Pharmacology Biochemistry & Behavior, Vol. 22, pp. 859–874, 1985. © Ankho International Inc. Printed in the U.S.A. 0091-3057/85 $3.00 + .00

The Alpha-Adrenergic Receptor: Radiohistochemical Analysis of Functional Characteristics and Biochemical Differences

JAMES R. UNNERSTALL,[1]* IMMACULADA FERNANDEZ† AND LUIS M. ORENSANZ†

Departments of Neuroscience, The Johns Hopkins University School of Medicine Baltimore, MD 21205 and †Departmento Investigación, Centro Ramón y Cajal, Madrid 34, Spain

UNNERSTALL, J. R., I. FERNANDEZ AND L. M. ORENSANZ. *The alpha-adrenergic receptor: Radiohistochemical analysis of functional characteristics and biochemical differences.* PHARMACOL BIOCHEM BEHAV 22(5) 859–874, 1985.—The partial agonist [^{3}H]para-aminoclonidine was used to label α_2-adrenergic binding sites in intact sections of the rat central nervous system using *in vitro* labeling receptor autoradiographic techniques. The distribution of α_2-agonist binding sites closely parallels the reported distribution of noradrenergic and adrenergic cell groups and their terminal fields, particularly the projections of the medullary catecholamine neurons. This distribution of α_2 binding sites confirms physiological studies which indicate that the anti-hypertensive actions of α_2-agonist compounds are mediated centrally in medullary and spinal centers involved in the control of parasympathetic and sympathetic outflow. Further, the high concentrations of α_2 binding sites in pontine and limbic areas such as the locus coeruleus, parabrachial nucleus, dorsal raphe, hypothalamus, amygdala, bed nucleus of the stria terminalis, septum and entorhinal cortex offer an anatomical basis for understanding the anxiolytic and antidepressant actions of drugs like clonidine. The antagonists [^{3}H]prazosin and [^{3}H]WB4101 were used to study the distribution of α_1-adrenergic binding sites in the rat forebrain and biochemical studies were performed to analyze the marked differences that were initially seen in the distribution of [^{3}H]prazosin and [^{3}H]WB4101 binding sites. Several pieces of evidence derived from both biochemical and autoradiographic studies suggest that [^{3}H]prazosin and [^{3}H]WB4101 act at distinctly different binding sites. However, both sites may represent components of an α_1-adrenergic receptor-effector complex since a high degree of overlap was seen in the binding site distribution of these two ligands and since kinetic interactions could be demonstrated in at least one region of the brain, the hippocampus. Differences noted in the relative displacements of [^{3}H]prazosin and [^{3}H]WB4101 binding in various forebrain regions could reflect differences in the coupling efficiency of the [^{3}H]prazosin and [^{3}H]WB4101 component of the hypothesized complex. Further, in some regions, [^{3}H]WB4101 labeled a binding site that is different from the α_1-receptor. Thus, [^{3}H]prazosin and [^{3}H]WB4101 binding sites seen in forebrain regions such as lamina V of the cortex, thalamic nuclei and dorsal raphe probably represent α_1-adrenergic receptors and confirm electrophysiological and biochemical studies which demonstrate that adrenergic transmission in these regions can be mediated through an α_1-receptor.

Clonidine [^{3}H]Para-aminoclonidine Norepinephrine Epinephrine [^{3}H]Prazosin [^{3}H]WB4101 Receptor autoradiography α-Adrenergic receptors

THE application of receptor binding techniques to the *in vitro* labeling of intact tissue sections for the autoradiographic analysis of drug and neurotransmitter binding sites is rapidly becoming a popular tool in the pharmacological armamentum. These procedures have several advantages. Conditions utilized to label the binding sites in the tissue sections can be carefully controlled and the pharmacologic and biochemical characteristics of the ligand binding can be reliably verified. Anatomical resolution is greatly enhanced. Thus, ligand binding sites can be studied not just as biochemical entities in a test tube but as integral components of functional neurochemical and neuroanatomical systems. Finally, autoradiography is quantitative. Thus, changes in binding parameters as a function of pathological, physiological and/or drug-induced state can be studied in discrete anatomical regions.

Receptor autoradiographic techniques can be applied to several different experimental questions. Mapping studies can be utilized to confirm the locus of drug action derived from behavioral or physiological experiments or to determine the potential for drug side effects based on the anatomical distribution of binding sites. These studies can

[1]Requests for reprints should be addressed to James R. Unnerstall, Ph.D., Department of Neuroscience, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205.

also be a rich source of experimental hypotheses concerning the physiological and behavioral effects of drugs, their locus of action and the function of the neuroanatomical network involved in these effects. Further, receptor autoradiography can reveal biochemical heterogeneity of neurochemical and receptor systems based on the ability to visualize differences in binding parameters in several regions simultaneously.

In this presentation, these types of questions will be discussed in relation to the study of the α-adrenergic receptor in the central nervous system (CNS). In the first section, experiments designed to study the distribution of the α_2-adrenergic receptor in the rat CNS will be reviewed. The analysis will center around a discussion of the binding site distribution in relation to the ascending and descending adrenergic system and its involvement in the several pharmacologic actions of α_2-adrenergic agents like clonidine. In the second section, ongoing work on the analysis of the possible heterogeneity of the α_1-adrenergic receptor as revealed by autoradiographic experimentation will be reviewed.

