dose of atropine sulfate was chosen to prevent rapid lethal effects yet not to interfere significantly with seizure development (Shih et al., 1996; McDonough and Shih, 1997). This dose (2 mg/kg, im) was not included in the final calculation of the anticonvulsant ED_{50} for the study of atropine sulfate. Five min after the onset of EEG seizure activity, atropine sulfate, biperiden HCl, trihexyphenidyl HCl, diazepam or midazolam was given intramuscularly. Animals were observed continuously for the first hour following exposure and treatment and periodically thereafter for at least 4 h. EEGs were monitored continuously throughout this time and for 30 min at 24 h. Seizure onset was operationally defined as the appearance of $\geq$10 sec of rhythmic high amplitude spikes or sharp wave activity in the EEG. Each animal was rated as having the seizure terminated (OFF) or not terminated (NOT OFF) based on the overall appearance of the EEG record at the end of the experimental day and during the 24-h observation. (Note: An animal was rated as OFF if the seizure was terminated and the EEG remained normal at all subsequent observation times.) Mortality was recorded 24 h after nerve agent exposure. Animals that survived 24 h were deeply anesthetized with an overdose of sodium pentobarbital (75 mg/kg, ip), and perfused through the aorta with saline followed by 10% neutral buffered formalin. The brain was blocked, embedded in paraffin, cut 6-10 um thick, stained with hematoxylin and eosin, and then evaluated by a board certified pathologist (S.M.D) who was unaware of the experimental history of a given subject. The procedures and criteria used for pathological evaluation have been published (McDonough et al., 1995, 2000). Briefly, six brain areas (cerebral cortex, pyriform cortex, amygdala, hippocampus, thalamus, caudate/putamen) were evaluated in each animal and each area was rated on a scale from 0 (no damage) to 4 (severe, >45% tissue involvement) for neuropathological damage. In addition to the individual brain areas, a total brain lesion score was obtained for each animal by summing the scores of the six areas.

Data Analysis. Dose-effect curves and the median effective dose (ED_{50}) for anticonvulsant activity of each individual drug were determined by probit analysis (Bliss, 1952) using 4-7 doses with 5-6 animals per group. A probit regression analysis (SPSS for Windows, Version 10.0, Chicago, IL) was used to estimate the ED_{50}s along with the 95% fiducial confidence limits for each drug treatment and agent combination. Additionally, the SPSS program also determined the relative mean potency of each treatment for each agent and each agent for each treatment at the 50% response along with the respective 95% confidence interval. These relative mean potencies are the ratio of the 50% response for two treatments for a given agent, or two agents for a given treatment. With this determination, if the 95% confidence interval of the resultant

ratio included the value of 1, the ED_{50}s for the two treatments (or agents) were considered similar; if the 95% confidence interval excluded the value of 1 then the ED_{50}s for the treatments (or agents) were significantly different. Using this statistic, the anticonvulsant ED_{50}s for each of the five drugs were also compared among different nerve agents and between the two different challenge doses of soman. Latencies for seizure onset between the different agents were not normally distributed and were evaluated utilizing the Kruskal-Wallis analysis of variance (ANOVA) on ranks followed by Dunn's multiple comparison test. Latencies for seizure terminations were evaluated using both one- and two-way ANOVA procedures followed by Tukey multiple comparison tests. The differences between proportions of animals surviving challenge with each agent, the proportion of animals surviving as a function of successful control of the seizure, as well as the incidence of neuropathology as a function of seizure control, all were evaluated using the Chi-square procedure with Yates correction (Winer, 1971). In all statistical analysis, $p<0.05$ was considered significant.

RESULTS

EEG seizure generation. All six OP nerve agents were capable of inducing brain seizure activity in this guinea pig model. When drug treatment failed to stop the seizure, epileptiform activity was evident virtually continuously throughout the 4-h experimental period and could still be observed in some animals 24 h after nerve agent exposure. Table 1 summarizes and compares the seizure-producing effects of the 6 different nerve agents in this guinea pig model. Under the conditions of this model, the agents soman, VR, tabun, cyclosarin and sarin produced greater incidences of seizures than did VX (Table 1). With sarin and VX challenge, the seizure activity spontaneously stopped in 2% and 6%, respectively, of the animals that developed seizures. In these cases epileptiform EEG activity would begin, wax and wane for a short time (30 sec to 4 min), then spontaneously terminate; these animals remained free of further epileptiform EEG activity for the rest of the experiment. Such spontaneous termination of epileptiform seizure activity was not observed with the other agents. There was no lethality in animals that never developed seizures or in which the seizures spontaneously stopped, even though there were prominent peripheral signs of anticholinesterase poisoning (ataxia, fasciculations, tremors, salivation). A Kruskal-Wallis one-way ANOVA on ranks showed that there were significant differences in the latencies for seizure onset between the six nerve agents ($H = 533.9$, $df = 5$, $p < .001$). EEG seizure onset was most rapid with tabun and slowest following VX. Post-hoc analysis with Dunn's test showed tabun-elicited seizures (median = 5.0 min) did not differ from cyclosarin (median = 6 min), but occurred significantly faster than with any other agent. Cyclosarin-elicited seizures did not differ in onset from those produced by sarin (median = 6.51 min), but were faster than those elicited by soman, VR or VX. Soman-elicited seizures (median = 7.61 min) did not differ in onset from sarin seizures, but were significantly faster than either VR- (median = 10.02 min) or VX-induced seizure (median = 20.04 min), which, in turn, were significantly different from each other (Table 1). In the cases of cyclosarin and VX exposure, the possibility existed that a small percentage (1% and 0.3%, respectively) of animals would die before any evidence of EEG activation. Likewise, with VX exposure, a small percentage (2%) of the animals died after developing seizure, but before receiving anticonvulsant treatment. A Chi-square analysis of the 24-hr mortality rates for the different agents showed that VX, sarin or tabun produced significantly ($p < 0.01$) less mortality when compared with the higher levels

produced by cyclosarin, soman or VR (except the contrast of tabun with VR which failed to reach significance), which did not differ between one another.

