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19. ABSTRACT (Continue on reverse if necessary and identify by block number) This project is assessing the effects of exposure to a chemical defense agent on anxiety and stress, by using rat models of anxiety (conditioned emotional response [CER]; conditioned suppression) and unconditioned non-specific stress (exposure to footshock). The specific experiments determined the plasticity of muscarinic cholinergic binding sites in the central nervous system. The neuroanatomical locus and neuropharmacological profile of changes in binding sites were assessed in brain areas enriched in cholinergic 'markers'. Acetylcholine turnover was measured to determine if the receptor response is compensatory or independent. The effects of acute exposure to doses of a chemical defense agent (soman--XGD) on lethality and behaviors were examined. The experiments involved training and conditioning adult rats to CER using standard operant/respondent techniques. The binding of radiolabelled ligands was studied in vitro using brain membranes and tissue sections (autoradiography). The major findings are that CER produces increases in acetylcholine turnover in brain areas involved in anxiety, and that primarily post-synaptic M1 receptors compensatorily decrease in response. These neurochemical phenomena are directly correlated with several behaviors, including onset and extinction of CER and non-specific stress. Followup experiments have been designed to test the interaction of CER, XGD and neurochemistry.					
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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). Tolerance to some aspects has also been observed. However, very few investigations have been comprehensive, in that they attempted to study chemistry and behavior after cholinergic 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 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. Their interactions with anxiety/stress have been determined, since stress has been linked to cholinergic function (Dillsaver, 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). 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 are outlined in the five behavioral experiments, and their respective followup neurochemical experiments, that follow.

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 consumes approximately four weeks (see Table 1). All animals have the same behavioral history prior to experimental manipulations. On test day during

food-reinforced responding (the baseline behavior), the CS (or equivalent or additional stimuli) was 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). Discrete trials of active 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 (3 of 4 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 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, 5 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 5 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 compensatory decrease in QNB binding sites. 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 [³H]-choline chloride, administered intravenously for 2-14 min (this corresponded to 13-1 min 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, cingulate 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 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 LD₅₀, 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 post-injection for lethality, and 2 hours post-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.

NEUROCHEMICAL STUDIES -- followed the behavior and drug treatment, such that a cell contained 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 behavior. 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 M₁ and/or to M₂ 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 M₁ (i.e., multiple undefined, but potentially definable) subtypes. If the resulting observations are consistent with prevailing reasoning, subsequent experiments will rely 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 M₁ and M₂ subtypes. [High designates sites with lower picomolar affinities, and low designates sites with high picomolar and suprananomolar affinities.] This will be 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, to comprise a second-messenger system, capable of producing a physiological effect; ii) exposure to diisopropylfluorophosphate (DFP) not only reduces B_{max} 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 M₂. In this context, based on reasoning that M₁ receptors are post-synaptic and the ones most likely to show plasticity, the PI will attempt to utilize 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), it will be important to evaluate multiple-affinity sites with neurochemical measures of receptors to ensure that there is a physiological correlation. For example, a decrease in a binding site parameter would predict a shift to the right in the dose-response of the receptor parameter. In addition, pharmacological profiling may 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 may indicate redundant sites (well established in the heart) or uncoupled sites. *In vitro* binding site autoradiography will be 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). Major forebrain areas and nuclei will be examined.

GENERAL METHODS

Behavioral: CER was trained-conditioned in adult F-344 littermate male rats (refer to Table 1 for summary). Several options to the basic protocol are outlined. Common operant terms, e.g., VI1, are defined in the table legend.

