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The effects of benzodiazepines and beta-carbolines on rat pup ultrasonic isolation calls

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THE EFFECTS OF BENZODIAZEPINES AND BETA-CARBOLINES ON RAT PUP ULTRASONIC ISOLATION CALLS

by

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Abstract

THE EFFECTS OF BENZODIAZEPINES AND BETA-CARBOLINES ON RAT PUP ULTRASONIC ISOLATION CALLS

by

Sara Thom

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The separation of rat pups from their mothers and littermates leads to increases in vocalization and locomotion. Chlordiazepoxide (1 + 3 mg/kg) reduces these vocalizations in one and two week old pups without reducing locomotor behavior. Substances active at the GABA-benzodiazepine receptor-chloride channel complex, pentylenetetrazol (15 + 30 mg/kg), methyl beta-carboline-3-carboxylate (3 + 9 mg/kg) and ethyl beta-carboline-3-carboxylate (1 microgram ICI) increase the number of vocalizations and decrease locomotor responses to isolation. These changes do not appear to be secondary to non-specific effect of thermoregulation or arousal. Temporal analysis of response changes and dose effects are reminiscent of adult rodent responses to conflict /punished response tests. These results are consistent with the hypothesis that rat pup separation distress behaviors in 2 week old pups may be mediated by the GABA-benzodiazepine receptor chloride channel complex.

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Without the support and cooperation of my husband and sons, this enterprize would surely have failed and I am deeply grateful for their continuing affection and patience.

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Introduction

"Anxiety Peptide found in brain, a peptide that occurs naturally in brain appears to act through the benzodiazepine receptor to bring about an increase in anxiety" was the title of a one page news article in the March, 1984 issue of Science . This announcement heralded a major breakthrough in the search for the specific chemical in the brain that might be responsible for "anxiety". The search for this elusive substance had been intense following the discovery in the 1970's that the brain contained specific receptors for benzodiazepines, a class of drugs known for their ability to reduce anxiety. Because the discovery of similar brain receptors for morphine had led to the discovery of the endogenous opiate-like substances, endorphin and enkephalin in the human CNS, the existence of a benzodiazepine receptor implied that there was a naturally occurring ligand in the brain that could have the same anxiety reducing effect,in other words, an "endogenous Valium". In the years that followed, various compounds were reported to interact with the benzodiazepine receptor and a number were proposed as endogenous ligands including inosine, hypoxanthine, and nicotinamide. However, until the recent announcement in Science , none were able to meet the necessary criteria.

Until recently, by far the most promising candidate

for the putative endogenous ligand was B-carboline-3-carboxylic acid ethyl ester (B-CCE). This substance, first described in 1977 by the Danish biochemist Klaus Braestrup, was initially isolated from human urine and later extracted from mammalian brain tissue. The chemical structure of this B-carboline was quite suggestive in that it resembled certain plant alkaloids that were known to have mood altering effects in humans. Its most outstanding feature however, was that it was eight times more effective at displacing benzodiazepine in vitro than Diazepam, the most frequently prescribed anxiolytic benzodiazepine. In other words, B-CCE was a substance that had been "found" in mammalian brain, was structurally similar to mood altering drugs and had an exceptionally high affinity for the receptor in question. All that remained was to determine whether of not this B-carboline had the pharmacological profile of the benzodiazepines, specifically, could it relieve anxiety in humans?

The following year, four volunteers were administered a more stable B-carboline, B-CCMA, intravenously so that blood levels of the substance could be monitored during the test of the drug's effect on anxiety. During the test, two of the volunteers experienced severe anxiety attacks, one subject was so anxious that he refused to allow members of the research team to approach him to take blood samples, the other demanded that the test be stopped and he was

administered an antidote. They reported experiencing "almost intolerable tension" and one described a feeling of "impending doom" (Dorow, Horowski, Paschelke, Amin and Braestrup, 1983).

It was this moment in the history of the benzodiazepine receptor research that an entirely new set of possibilities became apparent. Before the test of the drug in humans, the search had been directed toward finding a substance in the brain that was benzodiazepine-like, that is, anxiety reducing, anti-convulsant and sedative, now it became apparent that this elusive endogenous ligand might actually cause anxiety.

Although it was later reported that B-CCE was probably not an endogenous ligand but was produced artifactually from the Pictet-Spengler condensation of tryptophan during the extraction process, it's discovery was highly significant. For with the discovery of B-CCE, the psychopharmacology of the benzodiazepine receptor became unique. When this B-carboline was found to interact with the benzodiazepine receptor site and induce anxiety it was the first time in pharmacology that receptors were shown to respond in entirely different directions depending upon the nature of the ligand (Braestrup, Nielsen, Honore, Jensen and Petersen, 1983). These investigations became the foundation of what would later be called the benzodiazepine receptor model of anxiety, which was, in

brief, that since benzodiazepine receptors bind compounds that reduce anxiety as well as novel substances that can precipitate anxiety, these receptors may mediate both the affective and physiological effects of anxiety.

As early as 1979 another research team from Denmark published the results from a series of investigations into the interaction of benzodiazepines with g-aminobutyric acid (GABA) and these data provided the foundation for the now widely held belief that benzodiazepines exert the majority of their pharmacological effects via GABA-ergic processes (Haefely , Polc, Schaffner, Keller, Pieri and Mohler, 1979). Subsequent investigations indicated that the molecular basis of this interaction was a post synaptic complex of proteins called the GABA-benzodiazepine chloride channel receptor complex (GBRC) and this complex of proteins was the major site of action for a number of psychoactive compounds. Specifically, that benzodiazepine receptor agonists relieve anxiety via interaction with the GBRC, alternatively, B-CCE and other anxiogenic substances such as pentylenetetrazol cause anxiety via interaction with this receptor complex.

It was curiosity about the importance of this "supramolecular complex" for infant rat behavior that initiated this research. Since there was considerable evidence that by the time pups reach two weeks of age they have developed the full adult complement of benzodiazepine

receptors (Candy and Martin, 1979) as well as the GABA-ergic processes associated with the GBRC, we reasoned that pharmacological agents active at these receptors should have some effect on infant rodent behavior. Because of the continuing interest of this laboratory in how early disturbances in the mother-infant relationship may affect the young and predispose them to stress induced disease in later life, the possibility that this molecular complex might mediate some of the "distress behaviors" seen in young rats when separated from their mothers and siblings seemed a viable hypothesis. However, before this notion could be tested, it was necessary to know more about the pharmacology of the GBRC ligands, the effects of "anxiety producing" and "anxiety relieving" agents on behavior in adult rats as well as the nature of the "isolation distress" response in young rats.

These subjects are addressed in the following chapter and provide the foundation for the design of this series of studies which have as their goal an increase in our knowledge of the central mechanisms that mediate "distress behaviors" in young animals.

PHARMACOLOGY

The pharmacology of the GABA-benzodiazepine receptor chloride channel complex (GBRC) has been the subject of a number of articles, symposia and monographs and even a minimal review of this literature is beyond the range of this chapter. The primary purpose of this section is to provide a description of the GBRC and describe the chemical structure, kinetics, bioavailability and effects on behavior of the ligands selected for administration in these experiments. Because the nomenclature associated with the benzodiazepine literature is somewhat specific to this class of drugs, clarification of terminology has also been included when appropriate.

A brief review of the neurobiology of the GBRC is included as well. Although some of this material is not critical to an understanding of the results of these investigations, it was incorporated for the following reasons. First, from a practical point of view, the drugs that were selected for study were chosen on the basis of data from neurobiological investigations. Second, both the methodology and choice of subject were determined by neurobiological constraints. Moreover, knowledge of the actions of these drugs at different levels contributes

importantly to both the interpretations of the results of these studies and suggestions for future research. Finally, it is hoped that a brief review of the neurobiology of this unique molecular structure, thought by many to be critical for the experience of anxiety, will interest readers and increase their appreciation for the recent advances in this field.

Nomenclature

Benzodiazepine Agonists, Antagonists and Inverse Agonists

Agonists - substances that bind to specific receptor sites and initiate the characteristic pharmacological actions of a given ligand. Benzodiazepine agonists produce the anxiolytic, anti-convulsant and sedative effects of the minor tranquilizers (Squires and Braestrup, 1977). Examples include the (1,4) benzodiazepines chlordiazepoxide and diazepam, and the triazolopyridazine CL 218,872.

Antagonists - substances that compete with agonists at receptor sites and prevent the pharmacological actions of that drug. The most frequently prescribed benzodiazepine antagonist is the (1,4) benzodiazepine -3 -carboxylate RO 15 1788 (Bonetti et al. 1982).

Inverse Agonists - substances that bind with high affinity to benzodiazepine receptors, antagonize the pharmacological effects of the benzodiazepines and have potent intrinsic effects of their own that are the opposite of benzodiazepine agonists. Specifically, they increase anxiety, wakefulness, muscle spasticity and seizures. Because the intrinsic effects of these ligands are opposite those of the benzodiazepine agonists they have been called "inverse agonists", "contragonists" and "active agonists". For purposes of continuity, in this paper they will be refered to as inverse agonists. Examples include methyl B-carboline-3-carboxylate (B-CCM) and B-carboline-3-carboxylic acid ethyl ester (B-CCE) (Braestrup and Nielsen, 1981 ;Jones and Oakley,1981).

Type I - Type II Benzodiazepine Receptors

There is considerable evidence that the CNS contains at least two types of benzodiazepine receptors, Type 1 and Type 2 with separate ontogenies, binding affinities, requirements for solubilization, brain location and behavioral effects (For a review see Lippa ,Beer, Sano, Vogel and Meyerson , 1981). The data also suggest that the Type II receptor mediates the sedative/hypnotic properties of the BZD whereas the Type I receptor mediates the anxiety reducing effects of these compounds (But see File and Wilks, 1986). In support of this hypothesis, the Type 1

receptors bind preferentially with both anxiety reducing compounds such as the 1,4 benzodiazepines and the triazolopyridazine CL-218 872 as well as the anxiety producing beta-carbolines. Type I receptors are located post synaptically, distributed widely but unevenly in the brain and they are present in regions that mediate affect, including the rostral medial forebrain bundle in the limbic lobe as well as nuclei within the septum and the amygdala. For a recent review see (Hersh, Garret and Beer, 1985). These authors conclude that although the concept of biochemically heterogenous BZD receptors has become well established, the present techniques have not been sufficient to prove that Type I and Type II receptors independently subserve the anxiolytic or sedative actions of these drugs.

At issue, as well, seems to be the question of what characterizes heterogeneity and is, in part, an argument among neuroscientists over whether or not different conformational states of the same molecular species constitute true heterogeneity. For a detailed discussion of these questions, see Haefely (1985).

The significance of the Type I - Type II distinction for this study is a developmental one. Radioligand studies show only small amounts of Type I BZDP binding in brain homogenate during the first week post partum in rats. However, by day 16 adult levels are reported for this

receptor type (Lippa et al., 1981). Other radioligand surveys have shown that beta-carboline receptors have a developmental course that is essentially indistinguishable from that of Type I receptors (Medina, Novas, and deRobertis, 1983). Because the effect of the beta-carbolines on vocalization is central to this study, the age of the animal selected for study was based on evidence that these Type 1/beta-Carboline receptors in pups do not show adult levels of binding until the end of the second week post partum. Indeed, whether or not the Type 1 receptor solely mediates anxiety (Hersh et al, 1985) is not critical to these investigations. What is important is that these data show that any experiments designed to study the effects of B-carbolines on behavior in infant rats will maximize their effects by choosing pups as close to two weeks of age as the protocol will allow. An issue that will be discussed later in more detail.

The GABA-benzodiazepine receptor-chloride channel complex

The neurobiology of the GABA-benzodiazepine receptor-chloride channel complex (GBRC) has been the subject of a number of articles, monographs and symposia and a review of the literature is beyond the scope of this chapter (for review see Haefely and Polc, 1983). The purpose of this section is to describe the functional and structural characteristics of this supramolecular complex

with regard to the pharmacological effects of chlordiazepoxide, B-CCE, B-CCM, and pentylenetetrazol. The behavioral effects of these compounds will be discussed in greater detail in another section.

The GBRC is described as a complex of proteins with a molecular weight of greater than 200,000 located postsynaptically to neurons releasing g-aminobutyric acid. While many aspects of the molecular interactions within this complex remain unresolved, considerable evidence suggests that the GBRC contains at least three different functional components, a GABA receptor, a chloride channel and more than one benzodiazepine receptor (Figure 1). The allosteric nature of the interaction of compounds at each of these sites indicates that these components exist in close proximity (Tallman and Gallager, 1985) however it has not been established that these components exist on the same polypeptide chain.

Although considerable disagreement exists as to the precise nature of the submolecular events controlling the interaction of these components, since the late 1970's electrophysiologists and pharmacologists have generally agreed that at the cellular level the receptor complex works in the following way.

After the release of GABA into the synaptic cleft it is bound to its receptor protein in the GBRC; this causes the chloride channel to open with greater frequency

allowing the chloride anion to move more freely across the membrane. This results in hyperpolarization or reduced firing rate of critical neurons (inhibition) (Study and Barker, 1981). This hyperpolarization of the postsynaptic membrane via increased chloride flux is the process by which GABA is believed to exert the vast majority of its inhibitory effects throughout the brain.

The functional relationship of the benzodiazepine receptor to the GABA receptor is a modulatory one. When the BZD receptor is occupied by a benzodiazepine receptor agonist such as chlordiazepoxide (in the presence of GABA) the chloride channel opens with even greater frequency leading to increased hyperpolarization and inhibition (Study and Barker, 1981). In other words, the anxiolytic, sedative and anti-convulsant properties of the benzodiazepines are thought to be initiated by an increase in inhibition at GABA-ergic terminal processes. Alternatively, when the BZD receptor interacts with an inverse agonist such as B-CCE, the chloride channel opens less frequently and the post-synaptic membrane is polarized. This results in an increase in the rate of cell firing or a down regulation of GABAergic neurotransmission. Hypothetically then, the increases in anxiety and muscular tension as well as the convulsant properties precipitated by the Beta-carbolines have their origins in the reduction in GABAergic inhibition.

The chloride channel itself is described as a funnel shaped water filled pore containing charged amino acids that determine the rate of flow of chloride across the membrane (Tallman and Gallagher, 1985). A receptor site(s) near or within the channel itself which binds a number of structurally unrelated compounds is the t-butyl/bicyclophosphorothionate (TBPS) site. Various substances that interact with this site may either increase chloride conductance or decrease it, depending on their chemical structure. Barbiturates interact with the TBPS site and have inhibitory effects on the rate of cell firing, whereas the cage convulsant t-butylbicyclophosphorothionate and pentylenetetrazol cause increases in cell firing (Ticku and Olsen, 1979; Squires ,Saederup, Crawley, Skolnick and Paul, 1984).

In summary, there are several classes of structurally unrelated compounds that interact with the various receptor sites of the GBRC and initiate either anxiolytic and anticonvulsant effects or anxiogenic and proconvulsant effects via of regulation of GABA-ergic neurotransmission. For the purposes of this study, examples from the two major groups of anxiolytic/anxiogenic substances that exert their effects via this complex were selected. The first of these groups is composed of compounds that exert their effects via the BZD receptor sites; examples chosen include the agonist chlordiazepoxide and the inverse agonists B-CCE and

B-CCM. The second group contains substances that interact with the TBPS/barbiturate site and the compound selected for investigation was pentylenetetrazol.

Chlordiazepoxide

Chlordiazepoxide is a prototypic benzodiazepine, a class of pharmacological agents developed in the late 1950's by Roche laboratories, that was introduced by the medical community as a minor tranquilizer. The spectrum of pharmacologic activity of the (1,4) benzodiazepines chlordiazepoxide and diazepam includes anxiety reduction, muscle relaxation, sedation and protection against seizures. The most prominent of the pharmacological actions clinically has been their ability to reduce anxiety without causing drowsiness or sedation. The drugs are quite potent, exerting their effects in the 10 to 100nM range which indicates a site of action with a high affinity and specificity for individual structures (Harvey,1980).

When psychologists and psychoanalysts describe human emotional states they distinguish between "anxiety","fear" and panic attacks, distinctions that are important both historically (Freud, 1926) and diagnostically (Millon, 1981).

While clinicians disagree among themselves on precise definitions of these emotional states a very general distinction has been that fear is experienced under

conditions of actual danger whereas anxiety is experienced under conditions of the anticipation of danger (Boissier, Simon and Soubrie, 1976; Klein, 1976). Because benzodiazepines do not distinguish between "fear" and "anxiety" but are equally efficacious in the treatment of real or imagined fears (Harvey, 1980) in the current benzodiazepine literature the general term "anxiolytic" is used to mean both anxiety and fear reducing and "anxiogenic" to mean both anxiety and fear inducing. Because this present research is concerned with the effects of benzodiazepines in animals, this convention has been observed throughout.

Effects of Benzodiazepines on Behavior in Rats

Since a discussion of the effects of benzodiazepines on behavior in rodents is central to a number of issues raised by these studies, a separate section has been included in which the animal models of human anxiety are discussed. In general, however, benzodiazepines, including chlordiazepoxide, are active in a variety of animal tests used to predict the anxiolytic efficacy of new drugs. The most widely used of these tests is the Geller-Seifter procedure (Geller and Seifter, 1960). In this protocol, rats are trained to lever press for milk reinforcement. At discrete intervals a tone signals the onset of a conflict period during which responses are simultaneously reinforced

with milk and punished with electric foot shock. This is thought to create a conflict between the animal's biological need for nourishment and " fear" or" anticipation" of punishment. The ability of benzodiazepines and other drugs that reduce anxiety in humans to increase responding during the "conflict" period is well established (Cook and Sepinwall, 1975). Benzodiazepines also reliably alter behavior in a variety of other experimental protocols involving fear of novelty and intraspecific interactions including social interaction and aggression tests.

Pharmacokinetics

When injected intraperitoneally the drug enters the bloodstream rapidly and there is a fast uptake into the gray matter of the brain. Sullman and his associates (1982) in a review of the relative clinical efficacy of the major benzodiazepines reported that because many of the metabolites of chlordiazepoxide are benzodiazepines themselves with biological activity, the half-life of the anxiolytic,sedative effects of this drug is reported to last from 5 to 30 hours. The residue is excreted almost entirely in the urine (Harvey, 1980).

Effects on Neurotransmitters

While benzodiazepines affect both brain content and turnover of several neurotransmitters these effects are

considered to be secondary to their GABA-ergic activity (Tallman et al., 1980). Specifically, BZD alters GABA neurotransmission in both the locus coeruleus and the raphe which in turn causes alterations in norepinephrine and serotonin neurotransmission respectively. Acute administration of BZD causes decreases in turnover of norepinephrine throughout the brain whereas dopamine is decreased in some regions and increased in others. Serotonin turnover is also decreased. With repeated exposure to the drug (benzodiazepine) tolerance develops to the decrease in norepinephrine, however, this is not the case with serotonin (Stein et al., 1973). Since tolerance develops to the sedative effect of the benzodiazepine but not to their anxiety reducing effects, it has been suggested that the depressant effects of these agents are mediated by noradrenergic processes whereas tryptaminergic processes mediate the anxiolytic effects.(But also see File and Wilks, 1986). For a review of the effects of BZD on monoamines see Costa and Greengard (1975).

Pentylenetetrazol

While pentamethylenetetrazol (pentylenetetrazol, PTZ) is a CNS stimulant best known for its convulsant properties, sub-convulsant doses cause intense anxiety when administered to human research subjects (Rodin, 1985; Rodin and Calhoun, 1970; Lal and Sherman, 1980). In animals, PTZ

doses as low as a quarter of the amount needed to induced seizures are reported to have anxiety inducing or "anxiogenic" effects as well. For example, in conflict avoidance models of anxiety PTZ increased pro-conflict activity in rats (Corda,Blaker,Mendelson, Guidotti and Costa, 1983) and decreased punished drinking in a modified Geller-Seifter procedure in mice (Prado do Carvalho, 1983). In intracranial self stimulation procedures low doses of pentylenetetrazol led to decreases in self-stimulation in rats (Liebman, 1985) and when File and Lister (1984) administered PTZ to rats in a social interaction test the number of interactions were reduced in a dose dependent fashion. In a drug discrimination paradigm in which animals were trained to discriminate PTZ from saline all known anxiety inducing drugs generalized to pentylenetetrazol. Similarly, the anxiety induced by benzodiazepine withdrawal also generalized to PTZ (Lal and Oglesby, 1983). Furthermore, pentylenetetrazol is widely used to antagonize the anxiety reducing effects of the benzodiazepines (Cook and Sepinwall, 1975).

Pharmacokinetics

When dissolved in saline and injected intraperitoneally the drug enters the bloodstream and is rapidly and equally distributed throughout the tissues (Harvey, 1980). At convulsant doses (60-70 mg/kg) EEG changes are observed within 50 seconds of administration in

rodents and seizures can occur within the first minute following the injection. Although rapidly metabolized by the liver to inactive products, anxiogenic effects have been reported in rats up ot 30 minutes after administration (Lal and Oglesby, 1983).

Site of action

One site of action in brain for pentylenetetrazol is the t-butybicyclophosphorothionate (TBPS) site which is one of the receptor sites on the GABA-benzodiazepine receptor complex (Squires et al., 1984.

Beta Carbolines

B-CCE and B-CCM, the ethyl and methyl esters of B-carboline-3-carboxylate bind with high affinity to the benzodiazepine receptor in brain (Squires and Braestrup, 1977; Mohler and Okada, 1977) and present a distinctive pharmacological profile (Nielsen and Braestrup, 1980). In animals B-CCE antagonizes many of the pharmacological actions of benzodiazepines including opposing the anti-convulsant actions of benzodiazepines in mice and rats (Oakley and Jones, 1980; Tenen and Hersh, 1980; Cowen et al., 1980). The most provocative pharmacological characteristic of these B-carbolines however is their ability to have intrinsic effects of their own that are diametrically opposed to the effects of the BZD. For example, B-CCE is not only proconvulsant in rats (Cowen et

al., 1981) it is reported to have anxiety inducing (anxiogenic) effects in rats in a punished response procedure (Corda, et al., 1983),in the social interaction test (File, Lister and Nutt,1982) and in an interoceptive discriminative stimuli model of anxiety (Lal and Sherman, 1980). More recently, intravenous administration of B-CCE in Rhesus monkeys caused dramatic increases in serum levels of the stress related hormones including cortisol, epinephrine and norepinephrine with attendant increases in blood pressure and heart rate. These physiological changes were accompanied by a pronounced behavioral syndrome typically associated with fear in primates (Ninan, Insel, Cohen, Paul and Skolnick,1982). Finally, when a more stable congener of B-CCE, the methylamide B-CCMA, was administered intravenously to human volunteers they reported experiencing extreme anxiety (Dorow, Horowski, Paschelke, Amin and Braestrup,1983).

B-CCM, methyl B-carboline-3-carboxylate, a congener of B-CCE and B-CCMA also binds selectively to the BZD receptor. This compound presents a pharmacological profile similar to that of pentylenetetrazol; at high doses it has convulsant activity (Oakley and Jones, 1982; Braestrup et al., 1982) and at lower doses it has anxiogenic effects. Specifically, in the social interaction test, B-CCM causes a reduction in active interactions without a change in locomotor behavior (Hindley, Hobbs, Paterson and Roberts,

1985), it is anxiogenic in a shock suppressed drinking protocol (Corda et al., 1983) and a conflict test in mice (Carvalho et al., 1983). Furthermore, B-CCM generalized to PTZ in the interoceptive discriminative stimuli model of anxiety (Lal and Sherman, 1980). Finally, the methyl ester of the B-carboline-3-carboxylate was ten times more effective than B-CCE in evoking the pituitary release of the stress associated metabolite B-END-LI (Maiewski, Larscheid, Cook, and Mueller., 1985).

Pharmacokinetics

B-CCE and B-CCM are highly lipophilic and should readily cross the blood brain barrier. However, when these compounds are administered intravenously or intraperitoneally they are rapidly metabolized by serum esterase(s). B-CCE is degraded very rapidly when incubated with rat plasma in vitro at 37 degrees celcius (Mendelson et al., 1982). While B-CCM is degraded rapidly as well, it has a half life in vivo three times that of B-CCE and has been shown to be effective in experimental protocols where intraperitoneal injection is preferred (Hindley et al., 1985; Nutt and Little, 1986; Maiewski et al.,1985).

Site of Action

The convulsant and anxiety inducing activity of both B-CCE and B-CCM are antagonized by the specific benzodiazepine antagonist RO 15-1788. This finding taken

together with evidence from competitive binding surveys of the substances indicate that the pharmacological effects on these behaviors are mediated by the same receptor sites as the agonist (Nutt, Cowen and Little, 1981). Electrophysiological data indicates that these central effects are mediated by interaction with the BZD receptors on the GABA-benzodiazepine receptor complex whereby GABA-ergic neurotransmission is down-regulated.