Anticonvulsant drug efficacy. Table 2 summarizes the anticonvulsant efficacy (ED_{50} doses with 95% confidence limits) of atropine sulfate, biperiden HCl, trihexyphenidyl HCl, diazepam and midazolam for six nerve agents in this guinea pig model. Table 3 summarizes the statistical differences in the relative potency of a given drug across the different nerve agents.

Anticholinergic Drugs: The anticonvulsant ED_{50}s of atropine sulfate for VX- and sarin-induced seizures were not significantly different, but both were significantly lower than the atropine sulfate anticonvulsant ED_{50}s for cyclosarin-, tabun-, VR- and soman-induced seizures, which did not differ among one another. For biperiden HCl, the anticonvulsant ED_{50}s for VX- and sarin-induced seizures were not significantly different from one another and were significantly lower than the anticonvulsant ED_{50}s for tabun-, VR-, cyclosarin- or soman-induced seizures. The biperiden anticonvulsant ED_{50} for tabun-induced seizures was significantly lower than those for VR-, cyclosarin- or soman-induced seizures, which were not significantly different from one another. For trihexyphenidyl HCl, the anticonvulsant ED_{50} for sarin-induced seizures differed only from the anticonvulsant ED_{50} for VR-induced seizures, which, in turn, did not differ from the anticonvulsant ED_{50}s of trihexyphenidyl HCl for any of the other agents. When compared for any given agent, the anticonvulsant ED_{50}s of trihexyphenidyl HCl were significantly more potent than the anticonvulsant ED_{50}s of biperiden HCl, which, in turn, were significantly more potent than the anticonvulsant ED_{50}s of atropine sulfate.

Benzodiazepines: For diazepam, the anticonvulsant ED_{50}s for VX- or sarin-induced seizures were not significantly different from one another, but were significantly less than the anticonvulsant ED_{50}s for seizures induced by the other four agents. Diazepam anticonvulsant ED_{50}s for VR- and cyclosarin-induced seizures were not significantly different and both were significantly less than the diazepam anticonvulsant ED_{50} for soman-induced seizures, but not tabun-induced seizures, while the anticonvulsant ED_{50}s for tabun- and soman-induced seizures did not differ. For midazolam, the anticonvulsant ED_{50}s for sarin- and VX-induced seizures did not differ from each other, but were significantly less than the anticonvulsant ED_{50}s for seizures induced by the other four agents; the anticonvulsant ED_{50} of midazolam for cyclosarin-induced seizures was significantly less than those for VR- or soman-induced seizures, but not tabun-induced seizures which, in turn, had a similar anticonvulsant ED_{50} as that of VR-induced seizures. The midazolam anticonvulsant ED_{50} of soman-induced seizures was significantly greater than all others. When compared for any given agent, the anticonvulsant ED_{50}s of midazolam were significantly more potent than the anticonvulsant ED_{50}s of diazepam.

The times it took (latencies) for seizures to terminate following successful anticonvulsant treatment were tabulated for each drug - nerve agent combination, regardless of the dose of the treatment drug, and then analyzed utilizing a two-way ANOVA with nerve agent and treatment drug as the main factors. The analysis showed significant differences only between treatment drugs ($F_{4, 397} = 5.94$, $p<0.001$) and the nerve agent X treatment drug interaction ($F_{20, 397} = 3.10$, $p<0.001$). A Tukey test of the treatment drug effect showed that overall, midazolam terminated seizures significantly more rapidly than atropine, trihexyphenidyl or diazepam, and that biperiden controlled seizures significantly more rapidly than trihexyphenidyl. Also, there were no significant differences in the latencies for control of seizures elicited by the different nerve agents following treatment with midazolam or biperiden. In contrast, seizures elicited by VR were significantly slower in stopping following atropine treatment when compared with the other

treatment drugs, VX- and cyclosarin-triggered seizures were significantly slower in responding to treatment with trihexyphenidyl when compared with the other drugs, and soman-induced seizures were significantly slower in responding to diazepam when compared with the other drugs. These data are presented in Figure 2. An example of the rapid control of EEG seizure activity by midazolam is presented in Figure 3.

It was also noted that seizures elicited by all the agents could recur after an initial anticonvulsant effect. This occurred occasionally (<2%) with any of the anticholinergic drugs, but more frequently ($\simeq$ 5%) with the two benzodiazepine drugs, and was typically associated with drug doses $\leq$ the anticonvulsant ED_{50}. In all cases where seizure activity was observed to recur, the particular treatment was classified as not being successful. Figure 4 displays an example of seizure activity returning after an initial period of anticonvulsant control.

When animals were challenged with the 5 x LD_{50} dose of soman, there were remarkable differences in the response to this challenge dose when compared with the 2 x LD_{50} challenge of soman. Animals challenged with 2 x LD_{50} of soman never lost their righting reflex during the initial development of toxic signs and seizures, and EEG epileptiform activity was continuously evident. In contrast, when challenged with the 5 x LD_{50} dose of soman, the animals rapidly lost their righting reflex, and some animals then entered a comatose state in which respiratory efforts became erratic and of a diaphragmatic nature, the EEG recording became isoelectric, cyanosis developed, and the animals were totally unresponsive. This comatose state would last 1-4 min, and then EEG activity slowly returned as respiratory efforts became more frequent and regular; epileptiform seizure activity emerged as the EEG returned toward a normal activity pattern. Five animals challenged under these conditions died without any expression of EEG seizure activity during this comatose state, and two seizing animals died before anticonvulsant treatment could be administered.

Table 4 summarizes the anticonvulsant efficacy (ED_{50} doses with 95% confidence limits) of atropine sulfate, biperiden HCl, trihexyphenidyl HCl, midazolam, and diazepam following different challenge doses (2 or 5 x LD_{50}) of soman. For all the drugs there were no significant differences in the anticonvulsant ED_{50}s between the two different agent challenge conditions.

