to the region of the X chromosome that is somehow involved in the expression of Duchenne muscular dystrophy. DNA from this region will not only allow a rational approach to finding the *dmd* gene and its product but will also provide tightly linked probes that may be valuable for carrier detection and prenatal diagnosis in selected families.

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Corticotropin-Releasing Factor Receptors in Rat Forebrain: Autoradiographic Identification

Abstract. *Corticotropin-releasing factor (CRF) receptors were identified in rat forebrain by autoradiography with an iodine-125–labeled analog of ovine CRF substituted with norleucine and tyrosine at amino acid residues 21 and 32, respectively. High-affinity receptors for CRF were found in discrete areas of rat forebrain, including laminae I and IV of the neocortex, the external layer of the median eminence, the lateral nucleus of the amygdala, and the striatum. These results are consistent with earlier findings on the immunohistochemical distribution of CRF and suggest that endogenous CRF has a physiological role in regulating activity of the central nervous system.*

Corticotropin-releasing factor (CRF), a 41–amino acid peptide originally isolated from ovine hypothalamus (*1, 2*), stimulates the release of proopiomelanocortin-derived peptides from the pituitary gland (*1, 3*). In addition to its endocrine activity in the pituitary gland (*1, 3*), CRF has extrahypophysiotropic effects. Intracerebroventricular administration of CRF elicits autonomic (*4–6*), electrophysiological (*7, 8*), and behavioral (*9–10*) effects, suggesting that it plays a crucial role in integrating the organism's response to stress. Radioimmunoassay (*11*) and immunocytochemical (*12*) studies have demonstrated that CRF-like immunoreactivity is distributed throughout the central nervous system (CNS), and pharmacological (*4–10, 13*) and immunohistochemical (*11, 12*) evidence suggests that CRF functions as a neurotransmitter or neuromodulator in the CNS. These actions of CRF in the brain are presumed to be initiated by binding to high-affinity receptors. Recently we identified and characterized high-affinity receptors for CRF in anterior and intermediate lobes of rat pituitary glands (*14*) and in the anterior lobe of human pituitary glands (*15*); however, specific CRF receptors in the brain had yet to be demonstrated. In the study reported here we used an iodine-125–labeled ovine CRF analog substituted with norleucine and tyrosine at amino acid residues 21 and 32, respectively, or [Nle^{21}, ^{125}I-Tyr^{32}]CRF, to identify receptor binding sites for CRF in sections of rat brain with light microscopic autoradiography.

We previously demonstrated that the kinetics, pharmacology, and distribution of [Nle^{21}, ^{125}I-Tyr^{32}]CRF binding in the rat pituitary gland are correlated with the biological potency and sites of action of CRF, suggesting that the ligand labels the physiologically relevant CRF receptor (*14*). To define the characteristics of [Nle^{21}, ^{125}I-Tyr^{32}]CRF binding in rat brain, we incubated serial, slide-mounted tissue sections with increasing concentrations of CRF-related and -unrelated peptides. Autoradiograms of these brain sections and iodinated standards were generated with tritium-sensitive Ultrofilm (LKB). An analysis of [Nle^{21}, ^{125}I-Tyr^{32}]CRF binding in the striatum was performed by microdensitometry, and the film optical density was related to the molar concentration of radioactivity by use of the standard curve generated concomitantly with the autoradiograms (*16*). The binding of [Nle^{21}, ^{125}I-Tyr^{32}]CRF in rat striatum was saturable and on Scatchard analysis revealed a high-affinity component with an apparent dissociation constant of 6.2 ± 0.8 n*M* and maximum binding of 4.5 ± 1.5 fmole per milligram of protein (means ± standard errors; $n = 3$).

