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NEUROPHARMACOLOGICAL ANTAGONISM OF THE β-CARBOLINE-INDUCED "ANXIETY" RESPONSE IN RHESUS MONKEYS[1]

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Abstract

A behavioral and physiological syndrome of stress-related responses was reported in primates following treatment with the benzodiazepine receptor antagonist, β-carboline-3-carboxylic acid ethyl ester (β-CCE). The behavioral and physiological effects of β-CCE are similar to those observed during stressful or "anxiety"-related conditions characterized in rhesus monkeys under natural conditions. Pharmacological agents which are known to antagonize anxiety responses in other paradigms were tested for their ability to antagonize the actions of β-CCE. Dizaepam (1 mg/kg) completely blocked the effects of β-CCE (200 μg/kg) on anxiety-related behaviors, heart rate and blood pressure, plasma catecholamines, cortisol, and adrenocorticotrophic hormone. A presynaptically active dose of the α-adrenoreceptor agonist, clonidine (10 μg/kg), significantly attenuated the effects of β-CCE on all parameters, whereas the β-adrenoreceptor agonist, propranolol (3 mg/kg), failed to alter the increases in plasma catecholamines, cortisol, or ACTH. In addition to these adrenerergic agents, the serotonin antagonist, cyproheptadine (1 mg/kg), and the GABA-mimetic, 4,5,6,7-tetrahydroisoxazolo(5,4-C)pyrindin-3-ol (1 mg/kg), partially blocked the behavioral, physiological, and biochemical changes after β-CCE. Manifestation of the complete "anxiety" syndrome evoked by β-CCE in primates may require the functional activity of several neurotransmitter systems.

The discovery of a specific receptor site for benzodiazepines (BZDs) in mammalian brain has raised the possibility that this site may mediate physiological responses to "fear" or "anxiety" (Tallman et al., 1980; Paul et al., 1981). BZDs are the most widely prescribed psychopharmacologic treatment for neurotic anxiety in humans (Greenblatt and Shader, 1974). BZDs such as diazepam act as anxiolytics in a variety of animal models, including the thirsty-lick conflict test (Vogel et al., 1971), the social interaction test (File, 1980), the light-dark exploration test (Crawley, 1981; Crawley and Davis, 1982), and punished responding in pigeons (Wuttke and Kelleher, 1970) and squirrel monkeys (Barrett and Brady 1983; Patel and Migler, 1982).

Antagonists of the BZD receptor have recently been developed (Hunkeler et al., 1981; Czernik et al., 1982), including a class of β-carbolines which appear to have active antagonist properties (Braestrup et al., 1982; Skolnick et al., 1982). (There has been some confusion about whether β-carboline-3-carboxylic acid ethyl ester (β-CCE) should be labeled an agonist or an antagonist at the BZD receptor. Classically, a pharmacologic antagonist is a drug which binds to the same receptor as an agonist but reverses or prevents the agonist's effect. Ro 15-1788, for example, binds to the BZD receptor with high affinity and antagonizes the principal pharmacologic actions of the BZDs. We have chosen the term "active antagonist" for drugs such as β-CCE because, like traditional antagonists, they bind to BZD receptors and reverse or prevent BZD effects, but, in addition, they have potent intrinsic effects opposite to those of the agonists. Confusion arises because Ro 15-1788 also blocks the effects of β-CCE. Some investigators have concluded that drugs such as β-CCE should therefore be considered "inverse agonists": agonists because they have intrinsic effects and inverse because these effects are opposite to those of BZDs. Ultimately, the discovery of an endogenous ligand for the BZD receptor should help to resolve the question of whether β-CCE is an agonist or antagonist.) β-Carbolines have been reported to act as anxiogenics in the thirsty-lick conflict test (Corda et al., 1983), the social interaction test (File et al., 1982), and an interoceptive discriminative stimuli model (Lal and Sherman, 1980). In a recent report, β-carboline-3-carboxylic acid ethyl ester (β-CCE) was shown to produce an acute behavioral syndrome characterized by dramatic elevations in heart rate, blood pressure, plasma cortisol, and catecholamines in rhesus mon-

[1] β-Carboline-3-carboxylic acid ethyl ester was a gift from Drs. J. M. Cook and M. Cain (Department of Chemistry, University of Wisconsin, Milwaukee, WI). Diazepam was a gift from Hoffman-La Roche (Nutley, NJ). Clonidine was a gift from Boehringer Ingelheim Ltd. (Ridgefield, CT). Propranolol was a gift from Ayerst Laboratories (New York, NY). Cyproheptadine was a gift from Merck Sharp and Dohme (Rahway, NJ). THIP was kindly supplied by Dr. John Tallman. Elizabeth Birecree, Gregory Bunt, and Marcie Engel provided excellent technical assistance in behavioral observation.

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keys (Ninan et al., 1982). These effects were blocked by pretreatment with the selective BZD receptor antagonist Ro 15-1788. Recent studies in humans have demonstrated anxiogenic effects of β-carboline esters when administered to normal volunteers (Dorow et al., 1983). Therefore, administration of β-CCE to monkeys was proposed as a reliable and reproducible animal model of human anxiety. In the present studies, pharmacological characterization of the β-CCE-induced syndrome was undertaken to determine the relationship of the effects of β-CCE to other neurotransmitter systems.