Relationship between seizure termination and lethality or neuropathology. Table 5 shows the strong relationship between the control of seizures and protection against the lethal effect of nerve agent exposure. Animals were categorized by seizure outcome (OFF, NOT OFF) and lethality (ALIVE, DEAD) at 24 h following nerve agent exposure. Less than 4% of the animals that had their seizures successfully controlled (OFF) died, whereas 42% of animals that failed to have the seizures controlled (NOT OFF) died. This result was highly significant ($\chi^2 = 173.5$, df = 1, $p < 0.001$). Analysis of the individual drugs showed this was a consistent and robust finding regardless of treatment drug.

The brains of 593 animals were available for pathological evaluation. All the nerve agents were capable of producing neuropathology under the conditions of this study. The basic lesion was one of neuronal necrosis. In its most moderate form, the lesion consisted of scattered shrunken pyknotic/necrotic individual neurons. In the more severe gradations, greater numbers of neurons were progressively involved along with astroglial cells as well, resulting in parenchymal necrosis and spongiform or malacic appearance to the tissue. Figure 5 presents an example of the different lesion grades and microscopic appearance of these lesions. There was no qualitative difference in the appearance of a lesion produced by one nerve agent versus another within a given neural structure and there was no difference between agents in the brain

areas affected. No drug completely protected against neuropathology development, but control of seizure clearly had an influence on the incidence and severity of neuropathology (Tables 6 and 7). Animals that had their seizure successfully controlled (OFF) had a significantly lower incidence of neuropathology ($\chi^2 = 81.14$, df = 1, $p < 0.001$). Again, analysis of the individual drugs showed that this was a consistent and robust finding with each compound.

Table 7 displays the average brain lesion scores for individual brain areas. The data are collapsed across all nerve agents and then categorized by whether the seizure was rated OFF or NOT OFF and listed by treatment drug. Note that only the 254 animals that displayed lesions are represented in the table. The data clearly show that failure to control the seizure resulted in significantly greater numbers of animals with brain lesions, and in general the severity of the lesion was greater than in animals in which the seizures were successfully controlled by the treatment drug. Overall, the cortex and the amygdala were the brain areas most frequently damaged. The amygdala also experienced the most severe damage, followed by the cortex and caudate nucleus.

DISCUSSION

The present findings show that in the guinea pig model used in this study the anticholinergic drugs (atropine sulfate, biperiden HCl, and trihexyphenidyl HCl) and the benzodiazepines (diazepam and midazolam) are capable of stopping the epileptiform seizures induced by any of the six nerve agents tested. The effective anticonvulsant dose for any one of these drugs is relatively the same across all nerve agents, with the exception of sarin and VX, which proved to have notably lower anticonvulsant dose requirements under these conditions. The selected dose of any of these test drugs to fully control soman-induced seizure is equally or more effective in treating seizures induced by all other nerve agents. Among the anticholinergic drugs studied, the doses of trihexyphenidyl HCl required to terminate seizure activity are considerably lower than those required for biperiden HCl, and both of these are substantially more potent than atropine sulfate across all nerve agents. In the case of benzodiazepines, the anticonvulsant ED_{50} doses of midazolam are markedly lower than those required for diazepam across all nerve agents. In terms of speed of action, midazolam also was more rapid in controlling seizures induced by all the agents than was atropine, trihexyphenidyl or diazepam. The results show that all six nerve agents have the potential for producing brain lesions when they are given at doses that produce seizures. In addition, the data clearly demonstrate a strong relationship between effective anticonvulsant control of these seizures and protection from the acute lethal effects of these nerve agents as well as protection from their neurotoxic effects.

The present study shows that all six nerve agents tested could induce prolonged brain seizures (*status epilepticus*) in our guinea pig model, even though there was a distinction among these nerve agents. Tabun, sarin, soman, cyclosarin and VR induced brain seizures fairly rapidly (5 to 10 min) and with high incidence (>90%), while VX induced seizure with a long latency (20.5 min) and low incidence of seizure development (52%) under these conditions. With VX, the epileptiform EEG activity waxed and waned initially and spontaneously stopped in a small fraction of the animals that developed seizures. Such spontaneous termination of seizure activity was not observed with any other nerve agents studied. The reasons for these differences are not known. The lower incidence of seizure occurrence and longer latency to seizure onset coupled with the waxing and waning of the initial epileptiform activity may, thus, provide a wider window of opportunity for any anticonvulsant treatment of VX-induced seizures. What the results emphasize is the potent epileptiogenic effects of all the nerve agents when given at lethal

doses, and the fact that lethality was not observed in any of the animals that did not develop seizures.

The present study shows that atropine sulfate, biperiden HCl and trihexyphenidyl HCl can terminate seizure activity induced by all six nerve agents if given shortly (5 min) after seizure onset. The differences in potency between these three anticholinergics across agents is the same as first reported against soman (Capacio and Shih, 1991; Shih et al., 1991). When compared with any given agent, trihexyphenidyl is more potent than biperiden, which, in turn, is more potent than atropine, while biperiden controlled seizures significantly more rapidly than trihexyphenidyl. One notable aspect of this data is that atropine sulfate, the drug used almost universally as an antidote for anticholinesterase poisoning, was the least potent anticholinergic drug for control of nerve agent-induced seizures under the conditions of this study. Substantially higher doses of atropine were required for seizure control, and this is probably due to the slow uptake and penetration of this drug into the brain relative to peripheral tissues when it is given by the im route (Ketchum et al., 1973; Lipp and Dola, 1978; McDonough and Shih, 1993).