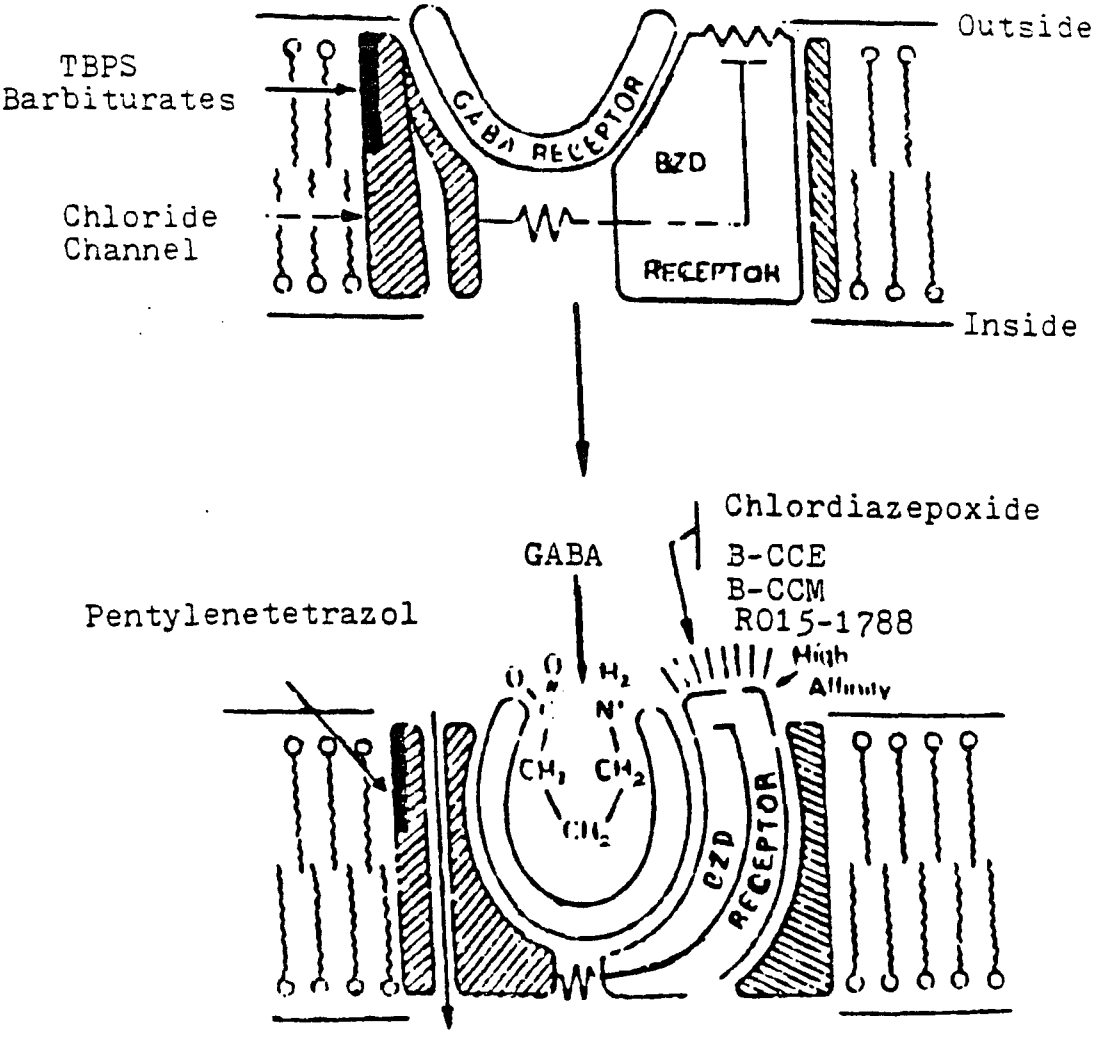
B-CCE has a high affinity (IC 50 1nM Nielsen and Braestrup, 1980) for the Type I benzodiazepine receptor, the subclass of receptor proposed by some (Lippa et al., 1981) to mediate the anxiolytic/anxiogenic effects of the BZD ligands.

Relationship to Endogenous Benzodiazepine Receptor Ligand

The relationship of the B-carbolines to the newly proposed endogenous "anxiety" peptide is central to this study. Both B-CCE and B-CCM share unique histochemical and pharmacological properties with the octo-deca-neuropeptide (ODN) proposed by Costa and his co-workers (1985) as the naturally occuring ligand for the benzodiazepine receptor. Not only does ODN displace benzodiazepines and show selectivity for displacing B-carbolines like B-CCE and B-CCM, it reduces the duration

of chloride channel opening when applied together with GABA . The down regulation of GABA action is antagonized by RO15-1788. Furthermore, behavioral experiments with rats show that B-carbolines and ODN injected intraventricularly both facilitate convulsions and elicit pro-conflict activity (Costa and Guidotti, 1985) .

Figure 1) Schematic representaion of GABA-benzodiazepine chloride channel complex function. Occupation of GABA receptor induces a change in the conformation of the benzodiazepine recognition site resulting in an increased affinity of the receptor for benzodiazepines. (Adapted from C. Braestrup.)



ANIMAL MODELS OF HUMAN ANXIETY

There are a number of excellent papers and monographs reviewing both animal models of behavior (Hanin and Usdin, 1977) and behavioral tests for the measurement of anxiety reducing drugs (Cook and Sepinwall, 1975; Thiebot and Soubrie, 1983). The purpose of this section is not to review this literature but to discuss the different categories of behavioral measures with particular emphasis on those that more directly apply to this project. In the interest of brevity, a distinction has been made between two types of animal tests, those in which anxiolytics have been shown to "reduce anxiety" or otherwise alter behavior and those tests used by pharmaceutical companies to screen for the anxiety reducing properties of drugs.

Three major categories or types of behavioral tests that are sensitive to the effects of anxiety reducing drugs were then selected for a more detailed discussion. These include the conflict or punished response models, exploration in a novel environment and social interaction protocols.

The reasons for these distinctions are first, these selections are helpful in assessing the legitimacy of the concept of "anxiety" in animals, i.e., the notion that benzodiazepines and B-carbolines are changing behavior in animals by altering an "emotional" or "motivational" state, rather than, for example, simply making them less sensitive

to pain, or drowsy, or hyperactive. This issue has been discussed at length in other papers (for review see Hanin and Usdin, 1977) and this discussion is designed only to provide a sample of the supporting evidence by describing and comparing the methodologies of these types of tests. These selections were also made on the basis of their general applicability for this project; for example, an understanding of the effects of benzodiazepines on adult rodents placed in a novel environment may be helpful in interpreting infant responses to isolation.

Since the discovery in the late 1950's that the first benzodiazepine, chlordiazepoxide had a "taming effect" in primates these compounds have been found to be active in a wide variety of behavioral tests in animals. The anxiolytic (1,4) benzodiazepines have been shown to alter behavior, physiology, and blood chemistry reliably in a variety of experimental protocols as diverse as conspecific aggression studies (Fox, Tuckosh and Wilcox, 1970; Malick, 1978; Quenzer, Feldman and Moore, 1974), intercranial self-stimulation studies (for review see Liebman, 1985) food neophobia (Cooper, 1980 ; File, 1980; Johnson, 1978), conditioned emotional response procedures (Millenson and Leslie, 1974; Iverson, 1983) and Sidman-avoidance procedures (Takaori , Yada and Mori, 1969). The significance of the range of the effectiveness of these tests is unclear, however it has been observed that all of

these tests, with the exception of the intercranial self-stimulation procedures, involve "conflict".

By far the most widely used test for "anxiety" in animals is the Geller-Seifter procedure or some modification thereof (Geller and Seifter, 1960; Howard and Pollard, 1977). This "conflict" or "punished response" test was developed specifically for its sensitivity to the anxiolytic effects of the benzodiazepines and it has continued to be used in one form or another by pharmaceutical companies since the mid 1960's. It has proven to be a highly reliable predictor of the ability of novel substances to relieve anxiety clinically. (Sepinwall and Cook, 1978).Because the characteristics of this test are relevant to later discussions concerning the merits of psychopharmacological models of human behavior, a brief description of the procedure has been included here.

In this protocol, rats are trained to lever press to obtain a sweetened milk reward. When the lever is pressed in the presense of a tone each lever press is rewarded with milk but it is also punished with a brief foot shock. In the absense of the tone the animal is never shocked when it bar presses, however it is only randomly and infrequently rewarded with milk in this condition. After several weeks of training, stable low rates of responding during the punishment period and stable high rates in the unpunished trials are reliably obtained.

When rats trained in this protocol are given low to moderate doses of benzodiazepines they show dramatic increases in the number of punished responses- without showing increases in unpunished responses. However, when they are given a higher dose of a benzodiazepine they reduce both their punished and unpunished responses. In other words, rats in this protocol demonstrate a low dose/ high dose response to this class of drugs that is similar to human responses to high and low doses of benzodiazepines. Specifically, low doses of benzodiazepines increase human behaviors inhibited by fear or anxiety whereas high (sedative) doses of the drug lead to decreases in all behavior. It is generally agreed that much of the success of the Geller-Seifter model can be attributed to the design feature that allows the unpunished behavior to act as a control for the potential sedative effect of the benzodiazepines and further aid research in the determination of doses of anxiolytics that might induce sedation clinically.

It is significant that with few exceptions only drugs known to have anxiety reducing effects clinically (benzodiazepines, meprobamate, barbiturates and possibly ethanol) cause increases in punished responses in this procedure (Stein 1981). Furthermore, since analgesic compounds such as morphine are not effective in this paradigm, the benzodiazepines and other anxiolytics are

thought not to increase these responses by reducing the pain of footshock. Further evidence that these compounds mediate behavioral change via alterations in emotional state is the report that well trained rats will stop bar pressing at the onset of the warning stimulus (tone) before they receive a shock. In other words, the benzodiazepines are thought to increase responding during the punishment trial because the fear initiated by the anticipation of pain has been reduced or eliminated. These tests are also referred to as "conflict" tests because this procedure has been interpreted as having created a conflict between the animal's biological need for nourishment and the fear or anticipation of punishment.

This punished response/conflict test has been highly successful in predicting the clinical anti-anxiety efficacy of new drugs. Furthermore, there is a very high correlation between the minimum anti-conflict dose in rats and the average daily dose of benzodiazepine prescribed clinically for the reduction of anxiety (Sepinwall and Cook, 1978). It is also of interest that the correlation between the number of binding sites occupied in brain and the degree of drug effectiveness in this test is high as well (Lippa et al., 1978).

Novelty Induced Behavior

Neophobia, or novelty induced fear or anxiety has

been assessed by measuring the amount of locomotion or food consumption in rodents when they are placed in an unfamiliar environment. There are a variety of different "environments" and procedures that have been designed to test specific aspects of these behavioral changes including test arenas that provide opportunities for exploration that are independent of locomotor behavior such as the holeboard test (File and Wardill, 1975), "Y" mazes , miniature staircases (Boissier, 1976) and light and dark chambers (Crawley and Goodwin, 1980). The unifying concept behind these procedures is 1) when animals are placed in an unfamiliar place they experience "fear" or "anxiety" which leads to reduced locomotor behavior and food consumption and/or 2) the procedure sets up a conflict between competing biological propensities to explore and to retreat from an unknown space (Crawley et al., 1980). Benzodiazepines and other clinically active anxiolytics reliably restore exploration and food consumption in these protocols. One of the characteristic findings of tests involving novel environments is that the differences in locomotor behavior between rats given anxiolytic doses of benzodiazepines and their controls can only be seen in the first few minutes of their exposure to the unfamiliar space. A finding that will be discussed later in greater detail.

The final class of procedures to be considered here

are those in which "normal" social interaction between conspecifics is disrupted by the investigator and anxiolytic drugs are administered to restore the behavior to normal. Typically, these increases are thought to be the result of changes in emotional state, i.e. increases in fearfulness and the drugs are presumed to reduce or eliminate this "anxiety". It has been noted however, that these procedures can also be interpreted as creating a conflict between competing responses.

These kinds of studies encompass a wider range of designs and are not usually grouped together. For example, they include procedures in which male rats are placed together and fighting is induced by shock or drugs (Malick, 1978; Quenzer et al., 1974), protocols in which rats are placed in novel environments under bright lights and their social interactions monitored (Niesink and Ree, 1983), and studies in which active and passive interactions are compared in adult males that have been isolated (File, 1980).

This last example, the "social interaction test" has been used primarily in England and is reported to be sensitive to the subtle differences in the effects of benzodiazepoine agonists, antagonists and inverse agonists (File and Lister, 1983; File, Lister and Nutt, 1982; File and Pellow, 1984).

In this protocol adult male rats, isolated for seven

days prior to the test are placed together in a familiar test area. Measures of the following behaviors are recorded for each rat: sniffing, following, grooming, mounting, boxing, kicking, and pushing. Separate scores are kept for passive contact time and infrared beams provide an automatic measure of overall motor activity. The results are analyzed in terms of the amount of time engaged in active social interaction versus the time spent in passive contact. Chronic administration of benzodiazepines causes an increase in social interaction behaviors without causing increases in locomotor behavior.

From the perspective of this research proposal, this procedure is notable for other reasons. First, data from these studies show that social interactions between adult rodents are responsive to benzodiazepine administration. Furthermore, and more importantly for this research, the anxiety inducing B-CCE is active in this model. While B-carbolines are effective in other animal models of anxiety, (Ninan et al., 1982) due to their rapid degradation in rat serum (Mendelson et al, 1982) they have not been as effective in procedures that require the monitoring of behavior over extended periods of time, such as the Geller-Seifter test.

Since the primary focus of this investigation is on the anxiety producing ligands of the GBRC, a review of this literature suggests that while there are a wide variety of

behavioral tests in which benzodiazepines are effective, fewer tests have proven themselves sensitive to the anxiety provoking B-carbolines. The test that has demonstrated an ability to distinguish subtle differences in the effects of benzodiazepine agonists (File, 1980) and antagonists, as well as inverse agonists in rodents is the social interaction test (File, Lister and Nutt, 1982). This suggests that a behavior in infant rats that might be responsive to these compounds is one that incorporates social interaction.

"Anxiety" in animals

Traditionally, the use of a word such as anxiety to describe animal behavior has been carefully proscribed. The standard issues raised by casual comparisons of human emotion with animal "motivational states" are well known to workers in this field and will not be reviewed here. The more specific problems associated with the concepts of anxiety in humans and fear in rodents are described in detail in Hanin and Usdin (1977).

In recent psychopharmacology literature there appears to be an increase in the use of the word anxiety to describe a variety of experimental animal behaviors. Moreover, traditional warnings about the dangers of anthropomorphism and convergent evolution are becoming

harder to find. Unquestionably, this is in large part the result of advances in neurobiology, histochemistry and pharmacology of the benzodiazepine receptor described earlier which demonstrate that human and rodent brains contain similar processes that bind equivalent amounts of radioactive benzodiazepines and other substances that interact with these receptors (for a recent review see Muller, 1987). What has probobly been even more influential in the general acceptance of the legitimacy of this extrapolation is, of course, the behavioral data. For even if eqivalent brain areas in human and rodents share similar densities of benzodiazepine receptors, unless a correlation between behavioral change and drug can be shown in both species, extrapolation of function is impossible. For this reason, the strength of the behavioral data has contributed significantly to the tendency of some researchers to be more aggressive in their use of the word anxiety to describe behavior in animals.. This strength is evidenced by the following; (1) the most frequently utilized animal models for testing the benzodiazepines are intuitively appropriate (from an anthropomorphic perspective) as measures of "fear" in rats (2) the most frequently cited test, Geller-Seifter, has been highly successful in predicting the ability of new drugs to relieve anxiety clinically (3) the correlation between the doses of benzodiazepine necessary to reduce

anxiety in patients and the dose required to cause changes in punished behavior in rats is "very high" (Sepinwall & Cook, 1978) finally, there is also a high correlation between the number of occupied binding sites in brain and drug effectiveness (Lippa et al., 1978). As a result of these and other behavioral data many psychopharmacologists have accepted the idea that the benzodiazepine receptors do mediate "fear" in humans and rats.

While the evidence, particularly from the behavioral studies, supports such a hypothesis, there are other problems associated with the scientific use of one word to describe behaviors in humans and rats. One of the more serious problems is that while the benzodiazepine receptor is clearly implicated in "fear"responses in both humans and animals, the specific pathways and other physiological processes involved remain to be elucidated and until that time, they must be presumed to be different in human and rats. For this and other reasons, (Hanin and Usdin, 1977) the use of the term anxiety in rats is misleading. Therefore, where I have sometimes used the word "anxiety" (in quotation marks) or more often, the expression "experimental anxiety" to describe some animal behavior, I do not mean to suggest that (1) the animal experiences "fear" or "anxiety" as it is experienced by humans or (2) that the physiological processes necessary for the experience of the motivational state of "fear" are

identical in human and rats. These expressions are used only as a simplified way of expressing the ideas implicit in a variety of animal behavior protocols designed to test drugs that reduce fear / anxiety clinically.

Finally, the purpose of the introductory chapter which included pharmacological and neurobiological evidence of the similarities of the human and animal benzodiazepine receptors was not to argue for a completely homologous model, but to show that there is clear evidence that there are specific receptors for benzodiazepines and beta-carbolines and that these receptors appear to mediate emotional states in humans and similar motivational states in rats in a dose related fashion.

SEPARATION

"So long as the required proximity to the attachment figure can be maintained no unpleasant feeling is experienced. When however, proximity cannot be maintained because the figure is lost... the consequent searching and striving are accompanied by a sense of disquiet, more or less acute. In this disquiet at separation and in the threat of separation Freud in his later work came to see the "key to an understanding of anxiety."

John Bowlby

If Freud is right, if the "key to an understanding of anxiety" is in the nature of the response to separation, then the study of the behaviors of infant animals isolated from their mothers may be informative. Certainly, the distress behaviors of human infants and neonates of many animal species when abruptly separated from their mothers are strikingly similar. While no one would suggest that what is experienced as anxiety by a human child is the same as that experienced by infant animals, there is mounting evidence that the internal state associated with the spontaneous crying and searching behaviors seen in both may be mediated by homologous neurobiological processes. As we

have seen, there are a number of pharmacological agents that either relieve or precipitate anxiety in humans that cause strikingly similar changes in behavior in rodents. Furthermore, histological, biochemical and anatomical data indicate that these agents are active at equivalent neurobiological receptor sites in brain (Braestrup et al., 1983). While it is difficult to translate very general statements such as "the key to an understanding of anxiety may be found in separation" into a testable hypothesis, the statement "behaviors characteristic of separated infants respond to the administration of anti-anxiety and pro-anxiety compounds" can be tested. For if the spontaneous crying and "searching" seen in isolated pups should prove to be sensitive to the effects of these agents, it would provide the basis for further investigation into the nature of the relationship between separation and anxiety.

Ultrasonic Vocalizations in Isolated Rat Pups

It is a commonplace that helpless young mammals if separated from their mothers will cry. This response is not learned but occurs spontaneously and in many species the cries begin immediately upon separation from the mother and siblings and continue until exhaustion.

Such is the case with infant rodents. From birth through the second week of life baby rats emit discrete

cries that have been called "distress vocalizations" when separated from their mothers and littermates (For review see Sales and Pye, 1974).

The first comprehensive studies of these rodent vocalizations were conducted by Zeppelius and Schleidt (1956). They suggested that these cries served to alert the mother to the infants separation and elicit a retrieval response, the calls were also thought to aid in the location of stray pups so that they could be returned to the nest. These suggestions were verified by a number of studies showing that with some exceptions, lactating dams of different rodent species orient toward and retrieve ultrasounding but not quiet pups (Beniest-Noirot, 1958; Noirot, 1964; Sewell,1970).

When rat pups of the Wistar strain are separated from their nest and littermates, they emit distress calls from a few hours after birth until approximately day 15 or eye opening. These cries are discrete, coincidental with expiration and laryngeal in origin (Roberts, 1975). They are inaudible to humans occurring in the ultrasonic range, between 30 and 50 Khz and have a developmental course characterized by changes in frequency, pattern and volume (Noirot, 1966; Sewell, 1970).

One of the most consistent findings in the early infant ultrasound research is that rat pups emit two distinctly different types of vocalizations. One type is

given in response to isolation and the other in response to tactile stimulation (Noirot, 1965; Okon, 1971;Sewell, 1968). These calls are reported to be distinguished by their length, rate, intensity and frequency changes over time and lactating dams are reported to respond differently to each type; "isolation calls" resulting in searching behaviors and "handling calls" leading to inhibition or changes in sequences of behavior (Noirot, 1966; Sewell, 1970).

Developmental studies show that the features of the environment that elicit isolation ultrasounding change over time suggesting that what constitutes "distress" in an infant rat changes considerably from birth through weaning. During the first seven to ten days of life, pups cannot regulate their body temperature and when placed in isolation at room temperature (22 degrees celcius) will distress call at high rates, however, if these same animals are isolated in a test container heated to nest temperature (35-37 degrees celcius) they will not vocalize at all (Allin and Banks, 1971; Okon, 1971). While temperature continues to influence the number of ultrasounds emitted in isolation during the second week of life, as the sensory system matures, the pups become responsive to other features of the environment. For example, novel odors or changes in the texture of the test surface cause isolated pups to increase the number of distress calls they emit.

(Oswalt and Meier, 1975).

By the time Wistar pups reach 14 days of age they are advanced developmentally. They are covered with fur and although their eyes are not yet open they sometimes leave the nest and move about the cage, returning to the nest area on their own, without being retrieved by the mother. When these pups are removed from their nest and littermates they may not vocalize immediately and when they do begin to ultrasound, the rate of calling is less than that reported for younger rats (Okon, 1972). Temperature is no longer the most salient feature of their environment and an isolated pup will continue to distress vocalize even in a warm test container. At this point in their development, the only stimulus that significantly reduces distress vocalizations is the return of the mother or a sibling (Hofer and Shair, 1978). Hofer and Shair found that a single anesthetized pup would reduce distress vocalizations from 60-90% and that both anesthetized dams and pups were equally effective in reducing the number of cries made by these pups. Taken together, these studies show that while young pups ultrasound primarily in response to temperature changes, older pups are responding to isolation or "social separation". These and other data suggest that vocalizations in rat pups are mediated by different mechanisms at different ages and the more mature 2 week old pup may more closely resemble the adult rat.

These studies also illustrate the necessity for using caution when discussing "anxiety" in infant rats. It is clear, for example, that in a 1 week old pup what might appear to be a fearful response to separation is simply a thermotaxic response to a change in ambient temperature. It is less clear what is happening at two weeks of age. The finding that two week old pups cry less in a novel environment when they are in the company of a sibling could be seen as evidence that isolated pups of this age experience "fear" and that a companion has fear relieving properties. However, there are alternative explanations. Moreover, should it be shown that anxiety reducing substances are capable of reducing isolation crying in these pups, this would not mean that these drugs are relieving "fear", only that the receptors and pathways that mediate the activity of these compounds in adult rats are developed enough to respond to these drugs in characteristically adult fashion. For example, when benzodiazepines and beta-carboline are administered to 4 day old pups, both drugs have the same effects on behavior, specifically, they cause seizure-like activity (Nutt & Little, 1986). It is only after maturation of the receptors and pathways involved that the divergent adult behavioral responses to these drugs is expressed.

The developmental data also introduce the issue of homologous versus analogous systems which arise when

behavior and evolution are discussed. As noted earlier, it is not my intention when using the words "anxiety" and "separation" to suggest that either the physiological processes necessary for or the emotional/motivational states themselves are homologous in human infants and rat pups. While altricial mammals and birds require parental care and any lengthy separation from these caretakers may constitute a serious threat, each species has developed unique strategys for maintaining parental/infant proximity. Both human infants and rat pups exhibit increased vocal responses and hyperactivity under certain conditions when separated from their mother, however these conditions vary for rodents and humans and reflect the nature of the evolutionary parental care strategy of each. For example, humans in primitive societies and other primates are in virtually constant contact with their young (Konner, 1972). This is possible because food and other requirements of human mothers and infants are provided by family members, often the father of the infant . In rodents, however, the mother must fend for her young which requires that she leave her offspring for as long as 1 or 2 hours at a time when they are as young as two weeks of age (Grota & Ader, 1969). In this parental care strategem the thermotaxic response of young pups which leads to huddling behavior serves to keep the pups together and conserve body heat during the mothers absense. Therefore, pups do not exhibit

"separation distress" when the mother leaves the nest. Increased crying and locomotion is typically observed only when pups are separated from both mother and littermates.

Although ultrasonic vocalizations in infant rats emitted in response to separation disappear around the end of the second week of life, they reappear in adult rodents in other situations involving social interactions (For review see Sales and Pye, 1974). According to Sewell (1967) adult laboratory rats emit two different types of ultrasonic calls that can be distinguished both by length and frequency. The "long" calls last up to 700 milliseconds at frequencys of 25 kHz and the "short" calls last from 3-60 milliseconds at 45-70 kHz . In the same report she also noted that the long calls are typically emitted by rats who are engaged in submissive behavior and the short calls emitted by animals engaged in aggressive or threat behavior. From the perspective of the present research proposal it is noteworthy that vocalization in adult rodents appears to be emitted in situations involving conflict; for example, when unfamiliar males are placed together in a novel environment or when males approach females for purposes of mating. In these instances, the conflict would be between the tendency to approach for the purposes of fighting/investigating in male/male encounters and for the purpose of mating in female/male encounters and the tendency to avoid an unfamiliar conspecific.

Furthermore, adult rodents also ultrasound in situations interpreted as fearful, including when they are handled by laboratory personel and when introduced into a novel environment (for review, see Sales and Pye, 1977). While there are alternative explanations of what causes adult rodents to ultrasound in these situations (Amsel et al.,1977; Bell 1974), because USV's in adults are primarily associated with agonistic and sexual encounters in mature animals, they are considered an important form of communication whose function is to signal changes in motivational state between conspecifics (Sewell. 1967).

Distress vocalization in infant animals have been studied in other species as well and it has been reported that a variety of pharmacological agents including opiate derivatives and imipramine cause reductions in vocalizations (for review see Panksepp, Meeker and Bean (1979). These data indicate that although the opioid system is implicated in the mediation of isolation calls in rodents (Herman and Panksepp, 1978; Panksepp et al.,1980) the exclusivity of this class of drugs for this behavior may be difficult to establish. Furthermore, the question of the nonspecific sedative effects of opiates on this behavior has not been fully resolved (Insel, Hill and Mayor, 1986).

Most recently, two other laboratories have independently reported that benzodiazepines reduce

ultrasonic vocalizations in isolated pups and have suggested that these reductions may be mediated by anxiolytic properties of these agents (Gardner, 1985; Insel et al., 1986). Insel and his group also administered an inverse agonist intraperitoneally to young pups (6-10) days of age and found that this led to inconsistent increases in USV's that reached statistical significance only after pups with high rates of calling were excluded from the results. The failure of this compound to increase USV's reliably was attributed to the variability in baseline ultrasounding and the rapid degradation of the inverse agonist ,B-CCE, in rat serum.

Need for New Animal Models of Human Anxiety

It is not coincidental that other laboratories have begun to search for new animal models of human anxiety. The discovery that the naturally occuring ligand for the BZDP receptor may actually cause anxiety rather than relieve it has changed the requirements of animal models radically. They must now not only be sensitive to the anxiety reducing properties of drugs they must be able to show "anxiogenic" effects as well.