Several neurotransmitter pathways in mammalian brain have been linked to anxiety-related behaviors, including the noradrenergic and serotonergic projections from the midbrain to regions of the limbic system and cerebral cortex. Stimulation of the nucleus locus ceruleus, either electrically or with the presynaptic α_2-adrenoreceptor antagonist, piperoxan, evokes anxiety-related behaviors in monkeys (Redmond et al., 1976; Redmond, 1977). Pretreatment with clonidine, at doses which inhibit the firing rate of locus ceruleus neurons, has been reported to block anxiety-related behaviors induced by threatening environmental stimuli (Redmond, 1981). Similarly, serotonin (5-hydroxytryptamine, 5-HT) antagonists have been shown to increase punished responding in conflict test paradigms (Geller and Blum, 1970; Graeff and Schoenfeld, 1970; Wise et al., 1972; Barrett and Brady, 1983). Direct injection of chlordiazepoxide into the serotonergic dorsal raphe nucleus produced anxiolytic effects in the conflict avoidance paradigm (Thiebot et al., 1982). As both the noradrenergic and serotonergic systems have been most directly implicated in anxiety-related behaviors, antagonists of norepinephrine (NE) and 5-HT were tested as possible antagonists of the actions of β-CCE. In addition, the functional coupling of the BZD binding site to the γ-aminobutyric acid (GABA)-chloride ionophore receptor complex suggests that GABA agonists might also block the behavioral and physiological actions of β-CCE (Herberg and Williams, 1983; Hoehn-Saric, 1983). The GABA-mimetics 4,5,6,7-tetrahydroisoxazolo(5,4-C)pyrindin-3-ol (THIP) and muscimol were reported to act as anxiolytics in the conflict test (Guidotti, 1981; Rasmussen et al., 1981) and in a shuttle-box test (Cuomo et al., 1981). To test the hypothesis that the "anxiogenic" effects of β-CCE are mediated through other neurotransmitters previously implicated in anxiety-related behaviors, the NE antagonists clonidine and propranolol, the 5-HT antagonist cyproheptadine, and the GABA agonist THIP were compared to diazepam for their ability to block the manifestations of the β-CCE-induced "anxiety" syndrome in monkeys.

Materials and Methods

Adult male rhesus monkeys (*Macaca mulatta*) weighing 7 to 9 kg were chair adapted over a period of several weeks and prior to each experiment. One day before each experiment, animals were restrained in chairs under ketamine anesthesia and allowed to adapt to this condition for at least 24 hr before drug administration. Previous behavioral and neuroendocrine experiments with "chair-adapted" rhesus monkeys have validated this procedure for nonstressful habituation to restraint chairs in other studies of stress-related hormones in monkeys (Kalin et al., 1983). All animals were fitted, under ketamine anesthesia, with femoral venous catheters, which were kept patent during the experiment by a slow infusion of sterile 0.9% NaCl.

One day after catheterization, each monkey was returned to the restraint chair. Values for heart rate, blood pressure, and behavioral parameters were assessed to determine a time point at which habituation to the chair was re-established, usually 20 to 30 min after placement in the chair. When a stable base line was obtained, each animal was administered β-CCE (200 μg/kg, i.v.) in 10% Emulfor vehicle (GAF Corp., New York, NY). One day later, the animal was pretreated with either vehicle, propranolol (3 mg/kg, i.v.), clonidine (10 μg/kg, i.v.), cyproheptadine (1 mg/kg, i.v.), THIP (1 mg/kg, i.v.) or diazepam (1 mg/kg, i.v.), 15 min before administration of β-CCE (200 μg/kg, i.v.).

TABLE I

Behavioral parameters related to agitation, stress, or anxiety in rhesus monkeys

Parameters were derived from Redmond (1977), Altmann (1962), Ninan et al. (1982), and further intensive observation of chair-adapted male rhesus monkeys in the NIMH primate facility. Occurrence of each parameter was scored for each 1-min interval within 15-min sampling blocks.

Agitation Parameters	
I. Body Movements	III. Head Movements
Struggles	Turns head
Jerky movements	Bares teeth-grimace
Twists whole body	Mouth open
Freezes	Bites hand
II. Hand Movements	IV. Other
Grasps self	Vocalization
Grasps chair	Hyperventilation
Hand wrings	Piloerection
Picks	Penile erection
Scratches	Urination
Scratches to bleeding	Defecation
	Drinking
	Eating
	Eyes closed

Doses of antagonists were chosen from previous reports and tested by pilot studies in the present paradigm to determine a nonsedating dose which had no intrinsic behavioral activity. Behavioral ratings and blood collections were taken every 15 min, in sequences beginning 30 min before β-CCE administration and ending 30 min after β-CCE administration.

Behavioral ratings were performed by a human observer who was "blind" to the treatment protocol, using a behavioral rating scale designed for this study. The categories of behaviors are listed in Table I. Each animal was constantly observed for the occurrence of each behavioral parameter during 1-min intervals. The number of occurrences of each behavior was totaled for each 15-min sequence.

Systolic, diastolic, and mean arterial pressures were monitored automatically at 5-min intervals through a femoral cuff with a Dinamap Research Monitor (model 1245, Applied Medical Research, Timonium, MD).

Three milliliters of blood were drawn at 15-min intervals and centrifuged immediately at 4°C. The plasma was frozen on dry ice and stored at −80°C. Plasma cortisol and adrenocorticotrophic hormone (ACTH) were quantitated by radioimmunoassay (Kao et al., 1975; Healy et al., 1983). Plasma epinephrine and NE were measured by high performance liquid chromatography with electrochemical detection (Goldstein et al., 1981).

Data were analyzed for each parameter over the series of vehicle, diazepam, clonidine, propranolol, cyproheptadine, and THIP pretreatment by analysis of variance (ANOVA), followed by a Newman-Keuls test for significance of individual means ($N = 6$ to 8 monkeys for each treatment).

