The 5-Hydroxytryptamine$_2$ Agonist, (±)-1-(2,5-Dimethoxy-4-Bromophenyl)-2-Aminopropane Stimulates the Hypothalamic-Pituitary-Adrenal (HPA) Axis. I. Acute Effects on HPA Axis Activity and Corticotropin-Releasing Factor-Containing Neurons in the Rat Brain[1]

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ABSTRACT

Corticotropin-releasing factor (CRF) is the major physiological regulator of adrenocorticotrophic hormone (ACTH) secretion from the anterior pituitary. *In vivo* and *in vitro* studies have suggested that hypothalamic CRF secretion is under stimulatory serotonergic control, although the receptor subtype(s) responsible have not been definitively determined. The acute effects of the 5-hydroxytryptamine$_2$ agonist, (±)-1-(2,5-dimethoxy-4-bromophenyl)-2-aminopropane (DOB), were examined on a number of biochemical indices of hypothalamic-pituitary-adrenal axis activity *in vivo*. DOB increased plasma ACTH and corticosterone concentrations at doses greater than 0.1 mg/kg. This effect is dose-dependent. Peak effects occurred 30 min postinjection and returned to basal levels by 4 hr after DOB injection. These effects of DOB are hypothesized to be mediated by the release of hypothalamic CRF because pretreatment with the CRF receptor antagonist (α-helical CRF$_{9-41}$) significantly attenuated the ACTH response to DOB. Median eminence CRF content was also decreased following DOB administration in the presence of the protein synthesis inhibitor, cycloheximide (200 mg/kg i.p.), suggestive of release of CRF from median eminence terminals as a result of DOB activation of CRF neurons. DOB administration was without effect on brain CRF concentrations in all of the 12 extrahypothalamic brain regions studied 60 min after injection. These results, taken together, support a stimulatory role for 5-hydroxytryptamine$_2$ receptors on hypothalamic CRF secretion.

CRF is the major physiological regulator of ACTH and β-endorphin secretion from the anterior pituitary (Vale *et al.*, 1981, 1983a; Rivier *et al.*, 1982a,b). Immunohistochemical and radioimmunoassay studies have revealed that CRF is distributed heterogeneously throughout the mammalian CNS. High concentrations are found in the hypothalamus, brainstem nuclei associated with autonomic functioning and in several limbic areas (Swanson *et al.*, 1983; Cummings *et al.*, 1983; Sakanaka *et al.*, 1987). Similarly, biochemical and autoradiographic studies have identified CRF receptors in the CNS (DeSouza *et al.*, 1985; DeSouza, 1987; Hauger *et al.*, 1988).

When administered directly into the CNS, CRF produces a multitude of behavioral (Britton, *et al.*, 1986a,b) and physiological alterations (Brown *et al.*, 1982; Fisher, 1989) that are not mediated by activation of the HPA axis, and are remarkably similar to those observed when laboratory animals are exposed to stress. These findings strongly suggest that CRF, acting as a neurotransmitter, may ultimately be responsible for integrating not only the endocrine, but also the autonomic and behavioral responses of an organism to stress. There is evidence that CRF is hypersecreted in some patients with major depressive illness (Nemeroff *et al.*, 1984, 1988; Banki *et al.*, 1987) and because there is evidence that 5-HT$_2$ receptors are involved in regulating mood and may be altered in psychiatric illness (Gonzalez-Heydrich and Peroutka, 1990), the investigation of 5-HT$_2$ receptor regulation of CRF-containing neurons is of potential clinical interest.

Past investigations have revealed a prominent serotonergic innervation of CRF perikarya in the paraventricular nucleus of the hypothalamus (Liposits *et al.*, 1987; Soghomonian *et al.*,

ABBREVIATIONS: CRF, corticotropin-releasing factor; ACTH, adrenocorticotrophic hormone; CNS, central nervous system; HPA, hypothalamic-pituitary-adrenal; 5-HT, 5-hydroxytryptamine; DOB, (±)-1-(2,5-dimethoxy-4-bromophenyl)-2-aminopropane; DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane.

1988). In addition, serotonin stimulates CRF secretion from the median eminence of the hypothalamus both *in vivo* (Gibbs and Vale, 1983) and *in vitro* (Holmes *et al.*, 1982; Nakagami *et al.*, 1986; Calogero *et al.*, 1989). Although there is evidence suggesting that a variety of 5-HT receptor subtypes may contribute to HPA axis activation *in vivo* (Lorens and van de Kar, 1987; Koenig *et al.*, 1988; Fuller and Snoddy, 1990; Gartside and Cowen, 1990; Owens *et al.*, 1990), the specific serotonergic receptor subtype(s) responsible have yet to be definitively determined. The *in vitro* evidence suggests that 5-HT$_2$ receptors represent the major serotonergic stimulus of CRF release from the median eminence (Calogero *et al.*, 1989). In order to determine whether this is the case *in vivo*, we have studied the acute effects of the potent and highly selective 5-HT$_2$ agonist, DOB, on plasma ACTH and corticosterone concentrations in addition to regional brain CRF concentrations in rats treated acutely with DOB.

