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STRESS, CHEMICAL DEFENSE AGENTS AND CHOLINERGIC RECEPTORS

FINAL REPORT

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The goal of this project is to assess the effects of exposure to a chemical defense agent (XGD; soman) on anxiety and stress, by using rat models of anxiety (conditioned emotional response, CER) and unconditioned non-specific stress. To test for changes in anxiety, extinction from CER was measured, based on the hypothesis that increased anxiety produced by environmental exposures would prolong extinction. The specific experiments determined the plasticity of muscarinic cholinergic binding-sites in the central nervous system. The effects of acute exposure to doses of soman on lethality and well-characterized behaviors were examined, and found to be consistent with previous reports. The binding of radiolabelled cholinergic (ACh) ligands to brain tissue was studied *in vitro*. The major findings are that CER produces increases in acetycholine turnover in brain areas involved in anxiety, and that primarily post-synaptic M_1 ACh receptors decrease. These neurochemical phenomena are directly correlated with several behaviors, including acquisition and extinction of CER and non-specific stress. If soman exposure increased anxiety under any conditions, this should be reflected in increased time of CER extinction and changes in receptors; the behavioral response was not observed, so receptor function was not assessed. Therefore, the effect of soman exposure on anxiety is not likely to be specific to the cholinergic system.

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INTRODUCTION

Muscarinic and nicotinic cholinergic receptors show plasticity with respect to behavior, agonists, antagonists and acetylcholinesterase (AChE) inhibitors, including chemical defense (CD) agents (many references, beyond the scope of this report). However, very few investigations have been comprehensive, in that they attempted to study neurochemistry and behavior after cholinomimetic insult. The conditioned emotional response (CER) paradigm provides a model which is especially appropriate to such a task, because cholinergic function has been correlated with conditioned suppression. In the CER paradigm, animals are trained to press a lever for food; the lever-pressing is the baseline response. Then a light-tone combination (which will become the conditioned stimulus, CS) is introduced into the lever-pressing sessions. Next, an aversive stimulus, footshock (the unconditioned stimulus, UCS) is delivered at the same time as the CS; presentation of the footshock disrupts lever-press responding. After several sessions in which the offset of the CS results in delivery of the UCS (i.e., pairing of CS-UCS) the animals are tested. During food-reinforced responding, the CS is presented continuously for up to 15 min, during which the CER conditioned animal responds very little and retreats to the corner of the chamber and exhibits collateral behaviors reminiscent of 'anxiety'. Thus, in behavioral terms, CER results in a reduction or elimination of the baseline behavior. To complete a comprehensive analysis of CER and stress in the context of these studies, several optional experiments based on the basic CER protocol have been designed and executed (see Table 1).

In behavioral terms, conditioned suppression indicates a reduction or complete elimination of a baseline behavior. Some key effects of agonists and CD agents on muscarinic and nicotinic 'receptors' are known; for example, both antagonists and agonists (particularly acetylcholinesterase inhibiters) disrupt behavior and peripheral nerovous system function (c.f., Romano et al., 1985). The potential interactions with anxiety/stress have been explored, since stress has been linked to cholinergic function (Dilsaver, 1988). The Principal Investigator (PI) has conducted studies of the effects of CER and its extinction (and reversal of CER by the anxiolytic diazepam) on cerebral cortical muscarinic cholinergic antagonist binding sites (Lane et al., 1982a,b,c; Lane, 1984; Lane, 1986). These studies demonstrated that after training, presentation of the conditioning stimulus (CS) initiated behavioral suppression and collateral emotional behaviors, and reduced cerebral cortical quinuclidinylbenzilate (QNB) binding sites. Repeated CS presentations (without footshock pairing) restored normal behavior and baseline numbers of cortical QNB sites over a similar time course. Acute diazepam had no effect on benzodiazepine sites, and resulted in only a modest reduction of QNB sites (or partially reversed the decrease in QNB sites attributed to CER). This suggests that CER-CS produced increased turnover of acetylcholine (ACh), with a compensatory decrease in QNB sites, and that diazepam reversed the effect (see Lane et al., 1982c; Lane, 1992). In naive animals, diazepam reduced ACh turnover (Zsilla et al., 1976), which is consistent with the data above (decreased turnover might be linked to the modest reversal in QNB binding sites, as observed). Therefore, CS presentation initiates increased ACh turnover, which is a

neurochemical component of anxiety, and cholinergic agonists exacerbate CER and stress. The inverse link between ACh turnover and cholinergic binding sites is implied. If conditioning of anxiety is contingent on cholinergic function, then agents which are capable of perturbing cholinergic function (principally the CD agent) should compound normal anxiety (vigilant preparedness) to a level which would compromise the organism. The systematic examination of these phenomena is outlined in a series of behavioral experiments, which are followed by their respective neurochemical experiments.

[BODY OF DOCUMENT (All Figures and Tables follow text prior to References)]

METHODS (Specific Experiments followed by General Methods)

BEHAVIORAL STUDIES: Behavioral designs are based on previous experience with CER and utilize cells of N=6 or greater in most instances. The training and conditioning procedure routinely requires approximately 4 weeks (see Table 1). All animals have the same behavioral history prior to experimental manipulations; they all undergo the same training and conditioning priot to test day, unless otherwise indicated (Table 1). On test day during food-reinforced responding (the baseline behavior), the CS (or equivalent, e.g., a no-CS control) or the superimposition of additional stimuli (e.g., unavoidable footshock stress) were presented to selected groups and the CER-CS animals exhibited CER (conditioned suppression and collateral emotional behaviors--bracing, freezing, shaking, urination, defecation, etc.).

Behavioral Experiment 1: LINK OF CER TO OTHER CHOLINERGIC-SENSITIVE BEHAVIORS

Training - Exposure on Test Day
I. CER-noCS
II. CER-CS

Previous studies by the PI have indicated behavioral suppression and decreased cortical QNB binding following CS presentation to CER-trained rats (Lane et al., 1982a,b,c; Lane, 1984; Lane, 1986). These effects are not observed in shock history (no CS pairing) or light-tone presentation (no shock history) controls. The aim of this experiment was to determine if decreased CS-associated QNB binding reflected changes within the ACh system specific to the CER paradigm, or whether binding changes reflect a more general modification of ACh-mediated behavioral processes, detectable as shifts in locomotor activity or avoidance learning ability. Separate groups were used for each test following CS or no CS. Locomotor activity was measured for a period of 60 min in 40 X 40 X 20 cm Digiscan activity monitors (8-beam system, Omnitech Electronics, Columbus, OH). The Digiscan instrument assesses 13 components of activity, including multiple forms of vertical activty, distance and velocity, revolutions, stereotypy and location within the chamber (see Table 13 for listing of each test and units of measurement). In many studies, the measures are limited to a few of the most common (see Table 2). Discrete trials of

active avoidance and passive avoidance training were conducted in a 30 X 30 X 60 cm acrylic chamber with a 10 X 10 X 6 cm platform in one corner and a grid floor wired for scrambled shock. Passive avoidance training consisted of a series of trials on which the rat was placed on the safe platform until a step-down response occurred (three of four paws off the platform) or 120 sec had elapsed. Step-down responses were followed by presentation of a 1-mA scrambled footshock (this intensity was predetermined by threshold tests; durations were ≤ 0.5 msec) for 10 sec, after which the rat was removed from the apparatus to a holding cage for a 60-sec intertrial interval. The criterion for acquisition of the passive avoidance response was two consecutive trials during which the rat remained on the platform for 120 sec. For step-up active avoidance, each rat was permitted to avoid a comparable 1-mA scrambled footshock in the chamber by reaching the safe platform within 10 sec of being placed in the apparatus. For latencies greater than 10 sec, the rat was shocked until it reached the platform or a maximum latency of 60 sec had elapsed. An interval of 60 sec elapsed between each trial until the rat had successfully avoided shock on 9 out of its last 10 trials. The three tests selected (active avoidance, passive avoidance and general activity measures) are sensitive to cholinergic pharmacologic modifications. If the decrease in QNB sites expected in the CS group is compensatory to cholinergic hyperactivity, then these rats should respond as though they had received muscarinic agonists, i.e., they should exhibit facilitated passive avoidance, disrupted active avoidance, and decreased locomotor activity. CER-no CS rats (no change in QNB sites--Lane et al., 1982c) provided the control group.

Behavioral Experiment 2: TIME COURSE OF RESTORATION-TO-NORMAL OF BINDING SITES

Training - Exposure on Test Day(s)
III. CER-CS (1-trial CS presentation)
IV. CER-CS (multiple trials of CS presentation without footshock pairing--extinction)

The purpose of this experiment was to determine the exact character and time course for cholinergic binding plasticity. Repeated once-daily trials of CS presentation (without pairing to footshock) were used to assess extinction of conditioned suppression. In the CER paradigm, five once-daily trials of respondent conditioning (where CS was paired with footshock) required 13-15 trials to extinguish (Lane, 1986). The extinction procedure demonstrated two features of CER: i) the control of behavioral suppression; and ii) a 34-40% reduction in cortical QNB binding sites for up to five trials, which can be exploited to characterize the receptor-mediated response. Based on previous extinction experiments, one should observe a concomitant return to normal in behavior and QNB binding sites over 10-15 trials. Muscarinic binding sites (see NEUROCHEMICAL STUDIES) were assessed over the time course of extinction at 1-15 trials of CS presentation, and at intervals between trials 1-2. Further, this design allowed for the assessment of whether binding sites return to normal between trials or whether they remain decreased and slowly return to normal during extinction.

Behavioral Experiment 3: COMPARISON OF ANXIETY AND NON-SPECIFIC STRESS

Training - Exposure on Test Day
V. CER-noCS
VI. CER-CS
VII. CER-noCS-shock
VIII. CER-CS-shock

The purpose of this experiment was to collect additional data concerning the role of CER and stress in altering cholinergic parameters. V versus VI and V versus VII determined the neurochemical distinctions between CER and non-specific stress. Group VIII was included to determine if the neurochemical effects of anxiety and stress are additive, interactive, or entirely different. CER animals were trained-conditioned, and on test day, 30 min into their food-reinforced responding, they experienced one of the four conditions. CS was presented continuously for 15 min. CER-noCS animals were not exposed to the CS. Random footshock trains were paired with either condition. The behavioral response in VI-VIII was suppression, collateral emotional behaviors, and in the case of shock, perhaps helplessness. Footshock was included as an unconditioned, unavoidable stress component and was likely to be an extremely potent environmental manipulation which masks or overwhelms the neurochemical response to anxiety.

Behavioral Experiment 4: ACETYLCHOLINE (ACh) TURNOVER IN RESPONSE TO CER-CS

Training - Exposure on Test Day
IX. CER-noCS
X. CER-CS

The purpose of this experiment was to demonstrate that CER-CS increases ACh turnover in selected but not all cholinergic-enriched central nervous system (CNS) sites, and is followed by a decrease in QNB binding sites. The prevailing interpretation of these results is that the increased presence of agonist (ACh) in the synapse caused a post-synaptic response, which could be interpreted as compensatory receptor plasticity. There are preliminary data which suggest that i) agonist assault (prolonged exposure) uncouples muscarinic receptors, and is followed by a reduction in binding sites (Burgoyne, 1983; Meeker and Harden, 1983); ii) the CER-CS presentation which reduces QNB sites can be reversed by acute diazepam (Lane et al., 1982c); and iii) acute diazepam reduces the turnover of ACh in naive rats (Zsilla et al., 1976). These data are consistent with CS presentation causing an increase in cholinergic function, i.e., ACh turnover, in selected CNS sites, which in turn results in a compensatory decrease in receptors. CER-trained-conditioned animals were surgically implanted with indwelling jugular catheters. On test day, during the 15-min continuous CS (or no-CS), the animals were pulse-labelled with 0.5 mCi [^{3}H]-choline chloride, administered intravenously for 2-15 min (since the total CS

presentation lasted 15 min, this corresponded to 13 min to 0 min (10 sec approximation) of CS presentation); then the animals were sacrificed by total freezing in liquid nitrogen. The incorporation of precursor radiolabel from choline into ACh was used to calculate turnover. The following brain areas were assessed: frontal, pyriform, cinguate and entorhinal-subicular cortices; nucleus accumbens, caudate-putamen, medial septum, hippocampus, and amygdala.

Behavioral Experiment 5: EXPOSURE TO THE CD AGENT

Before we could utilize dilute soman (XGD -- X designates the dilute, as opposed to the neat, form of GD) in behavioral or neurochemical tests, we had to assess the lethal dose characteristics of XGD in the F-344 strain of rats. Ten groups of 10 rats (body weights 257 ± 6 g) were randomly assigned to groups for subcutaneous injections of saline, or 0.10 log-dose increments of XGD (200 ug/ml diluted in saline) over the range of 30-200 ug/kg. For selected doses circa the predicted LD_{50}, 0.05 log-dose increments were also used to evaluate behaviors. LD values were plotted under linear, log-linear, and probit analyses. Based on the methods described by Romano et al. (1985), rats were evaluated 24 hours after injection for lethality, and 2 hours after injection for behavioral signs and activity. Once the threshold for lethality was defined, the remaining animals in the high-dose groups were reassigned to middle-range dose groups.

Behavioral Experiment 6: EFFECT OF CHRONIC SOMAN ON EXTINCTION OF CER

Rats were treated with one-half of the LD_{50} dose of soman every other day for five treatments (see Table 1). Afterward the animals were exposed to once-daily presentations of the CS, which was not paired with footshock (refer to Behavioral Experiment 2). Following the study, brains were harvested to determine acetylcholinesterase levels, to verify the effectiveness of soman in inhibiting the enzyme.

Behavioral Experiment 7: EFFECT OF ALPRAZOLAM ON REVERSING CER AND EFFECT ON OTHER BEHAVIORS

Consistent with a broad spectrum of benzodiazepines, alprazolam is known to attenuate anxiety, and was hypothesized to reverse CER (based on Lane et al., 1982; Lane, 1992). To test the effectiveness of the drug (see Table 1), alprazolam was assessed on a food-reinforced (FR10) behavior, on an accelerating rotorod, by Digiscan activity monitors, and on the CER paradigm with and without the superimposition of non-specific stress (footshock). The dose range was originally 0.25 to 4.0 mg/kg, i.p., but the highest dose was deleted due the profound effects of the 2.0 mg/kg dose. Animals which received 1.0 mg/kg, i.p., alprazolam were used for receptor studies.

Behavioral Experiment 8: ATTEMPTS TO STUDY THE ACQUISITION OF CER

In an attempt to observe a partial conditioned suppression (instead of the all-or-none CER); rats received 0.5 to 6 sessions of the pairing of CS-unconditioned stimulus, UCS (see Table 1), after which on test day they were exposed to the CS alone as described previously.

Behavioral Experiment 9: EFFECT OF CHRONIC SOMAN IN RATS PRETREATED WITH N-METHYL-ATROPINE

In an attempt to reduce the mortality of soman, Behavioral Experiment 6 was adapted to another group of rats which were given a dose of 10 mg/kg, i.p., n-methyl-atropine 60 min before soman, after which the CER extinction experiment was repeated (Behavioral Experiment 2). The soman would still have its central effects, but the muscarinic cholinergic antagonist should protect the animals against peripheral toxicity, and potentially reduce the mortality observed with the administration of chronic soman alone.

NEUROCHEMICAL STUDIES: These studies followed the behavior and drug treatment, such that a cell contained at least N=6 or more in some instances. The purpose of these experiments was to determine the general characteristics and neuroanatomical localization of changes in radioligand binding and functional receptors following drug and/or behavioral manipulations. In addition, the approach provided definitive information whenever possible directed toward several questions which remain unanswered: i) Are high- and/or low-affinity sites functional?; ii) Are M1 sites only post-synaptic (M2 appear to be both pre-synaptic and post-synaptic [Mash et al., 1985])?; iii) Is cyclic nucleotide and phosphoinositide metabolism linked to M_1 and/or to M_2 subtypes, and is it linked to pre-synaptic and/or post-synaptic sites?; and iv) Is phosphoinositide turnover in the CNS activated by a guanine nucleotide recognition protein, such as Gp, in mast cells (Cockcroft and Gomperts, 1985)?; Definitive answers to these questions will aid the data interpretation pursuant to the major objectives of this project.

Analysis of Binding Sites: Multiple-Affinity Muscarinic Binding Sites - Particulate Fractions [in cortex, striatum, hippocampus, and habenulo-interpeduncular (H-I -- diencephalon) system] and *In Vitro* Binding Site Autoradiography - [representative coronal sections of forebrain]: Multiple muscarinic binding sites were evaluated following behavioral and drug manipulations. These assays were utilized initially to examine pharmacological profiles and to characterize multiple affinities and M_i (i.e., multiple undefined, but potentially definable) subtypes. When the resulting observations were consistent with prevailing reasoning, subsequent experiments relied more on autoradiographic analysis of changes in binding-sites to glean anatomically definitive information. Total particulate fractions from areas rich in muscarinic sites were used. These studies concentrated on displacement of radioligands by specific agonists and antagonists over a broad range of concentrations to collect information on superhigh-, high- and low-affinity sites and M_1 and M_2 subtypes. [High designates sites with lower picomolar affinities, and low designates sites with high

picomolar and suprananomolar affinities.] This was important for three reasons: i) Based on the observations of Dam et al. (1982), i.e., low-dose oxotremorine activation of cerebral glucose utilization in cortical layers IV/Vb, there may exist high-affinity functional muscarinic receptors which must be examined in addition to low-affinity receptors, traditionally thought to be functional [discussed by Lane, 1984--functional in this context means a binding site, coupled to a G-protein and catalytic unit, comprising a second-messenger system, capable of producing a physiological effect]; ii) exposure to diisopropylfluorophosphate (DFP) not only reduces Bmax for muscarinic antagonist binding sites, but also substantially shifts agonist affinity (Ehlert et al., 1980); and iii) Mash et al. (1985) have demonstrated that at least a portion of neocortical pre-synaptic ACh sites are M_2. In this context, based on the reasoning that M_1 receptors are post-synaptic and the ones most likely to show plasticity, the PI utilized pharmacological profiles to differentiate changes in pre-synaptic versus post-synaptic sites, and to assign function and plasticity characteristics to the respective sites and subtypes in this fashion. There are preliminary data which demonstrate decreases in cortical muscarinic (QNB) sites. Based on the concept that low-affinity sites represent the functional receptors (discussed by Lane, 1984), multiple affinity sites with neurochemical measures of receptors were evaluated to ensure that there is a physiological correlation. For example, a decrease in a binding-site parameter predicted a shift to the right in the dose-response of the receptor parameter. In addition, pharmacological profiling would reveal whether the decrease in binding was restricted to the loss of a specific affinity site, which in turn would speak to the question regarding affinity of the functional receptor. Lack of correlation between binding sites and receptor response would indicate redundant sites (well established in the heart) or uncoupled sites. *In vitro* binding-site autoradiography was utilized to assess neuroanatomical loci of changes in primarily high-affinity binding sites at the light-microscopic level. This binding is already well characterized in normal rats (Wamsley et al., 1980; Clarke et al., 1985). All major forebrain areas and nuclei have been examined.

GENERAL METHODS

Behavioral: CER was trained-conditioned in adult F-344 littermate male rats (refer to Table 1 for summary). The subjects were obtained from Harlan, Indianapolis, IN; they were maintained on normal light-dark cycles and trained and tested during the normal work day, allowed access to water *ad libitum*, and given approximately 15 g rat chow daily to maintain their body weights. Several options to the basic protocol are outlined in Table 1.