Results

β-CCE (200 μg/kg) significantly increased behavioral parameters, heart rate, blood pressure, plasma NE, epinephrine, plasma cortisol, and ACTH, as compared to base line and vehicle controls. All values are expressed as mean ± SEM ($N = 6$ to 8 for each treatment; significant at $p < 0.05$ by the Newman-Keuls *a posteriori* test of individual means following ANOVA).

Figure 1 illustrates the consistency and reproducibility of the behavioral responses to β-CCE. Experiments 1 and 2 were separated by a time period of 1 month. Similar qualitative and quantitative responses to β-CCE were seen for each monkey on the two experiments; e.g., monkeys 8085, 8221, and 57 were high responders, whereas monkey 58 was a low responder. In addition, individual monkeys showed idiosyncratic responses; e.g., one monkey consistently scratched, another hand-wrung,

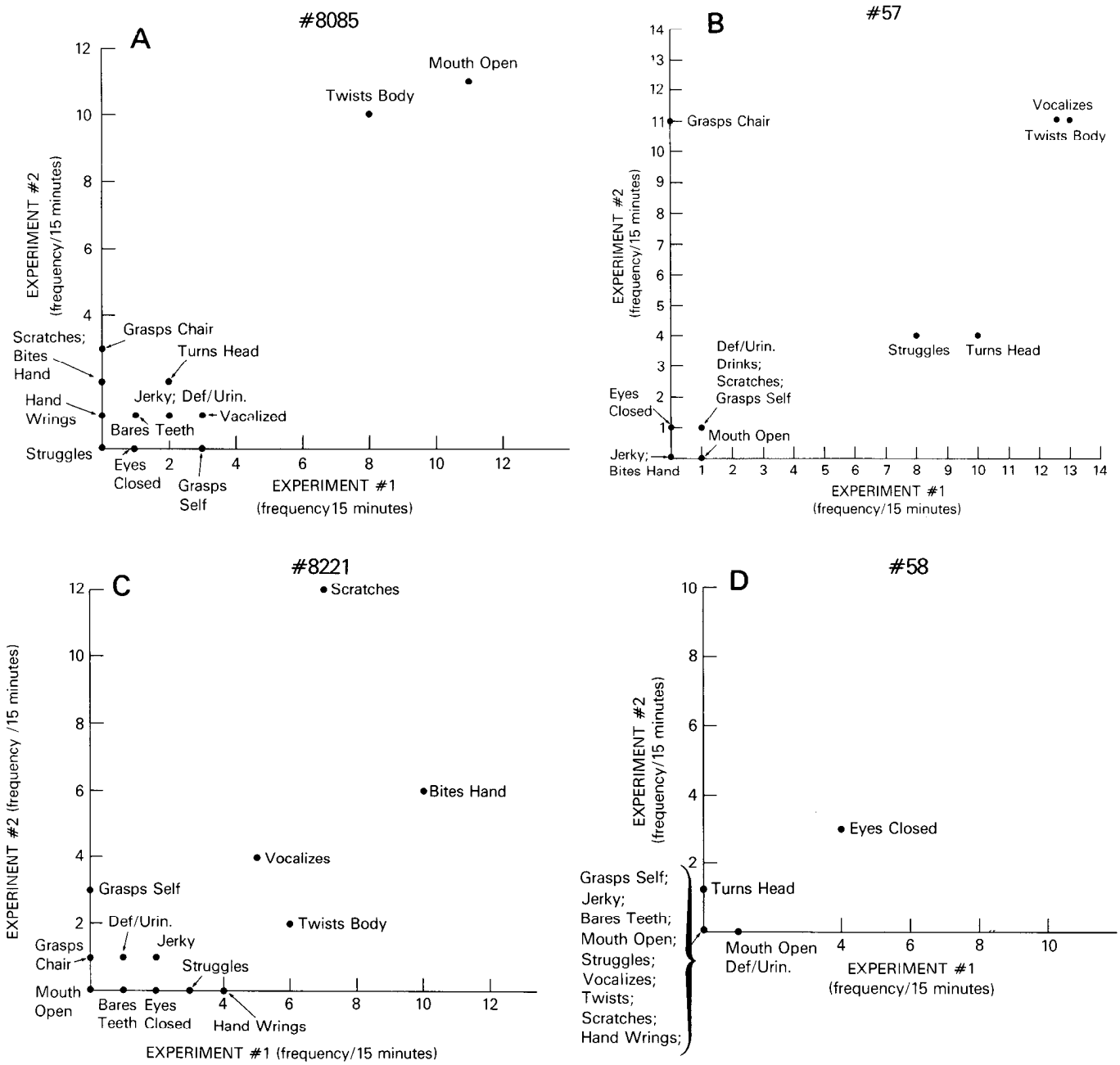

Figure 1. Behavioral responses to β-CCE, 200 μg/kg, i.v., in four chair-adapted male rhesus monkeys. The *abscissa* represents frequency of occurrence of behavioral parameters during the 15 min immediately following the first administration of β-CCE. The *ordinate* represents frequency of occurrence of behavioral parameters during the 15 min immediately following the second administration of the same dose of β-CCE, 1 month later. Each monkey showed an idiosyncratic response to β-CCE, on both the identity and intensity of the behaviors elicited. Both the types of behaviors and the frequency of their occurrence were consistent for a given monkey on each test day.

another repetitively turned his head, another drank from the water bottle in a stereotyped manner, and so on.