Methods

Animals. Male Sprague-Dawley rats (150–300 g) were housed two per cage, with food and water available *ad libitum* in an environmentally controlled animal facility (12 hr/12 hr light/dark cycles with lights on at 7:30 A.M.). Animals were handled daily to minimize stress on the day of sacrifice. The time of sacrifice was between 8:30 to 10:30 A.M. for all experiments.

Drug treatment. After 7 days of habituation to human handling, DOB, supplied as the hydrochloride salt, was administered by s.c. injection. The rats were sacrificed 60 min after injection by decapitation. After generation of the dose-response curves, the time course of effects following a single acute s.c. injection was studied. The dose chosen (0.35 mg/kg) represents the lowest dose resulting in robust activation of the HPA axis as defined from the data obtained in the dose-response experiments. For the time course experiments, two water-injected control rats were sacrificed at each time point. The data from these animals were subsequently pooled and used as the control group. Little variance was found between water-injected controls, regardless of the time of sacrifice.

Animals in which regional brain CRF concentrations were to be measured, received a single s.c. injection of DOB (0.35 mg/kg) or vehicle (H$_2$O) and were sacrificed 60 min later. The dose and time of sacrifice (time of peak plasma ACTH and corticosterone concentrations) were determined from the above dose-response and time course studies.

To determine whether a CRF receptor antagonist could inhibit the actions of DOB on the HPA axis, rats were pretreated with either water or the CRF antagonist (α-helical CRF$_{9-41}$, 0.4 μmol/kg s.c.) 30 min before receiving an acute s.c. injection of DOB (0.35 mg/kg) or water. Animals were sacrificed 30 min after the second injection.

To determine whether inhibition of protein synthesis alters the response of the HPA axis to DOB, rats were pretreated with cycloheximide (200 mg/kg i.p.) or vehicle (dimethylsulfoxide) 60 min before receiving an acute s.c. injection of DOB (0.35 mg/kg) or water. Animals were sacrificed 60 min after the second injection.

Sample preparation. After decapitation, trunk blood was collected into heparinized tubes on ice (corticosterone assay) or into tubes containing EDTA (ACTH assay), centrifuged (1000 × g, 4°C) for 5 min and the plasma frozen at −70°C until assay. The brains were removed quickly, taking care not to damage the median eminence, then frozen ventral side up on dry ice and stored at −70°C until dissection.

Fourteen brain regions were dissected on ice while still partially frozen by a modification of the technique of Glowinski and Iversen (1966) and Palkovits and Brownstein (1988). Samples were sonicated in 1 ml of ice-cold 1 M HCl containing 50 μM bacitracin, aprotinin (10 μg/ml) and phenylmethysulfonyl fluoride (1 μg/ml) and then placed in boiling water for 3 to 5 min (extraction time is dependent upon size of the tissue sample; M. J. Owens, unpublished observations). After microcentrifugation, duplicate aliquots of supernatant were placed in

10 × 75 mm borosilicate glass tubes, lyophilized and stored at −70°C until assayed. Aliquot size was determined for individual brain regions from prior experience so that CRF concentrations of the samples fell near the middle of the radioimmunoassay standard curve. Pellets were dissolved in 1 M NaOH and assayed for total protein with an automated protein analyzer utilizing the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

CRF radioimmunoassay. CRF concentrations in individual brain regions were measured in duplicate by modification of a previously described specific radioimmunoassay for CRF (Vale *et al.*, 1983b) using an antiserum (oC33, generously provided by Dr. Wylie Vale, Salk Institute, La Jolla, CA) raised in rabbits against ovine CRF. This antiserum recognizes the 33–41 amino acid sequence of CRF but does not recognize sauvagine, urotensin I or any other hypothalamic releasing hormone.

The lyophilized samples were reconstituted in 200 μl of radioimmunoassay buffer [SPEAB buffer: 100 mM NaCl; 50 mM Na$_2$HPO$_4$; 25 mM EDTA; 0.1% sodium azide; 0.1% bovine serum albumin (radioimmunoassay grade); and 0.1% Triton X-100, pH 7.3] and incubated at 4°C for 24 hr with 100 μl of oC33 antiserum at a dilution of 1:8000 in SPEAB buffer with 1.5% normal rabbit serum. Radiolabeled [^{125}I] Tyr^0-rat/human CRF trace was prepared in our laboratory by the chloramine-T method and subsequently purified by high-pressure liquid chromatography as described previously (Smith *et al.*, 1986). After dilution in SPEAB buffer, 50 μl (approximately 20,000 cpm) of labeled CRF was added to each tube. After incubation for 24 hr at 4°C, 10 μl of second antibody (goat antirabbit serum; Arnel Products, New York, NY) was added to precipitate bound CRF.

A standard curve was prepared utilizing rat/human CRF (Bachem, Inc., Torrance, CA) from 0.625 pg/tube to 5120 pg/tube. The sensitivity of the assay was 1.25 pg/tube with 50% displacement of radiolabeled CRF (IC$_{50}$) at 30 pg. The inter- and intra-assay coefficients of variation are 10 to 13 and 2 to 8%, respectively. CRF immunoreactivity measured in this assay and brain extracts subjected to high-performance liquid chromatography has been shown to cochromatograph with synthetic CRF (Smith *et al.*, 1986).