To characterize [Nle^{21}, ^{125}I-Tyr^{32}]CRF binding pharmacologically, we examined the relative potencies of 1 μ*M* concentrations of CRF-related and -unrelated peptides in inhibiting specific binding to slide-mounted sections of rat forebrain. In these competition studies, 1 μ*M* rat CRF and ovine CRF inhibited 105.5 ± 6.3 and 80.8 ± 0.2 percent, respectively, of the specific [Nle^{21}, ^{125}I-Tyr^{32}]CRF binding in rat striatum. Two analogs of ovine CRF, CRF-(1–39) and CRF-(1–22), which are weak in stimulating proopiomelanocortin-derived peptide secretion from the pituitary (*1*), displaced 31.4 ± 0.9 and 29.4 ± 3.6 percent, respectively; the unrelated peptide, arginine vasopressin, did not significantly inhibit [Nle^{21}, ^{125}I-Tyr^{32}]CRF binding at 1 μ*M*. These kinetic characteristics and pharmacological properties of the CRF binding site in rat striatum are comparable to those for the CRF receptor in rat anterior pituitary membrane homogenates (*14, 17*) and slide-mounted bovine pituitary sections (*14*), substantiating earlier suggestions (*13, 18*) that the structural requirements for CRF activity are shared by brain and pituitary receptors.

The autoradiographic mapping of [Nle^{21}, ^{125}I-Tyr^{32}]CRF binding sites in rat forebrain is shown in Fig. 1. Specific high-affinity binding sites for CRF were heterogeneously distributed, with the highest densities observed in laminae I and IV of the neocortex (Fig. 1, A and B) and in the external layer of the median

eminence of the hypothalamus (Fig. 1, C and D). High concentrations of CRF binding were also found in the striatum, lateral nucleus of the amygdala, cingulate cortex, nucleus of the diagonal band, anterior ventral nucleus of the thalamus, and in hypothalamic regions outside the median eminence. Lower levels of CRF binding were detected in the pyriform cortex and hippocampus, and specific binding was absent in the corpus collosum.

The distribution of CRF receptors in rat forebrain is generally consistent with immunohistochemical mapping (*12*) and with proposed sites of action of CRF (*6, 8, 10, 18*). For example, CRF cell bodies in the neocortex are concentrated in laminae II and III, with projections to laminae I and IV (*12*), areas rich in CRF receptors. The biological relevance of CRF receptors in the cerebral cortex is supported by the recent observation that CRF stimulates secretion of somatostatin from dispersed rat cerebral cortical cells (*18*). In the hypothalamus the highest concentrations of CRF receptors are in the external layer of the median eminence, where the most intense concentration of CRF fibers is also found (*12*). Microinjections of CRF into the lateral hypothalamus inhibit gastric acid secretion through modulation of the autonomic nervous system (*6*), and microinfusion of CRF into the arcuate-ventromedial area of the hypothalamus suppresses sexual behavior (*13*). The hypothalamus may be a primary site of action for integrating the endocrine, autonomic, and behavioral responses of CRF. Intracerebroventricular administration of CRF produces potent behavioral activation (*5, 8, 9*), and at high doses elicits seizures that electroencephalographically appear to originate in the amygdala (*8*). The presence of CRF receptors in the amygdala supports the latter finding. Additional studies are necessary to define more precisely the brain sites where centrally administered CRF produces its various effects.

In summary, these studies with [Nle^{21}, ^{125}I-Tyr^{32}]CRF have identified pharmacologically specific, high-affinity receptor binding sites for CRF in discrete areas of the rat forebrain. These areas are correlated with the immunohistochemical distribution of CRF pathways in rat forebrain and support a physiological role for endogenous CRF in regulating CNS activity. Studies to characterize CRF receptors and CRF-containing pathways in the brain provide a means for understanding the various functions of this peptide in different areas of the CNS.