Figure 2 shows three behaviors representative of the increase in "anxiety"-related behaviors after β-CCE administration. Behavioral parameters were included for statistical analysis only if at least four of the eight monkeys tested displayed a given behavior. The three parameters of Figure 2 represent behaviors displayed by six or seven of eight monkeys. ANOVA across treatment groups yielded values of $F_{7,44} = 3.84$, $p < 0.01$ for "vocalizations," $F_{7,44} = 4.59$, $p < 0.01$ for "body twisting," and $F_{7,44} = 9.03$, $p < 0.01$ for "mouth open." As tested by Newman-Keuls analysis, vehicle + β-CCE and propranalol + β-CCE were significantly above control values on all three parameters ($p < 0.05$ criterion for significance). Cyproheptadine + β-CCE was significantly above control values for some parameters. Clonidine, diazepam, cyproheptadine, and THIP effectively blocked most of the behavioral effects of β-CCE.

Heart rate and blood pressure changes are illustrated in Figure 3 (ANOVA: $F_{7,44} = 2.85$, $p < 0.05$ for heart rate; $F_{7,44} = 2.26$, $p < 0.05$ for blood pressure). Vehicle + β-CCE significantly elevated heart rate and mean arterial pressure. Diazepam + β-CCE was not significantly different from base line. Clonidine and propranolol significantly decreased heart rate and blood pressure. Cyproheptadine and THIP were not potent inhibitors of the β-CCE-induced elevation of heart rate and blood pressure.

Plasma epinephrine and NE were significantly elevated following β-CCE administration. (Fig. 4; ANOVA: $F_{7,44} = 2.67$, p

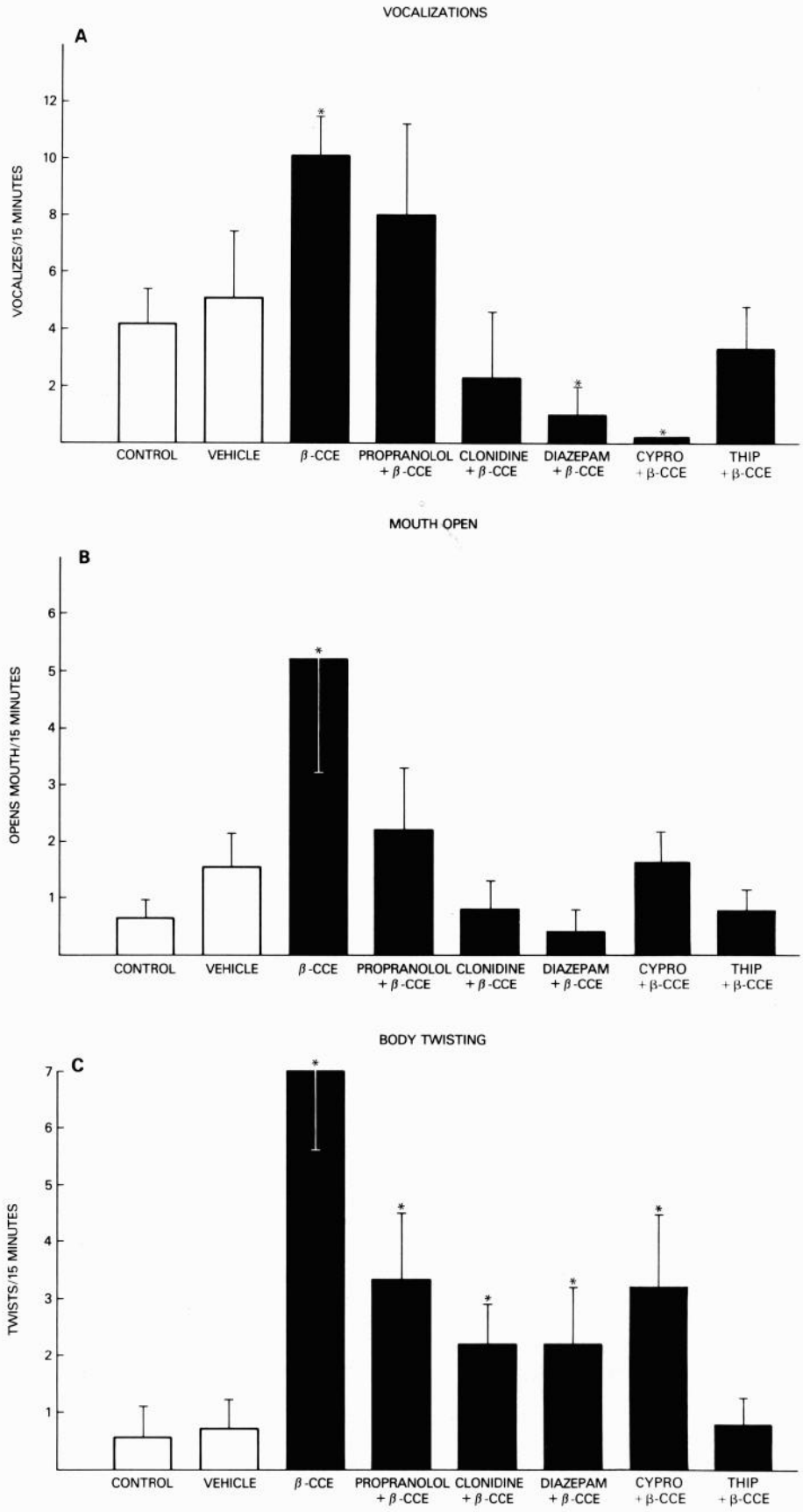

Figure 2. Three parameters of behaviors representative of the "agitation" induced by β-CCE. In Figures 2 to 5, the protocol involved behavioral, cardiovascular, and plasma sampling (*a*) 15 min before intravenous treatments (base line); (*b*) 15 min after vehicle, diazepam (1 mg/kg), clonidine (10 μg/kg), propranalol (3 mg/kg), cyproheptadine (1 mg/kg), or THIP (1 mg/kg); (*c*) 15 min after β-CCE (200 μg/kg), which was administered 15 min after the pretreatments in protocol *b*; and (*d*) 30 min after β-CCE. $N = 6$ to 7 for each treatment condition. Data in Figures 2 to 5 are represented as mean ± SEM and $p < 0.05$ (*) as compared to vehicle controls, by the Newman-Keuls *a posteriori* test for significance of individual means following significant values for one-way ANOVA. Scores for vocalization (*A*), mouth open (*B*), and body twisting (*C*) were total occurrences in the 15-min segment following β-CCE administration.