Receptor Binding: Binding of [^{3}H]-QNB, [^{3}H]-N-methylscopolamine (NMS), [^{3}H]-oxotremorine-M (OX), [^{3}H]-pirenzepine (PZ), was conducted using techniques similar to Lane et al. (1982c), Mash et al. (1985), Costa and Murphy (1983), Gillard et al. (1987), and Waelbroeck et al. (1987). Total particulate membranes were prepared by repeated homogenization and high-speed centrifugation. Ligand binding was assessed by Rosenthal (Scatchard) and displacement plots using ENZFITTER (Elsevier Biosoft), LIGAND (NIH) and INPLOT (GraphPad) iterative computer programs for 1-3 non-interacting sites (for examples, see Figure 1). Displacement plots utilized detailed 1-pM to 100-mM

concentrations of unlabelled drug. Rosenthal plots utilized 10-pM to 100-nM concentrations of radioligand. Muscarinic sites were converted to the low-affinity-agonist form and were uncoupled by treatment with 1 mM ethylenediaminetetraacetate/n-ethyl-malaemide (Mash et al., 1985). High-affinity sites were determined as those NMS sites sensitive to displacement by 1 uM carbachol. M_1 was differentiated from M_2 by [^{3}H]-QNB displacement by carbachol (2-site model), and M_2 was identified by subsaturating the high-affinity M_2 sites with [^{3}H]-OX (Mash et al., 1985). Binding of [^{3}H]-PZ was used to verify M_1 results. To assess the effects of non-specific "stress" (defined by the paradigm which utilizes random unavoidable footshock), high-affinity [^{3}H]-muscimol (for gamma-aminobutyric acid [GABA]) binding sites were measured, using the procedure of Booker et al. (1986).

In Vitro Binding-Site Autoradiography: Brains were sectioned coronally (20 um) with a Damon cryostat-microtome and were slide-mounted; they were then defatted and incubated with 0.2 nM - 1 nM [^{3}H]-NMS, 0.2 nM - 1 nM [^{3}H]-QNB, 2 nM - 10 nM [^{3}H]-PZ and 2 nM - 10 nM [^{3}H]-OX, according to the procedures described by Clarke et al. (1985). The use of PZ and carbachol displacement of [^{3}H]-QNB was one method used to differentiate M_1 and M_2 by subtraction. Displacement of [^{3}H]-NMS by 1 uM carbachol was used to identify high-affinity muscarinic sites (Wamsley et al., 1980). After washing and drying in cold-dessicated air, the slides were affixed to LKB Ultrofilm and stored for various periods of time in cassettes at room temperature, developed, and viewed at the light-microscopic level. Films contained [^{3}H]-microscales (Amersham) for quantitating optical densities. Sections were then stained (Kluver and Barrera, 1953). Areas for quantitation were defined according to histological identification of discrete areas, not according to binding distribution alone. Displacer controls (usually 10 uM atropine or unlabelled ligand) for non-specific binding were handled in parallel. To convert the microscale standard values (nCi/mg) to fmol/mg protein for binding, adjacent discrete sections of 20 micron-thick discretely isolated striata (caudate-putamen) were handled in parallel; these sections were analyzed for absolute binding by liquid scintillation spectrometry, and adjacent sections were analyzed for protein; and based on specific radioactivity of the ligands, the conversions were calculated. Computerized densitometry was performed on a DUMAS/BRAIN Image Analyzer.

Acetylcholine Turnover: Rats were sacrificed by total freezing in liquid nitrogen. Choline and acetylcholine (ACh) were quantitated by high performance liquid chromatography (HPLC), using platinum-electrode electrochemical detection of post-column enzymatically liberated H_2O_2 (Bioanalytical Systems, Inc.). [The enzymatic procedure, which uses choline oxidase, relies on the conversion of choline to hydrogen peroxide, which is stochiometrically related to the amount the original ACh or choline in the individual peaks, which were separated by HPLC; the incorporation of tritium, associated with ACh and choline, can be captured in the effulent.] Respective peaks corresponding to choline and ACh were collected manually and counted for [^{3}H]-incorporation. Specific activities (dpm/mole) of [^{3}H]-incorporation were utilized to calculate turnover (k x content of ACh at time equivalent to time-zero; in reality, the ACh content is that collected at each time point after injection of radiolabelled precursor). The rate constant k was compared for the

incorporation into and decline in specific activities of choline and ACh (see Smith et al., 1984a; Racagni et al., 1976; Jenden et al., 1974 for examples of kinetic models for turnover estimation) according to the following formula:

$$K_{ACh\ TO} = 2\,(ACh_{t2} - ACh_{t1}) \,/\, [(t_2 - t_1)\,(Ch_{t1} + Ch_{t2} - ACh_{t1} - ACht_2)]$$

Statistical Analysis: All data were analyzed initially according to fixed-effects factorial analyses of variance (ANOVA). Planned comparisons (i.e., tests of stated hypotheses) were conducted, using Student's t-test within appropriate interactions, and SNK and Duncan Multiple Range Tests. Post hoc comparisons were made using a Scheffe test. [$p < 0.01$ was adopted as a general criterion for statistically significantly differential experimental observations]

RESULTS AND DISCUSSION

BEHAVIORAL STUDIES

Behavioral Experiment 1: The ability of other behaviors to exemplify, reflect or detect cholinergic function was assessed (see Table 2). When compared with controls (no CS presentation), rats which had been exposed to the CS exhibited total suppression of food-reinforced responding, and showed emotional collateral behaviors. The CS-exposed rats also showed 24% lower total activity, 75% greater stereotypy, and 50% greater center time when compared with controls. Perusal of 10 other activity measures provided by the Digiscan apparatus (such as vertical activity and revolutions) failed to suggest differences between CS-exposed and control groups. The CS-exposed rats had greater difficulty learning the active-avoidance task, as suggested by the 15% greater number of trials required for acquisition when compared with controls. In contrast, the CS-exposed rats required 27% fewer trials to reach criterion for passive-avoidance than controls. Activity, active-avoidance and passive-avoidance observations were consistent with cholinergic hyperactivty, just as though the rats had received injections of muscarinic agonists prior to testing. These observations do not rule out changes in other neurotransmitter systems (c.f., Lane et al., 1982a,b; Lane, 1992). Finally the changes in stereotypy and thigmotaxis (wall-clinging, non-center time) cannot be explained at this time.

Behavioral Experiment 2: In this study (see Table 3), repeated presentations of the CS, which normally elicited CER, brought about extinction (restoration of normal responding and behaviors) in 10 days. When this experiment was last conducted, it took 15 days for extinction to occur. This is likely a function of the slightly different behavioral programming system used to do the most recent experiments. In spite of the difference in total time to extinction (10 days), the shape of the suppression curve was similar to the one generated over 15 days, e.g., the presentation of the fourth CS produced approximately 60% suppression, and by two-thirds of the way through all of the CS presentations, the CS

produced approximately 30% suppression, as observed here. These animals were harvested for binding-site analysis (see neurochemical observations that follow).

Behavioral Experiment 3: In this study (see Table 4), the impact of non-specific footshock stress was superimposed on CER. The CS presentation produced 100% suppression, as expected. Shock in the absence of CS presentation produced 100% suppression, suggesting that the behavioral expression of non-specific stress was not distinguishable from CER. When CS presentation and shock were combined, there was also 100% suppression, as predicted. However, CS and shock had to be analyzed neurochemically to differentiate the components of suppression due to each. These animals were harvested for binding site analysis (see neurochemical observations that follow). The binding of GABA will also be assessed to determine the effects of stress on a parameter (independent of the cholinergic systems), which is known to decrease with stress (Biggio et al., 1985).

Behavioral Experiment 4: In the acetylcholine turnover experiment (see Table 5), the response rates during recovery and on test day were lower than in the previous experiments. In spite of this difference, the CS presentation produced 100% suppression. The lower response was attributed to the presence of the indwelling jugular catheter backpack mounted above the scapula on the animals' backs, and to the fact that on test day, the catheter was removed from the backpack, was run out of the top of the chamber (for radiolabelled precursor administration), and was relatively loose during the VI1 and VI1-CS portions of the experiment. Thus, the tubing could have posed somewhat of a distraction to the animal. There was no alternative to this approach, since the animals could not be handled nor disturbed during the session, and the pulse times were short, i.e., less than 15 min. These animals were harvested for acetylcholine turnover (see neurochemical observations that follow).

Behavioral Experiment 5: In this study, all three plots (linear, log-linear, and probit) of soman (XGD) lethality yielded comparable results: LD_0 less than 40 ug/kg; LD_{10} = 50 ug/kg; LD_{30} = 66 ug/kg; LD_{50} = 83-86 ug/kg; LD_{90} = 118 ug/kg; and LD_{100} greater than 126 ug/kg. Behaviors, rated by a single individual blind to the dose, were plotted individually as log-dose versus mean score for the group for a particular behavior, and also were compiled as a cumulative mean score (Figure 2). Prostration, seizures and jerks, lacrimation, panting and eye protrusion were distinguished by their dose-dependent increases, while other measures were relatively unaffected. Activity was also measured in 8 X 8 cell array Digiscan activity monitors in 2-min time bins over a 10-min total period as a function of maximal response (refer to Table 6 and Figure 3). If there was an effect on any activity measure, it was generally decreased with increasing doses of soman, with the exception of rest time which increased. Samples were also collected for determination of brain acetylcholinesterase activity, which showed a progressive decline in activity consistent with partial or total impairment of the animals (Figure 7). We then proceeded to the next behavioral experiment, which involved the ability of chronic XGD to exacerbate

CER extinction, based on the hypothesis that excess cholinergic function will prolong anxiety.

Behavioral Experiment 6: Rats received five doses of soman during the shock (US-CS pairings) and recovery sessions (see Table 1 and Figure 5). During the initial shock sessions, the routine food-reinforced responding was initially decreased (Figure 5) compared to pre-respondent-conditioning sessions. Responding remained lower during the beginning of the soman injections, but returned to normal levels toward the end of the recovery sessions and during the extinction sessions (Figure 5). Chronic soman produced a CER extinction curve comparable to drug-free animals (Figure 6). The routine criterion used for extinction was a restoration of 80% of lever-pressing or 80% delivery of the 15 available food pellet reinforcers (because the VI1 schedule has random elements); the more stringent criterion referred to in Figures 6 and 22 required essentially full restoration of lever-pressing and delivery of reinfocers. Based on several experiments in drug-naive-untreated animals (Lane, 1986; and Lane, unpublished), the extinction curve from this experiment fell within the domain of all previous CER extinction experiments. The most likely explanation for these observations is that CER-CS produces a maximal hypercholinergic state, and that acetylcholinesterase inhibition could not further exacerbate the condition. To ensure that the chronic exposure to soman was producing the expected effect, brain levels of acetylcholinesterase were monitored in animals which died or survived (and thus were used in the CER extinction study). The treatment regimen produced the anticipated changes in enzyme activity -- a 48% reduction in surviving animals and 81% reduction in animals which did not survive (Figure 7). Since this response did NOT support our initial hypothesis, an additional experiment was attempted (see Behavioral Experiment 9 and Figure 22), which will be discussed later. Because of the behavioral results, no neurochemical experiments on cholinergic binding were conducted.

Behavioral Experiment 7: Alprazolam is an extremely potent [e.g., human daily doses are commonly in the range of 0.007 - 0.114 mg/kg] substituted benzodiazepine derivative which has anxiolytic, antidepressant, and antipanic efficacy. Prior to testing the effect of alprazolam on CER, a variety of other behaviors were evaluated, so that the anti-anxiety effects attributed to alprazolam could be evaluated with respect to changes in CER and/or non-specific stress (superimposed footshock -- CER-CS-Shock). As the dose of alprazolam increased, the effect on fixed-ratio-10 responses (FR10) food-reinforced responding decreased (Figure 8). This is consistent with our current understanding that higher doses of alprazolam(greater than 1.0 mg/kg) disrupt behaviors associated with a post-reinforcement pause (i.e., FR10). As the dose of alprazolam increases, performance on an accelerating rotorod apparatus, indicative of loss of coordination and/or sedation, decreased (Figure 9). The patterns of behavior, as evaluated by VI1 responding, were not identical for CER-CS and CER-CS-Shock (Figures 10-13). As the dose of alprazolam increased, the appetitive effect of the benzodiazepine was observed. For the most part, the imposition of a shock component had modest--if not erratic--effects on both pre-CS (component 1) and during-CS (component 2) response rates for VI1 food-reinforced responding. CER reversal, as detected by component 2 rates, appeared by 0.5 mg/kg

and was near maximal at a dose of 1.0 mg/kg (Figures 10-13). In addition, 13 measures of behavioral activity by a Digiscan apparatus (Figures 14-20 and Table 12) were assessed for both CER-CS and CER-CS-Shock paradigms. As before, the patterns of behavior were not identical for CER-CS and CER-CS-Shock. In general, the CER-CS paradigm produced a mixed pattern of behavior (i.e., 7 measures decreased 45-85%; 4 measures increased 118-1680% -- Table 12). The superimposition of shock was modest (i.e., 4 measures decreased 70-92%; 7 measures increased 87-425%; shock alone produced only 4 changes). [Figures 14-20 display dose-response curves for 0.25-2.0 mg/kg alprazolam, while Table 12 summarizes the effects on behavior at the highest dose of alprazolam.] These observations are consistent with the concept that alprazolam is a partial agonist at the benzodiazepine-GABA-picrotoxin complex, accounting for a combination of anxiolytic, appetitive and sedative effects. [NOTE: Asterisks are used in Figures 14-20 to demonstrate from separate experiments that drug-naive CER-CS animals perform at predominantly lower levels in selected activity measures.]

Behavioral Experiment 8: As an attempt to develop an alternative to the CER-extinction experiment, a CER-acquisition experiment was designed. Rats received reduced respondent-conditioning sessions, in anticipation that a sufficiently low exposure to US-CS pairings could elicit a partial CER. This was not observed, even after one-half of the first shock session, i.e., animals were essentially totally suppressed during CS presentation (component 2 -- Figure 21). In our hands, CER is an all-or-nothing phenomenon, and not amenable nor applicable to soman experiments. Accordingly no neurochemical experiments were performed.

Behavioral Experiment 9: Since Behavioral Experiment 6 did not demonstrate increased cholinergic hyperactivity, the study was repeated using the same dosing regimen of soman, in animals pretreated with 10 mg/kg n-methyl-atropine to protect peripheral tissues. Following treatment and recovery, the CER extinction experiment was conducted (see Behavioral Experiments 2 and 6). The CER extinction pattern was within the domain of all previous experiments, suggesting that n-methyl-atropine did not represent a significant beneficial effect (Figure 22). In addition, the mortality factors were compared for Behavioral Experiments 6 and 9: During the 9-day (every other day) dosing regimen, the patterns of mortality for the rats were similar (Table 16), strongly suggesting that the effects of prolonged exposure to sublethal dosing of nerve agent on the central nervous system is the most important component. Since interesting behavioral findings were not gleaned, no studies of CER-CS-Shock nor neurochemical studies were conducted.

NEUROCHEMICAL STUDIES:

The brains of animals from the Behavioral Experiments were harvested for receptor studies. Our results for binding-site analysis to total particluate fractions were consistent with several previous reports (e.g., Luthin and Wolfe, 1984; Cortes and Palacios, 1986; Frey et al., 1985; Mash and Potter, 1986; Lee and El-Fakahany, 1985; and Spencer et al., 1986). Therefore, we are confident that we are examining bona fide phenomena with

suitable methodologies. In general, i) all changes reflected changes in Bmax and not Kd; ii) the values for NMS were always a fraction of QNB binding (usually approximately 60 %), supporting the notion that NMS binds to external sites, while QNB binds to all, including lipophilic, sites; iii) the summations of M_1 and M_2 did not add up to 100%, supporting the understanding that there are more than two M_i sites; and iv) high-affinity M_2-like sites followed the patterns of M_2 sites.

Binding-Site Analysis of CER Extinction: In this followup to Behavioral Experiment 2 (see Tables 7 and 8), the initial CS presentation reduced QNB binding 42-44% in the cortex (compared to CER-CS-full-extinction and CER-noCS control values, circa 2000 pmol/mg protein -- $p < 0.01$; see also Table 9) . The binding gradually returned to normal over the time course of extinction. All of the changes could be accounted for by changes in PZ binding (M_1), suggesting predominantly post-synaptic sites. There were no changes in OX binding, but the percentage of M_2 sites appeared to fluctuate; in reality, it merely reflected a relatively larger portion of the total binding sites in the absence of M_1 sites. There were no changes in the diencephalon. Sites in the hippocampus behaved similarly to the cortex. The first CS presentation produced a 40% reduction in QNB binding and parallel decreases in NMS binding. The reduction could be implied to be attributed to PZ (M_1) sites, since OX binding did not change, although the percentage of M_2 sites did fluctuate in the predicted manner, as before. There were no changes in the striatum. This supports the hypothesis that only cholinergic areas involved in CER and anxiety would change.

Binding Site Analysis of CER Versus Non-Specific Footshock Stress: In this followup to Behavioral Experiment 3 (see Tables 9 and 10), CS presentation reduced QNB binding 44 % in the cortex (compared to CER-noCS controls, $p < 0.01$), while the addition of footshock further reduced QNB binding by only 4% (not different than CER-CS), again with parallel changes in NMS binding. Animals exposed to CER-noCS-shock were similar to CER-noCS, suggesting that random footshock, though effective in disrupting and suppressing baseline food-reinforced behavior (Table 4), had a non-cholinergic neurochemical profile. The changes were accounted for by PZ (M_1) binding; there were no changes in OX binding, but there were changes in the percentage of M_2 sites, as before. Muscimol binding was decreased 32 and 39 % by footshock in the cortex, suggesting that CS and shock components might be mildly additive (trend). There were no changes in the diencephalon. The hippocampus behaved like the cortex, with 46-53 % reduction in QNB binding, no changes in OX binding, but predicted fluctuations in the percentage of M_2, which implied that changes were in PZ sites (M_1). There were no changes in the striatum. There was not sufficient tissue to perform muscimol binding site analyses in these latter brain areas.

Effects of CER on Acetylcholine Turnover: In this followup to Behavioral Experiment 4 (see Table 11), the CS presentation produced increased turnover of ACh in the frontal cortex (90%), pyriform cortex (117%), hippocampus (98%) and amygdala (127%) [compared to CER-noCS controls, $p < 0.001$]. This is consistent with our hypothesis of cholinergic hyperactivity in cholinergic-enriched areas thought to be involved in anxiety.

In contrast, there were no changes in other cholinergic-enriched areas, e.g., caudate-putamen, not likely involved in anxiety. Values for ACh content and rate constants were consistent with many previous reports (e.g., Zsilla et al., 1976). These results are also consistent with the binding site analyses. These data, along with the other behavioral data in Table 2, are also consistent with the reports of Miczek (1973) and Izquierdo et al. (1989) who observed that muscarinic antagonists disrupt, and muscarinic agonists do not interfere, with CER, respectively.

Demonstration of the In Vitro Binding-Site Autoradiographic Technique: In the behavioral experiments above, brains were harvested for the *in vitro* binding site autoradiographic techniques. To demonstrate that we can utilize this sophisticated methodology, we have included two representative examples of the binding of 2 nM[^{3}H]-OX and 0.2 nM [^{3}H]-QNB to coronal sections of rat brain, scanned by the DUMAS/ BRAIN Image Analyzer (see Figure 4). In the preliminary data presented in Figure 4, the binding densities of [^{3}H]-OX appear much greater than [^{3}H]-QNB; however, the respective autoradiograms do not imply that OX binding is greater than QNB, since different concentrations of each radioligand (circa their Kd's) were used. The most important feature is not the absolute binding, but the relative binding. Therefore, as long as all the sections from the various behavioral groups are handled identically in parallel, then important information can be gleaned regarding binding and subtypes versus behavior for each brain area. The binding of [^{3}H]-PZ and [^{3}H]-NMS was also studied.