ACTH radioimmunoassay. Plasma ACTH concentrations were determined by a sensitive and specific radioimmunoassay using an antiserum raised in rabbits against thyroglobulin-conjugated ACTH$_{11-24}$ and a radiolabeled tracer prepared by the chloramine-T method. The assay typically has a sensitivity of 0.25 fmol/ml in plasma. Before assay all plasma samples were extracted using C$_{18}$ Sep-pak cartridges (Waters Associates, Milford, MA) to minimize nonspecific binding effects from undiluted plasma. Extracts were lyophilized and reconstituted in buffer (55 mM Na$_2$HPO$_4$, 0.02% sodium azide, 1.25% normal rabbit serum, 0.02% poly-L-lysine and 0.05% Triton X-100, pH 7.4) for assay at an antibody dilution of 1:4000. Recovery of ACTH from spiked plasma is 85 to 90%. The inter- and intra-assay coefficients of variation are 12 to 15 and 10 to 13%, respectively.

Corticosterone assay. Plasma corticosterone concentrations were determined using a modification of Murphy's competitive protein-binding radioimmmunoassay with a sensitivity of 5 ng/ml, as described previously (Chappell *et al.*, 1986). The inter- and intra-assay coefficients of variation are 10 to 12 and 5 to 7%, respectively.

[^{3}H]Leucine incorporation. Cycloheximide (200 mg/kg i.p.) has been shown previously to abolish cardiac protein synthesis (Lau and Slotkin, 1979). To determine whether this dose provides similar efficacy in CNS tissue, rats were given vehicle (dimethylsulfoxide) or cycloheximide (200 mg/kg i.p.) 60 min before receiving [^{3}H]leucine (1 mCi/kg s.c.). Rats were sacrificed 60 min after [^{3}H]leucine administration. Brains were homogenized in 10 volumes of ice-cold distilled water. A 100-μl aliquot of tissue homogenate was placed in a scintillation vial. Hyamine hydroxide (1 ml) was added to the vial and incubated in a water bath at 55°C for 4 hr. After incubation, 100 μl of glacial acetic acid and 10 ml of scintillation fluid was added before counting by liquid scintillation spectrophotometry. A separate 1-ml aliquot of tissue homogenate was added to 2.5 ml of cold 10% trichloroacetic acid in a centrifuge tube. These tubes were subsequently centrifuged at 30,000

× *g* for 10 min. The resulting pellet was resuspended in 2.5 ml of 10% trichloroacetic acid. This process was repeated twice. After the third centrifugation the resulting pellet was treated with hyamine hydroxide and prepared for spectrophotometry as above.

Drugs. Cycloheximide was purchased from the Sigma Chemical Co. (St. Louis, MO). Rat CRF, ovine CRF and α-helical CRF_{9-41} were purchased from Bachem Inc. (Torrance, CA). DOB was a gift from the National Institute of Drug Abuse (Baltimore, MD) and the Research Triangle Institute (Research Triangle Park, NC).

Statistics. One- and two-way analysis of variance were used to evaluate significant differences. In the cases in which a significant interaction was identified, Student's *t* test, Student-Newman-Keuls test for multiple comparisons or Dunnett's test for multiple comparisons to a single mean were used for posthoc comparisons. Results are expressed as the mean ± S.E.

Results

DOB dose-response curves. Acute administration of DOB dose-dependently increased plasma ACTH and corticosterone concentrations (fig. 1). However, no changes in median eminence or hypothalamic CRF concentrations were observed, although a trend toward decreased median eminence CRF content was noted (fig. 2). The lowest dose of DOB that produced maximal increases in plasma ACTH and corticosterone concentrations (0.35 mg/kg) was used in subsequent experiments.

Time course of the effects DOB on the HPA axis. Maximal elevations in plasma ACTH concentrations were observed by 30 min postinjection. Significant elevations in plasma corticosterone were observable 30 min postinjection with a maximal response 60 min postinjection (fig. 3). Plasma ACTH and corticosterone concentrations returned to base line by 4 hr