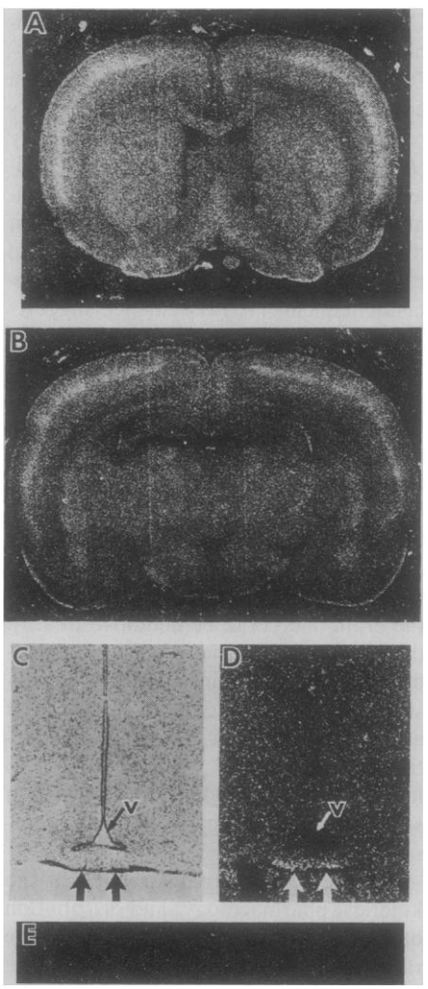

Fig. 1. Autoradiographic mapping of [Nle^{21}, ^{125}I-Tyr^{32}]CRF binding sites in rat forebrain. (A and B) Dark-field photomicrographs (tritium-sensitive Ultrofilm), showing the distribution of autoradiographic grains in coronal sections of rat forebrain at the regions of the striatum (A) and diencephalon (B). (C) Bright-field photomicrograph showing a high-power view of the median eminence of the hypothalamus at the base of the third ventricle (*v*). (D) Dark-field photomicrograph showing the autoradiographic grain distribution on emulsion-coated cover slips over the same area shown in (C). In dark-field illumination the autoradiographic grains appear as white spots and the tissue is not visible. Thus the brightest areas have the highest concentrations of binding sites. In (D), note that the concentration of grains in the external layer of the median eminence (arrows) is higher than in the remainder of the hypothalamus. (E) Dark-field photomicrograph showing the absence of specific receptor binding when 1 μM unlabeled [Nle^{21}, ^{125}I-Tyr^{32}]CRF is included in the incubation buffer. In the autoradiographic procedure used (*19, 20*), male Sprague-Dawley rats weighing 200 to 350 g were anesthetized with pentobarbital and perfused intracardially with 500 to 700 ml of a mixture of equal parts of phosphate-buffered saline and 0.32*M* sucrose (*p*H 7.4). The brains were then rapidly removed, embedded in homogenized brain paste, and frozen in powdered dry ice. Serial coronal sections (8-μm) of rat forebrain in the regions of the striatum and diencephalon were cut with a microtome (Harris Cryostat) at −16°C, thaw-mounted onto chrome alum gelatin–subbed microscope slides, and stored at −20°C until use. Sections were incubated with 0.1 n*M* [Nle^{21}, ^{125}I-Tyr^{32}]CRF for 60 minutes at room temperature in 50 m*M* tris HCl (*p*H 7.7) containing 5 m*M* $MgCl_2$, 2 m*M* EGTA, 0.1 percent bovine serum albumin, aprotinin (100 kallikrein-inhibiting units per milliliter), and 0.1 m*M* bacitracin. Blanks were incubated in the same medium with the addition of 1 μM [Nle^{21}, Tyr^{32}]CRF. Before exposure to [Nle^{21}, ^{125}I-Tyr^{32}]CRF, all brain sections were incubated at room temperature for two 15-minute periods in 50 m*M* tris HCl (*p*H 7.7) containing 5 m*M* $MgCl_2$ and 2 m*M* EGTA in order to displace endogenous CRF from its receptor. After incubation with [Nle^{21}, ^{125}I-Tyr^{32}]CRF, tissue sections were washed in Dulbecco's phosphate-buffered saline containing 1 percent bovine serum albumin at 4°C for two 20-minute periods, dipped in deionized water, and dried rapidly under a stream of cold, dry air. Tritium-sensitive Ultrofilm (*19*) or NTB-3 emulsion–coated glass (*20*) or Aclar plastic cover slips were apposed to the tissue sections. After 7 to 14 days of exposure at 4°C, the autoradiograms were developed and the tissue was stained with toluidine blue.

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Dopaminergic Neurotoxicity of 1-Methyl-4-Phenyl-1,2,5,6-Tetrahydropyridine in Mice

Abstract. *1-Methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) is known to cause an irreversible destruction of the dopaminergic nigrostriatal pathway and symptoms of parkinsonism in humans and in monkeys. However, MPTP has been reported to act only minimally or not at all in several other animal species. When MPTP (30 milligrams per kilogram of body weight) was administered parenterally to mice, a decrease in concentrations of neostriatal dopamine and its metabolites, a decrease in the capacity of neostriatal synaptosomal preparations to accumulate [^{3}H]dopamine, and a disappearance of nerve cells in the zona compacta of the substantia nigra were observed. In contrast, MPTP administration had no effect on neostriatal concentrations of serotonin and its metabolites. MPTP administration thus results in biochemical and histological changes in mice similar to those reported in humans and monkeys and similar to those seen in Parkinson's disease in humans. The mouse should prove to be a useful small animal with which to study the mode of action of MPTP.*