Qualitative Distribution of M_1 and M_2 Muscarinic Cholinergic Receptor Subtypes in Rat Brain: Fifty-eight areas of rat brain were examined for the distribution of M_1 and M_2 subtypes, and are presented as a progressive [0, +, ++, +++] rating (Table 13). Binding to the M_1 subtype was highest in cortical areas, striatum, nucleus accumbens, amygdala and hippocampus (CA1); binding to the M_2 subtype was highest in the cortex, septum and lateral geniculate (an area of the thalamus). Many other areas had much lower levels of binding or no detectable levels. These findings are consistent with the observations of Cortes and Palacios (1986) and Spencer et al. (1986), who observed a similar distribution in rat brain.

Autoradiographic Analysis of the CER and Stress Paradigms: Previous studies (see Tables 7 - 10) demonstrated that i) the muscarinic plasticity was restricted to the M_1 subtype; and ii) the most interesting behavioral comparisons were CER-no CS versus CER-CS versus CER-CS-Shock, in the absence or presence of drug treatment. Accordingly these were examined at the autoradiographic level. CS presentation caused a 26 - 40 % reduction in M_1 sites in seven brain regions; 51 other regions produced no changes (Table 14). The superimposition of non-specific stress (CER-CS-Shock) for the most part followed the CER-CS group, with the exception of a 77 % increase in the entorhinal-subicular cortex (Table 14). This latter observation is likely serendipitous, or lacks present explanation. The other decreases in M_1 subtype binding follow the results observed in total particulate fractions (see Tables 7 - 10).

Autoradiographic Analysis of the Effect of Alprazolam on CER and Stress Paradigms: As anticipated, alprazolam reversed (binding increased 45 - 110 %), but did not for the most part restore to normal the M_1 subtype binding in CER-CS animals (Table 15). This observation is consistent with the findings of Lane et al. (1982c) who studied the effect of another benzodiazepine anxiolytic, diazepam, on CER. As before, the Shock component followed the CER-CS and did not affect the results, except for a 45 % reduction in binding in the basolateral amygdala. As before, this presently lacks explanation.

ADDITIONAL DISCUSSION

Most of the observations, particularly for the control groups, are consistent with previous work (Lane et al., 1982c). Plasticity of QNB binding in the telencephalon was a function of the CER-CS group, not controls handled in parallel which controlled for the history of light-tone or shock. One can likely conclude that completely, behaviorally naive animals would have binding profiles similar to these latter two control groups.

The distribution of muscarinic receptor subtypes has been assessed by *in vitro* autoradiographic localization of specific radioligands and by *in situ* hybridization of oligonucleotide probes against specific genes for m1-m5 receptors (c.f., Levine et al., 1988; Vilaro et al., 1989). For the most part, our observations based primarily on binding to total particulate membrane fractions, were consistent with these findings; albeit there is not a concensus by other investigators on the respective distributions. Our observations support the contention that i) M_1 sites are relatively higher in the neocortex, hippocampus, caudate-putamen and amygdala; and ii) M_2 sites are found in the cortex, septum, caudate-putamen and amygdala. M_3 sites (AFDX-116-sensitive) were not assessed *per se*, since they are localized in more caudal regions, e.g., superior colliculus. For the data in Tables 7-10, the sites do not sum to 100 %, because there is a small contribution from the third subtype in the rostral brain areas. There is, however, good agreement between M_2 and carbachol-sensitive-NMS displacement. Finally since M_1 sites accounted for the plasticity in total binding with respect to CER behavior, the percentage of M_2 sites varied inversely, i.e., a decrease in M_1 sites would be perceived as an increase in M_2 sites.

CONCLUSIONS

POSITIVE CONCLUSIONS:

The M_1, and not the M_2, muscarinic cholinergic subtype of receptor shows plasticty in selected anxiogenic areas of the CNS, which have been previously implicated in anxiety, with respect to the CER. This phenomenon is predominantly independent of non-specific stress (the CER-CS-Shock component).

Alprazolam, an extremely potent anti-anxiety agent, reverses anxiety concomitant with neurochemical changes. This has important implications about tactical vigilance, and as an adjunct to other therapies which might be provided to the combat soldier.

NEGATIVE CONCLUSIONS (BASED ON ORIGINAL EXPERIMENTAL HYPOTHESES)

Soman doses NOT exacerbate the effects of CER on anxiety nor non-specific stress. As described before, this might be explained by the fact that anxiety, known to activate the cholinergic system, already activates the system to a maximal level; therefore, soman, the acetylcholinesterase inhibitor, does not appear to have a further hypercholinergic effect. Further attention shculd probably be focused on sublethal doses of nerve agents. The degree of incapacitation (particularly extent of seizures), and immediate versus prolonged recovery, will probably determine combat effectiveness.

PARADOXES

These experiments were designed to evaluate the plasticity of muscarinic cholinergic receptors, based on the premise that one could account for all binding sites. Despite the use of multiple radioligands, the loss of binding sites cannot be reconciled at this time. A future analysis of *in situ* hybridization of m1-m5 mRNA probes may resolve this aspect. However, it does not deal with the potential role of redundant muscarinic receptors in the CNS, as have been observed in the heart. This will require further study.

PUBLICATIONS

Because of the demanding nature of both the behavioral and neurochemical aspects of this project, no major scientific publications have been forthcoming. The results of these studies will generate several publications. It is our intention to submit several *opus magnum* publications to ***Brain Research*** in the near future. Naturally the proper DoD/USAMRDC acknowledgement will be included.

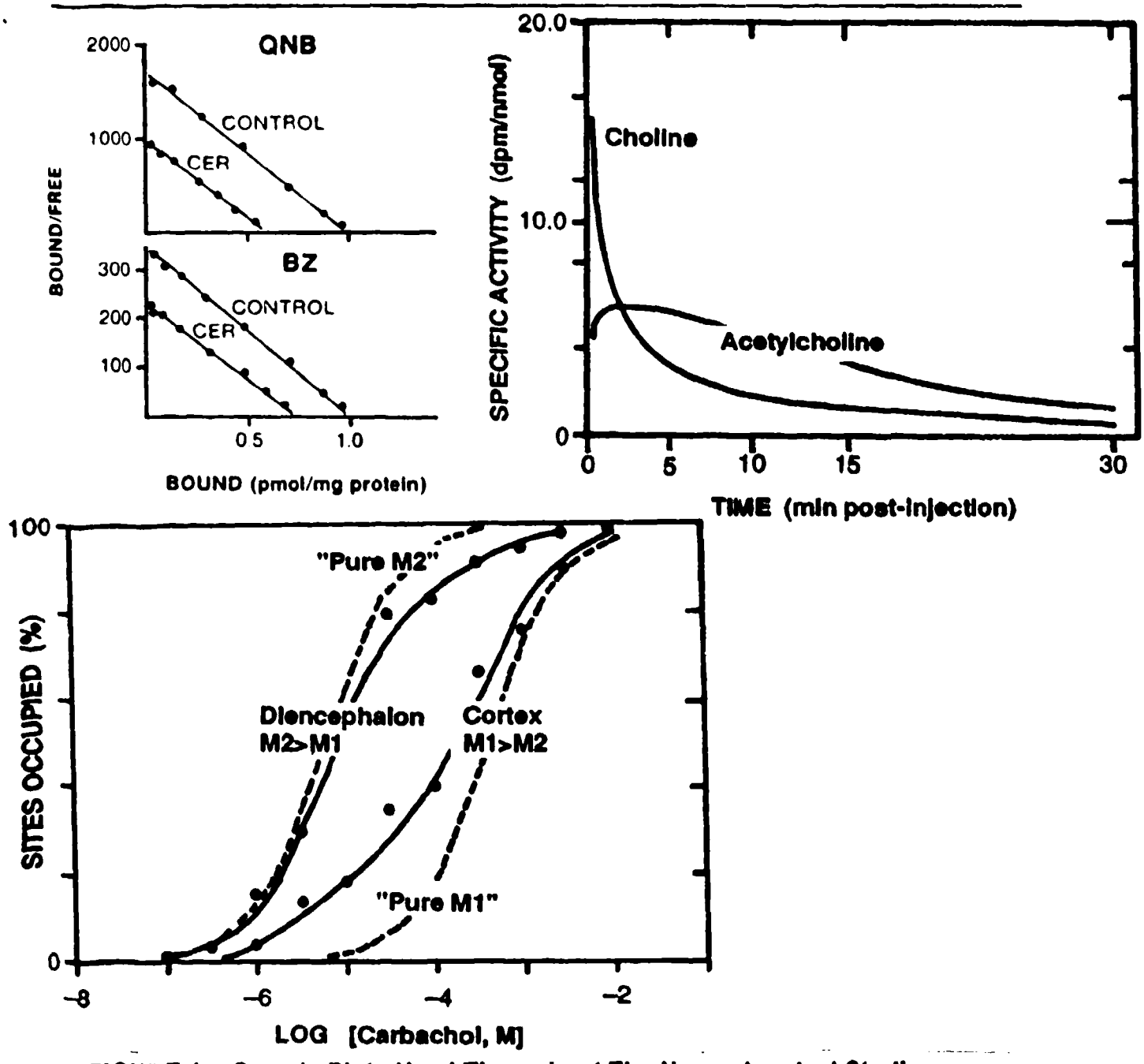


FIGURE 1 -- Sample Plots Used Throughout The Neurochemical Studies
Upper Left: Typical Rosenthal (Scatchard) plot for QNB and Diazepam (BZ) binding to brain membranes. The abscissa is binding in mol per mg-protein; the ordinate is the ratio of bound/free ligand. The x-intercept defines Bmax, while the slope = -1/Kd.
Upper Right: A plot of the specific activity of tritium incorporation from precursor choline into acetylcholine over the time course after injection. The plot indicates that there is a product-precursor relationship between the two metabolites. Times within the 0-15 min range are generally used to calculate fractional rate constants, and thus turnover of acetylcholine. **Lower:** Typical displacement plot for the binding-site occupancy for [^{3}H]-QNB, displaced by log doses of carbachol in the diencephalon and cortex. The dashed lines indicate mass action saturation isotherms for theoretically distinct "pure" M_1 and M_2 sites. ENZFITTER, LIGAND and INPLOT software were used to resolve the actual results into individual percentages of subtype components. Note the differential distribution in different brain regions -- see Methods.

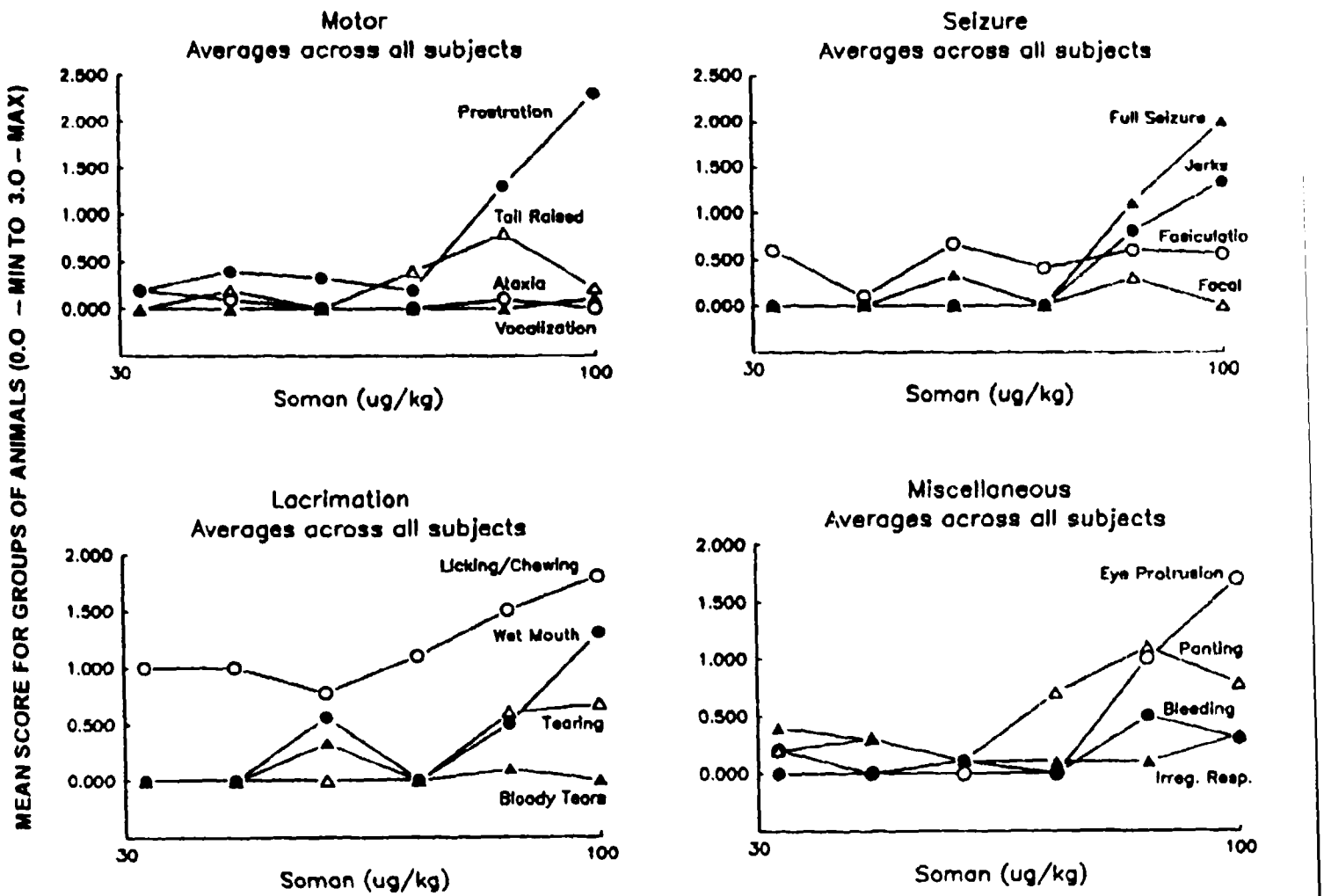


FIGURE 2 -- Behaviors Observed by a Single Blind Rater Following Exposure of Rats to Soman

The abscissa is log-dose of soman (XGD) over the 30-100 ug/kg range. The ordinate is the mean score for all animals on a given measure for behaviors scored in arbitrary units (0-3 according to increasing severity or appearance) by a single-blind rater. **Upper Left:** Motor function (prostration, tail raising, ataxia and vocalizations). **Upper Right:** Incidence and type of seizures (full body tonic-clonic, body jerks, fasiculations, focal seizures). **Lower Left:** Lacrimation (licking/chewing, wet mouth, tearing, bloody tears). **Lower Right:** Miscellaneous behaviors (eye protrusion, panting, bleeding, irregular respiration). Selected behaviors (e.g., prostration, full seizures, licking/chewing, eye protrusion, etc.) had the largest impact on the cumulative behaviors. Attempts at plotting these data on linear, log-linear, and probit axes did not yield meaningful information, although if one assumed that the maximum cumulative scores were comparable to LD_{100}, then threshold "toxic signs" of behaviors were observed circa 50-80 ug/kg.

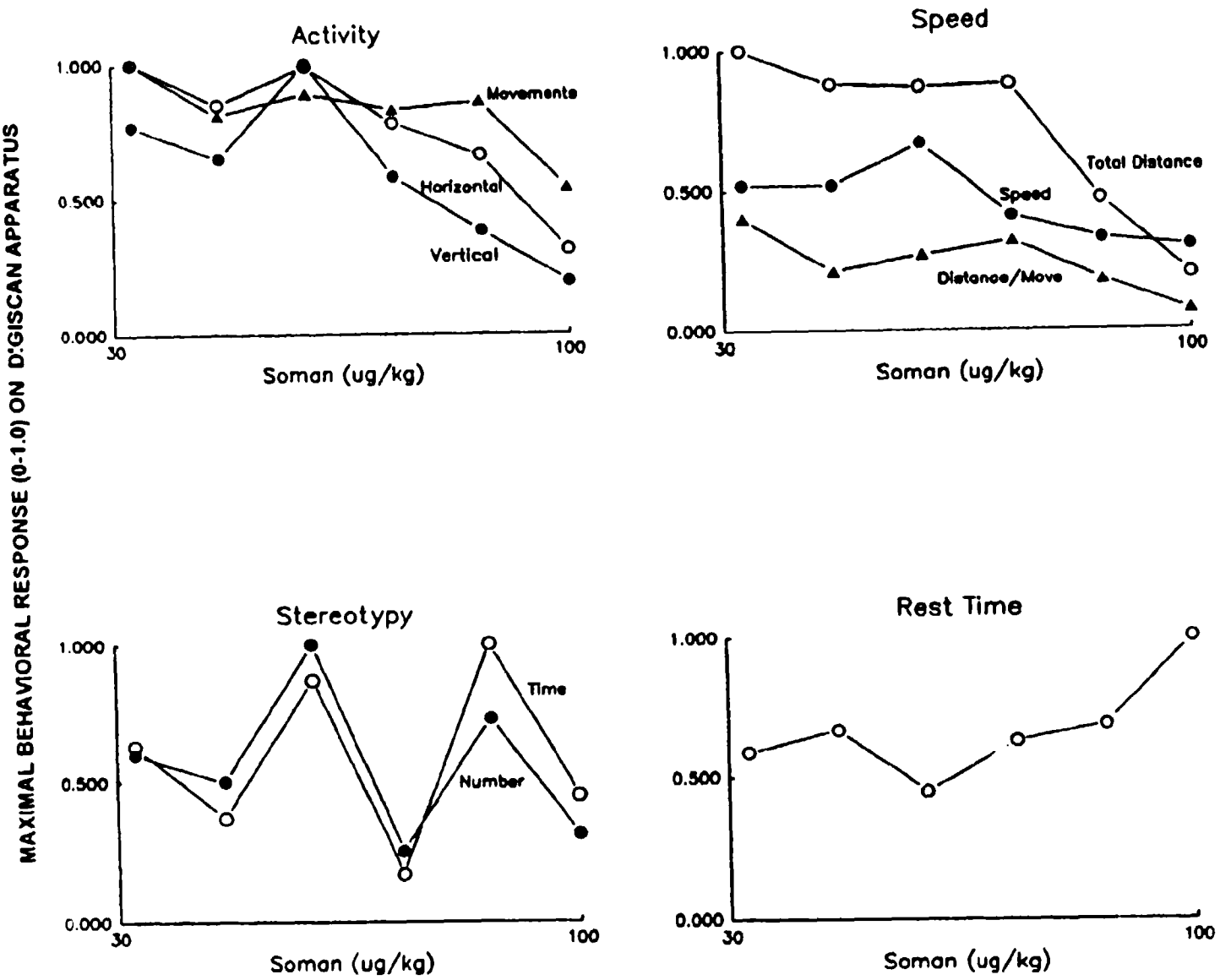


FIGURE 3 -- Behaviors Detected by Digiscan Activity Apparatus Following Exposure of Rats to Soman

The abscissa is log-dose of soman (XGD) over the 30-100- ug/kg range. The ordinate is the average of the maximal response (0 up to 1.0) in values for Digiscan activity measures; some measures are represented in arbitrary units; some measures are represented in space-movement or time variables. **Upper Left:** Activity (horizontal, vertical, total movements). **Upper Right:** Speed (total distance, speed, distance per movement). **Lower Left:** Stereotypy (time, number). **Lower Right:** Rest time.