Fig. 2. Tissue concentrations of CRF after increasing doses of DOB 60 min after s.c. administration. CRF concentrations in the median eminence (●) and hypothalamus (O) were not statistically altered by DOB administration, although a trend toward decreased CRF content in the median eminence was observed.
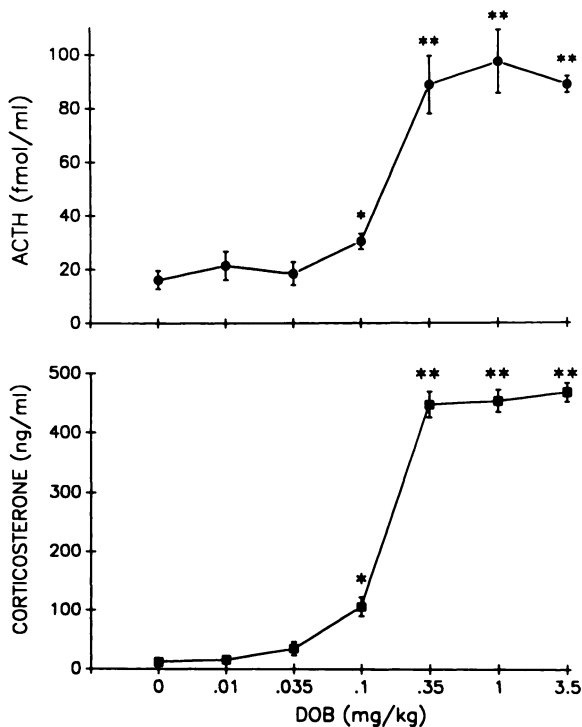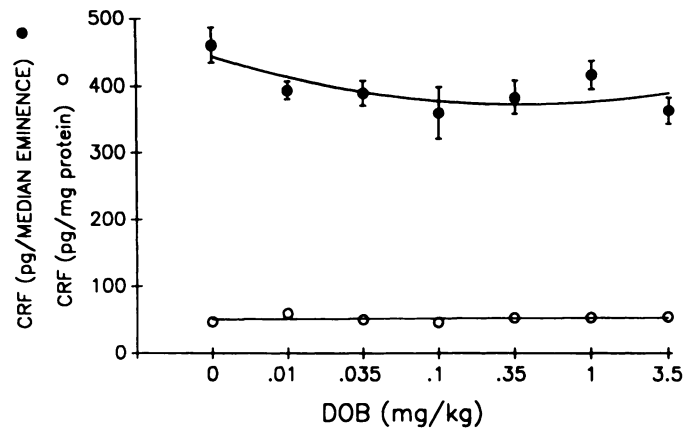
Fig. 1. ACTH and corticosterone responses to DOB administration. DOB dose-dependently increased plasma ACTH (●) and corticosterone (■) concentrations 60 min after a single s.c. injection. *n* = 6 at each dose. Results were analyzed by one-way analysis of variance followed by Dunnett's test for multiple comparisons to a single mean (control). *P < .05; **P < .01.
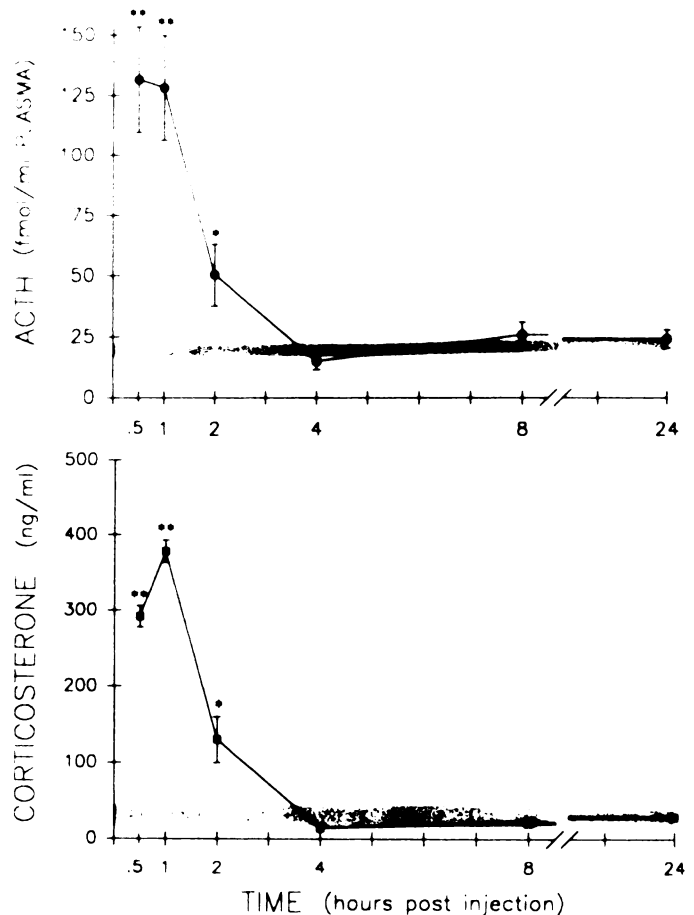
Fig. 3. Time course of plasma ACTH and corticosterone responses to DOB (0.35 mg/kg s.c.). Plasma ACTH (●) concentrations had peaked by 30 min postinjection and returned to control values by 4 hr postinjection. Plasma corticosterone (■) concentrations were significantly increased by 30 min and had peaked by 60 min postinjection. Corticosterone concentrations also returned to control values by 4 hr postinjection. Two water-injected rats were sacrificed with each group of DOB-treated rats and were subsequently pooled and used as the control group (shaded region). *n* = 10 for controls and *n* = 6 for each DOB-treated group. Results were analyzed by one-way analysis of variance followed by Dunnett's test. *P < .05; **P < .01.

postinjection. DOB administration was without effect on CRF concentrations in the median eminence or hypothalamus at all time points studied (fig. 4).