Diazepam is the drug used by U.S. and other military forces to treat seizures and convulsions that may develop as a result of nerve agent exposure (Moore et al., 1995; Sidell, 1997). However, there has been a continuing concern that diazepam, at the dose and route of administration that is available (3 x 10 mg given im at 10 min intervals), may not provide the optimal treatment for rapid control of nerve agent-induced seizures. A recent study has shown that midazolam can terminate soman-induced seizures more rapidly and with a smaller dosage than diazepam (McDonough et al., 1999). The present results further show that these differences between midazolam and diazepam extend to seizures induced by all six nerve agents. These differences between diazepam and midazolam in their potency and speed of action may be due to their rate of absorption and bioavailability achieved by the im route of administration (Capacio et al., 2001; 2002). Consistent with the outcomes of anticholinergic drugs, higher doses of diazepam and midazolam were required to control soman-, VR-, tabun- or cyclosarin-induced seizures than were required for sarin- and VX-induced seizures. Thus, sarin- and VX-induced seizures remain the most readily treated with an anticonvulsant medication among the six threat nerve agents studied.

At the outset of this study we identified drug potency, speed of anticonvulsant action, and broad spectrum of effect across nerve agents as the key features most desirable for a replacement of diazepam. Among the drugs tested in this study, midazolam stands out as the most rapidly acting and potent anticonvulsant drug for all six nerve agents studied, and thus meets all these key requirements. Furthermore, the effective doses of midazolam across all nerve agents are close to those reported to be effective in terminating human *status epilepticus* (0.1 – 0.3 mg/kg; Shorvon, 1994). In comparison, diazepam's anticonvulsant dose requirements, with the exception of VX, are substantially higher than human equivalent doses (0.3-0.6 mg/kg; Shorvon, 1994) reported to successfully terminate *status epilepticus*. Additionally, the latency to terminate on-going seizure data showed the slower action of diazepam relative to midazolam against soman-induced seizures. Although trihexyphenidyl displayed a broad spectrum of activity and high anticonvulsant potency, overall it too was slower than midazolam in terminating seizures.

Another interesting aspect of the data is that similar doses of the compounds were successful in terminating seizures elicited by different challenge doses of soman. In regards to the anticholinergic drugs, it may be reasonable to speculate that there is a functional threshold of cholinergic receptors that has to be activated by the increased ACh that follows ChE inhibition by a nerve agent to trigger epileptiform seizure activity, and the same amount of anticholinergic

is required to compete with and block these functional receptors (Shih et al., 1991; McDonough and Shih, 1997). Likewise, for the benzodiazepines, the maximum capacity of the inhibitory function of benzodiazepine receptors could be reached regardless of the amount of challenging dose of a nerve agent. In any case, these data show that similar doses of any of these compounds will treat seizures elicited by a range of nerve agent challenge doses.

The present findings show that all threat nerve agents have the potential for producing neuropathology. To our knowledge, this is the first study demonstrating that the nerve agents tabun, VR and VX have the ability to produce neuropathology similar to that well-documented for soman and sarin (Petras, 1981, 1984; Lemercier et al., 1983; McLeod et al., 1984; Kadar et al., 1995). Additionally, the data provide the strongest evidence to date that the prolonged seizure activity elicited by the nerve agents is the major factor in lesion production. Animals that had seizures controlled by the different anticonvulsant treatments (OFF) were significantly more likely to be totally free of neuropathology or, if it did occur, to have it greatly reduced in both incidence and severity. This is in agreement with previous findings in both rodents (Lallement et al., 1993, 1994; Martin et al., 1985; McDonough et al., 2000) and nonhuman primates (Hayward et al., 1990; Lallement et al., 1997a, b, 1998, 1999) that any treatment that can reduce or terminate seizure activity in soman-exposed animals has a protective effect on the development of neuropathology.

Furthermore, the present study shows that there is a strong association between anticonvulsant effect of the treatment and protection from the acute lethal effects of the nerve agents. This has been seen in previous studies from our laboratory with both anticholinergic as well as benzodiazepine drugs using soman challenge in this guinea pig model (McDonough et al., 1999, 2000) and has also been observed in nonhuman primate studies of anticonvulsant treatment of soman exposure (Lallement et al., 1997a, b, 1998, 1999; unpublished observations). Such an association between mortality and status epilepticus is well recognized in the clinical medical literature (Shorvon, 1994; Towne et al., 1994; Krumholz et al., 1995). The fact that control of nerve agent seizures is so strongly linked to protection from the lethal effects of nerve agents may explain the requirement for the high doses of atropine that have been routinely used in studies of the protective effects of carbamate pretreatment (Berry and Davis, 1970; Dirnhuber et al., 1979; Maxwell et al., 1988) or oxime therapies (Melchers et al., 1994; Worek and Szinicz, 1993; Worek et al., 1994; Koplovitz et al., 1995). These findings lend perspective to the older reports that inclusion of a benzodiazepine to standard atropine and oxime therapy would increase the protective ratios against OP nerve agent exposure (Rump et al., 1973; Johnson and Wilcox, 1975; Boskovic, 1981).

TABLE 1

Seizure-producing Effects of Tabun, Sarin, Soman, Cyclosarin, VX and VR in the Guinea Pig Model[a].

	Tabun	Soman	Sarin	Cyclosarin	VX	VR
Group size (N)	136	120	178	139	319	136
Seizure Occurrence	130/136 (96%)	120/120(100%)	163/178(92%)	130/139(94%)	166/319(52%)	132/136 (97%)
Seizure Onset Time (min)	5.18±0.13[b]	8.11±0.30[d]	7.15±0.13	6.32±0.25[c]	20.49±0.46	10.75±0.30[e]
Died after Treatment	21/130 (16%)	24/120 (22%)	19/163 (12%)	46/130 (35%)	27/166 (16%)	34/132 (26%)
24-h Cumulative Mortality	21/136 (15%)[g]	38/120 (32%)	19/178 (11%)[f]	48/139 (35%)	31/319 (10%)[f]	34/136 (25%)

[a] Guinea pigs received pyridostigmine (0.026 mg/kg, im) 30 min before receiving nerve agents (2.0 x LD_{50}), followed 1 min later by atropine sulfate (2 mg/kg, im) and 2-PAM (25 mg/kg, im). Anticonvulsant treatment was given intramuscularly 5 min after seizure onset.
[b] Significantly less than sarin, soman, VR or VX.
[c] Significantly less than soman, VR or VX.
[d] Significantly less than VR or VX.
[e] Significantly less than VX.
[f] Significantly less than soman, cyclosarin or VR.
[g] Significantly less than soman or cyclosarin.