While modifications of the conflict procedures (Vogel et al., 1971) and social behavior tests have been developed to measure the anxiety inducing properties of the inverse agonists such as B-CCE and B-CCM, these tests require

considerable training of test animals or they involve extensive observation time by trained personnel. Although single trial, single index tests have been reported that screen for the "anxiety" producing effects in mice (Crawley, et al. 1981), none has been reported for laboratory rats. Since the preponderance of neurophysiological, biochemical and pharmacological data available on the effects of these compounds is based on information derived from rat brain and behavior studies and since mice and rat brains are disimilar in critical ways, it is important that animal behavior models be developed in the laboratory rat. One of the purposes of this series of studies was to investigate the possibility that changes in the rate of ultrasonic vocalizations in isolated rat pups might provide such a model, specifically that the isolation call might provide a behavioral index for the effects of compounds that exert their actions via the GABA-benzodiazepine receptor-chloride channel complex.

In conclusion, a review of the distress vocalization literature supports the suggestion that isolation induced ultrasound in rat pups has characteristics that make it a suitable potential candidate for a behavioral model of anxiety in the developing rat. These characteristics include 1) the behavior itself is discrete and therefore lends itself to quantitative analysis 2) the vocal response to isolation is spontaneous and unlearned and no

assumptions of thirst, hunger or pain threshold are required, 3) the procedure necessary for the behavior to occur, isolation in a novel environment, is non-invasive and ecologically appropriate, 4) the vocal response to isolation can be distinguished from other vocalizations throughout development and although it appears to be mediated by ambient temperature in young pups, reports indicate that discrete neurobiologicalprocesses mediate this behavior from birth onward and 5) finally, since a lengthy separation from the mother in helpless mammals constitutes a deadly threat and "anxiety" in both adult rodents and humans is thought to be an emotional response to potential danger, isolation in infant rodents may be the first manifestation of this internal state, or stated more conservatively, the processes that mediate perceived threat of injury in adults may be the same processes underlying the threat of separation in infant rodents.

Project Design

While the primary objective of this project is to investigate the possibility that distress vocalizations in isolated rat pups might have potential as an animal model of anxiety, the studies themselves are designed to address the following questions; 1) Does the GABA-benzodiazepine receptor chloride channel complex play a role in the mediation of isolation distress vocalizations? 2) Drugs acting at individual receptor sites on this molecular complex have pharmacological profiles that include changes in arousal, muscle tone and susceptibility to seizures as well as changes in levels of "anxiety", therefore, are the changes in vocalization following the administration of these GBRC ligands the result of the anxiolytic/anxiogenic actions of these drugs or are they secondary to other effects?

Although the GABA-benzodiazepine receptor chloride channel complex has been proposed as the mechanism whereby "anxiety" is mediated in adult rats, and radioligand surveys show that in 2 week old rat pups the GBRC is actively binding adult levels of psychoactive drugs, few studies have reported the effects of these compounds on behavior in young animals. Since substances active at receptor sites on this complex both relieve and precipitate

experimental anxiety in adult rodents, we proposed that the anxiety reducing ligands (anxiolytics) might decrease distress vocalizations in rat pups separated from their mothers and littermates. Similarly, we proposed that substances shown to have anxiety inducing (anxiogenic) properties in humans and adult rodents might increase these cries. To test these hypotheses, five separate GBRC ligands including the benzodiazepine receptor agonist chlordiazepoxide, the prototypic BZD receptor antagonist R015-1788, the ethyl and methyl esters of B-carboline -3-carboxylate and pentylenetetrazol were administered to rat pups in a series of individual experiments.

The GBRC ligands chosen for this study were selected on the basis of their receptor affinity/specificity, pharmacokinetic factors and their ability to effect changes in adult rodent social interactions. They were administered to preweanling rat pups of selected ages at appropriate times preceding isolation in a novel environment and the effects of these compounds on ultrasonic vocalizations and locomotion were recorded and analyzed.

The purpose of this section is to 1) discuss some of the major factors influencing the design of the project as a whole as well as individual experiments within the project 2) provide a general methods section 3) and finally, to list the individual experiments giving a brief

description of each.

Major Factors Influencing Project Design

There are three major factors influencing the design of this project; developmental concerns, variability of vocalization response to isolation and pharmacokinetics.

The most important constraints on the overall design of this project are developmental. Because the CNS of the infant rat is undergoing continuous changes in development until at least two months after birth, there is a question of the degree of development of those systems that mediate the pharmacological effects of the drugs active at the GBRC.

While benzodiazepine binding in cerebral cortex is 56% of adult binding capacity at birth (as p mol/g of tissue) and adult levels are reached by day 14 (Candy and Martin, 1979), postnatal surveys of 3H B-CCE reveal a significantly different developmental pattern. The Bmax is very low for this B-carboline until the seventh day post partum and begins to rise during the second week surpassing that of the labeled benzodiazepines on day 14 (Medina et al., 1983). These results are consistent with the finding that Type I benzodiazepine receptors, a biologically and pharmacologically distinct subpopulation of BZD receptors, do not proliferate until after the seventh day and reach

adult levels by day 16 (Lippa et al., 1981). Since both B-CCE and B-CCM interact preferentially with this later developing receptor subtype, it is clear that a two week old pup is more likely to respond to physiologically appropriate doses of these B-carbolines than younger pups.

Receptors for GABA are present at birth, about 24% of the adult Bmax and they increase postnatally in a monotonic fashion reaching adult levels about day 28 (Aldinio, Balzano and Toffano, 1980). It is generally believed that the benzodiazepine/GABA linked receptors develop concurrently, (Tucker, 1985) therefore adult levels would be attained by the fourteenth day, however, there is no hard evidence for this claim.

These neurobiological data in combination with behavioral studies showing that pups begin to ultrasound in response to isolation in an unfamiliar place per se around the end of the second week (Okon, 1972) (Hofer and Shair, 1978) led to the decision to test pups that were 13-14 days of age.

This evidence that the vocal response to isolation in 2 week olds was in response to a combination of stimuli rather than the more reflexive response to temperature seen in younger pups was important for another reason. The second question addressed by this series of studies was: is the pharmacological action of the BZD and B-carbolines responsible for the changes in distress calling the same

action that reduces punished responding in adult rodent models of human anxiety? Implicit in this question is the assumption that there is a neural substrate that subserves "fear" in animals. That is, there are precise physiological processes including not only GABA-benzodiazepine receptors but other neurotransmitters and their pathways, cellular and intracellular mechanisms and neural networks that are integrated in a specific way that are necessary for the expression of "fearful" behavior in adult rodents. Moreover, the components of this neural substrate are presumed to exist in some immature, developing form in preweanling rats. Since changes in the developing rat's behavior are considered to be evidence for these brain changes, the appearance of adult-like responses to stimuli, such as the calming effect of a sibling, suggests that a more mature substrate might be mediating vocal responses to isolation in these older pups, perhaps the same substrate that mediates adult rodent responses to "fear" inducing procedures.

The decision to design the study so that two week old pups could be tested for their vocal response rather than younger pups had a number of implications. First, it meant that while we had to control the room temperature where we tested the pups, we did not have to test the benzodiazepines at one temperature and the B-carbolines at another(See Insel, Hill & Mayor, 1986). Next, it meant

that the second problem associated with the use of vocalization as a behavioral measure, variability, was exacerbated.

Variability

Pilot studies confirmed what the literature search had suggested-"response variability" was to be the most serious problem we would encounter in the design of these investigations. For not only was the behavior selected for the study, distress vocalization, notorious for its variability across litters, drugs active at the benzodiazepine receptor were known for their lack of consistent effects in experimental trials (Liebman, 1985; Wise, 1980). As reported earlier, Howard and Pollard (1977) noted that in the most widely used behavioral test for benzodiazepines, the Geller-Seifter procedure, "variability estimates for group data were conspicuously absent from many reports using this procedure." More recently, Babbini, Garardi and Bartaletti (1982) in a paper devoted to a discussion of the problems with these tests reported that 21% of rodents given a standard benzodiazepine were completely unresponsive to the anti-conflict action of this drug. Moreover, while the inter-litter vocal response of individual pups to separation is quite variable throughout infancy (Graham and

Letz, 1979), the amount of individual difference in responses within litters is greatest for very young pups (1-5 days) and older animals (12-15) days. For this reason, most investigations into the effects of specific drugs on ultrasonic vocalization have been done with pups between the ages of 8 and 12 days.

Since both the binding data and the behavioral studies indicated that the two week old pup was the appropriate candidate for study given our thesis and assumptions, the importance of controlling for variability in baseline vocalization rate became central to the design of the study. Some measures were initiated to control for the anticipated variability before the study began. These included changes in the methodology used to record vocalization scores and the use of longer recording periods. As work progressed other methods were initiated including the selection of a statistical model designed to accommodate extreme ranges in data points.

Data from pilot studies confirmed Okon's earlier report that a large portion of the variability seen in individual two week old pups could be traced to their differences in latency to begin to ultrasound after being placed in isolation. Some pups begin to call after only a few seconds while others do not ultrasound at all until 2 or 3 minutes have passed. With this in mind, instead of

recording the vocalizations of pups during the first 2 minutes of isolation which is the traditional method, each pup was isolated for a full 8 minutes and the number of calls made during each of the last 5 minutes was recorded before and after drug treatment. This reduced the variability of the data in the following ways; by having five "calls-per-minute" data points for each pup, a mean value of the calls could be derived which reduced extreme high and low values that were the result of individual fluctuation over time 2) by eliminating the first 3 minutes the variability attributed to individual differences in latency to call was reduced and finally 3) each pup served as its own control. In this way, each pup would have a percent change score derived from the number of calls made in isolation before and after treatment.

In the first 4 studies, pups were randomly assigned to treatment conditions regardless of their baseline rate of calling. In the final 3 studies (PTZ, B-CCE and B-CCM) pups from each litter were tested in isolation and then assigned a rank based on the total number of calls emitted during the test. They were then assigned to a drug or control group such that each group contained an equal number of pups with high and low rates of ultrasounding (defined simply as the highest and lowest in each litter). While this change in method doesn't eliminate variability, it guarantees an even distribution of variance. It also

permits a further analysis of the effects of anxiogenic compounds on pups with "intense" responses to isolation (high rate of calling) versus those with more moderate responses.

Midway through the project it became clear that the relationship of vocalization to locomotion was more important than had been anticipated. Therefore, we changed this procedure for the 3 final studies so that vocalization and locomotion were recorded simultaneously from the first through the third minute.

Pharmacological Factors

The pharmacokinetics of the B-carbolines contributed importantly to the design of the final investigations. Because B-CCE shares unique histochemical and pharmacological properties with the proposed endogenous anxiety producing substance and because of its extremely high affinity for the benzodiazepine receptor, B-CCE was the drug of choice for this study. Since its half life in rat serum in vitro is approximately 1 minute due to its rapid degradation by serum esterase(s), it was injected into the fourth ventricle of intact conscious pups using a method described by Velluci and Webster (1984) and the pups were tested immediately. For purposes of comparison, test procedures used to test the effects of CDZ and PTZ on

vocalization were replicated using another B-carboline. B-CCM was injected intraperitoneally and pups were returned to the nest and littermates for 15 minutes before the test in isolation. Although the affinity of this B-carboline is less dramatic than that of B-CCE, its half-life in rat serum is considerably greater. Moreover, it is probable that the enzyme systems that degrade the B-carbolines (Mendelson et al., 1982) are not fully functional in pups of this age and these drugs may be active longer than they are in the adult.

Methods

Subjects and housing

The subjects were preweanling Wistar rats of specific ages, born and raised in our laboratory and housed in polystyrene terraria (40 x 20 x 24cm). Food (Purina Rat Chow 35008) and water were made available ad libitum and the animals were maintained under conditions of regulated temperature and humidity. The litters were culled to 9-11 within 72 hours of birth and left otherwise undisturbed until the day of testing.

Ultrasound Detection

A superheterodyne detector (Mark V, Holgate of Totten, England) which transduced ultrasound to frequencies within the range audible to humans was used to detect the isolated pup's ultrasounds. The detector microphone was

suspended approximately 12 cm above the center of the test chamber floor and the transduced ultrasonic pulses were recorded manually on a Panasonic electronic calculator Model JE 8358U.

Drugs

Chlordiazepoxide (Hoffmann-LaRoche) and pentylenetetrazol (Sigma Drug Co.) were dissolved in physiological saline. RO 15-1788 (Hoffmann-LaRoche) was suspended in a vehicle of 40% propylene glycol, 10% ethanol and 50% saline and the stock solution was then further diluted in saline to the required concentration. B-CCM (Research Biochemicals Inc.) was dissolved in physiological saline containing 1 drop of Tween 80 per ml of saline. Chlordiazepoxide, pentylenetetrazol, RO 15-1788 and B-CCM were administered to pups intraperitoneally in a volume of 1 ml per 100 grams of body weight. Disposable syringes with (30) gauge needles were used once to eliminate sources of error attributable to contamination. B-CCE (Beta-carboline-3-carboxylic acid ethyl ester) was mixed with 0.1 ml acetic acid and added to 5 ml of distilled water so that 10 micro liters of the final solution contained the dose to be administered. Control treatment consisted of equal volume injections of 10 microliters of the vehicle alone (from Velluchi and Webster, 1986). For the ICI injection a standard procedure was employed in which 10 microliters volume were slowly injected into the

fourth ventricle of the intact conscious rat pup via a Hamilton syringe with a 27 gauge needle 3.5 mm in length.

Procedures

Although each of the seven experiments described in this chapter used similar methods, the procedures changed as work progressed. For this reason, a "general procedure" has not been included. Instead, for purposes of convenience, the seven experiments are listed and an abbreviated description of the method used in each study is included.

Pre test Procedures

All pups tested received the following treatment. Two hours prior to testing the dam was removed from the home cage and placed in a holding container. Pups were weighed and numbered with felt tipped pens and replaced in their home cage which was situated on a thermistor controlled heated surface near the test area. Pups weighing not less than 20 nor more than 35 grams were tested between 1400 and 1700 hours.

Experiment #1

The effects of 1, 3 and 9 mg/kg of chlordiazepoxide on ultrasonic vocalizations (USV's) and locomotor behavior in 7 and 14 day old pups were determined. Although recently, another laboratory reported decreases in USV's in younger

pups following the administration of similar doses of benzodiazepines (Insel, Hill & Mayor, 1986), no studies have been published examining the effects of chlordiazepoxide on separation in two week old pups.

Procedure

Each pup was removed separately from its litter and placed in the center of the test chamber, a 10 x 13 cm aluminum cylinder where it remained for eight minutes. The number of ultrasounds emitted during the last five minutes was recorded via a microphone suspended 12 cm above the center of the chamber. After pups from each litter were tested in this way, they were assigned randomly to one of 4 drug or vehicle conditions, each was injected and returned to the home cage and littermates for 15 minutes. At the end of this time, the test procedure was repeated for each animal. Each pup was tested in this way only once and served as its own control. To determine the effects of chlordiazepoxide on locomotion, after isolation, each pup was placed in a 10 x 22 x 15 cm plexiglas container whose floor was divided into nine squares and the number of squares entered during each minute was recorded for three minutes.

Experiment #2

The effects of 1 and 3 mg/kg of chlordiazepoxide on ultrasonic vocalizations in pups between the ages of 7 and 14 days were examined. In this experiment, the test

procedure was the same except that pups were not tested for the effects of the drug on locomotor behavior. The purpose of this experiment was to investigate the development of the suppression of vocalization response to chlordiazepoxide within and between litters. To do this, 3 pups from a single litter were tested on a given day, say day 10, 3 others from the same litter were tested on day 12 and the remaining three tested on day 14. Pups from other litters were tested on days 8, 10 and 12 or 7, 9 and 11 such that 12 pups were tested at each of 8 ages (N= 96).In this experiment only, after a pup was tested it was ear-notched to assure that it would not be tested again and returned to the litter.

Experiment #3

The effects of 3 mg/kg of chlordiazepoxide on ultrasonic vocalization and locomotion during the first 3 minutes of isolation.

Procedure

The purpose of this experiment was to record simultaneously vocalization and locomotion during the first 3 minutes of separation from the home nest and littermates for purposes of comparison. Therefore the procedure leading up to the isolation period remained the same, however, the test itself was modified. In this experiment the test chamber was changed from a metal cylinder to a 22 x 24 x 26 cm polystyrene container whose floor was divided

into nine squares. Each pup was placed at one end of the test chamber and the number of ultrasonic vocalizations and the number of squares entered for each of the first three minutes in isolation were recorded simultaneously. Following this baseline test, each pup was returned to its home nest until all the pups from the litter were tested. Pups were then administered either 3 mg/kg of CDZ or saline and placed again in their home nest. After 15 minutes, the test was repeated.

Experiment #4

The effects of the benzodiazepine antagonist RO 15-1788 on ultrasonic vocalization in pups who had been pretreated with 3 mg/kg of chlordiazepoxide. The purpose of this study was to determine if the effects of chlordiazepoxide upon ultrasonic vocalization were being mediated by the benzodiazepine receptors.

Procedure

In the fourth and final experiment in which chlordiazepoxide was administered, pups were tested twice in isolation as usual, once to determine a baseline rate of calling and again following the administration of either 3 mg/kg of CDZ or an equal amount of saline, then they were given a second intraperitoneal injection of the BZD antagonist RO 15-1788 (5 mg/kg) and returned to the nest and littermates for 5 minutes. After this time, the test was repeated and the number of USV's recorded for each pup.

Experiment #5

The effects of 5, 15, and 30 mg/kg of pentylenetetrazol on ultrasound and locomotion in isolated 2 week old pups. The purpose of this study was to see if the standard anxiogenic compound pentylenetetrazol, known to be active at the GBRC would alter ultrasound and/or locomotor behavior in pups this age.

Procedure

In this study the test chamber was the 22 x 24 x 26 cm polystyrene container whose floor had been divided into nine squares. Each pup was placed at one end of the chamber and the number of USV's were recorded for a full eight minutes. The number of squares and the number of USV's were simultaneously recorded for the first 3 minutes of isolation. Following this baseline test, each pup was returned to its home nest until all the pups from the litter were tested. To control for baseline vocalization response variability, pups were assigned to a drug or control group such that each group contained an equal number of pups with high and low rates of ultrasounding. Pups were injected with one of the 3 doses of PTZ or saline and returned to the home cage for 15 minutes. At the end of this time, the test was repeated for each animal. Each pup was tested in this way only once and served as its own control.

Experiment #6

The effects of B-CCE, ethyl beta-carboline-3-carboxylate, on ultrasound and locomotion in two week old pups isolated in a novel environment. The purpose of this experiment was to see if a substance active at the benzodiazepine receptor, reported to have anxiety inducing effects in humans and animal models of anxiety would increase USV's in two week old isolates.

Procedure

Each pup was placed at one end of the polystyrene container and the number of USV's and squares crossed was recorded simultaneously for the first three minutes in isolation. The number of USV's alone were recorded for the next 2 minutes. Following this baseline test, each pup was returned to its home nest until all the pups from the litter were tested. Pups were then assigned to a drug or control group such that each group contained an equal number of pups with high and low rates of ultrasounding. Because B-CCE is rapidly degraded in rat serum, a 10 micro liter volume of solution containing either 1 micro gram of B-CCE or vehicle alone was slowly injected into the fourth ventricle of the conscious rat pup using a Hamilton syringe with a 27 gauge needle. Immediately following the injection, the pups were placed in isolation and the test repeated.

Experiment #7

The effects of B-CCM, methyl beta-carboline-3-carboxylate, on ultrasound and locomotion in two week old pups isolated in a novel environment. The purpose of this experiment was to 1) further investigate the effects of B-carbolines on USV's 2) determine the effects of this substance on locomotor behavior 3) replicate the procedures used to test other compounds to facilitate behavioral comparisons.

Procedure

1. In this study each pup was placed at one end of the polystyrene chamber and the number of USV's and squares crossed were simultaneously recorded for the first 3 minutes of isolation. USV's were recorded for another two minutes. Following this baseline test, each pup was returned to its home nest until all the pups from the litter were tested. Pups were then assigned to a drug or control group such that each group contained an equal number of pups with high and low rates of ultrasounding. Pups were administered one of 3 doses of B-CCM or vehicle and returned to the home cage for 15 minutes. At the end of this time, the test was repeated for each animal.

DATA ANALYSIS

Because of the variability of ultrasonic vocalization response to isolation/novelty the data were analyzed in

the following ways. First, to determine the main drug effects, the traditional method for assessing treatment effects on distress vocalizations was used throughout, that is, the number of calls made in isolation before and after treatment were calculated for each animal and reported as a "percent change". These individual percent changes were summed and the mean and standard error determined for each drug dose and its controls. These scores were then submitted to the Bartlett test for homogeneity of error variance which indicated that a nonparametric statistical model was appropriate for these data. These values were then submitted to Kruskal-Wallis analysis of variance for overall effects and were followed, when appropriate, by Mann-Whitney U tests for individual comparisons.

The Kruskal-Wallis test is considered the most efficient of the nonparametric tests for k independent samples, having a power efficiency of =95.5% when compared with the most powerful parametric test, the F test. This test was selected to analyze these data because it converts raw scores to ranks before submitting them to analysis, a process designed to test values that cover an exceptionally large range.

As the following example will attest, "percent change" scores may be characterized by extreme scores skewed in one direction. For example, a pup that increases its calls per minute after treatment from 5 to 55 calls will have a

percent change score of 1000%. Contrast this with the pup that decreases its calls from 55 to 0, a net percent change score of only 100%. In another words, while the maximum decrease in percent is 100%, there is no maximum increase, it is unlimited. Because the Kruskal-Wallis test converts all scores to ranks, it controls for both the extreme range in responses and the distortion in scores caused by the use of "percent change" as the measure .

The effects of the drugs on ultrasound and activity over time were assessed by first determining the exact number of ultrasounds emitted or squares entered within each minute for each animal throughout the isolation period. Minutes 1, 3 and 5 were then selected as sample minutes and the number of USVs and squares entered within each of these minutes before and after treatment were presented as "percent change" over time. These values were then submitted to the Kruskal-Wallis analysis of variance by ranks, followed by Mann-Whitney U tests for individual groups.

Finally, to further assess the temporal effects of these drugs, the percent of animals showing an increase in ultrasound following the administration of anxiogenic compounds was calculated for each sample minute.

ORGANIZATION OF MATERIAL

The presentation of the results of these experiments

has been organized pharmacologically. Instead of discussing each investigation separately in chronological order, all experiments in which a given drug was administered were grouped together into one of 3 chapters titled either Chlordiazepoxide, Pentylenetetrazol or Beta-carbolines.

Because the background material for these studies was presented in the Pharmacology, Animal Models and Separation chapters, the introductory material for these sections has been omitted to avoid repetition. Instead, each chapter begins with a brief description of the experiments to be discussed followed by a summary of their purpose. Where relevant, a review of the literature of the effects of specific drugs on infant rodent behavior is included as well as separate results and discussion sections,appropriate tables and figures.

The Effects of Chlordiazepoxide and RO 15-1788 on Ultrasound in One and Two Week Old Pups Isolated in a Novel Environment

The Effects of Benzodiazepines on Behavior in Infant Rats

At present there are few reports on the behavioral effects of benzodiazepines in infant rats. The majority of developmental investigations are behavioral teratology studies, protocols in which pregnant or nursing dams are given benzodiazepines and their pups tested later for evidence of perceptual or cognitive deficits. (For review see Tucker, 1985). Other studies have reported the effects of benzodiazepines on drug induced seizures in immature rats. Recently, however, Barr and Lithgow (1983) reported that chlordiazepoxide and flurazepam elicited behavioral convulsions in 3 to 18 day old pups. Since these benzodiazepines are noted for their anti-convulsant/ sedative activity in adult rats, this report of paradoxical effects in infant rats was quite surprizing. Later that year, Pappas and Walsh (1983) reported that while benzodiazepines did cause a loss of righting reflex, increases in motor activity, twitches and jerks in rat pups, they questioned whether or not these behaviors constituted true convulsions. Most recently, File and

Wilkes (1986) reported that benzodiazepines appear to have two separate effects on behavior in young pups. First, they appear to increase locomotor behavior and secondly they cause myoclonic jerks accompanied by a loss of righting reflex, behaviors associated with seizures in young animals.

In light of these paradoxical activating effects on locomotor behavior it was surprising that two laboratories independently reported that in same age pups both diazepam (0.5 mg/kg)(Insel et al., 1986) and chlordiazepoxide (1 and 3 mg/kg) (Gardner, 1985) reduced distress vocalizations. As a result of these and other data, both laboratories suggested that the effects of these BZD on vocalization might be the result of the "anxiolytic" action of these drugs.

Ultrasonic Vocalizations

This was not the first time that these rodent vocalizations in isolation had been proposed as measures of a change in internal "state". While it is generally accepted that the function of these calls is to reunite mother and infant (Noirot, 1964; Sewell, 1970), the calls themselves have been proposed as measures of "distress" (Zeppelius and Schleidt, 1956) emotionality (Graham and Letz, 1979) and arousal (Amsel et al., 1977; Bell et al.,

1974). Indeed, vocalization is the behavior most consistently reported as an index of "emotional distress" following mother/infant separation across mammalian species. In Wistar pups these calls can be elicited reliably from a few hours after birth through approximately day 15 or eye opening. The calls themselves are laryngeal, coincidental with expiration and are emitted at ultrasonic frequencies between 30 and 50 Khz (For review see Sales and Pye, 1977).

Behavior Other Than Vocalization Seen in Isolation

The behavior of isolated 2 week old Wistar pups has been fully characterized elsewhere (Hofer and Shair, 1987). The purpose of this abbreviated description is to facilitate the comparison of the effects of chlordiazepoxide on behaviors other than vocalization.

Two week old pups placed in an unfamiliar restricted area for six minutes engaged in a variety of behaviors including locomotion, vocalization, rising (standing on hind legs with forepaws on the wall of the container) wall climbing, self-grooming and sitting still. While there is considerable variability in the behaviors seen in individual pups, frequently animals of this age will ultrasound very little during the first three minutes of isolation, engaging instead in a combination of behaviors

that have been described as "investigatory" (Allin and Banks, 1972). Specifically, they move around the perimeter of the container, rising frequently and appearing to sniff the air, walls and floor of the test container. After from 1 to 3 minutes of these behaviors, these pups reduce their locomotor behavior significantly and begin to vocalize. Allin and Banks have suggested that this sequence of behavior may have an adaptive value for pups that find themselves outside the nest. They argue that since vocalization would be heard by nearby predators, it was more adaptive for a well-developed animal to try and find its way back to the nest using olfactory cues and locomotor skills; and only after these avenues have been exhausted should a pup announce its whereabouts by vocalizing. In any case, the importance of this analysis for these studies is to show that this sequence of behavior is different from that seen in pups that are younger than 10 days of age. These young animals at room temperature (25 C) typically begin to ultrasound immediately upon being placed in the test container and the number of calls actually declines after the third minute (Blass and Kehoe, 1982). Because pups of this age are at risk for hypothermia, it is argued that it is more adaptive for them to signal immediately. The significance of this developmental difference in the response to isolation will be discussed later.