Acute effects of DOB on CRF concentrations in rat brain. Rats sacrificed 60 min after a single s.c. injection of DOB (0.35 mg/kg) exhibited significant elevations of plasma ACTH and corticosterone concentrations as expected from the dose-response and time course data (ACTH: control, 23.4 ± 3.8; DOB, 117.2 ± 18.7 fmol/ml of plasma; P < .01. Corticosterone: control, 11 ± 4; DOB, 327 ± 25 ng/ml of plasma; P < .01). Acute DOB administration was without effect on CRF concentrations in all brain regions studied. These included the median eminence, hypothalamus, bed nucleus of the stria terminalis, amygdala, septum, hippocampus, piriform cortex, cingulate cortex, prefrontal cortex, frontal/parietal cortex, cerebellum, raphe nuclei, locus ceruleus and nucleus of the solitary tract.

Effects of CRF antagonist pretreatment on DOB-induced alterations in HPA axis activity. Rats pretreated with the CRF antagonist (α-helical CRF_{9-41}, 0.4 μmol/kg s.c.) 30 min before an acute dose of DOB did not exhibit the increases in plasma ACTH concentrations observed previously (fig. 5). However, CRF antagonist pretreatment was without effect on DOB-induced increases in plasma corticosterone concentrations. As observed in earlier experiments, neither CRF antagonist-pretreatment nor DOB administration alone altered median eminence CRF content (data not shown).

Effects of protein synthesis inhibition on HPA axis activity after DOB administration. Cycloheximide (200 mg/kg i.p.) administration results in a substantial decrease in new protein synthesis, as evidenced by [^{3}H]leucine incorporation into proteins. Indicative of protein synthesis inhibition, cycloheximide treatment reduced [^{3}H]leucine incorporation into brain tissue to 15% of that observed in vehicle-treated controls by 60 min after a single injection (data not shown). As shown in figure 6, rats pretreated with cycloheximide (200 mg/kg i.p.) 60 min before receiving a single DOB injection did not exhibit the increases in plasma ACTH and corticosterone con-

Fig. 5. Plasma ACTH and corticosterone responses to DOB (0.35 mg/kg s.c.) after pretreatment with a CRF antagonist. Pretreatment with the CRF antagonist (α-helical CRF_{9-41}, 0.4 μmol/kg s.c.) significantly attenuated the ACTH, but not the corticosterone, response to DOB administration. n = 6/group. Results were analyzed by two-way analysis of variance (significant interaction, F = 4.4, dF = 23 for ACTH; F = 149, df = 23 for corticosterone) followed by the Student-Newman-Keuls test for multiple comparisons. **Significantly different from appropriate control, P < .01. †,Significantly different from control/DOB, P < .05.
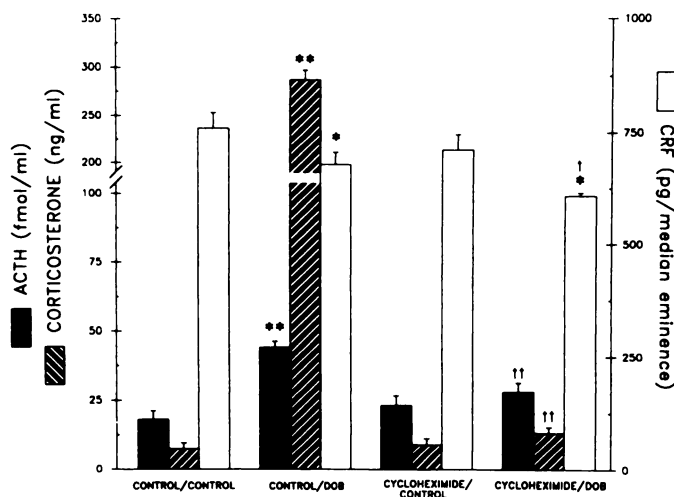
Fig. 6. Plasma ACTH, plasma corticosterone and median eminence CRF responses to DOB (0.35 mg/kg s.c.) after pretreatment with the protein synthesis inhibitor, cycloheximide. Pretreatment with cycloheximide (200 mg/kg i.p.), abolished the ACTH and corticosterone responses to DOB. In the median eminence, DOB administration resulted in significantly decreased median eminence CRF content in both vehicle- and cycloheximide-pretreated rats. However, the decreases in CRF content were significantly greater after cycloheximide-pretreatment compared to vehicle-pretreatment. n = 6/group. Results were analyzed by two-way analysis of variance (significant interaction, F = 12.4, dF = 23 for ACTH; F = 150, dF = 23 for corticosterone; F = 4.4, dF = 23 for median eminence CRF content) followed by the Student-Newman-Keuls test. *Significantly different from appropriate control, P < .05. **Significantly different from appropriate control, P < .01. †,Significantly different from control/DOB, P < .05. †† indicates significantly different from control/DOB, P < 0.01.
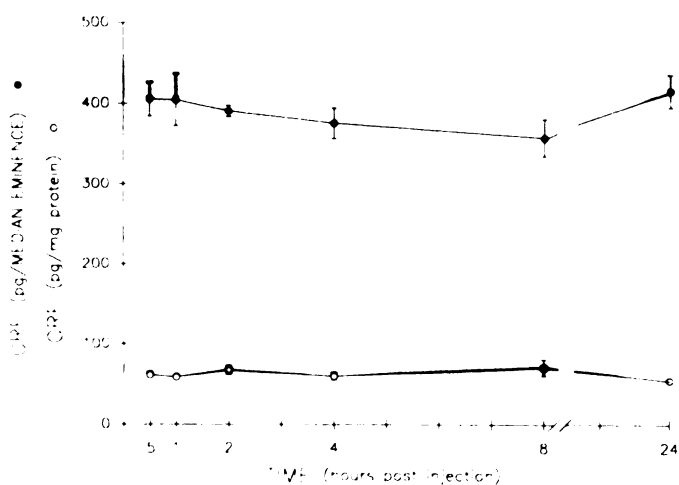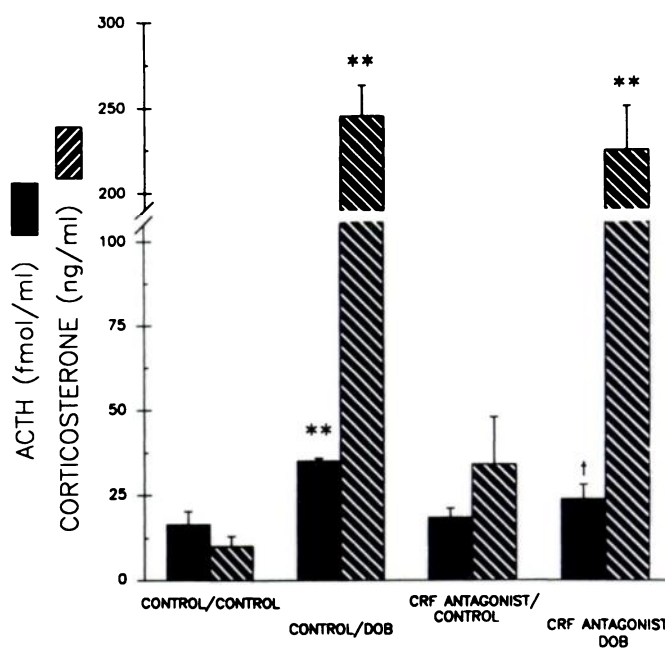

