Beta-Adrenergic–Receptor Localization by Light Microscopic Autoradiography

Abstract. *β-Receptors were identified in rat brain by a light microscopic autoradiographic technique. The procedure involved binding ^{3}H-labeled dihydroalprenolol to β-receptors in intact slide-mounted tissue sections and generating autoradiograms by the apposition of emulsion-coated cover slips. Biochemical analysis of the binding indicated that these conditions provided a high degree of selective labeling of β-receptors. High densities of receptors were found in superficial layers of the cerebral cortex, throughout the caudate-putamen, in the periventricular nucleus of the thalamus, in the molecular layer of the cerebellum, and in other areas. These results are in agreement with other electrophysiological and histochemical data. This radiohistochemical approach should be an important addition to other methods for mapping functional catecholamine neuronal pathways and sites of hormonal action.*

A wide variety of pharmacological and physiological experiments have demonstrated the presence of β-adrenergic receptors throughout various organs in the body (*1*). Recently, Lands *et al.* (*2*) and others (*3*) have subdivided the β-receptors into β_1 and β_2 populations. In recent years, the recognition binding site of the β-receptor has been identified in a variety of tissues by the direct binding of radiolabeled agonists and antagonists. These binding studies have revealed the expected variation in β-receptor density throughout the body (*4, 5*). Being able to examine the distribution of receptors in tissues with a high degree of anatomic resolution is an important goal.

We have developed and adapted a general method for the light microscopic autoradiographic location of drug and neurotransmitter receptors in tissues (*6*). The method involves the direct binding of radiolabeled drugs to specific receptor sites in slide-mounted tissue sections and the subsequent generation of autoradiograms by the apposition of emulsion-coated cover slips. This procedure has proved valuable for locating a wide variety of drug and neurotransmitter receptors in the brain (*7, 8*). In this report we discuss the use of light microscopy for locating β-adrenergic receptors in rat brain.

Before beginning autoradiographic experiments, we performed detailed biochemical experiments with slide-mounted tissue sections to identify the conditions for binding to the authentic β-receptor. [^{3}H]Dihydroalprenolol (DHA) was selected as the receptor-labeling ligand because it gave the best specific-to-nonspecific ratios among the commonly used ligands (see legend to Fig. 1). We found that the binding of [^{3}H]DHA to the slide-mounted tissue sections had the same kinetic characteristics, regional localization, and pharmacological properties that have been described for the β-receptor in tissue homogenates (*4, 5, 9*). Thus the preparation of tissues for the autoradiographic procedure does not alter the binding characteristics of [^{3}H]DHA in any measurable way (see legend to Fig. 1). This has been true for many other receptors examined by this procedure (*6–8*).

Autoradiographs revealed a widespread distribution of β-receptors in the brain (Fig. 1 and Table 1). In general, our regional distribution agreed with those binding studies with homogenates in vitro in that the greatest concentrations of receptors were observed in the caudate nucleus and cerebral cortex of the rat (*4, 5, 9*). White matter areas and fiber bundles had negligible specific binding. Although specific binding was widespread and less dense in many areas such as the hippocampus, thalamus, hypothalamus, and brainstem, certain smaller areas showed greater concentrations of binding sites. All results were reproducible as they were found in several sections from the same animal as well as in sections from different animals.

In the cerebellum, an elevated level of binding was observed throughout the molecular layer. A very low level of binding was observed in the granule cell layer. In the rostral pons, the locus ceruleus had an elevated level of binding, as did the adjacent gray matter of the floor of the fourth ventricle. In the cerebral cortex, the highest grain densities were found in laminae I, II, and III. In the thalamus, an elevated grain density was observed in the periventricular nucleus, pars rotundocellularis, and ventral medullary lamina.

The distributions of β-receptors that we observed in the cerebellum, cerebral cortex, and hippocampus are not in agreement with the distribution of a fluorescent derivative of a β-blocker used in earlier studies (*10*) aimed at identifying the receptor. However, the earlier studies did not have the detailed kinetic and pharmacological data on β-receptor binding that we have presented here. The affinity of the fluorescent drug utilized was relatively low and it would, in our experience, be very difficult to obtain a high degree of selective localization of receptors after administration in vivo. Recent studies in other laboratories with the same fluorescent drug indicate that the fluorescence observed under those conditions was not associated with β-receptors (*11, 12*).