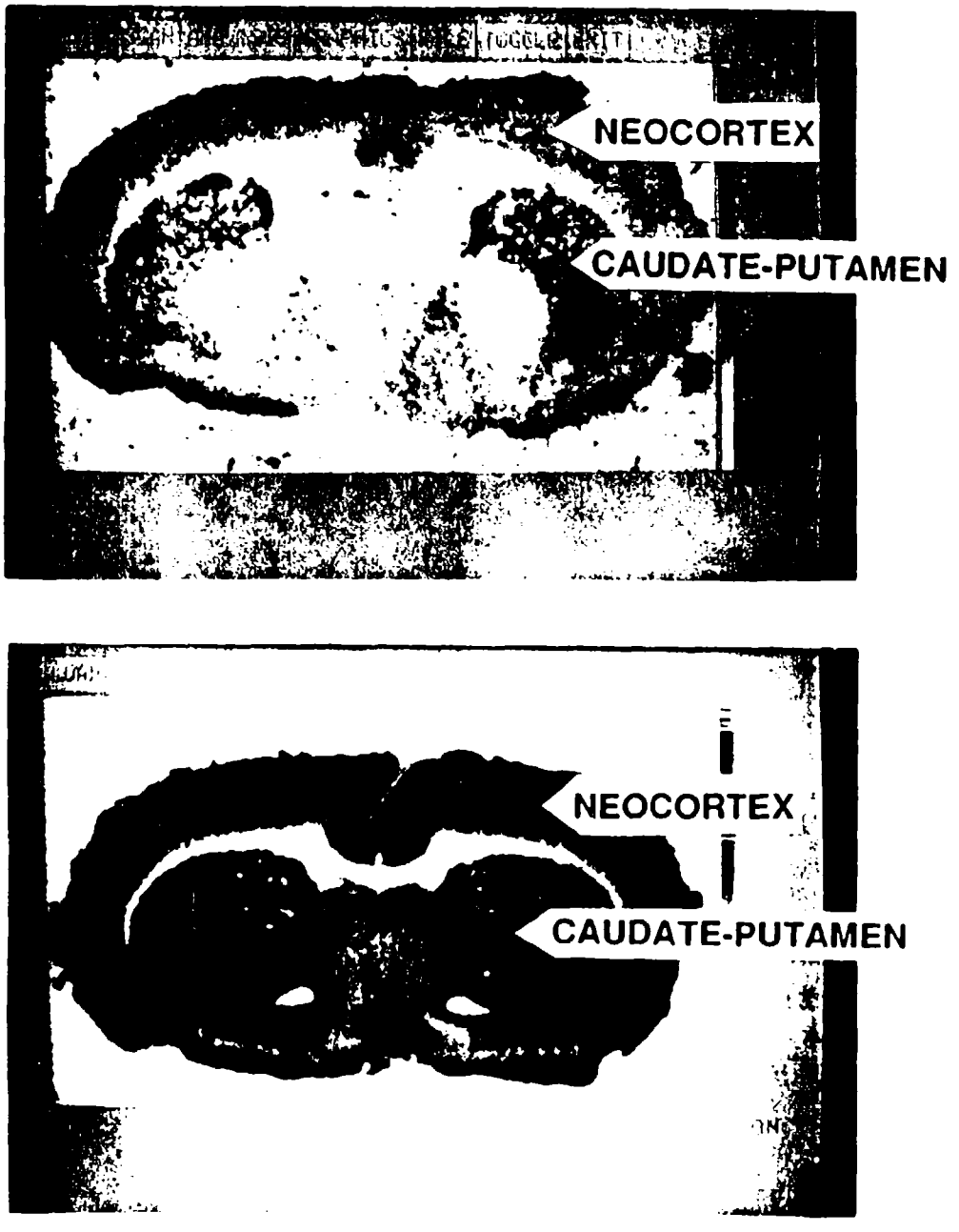


FIGURE 4 -- *In Vitro* Binding-Site Autoradiography
The two photographs are digitized images from LKB Ultrofilm, produced by the DUMAS/BRAIN Image Analyzer. These are coronal sections at the level of the striatum from the brains of adult male naive animals. **Upper:** Localization of the binding of 0.2 nM [^{3}H]-QNB. **Lower:** Localization of the binding of 2.0 nM [^{3}H]-Oxotremorine-M
The highest levels of binding for both radioligands are observed in the cerebral cortex and in the caudate-putamen.

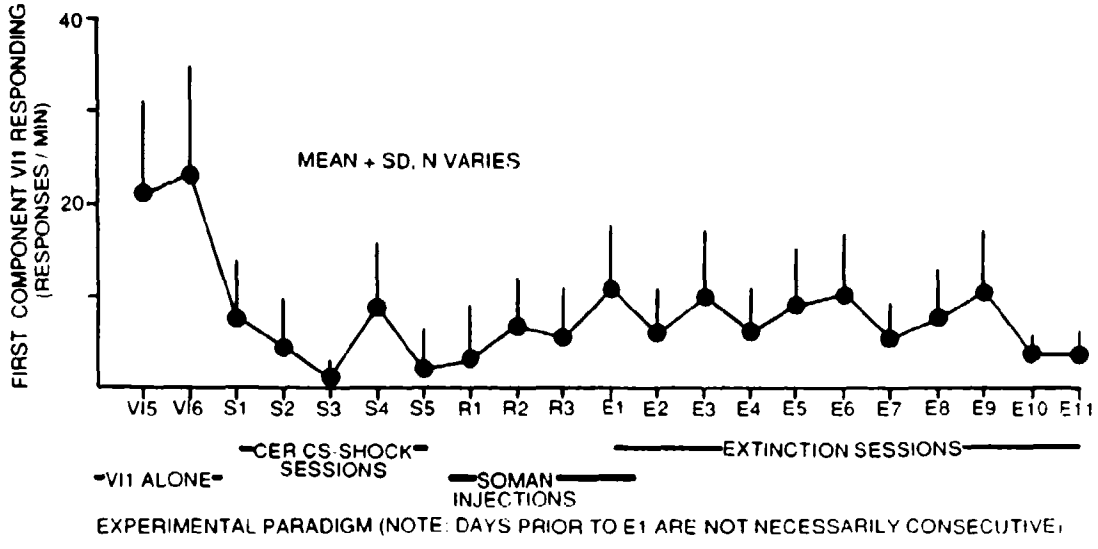


FIGURE 5 -- Survey of the Conditioned Emotional Response (CER) Extinction Paradigm Before and During the Various Key Phases

The ordinate indicates the response rates during component 1 of VI1 responding for food reinforcement. This component has no superimposed conditioned stimulus (CS), i.e. is pre-CS food-reinforced responding only. The abscissa shows progressive but not necessarily daily sessions: VI1 alone indicates component 1 for sessions 5-6; CER CS-shock indicates respondent conditioning sessions 1-5, where the animals were presented with the CS of varying lengths, followed at the offset by footshock; this was followed by recovery sessions 1-3 with VI1 component 1 alone; this was followed by extinction sessions 1-11 where the CS was presented once daily (during subsequent VI1 responding, called component 2, the CS was superimposed). Soman injections (at one-half the LD_{50} equivalent) were administered every other day between S5 and E1. Component 2 response rates are not shown, since they would be 0, indicating CER; or comparable to component 1, indicating extinction. The variances are great for this responding because these data include all rats, some of which were progressively debilitated by the soman.

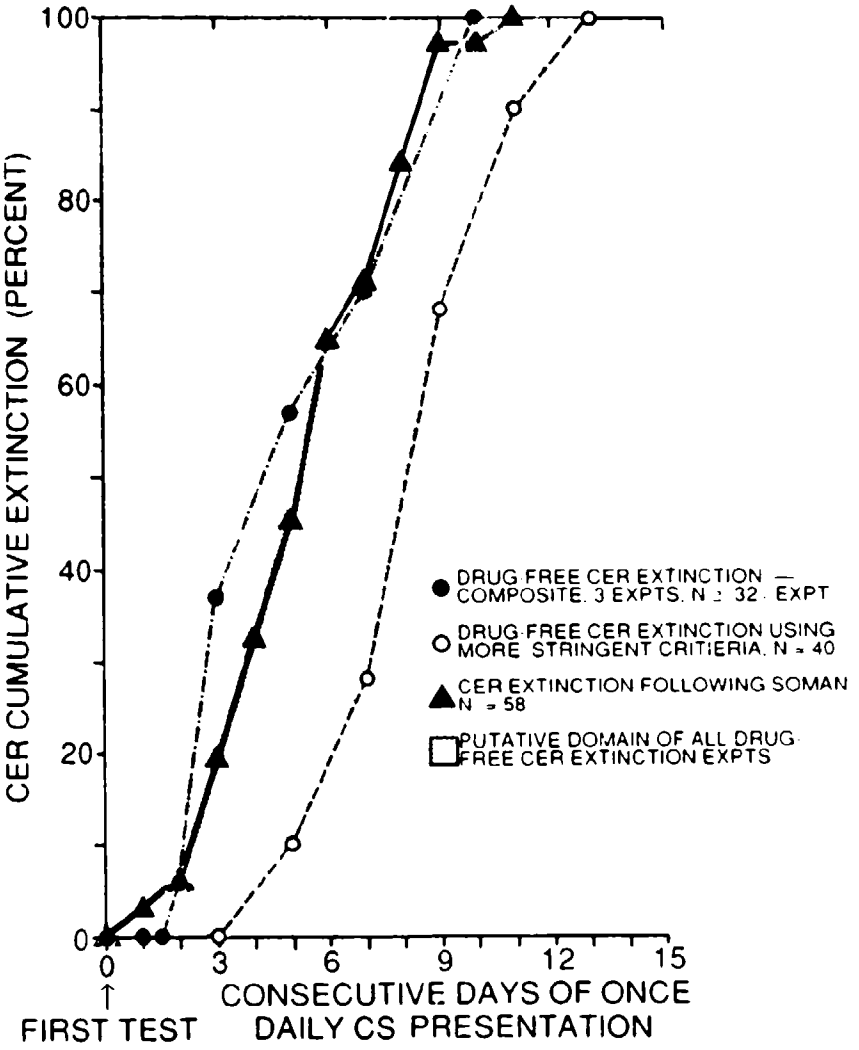


FIGURE 6 -- Conditioned Emotional Response (CER) Paradigm Extinction in Drug-Free and Soman-Treated Rats
The abscissa shows once daily presentations of the conditioned stimulus (CS) without subsequent pairing to footshock, which progressively brings about extinction of CER and restoration of normal food-reinforced responding. The ordinate accumulates the number of animals which demonstrate extinction, presented as a percentage of total. The closed and open circles define a putative domain for drug-free extinction, while the closed triangles show the pattern of extinction following soman-treatment. There is no significant difference, indicating that a soman-induced hypercholinergic state does not exacerbate CER. The more stringent criteria for the data represented by open circles is defined in the Methods. Data from closed circles are from Lane, 1986; data from open circles, where the criterion is increased from 80-100% reversal, are from Lane (unpublished).

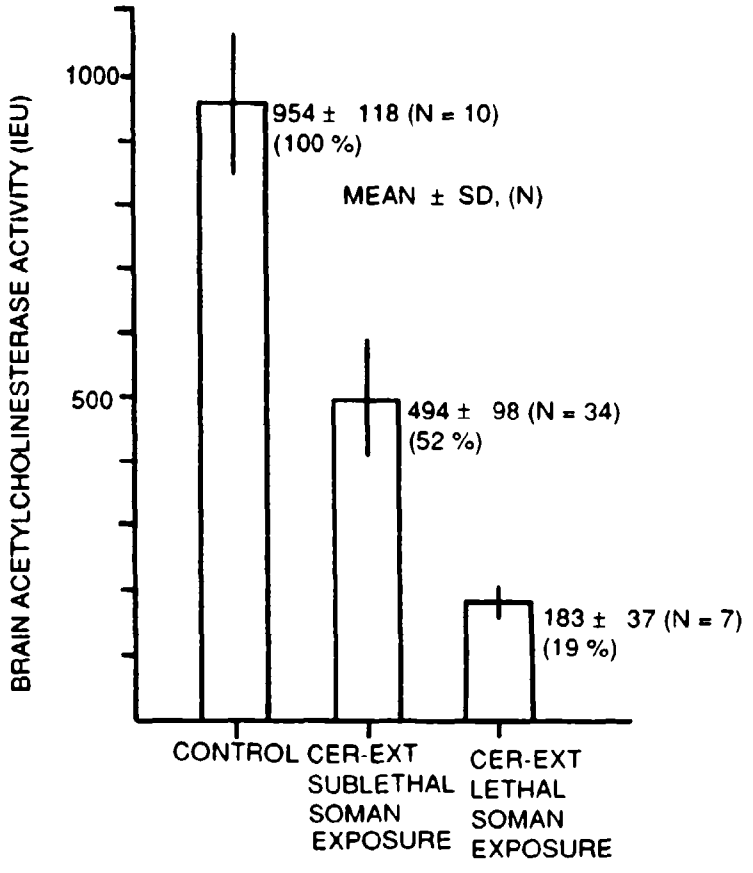


FIGURE 7 -- Brain Acetylcholinesterase Activity During the CER Extinction Paradigm
Following repeated injections of soman, some animals died at various days during extinction, and had the lowest levels of enzyme activity. A majority of the animals survived to complete the extinction criterion, but had significantly suppressed and intermediate enzyme activity. Controls received no soman treatment.

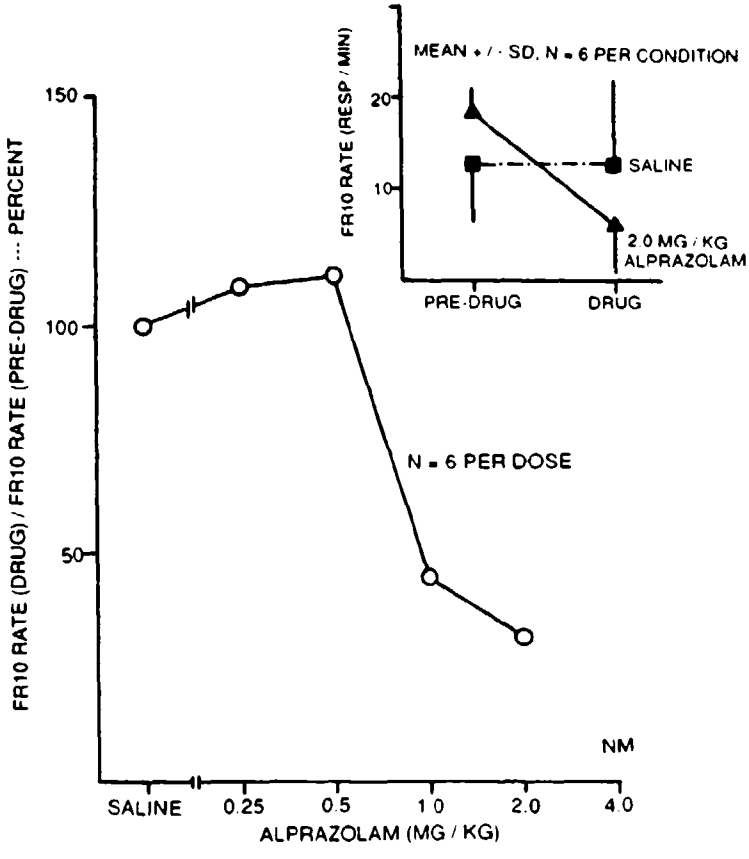


FIGURE 8 -- Dose-Response for the Effects of Single Doses of Alprazolam on Food-Reinforced Responding
The large graph indicates the ratio of FR10 rate (pre-drug) to FR10 (drug), presented as a percentage. The small graph shows two representative response pattens, from which the ratios were derived. Doses up to 0.5 mg/kg have no effect, circa 100%; while higher doses suppress responding below 50%, consistent with stupor. The highest dose (4.0 mg/kg) was not measured (NM).

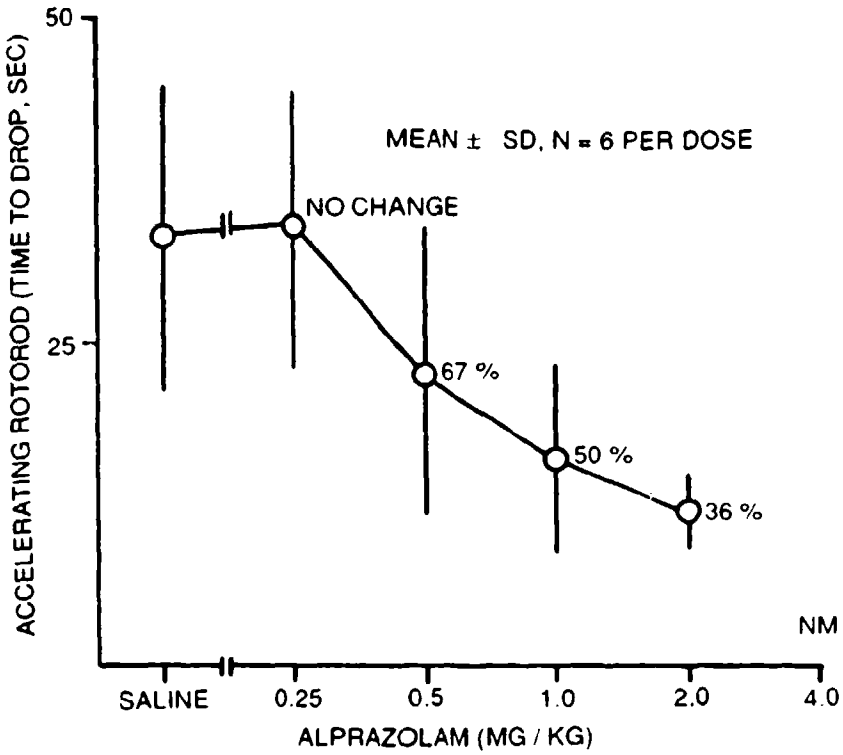


FIGURE 9 -- Dose-Response for the Effects of Single Doses of Alprazolam on Rotorod Performance
Doses up to 0.5 mg/kg do not significantly affect performance, while higher doses have a predictable disruptive effect. The highest dose (4.0 mg/kg) was not measured (NM).