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) iterative computer programs for 1-3 non-interacting sites (for examples, see Figure 1). Displacement plots utilized detailed 1-pM to 100-nM 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 uncoupled by treatment with 1 mM ethylenediaminetetraacetate/n-ethyl-maleimide (Mash et al., 1985). High-affinity sites were determined as those NMS sites sensitive to displacement by 1 μ M carbachol. M₁ was differentiated from M₂ by [3 H]-QNB displacement by carbachol (2-site model) and M₂ was identified by subsaturating the high-affinity M₂ sites with [3 H]-OX (Mash et al., 1985). Binding of [3 H]-PZ was used to verify M₁ 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 μ m) with a Damon cryostat-microtome and slide-mounted; they were then defatted and incubated with 0.2 nM to 1 nM [3 H]-NMS, 0.2 nM to 1 nM [3 H]-QNB, 2 nM to 10 nM [3 H]-PZ and 2 nM to 10 nM [3 H]-OX, according to Clarke et al. (1985). PZ and carbachol displacement of [3 H]-QNB was one method used to differentiate M₁ and M₂ by subtraction. Displacement of NMS by 1 μ M carbachol was used to identify high-affinity muscarinic sites (Wamsley et al., 1980). After washing and drying in cold-desiccated air, the slides were affixed to LKB Ultrafilm and stored in cassettes at room temperature for varying periods of time, 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. 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 H₂O₂ (Bioanalytical Systems, Inc.). Respective peaks corresponding to choline and ACh were collected manually and counted for [³H]-incorporation. Specific activities (dpm/mole) of [³H]-incorporation were utilized to calculate turnover ($k \times$ content of ACh). The rate constant k was compared for the incorporation into and decline in specific activities of choline and ACh (see Smith et al., 1984a; Fiacagni et al., 1976; Jenden et al., 1974 for examples of kinetic models for turnover estimation) according to the following formula:

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

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 detect cholinergic function was assessed (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 ten 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 hyperactivity, 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). 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 non-specific stress was not independent of CER. When CS presentation and shock were combined, there was also 100 % suppression, as predicted, confirming that the CS was still a potent stimulus. However, CS and shock did not behaviorally characterize the suppression. 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 responding 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: LD₀ less than 40 ug/kg; LD₁₀ = 50 ug/kg; LD₃₀ = 66 ug/kg; LD₅₀ = 83-86 ug/kg; LD₉₀ = 118 ug/kg; and LD₁₀₀ 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 compiled as a cumulative mean score. Activity was measured in 8 X 8 cell array Digiscan activity monitors in 2-min time bins over a 10 min total period. (Refer to Table 6 and Figures 2 and 3). Samples were also collected for determination of cholinesterase activity, but the results were not yet available. We are now ready to begin the next behavioral experiment, which

involves the ability of chronic XGD to exacerbate CER extinction, based on the hypothesis that excess cholinergic function will prolong anxiety.

NEUROCHEMICAL STUDIES

The brains of animals from Behavioral Experiments 2-4 were harvested for receptor studies. Our results for binding site analysis to total particulate 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 Horvath, Traber and Spencer, personal communications). 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 a¹, including lipophilic, sites; iii) the summations of M₁ and M₂ did not add up to 100 %, supporting the understanding that there are more than two M₁ sites; and iv) that high-affinity M₂-like sites followed the patterns of M₂ 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 (M₁), suggesting predominantly post-synaptic sites. There were no changes in OX binding, but the percentage of M₂ sites appeared to fluctuate; in reality, it merely reflected a relatively larger portion of the total binding sites in the absence of M₁ 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 (M₁) sites, since OX binding did not change, although the percentage of M₂ 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 (M₁) binding; there were no changes in OX binding, but there were changes in the percentage of M₂ 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 M₂, that implied that changes were in PZ sites (M₁). 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.

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. Due to focus on the other aspects of the project and manpower shortages, we have not completed these analyses. However, to demonstrate that we can utilize this sophisticated methodology, we have included two representative examples of the binding of 2 nM [³H]-OX and 0.2 nM [³H]-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 [³H]-OX appear much greater than [³H]-QNB; however, the respective autoradiograms do not imply that OX binding is greater than QNB, since different concentrations of each radioligand (circa their K_d'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. As time permits, we will begin analyzing the harvested tissues, for comparison with the results presented in Tables 7-10.

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

consensus by other investigators on the respective distributions. Our observations support the contention that i) M₁ sites are relatively higher in the neocortex, hippocampus, caudate-putamen and amygdala; and ii) M₂ sites are found in the cortex, septum, caudate-putamen and amygdala. M₃ 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 M₂ and carbachol-sensitive-NMS displacement. Finally since M₁ sites accounted for the plasticity in total binding with respect to CER behavior, the percentage of M₂ sites varied inversely, i.e., a decrease in M₁ sites would be perceived as an increase in M₂ sites. In all, our observations now allow us to focus future attention on M₁ sites with respect to CER.