TABLE 2

The Anticonvulsant Potency of Some Anticholinergic and Benzodiazepine Drugs against Nerve Agent-Induced Seizures in the Guinea Pig Model[a].

ED_{50}s (mg/kg, im)[b]	Tabun	Sarin	Soman	Cyclosarin	VX	VR
Atropine Sulfate	10.37 (7.51 - 14.32)	5.11 (3.63 - 7.15)	11.84 (9.85 - 14.16)	10.28 (7.64 - 13.86)	3.99 (3.05 - 5.27)	11.75 (9.60 - 14.35)
Biperiden HCl	0.19 (0.13 - 0.29)	0.10 (0.07 - 0.14)	0.57 (0.39 - 0.84)	0.53 (0.34 - 0.87)	0.10 (0.07 - 0.15)	0.40 (0.26 - 0.61)
Trihexyphenidyl HCl	0.06 (0.03 - 0.16)	0.02 (0.007 - 0.05)	0.06 (0.02 - 0.14)	0.06 (0.02 - 0.15)	0.04 (0.01 - 0.10)	0.14 (0.06 - 0.36)
Diazepam	3.34 (2.45 - 4.61)	0.80 (0.53 - 1.19)	4.83 (3.58 - 6.73)	2.61 (1.88 - 3.65)	0.47 (0.30 - 0.72)	2.35 (1.69 - 3.20)
Midazolam	0.06 (0.03 - 0.09)	0.01 (0.007 - 0.019)	0.37 (0.22 - 0.63)	0.04 (0.03 - 0.06)	0.01 (0.009 - 0.02)	0.09 (0.05 - 0.16)

[a] Guinea pigs received pyridostigmine (0.026 mg/kg, im) 30 min before receiving nerve agents (2.0 x LD_{50}), followed 1 min later by atropine sulfate (2 mg/kg, im) and 2-PAM (25 mg/kg, im). Anticholinergic or benzodiazepine drugs were administered (im) 5 min after seizure onset.

[b] ED_{50}s calculated based on terminating cortical EEG seizure activity; 95% confidence limits in parentheses.

TABLE 3

Differences in Relative Anticonvulsant Potency[a] of the Tested Treatment Drugs against Seizures Elicited by the Different Nerve Agents[b]

Treatment Drug	Nerve Agent					
Atropine	VX	GB	GF	GA	VR	GD
Biperiden	VX	GB	GA	VR	GF	GD
Trihexyphenidyl	GB	VX	GD	GF	GA	VR
Diazepam	VX	GB	VR	GF	GA	GD
Midazolam	GB	VX	GF	GA	VR	GD

[a] Anticonvulsant ED_{50}s of a treatment drug against the different nerve agents are rank ordered lowest (left) to highest (right); lines under different nerve agents indicates no statistical difference in potency between the ED_{50}s.

[b] Nerve agents are indicated by their military designation for presentation purposes; GA=tabun, GB=sarin, GD=soman, GF=cyclosarin.

TABLE 4

Anticonvulsant Potency (ED_{50}s[a]) of Some Anticholinergic and Benzodiazepine Drugs against Different Challenging Doses of Soman in the Guinea Pig Model[b].

Soman Dose	Atropine Sulfate	Biperiden HCl	Trihexyphenidyl HCl	Midazolam	Diazepam
2 x LD_{50}	11.84 (7.51 - 14.32)	0.57 (0.39 - 0.84)	0.06 (0.02 - 0.14)	0.37 (0.22 - 0.63)	4.83 (3.58 - 6.73)
5 x LD_{50}	12.20 (7.53 - 18.14)	0.48 (0.25 - 0.73)	0.18 (0.01 - 1.51)	0.42 (0.29 - 0.65)	6.95 (4.81 - 8.44)

[a] ED_{50} (mg/kg, im; with 95% confidence limits in parentheses).

[b] Guinea pigs received pyridostigmine (0.026 mg/kg, im) 30 min before receiving nerve agents (2.0 x LD_{50}), followed 1 min later by atropine sulfate (2 mg/kg, im) and 2-PAM (25 mg/kg, im). Anticonvulsant treatment was given intramuscularly 5 min after seizure onset.

TABLE 5

Acute (24-h) Survival as a Function of Seizure Control

	24-h Survival		
	ALIVE	DEAD	TOTAL
SEIZURE OFF	411 (96%)	16 (4%)	427
SEIZURE NOT OFF	232 (58%)	169 (42%)	401

$\chi^2 = 173.5$, df = 1, $p < 0.001$

TABLE 6

Incidence of Neuropathology as a Function of Seizure Control

	No Pathology	Pathology	TOTAL
SEIZURE OFF	284 (78%)	77 (22%)	361
SEIZURE NOT OFF	55 (24%)	177 (76%)	232

$\chi^2 = 81.14$, df = 1, $p < 0.001$

TABLE 7

Average Brain Lesion Score by Brain Area Collapsed Across All Nerve Agents and Categorized by Seizure Control and Treatment Drug.