As discussed in the preceding chapter, ambient

temperature alone determines the number of vocalizations emitted in isolation during the first week of rodent life (Okon, 1972). Furthermore, Pappas and Walsh (1983) using a time sampling procedure to assess the effects of CDZ on "head and limb" movements in isolated pups found that it produced very different responses depending upon whether the pups were tested at room (25 C) or nest temperature (35 C) up to 12 days of age. Therefore, in recognition of the importance of the interaction of CDZ and temperature at different ages as well as other developmental concerns, we began these investigations by looking into the effects of chlordiazepoxide on vocalization and locomotion at two different ages.

In the first experiment, 3 doses of CDZ and saline were administered to 7 and 14 day old pups and the effects on vocalization and locomotion were recorded. In the second investigation selected doses of CDZ and saline were given to pups that were either 7 ,8 ,9 ,10, 11, 12, 13, or 14 days of age to determine the developmental course of the drug's effect on vocalization. In the third experiment, 3 mg/kg of CDZ was administered to 14 day olds and the effects of the drug on vocalization and locomotion were recorded simultaneously during the first three minutes of isolation. In the fourth and final experiment, the BZD antagonist RO 15-1788 was administered to pups following an injection of chlordiazepoxide to see if the actions of this

drug were mediated by the benzodiazepine receptor.

Results

Experiment #1

One week old pups tested in isolation gave a mean (+-SEM) of 76.7+-7.4 calls per minute during the five minute baseline trial and entered a mean (+-SEM) 14.9 +-1.5 squares during the 3 minute baseline activity trial.

Two week old pups gave a mean (+-SEM) of 51.9 +-8.4 calls per minute during their five minute baseline trial and entered a mean (+-SEM) 20.9 +-2.0 squares during the three minute baseline activity trial.

Kruskal-Wallis one way analysis of variance by ranks revealed that in 1 week old pups the administration of chlordiazepoxide was followed by significant decreases in the number of ultrasounds in isolation ($H(3) = 14.6$, $p<.01$). Comparisons of individual doses with saline controls (Mann-Whitney U test) showed that all three doses significantly reduced vocalization ,$p<.01$, $p< .01$, and $p< .001$) respectively as shown in Figure 2.

Figure 3 shows the percent change in vocalizing after the administration of either 1, 3, or 9 mg/kg of chlordiazepoxide to 2 week old pups. Analysis of variance by ranks showed that at 2 weeks of age, chlordiazepoxide

significantly reduced the number of USVs when compared to controls ($H(3) = 16.2$, $p < .005$) and Mann-Whitney U comparisons of individual doses with controls revealed that 1, 3, and 9 mg/kg significantly reduced vocalizations $p < .001$ for all three doses . At this age, 1 and 3 mg/kg reduced ultrasonic calls by an average of 60.8% and 70.2% respectively without significantly decreasing activity. While 9 mg/kg led to the largest decrease, 96.4%, all but one of these animals showed major reductions in activity levels and hypothermia.

Figures 2 and 3 also show that 1 and 3 mg/kg did not cause significant decreases in locomotion at either 1 or 2 weeks of age. However, the administration of 9 mg/kg was followed by extreme reductions in behavior at both ages.

To see if there was an age / drug dose interaction, the data were submitted to a 2 way ANOVA . This test revealed that the age differences in drug response was significant at the .05 level, and the drug effect was significant at the .01 level. T tests indicated that 1 mg/kg dose responses on day 7 were statistically different from those reported for day 14 $p < .01$. The 3 mg/kg response was significantly different between the two ages with a probability greater than .001. However, there was no statistical difference betweeen the effects of 1 or 3 mg/kg at either age.

Experiment # 2

Figure 4 shows the mean (+-SEM) number of calls emitted during the five minute baseline trial for 96 pups tested on one of 8 days.

Figure 4a (See appendix)shows the percent change in USV following the administration of 3 mg/kg of chlordiazepoxide from day 7 through day 14. Because the design of this study was flawed by the repeated disturbance of litters and this "handling" effect could have altered the results, the data from this study has been placed in the appendix.

Experiment #3

As the study progressed it became apparent that it was necessary to know what the effects of these drugs were on vocalization and locomotion during the same time period. Therefore 2 week old pups were tested so that ultrasound and locomotion were recorded simultaneously from the first through the third minute in isolation. Pups emitted a mean (+-SEM) 30.3 +-7.5 calls per minute and entered a mean (+-SEM) 9.7 +-1.2 number of squares per minute during the first 3 minutes of this trial. Using this procedure, the administration of 3 mg/kg of CDZ lead to a mean (+-SEM) of 69.1% reduction in vocalizations and a mean increase in the percent of squares entered of 70.1 +-41.3 as shown in figure 5. Analysis of variance by ranks found that the

decrease in USV's was significant (H (1)3.17 , p<.05) when compared to saline controls, however the increase in the number of squares entered failed to reach significance.

Experiment #4

Figure 6 compares the number of calls made by pups following treatment with chlordiazepoxide or saline with pups receiving a combination of chlordiazepoxide and RO 15-1788. Kruskal-Wallis analysis of variance by ranks comparing the number of calls made by pups following the administration of chlordiazepoxide alone with pups receiving 5 mg/kg of RO15-1788 and CDZ or saline was significant ($H(2) = 7.57$, $p<.025$). Mann-Whitney U comparisons between the benzodiazepine group and the pups injected with chlordiazepoxide and RO15-1788 was also significant ($U=24$, $p<.025$). To reduce the possibility that repeated testing alone might be responsible for the antagonistic effects of RO15-1788 the tests were repeated with pups receiving either 2 consecutive saline injections or a chlordiazepoxide injection followed by saline. These groups were not significantly different from their controls. Taken together, these data show that RO15-1788 antagonized the effects of CDZ on ultrasound which indicates that these actions were mediated by benzodiazepine receptors.

It should be mentioned here that the adult dose of RO

15-1788 given to antagonize 3 mg/kg of chlordiazepoxide is 10 mg/kg. However, in a pilot study in which five pups received injections of 3mg/kg of CDZ and 10 mg /kg of RO 15-1788 all the pups tested showed further decreases in both ultrasounds and locomotion. For this reason 5 mg/kg was used to antagonize the effects of chlordiazepoxide in this test.

Effects of CDZ on Behavior other than Vocalization and Locomotion

Following the administration of chlordiazepoxide (1 and 3 mg/kg) pups engaged in a variety of behaviors that were infrequently or never observed in control animals. The most obvious were large myoclonic jerks and twitches that sometimes resulted in the pup being thrown against the side of the container. Although full tonic-clonic seizures were not observed at these doses, these behaviors resembled the pre-seizure activity seen in pups given convulsant doses of pentylenetetrazol.

Ataxia was also observed, frequently in animals showing increased locomotor behavior where it appeared as clumsiness leading to an occasional loss of balance. Less frequently observed was an increase in startle reflex.

Of particular interest was a group of behaviors that might have gone unnoticed in the absense of a microphone.

These included mouthing accompanied by sucking noises, increases in salivation as well as mouth wiping, responses that were clearly distinguishable from face washing and have been associated with serotonergic activity.

Discussion

These experiments show that 1) ultrasonic vocalizations in isolated rat pups are significantly reduced following the administration of 1 and 3 mg/kg of chlordiazepoxide at both 7 and 14 days 2) locomotor behavior is not significantly reduced by these doses of the drug at either age 3) 9 mg/kg of CDZ however, reduced both ultrasounds and locomotion at both ages 4) the benzodiazepine antagonist, RO15-1788 (5 mg/kg) blocked the reduction of USV seen following the administration of CDZ whereas 10 mg/kg of RO15-1788 caused further reductions in USV and locomotion.

Although benzodiazepines are best known for their anti-anxiety properties they have a spectrum of pharmacologic activity that includes sedation, muscle relaxation and protection against seizures. Therefore, one of the central questions in any discussion of the effects of these drugs on distress vocalizations is which of these pharmacologic actions is responsible for these drug

actions. While these studies were designed to test the hypotheses that BZD receptor ligands with anxiety reducing properties would decrease distress calling whereas those with anxiety causing action would increase these calls, these experiments do not claim to demonstrate that these changes are the result of "anxiolytic actions" of the drug. It is evident, for example, that chlordiazepoxide could reduce USV's via its sedative or muscle relaxant properties. However, while these behavioral studys cannot directly demonstrate that some pharmacologic property of the BZD is altering behavior, each experiment may support or fail to support either the anxiolytic or some alternative hypothesis. While there are a number of possible explanations for the effects of CDZ on distress calling, there are two major alternative hypotheses that should be addressed. The first of these is that these reductions in vocalization are secondary to a change in the level of arousal (Amsel et al., 1977)(Bell, 1974) and the second is that benzodiazepines reduce USV's by altering body temperature or temperature perception.

The theory that these changes in behavior can be explained by a simple decrease in levels of arousal is not supported by these data. For if sedative effects alone were responsible for these changes, then both vocalization and locomotion should be reduced. Although the finding that 1 and 3 mg/kg of CDZ reduces USV's without changing

activity levels may affect distress calling at higher doses, it suggests that at lower doses vocalizations are being reduced by some other pharmacologic property of the drug. The effects of the highest dose of CDZ, 9 mg/kg which were to eliminate ultrasound in all but one animal, and dramatically decrease activity, was also suggestive. In each animal, vocalization was completely eliminated before the locomotor response became seriously depressed. This further suggests that there are two separate effects on behavior, and that while the sedative or "arousal reducing" action of the high dose of the benzodiazepine may contribute to USV reduction, some other property, possibly the anxiolytic action, is responsible for the reduction in calls seen at lower doses. Moreover, the separation of these effects on behaviors at high and low doses is consistent with the effects of the BZD on punished and unpunished responses in conflict tests, i.e. benzodiazepines and other anxiolytic drugs reduce only punished responses at low doses and decrease both at higher dose levels. Finally, the observed increases in twitching, myoclonic jerks and sucking are behaviors that are characteristically observed following the administration of stimulants as opposed to drugs known to have sedative effects.

The hypothesis that benzodiazepines may be reducing vocalizations by some thermoregulatory mechanism is more

difficult to dismiss. Because temperature is an important factor in determining the number of calls that a younger pup will make in isolation, we took axillary temperatures of a sample number of 2 week olds before and after testing and found that although 9 mg/kg reduced body temperature a mean of 2.3 degrees Celsius, the body temperatures of pups receiving 3 mg/kg ranged from 33.8 to 35.5 degrees , temperatures that were not significantly different from controls. However, this does not eliminate the possibility that the sensory processes of these pups were altered (possibly via serotonergic processes) in such a way that their perception of the ambient temperature was modified. Sophisticated studies of the effects of drugs on temperature require a "heat seeking" test whereby animals are allowed to choose among a variety of ambient temperatures which addresses the issue of how warm the animal "feels" as opposed to its actual body temperature. Although these tests have not been done, Gardner, (1985) addressed the issue of thermoregulation by administering 2 drugs that cause hypothermia in rats, clonidine and prazosin and reported that both caused increases in vocalizations in pups. However, he also found that 6 mg/kg of chlordiazpoxide, which dramatically reduced vocalizations, also led to reductions in body temperature as well. Since drugs that both decrease and increase USV lead to reduced body temperatures, Gardner suggests that

vocalizations in rodent pups were probably being reduced by some other pharmacological property of the benzodiazepines.

In the only other experiments investigating the relationhip between benzodiazepines and vocalizations (Gardner, 1985;Insel et al., 1986) the animals were not tested at different ages and their procedures were different in several ways, including the amount of time spent in isolation and the use of temperature as a control. In spite of these differences in protocol the results from the present studies are in agreement with reports from these other laboratories in the following ways: first, benzodiazepines, at comparable doses reduce vocalizations in pups isolated at room temperature without reducing activity, second, we reported that 9 mg/kg caused hypothermia and sedation which agreed with Gardners finding that 6 mg/kg had these effects, finally, all three studies reported that the BZD antagonist RO 15-1788 blocked these actions. Data from this and Insel's laboratory both indicated that 10 mg/kg of RO15-1788, the dose of BZD antagonist used to block the effects of the BZD in adult animals, had benzodiazepine-like effects in pups.

In summary, the data from the present studies show that CDZ acting at the benzodiazepine receptor reduces vocalization in isolated two week old rat pups. Because these changes in rate of calling occur in the absence of sedative and thermoregulatory effects. these reductions in

ultrasonic vocalizations may be the result of the anxiolytic properties of these drugs.

Fig. 2. The effects of chlordiazepoxide on ultrasonic vocalizations and locomotor behavior in 1 week old pups isolated in a novel environment. Values represent means (+ - SEM) of percent change from baseline trials after 5 minutes in isolation for ultrasounds and 3 minutes for locomotion. The results were obtained 20 minutes following the administration of either chlordiazepoxide, 1, 3, or 9 mg/kg. The * indicates a significant difference ($p < .01$) from saline controls on post hoc Mann-Whitney U Test.

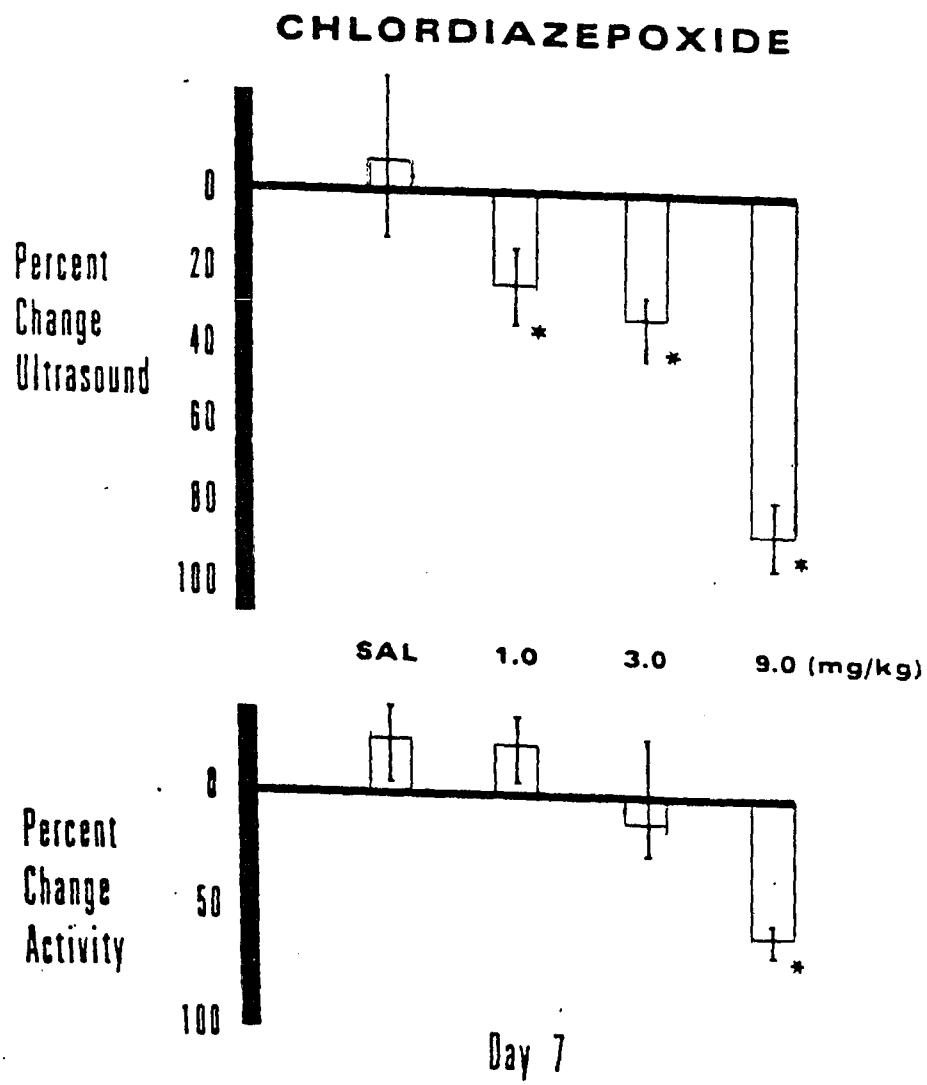

CHLORDIAZEPOXIDE
Percent Change Ultrasound
0
20
40
60
80
100
*
*
*
SAL
1.0
3.0
9.0 (mg/kg)
Percent Change Activity
0
50
100
*
Day 7

Fig. 3. The effects on chlordiazepoxide on ultrasonic vocalizations and locomotor behavior in 2 week old isolated pups. Values represent means (+ - SEM) of percent change from baseline trials after 5 minutes in isolation for ultrasunds and 3 minutes for locomotion. The results were obtained 20 minutes following the administration of either chlordiazepoxide, 1, 3, or 9 mg/kg. The * indicates a significant difference ($p<.01$) from saline controls on post hoc Mann-Whitney U test.

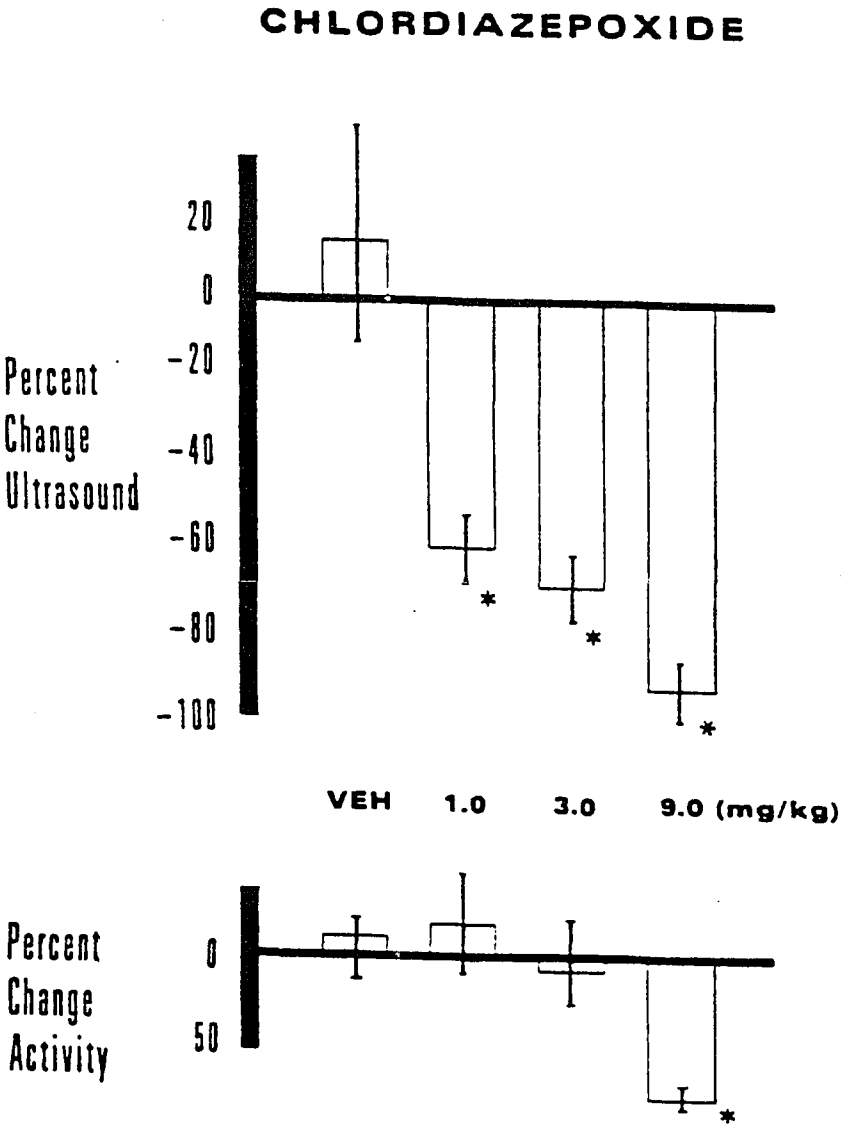

CHLORDIAZEPOXIDE
Percent Change Ultrasound
20
0
-20
-40
-60
-80
-100
*
*
*
VEH
1.0
3.0
9.0 (mg/kg)
Percent Change Activity
0
50
*
Day 14

Fig. 4. The mean number of calls emitted during a 5 minute isolation period for pups at different ages (N= 96). Results were obtained by placing naive pups in isolation and recording the total number of calls emitted during the 5 minute trial.

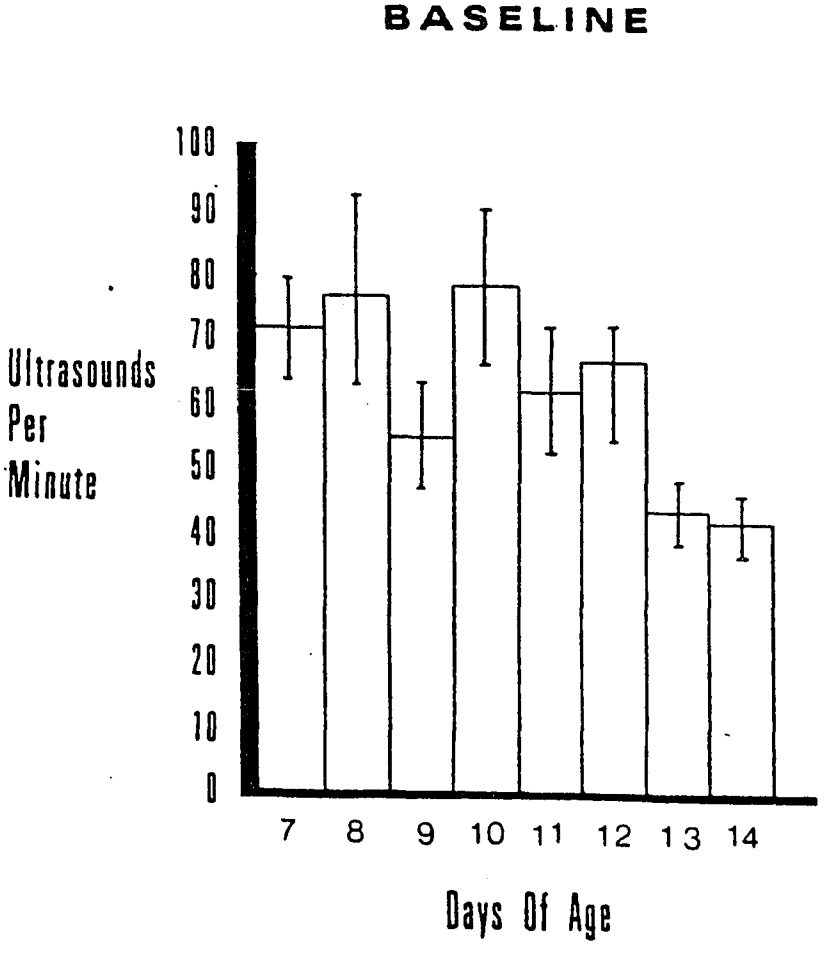

BASELINE
Ultrasounds Per Minute
100
90
80
70
60
50
40
30
20
10
0
7
8
9
10
11
12
13
14
Days Of Age

Fig. 5. The effects of chlordiazepoxide on ultrasonic vocalization and locomotion in 2 week old pups recorded simultaneously. Values represent means (+ - SEM) of percent change from baseline trials for vocalization and locomotion during 3 minutes in isolation. The results were obtained 20 minutes following the i.p. administration of chlordiazepoxide, 3 mg/kg. The * indicates a significant difference ($p < .05$) from saline controls on post hoc Mann-Whitney U test.