CONCLUSIONS

The implications for the experiments are clear—the CER model mimics cholinergic hyperactivity in CNS sites involved with anxiety. This condition is analogous to the impact of exposure to sublethal doses of CD agents. The combination of XGD treatment and CER on behavior and neurochemistry will now be explored. Experiments will now focus on QNB, PZ and OX binding in the cortex and hippocampus. The use of NMS and other brain regions (diencephalon and striatum) will be deleted, since they yielded no interesting information, other than to rule out a generalized effect on all cholinergic systems. This will allow time to complete some receptor function experiments and analysis of other M₁ subtype sites. The PI cannot predict *a priori* the interactions of XGD, CER and neurochemistry, but studies will be continued along the same general plan, and be modified as needed, contingent on future results. In general, the plan of hypothesis testing has proved to be valid and fruitful.

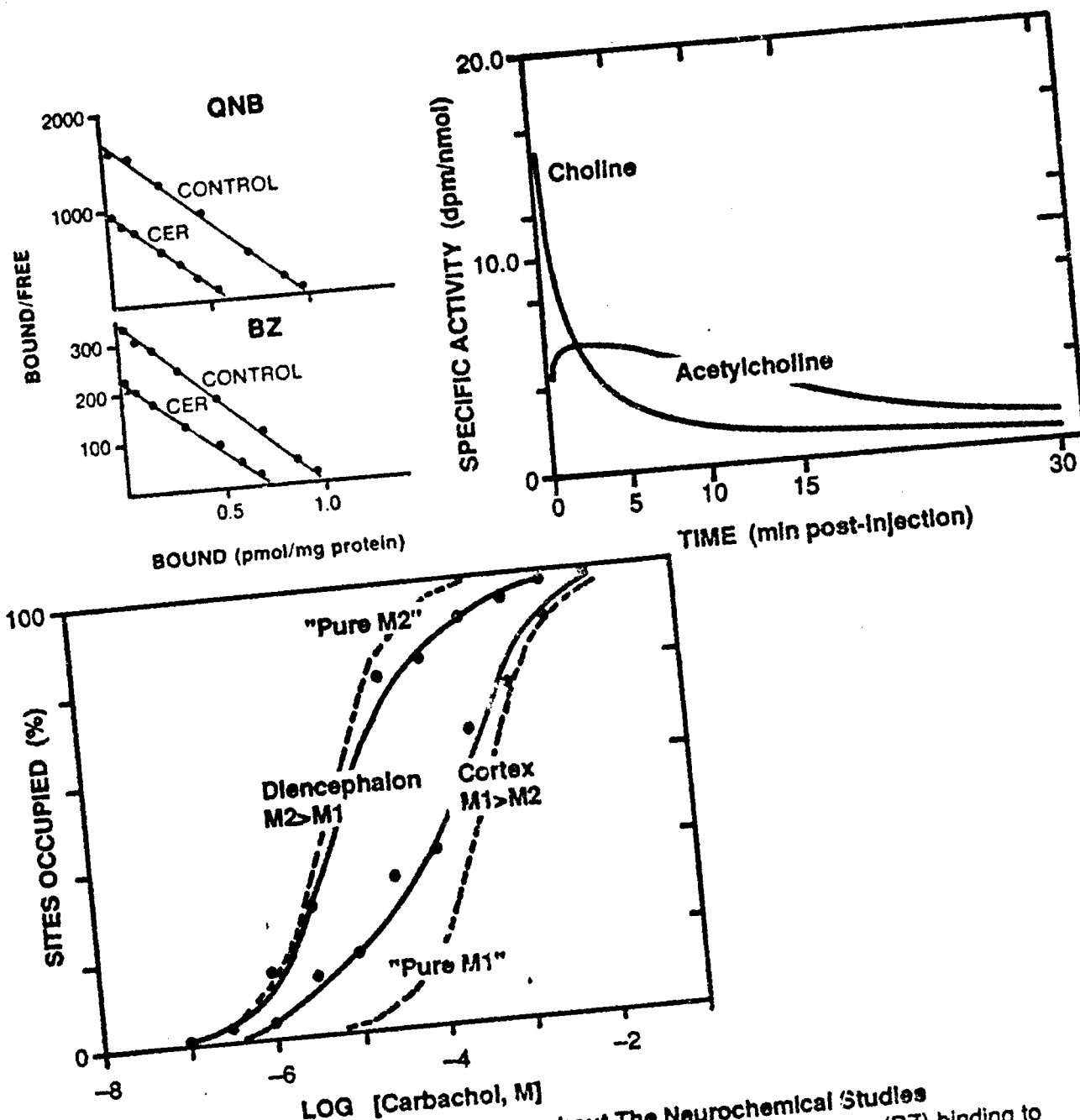


FIGURE 1 – Sample Plots Used Throughout The Neurochemical Studies
Upper Left: Typical Rosenthal (Scatchard) plot for QNB and Diazepam (BZ) binding to brain membranes. The abscissa is binding in mol per mg protein; the ordinate is the ratio of bound/free ligand. The x-intercept defines B_{max} , while the slope = $-1/K_d$.
Upper Right: A plot of the specific activity of tritium incorporation from precursor choline into acetylcholine over time course post-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 $[^3H]$ -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 is used to resolve the actual results into its subtype components. Note the differential distribution of subtypes in different brain regions.