THE α_2-ADRENERGIC RECEPTOR: INVOLVEMENT IN AN INTEGRATED NEURONAL SYSTEM

Several experimental approaches have confirmed the fact that drugs like clonidine and other α_2-adrenergic agents produce their anti-hypertensive effects by an action in the CNS, primarily within the dorsal medulla (including the nucleus of the solitary tract and the dorsal motor nucleus of the vagus) ventrolateral medulla and the intermediolateral cell column of the thoracic spinal cord (for an extensive listing of the pertinent references, see [112]). The use of clonidine in several experimental and clinical paradigms have revealed many other centrally-mediated effects of α_2-adrenergic agents. Clonidine has potent anxiolytic activity [32, 39, 41, 42, 79, 104, 110] and it has been used to reverse the behavioral deficits induced by uncontrollable stressors in animal models [117]. Further, clonidine is an effective analgesic [6, 22, 24, 51, 68–71, 78, 96, 101] and it can attenuate many of the symptoms of opiate withdrawal [1, 5, 24, 25, 27–29, 45, 100, 109, 116].

Since many of the behavioral, somatosensory and autonomic responses affected by clonidine can be simultaneously elicited during the response of an organism to physical or "psychic" stress, it is probable that the several effects of α_2-adrenergic agents are not isolated phenomena but rather manifestations of a drug action at several sites within an integrated neuronal system. Autoradiographic analysis of the distribution of high-affinity α_2-agonist binding sites in the rat CNS has confirmed this hypothesis and has indicated a significant relationship between the distribution of binding sites and components of the norepinephrine (NE) and epinephrine (EPI) neuronal systems involved in these effects [112].

METHOD

Animals and Tissue Preparation

Details of the procedures utilized have been previously published [112]. Briefly, male Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI), 175–225 g body weight, were anesthetized with 65 mg/kg Na^+pentabarbitol (intraperitoneally) and rapidly perfused intracardially with a solution of isotonic phosphate-buffered saline and 0.32 M sucrose (50:50 v/v). The brains and spinal cords were removed on ice, mounted onto brass microtome chucks using brain paste, frozen over dry ice and stored at −80°C until use (1 to 2 weeks).

For sectioning, the mounted tissue was removed to the cryostat (Harris Mfg. Corp., N. Billerica, MA) and allowed to equilibrate to cryostat temperature (−17 to −20°C). Eight μm coronal brain sections and 10 μm horizontal spinal cord sections were thaw-mounted onto subbed (chrome alum and gelatin) microscope slides, air dried and stored at −20°C until use. A minimum of 8 animals was used for the subsequent autoradiographic analyses.

Preparation of Autoradiograms

The partial α_2-adrenergic agonist [^{3}H]para-aminoclonidine ([^{3}H]PAC, 45–56 Ci/mmol, New England Nuclear Corp., Boston, MA) was used to label the binding sites in the intact tissue sections. After being brought to room temperature, sections were first preincubated in a modified Krebs-phosphate buffer (pH 7.4 at 25°C, containing 119 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 10 mM Na_2HPO_4 and 1.3 mM $CaCl_2$) in the presence of 100 μM Na^+GTP for 30 min at room temperature. This step is utilized to dissociate endogenous ligand from the receptors that may interfere with the ligand binding. The sections were then washed in the incubation buffer (0.17 M Tris·HCl containing 10 mM $MgCl_2$, pH 7.6 at 25°C) for 10 min at room temperature. The sections were then incubated in the incubation buffer containing 1.0 nM [^{3}H]PAC for 60 min at room temperature. Serial sections were incubated with 1.0 nM [^{3}H]PAC in the presence of 10 μM phentolamine in order to assess non-specific binding. Finally, sections were washed in the incubation buffer alone for 10 min at 4°C. Under these conditions, [^{3}H]PAC labels α_2-adrenergic receptors with a high degree of specificity [112]. Given K_D's of 0.24 and 18 nM for the high- and low-affinity binding sites, approximately 78% of the high-affinity agonist binding sites and less than 5% of the low-affinity binding sites will be labeled using these conditions.

After the final wash, the sections were briefly dipped in cold deionized water to remove buffer salts and dried under a stream of cool, dried air. The slides were stored in dessicated boxes at 4°C for 24 hr prior to the apposition of emulsion. Either [^{3}H]Ultrofilm (LKB Products, Inc., Rockville, MD) was exposed to the slides stored in X-ray cassettes or emulsion-coated coverslips (Kodak NTB3, diluted 1:1 with distilled water) were affixed to the labeled slides. Exposure time was 8 to 10 weeks. Emulsions were developed and tissues stained according to previously published procedures [113,119]. Some horizontal sections of the thoracic spinal cord were counterstained for acetylcholinesterase [75] in order to more easily visualize the intermediolateral cell column.

Direct darkfield and brightfield photomicrographs were obtained using a Leitz Orthoplan microscope fitted with a Hinsch-Goldman box in order to view the autoradiographic grains under incident light illumination. Images obtained using [^{3}H]Ultrofilm were analyzed using the LAI Quantitative Biomed Image Analysis System (Loats Associates, Inc., Westminster, MD). Gray scale, reverse gray scale and color-coded images were taken directly from the image screen using a 35 mm camera fitted with a macro lens. Optical densities were calibrated using brain paste standards that were included with the labeled tissue sections [113].