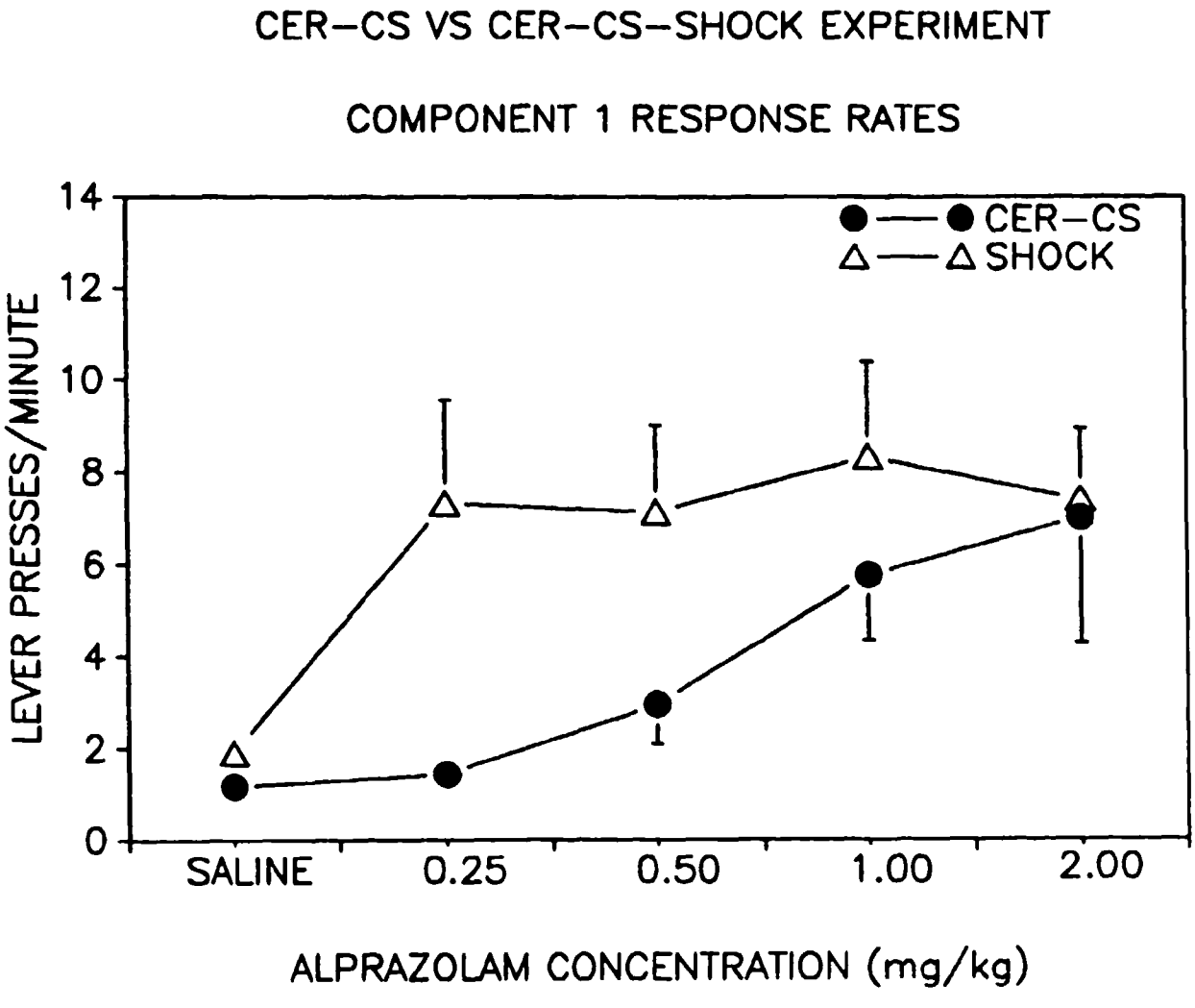


FIGURE 10 -- The Effect of Alprazolam on Conditioned Emotional Response (CER-CS) Versus Non-specific Stress (CER-CS-Shock) on Food-Reinforced Response Rates in the Absence of the Conditioned Stimulus and Shock (Component 1)
Data represent mean ± S.E.M.; CER-CS cells contained N=8 per dose; CER-CS-Shock cells contained N=5-6 per dose.

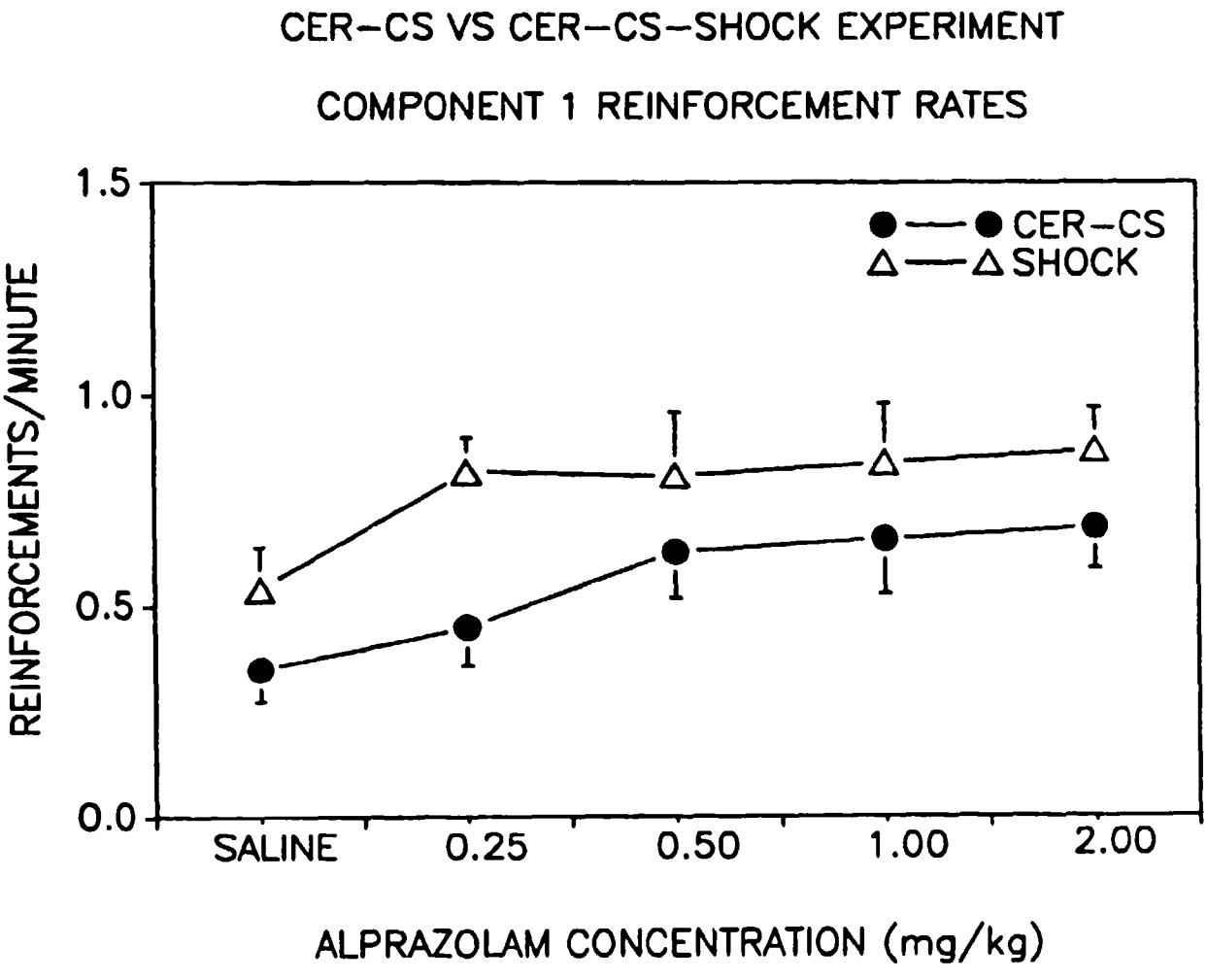


FIGURE 11 -- The Effect of Alprazolam on Conditioned Emotional Response (CER-CS) Versus Non-specific Stress (CER-CS-Shock) on Reinforcemnts Delivered in the Absence of the Conditioned Stimulus and Shock (Component 1) Data represent mean ± S.E.M.; CER-CS cells contained N=8 per dose; CER-CS-Shock cells contained N=5-6 per dose.

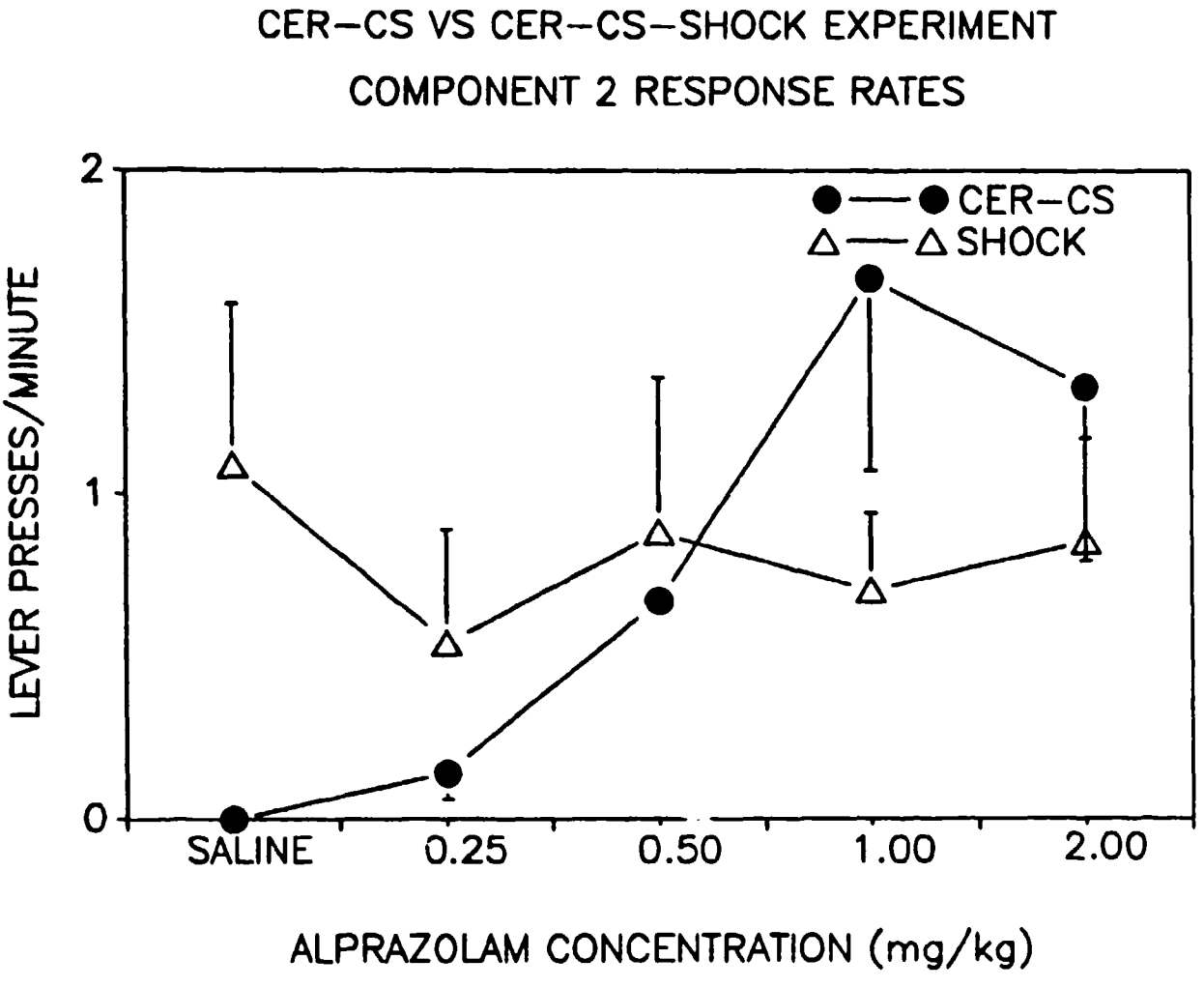


FIGURE 12 -- The Effect of Alprazolam on Conditioned Emotional Response (CER-CS) Versus Non-specific Stress (CER-CS-Shock) on Food-Reinforced Response Rates in the Presence of the Conditioned Stimulus or Conditioned Stimulus Plus Shock (Component 2)
Data represent mean ± S.E.M.; CER-CS cells contained N=8 per dose; CER-CS-Shock cells contained N=5-6 per dose.

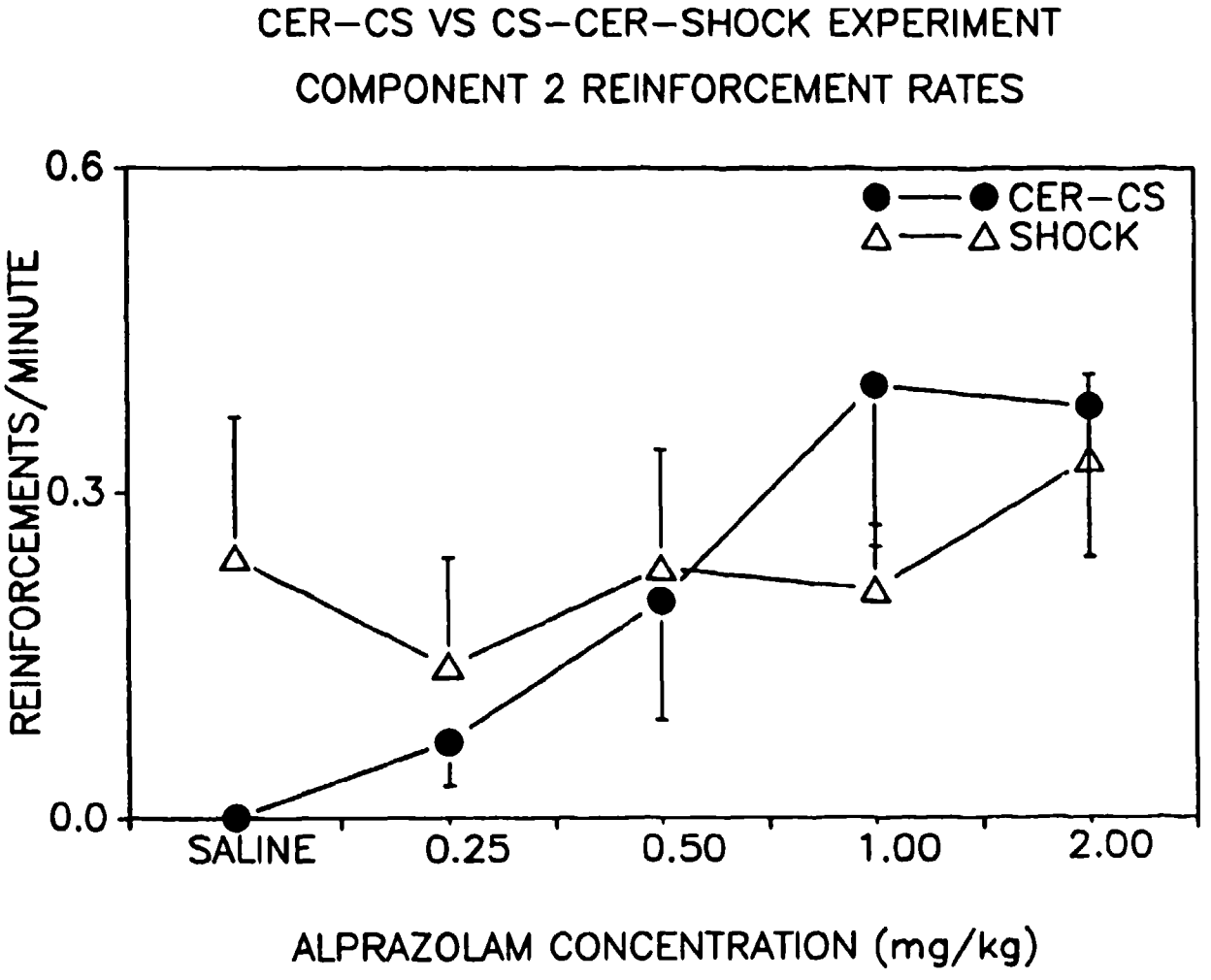


FIGURE 13 -- The Effect of Alprazolam on Conditioned Emotional Response (CER-CS) Versus Non-specific Stress (CER-CS-Shock) on Reinforcements Delivered in the Presence of the Conditioned Stimulus or Conditioned Stimulus Plus Shock (Component 2)
Data represent mean ± S.E.M.; CER-CS cells contained N=8 per dose; CER-CS-Shock cells contained N=5-6 per dose.

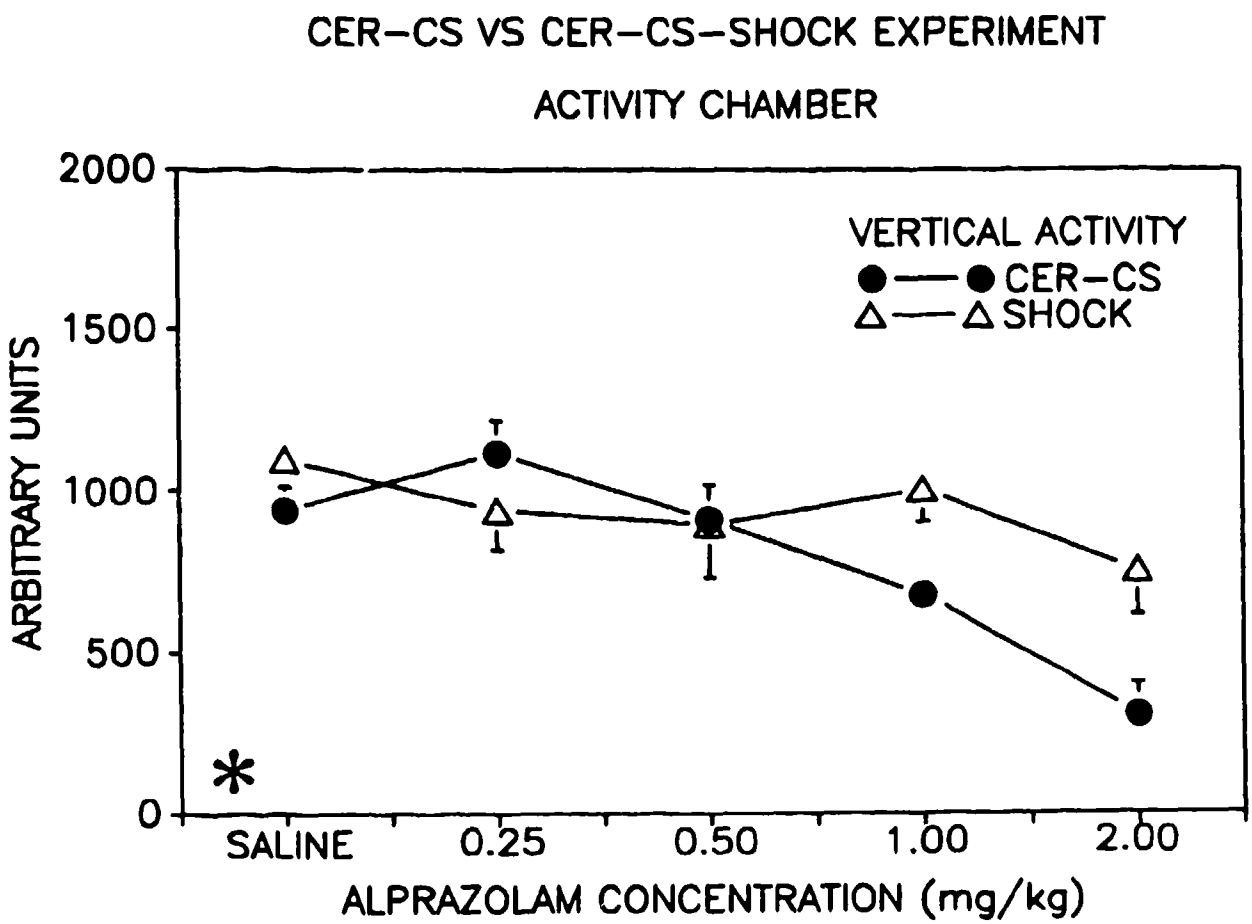


FIGURE 14 -- The Effect of Alprazolam on Conditioned Emotional Response (CER-CS) Versus Non-specific Stress Superimposed on CER (CER-CS-Shock) on Vertical Activity

Data represent mean ± S.E.M.; CER-CS cells contained N=8 per dose; CER-CS-Shock cells contained N=5-6 per dose. [The asterisk represents data from a separate experiment for drug-naive CER-CS animals.]