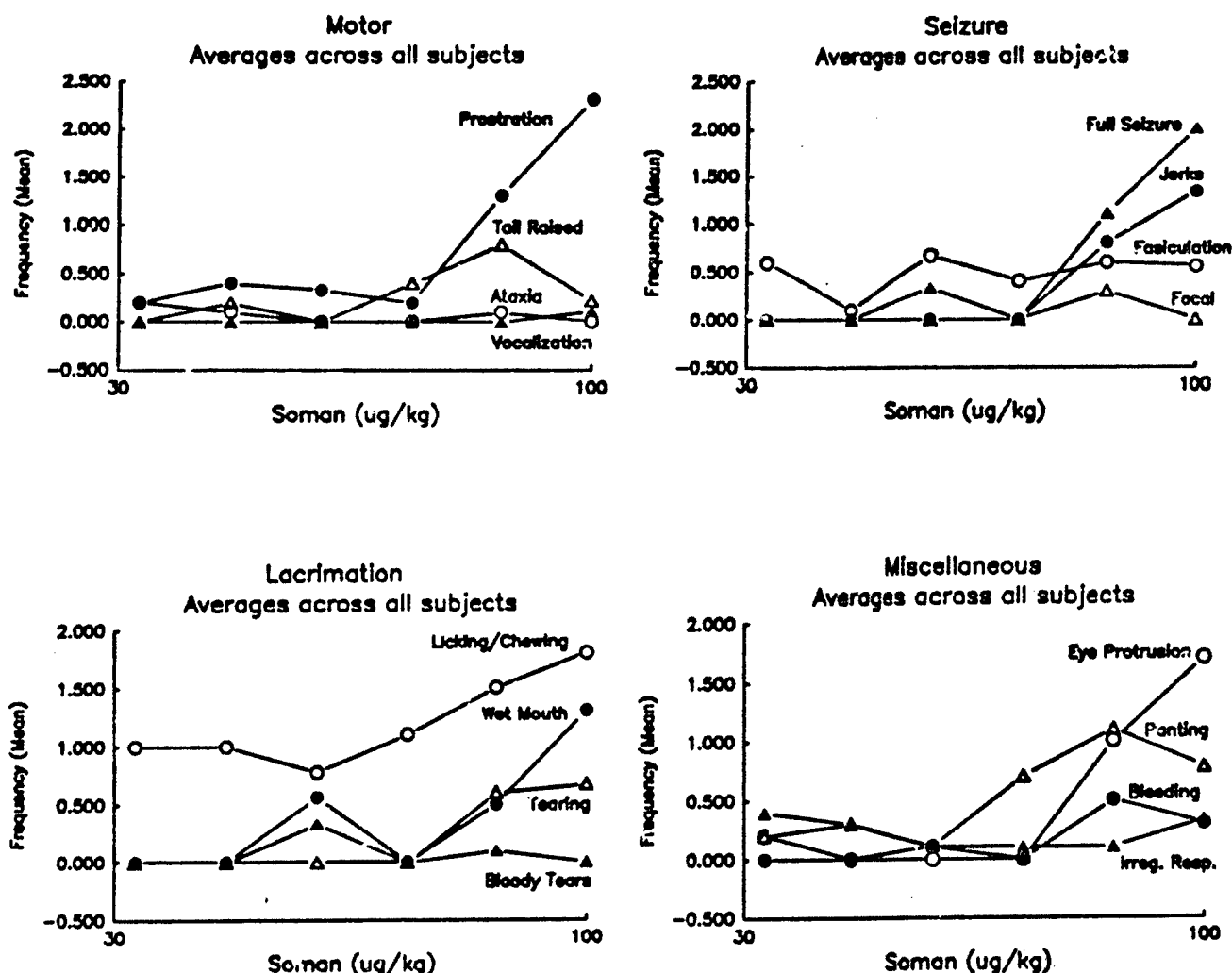


FIGURE 2 -- Behaviors Observed by Single Blind Rater Following Exposure of rats to Soman

Each plot shows the averages across all rats within the group. The abscissa is log-dose of soman (XGD) over the 30-100 ug/kg range. The ordinate is the mean frequency of observable behaviors scored as 0 - 3 units.

Upper Left: Motor function (prostration, tail raising, ataxia and vocalizations)

Upper Right: Incidence and type of seizures (full body tonic-clonic, body jerks, fasciculations, focal seizure)

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 LD₁₀₀, then threshold "toxic signs" of behaviors were observed circa 50 - 80 ug/kg.