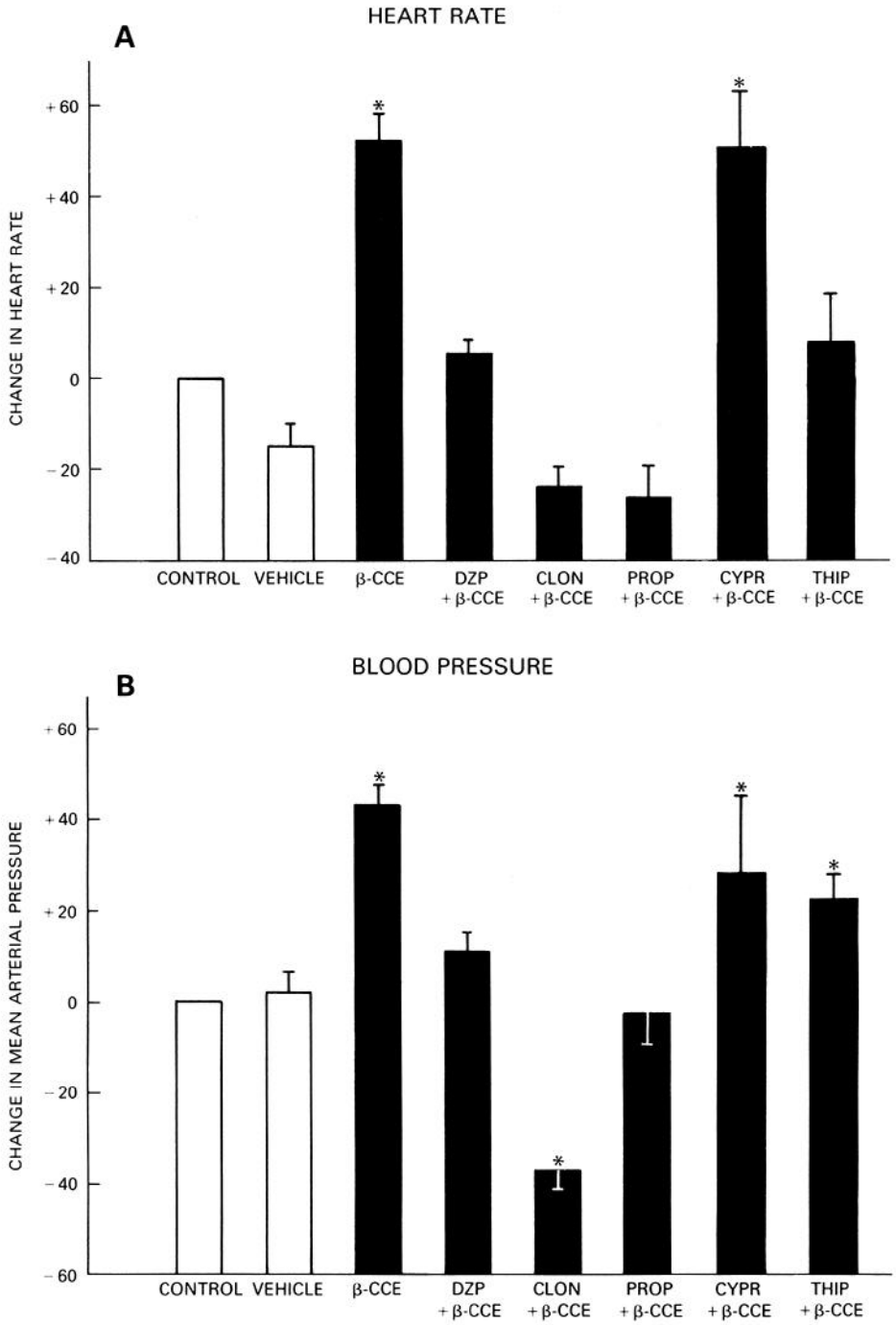

Figure 3. Heart rate (*A*) and blood pressure (*B*) were measured through femoral cuffs. Values presented were obtained by the protocol described in the legend to Figure 2, at the time point 15 min after β-CCE administration.

< 0.05 for epinephrine; $F_{7,44} = 2.55$, $p < 0.05$ for NE). Epinephrine values following clonidine + β-CCE and diazepam + β-CCE were not significantly different from controls. By contrast, propranolol pretreatment followed by β-CCE resulted in epinephrine values that were significantly above control values. Cyproheptadine and THIP had no effect on the β-CCE-induced elevations of plasma epinephrine and NE.

As previously reported, β-CCE significantly elevated plasma cortisol and ACTH (Fig. 5; ANOVA: $F_{7,44} = 2.91$, $p < 0.05$ for cortisol; $F_{7,44} = 2.75$, $p < 0.05$ for ACTH). Diazepam, clonidine, cyproheptadine, and THIP prevented the β-CCE-induced increase in cortisol, whereas diazepam, clonidine, and cyproheptadine prevented the β-CCE-induced increase in ACTH. THIP did not block the effects of β-CCE on ACTH. Propranalol did not block the effects of β-CCE on either cortisol or ACTH.