RESULTS AND DISCUSSION

α_2-Binding Sites and Autonomic Control

[^{3}H]PAC binding sites were found in several areas of the

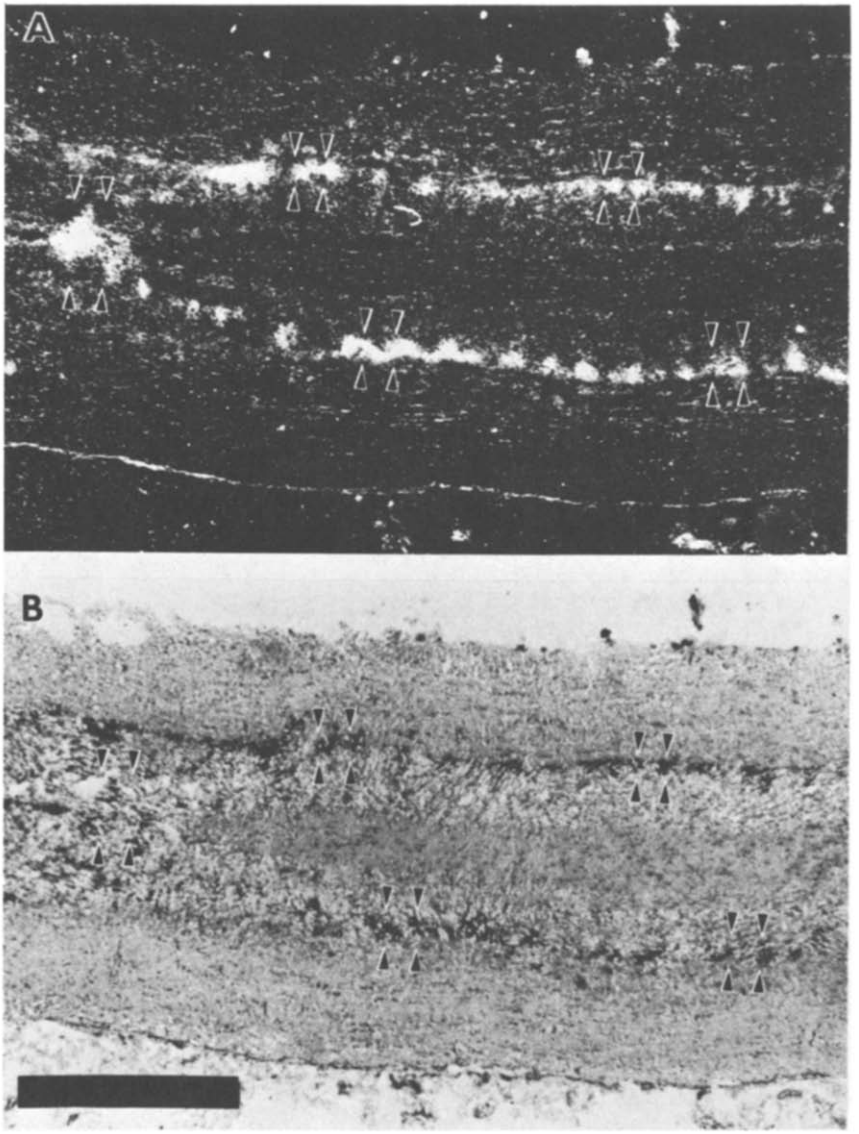
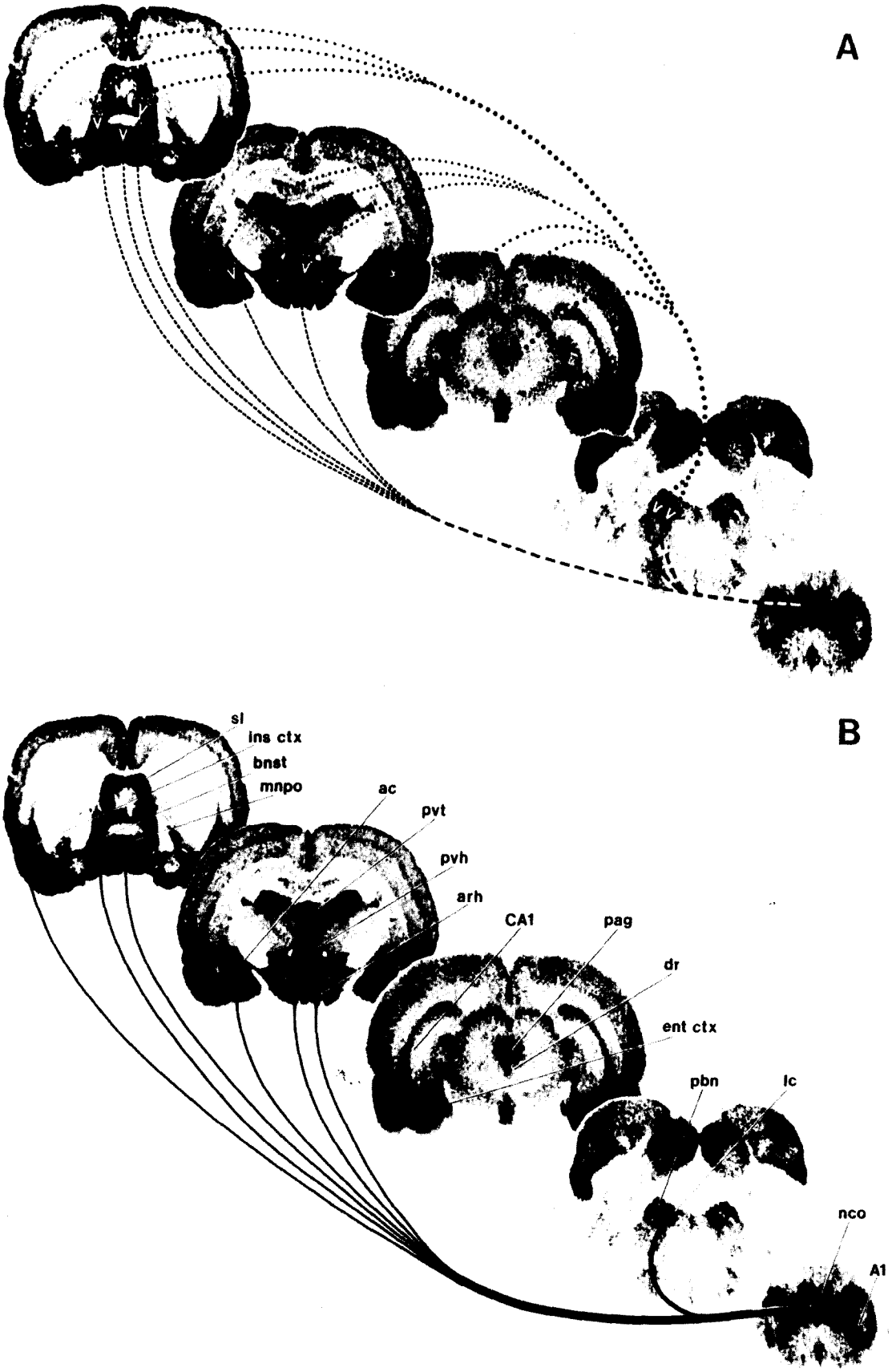