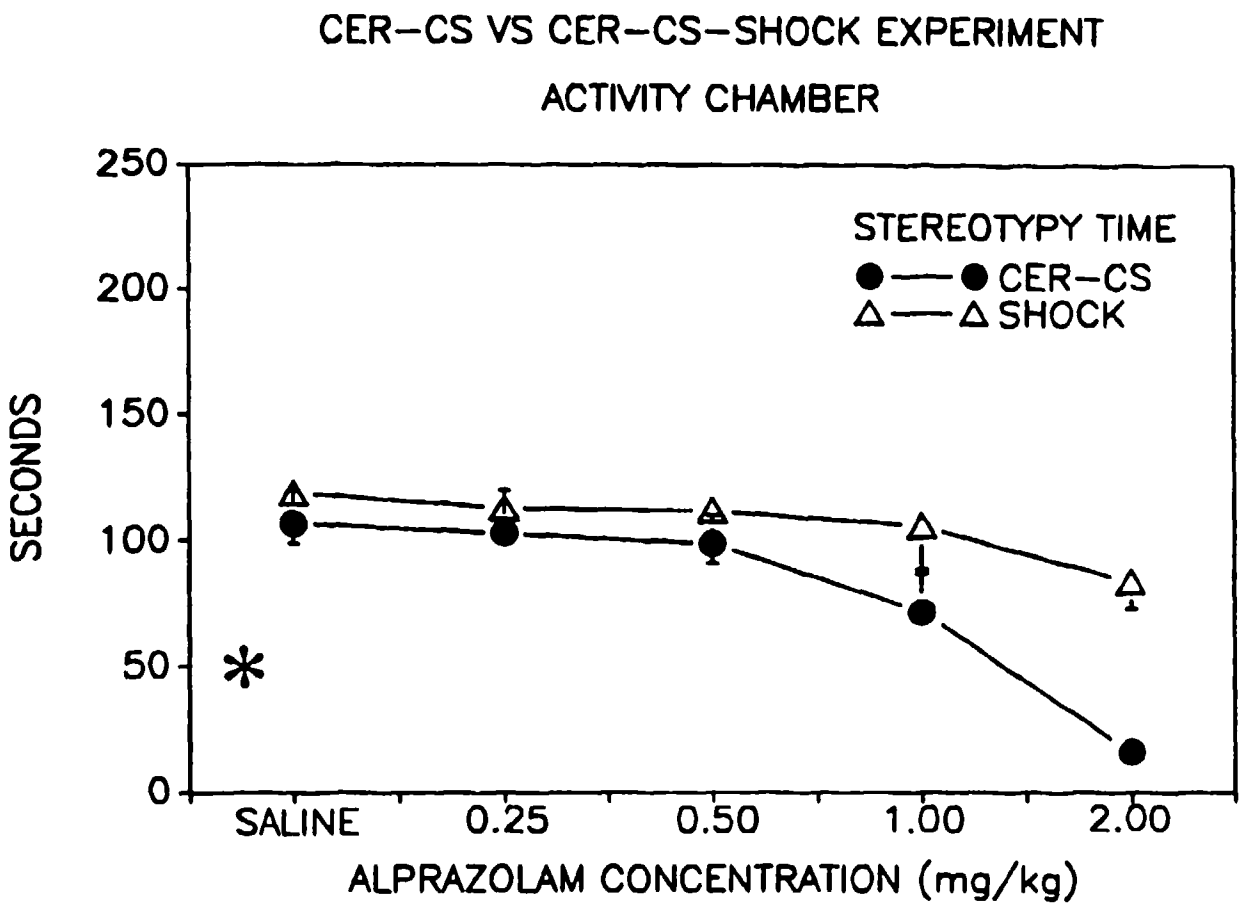


FIGURE 15 -- The Effect of Alprazolam on Conditioned Emotional Response (CER-CS) Versus Non-specific Stress Superimposed on CER (CER-CS-Shock) on Stereotypy Time

Data represent mean ± S.E.M.; CER-CS cells contained N=8 per dose; CER-CS-Shock cells contained N=5-6 per dose. [The asterisk represents data from a separate experiment for drug-naive CER-CS animals.]

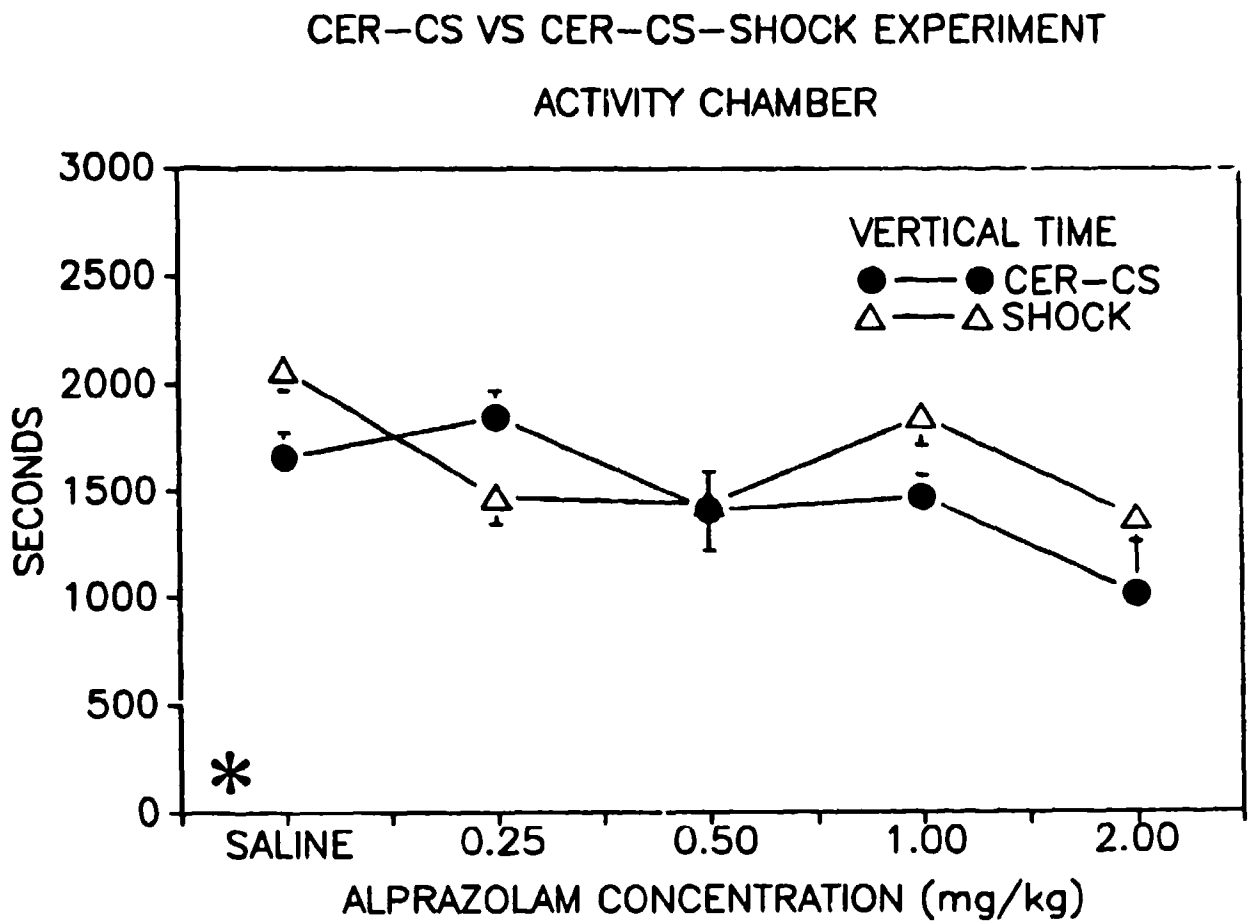


FIGURE 16 -- The Effect of Alprazolam on Conditioned Emotional Response (CER-CS) Versus Non-specific Stress Superimposed on CER (CER-CS-Shock) on Vertical Time

Data represent mean ± S.E.M.; CER-CS cells contained N=8 per dose; CER-CS-Shock cells contained N=5-6 per dose. [The asterisk represents data from a separate experiment for drug-naive CER-CS animals.]

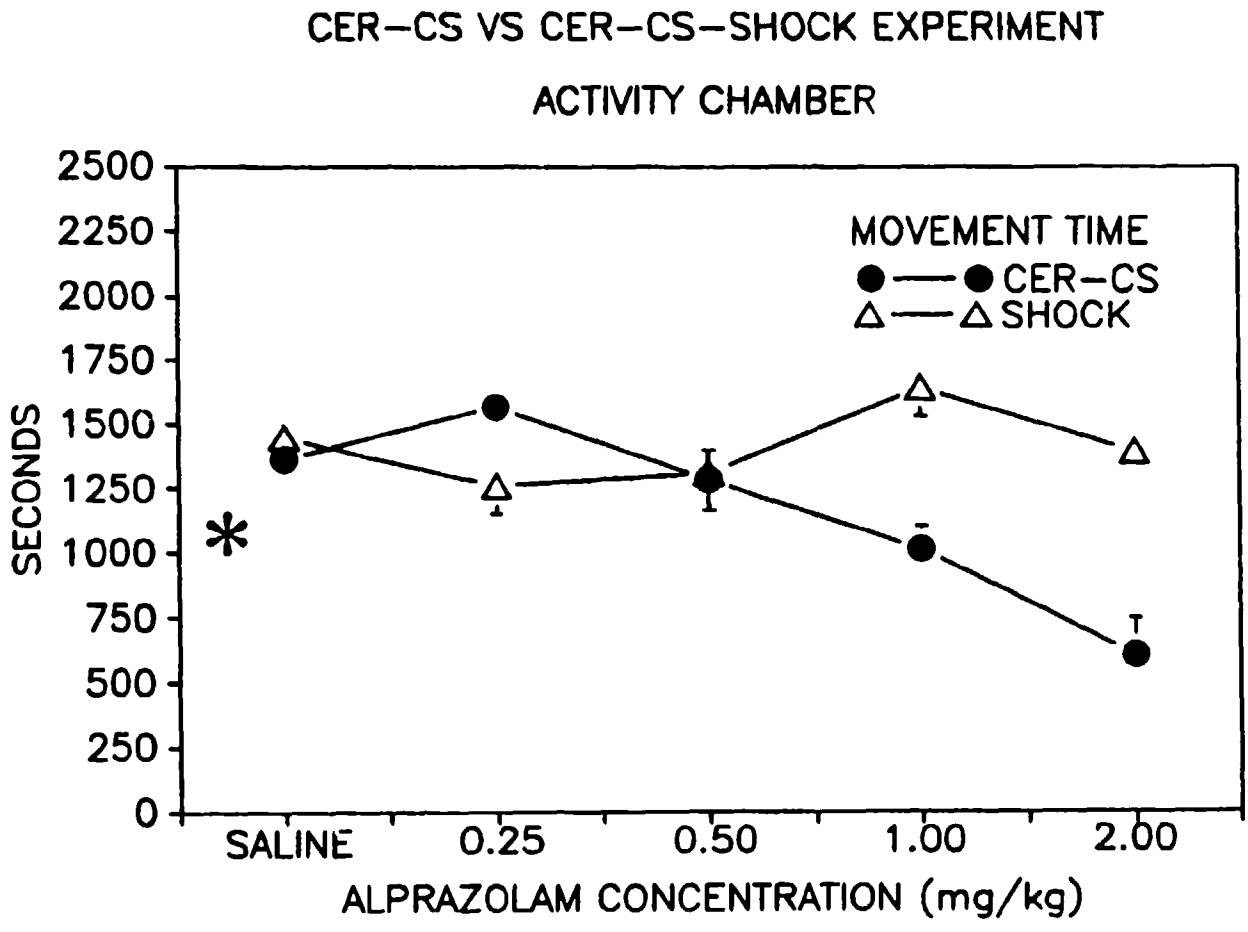
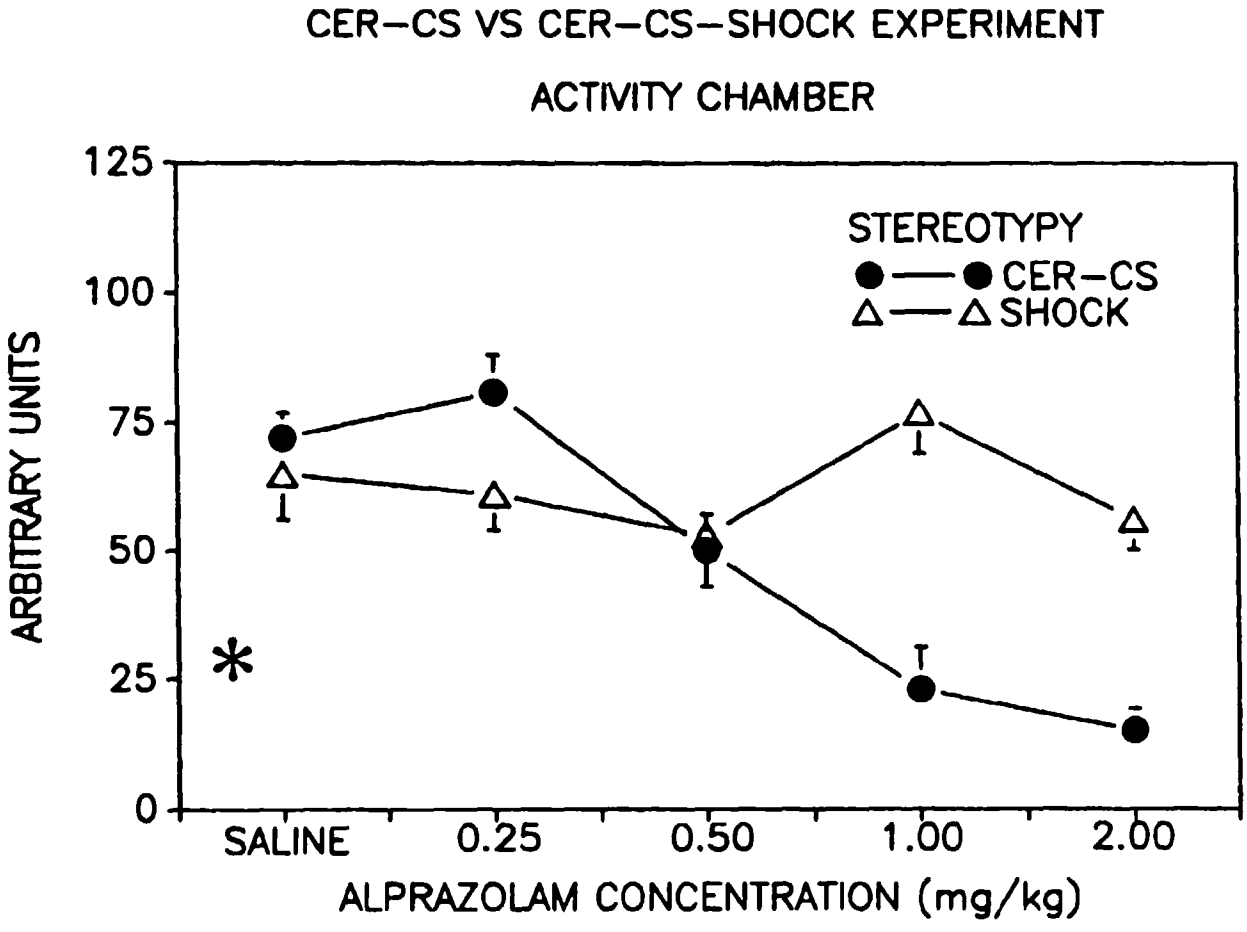


FIGURE 17 -- The Effect of Alprazolam on Conditioned Emotional Response (CER-CS) Versus Non-specific Stress Superimposed on CER (CER-CS-Shock) on Movement Time

Data represent mean ± S.E.M.; CER-CS cells contained N=8 per dose; CER-CS-Shock cells contained N=5-6 per dose. [The asterisk represents data from a separate experiment for drug-naive CER-CS animals.]



FIGUPE 18 -- The Effect of Alprazolam on Conditioned Emotional Response (CER-CS) Versus Non-specific Stress Superimposed on CER (CER-CS-Shock) on Stereotypy

Data represent mean ± S.E.M.; CER-CS cells contained N=8 per dose; CER-CS-Shock cells contained N=5-6 per dose. [The asterisk represents data from a separate experiment for drug-naive CER-CS animals.]

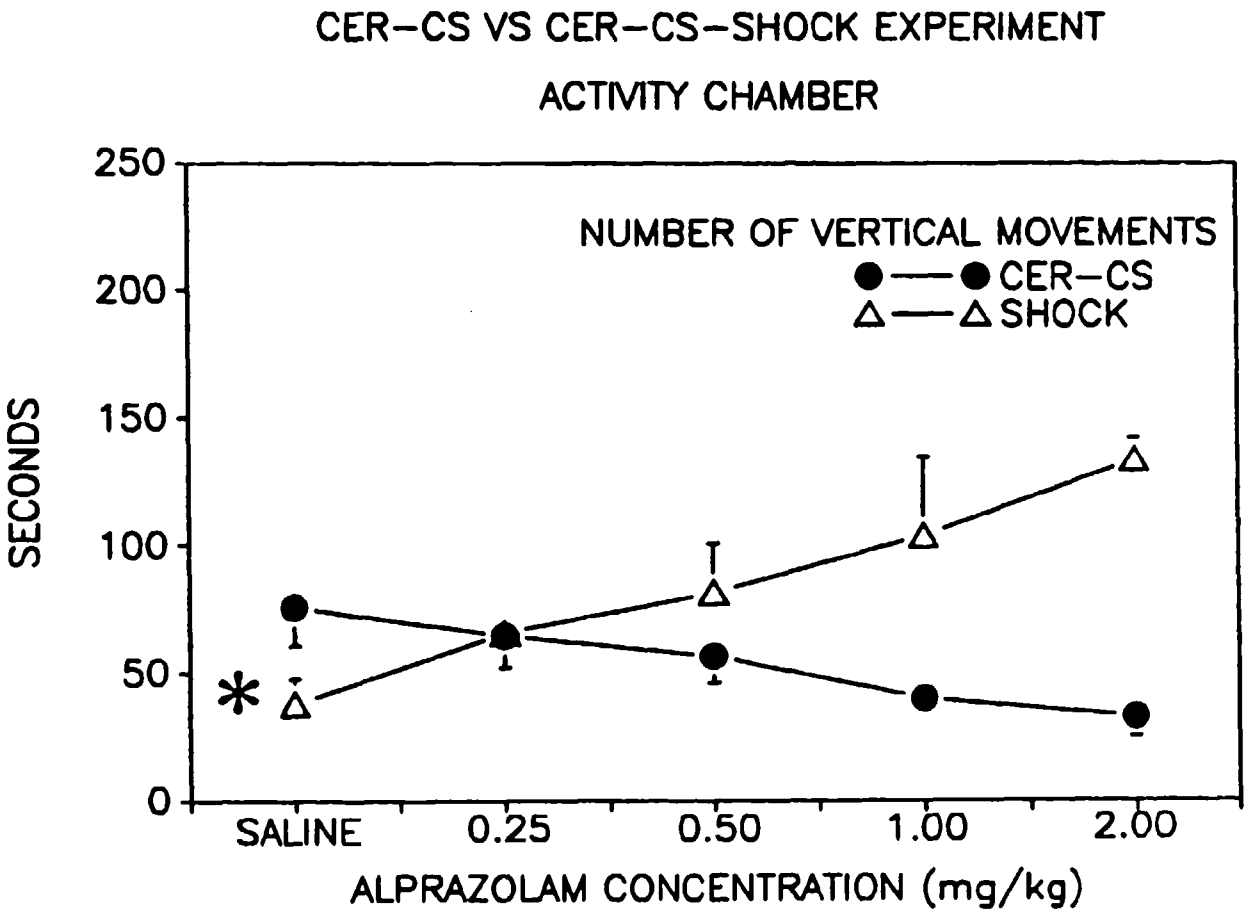


FIGURE 19 -- The Effect of Alprazolam on Conditioned Emotional Response (CER-CS) Versus Non-specific Stress Superimposed on CER (CER-CS-Shock) on Number of Vertical Movements

Data represent mean ± S.E.M.; CER-CS cells contained N=8 per dose; CER-CS-Shock cells contained N=5-6 per dose. [The asterisk represents data from a separate experiment for drug-naive CER-CS animals.]