The localization of receptors that we found in these areas is in agreement with several experimental observations. Electrophysiological studies suggest that the noradrenergic receptor of the β type is found in the cerebellum on Purkinje cells (*13*). In agreement with this, we found the receptors in the molecular layer, the region containing the dendrites of the Purkinje cells, and the Purkinje cell layer. Electrophysiological and biochemical studies of the hippocampus indicate the presence of β-receptors in areas CA_1, CA_2, and CA_3 (*12, 14*). In agreement with this result, we found receptors in these areas between cells and in the stratum oriens and stratum radiatum, areas con-

Table 1. Distribution of β-receptors in various brain regions. Data are autoradiographic grains per 1000 μm^2 (mean ± standard error; data are from three slides, with each slide providing a mean average of four separate microscopic fields). Blank values obtained from slides incubated with an excess of propranolol were 15.2 ± 2.5 and were subtracted from the above values. White matter areas were not significantly different from gray matter areas in blank slides. Slides were exposed 11 weeks. Measurements in the cortex were made in area 4 according to Krieg (*21*) at the level A8380 of Konig and Klippel (*22*). Laminae I and III had densities similar to that in II. Measurements in the caudate-putamen were made between the fiber bundles and were done in the same sections as were those in the cortex; measurements in the hippocampal formation and thalamus were made at level A4110 (*22*). Receptor densities in pyramidal cell fields CA_2 and CA_3 were about the same as that in CA_1.

Region	Grain density
Cortex	
Lamina II	45.9 ± 5.9
Lamina VI	17.9 ± 0.6
Caudate-putamen	81.7 ± 7.6
Hippocampal formation	
CA_1	21.0 ± 1.9
CA_4	6.4 ± 1.1
Molecular layer of dentate gyrus	28.1 ± 2.3
Periventricular nucleus of the thalamus	38.9 ± 1.9
Cerebellum	
Granule cell layer	6.0 ± 1.3
Molecular and Purkinje cell layers	38.2 ± 3.0
Locus ceruleus	19.0 ± 1.3
Nucleus tractus mesencephali	6.3 ± 0.6

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taining the dendrites of the pyramidal cells. The neurons in the locus ceruleus have been found to be inhibited by isoproterenol, a β-agonist (*15*). This is in agreement with the elevated levels of receptor binding we observed there; however, a β-antagonist, sotalol, did not block the inhibition. Additional physiological studies are needed to clarify the issue.

Fluorescence histochemical studies indicate a noradrenergic innervation of the Purkinje cells in the cerebellum and of the pyramidal cells in the hippocampus (*16*). Catecholamine fluorescence is also increased in the dorsomedial thalamus (*16*), an area we found to have high densities of receptors. In the cat frontal cerebral cortex, β-adrenergic-sensitive adenylate cyclase was concentrated in superficial layers (*17*); this corresponds to our observed high level of β-receptor binding in laminae I to III and lower levels in other laminae. Dopamine-β-hydroxylase-positive fibers and varicosities were also observed in these layers (*18*).

As in other receptor studies, we did not find a simple one-to-one correlation between receptor densities and norepinephrine levels. For example, we and others have found high concentrations of β-receptors throughout the caudate-putamen, an area with low levels of norepinephrine (*4, 5, 9*). There is no obvious explanation for these discrepancies, but many hypotheses can be considered. One suggestion has been that a portion of the receptors are located on nonneuronal membranes (*19*), which are not associated with synaptic transmission. An explanation for a lack of receptors in innervated areas could be that certain binding studies do not show all relevant receptors and that, for example, α-receptors must be included in any discussion of noradrenergic transmission. Another possible explanation has to do with the relatively large dendrite tree of certain cells (*5, 20*); even though norepinephrine levels in cerebellum are relatively low, each Purkinje cell is supposedly innervated by a noradrenergic fiber (*13, 16*). Thus, the Purkinje cells may be "committed" to making receptor, which is found throughout the large dendrite tree of the cells. No single explanation for all cases seems satisfactory at this time.

Although these studies were performed on brain tissue, the methods are applicable to a wide variety of tissues. Radiohistochemical studies of the localization of β-receptors should provide a detailed knowledge of the sites of action of β-adrenergic drugs. These radiohistochemical studies of β-receptors as well as studies of α-receptors (*8*) should be important additions to other methods