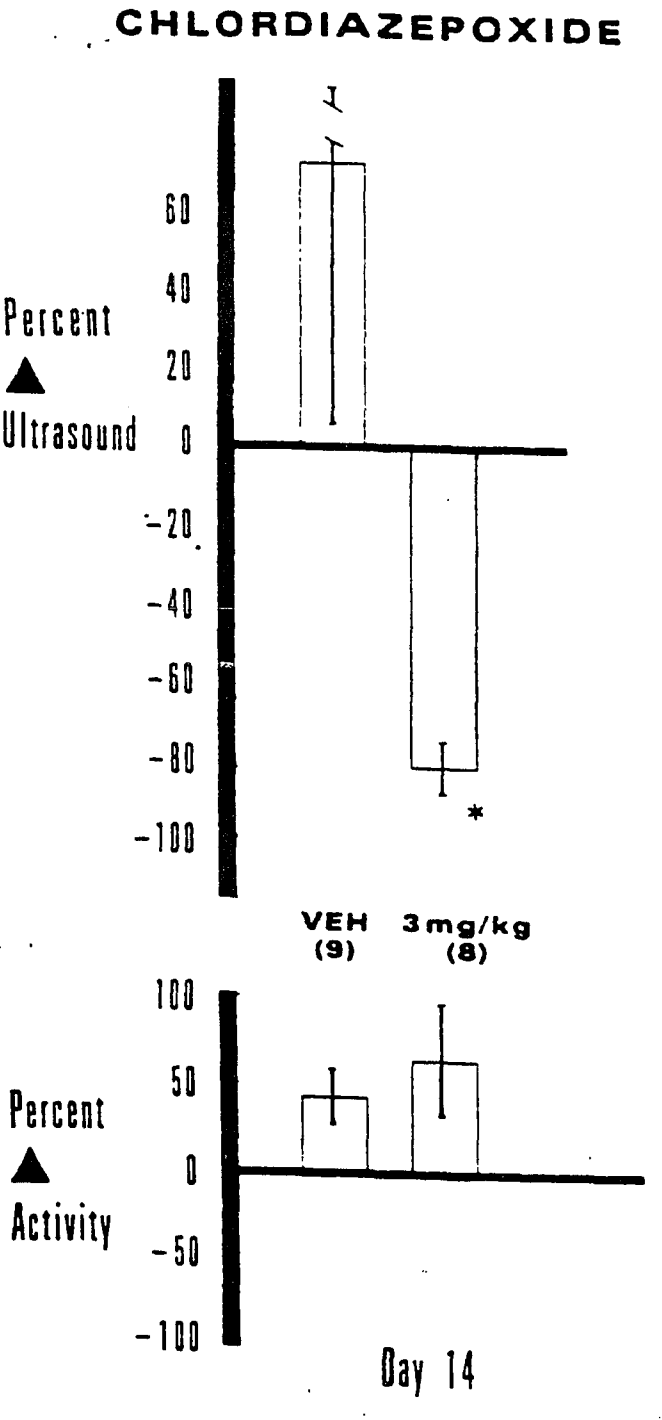
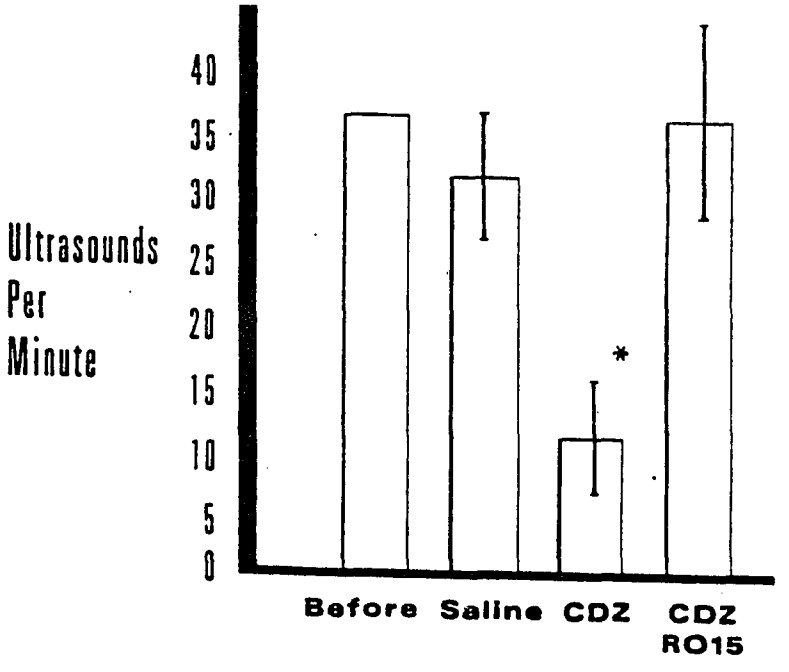

CHLORDIAZEPOXIDE
Percent
Ultrasound
60
40
20
0
-20
-40
-60
-80
-100
*
VEH (9)
3mg/kg (8)
Percent
Activity
100
50
0
-50
-100
Day 14

Fig. 6. The effects of chlordiazepoxide and RO 15-1788 on vocalization in isolated 2 week old pups. Values represent means (+ – SEM) of all pups tested during the baseline trail, as well as the means (+ - SEM) of percent change from baseline trial following the administration of either chlordiazepoxide (3 mg/kg) and RO 15-1788 (5 mg/kg), chlordiazepoxide (3 mg/kg) and saline or 2 injections of saline . The results were obtained 5 minutes following the administration of the last injection. The * indicates a significant difference from pups receiving RO15-1788 ($p<.025$) on post hoc Mann-Whitney U test.

RO15-1788


Ultrasounds
Per
Minute
40
35
30
25
20
15
10
5
0
*
Before
Saline
CDZ
CDZ
RO15

The Effects of Pentylenetetrazol on ultrasound and Locomotor Behavior in Two Week Old Rats Isolated in a Novel Environment.

In this experiment, pentylenetetrazol (5, 15, and 30 mg/kg) was administered intraperitoneally to 2 week old pups isolated in a novel environment and measures were obtained of the differences between vocalization rates and locomotion in pups before and after treatment.

The purpose of this study was to see if pentylenetetrazol at doses reported to have anxiogenic effects in adult rats would 1) increase ultrasonic vocalizations in infant rats and 2) increase locomotor behavior as measured by the number of squares entered in a novel environment.

While pentamethylenetetrazol (PTZ) is a CNS stimulant best known for its convulsant properties, subconvulsant doses cause intense anxiety when administered to human research subjects (Rodin, 1985). In animals, PTZ doses as low as one-quarter of the amount needed to induce seizures are reported to have anxiety inducing or "anxiogenic" effects as well. Moreover, pentylenetetrazol is widely used to challenge the anxiety reducing effects of the benzodiazepines (Cook and Sepinwal, 1975) in adult rodent models of human anxiety.

In infant rats, the effects of pentylenetetrazol on

behavior other than seizures is not well known. Although there are a number of studies reporting the effects of convulsant doses of PTZ (50-70 mg/kg) on behavior, few report behavioral effects of doses said to be anxiogenic in adults (5-30 mg/kg). From birth through day 7, PTZ at convulsant doses causes extreme hyperactivity and tonic seizures (Pappas and Walsh, 1983; Vernadakis and Woodbury, 1969), however, from the eighth through the sixteenth day it causes tonic-clonic seizures (Mares and Seidle, 1982). Although Pappas and Walsh (1983) were concerned primarily with the effects of convulsant doses of PTZ on behavior, they administered a lower "anxiogenic" dose to isolated 4, 8 and 16 day old rats. They found that while a convulsant dose was "activating", that is, it increased head and limb movements at all three ages, the lower dose of PTZ (25 mg/kg) had no effect on head or limb movements.

RESULTS

When two week old pups were placed alone in an unfamiliar test container they emitted a mean (+- SEM) of 26 +-4 calls per minute during the eight minute baseline trial and entered a mean (+- SEM) of 7 +-2 squares per minute during the three minute activity trial.

Figure 1 shows the relationship between vocalization and locomotor behavior in two week old pups isolated in a novel environment over time. In this figure the means and

standard errors of the number of calls given during the first, third, fifth and eighth minute of isolation are compared with the mean number of squares entered for the first and third minutes of all pups during their baseline trial. This time sample analysis shows that two week old pups emit only 19 (+- 4) ultrasounds during the first minute of isolation and that there is a gradual increase in USV's from the first to the eighth minute of 46% (Kruskal-Wallis H (3) = 7.83 , $p<.05$) ($u=18.5$, $p<.05$). Figure 1 also shows that locomotor behavior declines from the first to the third minute (Kruskal-Wallis H (1) = 3.83 , $p<.05$). These data are in agreement with previous observations that when many two week old pups are separated from their littermates they initially engage in locomotor behavior and only after the first or second minutes have passed do they begin to emit ultrasounds.

Figure 2 shows that after the 8 minute isolation period pups injected with pentylenetetrazol had significant mean increases in vocalization (Kruskal-Wallis H (3)=10.29, $p<.05$). Posthoc analysis showed that the increases following 15 and 30 mg/kg were significant when compared to controls ($p < 0.05$).

Since "time in isolation" is an important factor determining how many USV's a pup will make when separated from its littermates, the question arises as to the effect of PTZ on the USV's over time. Does pentylenetetrazol

simply enhance the normal response to isolation as shown in Figure 1? If so, the increases in USV's following the administration of PTZ should increase gradually from the first through the eighth minute. Figure 3 illustrates that this is not the case. When the number of ultrasounds after drugs or saline administration is compared over time, it is evident that although the saline treated pups show a pattern of response similar to that of the untreated pups in Figure 1, pups receiving PTZ exhibit a response that is different. This figure illustrates that the most dramatic increases occur within the first minute of isolation for the groups receiving both 15 and 30 mg/kg of the drug ($p<.05$ and $p<.001$). While vocalizations continue to be significantly increased during the third minute of the test for pups receiving the higher dose ($p<.02$) by the fifth minute the differences between the groups have disappeared at all doses. This analysis suggests that the effect of pentylenetetrazol on ultrasonic vocalization may be short lived and that the primary effect of the drug is to decrease the latency to ultrasound when isolated in a novel environment. Because PTZ is reported to have longer lasting effects on behavior in adult rats, these data require further analysis.

To further assess the effects of PTZ on ultrasound, the number of pups that increased their USV's following pentylenetetrazol administration were compared with the

number receiving saline. Figure 4 shows that 18 out of 20 pups given either 15 or 30 mg/kg of PTZ increased their USV's through the third minute of isolation compared to only 2 of the saline treated pups. Furthermore, using this measure, even 5 mg/kg caused 80% of the pups to increase calling during the first and fifth minute of separation. These data, taken together, suggest that while the primary effect of PTZ on ultrasound is to cause dramatic increases during the first minute of isolation, it may also lead to more modest increases that extend throughout the isolation period.

The effects of pentylenetetrazol on locomotor behavior during the first minute of isolation are shown in Figure 2. The administration of 5, 15 and 30 mg/kg was followed by significant decreases in locomotor activity ($p<.05$, $p<.05$ and $p<.01$) respectively after the first minute in isolation. By the third minute, however, differences between groups were no longer significant.

Pentylenetetrazol did not cause seizures at any of these doses, prior testing having revealed a CD (i.e. dose at which 50% of the pups had tonic-clonic seizures) of 60 mg/kg.

DISCUSSION

This experiment shows that doses of Pentylenetetrazol reported to be anxiogenic in adult rats 1) cause dramatic

increases in distress vocalizations and 2) significantly reduce locomotor behavior during the acute phase of separation in two week old rats. Because Pentylenetetrazol is a CNS stimulant, increases in ultrasonic vocalizations were not altogether unexpected, however the reduction in locomotor behavior was not anticipated. This latter finding has a number of implications, the most significant being that it can be interpreted as evidence supporting the hypothesis that PTZ is exerting its pharmacologic effects on isolation distress behaviors via its "anxiety" inducing properties as mediated by the proposed GABA-benzodiazepine receptor-chloride channel complex. The anxiogenic hypothesis is supported by decreases in locomotor behavior in the following ways.

First, since pentylenetetrazol increases one behavior, vocalization, and decreases another, locomotion, we can dismiss with some confidence the possibility that the dramatic increases observed in ultrasounds were secondary to non-specific stimulant effects of the drug. Secondly, other laboratories have reported that low doses of PTZ cause reductions in locomotor behavior when adult rats are tested in a novel environment (File and Lister, 1984). This suggests that this change in behavior is not the result of some aspect of the experimental design or statistical analysis, but a legitimate drug effect. Since a reluctance to explore a novel environment is considered

evidence of "anxiety" in adult rodents and measures of this behavior in mice (Crawley and Goodwin, 1980) have been proposed as animal models of anxiety, these data are consistent with the hypothesis that PTZ is effecting these changes in behavior via way of processes believed to mediate "anxiety".

However, there are a number of ways that pentylenetetrazol could cause increases in vocalizations while decreasing locomotor activity other than via its anxiogenic properties. While these changes are probably not secondary to stimulant effects, they may be secondary to thermoregulatory effects, muscle spasticity or tension or disturbed equilibrium via cerebellar GABA-ergic processes. Two of the more interesting possibilities are the disturbed equilibrium and the thermoregulatory hypotheses. While it is difficult to test, pentylenetetrazol may be affecting other GABA-ergic processes, possibly cerebellar, thereby altering the pups' sensitivity to disturbances in body position. Since pups ultrasound when they are handled or turned over, and there is evidence that these "handling calls" are mediated by processes other than those mediating isolation (Noirot, 1965;Sewell, 1968;Okon, 1971), it is clear that something about vestibular or equilibrium disturbances can cause increases in ultrasound. That the PTZ increases in vocalization occur primarily in the first 1 or 2 minutes

after the pups have been placed in isolation is consistent with this hypothesis: as is the reduction in locomotor behavior during the first minute of isolation. Therefore, until further tests can be undertaken, the possibility that PTZ may be mediating these changes in behavior via processes associated with vestibular or equilibrium disturbance cannot be dismissed.

Because vocalizations are sensitive to the effects of temperature, we tested the possibility that PTZ might be altering rates of calling by changing body temperature. Axillary temperatures were recorded following an eight minute isolation period for 15 pups receiving either saline, 15 or 30 mg/kg of PTZ. The mean temperature following saline injection was 34.5 degrees Celcius and for the respective doses of PTZ 33.9 C and 34.2 C. The differences between the groups were not significant indicating that thermoregulatory effects of the drug were not responsible for the changes in vocalization and activity rates following the administration of pentylenetetrazol.

Since GABA is the major inhibitory neurotransmitter in brain, any substance that acts to decrease its effectiveness, such as pentylenetetrazol, must have many CNS effects. Therefore, any discussion of the effects of PTZ on ultrasound and activity must begin with the caveat that there are many ways this substance can alter behavior

other than by increasing fear or anxiety. At the same time, it should be noted that there is indirect evidence from this study supporting the hypothesis that pentylenetetrazol is affecting these changes in behavior by way of processes believed to mediate anxiety. For example, the most prominent effect of PTZ is to dramatically increase vocalization during the first minute of isolation. This decrease in latency to respond to the fearful stimuli (isolation/novelty) further supports an anxiogenic hypothesis. While there may be alternative explanations for the effect of PTZ on acute responses to isolation, in exploratory models of anxiety where rodents are exposed to novel surrounding or unfamiliar food, the largest effects of anxiety reducing drugs occur during the first and second minute of exposure (Crawley et al., 1981; Soubrie et al., 1976). In other words, it is the latency to begin exploration that is most affected by drugs presumed to reduce anxiety. This is the case even when the half-life of the drug being tested is 30 hours. Therefore, these data, showing a dramatic increase in USV's and a decrease in locomotor behavior during the first and third minute in isolation are consistent with data from other laboratories that have examined the temporal relationship between anxiolytic/anxiogenic drugs and behavior.

Finally, the ability of pentylenetetrazol to both dramatically increase ultrasonic distress vocalization

responses to isolation and decrease locomotor behavior in a novel environment in the absence of stimulant or thermoregulatory effects is consistent with the hypothesis that pentylenetetrazol may be exerting its pharmacologic effects on these behaviors via its "anxiety" inducing properties as mediated by the proposed GABA-benzodiazepine receptor-chloride channel complex.

Fig. 7 The effects of isolation in a novel environment on ultrasonic vocalization and locomotor behavior in two week old pups over time. Values represent the (+ - SEM) number of calls and number of squares entered during minutes 1, 3, 5, and 8 for all pups in their baseline trials (N=39). The * indicates a significant difference ($p < .05$) from minute 1 on post hoc Mann-Whitney U test.

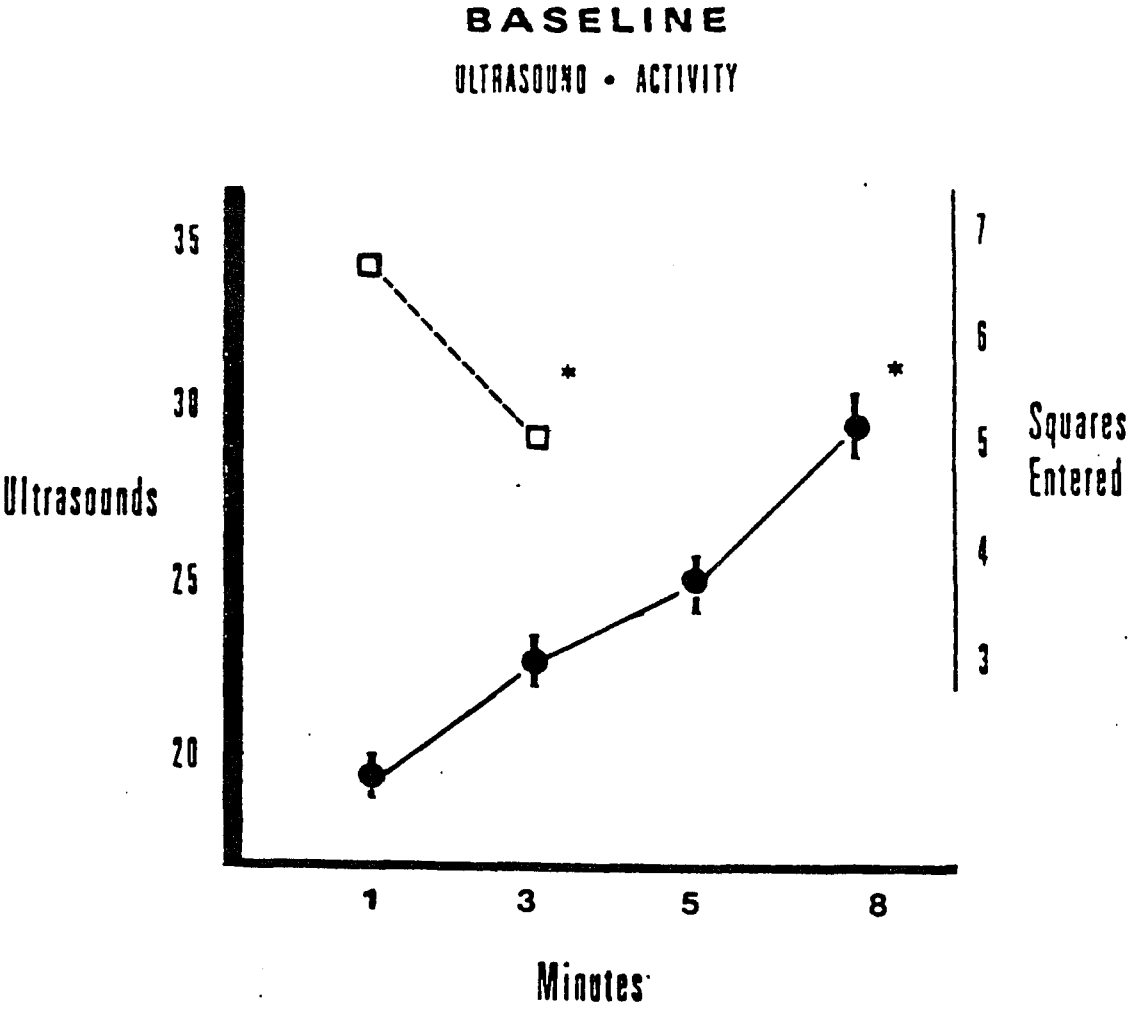

BASELINE
ULTRASOUND • ACTIVITY
35
30
25
20
Ultrasounds
7
6
5
4
3
Squares Entered
1
3
5
8
Minutes

Fig. 8 The effects of pentylenetetrazol on ultrasonic vocalization and locomotor behavior in isolated two week old pups. Values represent means (+ - SEM) of percent changes from baseline trials after eight minutes in isolation for ultrasounds and one minute in isolation for activity. The results were obtained 20 minutes following the administration of either pentylenetetrazol (5, 15, or 30 mg/kg) or saline. The * indicates a significant difference ($p < .05$) from saline controls on post hoc Mann-Whitney U test.

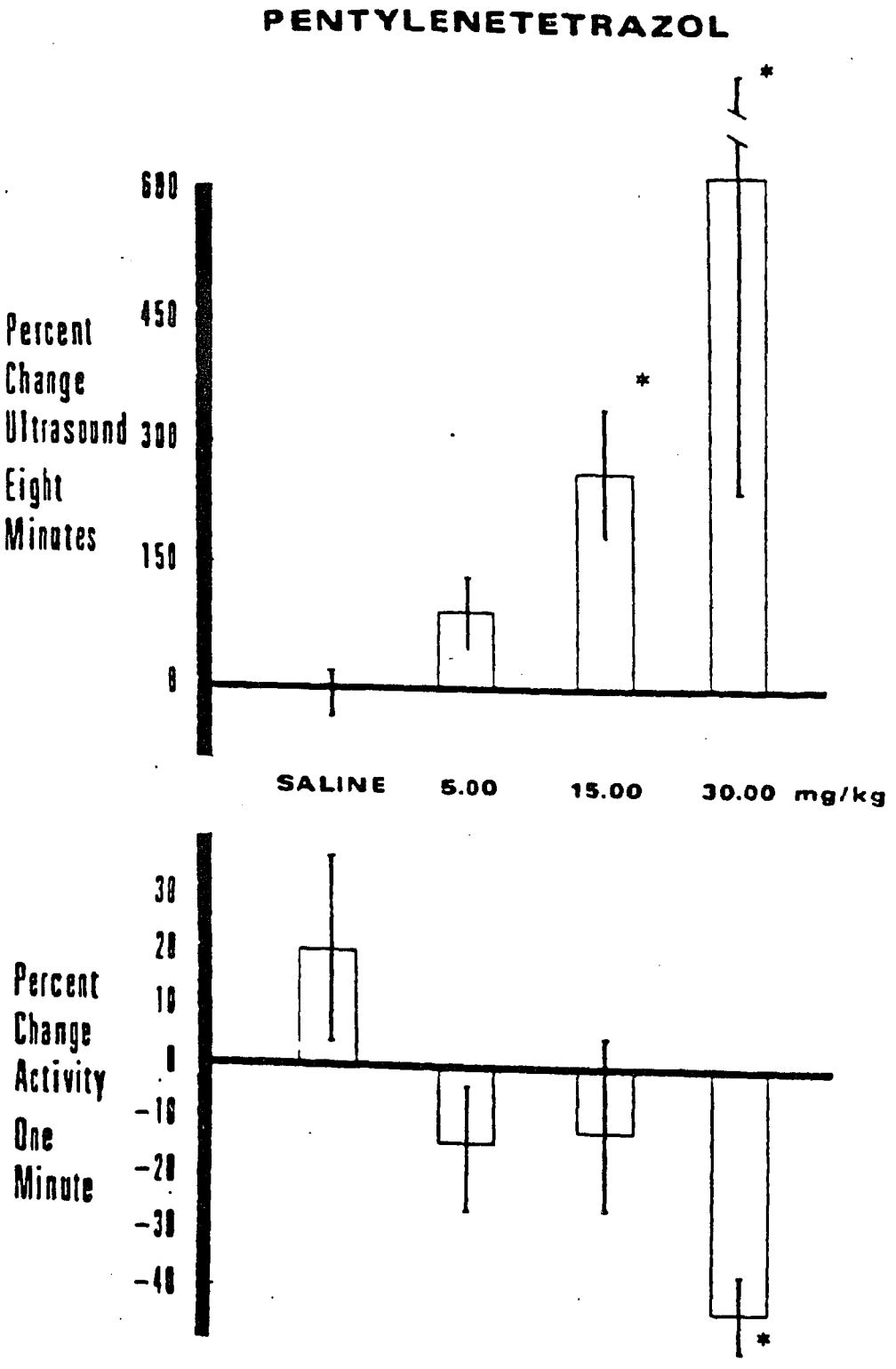

PENTYLENETETRAZOL
600
450
300
150
0
Percent
Change
Ultrasound
Eight
Minutes
*
*
SALINE
5.00
15.00
30.00 mg/kg
30
20
10
0
-10
-20
-30
-40
Percent
Change
Activity
One
Minute
*

Fig. 9 The effects of pentylenetetrazol on ultrasound in isolated two week old pups over time. The values represent means (+ - SEM) of the numbers of vocalizations emitted during each minute, for minutes 1, 3, 5, and 8 following the administration of 5, 15, or 30 mg/kg of PTZ or saline. The * indicates a significant differences ($p<.05$ or greater) from saline controls in post hoc Mann-Whitney U test.