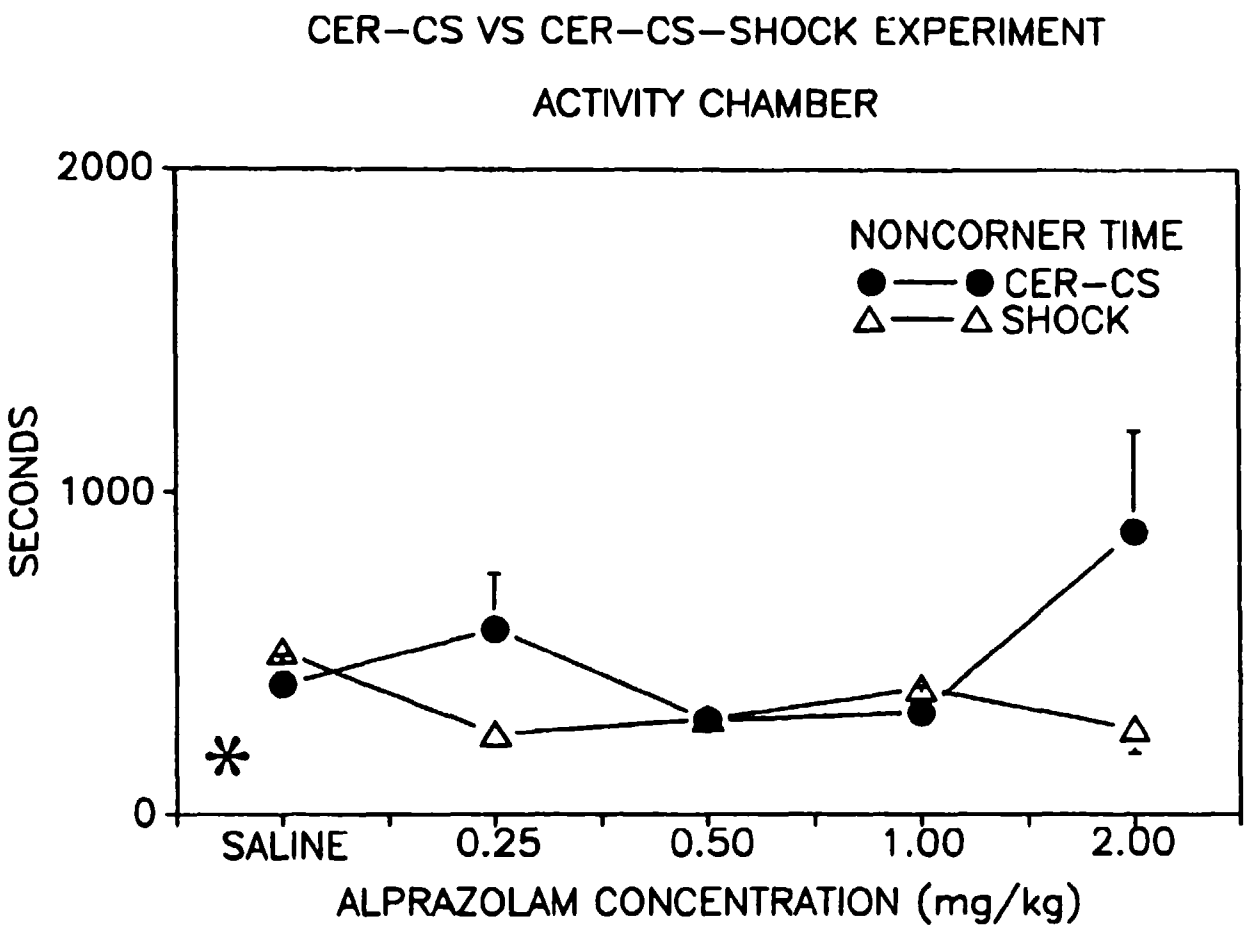


FIGURE 20 -- The Effect of Alprazolam on Conditioned Emotional Response (CER-CS) Versus Non-specific Stress Superimposed on CER (CER-CS-Shock) on Non-Corner Time

Data represent mean ± S.E.M.; CER-CS cells contained N=8 per dose; CER-CS-Shock cells contained N=5-6 per dose. [The asterisk represents data from a separate experiment for drug-naive CER-CS animals.]

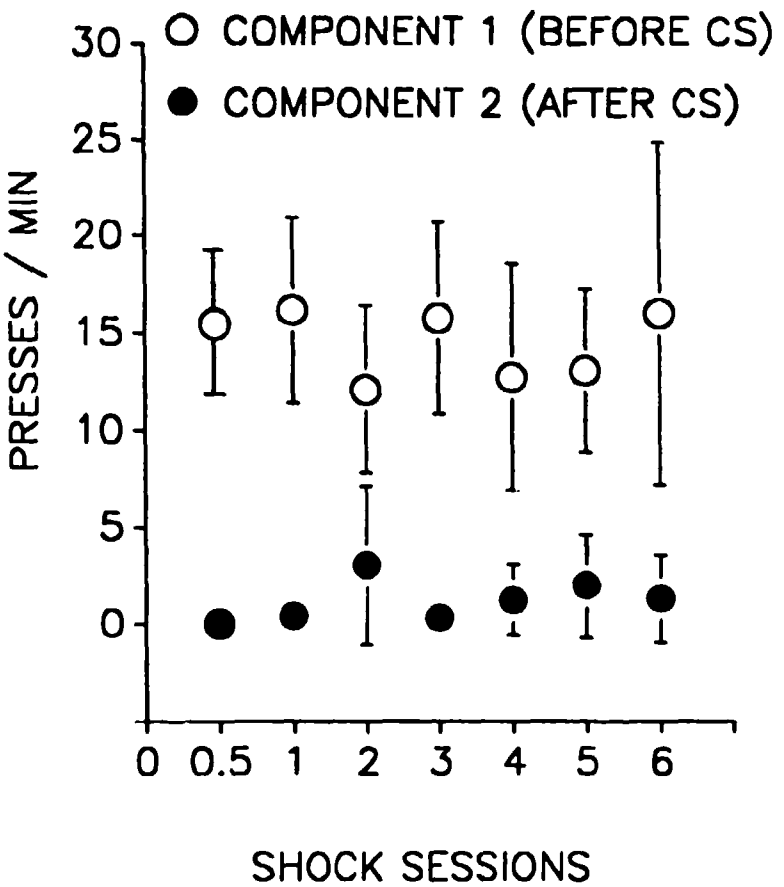


FIGURE 21 -- Acquisition of the Conditioned Emotional Response (CER) Paradigm The abscissa shows once-daily sessions of the presentation of the conditioned stimulus (CS) with subsequent offset of the CS paired with footshock, which on test day elicits CER. The ordinate indicates lever presses per minute before the CS (component 1 -- open circles) and after/during the CS (component 2 -- filled circles). The 0.5 session indicates that the subjects received one-half of the first-day session, normally 105 min in length. Since this is a random paradigm, a one-half session includes CS durations of 2-8 min, randomly paired with approximately 10 footshocks. N = 7-8 per group.

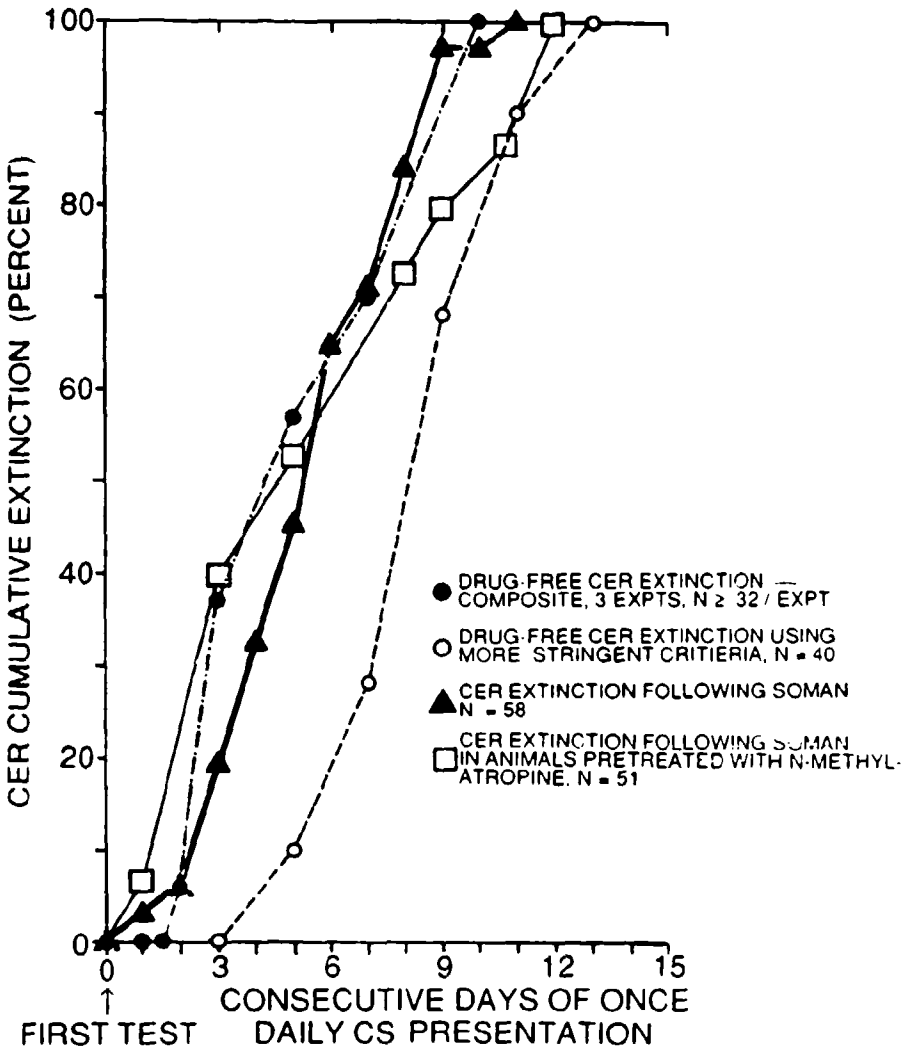


FIGURE 22 -- Conditioned Emotional Response (CER) Paradigm Extinction in Drug-Free and Soman/N-Methylatropine-Treated Rats

The abscissa shows once-daily presentations of the conditioned stimulus (CS) without subsequent pairing to footshock, which progressively brings about extinction of CER and restoration of normal food-reinforced responding. The ordinate accumulates the number of animals which demonstrate extinction, presented as a percentage of total subjects. The closed and open circles define a putative domain for drug-free extinction, while the closed triangles show the pattern of extinction following soman treatment. The open squares show the pattern following soman-treatment in animals pretreated with n-methylatropine. There are no significant differences in any of the groups, indicating that a soman-induced hypercholinergic state, irrespective of adjunctive treatments, does not exacerbate CER. The more stringent criteria for values expressed by open circles, i.e., 80-100% reversal of CER, are defined in the Methods.

TABLE 1 -- TRAINING AND CONDITIONING PROTOCOLS FOR CER RATS USED IN MULTIPLE BEHAVIOR, EXTINCTION, NON-SPECIFIC STRESS, ACETYLCHOLINE TURNOVER, ACQUISITION OF CER, AND ALPRAZOLAM STUDIES

Days Involved With Component or Treatment	Component or Treatment
5 days	Food lever shaping
4 days	60 min FR1 sessions (see legend)
6 days	60 min VI1 sessions until stable responding occurs
5 days	60 min VI1 sessions during which the animals are habituated to the light-tone combination which will become the CS
8 days - Option D ONLY (all other studies omit this step	Surgical implantation of chronic indwelling jugular catheters for precursor (choline) administration on testday
5 days - Routine procedure for inducing CER	Respondent conditioning sessions: Morning - 60 min VI1 sessions; Afternoon - light-tone (CS) paired with footshock
6 days - Option E ONLY (Behavioral Experiment 8)	Respondent conditioning sessions: Morning - 60 min VI1 sessions; Afternoon - light-tone (CS) paired with footshock for 1, 2, 3,...days; rats received 0.5 to 6 sessions of US-CS pairings to test acquisition of CER
5 days - Option F ONLY (Behavioral Experiment s 6--no atropine; and 9--atropine pretreatment)	Beginning on shock day 5, rats received five treatments one-half of the LD_{50} equivalent of soman every other day
5 days	Recovery: 60 min VI1 sessions until stable responding returns to pre-conditioning rates
Test day - 45 min - in general and Option A	30 min VI1 sessions, followed by 15 min continuous CS (or equivalent for control groups)
Test day - after 45 min - Option A ONLY (Behavioral Experiment 1)	Individual rats immediately tested for Digiscan activity measures, passive avoidance or active avoidance
Test day - Option B ONLY (Behavioral Experiments 2--no drug; and 6 and 9--soman treatments)	During VI1 sessions, rats receive once daily or more frequent presentations of CS for 15 days (extinction)
Test day - Option C ONLY (Behavioral Experiment 3)	During CS or equivalent presentations, groups of animals receive random footshocks to mimic non-specific, unavoidable stress
Test day - Option D ONLY (Behavioral Experiment 4)	During CS or equivalent presentations, rats received 0.5 mCi [^{3}H]-choline i.v. at 2-15 min, scheduled so that sacrifice after injection occurred at the end of the 15 min continuous CS
Test day - Option G ONLY (Behavioral Experiment 7)	30 minutes prior to CS or CS-Shock or equivalent, rats received saline or 0.25 - 2.0 mg/kg alprazolam

For the most part, the CER training and conditioning protocol followed the same scheme: stabilization of food-reinforced responding; habituation to the novelty value of the light-tone; respondent conditioning of CS-US pairings; and presentation of the CS or equivalent on test day. Options A-G indicate changes in the respective experiments which modify the general scheme, while retaining the main components of the CER paradigm. [NOTE: Options which deviate from the basic protocol have been referenced against Behavioral Experiment numbers in text.]

TABLE 2 -- EFFECTS OF CER ON OTHER BEHAVIORS WHICH REFLECT CHANGES IN CHOLINERGIC FUNCTION

Behavior	CS Exposure (CER)	No CS Exposure (Control)
CER Suppression Index (Response Rate During 15 min CS / Response Rate During 30 min VI1 Before CS)	0	1.0
Collateral Behaviors (see Legend)	"Emotional"	Normal
Total Locomotor Activity (Distance in cm)	2063 ± 287*	2710 ± 295
Stereotypy (Arbitrary Units)	77 ± 12*	44 ± 11
Center Time (Antithigmotaxis - Total sec)	240 ± 38*	147 ± 29
Step-Up Active Avoidance (Trials to Criterion)	16.2 ± 0.9*	14.1 ± 1.0
Step-Down Passive Avoidance (Trials to Criterion)	6.8 ± 0.4*	9.3 ± 0.7

Data represent means ± S.D. for N=7-8 per group. *$p<0.05$. "Emotional" behaviors include bracing, freezing, urination, defecation, shaking -- these were not quantitated in this study, but have been assessed with ordinal scales in the past, and have positively correlated with CER suppression.

TABLE 3 -- EFFECTS OF REPEATED CS PRESENTATIONS (EXTINCTION) ON CER BEHAVIOR

Behavioral Group	VI1 Responding Before CS Presentation (or Equivalent)		VI1 Responding During CS Presentation(or Equivalent)		Percent Suppression +
	Responses per Minute	Reinforcers per Minute	Responses per Minute	Reinforcers per Minute	
CER first CS presentation at 0 hours	8.8 ± 0.9	0.98	0	0.01	100%
Second CS presentation at 12 hours	9.5 ± 1.3	0.90	0	0.05	100%
Third CS presentation at 24 hours	7.4 ± 0.9	1.0	0.1 ± 0.1	0.55	94%
Fourth CS presentation at 48 hours	8.0 ± 1.0	0.98	1.9 ± 0.4	0.53	63%
Additional once daily CS presentations - groupings between 72-96 hours	10.2 ± 1.3	1.0	2.0 ± 0.3	0.53	43%
Additional once daily CS presentations - groupings between 120-144 hours	7.2 ± 0.8	0.88	2.1 ± 0.4	0.70	30%
Additional once daily CS presentations up until 216 hours when extinction had reversed CER	6.5 ± 0.9	0.82	3.5 ± 0.5*	0.96*	0%

Data represent means or means ± S.D. for N=6-13 per group. +The computer-controlled program used to control and record the CER behaviors did not have the option of a limited hold; thus, an "accidental" response after an average of 1 min would deliver a reinforcement pellet; accordingly, more flexible criteria must be utilized to assess suppression and its reversal. Suppression was defined for each animal which fails to receive 12 of 15 possible reinforcers -- under these conditions, some animals responded just enough to activate the reinforcer-delivery criterion, but still received only a small number of total food pellets during this component.

*When reinforcement returns to the normal rate circa one per minute, independent of the absolute response rate, reversal of suppression is operationally defined.

TABLE 4 -- EFFECTS OF CS PRESENTATION AND NON-SPECIFIC FOOTSHOCK STRESS ON CER BEHAVIOR

Behavioral Group	VI1 Responding Before CS Presentation (or Equivalent)		VI1 Responding During CS Presentation(or Equivalent)		Percent Suppression +
	Responses per Minute	Reinforcers per Minute	Responses per Minute	Reinforcers per Minute	
CER- no CS (Control)	13.2 ± 2.0	1.0	11.7 ± 1.7	0.95	0%
CER-CS	10.7 ± 2.2	1.0	0.2 ± 0.6*	0	100%
CER-no CS-Shock	12.8 ± 1.5	1.0	1.0 ± 0.6	0.37	100%
CER-CS-Shock	11.0 ± 3.1	1.0	0.72 ± 0.20	0.32	100%

Data represent means or means ± S.D. for N=7 per group. *One animal responded at 1.7 responses per min, but overall there was, by definition, total suppression. +Suppression was defined for each animal which fails to receive 12 of 15 possible reinforcers -- under these conditions, some animals responded just enough to activate the reinforcer-delivery criterion, but still received only approximately 5 total food pellets during this component.

TABLE 5 -- EFFECTS OF CS PRESENTATION ON CER BEHAVIOR IN THE ACETYL-CHOLINE TURNOVER EXPERIMENT

Behavioral Group	VI1 Responding During 3 Days of Recovery Prior to Test day		VI1 Responding Before CS Presentation (or Equivalent)		VI1 Responding During CS Presentation (or Equivalent)		Percent Suppression
	Responses per Minute	Reinforcers per Minute	Responses per Minute	Reinforcers per Minute	Responses per Minute	Reinforcers per Minute	
CER- no CS (Control)	4.6 ± 0.7	0.55	2.8 ± 0.5	0.48	2.8 ± 0.3	0.80	0%
CER-CS	4.5 ± 0.6	0.46	2.4 ± 0.3	0.45	0.09 ± 0.01	0.01*	100%

Data represent means or means ± S.D. for N=28-29; *2 of 29 animals in the CER-CS group received but did not necessarily consume one reinforcement pellet during the CS presentation.

TABLE 6 -- SUMMARY OF THE EFFECTS OF SOMAN (XGD) ON F344 RATS WITH RESPECT TO LETHALITY, BEHAVIORS, AND ACTIVITY

Dose (ug/kg) Subcutaneous Injection	Percent Lethality	Mean Total Behaviors (Arbitrary Units)	Activity (Arbitrary Units)	Remarks
Saline (0)	0	—	4.96	Normal, unremarkable behaviors noted
31 ug/kg	0	1.4	4.69	
40	0	1.7	3.92	
50	10	3.6	4.69	
63	44	3.1	3.80	
71 (0.05 increment)	—	4.7	—	
80	60	10.0	2.88	Decrease in general activities noted
89 (0.05 increment)	—	11.2	—	
100	72	11.5	1.61	
112 (0.05 increment)	—	12.3	—	
126	100	—	—	no survivors
159	100	15.0	—	one survivor at 2 hours after injection, then subsequently lethal
200	100	—	—	no survivors

The activity measures were the summations of averages over six activity and speed parameters, based the highest value observed being equated asymptotically to 1.0, and the remaining values adjusted as fractions thereof, and summed accordingly. Measures of stereotypy showed no pattern, and were excluded from these analyses. Rest time is likely to be inversely related to activities, and was thus excluded.