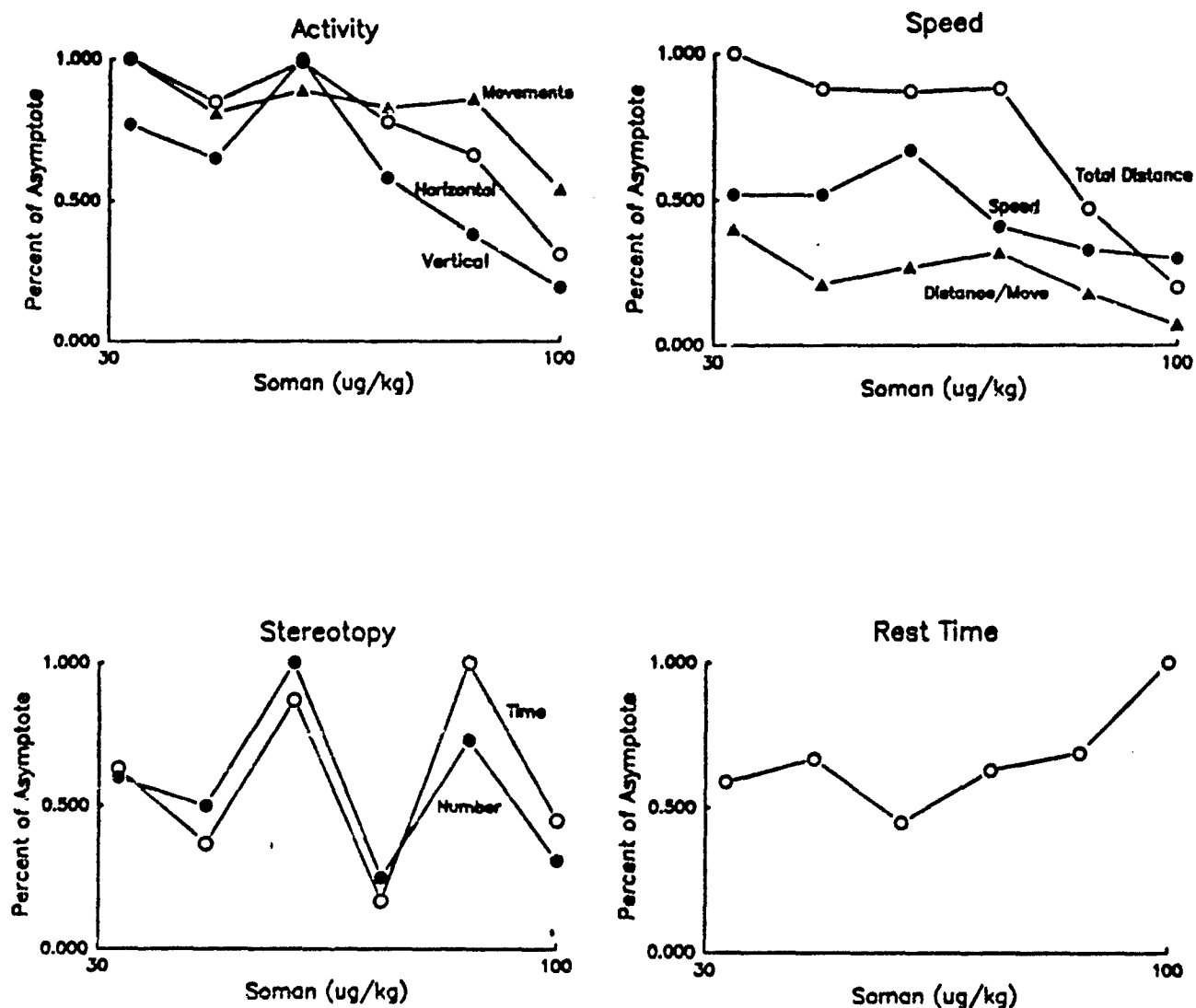


FIGURE 3 -- Behaviors Detected by Digiscan Activity Apparatus Following Exposure of Rats to Soman

Each plot shows the averages across all rats within the group. The abscissa is log-dose of soman (XGD) over the 30-100 ug/kg range. The ordinate is summation of average asymptotically fractionated value for Digiscan activity measures.