FIG. 1. Binding of [^{3}H]PAC in the intermediolateral cell column of the rat thoracic spinal cord. In (A) a darkfield photomicrograph taken from a horizontal section through the upper thoracic cord is shown. Bright areas represent regions of high binding site density. In (B), a brightfield photomicrograph of the underlying tissue is shown. The tissue was stained for acetylcholinesterase (dark stain) in order to visualize the intermediolateral cell column and counterstained with Toluidine blue. The dark arrowheads point to the same regions of the intermediolateral cell column. Note the striking similarity between the distribution of cholinesterase tissue and the "beaded" distribution of the autoradiographic grains. This illustrates that α_2-binding sites are associated with putative preganglionic neurons and indicates that the intermediolateral cell column may be a primary locus for the antihypertensive and anti-opiate withdrawal actions of clonidine. Bar=1000 μm.

medulla and thoracic spinal cord known to regulate parasympathetic and sympathetic function through their participation in the baroreceptor reflex loop [46, 74, 84–86, 94, 102, 108, 118]. These regions include the nucleus commissuralis, nucleus of the solitary tract and dorsal motor nucleus of the vagus in the dorsal medulla; the ventrolateral medulla including the nucleus ambiguus and nucleus retroambiguus; raphe pallidus and raphe obscurus in the ventromedial medulla; and the intermediolateral cell column of the thoracic spinal cord (Figs. 1 and 2). These regions include the A1, A2 and A5 NE cell groups and the C1 and C2 EPI cell groups [2, 17, 34, 43, 72, 82, 88, 94, 105]. These regions also have a high density of catecholamine nerve terminals arising from local circuitry within the dorsal medulla [2, 15, 34, 43, 105, 107] and NE and EPI innervation of the dorsal medulla, midline raphe nuclei and intermediolateral cell column arising from the ventrolateral medulla [2, 10, 43, 46, 84, 86, 94, 111, 118]. These results corroborate other experimental data which demonstrate that α_2-agonists can exert their effect on cardiovascular function


A
B
sl
ins ctx
bnst
mnpo
ac
pvt
pvh
arh
CA1
pag
dr
ent ctx
pbn
lc
nco
A1

in several brainstem and spinal regions involved in the baroreceptor reflex loop and indicate a strong correlation between adrenergic function and α_2-adrenergic receptors in these regions [112].

Supramedullary Integration of Cardiovascular and Affective Function

While the final common pathway involved in the regulation of cardiovascular function is found in the medulla and intermediolateral cell column of the thoracic spinal cord, other forebrain areas, particularly regions in the dorsal pons, hypothalamus amygdala and limbic forebrain can exert a powerful influence on autonomic function [73]. More important, several of these same regions may serve as neuronal units acting to integrate autonomic, arousal and sensory information. Thus, the possibility arises that many of these same areas may be involved in the expression of not only the cardiovascular actions of clonidine and related agents but also the affective and behavioral effects of this class of drugs.