TABLE 7 – EFFECTS OF MULTIPLE PRESENTATIONS OF CS ON CER (EXTINCTION) ON MUSCARINIC BINDING PARAMETERS IN RAT CEREBRAL CORTEX AND DIENCEPHALON

Brain Region and Behavior	Displacer										
	QNB		NMS			PZ			OXO-M		
	Bmax	Kd	Bmax	Kd	%High Affinity	Bmax	Kd	%M1	Bmax	Kd	%M2
Cortex											
0 Hours	1180 ± 138*	0.19	645 ± 48*	0.12	80	795 ± 80*	16	38	1404 ± 230	1.9	62
12	1201 ± 117*	0.18	670 ± 75*	0.13	78	780 ± 67*	14	38	1385 ± 201	2.1	60
24	1236 ± 110*	0.20	695 ± 70*	0.12	75	822 ± 85*	13	42	1400 ± 165	2.3	58
48	1384 ± 200*	0.23	712 ± 89*	0.15	64	862 ± 95*	17	45	1340 ± 170	2.4	51
72-96	1550 ± 172	0.17	880 ± 98	0.12	50	1060 ± 112	18	53	1405 ± 190	1.8	48
120-144	1802 ± 234	0.19	1013 ± 120	0.11	55	1193 ± 127	15	57	1346 ± 202	2.0	42
216	2040 ± 275	0.18	1106 ± 189	0.10	59	1326 ± 156	16	56	1405 ± 170	1.9	43
Diencephalon											
0	900 ± 98	0.20	670 ± 73	0.13	43	500 ± 43	16	10	902 ± 101	2.0	100
12	890 ± 75	0.20	642 ± 47	0.11	45	480 ± 57	16	0	880 ± 78	1.9	100
24	967 ± 54	0.23	650 ± 72	0.15	46	520 ± 45	17	8	870 ± 96	2.2	100
48	1030 ± 104	0.24	700 ± 83	0.17	50	558 ± 67	14	0	923 ± 120	2.5	100
72-96	1030 ± 98	0.21	680 ± 62	0.12	43	550 ± 67	15	0	900 ± 110	2.0	100
120-144	975 ± 100	0.19	702 ± 80	0.12	40	503 ± 60	15	6	912 ± 124	2.3	100
216	1000 ± 123	0.20	670 ± 73	0.15	44	516 ± 63	16	8	1055 ± 157	2.2	100

Bmax values are fmol/mg protein; Kd values are nM; % high-affinity M_2-like sites are carbachol-sensitive; % M_1 and/or %M_2 sites may not equal 100% since more than two M_i subtype binding sites are recognized. Data represent means or means ± S.D. for N=6-13 per behavioral group. *$p<0.01$.

TABLE 8 – EFFECTS OF MULTIPLE PRESENTATIONS OF CS ON CER (EXTINCTION) ON MUSCARINIC BINDING PARAMETERS IN RAT HIPPOCAMPUS AND STRIATUM

Brain Region and Behav-ior	Displacer										
	QNB		NMS			PZ – NOT MEASURED			OXO-M		
	Bmax	Kd	Bmax	Kd	%High Affinity	Bmax	Kd	%M1	Bmax	Kd	%M2
Hippocampus											
0	1280 ± 134*	0.18	710 ± 83*	0.13	42				1008 ± 180	2.0	70
12	1301 ± 146*	0.20	708 ± 65*	0.12	40				1030 ± 124	2.3	72
24	1210 ± 140*	0.23	700 ± 83*	0.14	35				980 ± 120	2.0	70
48	1371 ± 170*	0.19	767 ± 87*	0.12	25				976 ± 98	1.8	65
72-96	1680 ± 203*	0.22	944 ± 101	0.12	26				1001 ± 129	1.9	55
120-144	1934 ± 205	0.18	1062 ± 143	0.14	20				1050 ± 178	2.1	52
216	2115 ± 236	0.19	1202 ± 239	0.14	22				1024 ± 156	2.0	50
Striatum											
0	2410 ± 300	0.20	1550 ± 201	0.14	35				1108 ± 145	1.8	33
12	2502 ± 268	0.21	1523 ± 210	0.12	30				1200 ± 207	2.3	30
24	2307 ± 304	0.23	1600 ± 178	0.15	37				1180 ± 230	2.1	38
48	2400 ± 368	0.18	1560 ± 240	0.12	34				1075 ± 90	2.2	34
72-96	2267 ± 305	0.19	1498 ± 204	0.132	30				1150 ± 140	1.9	30
120-144	2401 ± 308	0.18	1557 ± 208	0.14	37				1208 ± 234	2.0	35
216	2368 ± 312	0.19	1604 ± 178	0.12	28				1100 ± 120	2.5	30

Bmax values are fmol/mg protein; Kd values are nM; % high-affinity M_2-like sites are carbachol-sensitive; % M_1 and/or %M_2 sites may not equal 100% since more than two M_i subtype binding sites are recognized. Data represent means or means ± S.D. for N=6-13 per behavioral group. *$p<0.01$. PZ not measured because of insufficient tissue.

TABLE 9 – EFFECTS OF CER VERSUS NON-SPECIFIC FOOTSHOCK STRESS ON MUSCARINIC AND GABA-ERGIC BINDING PARAMETERS IN RAT CEREBRAL CORTEX AND DIENCEPHALON

Brain Region and Behavior	Displacer												
	QNB		NMS			PZ			OXO-M			MUSCIMOL	
	Bmax	Kd	Bmax	Kd	%High Affinity	Bmax	Kd	%M1	Bmax	Kd	%M2	Bmax	Kd (High Affinity)
Cortex													
CER-no CS	2203 ± 302	0.20	1109 ± 145	0.13	59	1350 ± 203	16	60	1340 ± 203	2.0	40	2950 ± 440	15
CER-CS	1230 ± 140*	0.18	670 ± 78*	0.12	80	804 ± 98*	17	30	1440 ± 157	1.8	65	2800 ± 304	14
CER-no CS-Shock	2105 ± 302	0.21	1050 ± 170	0.12	62	1450 ± 178	16	35	1478 ± 167	1.7	38	2005 ± 406*	16
CER-CS-Shock	1140 ± 130*	0.19	908 ± 100*	0.11	70	703 ± 86*	14	28	1398 ± 201	1.9	32	1810 ± 196*	14
Diencephalon													
CER-no CS	1080 ± 78	0.21	706 ± 80	0.10	38	508 ± 63	14	0	908 ± 120	2.0	100	1605 ± 230	14
CER-CS	1009 ± 120	0.21	670 ± 65	0.11	44	550 ± 65	13	4	980 ± 145	2.0	90	1657 ± 178	16
CER-no CS-Shock	980 ± 102	0.21	701 ± 83	0.12	46	554 ± 78	12	6	900 ± 139	2.0	100	1589 ± 245	17
CER-CS-Shock	1002 ± 134	0.19	698 ± 87	0.10	40	524 ± 72	14	0	875 ± 93	1.8	100	1700 ± 234	16

Bmax values are fmol/mg protein; Kd values are nM; % high-affinity M_2-like sites are carbachol-sensitive; % M_1 and/or %M_2 sites may not equal 100% since more than two M_i subtype binding sites are recognized; high-affinity muscimol binding represents the GABA receptor. Data represent means or means ± S.D. for N=7 per behavioral group. *$p<0.01$.

TABLE 10 -- EFFECTS OF CER VERSUS NON-SPECIFIC FOOTSHOCK STRESS ON MUSCARINIC ANΓ GABA-ERGIC BINDING PARAMETERS IN RAT HIPPOCAMPUS AND STRIATUM

Brain Region and Behav-ior	Displacer												
	QNB		NMS			PZ – NOT MEASURED			OXO-M			MUSCIMOL – NOT MEASURED	
	Bmax	Kd	Bmax	Kd	%High Affinity	Bmax	Kd	%M1	Bmax	Kd	%M2	Bmax	Kd (High Affinity)
Hippocampus													
CER-no CS	2150 ± 306	0.20	1185 ± 120	0.15	22				1007 ± 120	2.0	50		
CER-CS	1180 ± 124*	0.18	719 ± 83*	0.12	40				1100 ± 126	2.1	62		
CER-no CS-Shock	2234 ± 300	0.19	1190 ± 146	0.14	20				1108 ± 148	1.8	45		
CER-CS-Shock	1008 ± 138*	0.21	955 ± 103	0.15	25				1080 ± 160	2.0	40		
Striatum													
CER-no CS	2508 ± 340	0.20	1702 ± 188	0.12	33				1140 ± 155	2.0	30		
CER-CS	2480 ± 310	0.21	1660 ± 203	0.13	35				1080 ± 129	2.0	37		
CER-no CS-Shock	2378 ± 230	0.18	1590 ± 200	0.11	37				1100 ± 110	2.0	41		
CER-CS-Shock	2402 ± 360	0.17	1678 ± 203	0.12	30				1095 ± 201	2.3	30		

Bmax values are fmol/mg protein; Kd values are nM; % high-affinity M_2-like sites are carbachol-sensitive; % M_1 and/or %M_2 sites may not equal 100% since more than two M_i subtype binding sites are recognized; high-affinity muscimol binding represents the GABA receptor. Data represent means or means ± S.D. for N=7 per behavioral group. *$p<0.01$. PZ and MUSCIMOL were not measured because there was not sufficient tissue.

TABLE 11 -- EFFECTS OF CS PRESENTATION ON CER VERSUS CONTROL IN ACETYLCHOLINE TURNOVER IN NINE DISCRETE RAT BRAIN REGIONS

Brain Region	Behavioral Condition	ACh Content (nmol/mg protein)	Apparent Fractional Rate Constant (K – per hour)	ACh Turnover * (nmol/mg-hour)
Frontal Cortex	CER-no CS (Control)	120 ± 15	4.1	492 ± 62
	CER-CS	130 ± 17	7.2	936 ± 122* (90%)
Pyriform Cortex	CER-no CS	168 ± 40	6.8	1143 ± 272
	CER-CS	188 ± 31	13.2	2482 ± 410 (117%)
Cingulate Cortex	CER-no CS	79 ± 10	5.1	403 ± 51
	CER-CS	86 ± 8	4.7	404 ± 38
Entorhinal-Subicular Cortex	CER-no CS	119 ± 25	4.7	559 ± 118
	CER-CS	132 ± 20	4.0	528 ± 80
Nucleus Accumbens	CER-no CS	200 ± 35	6.8	1360 ± 238
	CER-CS	204 ± 40	7.5	1530 ± 300
Caudate-Putamen	CER-no CS	598 ± 55	3.1	1854 ± 171
	CER-CS	615 ± 48	3.0	1845 ± 146
Medial Septum	CER-no CS	203 ± 24	7.5	1523 ± 180
	CER-CS	215 ± 18	6.9	1484 ± 124
Hippocampus	CER-no CS	120 ± 11	5.0	600 ± 55
	CER-CS	135 ± 20	8.8	1188 ± 176* (98%)
Amygdaloid Complex	CER-no CS	456 ± 55	4.5	2052 ± 248
	CER-CS	501 ± 62	9.3	4659 ± 577*(127%)

*Since (K) is a derived function, it has no inherent variance; therefore, ACh turnover measures reflect variances in ACh content from the CER-CS and CER-noCS groups, respectively. Data represent means ± S.D. for N=28-29, collapsed into the two behavioral groups, which represent N=7-8 within each of four neurochemical groups used for each time point after injection, for both the CER-CS and the CER-noCS groups. For the most part, times after injection of radiolabelled precursor, [^{3}H]-choline, corresponded to 2, 5, 10 and 15 min of CS (or equivalent time) presentation. Because of limited technical problems, 3 subjects received injections at 3 or 7 min; since the time course of the precursor-product relationship is utilized to generate the apparent fractional rate constant (K), this procedural difference does not taint the protocol.
*$p < 0.001$.

TABLE 12 -- THE EFFECTS ON ACTIVITY MEASURES OF SALINE VERSUS 2 MG/KG ALPRAZOLAM ON ANIMALS IN CER-CS (CONDITIONED SUPPRESSION) AND CER-CS-SHOCK (NON-SPECIFIC STRESS) PARADIGMS

Activity Measures	CER-CS PARADIGM		CER-CS-SHOCK PARADIGM	
	Saline	2 mg/kg Alprazolam	Saline	2 mg/kg Alprazolam
Vertical Activity (arbitrary units)	940 ± 71	305 ± 97 *	1098 ± 45	752 ± 138 +, @
Total Distance (cm)	2642 ± 289	988 ± 282 *	3227 ± 317	2613 ± 283 @
Total Revolutions	1 ± 1	1 ± 1	2 ± 1	3 ± 1
Stereotypy Time (sec)	107 ± 13	16 ± 7 *	119 ± 20	84 ± 11 @
Average Speed (cm/sec)	1.64 ± 0.15	4.24 ± 0.81 *	0.78 ± 0.05 #	0.56 ± 0.11 @
Average Distance (cm)	13.53 ± 2.75	28.19 ± 7.40 *	5.81 ± 0.68 #	4.05 ± 1.81 @
Vertical Time (sec)	1657 ± 120	1016 ± 249	2071 ± 101 #	1378 ± 116 +
Movement Time (sec)	1364 ± 55	608 ± 140 *	1448 ± 82	1393 ± 57 @
Stereotypy (arbitrary units)	72 ± 5	15 ± 4 *	65 ± 9	56 ± 6 @
Vertical Movement Number	76 ± 15	33 ± 8 *	38 ± 10 #	134 ± 8 +, @
Total Movement Number	230 ± 9	127 ± 25 *	249 ± 9	251 ± 24 @
Center Time (sec)	10 ± 4	178 ± 146 *	11 ± 3	15 ± 2 @
Non-corner Time (sec)	402 ± 91	879 ± 34 *	504 ± 67	264 ± 75 +, @

* $p < 0.01$ for CER-CS-Saline versus CER-CS-Alprazolam.
+ $p < 0.01$ for CER-CS-Shock-Saline versus CER-CS-Shock-Alprazolam.
# $p < 0.01$ for CER-CS-Saline versus CER-CS-Shock-Saline.
@ $p < 0.01$ for CER-CS-Alprazolam versus CER-CS-Shock-Alprazolam.
Data represent mean ± S.D., N = 6 per group.

TABLE 13 -- QUALITATIVE DISTRIBUTION OF M_1 AND M_2 MUSCARINIC CHOLINERGIC RECEPTOR SUBTYPES IN AREAS OF RAT BRAIN AS IDENTIFIED BY IN VITRO BINDING SITE AUTORADIOGRAPHY

Brain Area	Binding Site Density (0, +, ++, +++) @	
	M_1 Receptor Subtype	M_2 Receptor Subtype
Frontal cortex	+	+++
Parietal cortex	+++	+++
Temporal cortex	+++	+++
Entorhinal-subicular cortex	+++	+++
Piriform cortex	+++	+
Cingulate cortex	+	+
Caudate-putamen	+++	+
Nucleus accumbens	+++	+
Globus pallidus	0	0
Septal nuclei	0	+++
Nucleus basalis of Meynert	0	+
Basolateral amygdala	+	+
Medial amygdala	+++	+
Central amygdala	0	+
Hippocampus - CA1 region	+++	+
Hippocampus - 4 other regions	+	+
Dentate gyrus	+++	0
Habenular nuclei	0	+
Lateral geniculate nucleus	0	+++
Thalamus - 21 regions	0	+ to ++
Hypothalamus - 13 regions	0	0 to +

@ Relative binding densities were progressively rated on a scale of 0 to +++, based on the respective binding of 2 nM [^{3}H]-pirenzepine (M_1) or 2 nM [^{3}H]-oxotremorine-M (M_2) to brain sections, corrected for non-specific binding by the addition of 1 uM atropine to sections handled in parallel. These results are consistent with the observations of Cortes and Palacios (1986) and Spencer et al. (1986).

TABLE 14 -- QUANTITATIVE DISTRIBUTION OF THE M_1 MUSCARINIC CHOLINERGIC RECEPTOR SUBTYPE IN AREAS OF RAT BRAIN AS IDENTIFIED BY IN VITRO BINDING SITE AUTORADIOGRAPHY IN CER AND STRESS PARADIGMS

Brain Area	Binding Site Density (fmol / mg protein) for M_1 Sites		
	CER-no CS	CER-CS	CER-CS-Shock
Frontal cortex	663 ± 41	451 ± 37 *	402 ± 35
Parietal cortex	570 ± 22	342 ± 45 *	319 ± 41
Temporal cortex	712 ± 35	427 ± 48 *	427 ± 53
Entorhinal-subicular cortex	605 ± 32	363 ± 26 *	643 ± 65 #
Piriform cortex	812 ± 73	488 ± 51 *	502 ± 43
Basolateral amygdala	407 ± 40	301 ± 36 *	358 ± 62
Hippocampus - CA1 region	588 ± 62	365 ± 41 *	404 ± 51

Binding to M_1 sites was approximated by using 2 nM [^{3}H]-PZ. Binding was converted to fmol/mg protein from the standard microscales (nCi/mg) by handling discrete brain sections in parallel, and determining the absolute amount of binding in dpm (later converted to fmol from the specific activity of the ligand), and the amount of protein present from adjacent sections.

Fifty-one other regions (see Figure 13) were examined and showed no differences in binding.

Data represent the mean ± S.D., N = 6-8 per group.

* $p < 0.01$ for CER-no CS versus CER-CS.

# $p < 0.01$ for CER-CS versus CER-CS-Shock.

TABLE 15 -- QUANTITATIVE DISTRIBUTION OF THE M_1 MUSCARINIC CHOLINERGIC RECEPTOR SUBTYPE IN AREAS OF RAT BRAIN AS IDENTIFIED BY IN VITRO BINDING SITE AUTORADIOGRAPHY -- EFFECT OF ALPRAZOLAM

Brain Area	Binding Site Density (fmol / mg protein) for M_1 Sites		
	CER-CS-Saline	CER-CS-Alprazolam	CER-CS-Shock-Alprazolam
Frontal cortex	433 ± 32	677 ± 72 *	750 ± 90
Parietal cortex	355 ± 43	515 ± 62 *	602 ± 71
Temporal cortex	433 ± 42	642 ± 45 *	588 ± 65
Entorhinal-subicular cortex	339 ± 50	551 ± 66 *	580 ± 49
Piriform cortex	430 ± 91	732 ± 81 *	699 ± 75
Basolateral amygdala	390 ± 32	821 ± 42 *	450 ± 50 #
Hippocampus - CA1 region	312 ± 55	584 ± 63 *	607 ± 70

Binding to M_1 sites was approximated by using 2 nM [^{3}H]-PZ (see legend of Figure 14 for description of the conversion of data from microscale standards to fmol/mg protein). Fifty-one other regions (see Figure 13) were examined and showed no differences in binding.

Data represent the mean ± S.D., N = 6-8 per group.

* $p < 0.01$ for CER-CS-Saline versus CER-CS-Alprazolam.

# $p < 0.01$ for CER-CS-Alprazolam versus CER-CS-Shock-Alprazolam.

TABLE 16 -- CUMULATIVE PERCENT MORTALITY IN RATS FOLLOWING CHRONIC DOSING WITH SOMAN ALONE OR SOMAN PLUS N-METHYL-ATROPINE

Treatment	Soman Injection Schedule (Day)				
	First	Third	Fifth	Seventh	Ninth*
Soman alone	15.9%	17.0%	23.9%	34.0%	34.0%
Soman, following pretreatment with n-methyl-atropine	11.8%	19.7%	28.9%	32.9%	32.9%

* Rats that survived at least four injections remained healthy and were available to complete the experiment.

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