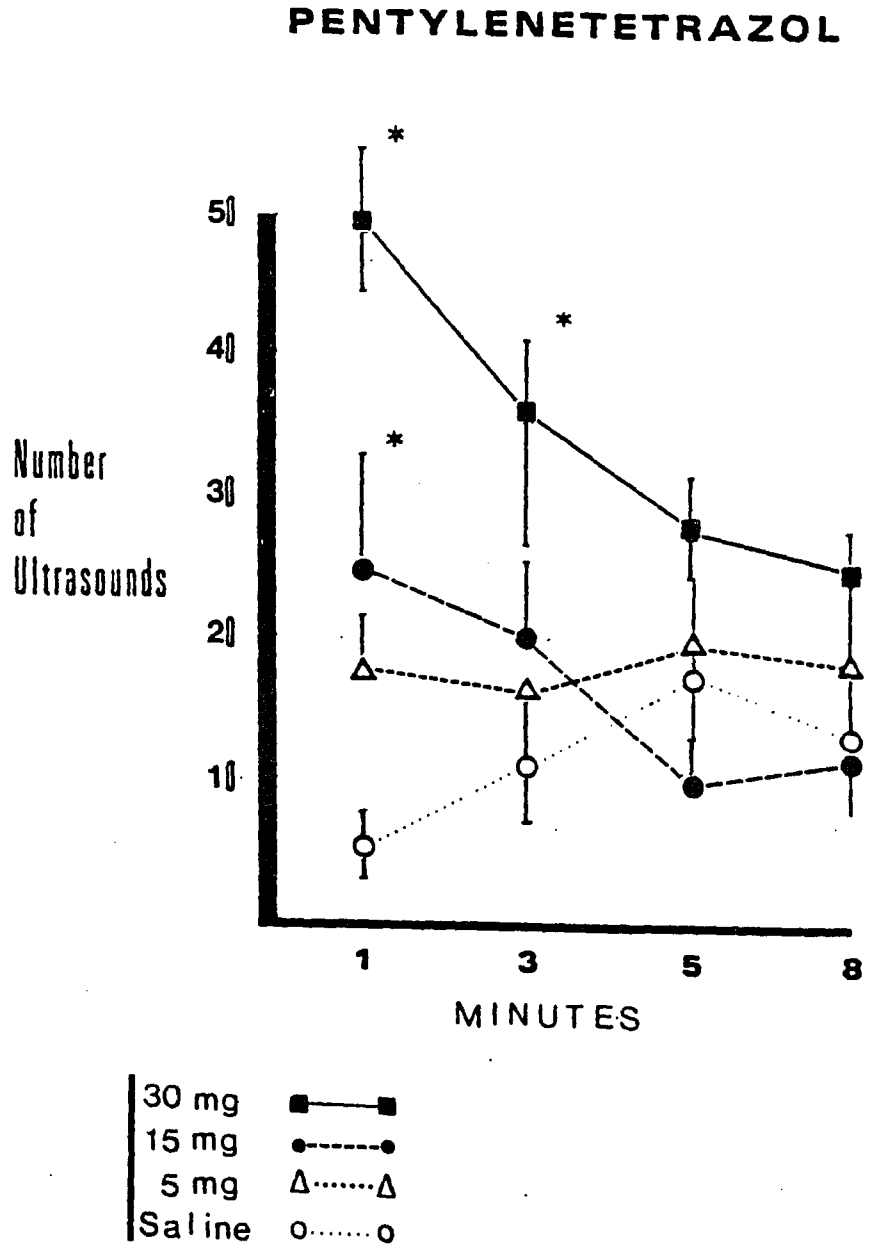
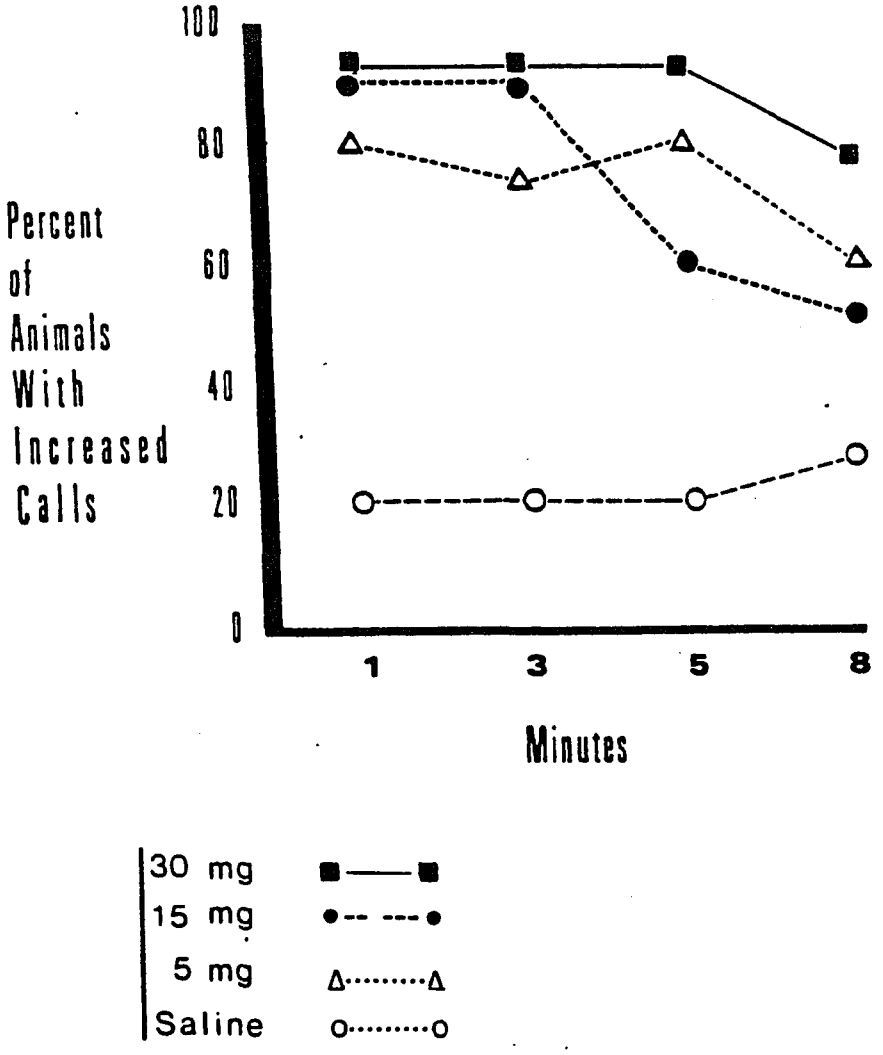

PENTYLENETETRAZOL
Number of Ultrasounds
50
40
30
20
10
*
*
*
1
3
5
8
MINUTES
30 mg
15 mg
5 mg
Saline

Fig. 10 The percent of animals with increased vocalization following i.p. administration of 5, 15, or 30 mg/kg of pentylenetetrazol or saline during the first, third, fifth or eigth minute of isolation.

PENTYLENETETRAZOL



The Effects of B-CCE and B-CCM on Ultrasound and Locomotion in Two Week Old Rats Isolated in a Novel Environment

In the first of two experiments B-CCE (1 microgram) was administered intracisternally to conscious 2 week old rats and changes in isolation induced vocalization and locomotion recorded. In the second experiment B-CCM (1, 3 and 9 mg/kg) was injected intraperitoneally in 2 week old rat pups and changes in isolation induced ultrasound and locomotion recorded.

The purpose of these investigations was to see if benzodiazepine receptor inverse agonists at doses reported to induce experimental anxiety in adult rodents would 1) alter ultrasonic vocalization rates in isolated 2 week old pups and 2) attenuate locomotor behavior as measured by the number of squares entered in a novel environment.

B-CCE

The methods of testing pups receiving intracisternal injections were essentially the same as those previously described, with the following exceptions. After the pups were weighed and numbered an electric shaver was used to remove the hair from a 1 x 1 cm portion of the skull immediately above the cisterna magna . The pups were then returned to their home nest and littermates for 2 hours. At the end of this time they were tested in isolation as

previously described to determine the baseline amounts of ultrasound and activity in isolation and returned to the nest for another 15 minutes. Following this period, a single pup was removed and injected intracisternally using the following standard procedure; 10 micro liter volume of solution containing either 1 microgram of B-CCE or vehicle alone was slowly injected into the cisterna magna of the conscious rat pup using a Hamilton syringe with a 27 gauge needle 3.5 mm in length. Immediately following the administration of the drug or vehicle, pups were placed singly in the test container and the number of squares entered and calls emitted were recorded for three and five minutes respectively. Each pup was used only once and served as it's own control.

Results

B-CCE

Intracisternal injection of 1 microgram of B-CCE led to a significant mean (+- SEM) increase in vocalization of 800% (+- 410%) during the first minute of isolation (Kruskal-Wallis $H(1)= 3.84$, $p<.05$) as shown in Figure 1. Posthoc analysis showed that this increase was significant when compared to vehicle injected controls ($p > 0.05$). During this same period locomotor behavior was significantly decreased in pups receiving B-CCE when

compared to non-injected controls (Kruskal-Wallis H(3.84, $p<.05$). However, as Figure 1 reveals, the pups injected intracisternally with the control vehicle also showed significant decreases in locomotor behavior. Since pups in this protocol were tested immediately following injection, it is possible that the mechanical insult of the injection alone was responsible for this reduction in activity. Alternatively, some chemical property of the vehicle could have led to the decrease.

Since previous studies have shown that the number of calls emitted in isolation increases in a monotonic fashion from the first through fifth minute (Fig. 1 of the PTZ study), the question arises as to the effects of B-CCE on USV's over time. If B-CCE simply enhances the normal vocal response to isolation then the USV increases that followed the B-carboline injection should also increase from the first to the fifth minute. Fig 2 illustrates that this is not the case. When the percent change values for B-CCE and vehicle injected pups are compared, it is clear that although the control pups show a 50% increase in USV's over baseline, these increases remain constant throughout the test, a pattern similar to that seen in naive animals. However, the pups injected with B-CCE present a very different response. This figure shows that while the most dramatic increases occur within the first minute of isolation of these pups, by the third minute the

differences between the groups have disappeared . This analysis suggests that the effect of B-CCE may be short lived and that the primary effect of the drug is to decrease the latency to ultraound when isolated in a novel environment.

To further assess the effects of B-CCE on ultrasound over time, the percent of animals within each treatment group that showed an increase in vocalizations following treatment were determined. Figure 3 shows that by the third minute, the number of B-CCE and control pups with increased ultrasound were approximately the same, further evidence that the effect of B-CCE on ultrasound in this protocol is short lived.

B-CCM

Intraperitoneal injections of 1, 3 and 9 mg/kg of B-CCM were followed by mean (+-SEM) increases of 630% (+-410%) 1298% (+-610%), and 1431% (+-458%) respectively, increases that were significantly different (Kruskal-Wallis $H(2)= 9.03$, $p< 0.05$). Posthoc analysis showed the increases following 3 and 9 mg/kg to be significant($p< 0.05$) as shown in Figure 4.

Figure 5 revealed that when the effects of B-CCM were assessed over time, after the first minute in isolation, the percent change in ultrasound decreased rapidly for all doses except the highest. While the administration of 9

mg/kg continued to result in hundredfold increases through the third minute, there were no longer significant differences between the scores of pups receiving 3 mg/kg and their vehicle controls during this minute. Furthermore, Figure 5 shows that the decline was precipitous for the 3 mg/kg dose pups, falling from a 1300% increase during the first minute to only 180% during the third minute.

When the percent of animals that increased their ultrasounds following drug or vehicle administration were compared over time as seen in Figure 6, it is clear that the actual number of animals with increased vocalizations declined more rapidly for pups given B-CCM than for controls. Taken together, while these data are only suggestive, the possibility that the higher doses of B-CCM may be having a biphasic effect on vocalization must be considered.

Figures 7 and 8 compare the effects of the three anxiogenic compounds on vocalizations and locomotor behavior. Figure 7 shows the percent change in ultrasound and locomotion for pups following the administration of either pentylenetetrazol (9 mg/kg), B-CCM (9 mg/kg), B-CCE (1 microgram per kilogram) and their respective controls after 1 minute in isolation. Figure 8 shows the percentage of animals in each drug group having increases in vocalization following the administration of either

pentylenetetrazol (9 mg/kg), B-CCM (9 mg/kg), B-CCE (1 microgram/kg) or saline after 1, 3 , and 5 minutes of isolation for purposes of comparison. Since these figures represent values that have been previously described and their purpose is to illustrate differences and similarities between the actions of these drugs, further description and interpretation is reserved for the discussion.

Discussion

This experiment shows that doses of B-CCE and B-CCM reported to be anxiogenic in adult rats 1) cause dramatic increases in distress vocalizations and 2) significantly reduce locomotor behavior during the first minutes of separation in two week old rats. These data also show that the changes in behavior following the administration of these beta-carbolines are short lived and that there is a striking similarity between the effects of B-CCE, B-CCM and pentylenetetrazol on both vocalization and locomotion despite differences in protocol and route of administration.

While the methyl B-carboline and pentylenetetrazol were injected intraperitoneally fifteen minutes before testing, B-CCE was injected into the cisterna magna only seconds before the pups were isolated. Not only were these drugs structurally dissimilar, their routes of

administration and post-injection test times were different in important ways. Considering the nature of these differences and their potential for interactive effects the similarities between the behavior of pups receiving B-CCE, B-CCM and pentylenetetrazol were surprising.

For example, all three compounds had their most pronounced effects on distress vocalizations during the first minute in isolation and none caused significant increases in this behavior after the third minute. Furthermore, at every dose, both B-carbolines had ceased to increase USV's by the final minute of the isolation trial as shown in Figures 2 and 5.

Further comparisons of these figures also reveals a striking similarity between the decline in the percent increase from the first to the third minute in pups injected with 3 mg/kg of B-CCM and those injected centrally with 1 microgram of B-CCE. Similarly, although the effects of the ICI vehicle on motor behavior make comparisons difficult, animals injected with B-CCE and B-CCM show decreases in locomotor behavior that continue to be significant through the third minute.

The only indication of a possible effect of the differences in procedure is seen when Figures 3 and 6 are compared. When the percent of animals with increased ultrasounds during the fifth minute are compared for B-CCE and B-CCM, the differences are clear. During this final

minute of the isolation period, 56% of the pups that were administered B-CCE intracisternally were continuing to ultrasound more than they did during the same minute of their baseline trial, whereas only 10% of the B-CCM injected pups had increased vocalizations by this time. Although there are alternative explanations for these data, one interpretation is that intracisternal injection of B-carbolines may result in longer lasting central effects on behavior, an analysis of the data that is consistent with reports that B-CCE and B-CCM are rapidly metabolized by blood esterases.

Another question of interest is how these individual compounds compare with one another in their ability to increase vocalizations. While it is important to exercise caution in comparisons of this sort because any differences in effectiveness may be the result of a number of factors including drug dosage, pharmacokinetics or procedure, nevertheless, a figure showing these values is informative. Figure 7 compares the percent change in ultrasound and locomotion during the first minute of isolation for pups administered the highest doses of either pentylenetetrazol , B-CCM or B-CCE . While all three of these putative "anxiogens" caused significant increases in vocalizations when compared to their controls, pentylenetetrazol (30 mg/kg) administration was followed by the most consistent and dramatic increases. B-CCM (9

mg/kg) injected intraperitoneally was also followed by first minute increases that were very pronounced while the vocalization responses in pups injected intracisternally with B-CCE were the least consistent of the three. While these differences are noteworthy thay are not inconsistent with what is known about the pharmacolgical activity of these drugs in other protocols. For example, pentylenetetrazol is well known for its efficacy and reliability as an "anxiogen" at low doses and as a "convulsant" at higher doses in a variety of protocols. Alternatively, beta-carbolines, like other agents active at benzodiazepine receptors, typically produce less consistent results (Insel et al., 1986; Liebman, 1985) That the methyl beta carboline injected intraperitoneally appears to be more effective at increasing vocalization than B-CCE injected intracisternally may or may not be noteworthy. Again, there are many factors that may lead to differences in responses, not the least of these is the skill of the person administering the injection. For although intracisternal injections in pups this age are straightforward and relatively simple, the possibility of error is much greater when using this route of administration.

Since both pentylenetetrazol and B-CCM have opposing actions on vocalization and locomotion, the question arises as to whether the pups with the highest rates of calling

are the same animals that have the greatest reduction in locomotor behavior. This was determined by computing a Spearman rank correlation between the percent change vocalization scores and the percent change locomotion scores for individual animals for each dose of these drugs during the first and third minutes of their isolation tests. The correlations for the first minute for pups receiving pentylenetetrazol or saline were as follows; saline (R=-.294), 5 mg/kg (R = .549) and 15 mg/kg (R=-.359) were not significant, however 30 mg/kg (R=-.648) was significant at the .05 level. For the third minute, this correlation was reduced (R=-.334) and was no longer significant. These data show that at lower doses of pentylenetetrazol the pups that show increases in vocalization are not necessarily the same ones that have reductions in locomotor behavior, a finding that was not altogether unexpected. Hofer and Shair (1987) in an analysis of specific "isolation distress behaviors" reported that they were unable to find a correlation between vocalization, locomotion, grooming, rising or increased heartrate in individual pups. While all of these behaviors were dramatically increased when pups were separated from their nest and littermates, no two behaviors were found to occur together with any consistency. This suggests that pups experiencing the same degree of "distress" may respond differently, some vocalizing at a

high rate while they continue to move about the test area, others becoming very still and increasing their vocalization more moderately. While these present results are consistent with these and other data from Hofer and Shair (1978), it is suggestive that at the highest dose of pentylenetetrazol there is a significant negative correlation (p,.05), which indicates that at doses where the pups are most responsive to both actions of the drug the animals with smallest increases in vocalization had the largest reduction in locomotor activity.

To see if the beta-carbolines had similar effects, the data from the B-CCM study was submitted to a Spearman rank correlation test, and the results for the first minute were as follows; the correlation for vehicle (=.286), 1 mg/kg (R=-.073) and 3 mg/kg (R= -.017) were not significant, however 9 mg/kg (R=.534) was significant at the .05 level. The actions of the vehicle and lower drug doses on correlations between locomotor behavior and vocalization were similar to pentylenetetrazol results in that they were not significant. However, the highest dose of B-CCM led to a significant (p,.05) positive correlation. This showed that following the administration of B-CCM the pups with the largest increases in vocalization also had the greatest reduction in locomotor behavior. This result is opposite that seen in pups who have received the highest dose of pentylenetetrazol, a finding that is difficult to interpret

and may merit further investigation. Although speculative, it could be seen as evidence that these drugs are influencing locomotor behaviors via separate systems.

Another question that arises concerns the issue of whether these drugs have different effects on pups that cry very little in isolation (hypothetically, pups that are less "distressed" by separation) and those that vocalize a lot (those that are more "distressed"). To address this issue the relationship between the number of ultrasounds made in isolation before and after treatment was examined. This was done by computing a Spearman rank correlation between the number of vocalizations made in the baseline test with the number of calls made following treatment with either saline or pentylenetetrazol for individual pups. A significant negative correlation would show that the pups with the lowest numbers of vocalizations before being administered a drug were responsible for the highest increases, an absence of correlation would indicate that both high and low calling pups increased their rates of calling approximately the same. Pups receiving saline alone had a correlation of R=+.931 which was significant at the .001 level indicating that in pups not receiving anxiogenic drugs, changes in vocalization were clearly a function of initial rates of calling. Specifically, pups that vocalized a lot the first time they were tested were very likely to vocalize a lot when they were tested again,

similarly, pups that cried very little during their first isolation test were likely to continue to cry very little when tested the second time. When the animals were given pentylentetrazol the correlations were as follows; 5 mg/kg (R= .806), 15 mg/kg (R=.646) and 30mg/kg (R=-.079). To determine whether the increases in vocalization following the administration of B-CCM were significantly correlated to baseline vocalization rates in a similar fashion, a Spearman rank correlation between these scores was computed for each drug dose and vehicle. Pups given the vehicle alone had a positive correlation of R=.759 which was significant at the .05 level. When the pups were given B-CCM the correlations were as follows; 1 mg/kg (R=.413), 3 mg/kg (R=.352) and 9 mg/kg (R= .308, $p<.05$).

A comparison of these correlations between pentylenetetrazol and B-CCM reveals a similarity in the pattern of responses across doses. The before and after test vocal responses for pups given either saline or the vehicle control were highly correlated indicating that in the absence of drugs pups tend to be consistent in their vocal responses to isolation over trials. Each increasing dose of drug (B-CCM and pentylenetetrazol) reduced the correlation and the highest dose of both drugs led to the lowest correlation for both. These data suggest that individual pups tend to have a characteristic vocal response to isolation and that at low to moderate doses

these drugs generally enhance this response. Furthermore, because these correlations are much lower at the highest doses does not mean that this effect has changed, most likely, these changes are the results of ceiling effects. That is, pups who are crying at high rates the first time they are tested are limited in the number of calls they can emit following the administration of a drug, regardless of its potency.

Another question suggested by these data is whether or not these results support the hypothesis that B-carbolines may be increasing distress vocalizations via their "anxiogenic" properties as mediated by the proposed GABA-benzodiazepine receptor chloride channel complex. A hypothesis supported by the following results.

First, because both B-carbolines cause increases in one behavior, vocalization, and decreases in another, locomotion, the hypothesis that increases in USV's are secondary to non-specific effects on arousal can be dismissed with some confidence. Furthermore, B-carbolines, like pentylenetetrazol, have been reported to cause "decreases in locomotor behavior that failed to reach significance" (File et al., 1982) suggesting that this behavioral change may be characteristic of low doses of B-carbolines in adult animals as well. As noted earlier, since a reluctance to explore a novel environment is considered evidence of "anxiety" in rodents (Crawley and

Goodwin, 1980), these data are consistent with the hypothesis that B-CCE, B-CCM and pentylenetetrazol may be effecting these changes in behavior via processes believed to mediate "anxiety".

Further evidence that these changes in behavior may be mediated by anxiogenic properties of these compounds was found in the assessment of the effects of the B-carbolines over time. The most prominent feature of the vocalization increases is that they occur during the first three minutes in isolation, behavior that can be interpreted as a decreased latency to respond to a fearful stimulus (isolation/novelty). While there are alternative explanations for these acute responses, in exploratory models of anxiety where rodents are exposed to novel surroundings, the only significant effects of anxiety reducing drugs occur during the first three minutes of exposure (Crawley and Davis, 1982) (Soubrie et al., 1976). In other words, it is the latency to begin exploration that is most affected by drugs presumed to reduce "anxiety" and this is the case regardless of the half-life of the drug being tested. Therefore, the data showing that the only significant increases in vocalization occur during the first three minutes of isolation are in agreement with data from other laboratories that have investigated the temporal relationship between anxiolytic/anxiogenic drugs and behavior, and further support the anxiogenic hypothesis.

There are, however, a number of ways that B-carbolines may cause increases in vocalization other than through some "anxiogenic" process. A few of the more important possibilities include increased muscle spasticity / tension, vestibular or equilibrium disturbances (as discussed in the chapter on pentylenetetrazol) or some dysphoric pre-convulsant state that might be composed of aspects of all of the above.

Perhaps the most problematic of these for any study addressing the "anxiety in animals" issue, is the pre-seizure dysphoria hypothesis. Since all anxiogenic beta-carbolines are either frankly convulsant at high doses or proconvulsant in certain protocols, the administration of any of the pro-anxiety B-carbolines is open to the criticism that any changes in behavior may be the result of some pre-convulsant state of dysphoria. This criticism is supported by the finding that sub-convulsant doses of several B-carbolines including B-CCE, B-CCM and FG 7142 can cause EEG changes characteristic of animals with chemically induced seizures (Skolnick et al., 1983; Pellow, 1985). It is important to note here, however that one of the most prominent theories about the neurophysiological substrate of anxiety proposes that <u>all</u> anxiety is the result of seizure-like activity in the septo-hippocampal area of the brain (Haefely et al., 1983). The theory is based, in part, on the fact that until quite recently, all known

anxiety reducing drugs protected against seizures and all pro-anxiety compounds at higher doses caused seizure activity in the brain or convulsions. Furthermore, the synaptic events underlying these effects were considered inseparable. Therefore, it is difficult to eliminate the possibility that B-carbolines may be changing behavior in this paradigm via some pre-convulsant dysphoric state. However, to guard against this possibility, the doses chosen for this study (1 microgram of B-CCE) and 1 and 3 mg/kg of B-CCM were selected because they were below the doses above which changes in the EEG were observed in adult rats. Finally, no animals in any of these conditions, at any dose were seen to have tremors, myoclonic jerks, or any other behaviors associated with convulsant doses of these drugs.

Finally, the ability of the ethyl and methyl esters of B-carboline-3-carboxylate to dramatically increase ultrasonic distress vocalization responses to isolation and decrease locomotor behavior in a novel environment in the absense of stimulant effects is consistent with the hypothesis that B-CCE and B-CCM may be exerting their pharmacologic effects on these behaviors via their "anxiety" inducing properties as mediated by the proposed GABA-benzodiazepine receptor-chloride channel complex.

Fig. 11. The effects of B-CCE on ultrasonic vocalizations and locomotion in 2 week old isolated pups. The values represent means (+ - SEM) of percent change from baseline trials after 1 minute in isolation for ultrasound and locomotion. The results were obtained immediately following the ICI administration of either 1 microgram of B-CCE or vehicle and compared to non-injected pups. The * indicates a significant difference ($p<.05$) from vehicle injected controls on post hoc Mann-Whitney U test.

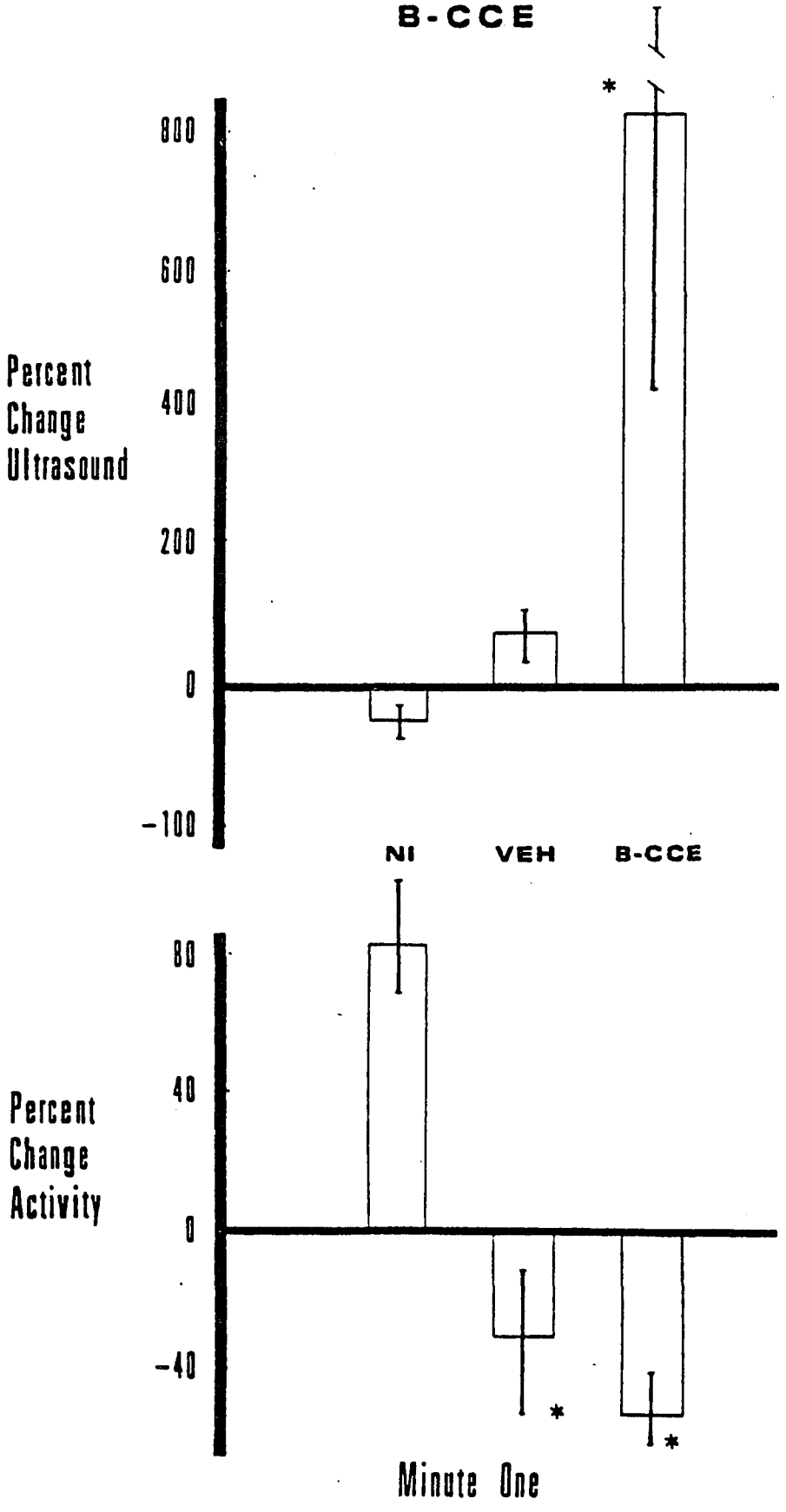

B-CCE
800
600
400
200
0
-100
Percent
Change
Ultrasound
NI
VEH
B-CCE
80
40
0
-40
Percent
Change
Activity
Minute One

Fig. 12. The effects of ICI administration of B-CCE on ultrasonic vocalization in 2 week old isolated pups over time. The values represent means (+ - SEM) of percent change in the number of vocalizations from the baseline trials after 1, 3 or 5 minutes in isolation. The results were obtained immediately following the ICI administration of either 1 microgram of B-CCE or vehicle. The * indicated a significant difference ($p<.05$) from vehicle injected controls on post hoc Mann-Whitney U test.