Mean Brain Lesion Scores[a]

		Brain Area					
	Drug[b]	Cortex	Amygdala	Piriform	Hippocampus	Caudate	Thalamus
OFF	ATR	1.5(6)	1.4(3)	1.8(4)	1.1(8)	1.0(5)	1.7(3)
	BIP	1.1(26)	1.7(10)	1.1(8)	1.1(7)	1.2(5)	1.1(15)
	THX	1.0(5)	3.0(3)	0(0)	1.4(5)	1.0(1)	1.0(1)
	DIZ	1.3(9)	2.0(8)	1.0(5)	0(0)	0(0)	1.0(2)
	MDZ	1.1(7)	2.4(7)	0(0)	1.4(5)	0(0)	1.0(1)
	Total	1.2(53)	2.1(31)	1.2(17)	1.5(25)	1.1(11)	1.1(22)
NOT OFF	ATR	1.2(14)	2.6(11)	1.8(12)	1.0(8)	2.0(8)	1.9(14)
	BIP	3.5(43)	3.8(41)	2.0(38)	1.1(33)	3.0(41)	3.3(34)
	THX	2.9(20)	3.8(21)	1.7(13)	1.3(18)	3.2(18)	2.0(19)
	DIZ	2.8(54)	3.3(55)	1.7(48)	1.0(2)	1.7(19)	1.7(44)
	MDZ	2.3(36)	3.7(33)	1.6(25)	1.5(15)	2.9(21)	1.7(28)
	Total	2.7(167)	3.5(161)	1.8(136)	1.2(76)	2.7(107)	2.1(139)

[a] Severity of damage assessed on a 0-4 scale (McDonough et al., 1995, 2000). Only lesioned animals are included; the number in parenthesis indicate number of animals involved.

[b] ATR=atropine sulfate; BIP=biperiden HCl; THX=trihexyphenidyl HCl; DIZ=diazepam; and MDZ=midazolam.

FIGURE LEGENDS

Figure 1. Chemical structures of tabun (GA), sarin (GB), soman (GD), cyclosarin (GF), VX and VR.

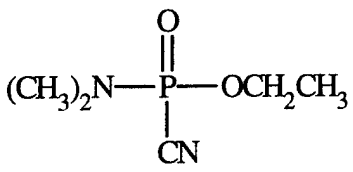
Figure 2. Mean latency (± SEM) for seizure termination categorized by treatment drug and nerve agent. All animals in which seizures were terminated, regardless of treatment drug dose, are included in each nerve agent – treatment drug combination group. The average group size ≃ 15, range 6-35.

Figure 3. . An example of the control of soman-induced seizure by midazolam. A. Baseline EEG activity. B. Seizure onset (arrow). C. Injection of midazolam 5 min after seizure onset (arrow). D. 10 min after midazolam injection: note the rapid diminution of both the frequency and amplitude of the discharge. Seizure was judged off at the arrow, 10 min 55 sec after midazolam injection. E. - G. EEG remains free of epileptiform activity 2, 4 and 24 h after midazolam treatment.

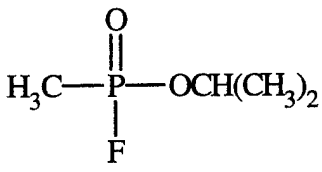
Figure 4. An example of the temporary control of a soman-induced seizure by diazepam. A. Baseline EEG activity. B. Seizure onset (arrow). C. Injection of diazepam 5 min after seizure onset (arrow). D. 5 min after diazepam injection: note the attenuation of the frequency and amplitude of the discharge. E. 20 min after diazepam injection: note continuing diminution of both the frequency and amplitude of the discharge. F. Epileptic activity fades so as to be indistinguishable from background EEG. Seizure was judged off at the arrow, 40 min 15 sec after diazepam injection. G. EEG remains free of epileptiform activity 2 h after diazepam treatment. H. Example of repeated epileptiform bursts noted in some animals on recording 24 h after initial seizure control; because of this activity the animal was judged as having the seizure not terminated.

Figure 5. Examples of the different gradations of lesion produced by the nerve agents in cerebral cortex; all sections except that shown in B are from the dorsal frontal or parietal cortex at the approximate A-P level of the optic chiasm. A. No lesion; animal was exposed to cyclosarin and seizure was successfully controlled by atropine. B. Score 1 – (1-10% necrotic neurons) characterized by scattered dark pyknotic neurons in this case in the deep layers of the cingulate cortex; animal was exposed to VX and seizure was not successfully controlled with trihexyphenidyl. C. Score 2 – (11-25% necrotic neurons) the necrosis is primarily concentrated in the superficial border of cortical layer 2; animal was exposed to tabun and seizure was not successfully controlled with diazepam. D. Score 3 – more extensive damage (26-45% necrotic neurons) again involving primarily cortical layer 2 accompanied by diffuse neuropil damage in cortical layer 1; animal was exposed to VR and seizure was not successfully controlled with atropine. E. Score 4 – severe damage (>45% necrotic neurons) to most neurons within cortical layer 2 along with neuropil damage that extends from the surface into cortical layer 3; animal was exposed to sarin and seizure was not successfully controlled with midazolam.

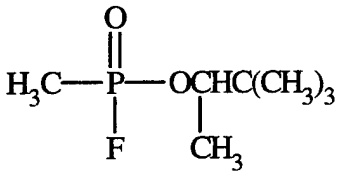
Tabun (GA)



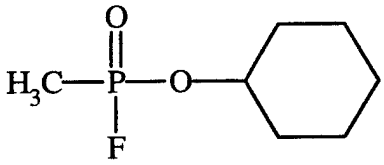
Sarin (GB)



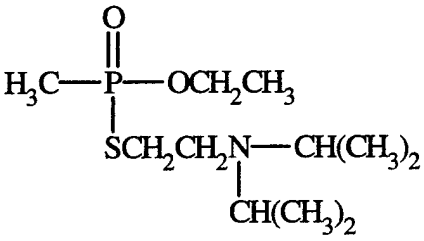
Soman (GD)