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

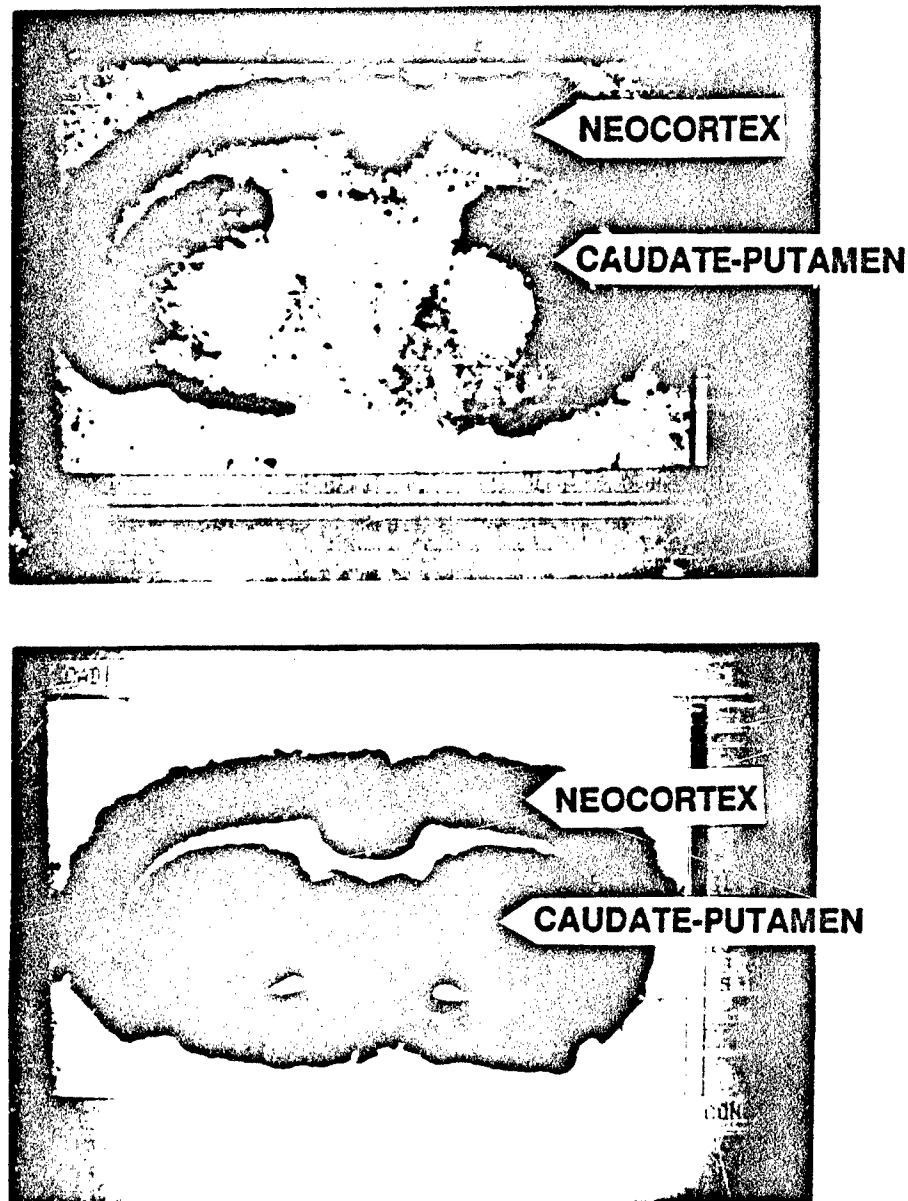


FIGURE 4 -- *In Vitro* Binding Site Autoradiography

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 rats.

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.

TABLE 1 -- TRAINING AND CONDITIONING PROTOCOLS FOR CER RATS USED IN MULTIPLE BEHAVIOR, EXTINCTION, NON-SPECIFIC STRESS AND ACETYLCHOLINE TURNOVER 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	Respondent conditioning sessions: Morning - 60 min VI1 sessions; Afternoon - light-tone (CS) paired with footshock
5 days	Recovery: 60 min VI1 sessions until stable responding returns to pre-conditioning rates
Testday - 45 min - in general and Option A	30 min VI1 sessions, followed by 15 min continuous CS (or equivalent for control groups)
Testday - post-45 min - Option A ONLY	Individual rats immediately tested for Digiscan activity measures, passive avoidance or active avoidance
Testday - Option B ONLY	During VI1 sessions, rats receive once daily or more frequent presentations of CS for 10 days (extinction)
Testday - Option C ONLY	During CS or equivalent presentations, groups of animals receive random footshocks to mimic non-specific, unavoidable stress
Testday - Option D ONLY	During CS or equivalent presentations, rats received 0.5 mCi [³ H]-choline i.v. at 2-15 min, scheduled so that sacrifice post-injection occurred at the end of the 15 min continuous CS

All rats received the same general protocol, subject to the four options which demonstrate the idiosyncrasies of each of the four experiments. FR1 - this paradigm delivers 1 food pellet after each lever press by the rat, and tends to elicit high rates of erratic responding; VI1 - this paradigm delivers 1 food pellet after the first lever press by the rat on a random schedule that averages 1 min, and tends to elicit moderate rates of stable responding; CS - the light-tone conditioning stimulus; Option C - groups of rats received 90 random 0.5 msec, 1 mA footshocks

TABLE 2 - EFFECTS OF CER ON OTHER BEHAVIORS WHICH DETECT 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 $\pm$ 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.