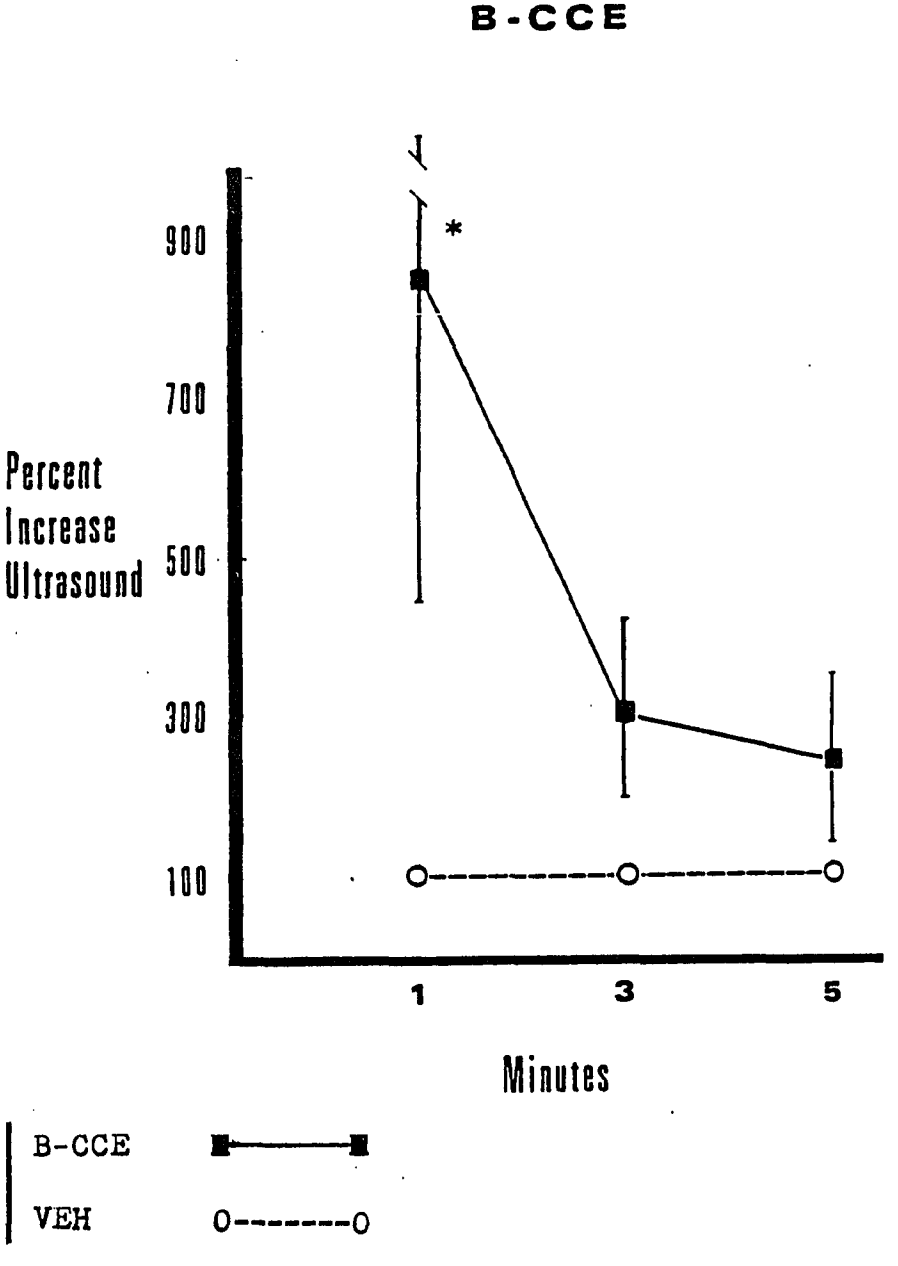

B-CCE
900
700
500
300
100
Percent
Increase
Ultrasound
*
1
3
5
Minutes
B-CCE
VEH
O-------O

Fig. 13. The percentage of animals with increased vocalization after 1, 3 and 5 minutes in isolation following ICI administration of B-CCE compared to their controls. The values represent the percentage of pups in each treatment condition that showed an increase in vocalization over their individual baseline trials. The results were obtained immediately following the ICI administration of either B-CCE or its vehicle alone and compared to non-injected controls.

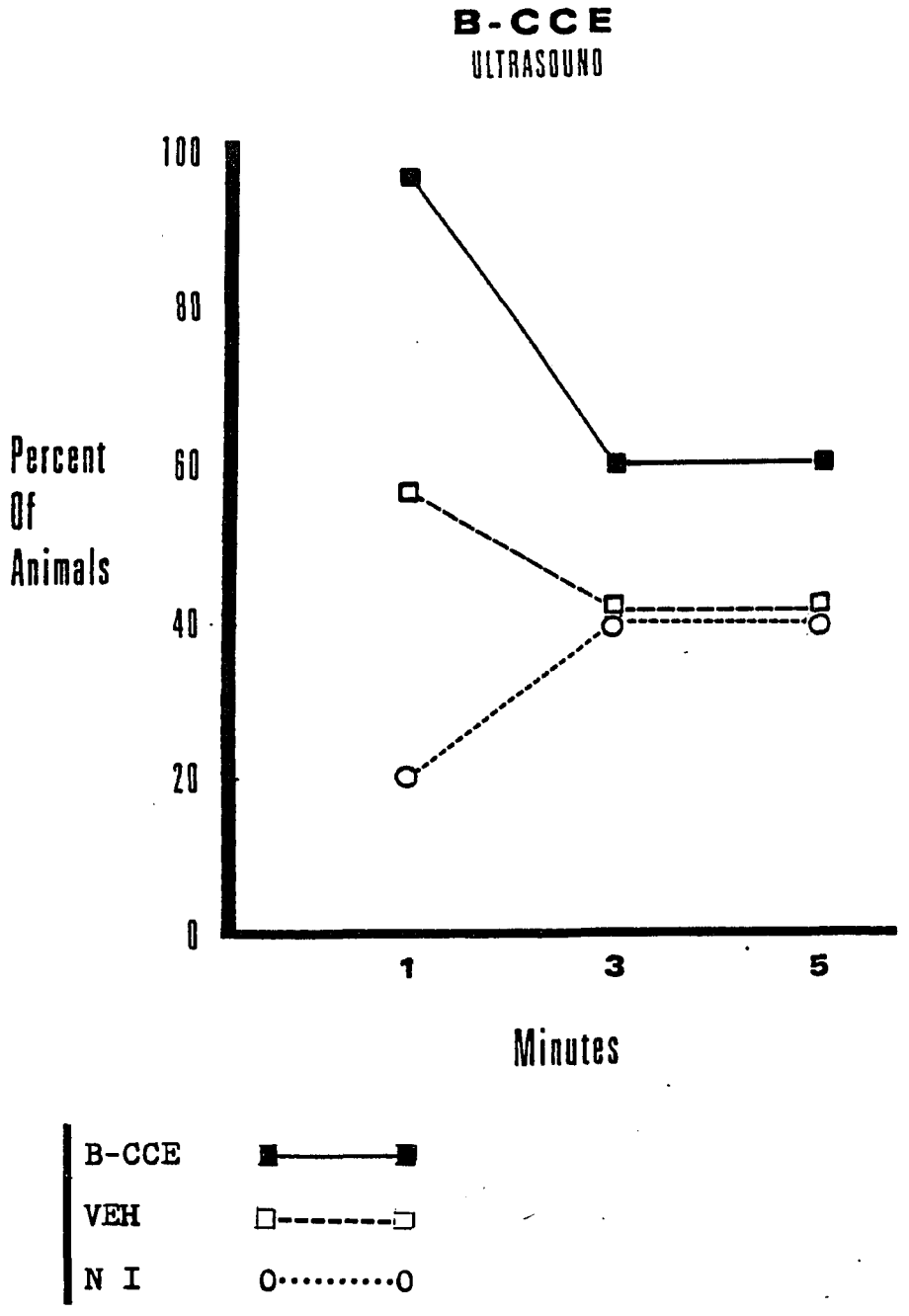

B-CCE
ULTRASOUND
100
80
60
40
20
0
Percent
Of
Animals
1
3
5
Minutes
B-CCE
VEH
N I

Fig. 14. The effects of B-CCM on ultrasonic vocalization and locomotion in 2 week old isolated pups. The values represent means (+ - SEM) of percent change from baseline trials after 1 minute in isolation for ultrasound and locomotion. The results were obtained 15 minutes following the administration of either 1, 3 or 9 mg/kg of B-CCM or vehicle. The * indicates a significant difference ($p<.05$) from vehicle controls on post hoc Mann-Whitney U test.

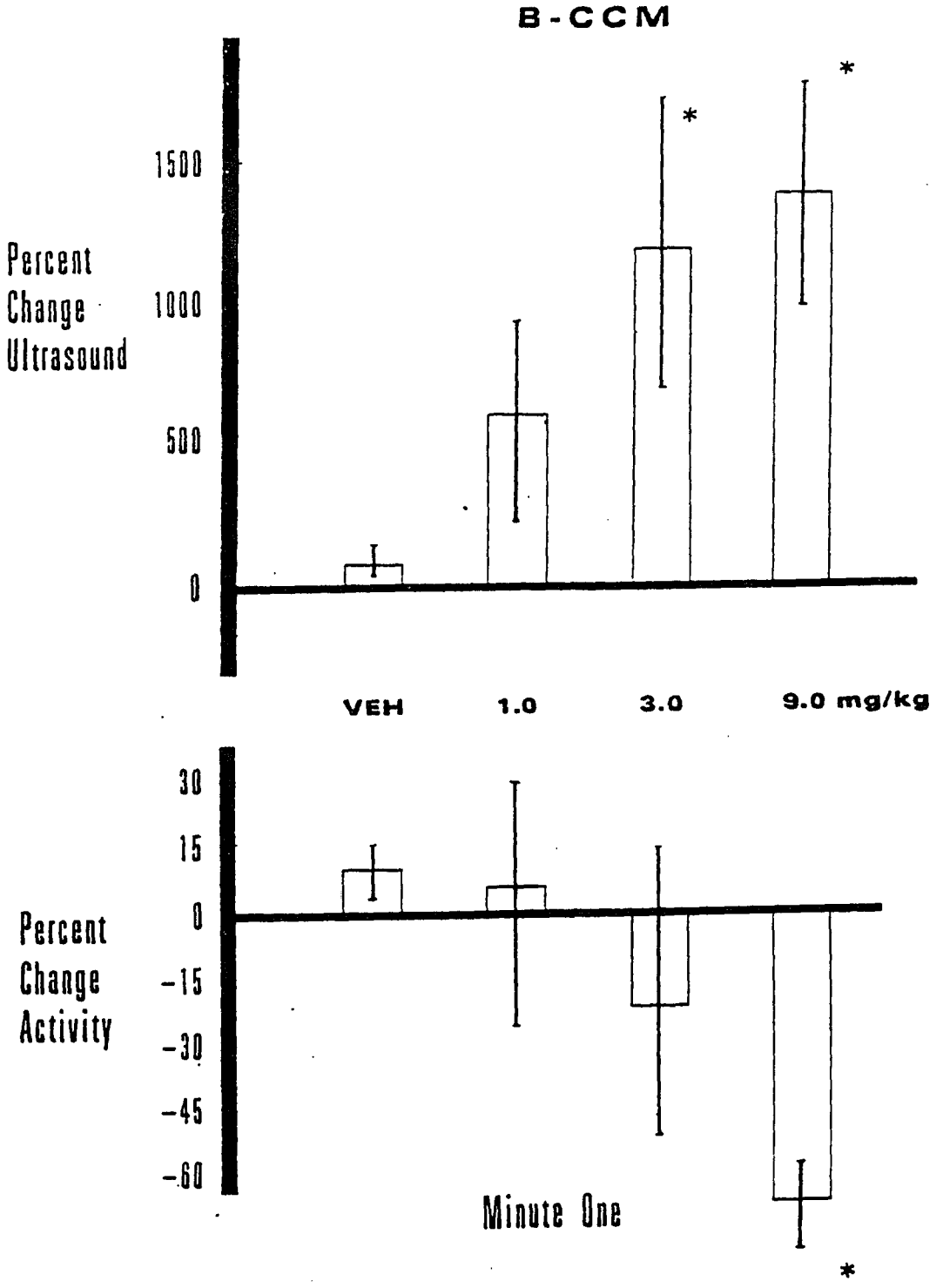

B-CCM
Percent Change Ultrasound
1500
1000
500
0
*
*
*
VEH
1.0
3.0
9.0 mg/kg
Percent Change Activity
30
15
0
-15
-30
-45
-60
Minute One
*

Fig. 15. The effects of B-CCM on ultrasonic vocalization in 2 week old isolated pups over time. The values represent means (+ -SEM) of percent change in the number of vocalizations from baseline trials after 1, 3 and 5 minutes in isolation. The results were obtained 15 minutes following the i.p. administration of either B-CCM, 1, 3 or 9 mg/kg or vehicle. The * indicates a significant difference ($p<.05$) from vehicle controls on post hoc Mann-Whitney U test.

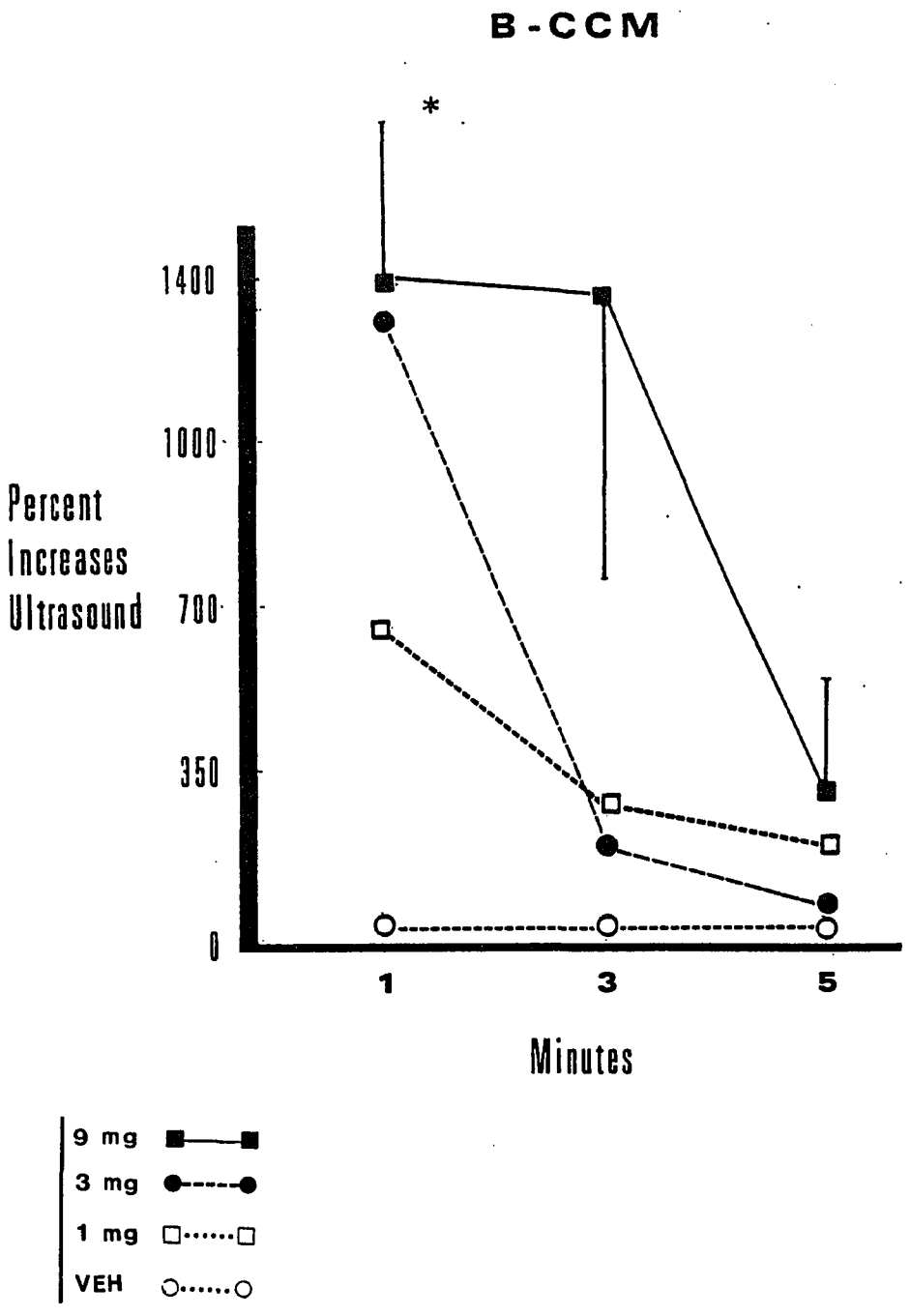

B-CCM
*
1400
1000
700
350
0
Percent
Increases
Ultrasound
1
3
5
Minutes
9 mg
3 mg
1 mg
VEH

Fig. 16. The percentage of animals with increased vocalizations after 1, 3 or 5 minutes in isolation following the administration of B-CCM or vehicle. The values represent the percentage of pups in each treatment condition that showed an increase in vocalizations over their individual baseline trials. The results were obtained 15 minutes following the administration of either B-CCM, 1, 3 or 9 mg/kg or vehicle.

B-CCM

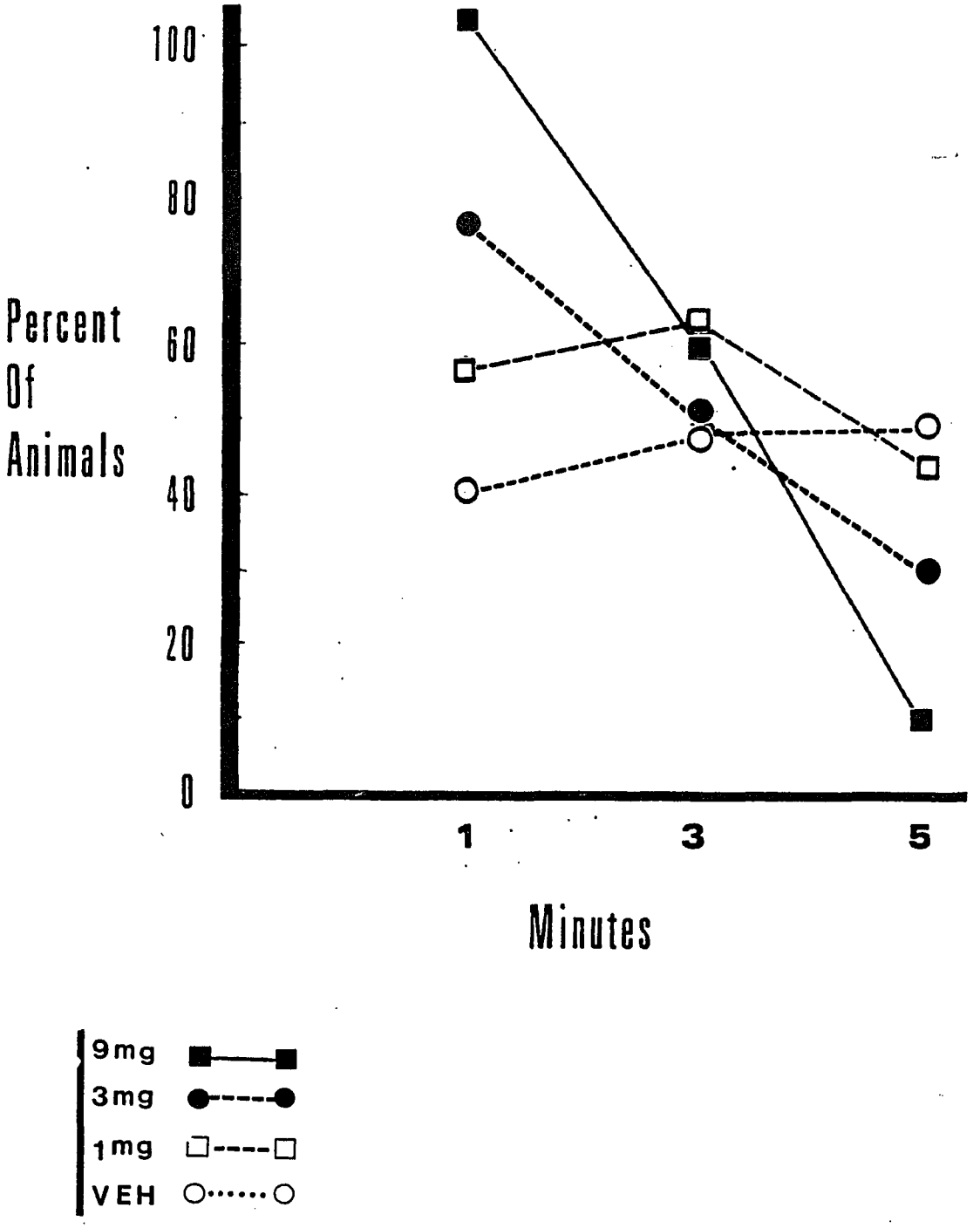

100
80
60
40
20
0
Percent
Of
Animals
1
3
5
Minutes
9mg
3mg
1mg
VEH

Fig. 17. A comparison of the effects of pentylenetetrazol, B-CCM and B-CCE on ultrasound and locomotion in 2 week old isolated pups. The values represent means (+ - SEM) of percent change from baseline trials for each drug and its control after 1 minute in isolation. The results were obtained following either the i.p. injection of 30 mg/kg of pentylenetetrazol, 9 mg/kg of B-CCM or the ICI administration of 1 microgram of B-CCE.

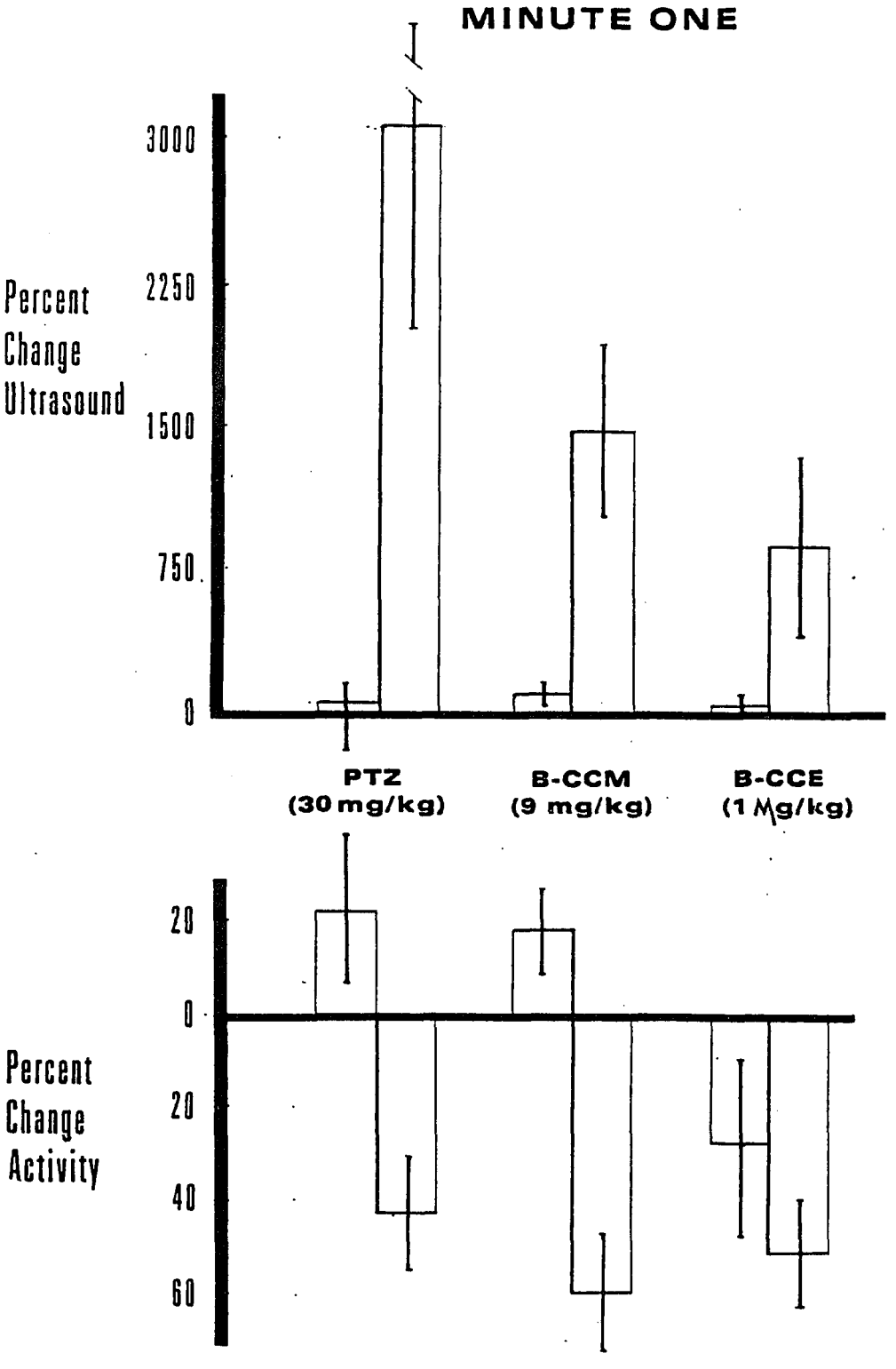

MINUTE ONE
Percent Change Ultrasound
3000
2250
1500
750
0
PTZ (30 mg/kg)
B-CCM (9 mg/kg)
B-CCE (1 Mg/kg)
Percent Change Activity
20
0
20
40
60

Fig. 18. A comparison of the effects of pentylenetetrazol, B-CCM and B-CCE on the percentage of animals with increased vocalizations over time. The values represent the percentage of pups in each drug treatment condition that showed an increase in vocalizations over their individual baseline trials during the first, third or fifth minute of isolation. The results were obtained following either the i.p. injection of 30 mg/kg of pentylenetetrazol, 9 mg/kg of B-CCM or the ICI administration of 1 microgram of B-CCE.