Fig. 4. Time course of median eminence and hypothalamic CRF concentrations after DOB (0.35 mg/kg s.c.) administration. Neither median eminence (●) nor hypothalamic (○) CRF concentrations were altered by DOB at any time point studied. Two water-injected rats were sacrificed with each group of DOB-treated rats and were subsequently pooled and used as the control group (shaded region). n = 10 for controls and n = 6 for each DOB-treated group. Results analyzed by one-way analysis of variance.

centrations observed in vehicle-pretreated rats. Although median eminence CRF content was decreased in DOB-treated rats irrespective of vehicle- or cycloheximide-pretreatment, median eminence CRF content after DOB administration was significantly lower in cycloheximide-pretreated rats (cycloheximide/DOB) compared to rats receiving vehicle pretreatment (control/DOB).

Discussion

In 1983 it was demonstrated that a series of phenylisopropylamine hallucinogens competed for [^{3}H]ketanserin-labeled 5-HT$_2$ receptors with affinities that correlated closely with their potencies as hallucinogens in humans and as discriminable stimuli in rats trained to recognize the hallucinogen 4-methyl-2,5-dimethoxyphenylisopropylamine (Glennon and Titeler, 1984; Shannon et al., 1984). 5-HT$_2$ antagonists blocked the phenylisopropylamine discrimination in rats; these results implied that the phenylisopropylamines were acting as agonists at the rat brain 5-HT$_2$ receptors (Glennon et al., 1983). Among these phenylisopropylamines, the 4-halo-substituted compounds: 4-bromo-2,5-dimethoxyphenylisopropylamine, DOB and 4-iodo-2,5-dimethoxyphenylisopropylamine, DOI were found to be the most potent in behavioral and biochemical studies.

Recently these compounds have been radioactively tagged and have been used to biochemically characterize the 5-HT$_2$ receptor (Glennon et al., 1986, 1988; Johnson et al., 1987; Titeler et al., 1987; Appel et al., 1990). Both [^{3}H]DOB and [^{125}I-DOI binding fit a two-site model typical of agonist binding to guanine nucleotide-linked receptors. These compounds also bind to the 5-HT$_{1C}$ receptor which shows considerable sequence homology with the 5-HT$_2$ receptor (Yagaloff and Hartig, 1985; Lubbert et al., 1987; Pritchett et al., 1988; Appel et al., 1990). However, 5-HT$_{1C}$ receptors are concentrated primarily in the choroid plexus of the rat and a physiological function for them has yet to be determined.

At the time of these experiments no data had been published regarding physiological responses to systemic administration of phenylisopropylamine hallucinogens. The only reports were from behavioral discriminative stimulus studies utilizing individual enantiomers of DOB, DOI and DOM (4-methyl-2,5-dimethoxyphenylisopropylamine) (Glennon et al., 1988). After accounting for our use of racemic DOB, 0.10 mg/kg was chosen as the median dose in the dose-response experiment. The doses ranged over 3 orders of magnitude. As shown in figure 1, significant increases in plasma ACTH and corticosterone concentrations were observed beginning at 0.1 mg/kg with maximal responses seen by 0.35 mg/kg. These results are in agreement with the recent report of Nash et al. (1989) who reported that acute DOI administration increased plasma corticosterone concentrations. Similarly, Bagdy et al. (1989) reported that DOI dose-dependently increased plasma ACTH and corticosterone concentrations in the rat. The lack of changes in median eminence CRF content (fig. 2) was surprising considering the robust increases in ACTH and corticosterone concentrations observed with increasing doses. However, the work of Plotsky and colleagues (1984), utilizing portal vessel cannulation to directly sample portal vessel CRF concentrations, suggests that only small amounts of CRF are required to elicit pituitary-adrenal activation, at least in response to hemorrhagic stress.