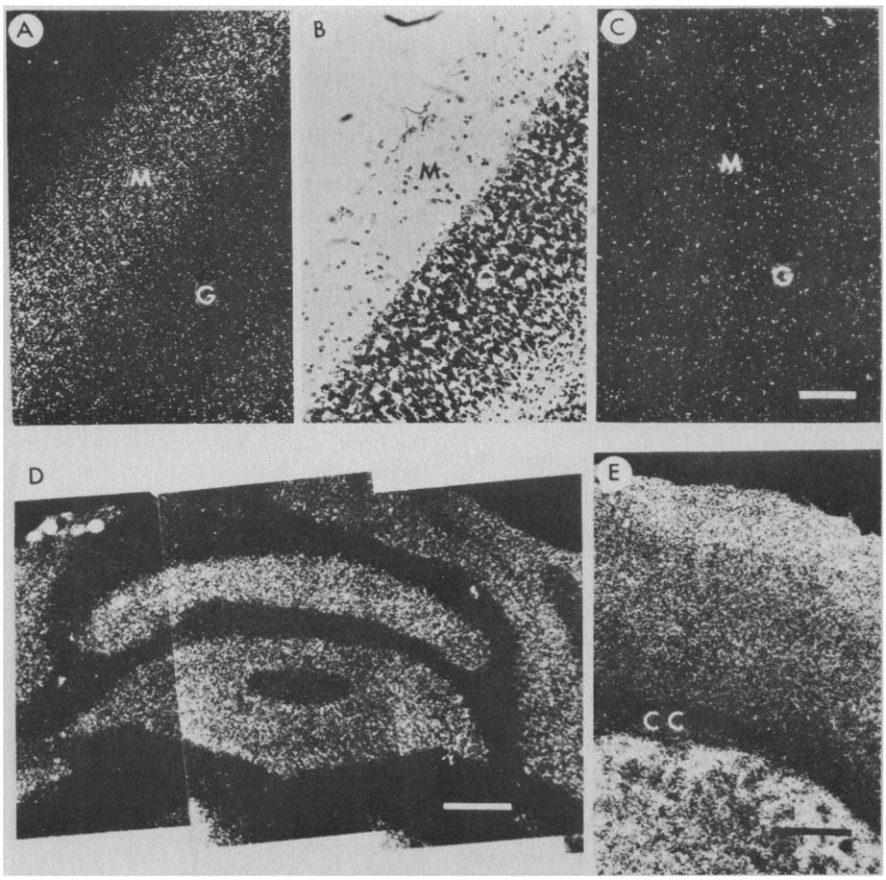

Fig. 1. Autoradiographic localization of β-receptors in rat brain. (A–C) High-power micrographs of rat cerebellum (scale bar, 100 μm). The bright-field micrograph in (B) showing the tissue and the dark-field micrograph in (A) showing the autoradiographic grains are from the same slide. The dark-field micrograph in (C) is from an adjacent section incubated with an excess of unlabeled propranolol to produce a blank. Receptors are concentrated in the molecular layer (*M*), with the granule cell layer (*G*) having a smaller quantity. (D) A lower power dark-field micrograph of rat cerebellum; the high grain densities stand out clearly in the molecular layer (scale bar, 500 μm). (E) Dark-field micrograph showing receptor distribution over the cerebral cortex, the corpus callosum (*CC*), and the caudate-putamen (scale bar, 500 μm). All slides were exposed 11 weeks. Experimental procedures and quantitative and pharmacological details are as follows. Ten-micrometer sections of rat brain were thaw-mounted on microscope slides. The areas of tissue examined included the cerebellum and portions of the forebrain. Before tissue dissection, the rats were perfused with very low concentrations of paraformaldehyde, 0.1 percent in phosphate-buffered saline. The slide-mounted tissue sections were incubated (0.17*M* tris-HCl buffer, *p*H 7.7, containing 10 m*M* $MgCl_2$) with [^{3}H]DHA (45 Ci/mmole, New England Nuclear; used under subdued light) under varying conditions to identify the optimal conditions for labeling the authentic β-receptor and for finding maximal specific-to-nonspecific ratios. After various incubations and washing conditions, the sections were scraped from the slides and the binding was assayed by scintillation spectrometry. A study of the equilibrium binding kinetics of [^{3}H]DHA for the slide-mounted tissue sections revealed a dissociation constant of about 2 n*M* and maximal binding of about 23 pmole/g tissue in forebrain sections. The binding of 2 n*M* of [^{3}H]DHA in sections of forebrain was displaced by a number of β-adrenergic drugs. These included (IC_{50} values in parentheses): L-propranolol ($5 \times 10^{-9}M$); D-propranolol ($10^{-6}M$); (−)isoproterenol ($10^{-7}M$); (−)epinephrine ($1.4 \times 10^{-6}M$); (−)norepinephrine ($1.6 \times 10^{-6}M$); and salbutamol ($2 \times 10^{-5}M$). The relative potencies of isoproterenol, (−)epinephrine, and (−)norepinephrine indicate that the bulk of the binding in these forebrain sections is to an authentic β_1-receptor as expected. In the cerebellar slices, however, the pharmacological characteristics revealed that the receptor was predominantly of the β_2 type (isoproterenol was more potent than epinephrine, which was more potent than norepinephrine). Zinterol, a predominantly β_2 drug, was also more potent in the cerebellum (IC_{50} in cerebellum was $3 \times 10^{-8}M$ and in forebrain was $10^{-6}M$). For routine autoradiographic experiments the following conditions were selected. Mounted tissue sections were incubated with 2 n*M* [^{3}H]DHA for 30 minutes at room temperature; 30-minute incubation times gave a maximal association of drug to receptor. The slides were washed twice at 2°C in buffer without drug for two 10-minute periods; these washing conditions resulted in specific-to-nonspecific ratios of 5:1 without a large loss of specific binding. To generate control slides, adjacent sections were incubated with [^{3}H]DHA and $10^{-5}M$ DL-propranolol.

for mapping functional catecholamine neuronal pathways and sites of neurohormonal action.