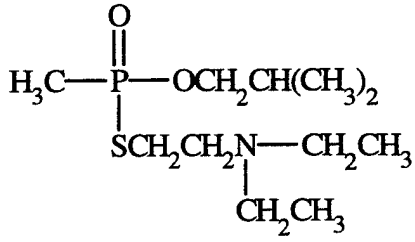
GF

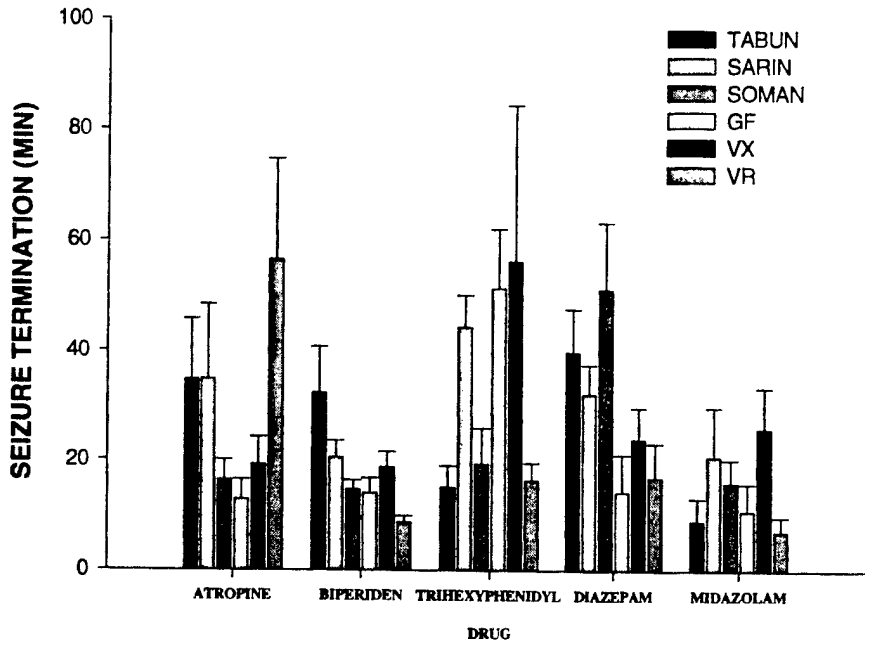


VX



VR




100
80
60
40
20
0
SEIZURE TERMINATION (MIN)
TABUN
SARIN
SOMAN
GF
VX
VR
ATROPINE
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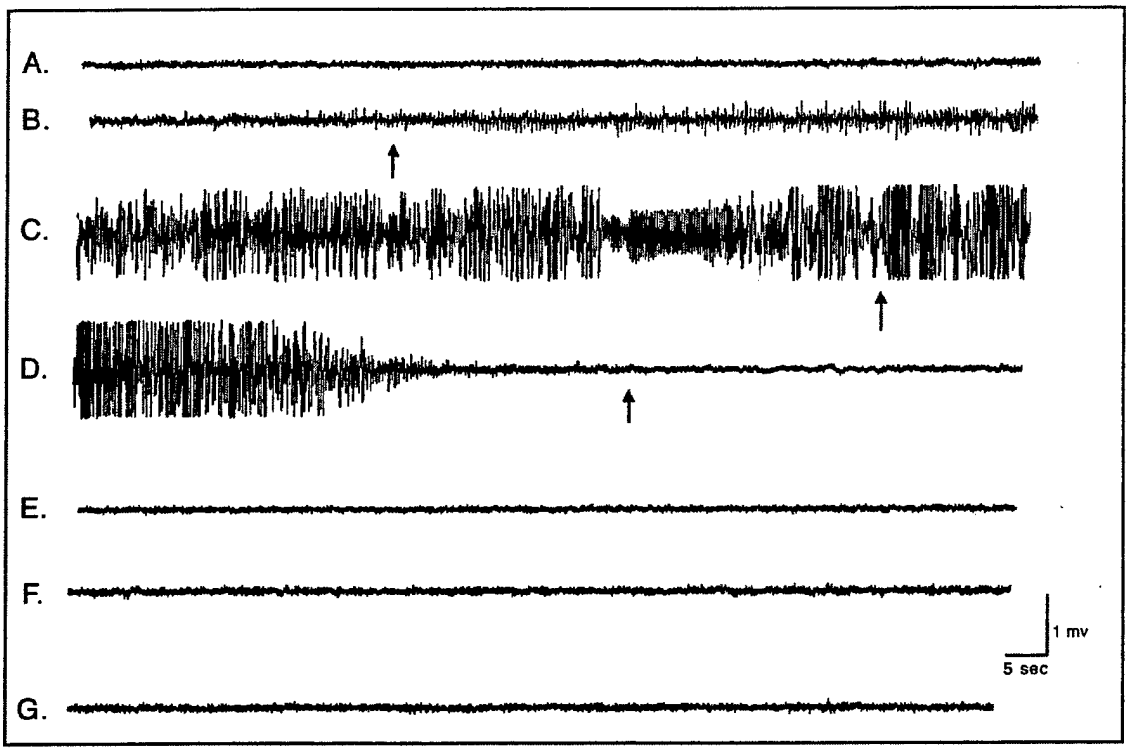
TRIHEXYPHENIDYL
DIAZEPAM
MIDAZOLAM