Discussion

Four categories of β-carboline effects in rhesus monkeys are diagrammed in Figure 6. Intravenous administration of β-CCE induced a behavioral syndrome analagous to behaviors exhibited by rhesus monkeys in naturalistic "anxiety-provoking" situations (Altmann et al., 1962; Redmond, 1977). A large increase in plasma cortisol and ACTH following administration of β-CCE reflects activation of the hypothalamic-pituitary-adrenal (HPA) axis. Increased heart rate and blood pressure, and elevated plasma catecholamines reflect an activation of the sympathetic nervous system and adrenal medulla. Taken together, these behavioral and physiological effects of β-CCE appear to represent a state of stress which may be specifically related to anxiety.

Diazepam was effective in antagonizing these four categories

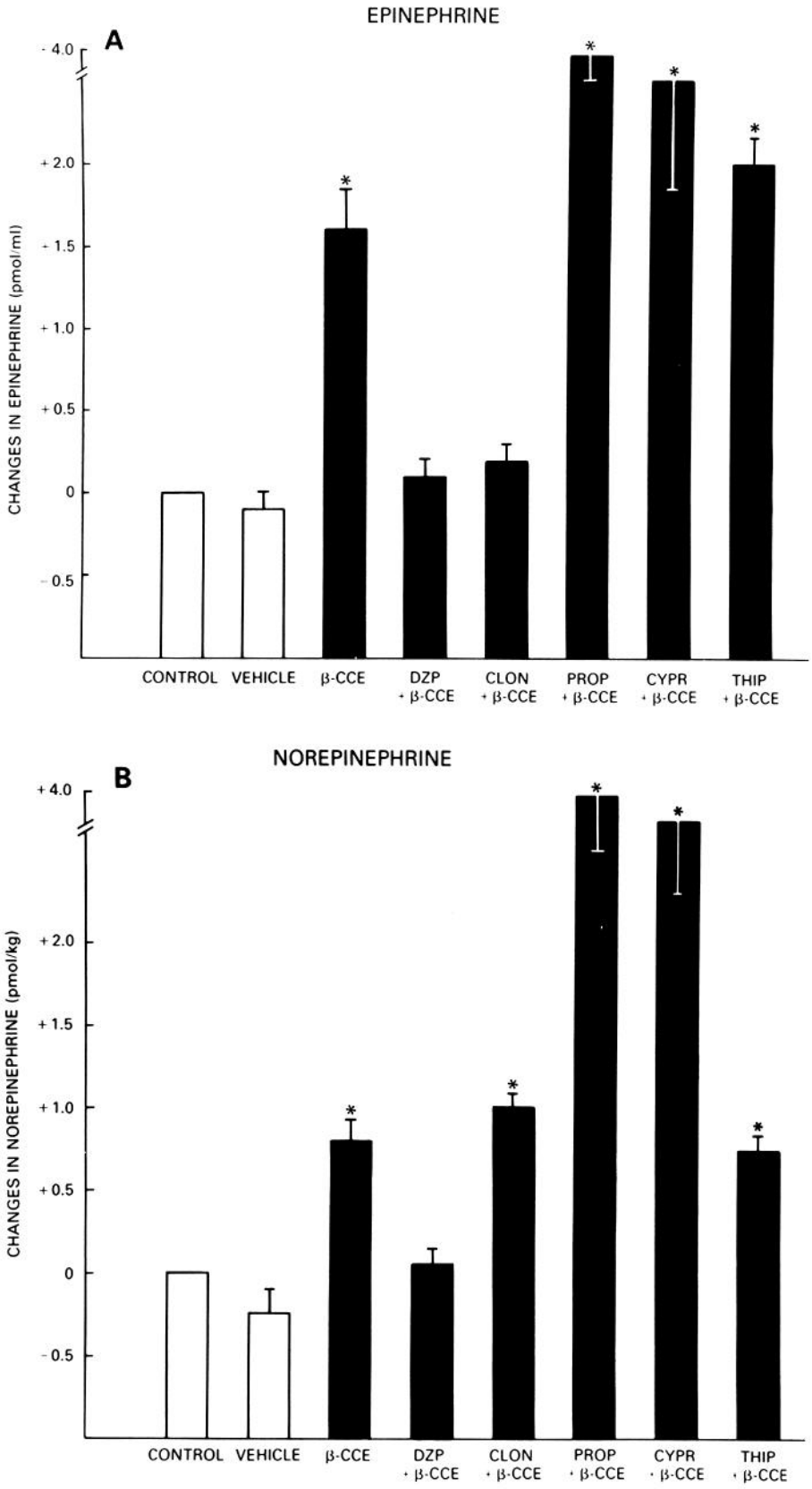

Figure 4. Plasma epinephrine (*A*) and NE (*B*) were measured by high pressure liquid chromatography with electrochemical detection. Values presented were obtained from plasma drawn from a femoral intravenous catheter, 15 min after β-CCE administration, as described in the protocol in the legend to Figure 2.

of responses to β-CCE. The dose of diazepam used was well within the clinically therapeutic range and did not produce significant sedation in rhesus monkeys in the present experiments as measured by frequency of "eyes closed" during the 15-min interval following diazepam pretreatment.