ANXIOGENICS

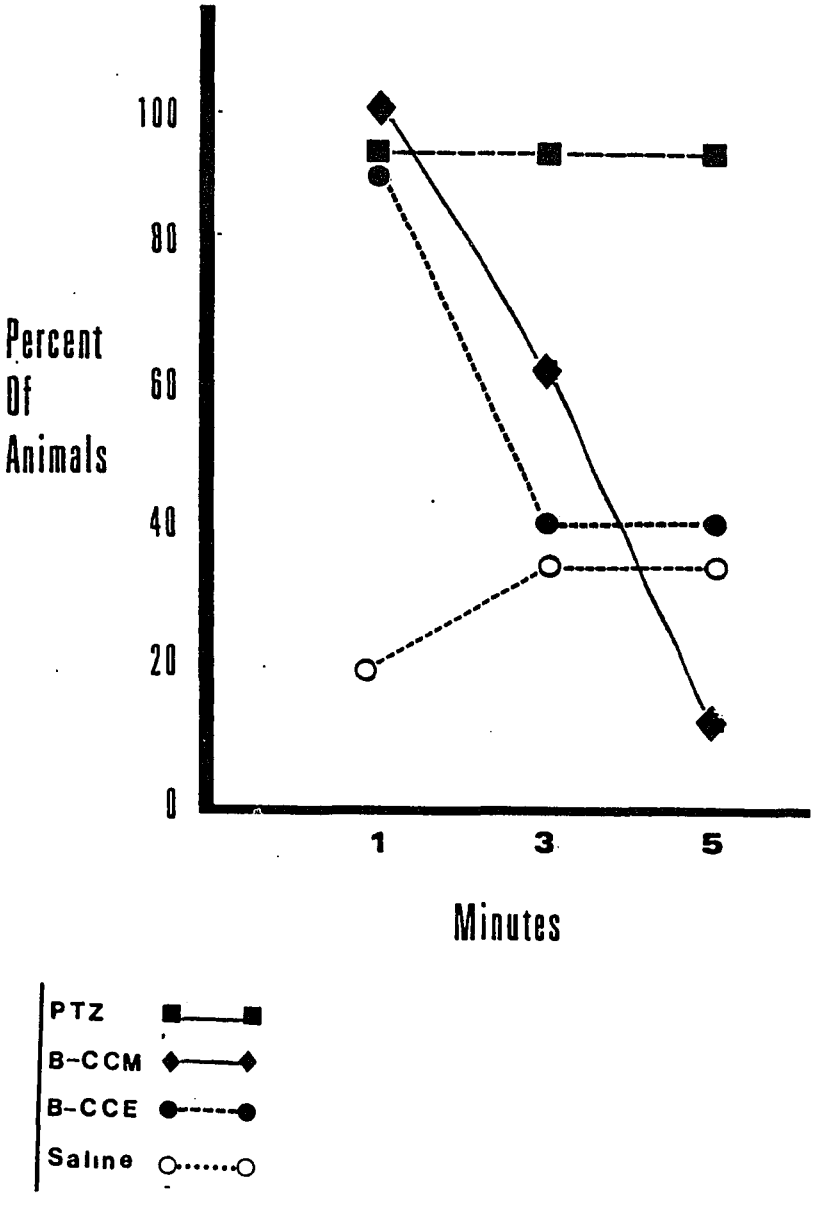
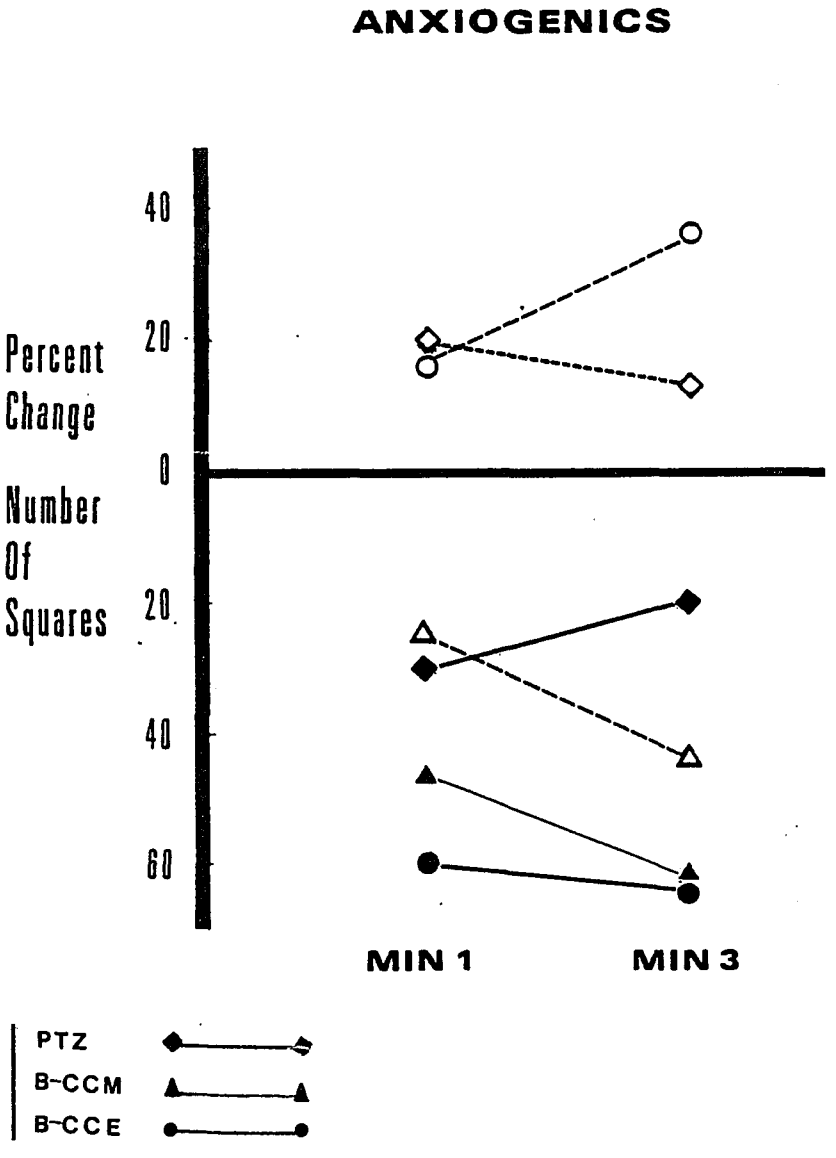


Fig. 19. A comparison of the effects of pentylenetetrazol, B-CCM and B-CCE and their controls on locomotor behavior in 2 week old pups over time. The values represent the mean (+ -SEM) percent change from baseline trials in the number of squares entered during the first and third minutes of isolation.


ANXIOGENICS
Percent Change
Number Of Squares
40
20
0
20
40
60
MIN 1
MIN 3
PTZ
B-CCM
B-CCE

Summary of Findings

A summary of the data from these studies reveals that ultrasonic calls of rat pups placed alone in a novel environment decreased following the administration of the anxiolytic chlordiazepoxide (1 , 3 , and 9 mg/kg) at 7 and 14 days of age. One and 3 mg/kg reduced vocalizations 22% and 28% respectively in 7 day olds and 61% and 70% in 14 day olds. These changes following chlordiazepoxide administration were not accompanied by decreases in locomotor behavior. The highest dose, 9 mg/kg, however, led to greater than 90% decreases in both vocalization and locomotor behavior at 1 and 2 weeks of age. The benzodiazepine receptor antagonist RO 15-1788 (5 mg/kg) significantly reduced the effects of chlordiazepoxide on vocalization demonstrating that its actions were mediated by the central benzodiazepine receptors.

Ultrasonic calls in isolation <u>increased</u> dramatically following the administration of the anxiogenic pentylenetetrazol (15 and 30 mg/kg). The 30 mg/kg dose was followed by a 3000% increase during the first minute in isolation. Locomotor behavior was significantly decreased during this time as well by pentylenetetrazol (5, 15, and 30 mg/kg).

Intracisternal administration of the anxiogenic B-CCE

(1 microgram/kg) was followed by an increase in vocalization rate of 800% when compared to controls during the first minute of isolation. Locomotor behavior was significantly reduced for pups that received both B-CCE and the vehicle control injection when compared to non-injected controls.

Intraperitoneal injections of B-CCM (3 and 9 mg/kg) were followed by increased vocalization during the first minute of isolation of 1298% and 1431% respectively. Locomotor behavior decreased during the first and third minute of isolation for pups receiving B-CCM (9 mg/kg) when compared to controls.

Conclusion

While the ultimate aim of these studies has been to investigate the possibility that "distress vocalizations" in rat pups isolated in a novel environment might have potential as an animal model of anxiety, the studies themselves were designed to address the following questions.

1. Do these data support the hypothesis that the GABA-benzodiazepine receptor chloride channel complex plays a role in the mediation of separation /isolation behaviors in rat pups?

The drugs administered in these tests,

chlordiazepoxide, RO 15-1788, B-CCE and B-CCM, and pentylenetetrazol bind to sites in the brain other than those on the GBRC, therefore, the actions of any or all of these drugs on vocalization and / or locomotion may be the result of effects on receptors completely independent of the GABA-benzodiazepine receptor chloride channel complex. Nevertheless, the data from these studies are in good general agreement with the hypothesis that the GBRC may play a role in the mediation of separation / isolation behavior in infant rats. For example, chlordiazepoxide, active at this receptor complex reduced distress calling by 60-70% in a manner and at doses consistent with previously reported effects of this drug in infant rats (Gardner, 1985; Insel et al., 1986; Pappas and Walsh, 1983). Moreover, since these reductions are blocked by the administration of the benzodiazepine receptor antagonist RO 15-1788, these actions of this drug probably are the result of pharmacologic activity at these central sites. Finally, both pentylenetetrazol and the Beta-carbolines, known to be active at sites independent of one another on the GBRC cause significant increases in these distress calls during the first three minutes of isolation.

2. Since drugs active at the GBRC effect levels of arousal, muscle tone and susceptibility to seizures as well as levels of "anxiety", do these results support the

hypothesis that these changes in USV following the administration of these agents are the result of drug actions on neurophysiological processes that mediate "experimental anxiety" in adult rodent models?

Again, the results of these investigations can be interpreted as supporting this hypothesis. In each study of a specific compound, the response of the isolated pup to the drug was consistent with this idea. Specifically, the anxiety reducing chlordiazepoxide quieted the pups and the anxiety provoking substances led to increased calling, and in every instance except one, the changes in USVs were independent of the changes in locomotion.

The most convincing support for the "anxiety hypothesis" came from the unexpected finding that both pentylenetetrazol and beta-carbolines caused a reduction in locomotor behavior. This was significant for two reasons . First, since the major alternative explanation for the effects of these compounds on behavior was that they were simply secondary to a change in "arousal". The fact that USVs were dramatically increased while locomotor behavior was significantly decreased was powerful evidence against this alternative explanation. Secondly,the changes in locomotor behavior that were seen over time were strikingly similar to adult rodent responses to a novel environment, responses that are typically interpreted as evidence of

"fear of novelty". Taken together, the locomotor data are perhaps the most important for supporting the hypothesis that the pharmacological actions responsible for the changes in USV and locomotion in pups may be the same as those that mediate experimental anxiety in adult rodents.

Other evidence supporting the anxiety hypothesis comes from data showing that these drugs do not significantly change the body temperature of pups this age. Because temperature is such an important determinant in the rate of calling in isolation in younger pups, an important alternative explanation for the effects of these drugs is that these changes in behavior are secondary to changes in body temperature. The finding that the highest dose (9 mg/kg) of chlordiazepoxide caused a 2 degree Celcius reduction in body temperature while eliminating ultrasound taken together with data from Gardner (1985) who reported that clonidine which also reduces body temperature caused significant increases in ultrasound suggests that some other mechanism is probably responsible for the changes in ultrasonic vocalizations and locomotion.

Finally, both isolated pups and adult rats tested in a variety of other animal models of anxiety have a characteristic low dose / high dose response to benzodiazepine receptor agonists. In adult rats low doses of benzodiazepines lead to increases in punished responses while at high doses both punished and unpunished responses

are significantly decreased. These results are said to reflect the ability of these agents to reduce "anxiety" at low doses whereas higher doses result in "sedation". A comparable phenomenon is seen in the data from the chlordiazepoxide study in which 1 and 3 mg/kg led to a 60-70% reduction in USV with no reduction in locomotor behavior whereas at 9 mg/kg both USVs and locomotion are essentially eliminated. While most drugs have different effects at high and low doses , the vocal and locomotor response to high and low doses of chlordiazepoxide is entirely consistent with the responses of adult rats to high and low doses of the benzodiazepines in the Geller-Seifter procedure, exploration models and social interaction models of anxiety as well.

3. While vocalization and locomotion in isolated rat pups must meet a number of other criteria before they can be considered potential animal models of "anxiety", what do the data from these studies say about the hypothesis that vocal and locomotor responses to isolation may provide a reliable and reproducible "model of anxiety"?

In general, these studies are in excellent agreement with this idea. As previously discussed, not only are these behaviors responsive to drugs active at the GBRC, but the data suggest that some pharmacological property of the benzodiazepine ligand other than its sedative effect is

mediating this drugs action on vocalization. Furthermore, the low dose / high dose response is strikingly similar to that seen in other animal models of behavior in which the low dose response to the drug is interpreted as "anxiolytic". Further evidence in support of the anxiety hypothesis are the findings that doses of these drugs that cause both increases and decreases in vocalizations in infant rats are entirely consistent with doses of these drugs that either precipitate or relieve experimental "anxiety" in adult rodents.

4. How do the results from these studies agree or disagree with data from other laboratories?

These data are in good general agreement with the reports from both Gardner (1985) and Insel and his colleagues (1986) that benzodiazepines reduce USVs without reducing locomotor behavior and that pentylenetetrazol causes inceases in distress vcalizations (Insel et al., 1986)

However, the results of the administration of the beta carbolines are in certain respects different. When Insel and his coworkers gave B-CCE intraperitoneally to 6-10 day old pups they reported "inconsistent" increases in isolation calls and it was only after pups with high rates of calling were separated from the data pool that they were

able to report statistically significant increases in USVs. Alternatively, when 2 week old pups from this laboratory were given B-CCE intracisternally or B-CCM intraperitoneally, significant increases in distress vocalizations were seen.

Because the procedures used to test the pups, the routes of administration of the drugs, the strain and age of the animals tested were different in these two laboratories, it would be speculative to suggest that any single factor was responsible for the differences in these results. However, the simplest explanation may be found in a comparison of 6-10 day olds typical vocal response rate to isolation to that of 14 day olds. At room temperature younger pups begin to ultrasound immediately at high rates and the number of calls actually decreases over time after only a few minutes in isolation (Kehoe and Blass, 1986). In 13 / 14 day olds however, many pups do not ultrasound at all during the first 2 minutes of separation (Okon, 1971) but appear to engage in more "exploratory" behavior (Allin and Banks, 1971). Since these anxiogenic compounds have their largest effect on USVs during the first 2-3 minutes of isolation and these effects disappear by the fifth minute, it would be more difficult to show statistically significant increases in younger animals. This potential "ceiling" effect, however, is essentially eliminated when 13 /14 day old pups are tested in this protocol because

their baseline ultrasound rates are normally so low. Alternatively, since the binding data indicate that the receptors thought to mediate experimental anxiety in adult rodents are reaching adult levels by this time, more pups may be responding fully to the action of these drugs. Finally, it is also likely that the difference in route of administration contributed significantly to these differences in results. We administered this beta-carboline intracisternally because it has been reported to have a helf-life of less than 1 minute in rat serum whereas Insel and his colleagues injected it intraperitoneally.

Another difference in our results was found in the pups locomotor response to the anxiety provoking agents. Both pentylenetetrazol and B-CCE caused statistically significant reductions in the number of squares crossed in the first three minutes of isolation in our study of 14 day olds whereas Insel and his coworkers did not report that these drugs reduced locomotion in their younger animals. While this difference in results may also be attributible to the differences in behavioral responses to isolation in pups of different ages, developmental studies by Pappas and Walsh (1983) suggest that locomotor responses to drugs acting at the benzodiazepine receptor undergo important developmental changes around day 12. Therefore, in the

absence of other information, the significance of these differences between pup's responses to PTZ and B-CCE at 1 and 2 weeks of age remains unclear.

5. To what degree are the data from these studies in agreement with existing theories concerning "distress vocalizations"?

Although there are a number of theories indirectly addressing issues associated with separation / isolation, there are only two major hypotheses directly related to issues addressed in this paper concerning distress vocalizations. The first of these was proposed by Bell in 1974. He suggested that vocalization simply reflected a high level of arousal in infants and the sound of these calls led to increased levels of arousal in dams.

As previously discussed, data from these studies and from other laboratories (Gardner, 1985 and Insel et al., 1986) are clearly not in agreement with this hypothesis. Although it is clear that sedative doses of chlordiazepoxide will eliminate distress vocalizations, there are lower doses (1 and 3 mg/kg) that reduce these calls from 60-70% without reducing motor behavior. Perhaps more significantly, the "anxiety" producing pentylenetetrazol, B-CCE and B-CCM which are CNS stimulants, capable of inducing seizures at higher doses actually reduce locomotor in isolated pups. The fact that

these drugs have divergent effects on these two behaviors argues effectively that in infant rodents these distress vocalizations are mediated by some processes other than those that mediate the sedative effects of these drugs.

The second hypothesis concerning distress vocalizations was proposed by Panksepp and his colleagues in 1980. In this paper he suggested that "social motivation" in humans and social animals was a "direct manifestation of innate neural circuits which are as spontaneously responsive as those which govern other basic motivated behavior patterns such as feeding and drinking ". He further proposed that brain opioids mediated social affect and social attachment and presented evidence supporting this hypothesis.

Since then other researchers have verified that vocalization in rodents can be significantly reduced by the administration of low doses of opiates (Herman and Panksepp, 1986; Kehoe and Blass, 1986). However, Insel reported recently (1986) that their laboratory was unable to reduce USVs with opiates without also reducing locomotor behavior, suggesting these effects might be secondary to the sedative effects of these drugs. Moreover, opiates reduce respiration rates dramatically and since ultrasonic vocalizations are coincidental with expiration, this may contribute importantly to their effects on ultrasound.

While the data from this laboratory do not dispute the efficacy of opiates for reducing distress vocalizations in isolated rodents, they support the hypothesis that a mechanism other than the opiate receptors may be able to influence separation responses of infant rodents in important ways.

To the degree then, that the GABA-benzodiazepine receptor complex is a mechanism that appears to play a role in the mediation of isolation distress responses, these theories could be said to be alternative hypotheses. However, recent advances in our understanding of the interactive nature of the various neurotransmitters and neuromodulators suggest that there is probably no single primary neural substrate responsible for a behavior as complex as social attachment. In any case, our ability to tease apart the contributions of specific substrates is not yet sophisticated enough to test such a premise. Therefore the idea that there are two specific neurophysiological systems, the GBRC / anxiety system and the opioid / social affect system competing with one another as the "primary" determinant of infant responses to isolation may not be the most fruitful approach to this problem. Perhaps a more productive question would address the possibility that these "systems" interact, a question that is currently under investigation in this laboratory.

In conclusion, these data support in interesting and unexpected ways the hypothesis that changes in levels of ultrasonic vocalizations in rat pups following the administration of drugs active at the GABA-benzodiazepine receptor chloride channel complex may be mediated by the same pharmacological actions that mediate experimental "anxiety" in adult rodents. Secondly, they support and extend research from two independent laboratories investigating the role of the benzodiazepine receptor in the mediation of distress vocalization. They also present new evidence that changes in distress vocalization following the administration of these psychoactive drugs are not secondary to the effects of these agents on arousal or thermoregulation. These studies also present evidence that although ultrasounds in 1 week old pups are responsive to benzodiazepine ligands, 2 week old Wistar pups because of the divergent effect of these drugs on vocalization and locomotion may offer more advantages as a test animal. Finally, taken together, these data support the hypothesis that distress vocalizations in isolated two week old rat pups may prove to be a reliable and reproducible animal model of anxiety in which to test the recently discovered endogenous benzodiazepine ligands.

Appendix

Fig. 3a. The effect of 3 mg/kg of chlordiazepoxide on ultrasonic vocalization in pups of different ages.

These values represent mean (+ −) SEM) percent changes in the number of vocalizations emitted in the 5 minute baseline isolation trials and the 5 minute drug isolation trials of 96 pups tested at different ages. The results were obtained 20 minutes following the i.p. administration of 3 mg/kg of chlordiazepoxide. Changes in vocalization were not significant for any of the ages ($F\ 7, 38 = 2.19$).

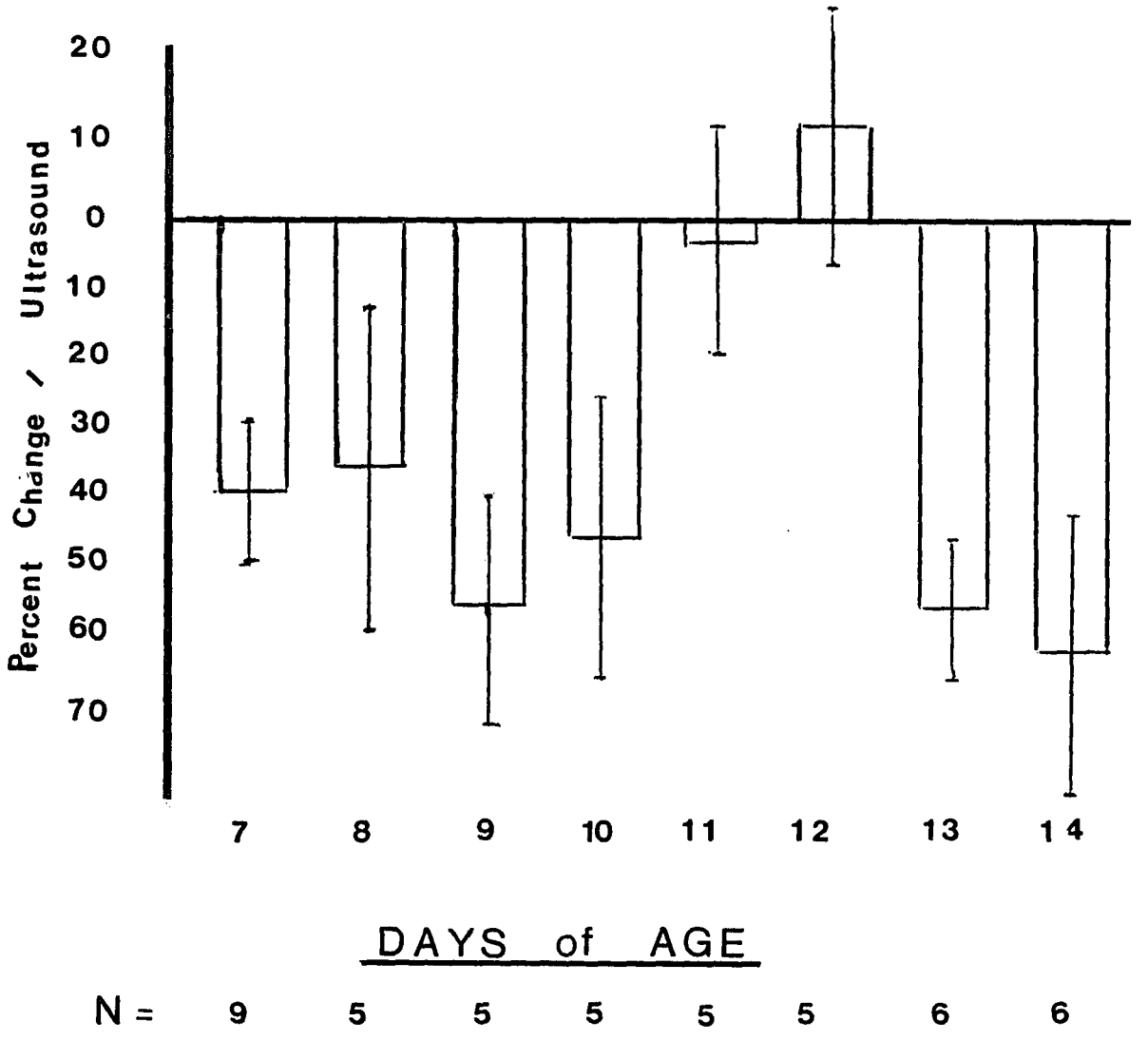
The methods used to derive these data (see Project Design methods section) led to repeated disturbances of the litters. This problem, plus the fact that some litters were disturbed only once and others as many as three times led to the decision to remove these data from the main body of the dissertation.

The primary purpose of the experiment was to determine when chlordiazepoxide began to exert its maximum effect on vocalization reduction in isolated pups. However,by looking at pups in the same litter at different ages I also hoped to learn something not only about the action of the drug at different ages, which I had assumed would be very straightforward, but to learn about the characteristics of litters that were more responsive or less responsive to the

drug.

Early in the experiment I noticed that pups of 11 and 12 days did not appear to be as consistently responsive to the vocalization reducing action of the drug as older or younger pups as shown in figure 3a. Although the differences in responses to isolation were not found to be statistically significant (F 7, 38+ 2.19), they do constitute a trend. As noted earlier, the design of this study was flawed, therefore this unusual distribution of scores may simply be a reflection of some aspect of the design error. However, when I returned to the literature to see if there were any reports of unusual responses to benzodiazepines in pups of this age, there was one reference that was suggestive. As noted earlier, there are very few studies of the effects of benzodiazepines on behavior in pups of any age, however Pappas and Walsh (1983) did note in the introduction of one study that benzodiazepines cause increases in activity that disappear "at 12 days". In the same study they suggest that this effect on locomotion may be mediated by the alpha two adrenoreceptors and since the benzodiazepines are known to inhibit firing in the locus coeruleus (Grant, Huang & Redmond, 1980), they suggest that it is possible that as the norepinephrin pathways mature, the effects of the benzodiazepines could be interrupted. Although it may only be coincidental that vocalization responses to

benzodiazepines also appear to be interrupted on day 11 and 12, the data are suggestive and have been included for this reason.


20
10
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70
Percent Change / Ultrasound
7
8
9
10
11
12
13
14
DAYS of AGE
N = 9 5 5 5 5 5 6 6

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