1-Methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) is a commercially available compound that can be formed as a by-product in high yield in the synthesis of 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP), a potent analgesic structurally similar to two other widely used analgesics, meperidine (Demerol) and alphaprodine (Nisentil). The inadvertent ingestion of small amounts of MPTP, mixed with varying amounts of MPPP and perhaps other agents, has caused an irreversible parkinsonism in several young individuals, who were most likely attempting to simulate the actions of heroin with MPPP (*1*). Also, intravenous injection of MPTP alone to monkeys has produced symptoms and pathology consistent with parkinsonism, including a severe dopamine depletion and a highly selective degeneration of the nerve cells of the substantia nigra (*2*). Therefore, the agent responsible for the parkinsonism observed in the young drug addicts was MPTP or a metabolite.

The discovery that a simple substance of this nature administered systemically can selectively destroy a specific neuronal system and closely reproduce the pathology of Parkinson's disease has implications for the etiology of the naturally occurring human disease. It suggests that a similar neurotoxin, exogenous or endogenous, may be involved in the pathogenesis of the disease. Such a suggestion is particularly timely in view of the recent findings of a low concordance rate for Parkinson's disease among monozygotic twins (*3*).

Surprisingly, MPTP was reported to have no neurotoxic effect in several other animal species, including rats and cats (*4*). We now report that MPTP administration to mice (two to ten daily injections) resulted in a decrease in the dopamine content of neostriatal brain tissue, inability of this tissue to accumulate [^{3}H]dopamine, and a severe loss of nerve cells in the zona compacta of the substantia nigra.

Male Swiss-Webster mice weighing between 25 and 35 g were injected intraperitoneally with MPTP dissolved in distilled water (*p*H adjusted to 8.5 with dilute hydrochloric acid). The dose was 30 mg (0.17 mmole) per kilogram of body weight, and the injection volume was 1.0 ml per 100 g of body weight. Control animals received either vehicle injection or no injection at all. Since dopamine concentrations in these animals did not differ, both groups were combined and considered as controls. Some animals received multiple MPTP injections 24 hours apart. Concentrations of dopamine in the mouse neostriatum were assayed at various times after the last injection. Each animal was stunned by a blow to the head, the brains were removed, and the neostriata were rapidly dissected from the rest of the brain. The neostriata were then weighed and homogenized in 0.1*M* perchloric acid containing dihydroxybenzylamine (DHBA) as an internal standard and centrifuged at 27,000*g* for 15 minutes. The supernatant, usually 20 μl in volume (equivalent to 0.2 mg of original tissue), was used for assays of dopamine, serotonin, and their metabolites (*5*).

An LC-304T liquid chromatograph (Bioanalytical Systems) connected to a dual pen recorder (Kipp and Zonen) was used for all assays. The operating potential was 750 mV, the temperature was 25°C, and full scale on the detector was usually set to 2.0 nA. A Biophase ODS 5-μm column (Bioanalytical Systems) was used for all separations. The flow rate of the mobile phase was 1.5 ml per minute. The mobile phase was made up as follows: 35 ml of acetonitrile was added to 965 ml of 0.15*M* monochloroacetate buffer (*p*H 3.0) containing 193 mg of sodium octyl sulfate. This mixture was filtered and degassed, tetrahydrofuran (18 ml) was carefully added, and the mobile phase was sealed until use.

Data were calculated from standard curves made on the same day on the basis of five to six data points in the same concentration range as that present in the tissue. Concentrations of dopamine and its major metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were calculated on the basis of the original tissue weight, and the results were expressed in micrograms per gram of tissue ± standard error of the mean (S.E.M.).

In some experiments, the neostriata from control and MPTP-treated mice were homogenized in 0.3*M* sucrose. To a sample of this homogenate, an equal volume 0.2*M* perchloric acid containing DHBA was added. This mixture was then centrifuged, and levels of dopamine and its metabolites were assayed as de-