High concentrations of [^{3}H]PAC binding sites are found in the locus coeruleus, dorsal parabrachial nucleus, paraventricular, periventricular and arcuate nuclei of the hypothalamus, medial preoptic nucleus, bed nucleus of the stria terminalis, lateral septum, central nucleus of the amygdala, insular and entorhinal cortices (Fig. 2). Several of these regions have been shown to be sensitive to changes in cardiovascular function and to have direct projections to medullary and spinal cardiovascular centers. These projections include efferents from the insular cortex [90], central nucleus of the amygdala [35, 40, 98], bed nucleus of the stria terminalis [99], periventricular and paraventricular hypothalamus [7, 8, 66, 95, 106], arcuate nucleus of the hypothalamus [65], medial preoptic nucleus [91] and locus coeruleus [48,76]. These same regions are reciprocally innervated by adrenergic projections from the nucleus commissuralis, nucleus of the solitary tract and ventrolateral medulla [14, 47, 61, 81]. These ascending medullary afferents include A1/C1 projections to the locus coeruleus, bed nucleus of the stria terminalis, medial preoptic nucleus, supraoptic, paraventricular, periventricular and posterior nuclei of the hypothalamus [9, 11, 12, 20, 53, 93, 94], and A2/C2 projections to the locus coeruleus, dorsal parabrachial nucleus, medial and anterior hypothalamic nuclei, bed nucleus of the stria terminalis, lateral septum and central nucleus of the amygdala [23a, 34, 37, 43, 44, 54, 60, 81, 93, 105]. Further, pontine regions (locus coeruleus, dorsal parabrachial nucleus, dorsal raphe nucleus) which are innervated by medullary catecholamine neurons in turn innervate many of these same forebrain regions plus other areas, such as the dorsomedial thalamus, hippocampus and pyriform, insular, cingulate and entorhinal cortices that are involved in the integration of cognitive and viscerosensory information [38, 60, 64, 89, 92]. A schematic of these integrated pathways is shown in Fig. 2.

Thus, these data confirm the hypothesis that α_2-adrenergic drugs can act directly in those regions of the CNS that regulate parasympathetic and sympathetic outflow. Further, a strong correlation exists between the distribution of α_2-binding sites labeled with [^{3}H]PAC and NE and EPI projections, particularly those projections from the medullary adrenergic cell groups. These data also suggest which brain regions may be involved in some of the other pharmacologic actions of clonidine, such as analgesia (substantia gelatinosa of the spinal cord and the trigeminal, periaqueductal gray, dorsomedial and intralaminar nuclei of the thalamus), opiate withdrawal (intermediolateral cell column of the spinal cord, dorsal and ventrolateral medulla, locus coeruleus, arcuate nucleus of the hypothalamus, amygdala) and stress and anxiety (locus coeruleus, dorsal parabrachial nucleus, para- and periventricular nuclei of the hypothalamus, bed nucleus of the stria terminalis, septum, amygdala, hippocampus, cingulate and entorhinal cortices). However, when analyzed in the light of the functional and neuroanatomical evidence which links limbic, pontine and medullary regions into a unified system involved in behavioral and physiological adaptation to environmental stressors [64,89], these data may not only help in understanding clonidine's pharmacologic profile but also suggest that information processed through α_2-adrenergic receptors plays a critical neurochemical role within this functional neuroanatomic network.

THE α_1-ADRENERGIC RECEPTOR: POSSIBLE HETEROGENEITY IN THE CNS?

[^{3}H]Prazosin ([^{3}H]PRZ) has been extensively utilized to label α_1-adrenergic binding sites in homogenate binding assays [4, 31, 36, 56, 59, 97, 103] and autoradiographic localization experiments [3, 18, 19, 77, 114]. Previously, the antagonist [^{3}H]WB4101 ([^{3}H]WB) had been used as the ligand of choice for labeling α_1 binding sites [115] and had been used by this lab to map α_1-receptors in the rat central nervous system [120,121]. However, several subsequent homogenate binding assays demonstrated a lack of specificity of [^{3}H]WB [33, 49, 80]. A similar lack of receptor specificity was found in some physiologic preparations [50] but not in others [23].

Our initial autoradiographic studies focused on delineating these differences in the CNS [114]. Findings similar to those seen in peripheral physiologic experiments were observed in the CNS. For example, marked differences were seen in the distribution of [^{3}H]PRZ and [^{3}H]WB binding sites in regions such as the neocortex, hippocampus, dentate gyrus and thalamus (Fig. 3) while identical binding site dis-

FACING PAGE

FIG. 2. Distribution of [^{3}H]PAC binding sites in the rat brain. The images are taken from gray-scale computer digitized images produced from [^{3}H]Ultrofilm using the LAI Image Analysis System. Thus, dark areas represent regions of high binding site density. In (A), the medullary and pontine catecholamine projections to the forebrain are schematized. In (B), the descending pathways from the forebrain and pons to the cardiovascular areas of the medulla are schematized. See the discussion for details and references. These data indicate that α_2-adrenergic binding sites are localized in anatomical regions that have been characterized as mediating the autonomic, sensory and behavioral functions affected by clonidine-like drugs and that α_2-receptors may form a vital neurochemical link in the integration of autonomic, viscerosensory and affective information. Abbreviations: A1—A1 noradrenergic cell group of the ventrolateral medulla; ac—central nucleus of the amygdala; arh—arcuate nucleus of the hypothalamus; bnst—bed nucleus of the stria terminalis; CA1—CA1 field of the hippocampus; dr—dorsal raphe nucleus; ent ctx—entorhinal cortex; ins ctx—insular cortex; lc—locus coeruleus; mnpo—medial nucleus of the preoptic; nco—nucleus commissuralis; pag—periaqueductal gray; pbn—dorsal parabrachial nucleus; pvh—paraventricular nucleus of the hypothalamus; pvt—periventricular nucleus of the thalamus; sl—lateral septum.