The α_2-adrenergic receptor agonist clonidine antagonized all four categories of responses to β-CCE. Clonidine appears to antagonize most aspects of the β-CCE syndrome as effectively as diazepam. The dose of clonidine used in this study produced some sedation as defined above. This dose has been reported to act preferentially on the presynaptic noradrenergic autoreceptor (Aghajanian, 1978), reducing the firing rate of locus

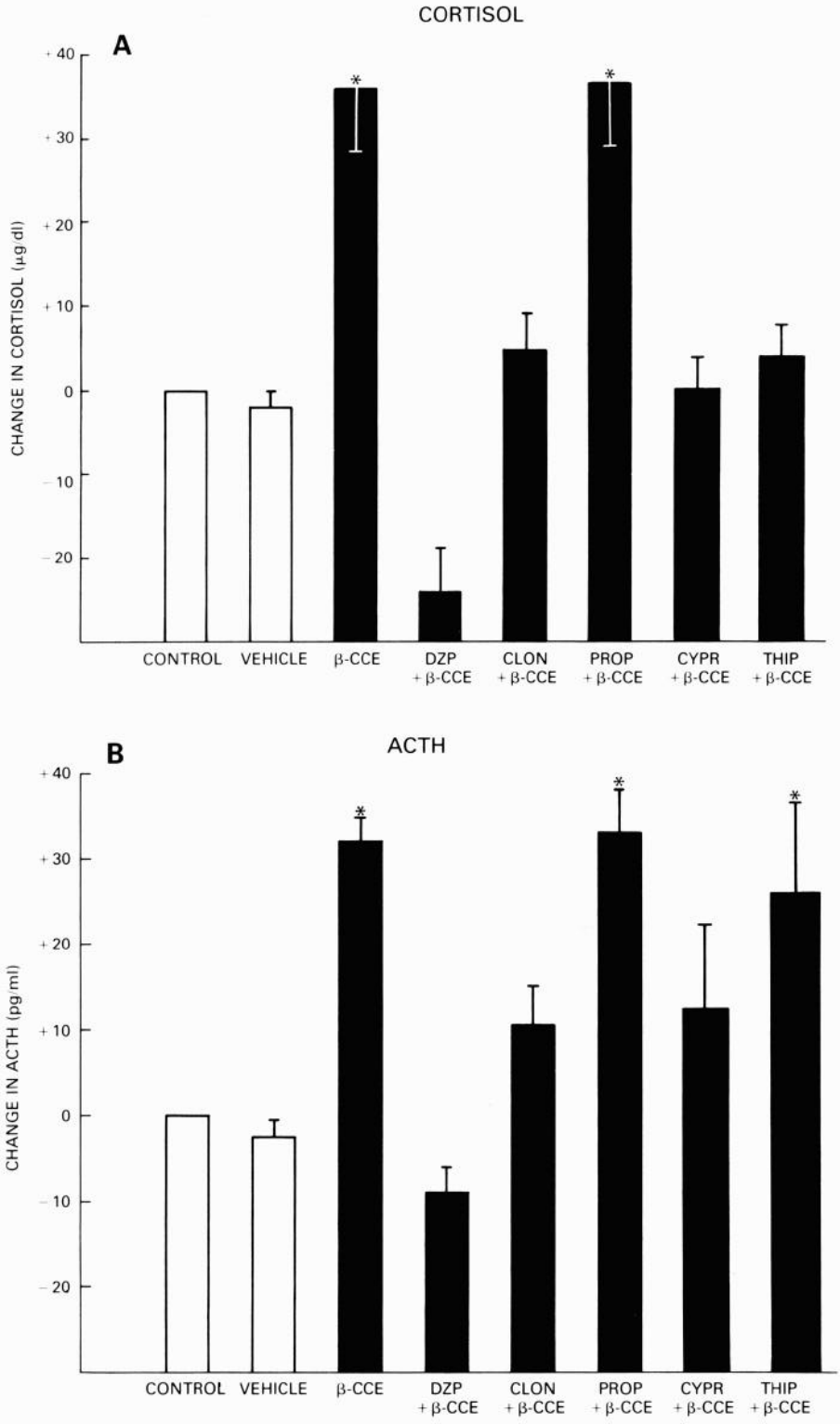

Figure 5. Plasma cortisol (*A*) and ACTH (*B*) were measured by radioimmunoassay. Values presented were obtained from plasma drawn from a femoral intravenous catheter, 15 min after β-CCE administration, as described in the protocol in the legend to Figure 2.

ceruleus neurons. The findings of the present study are in accordance with Redmond's (1977) hypothesis that the locus ceruleus is necessary for the elicitation of anxiety-related responses in primates. However, the antagonism of β-CCE effects by clonidine may involve a more generalized behavioral state of nonresponsivity to novel environmental situations, as shown by Foote et al. (1980).

Propranolol antagonized some of the behavioral effects of β-CCE, but was more effective as an antagonist of the cardiovascular activation. However, propranolol did not block the increase in plasma catecholamines, cortisol or ACTH. Propanalol acts at both the peripheral and central β-adrenergic receptors. The lesser blockade of β-CCE effects by propranalol suggests that the β-adrenergic receptor is less involved in these effects.

Cyproheptadine inhibited most of the behavioral effects of β-CCE. Although heart rate, blood pressure, and plasma catecholamine changes after β-CCE were not blocked by cyproheptadine, this serotonergic antagonist was effective in inhibiting the β-CCE-induced rise in both cortisol and ACTH. This indicates that serotonergic systems may be involved in mediat-