In those rats in which regional brain CRF concentrations

were to be assayed, we sought to sacrifice the animals at the time of maximal response. Significant elevations of plasma ACTH and corticosterone were seen by 30 min postinjection (fig. 3). ACTH concentrations peaked by 30 min postinjection and remained at maximum concentrations at 1 hr postinjection. ACTH concentrations were declining by 2 hr postinjection and had returned to base line by 4 hr postinjection. Corticosterone concentrations followed a similar pattern, the only difference being that peak concentrations were not reached until 1 hr postinjection. Metabolic inactivation of DOB plus an intact negative feedback system probably explains the results of the ACTH and corticosterone time course data in which plasma hormone concentrations had returned to base line by 4 hr postinjection. However, it is not implausible that 5-HT$_2$ receptor tachyphylaxis produced by DOB may partially account for the approximate 2 to 3 hr length of action of DOB on plasma ACTH and corticosterone concentrations. The 5-HT$_2$ receptor is a G-protein coupled receptor (Pritchett et al., 1988) that could conceivably become quickly desensitized when exposed to receptor agonists as is the case with the beta adrenergic receptor (Benovic et al., 1988). Median eminence and hypothalamic CRF concentrations were unchanged at all times studied (fig. 4).

The time course and dose-response results led us to choose a dose of 0.35 mg/kg (smallest dose eliciting maximal ACTH and corticosterone responses) and a time of sacrifice of 1 hr postinjection (peak ACTH and corticosterone responses). As expected, significant elevations in ACTH and corticosterone concentrations were observed. However, CRF concentrations were unchanged in all brain regions studied. Regional brain CRF concentrations have been shown previously to undergo changes after pharmacological or environmental pertubation (Owens et al., 1989; Chappell et al., 1986). These changes are thought to represent changes in the activity of the CRF-containing neurons.

The lack of change in median eminence CRF concentrations initially made it difficult to attribute the plasma endocrine responses to DOB administration to be due to alterations in the activity of hypothalamic CRF neurons. Two different approaches were then undertaken to further define the role of CRF, if any, in the DOB-induced activation of the pituitary-adrenal axis. First, if CRF secretion from the median eminence and subsequent activation of pituitary corticotrophs is responsible for the observed ACTH and corticosterone responses, blockade of pituitary CRF receptors with a CRF antagonist should block the endocrine response to DOB. As shown in figure 5, CRF antagonist pretreatment significantly attenuated the ACTH rise in DOB-treated rats (CRF antagonist/DOB). This suggests that median eminence CRF secretion is responsible for the previously described ACTH increases. However, the corticosterone responses were unaffected by prior CRF antagonist administration. Median eminence CRF concentrations were unchanged (data not shown).

These results suggest that increased plasma ACTH concentrations after DOB-treatment are, at least in part, due to 5-HT$_2$ receptor activation of hypothalamic CRF neurons. Moreover, the magnitude of CRF release necessary for the observed ACTH responses appears to be small because the content of CRF in the median eminence was not decreased measurably. The observed corticosterone response after CRF antagonist pretreatment suggests that 5-HT$_2$ receptor activation downstream of the anterior pituitary corticotrophs may somehow

increase plasma corticosterone concentrations. However, there is no evidence in the literature for direct serotonergic stimulation, and certainly not specific 5-HT$_2$ receptor activation, of corticosterone secretion from the adrenal cortex. However, increased central sympathetic outflow as a result of central 5-HT$_2$ receptor activation cannot be ruled out, but only small numbers of 5-HT$_2$ receptors are directly associated with autonomic nuclei in the brainstem. Similar increases in plasma corticosterone, not thought to be directly related to hypothalamo-pituitary activation, after DOI administration have also been reported (Alper, 1990; Calogero et al., 1990).

Several points need to be clarified here. First, α-helical CRF$_{9-41}$ is generally a poor antagonist of CRF's actions in vivo when administered peripherally (Rivier, et al., 1984b; C. Rivier, personal communication). Whereas poor efficacy would explain why CRF antagonist-pretreated rats exhibited elevated plasma corticosterone concentrations after DOB, it does not explain why ACTH concentrations in CRF antagonist-pretreated rats that received DOB were not statistically different from controls, although the rats receiving DOB did have slightly higher mean ACTH concentrations. As for the second point, if DOB could bypass the CRF neuron and act directly on the pituitary to activate the pituitary-adrenal axis, CRF antagonist pretreatment should not alter ACTH concentrations in DOB-treated rats. Moreover, Hashimoto et al. (1982) found that serotonin had no effect on ACTH secretion in pituitary cell cultures.