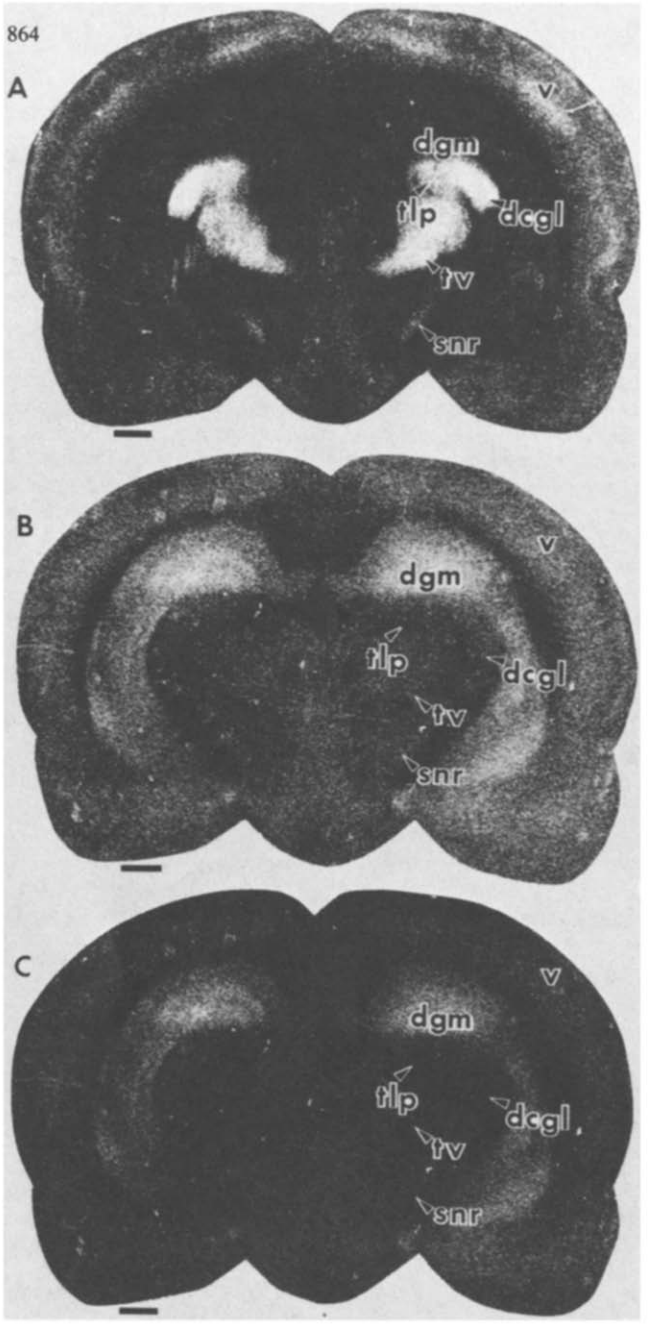


FIG. 3. Distribution of (A) [^{3}H]PRZ binding sites, (B) [^{3}H]WB binding sites and (C) [^{3}H]WB binding sites in the presence of 10 μM phentolamine. The pictures are darkfield photomicrographs taken directly from [^{3}H]Ultrofilm; thus, bright areas represent regions of high binding site density. Serial sections were labeled with 0.5 nM [^{3}H]PRZ or [^{3}H]WB in an incubation buffer consisting of 0.17 M Tris·HCl containing 10 mM $MgCl_2$ (pH 7.6 at 25°C). Under these conditions, given the K_d's obtained for these two buffers in Tris buffer (approximately 0.45 nM), approximately 50% of each ligand's binding sites should have been labeled. Note the difference in the distribution of binding sites seen with these two ligands. High levels of [^{3}H]PRZ binding sites were seen in lamina V of the cortex and thalamic nuclei. High levels of [^{3}H]WB binding sites were seen in the hippocampus and dentate gyrus. [^{3}H]WB binding sites in the hippocampus and dentate gyrus were not completely displaced by phentolamine. Also, note the difference in [^{3}H]WB binding in the cortex and thalamic nuclei between what is shown here and in Fig. 9. Abbreviations: dcgl—dorsolateral geniculate nucleus; dgm—molecular layer of the dentate gyrus; snr—substantia nigra, pars reticulata; tlp—posterolateral nucleus of the thalamus; tv—ventral nucleus of the thalamus; v—lamina V of the cortex. Bar=1000 μm.

tributions were seen in other regions, such as the dorsal raphe nucleus (Fig. 4). Further, in some regions, such as CA1 of the hippocampus and the molecular layer of the dentate gyrus, [^{3}H]WB binding was not completely displaced by even 10 μM concentrations of the general α-antagonist phentolamine (Fig. 3). Also, when characterizing the binding of [^{3}H]PRZ and [^{3}H]WB in intact tissue sections, marked differences were observed in the pharmacologic profiles of these two ligands (Fig. 5). In order to further characterize these differences, parallel binding assays were performed in hippocampal and thalamic homogenates using these two ligands. These results were applied to the re-evaluation of the autoradiographic profiles of [^{3}H]PRZ and [^{3}H]WB binding in intact tissue sections.