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Hormone-Induced Sexual Differentiation of Brain and Behavior in Zebra Finches

Abstract. *The male zebra finch sings, whereas the female does not. This behavioral dimorphism is correlated with the presence of morphological sex differences within the neural substrate that mediates this behavior, the song system. When a female chick is exposed to 17β-estradiol her song system is subsequently masculinized. Either testosterone or 5α-dihydrotestosterone may then induce such a female to sing when an adult.*

Adult vertebrates are sexually dimorphic with respect to various outward signs of brain function, among which are patterns of sexual behavior and of gonadotropin secretion. In mammals and birds, the sexuality of the brain develops under an influence of androgens or estrogens exerted during the perinatal period. This suggests that sex differences in the neural substrates that mediate these brain functions are also determined at this time (*1*). The sites of brain sexual differentiation in mammals have been implicated indirectly by brain lesion, brain stimulation, steroid autoradiography, and local hormone implants (*2*). Sex differences in brain structure have also been documented (*3*). However, since the neural circuits that mediate sexually dimorphic brain functions in mammals have been characterized only roughly, the specific hormone target sites within the developing mammalian brain and the cellular or functional consequences of their sexual differentiation are not well understood.

We have examined hormone influences on the development of song and of the brain nuclei that control this behavior in zebra finches (*Poephila guttata*). Among vertebrates, the avian song system is one of a few examples of discrete neural systems that mediate specific behaviors, and thus is particularly advantageous for neurobiological investigation. The song system consists of a chain of distinct brain nuclei that directly participate in the efferent motor pathway responsible for song (*4*). In the zebra finch, only males sing, and all brain nuclei of the song system are much larger in males than in females (*5*). The studies described in the present report demonstrate that 17β-estradiol (E_2) and 5α-dihydrotestosterone (DHT) influence the establishment of these differences in functional capacity and in brain architecture. This is the first such clear-cut example of a direct association between the sexual differentiation of brain and behavior.

The goals of our study were to investigate: first, if androgen or estrogen influences sexual differentiation of the song system (*6*); second, which hormone acts where; third, when the song system is sensitive to hormone; and fourth, if sexual differentiation of the functional capacity for song follows morphological masculinization of the song nuclei. Our

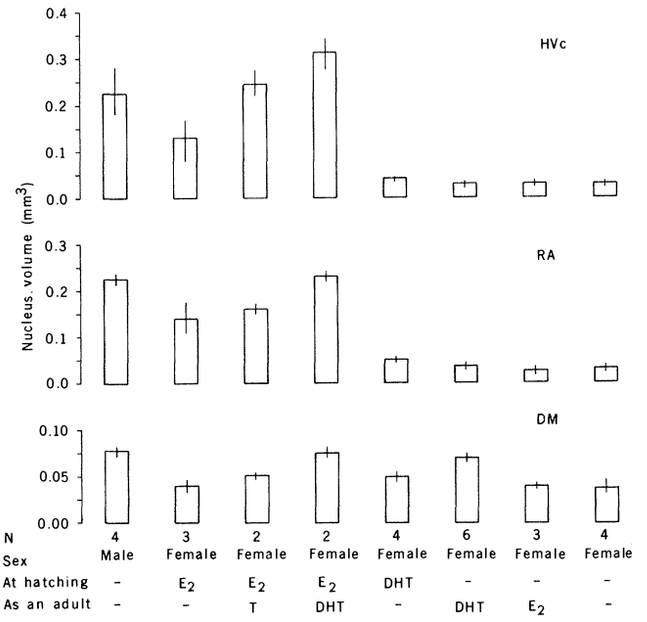

Fig. 1. Effect of various hormone treatments on the volume of HVc, RA, and DM. Mean volumes with ranges are plotted on the ordinate in cubic millimeters (*9*). Below each column is the number (*N*) of birds used and the hormone treatment given to that group; *E_2*, *DHT*, and *T* designate, respectively, 17β-estradiol, 5α-dihydrotestosterone, and testosterone. Bars denote no treatment.

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