A different experimental paradigm was then used to assess whether median eminence CRF secretion was responsible for the ACTH and corticosterone responses to DOB. It was hypothesized that CRF is responsible for the pituitary-adrenal activation after DOB, but that either new synthesis of CRF or axonal transport of CRF were rapidly replenishing median eminence CRF stores such that radioimmunoassay of median eminence extracts revealed no differences between treatment groups. If this is true, blockade of new CRF synthesis might allow for measurable differences in median eminence concentrations of CRF after DOB-induced activation of the HPA axis. To test this hypothesis, rats were pretreated with the protein synthesis inhibitor, cycloheximide, 1 hr before receiving an injection of either water or DOB.

After cycloheximide pretreatment, CRF content in the median eminence was significantly lower in rats receiving DOB (cycloheximide/DOB) compared to controls (cycloheximide/control) (fig. 6). This supports the hypothesis that DOB does stimulate CRF secretion. However, making this finding more difficult to interpret, DOB-treated rats which did not receive cycloheximide (control/DOB) exhibited lower CRF content compared with their appropriate control group (control/control). This represented the first time DOB administration alone resulted in measurable decreases in median eminence CRF content. Although the finding that control/DOB rats exhibited decreased median eminence CRF content relative to their controls (control/control) makes the initial hypothesis that inhibition of protein synthesis would allow for detection of DOB-induced release of CRF (cycloheximide/DOB) more difficult to interpret, the cycloheximide/DOB group did exhibit significantly lower amounts of CRF in the median eminence compared to the control/DOB group (which was statistically equivalent to the cycloheximide/control group). Why DOB administration alone resulted in measurable decreases in CRF content in this experiment is unclear, but the results in cycloheximide pretreated rats do suggest that replenished stores of newly synthe-

sized CRF may explain the general lack of measurable differences in median eminence CRF content seen in the presence of robust ACTH and corticosterone increases.

In a related experiment that supports the present findings, Berkenbosch and colleagues (Berkenbosch and Tilders, 1988; Berkenbosch et al., 1989) reported that 5 μg of colchicine given intracisternally blocked fast axonal transport of hypothalamic CRF by > 90%. They concluded that hypothalamic CRF neurons refill their median eminence stores after activation. They have calculated that CRF was replenished at a rate of 9.2%/hr in adrenalectomized rats and 23%/hr during 3 hr of hypoglycemic stress. These results suggest that substantial quantities of CRF can be replenished rather quickly after CRF neuronal activation and may explain the previously unmeasurable changes in median eminence CRF concentrations after 5-HT$_2$ receptor stimulation.

As shown in figure 6, cycloheximide pretreatment unexpectedly abolished the DOB-induced increases in ACTH and corticosterone concentrations. Although not directly related to 5-HT regulation of CRF neurons, these findings suggest that protein synthesis is necessary for synaptic secretion mechanisms to function properly as inhibition of new protein synthesis by cycloheximide should not affect CRF or ACTH processed previously and ready for release. Cycloheximide binds to the ribosome inhibiting enzymatic translocation of mRNA thereby disrupting new protein synthesis. It would not be expected to significantly disrupt cellular secretion mechanisms at this time point (2 hr between cycloheximide injection and sacrifice). One possible explanation of this finding could be that those proteins necessary for neurotransmitter secretion are turned over very quickly and need to be newly synthesized within the 2-hr time frame studied here. However, if this were true one would expect the rats to have died long before 2 hr had elapsed due to a generalized inhibition of cell to cell communication.

Overall, the results suggest that 5-HT$_2$ receptor activation stimulates hypothalamic CRF secretion. However, it cannot be ruled out that DOB can increase circulating glucocorticoid concentrations via non-CRF mechanisms as well. Whereas the synergistic action of arginine vasopressin on CRF-stimulated ACTH release is well established, AVP alone possesses ACTH-releasing activity both in vitro and in vivo, but at lower efficacy than CRF (Rivier and Vale, 1983, 1985; Rivier, et al., 1984a. Additionally, oxytocin potentiates CRF-stimulated ACTH secretion; however, it is less clear whether oxytocin alone can stimulate ACTH release (Gibbs, 1985; Gibbs et al., 1984; Schwartz and Vale, 1988). In either case, if the observed increases in plasma ACTH after DOB administration were due to release of vasopressin or oxytocin alone, the CRF receptor antagonist would not be expected to nearly abolish the DOB-induced increases in plasma ACTH (fig. 5). Although we (Owens and Nemeroff, 1989) could not replicate the in vitro findings of others reporting direct 5-HT stimulation of hypothalamic CRF release (Nakagami et al., 1986; Calogero et al., 1989), they support the in vivo results presented here. Additionally, results of the CRF radioimmunoassay suggest that extrahypothalamic CRF neurons are not measurably altered (as evidenced by peptide concentrations) by acute DOB administration at the dose and time point studied here. However, as appears to be the case in the median eminence, CRF concentrations may be replenished with newly synthesized peptide in other brain regions as well. Changes in CRF receptor concentrations and/or mRNA expression after 5-HT$_2$ agonist administration will

further clarify the role of 5-HT$_2$ receptors in regulating CRF neurons, particuarly those of the endocrine hypothalamus.

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