METHOD

Tissue Preparation for Homogenate Binding Assays

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI), 175–225 g body weight, were used in all experiments. The animals were sacrificed by decaptiation, the brains rapidly removed and the hippocampi and thalamus were dissected out in ice-cold phosphate buffered saline. The tissues were homogenized (Brinkman Polytron, setting 6) for 10 sec in 20 vol of incubation buffer (50 mM Na^+-K^+-phosphate buffer, pH 7.4 at 25°C) and centrifuged at 49,000 $\times$ g for 10 min at 4°C. The crude pellet was resuspended in 50 vol of incubation buffer and spun down. The pellets were resuspended in 200 vol (hippocampal membranes) or 400 vol (thalamic membranes) of incubation buffer for the assay.

[^{3}H]PRZ and [^{3}H]WB Binding Assays

Assays were initiated by adding 1.0 ml of tissue to test tubes containing the incubation buffer and appropriate concentrations of radioactivity and displacing drug. In a final volume of 5.0 ml, the final concentration of hippocampal membranes was 1.0 mg wet weight/ml, while the final concentration of thalamic membranes was 0.5 mg wet weight/ml. For saturation experiments, ligand concentrations ([^{3}H]PRZ, 17.4 Ci/mmol; [^{3}H]WB, 27 Ci/mmol, New England Nuclear) were varied from 5.0–1000 pM. For competition and kinetic determinations, ligand concentrations were held constant at 200 pM. Non-specific binding was determined in the presence of 10 μM phentolamine. Phentolamine displaced approximately 75% of the total binding of both ligands with appropriate asymptotes between 1.0 and 10 μM and IC_{50} values of approximately 30 nM.

The phosphate-based buffer system was used in these experiments since preliminary experiments revealed a 5–10 fold increase in the affinity of these compounds when using the phosphate buffer as opposed to a Trizma-based buffer. These effects were independent of the cation used or the tonicity of the media. The effect of Tris on both [^{3}H]PRZ and [^{3}H]WB binding appeared to be competitive since parallel saturation experiments using either a Tris- or a phosphate-based buffer revealed significant changes in the K_D's of both ligands with no change in the maximal number of binding site labeled (data not shown).

Membranes were incubated for 60 min at 25°C. The assay was terminated by rapid filtration over glass fiber filters (Whatman GF/B) followed by 3$\times$5 ml washes with cold buffer. The filters were deposited in scintillation vials, solubilized with Formula 963 (New England Nuclear) and counted for 10 min at 45–51% counting efficiency using standard liquid scintillation counting techniques.