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

Behavioral Group	V11 Responding Before CS Presentation (or Equivalent)		V11 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 Testday		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 (KGD) 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 post-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 FOUR RAT BRAIN REGIONS

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	882 ± 95*	17	45	1340 ± 170	2.4	51
72-96	1550 ± 172	0.17	380 ± 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	1100 ± 189	0.10	59	1326 ± 158	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 M2-like sites are carbachol-sensitive; % M1 and/or %M2 sites may not equal 100 % since more than two M1 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 FOUR RAT BRAIN REGIONS

Brain Region and Behavior	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 M₂-like sites are carbachol-sensitive; % M₁ and/or %M₂ sites may not equal 100 % since more than two M₁ 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 FOUR RAT BRAIN REGIONS

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	18	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	18	35	1478 ± 167	1.7	38	2005 ± 406*	18
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	18
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 M₂-like sites are carbachol-sensitive; % M₁ and/or %M₂ sites may not equal 100 % since more than two M₁ 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 AND GABA-ERGIC BINDING PARAMETERS IN FOUR RAT BRAIN REGIONS

Brain Region and Behavior	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 M₂-like sites are carbachol-sensitive; % M₁ and/or %M₂ sites may not equal 100 % since more than two M₁ 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 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%)

Data represent means ± S.D. for N=28-29 collapsed into two behavioral groups and N=7-8 within neurochemical groups used for each time point post-injection. Since (K) is a derived function, it has no inherent variance; ACh turnover measures reflect variances in ACh content. *p< 0.001.

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