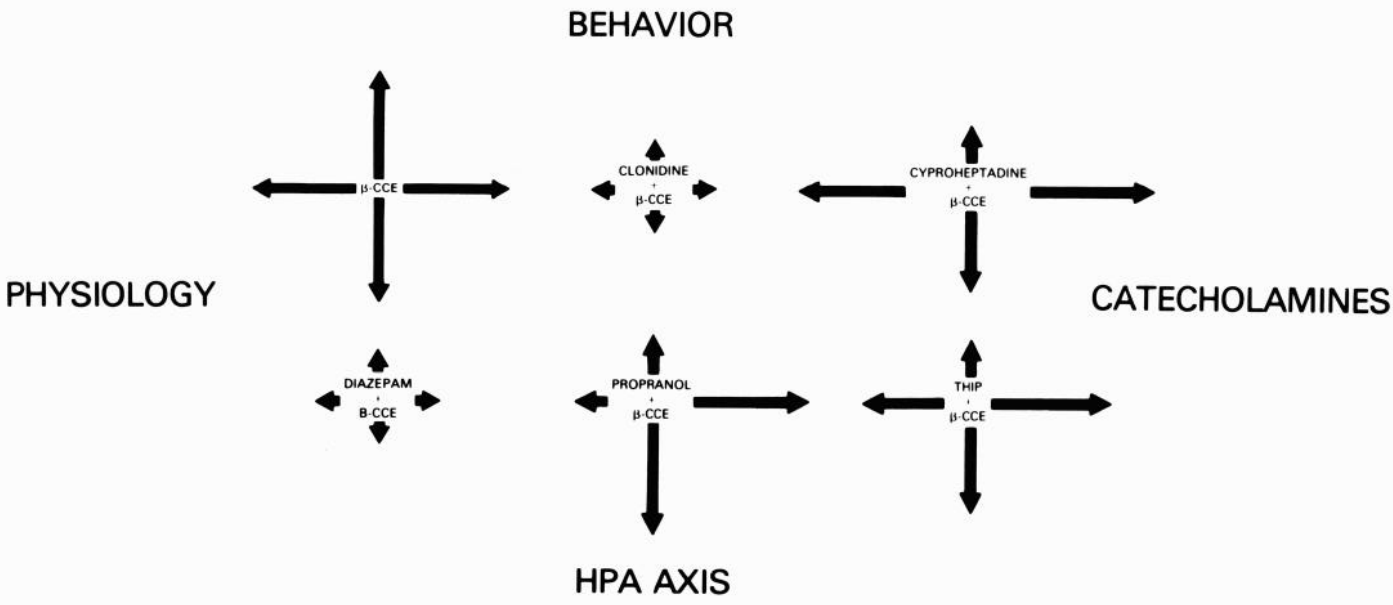

Figure 6. Diagrammatic representation of neuropharmacological treatments on responses to β-CCE. β-CCE significantly increased behavioral parameters of agitation (*BEHAVIOR*), heart rate and blood pressure (*PHYSIOLOGY*), plasma epinephrine and NE (*CATECHOLAMINES*), and plasma cortisol and ACTH (*HPA AXIS*). Diazepam, an agonist at the BZD receptor, and clonidine, an α-adrenergic presynaptic agonist which reduces noradrenergic activity, blocked all of these actions of β-CCE. Propranalol, a β-adrenergic antagonist, partially blocked behavioral and physiological effects of β-CCE but had no effect on the increases in plasma catecholamines, cortisol, and ACTH. The 5-HT antagonist, cyproheptadine, and the GABA agonist, THIP, partially reduced behavioral responses but were relatively less effective on other measures of β-CCE actions.

ing some of the behavioral and HPA axis activation components of the β-CCE actions. Further studies with some of the newer, more specific central antagonists of 5-HT will be necessary to carefully elucidate the role of brain serotonergic pathways in the β-CCE syndrome.

THIP inhibited the majority of the behavioral effects of β-CCE. THIP was partially effective in blocking cardiovascular actions of β-CCE. However, plasma catecholamine elevations after β-CCE were not blocked by THIP. THIP blocked the β-CCE-induced elevation in cortisol, but not ACTH, an anomalous finding which remains paradoxical at this time. GABAergic systems, therefore, appear to contribute to some of the behavioral and endocrine actions of β-CCE. Since it is not clear how effective parenterally administered THIP is as a centrally active GABA agonist, and since a complete dose-response study of THIP has not been completed, these results cannot be interpreted as indicating a lack of involvement of GABAergic mechanisms in the β-CCE syndrome.

Our results suggest that the set of behavioral, physiological, and neurochemical changes induced by β-CCE depends on intact noradrenergic pathways, as well as inputs from serotonergic and GABAergic systems. Involvement of several neurotransmitter systems in "anxiety"-related behaviors is not surprising, since a wide spectrum of internal and external sensory cues are integrated and many motor systems are activated in the perception and response to anxiety-provoking environmental stimuli. Furthermore, the relatively ubiquitous distribution of GABAergic synapses in brain suggests that other neurotransmitter pathways may be affected by β-CCE. BZD receptors are located on cell bodies within the locus ceruleus, dorsal raphe, and throughout the limbic system. The profound behavioral and physiological effects of BZD receptor antagonists may be the sum total of altered firing rates in a variety of interacting transmitter systems.

The present study does not address the issue of whether β-CCE induces anxiety or a more generalized stress response. Nonetheless, it should be emphasized that the autonomic responses reported herein are elicited by a variety of stressors, including immobilization and footshock (Stolk et al., 1974; Saavedra et al., 1979; File and Peet, 1980; Stone, 1980). The current evidence that the β-CCE-induced syndrome is related to "anxiety" or "fear" is indirect and derived from pharmacological data in animal experiments. The pro-conflict effects of β-CCE and the anti-conflict action of BZDs support the specificity of this model. These data, coupled with the very limited clinical studies of β-carboline esters administered to humans (Dorow et al., 1983), support the validity of the model but require further studies to distinguish between "anxiety" and generalized "stress."

In conclusion, noradrenergic, serotonergic, and GABAergic agents differentially antagonized many of the parameters of the β-carboline response. In particular, clonidine was as effective as diazepam in blocking the behavioral, physiological, and neurochemical activation induced by intravenous β-CCE administration in rhesus monkeys. The paradigm of administration of BZE receptor active antagonists to rhesus monkeys may be a useful model system for delineating neurochemical and anatomical pathways mediating human anxiety.

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