DRUG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1 mv
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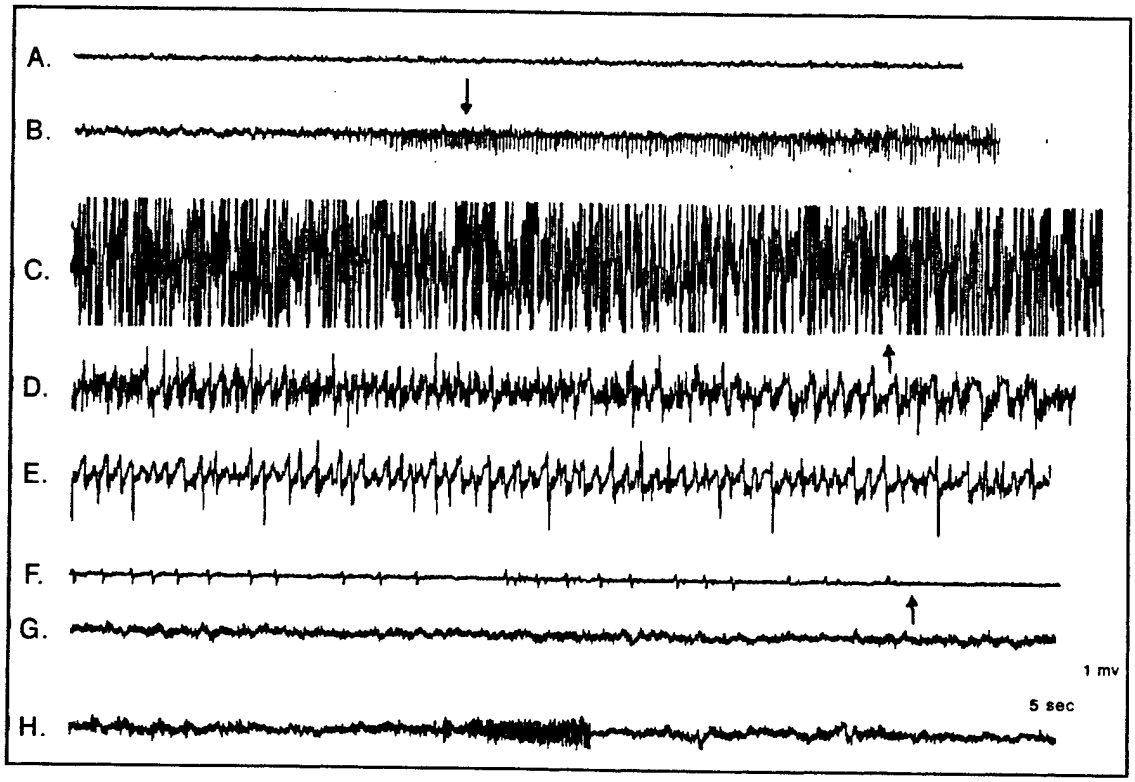
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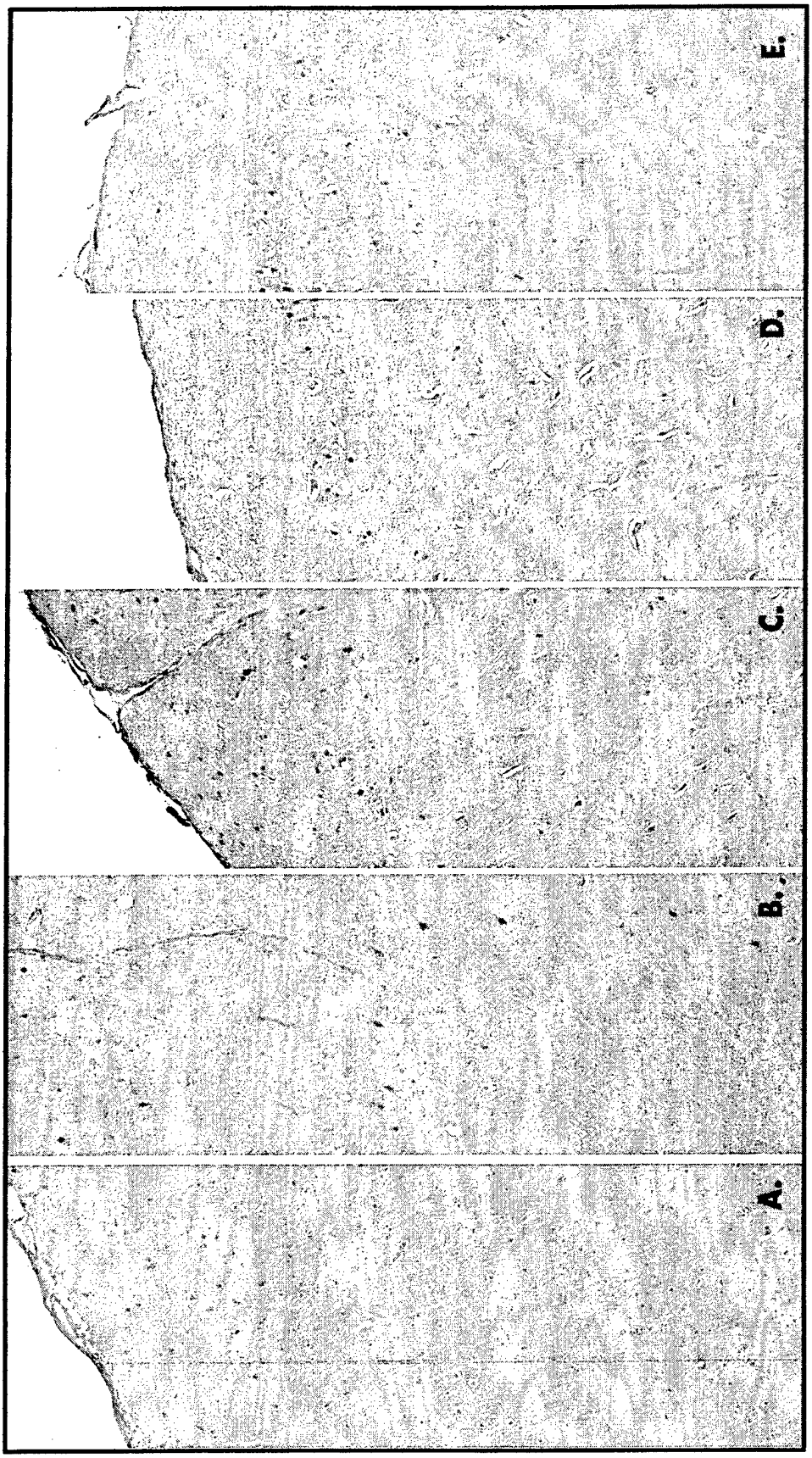
E.
D.
C.
B.
A.

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