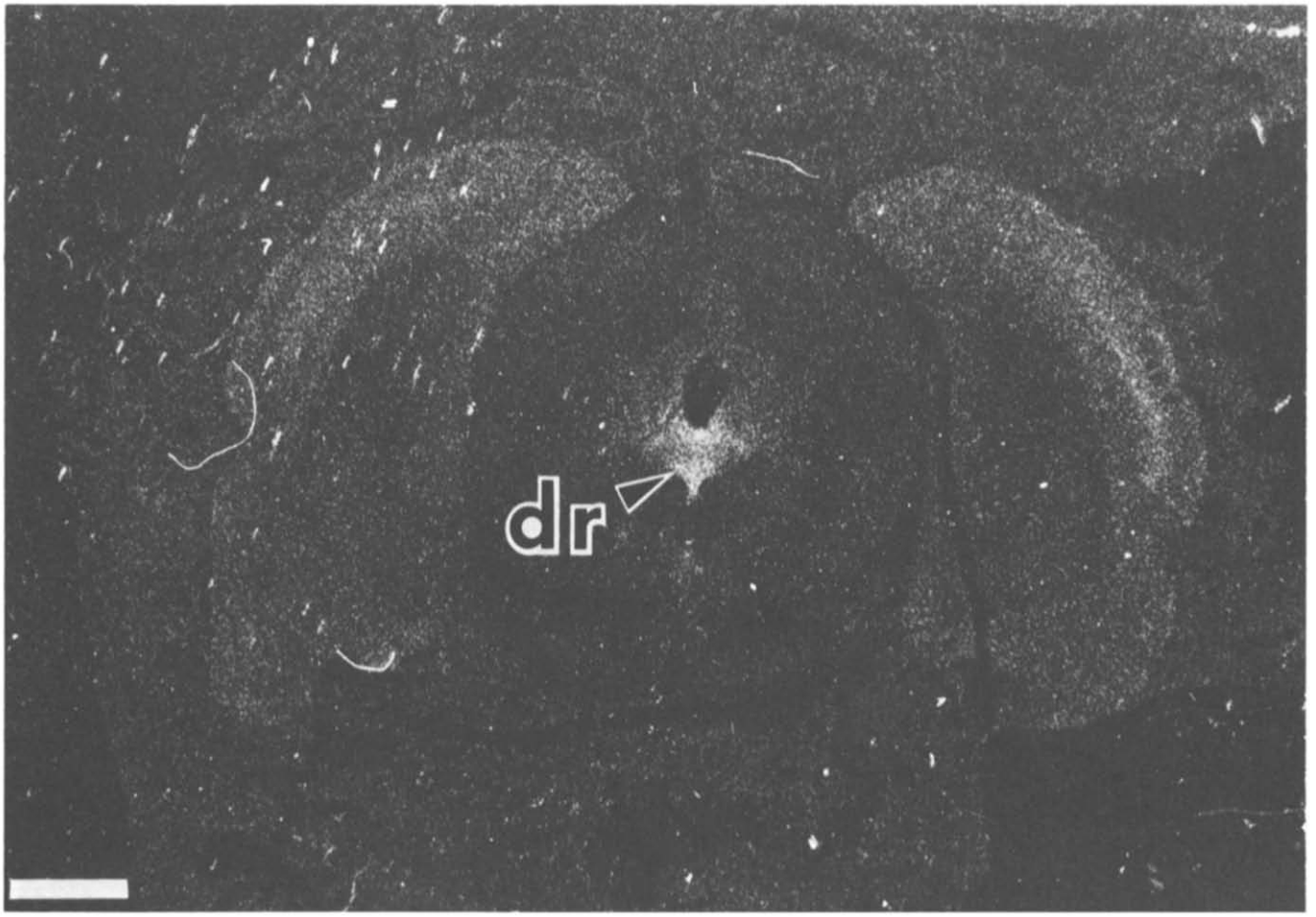


FIG. 4. [^{3}H]PRZ binding sites in the midbrain. The photograph is a darkfield photomicrograph taken directly from [^{3}H]Ultrofilm; thus, bright areas represent regions of high binding site density. Note the high density of binding sites in the dorsal raphe (dr) and in the floor of the cerebral aqueduct. A similar distribution was seen with [^{3}H]WB. This distribution was independent of the conditions used to label the binding sites. The distribution of binding sites seen in the thalamus (Fig. 3) and the dorsal raphe correlate well with pharmacologic and physiologic data obtained in these regions (see conclusions for references). Bar=1000 μm.

Data Analysis

Saturation and competition data were analyzed using the BASIC program EBDA [55] which provides initial parameter estimates and formats the data for analysis by the program SCAFIT [63]. Replicate experiments were analyzed simultaneously using SCAFIT. Non-specific and between experiment correction parameters were allowed to vary.

Autoradiographic Experiments

Coronal tissue sections through the rat forebrain were prepared as described in the section on [^{3}H]PAC binding. Serial sections were incubated for 60 min at room temperature in a modified Krebs phosphate buffer (pH 7.4 at 25°C, described in the previous section) containing 2.0 nM of either [^{3}H]PRZ or [^{3}H]WB and appropriate concentrations of the various displacers (30 nM unlabeled prazosin against [^{3}H]WB binding; 10 nM unlabeled WB4101 against [^{3}H]PRZ binding). Non-specific binding was determined in the presence of 10 μM phentolamine. Under these conditions, based on the parameter estimates obtained from the homogenate binding assays for the high-affinity components of each ligand and displacer (see below), both [^{3}H]PRZ and [^{3}H]WB will label >97% of their respective binding sites, WB4101 will displace 89.8% of [^{3}H]PRZ binding and prazosin will displace 89.2% of [^{3}H]WB binding (assuming a single class of high-affinity sites (see [30] for the equations used to calculate receptor occupancies). The parameter estimates obtained in homogenate binding assays were confirmed in tissue sections using the Krebs phosphate buffer prior to performing the autoradiographic analysis (data not shown).

Following the incubation, the labeled sections were given 2×5 min washes in cold buffer in order to reduce the non-specific binding. The sections were dipped in cold water, dried and prepared for the exposure of [^{3}H]Ultrofilm as previously described. Exposure time was 10 to 12 weeks. Autoradiographic images were analyzed using the LAI Image Analysis System. Optical densities were converted to relative dpm's based on standards exposed along with the experimental tissues in order to accurately compare the relative displacements by the various compounds within the brain regions being studied.

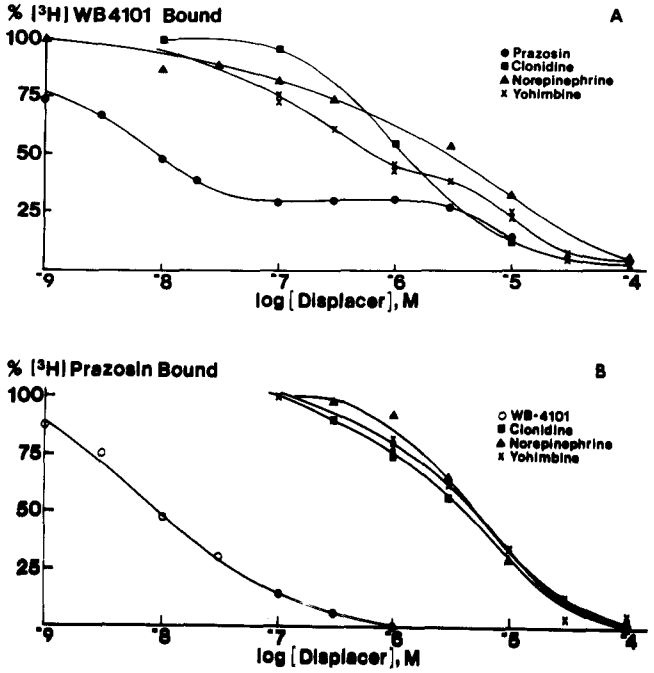


FIG. 5. Displacement of (A) [^{3}H]WB binding and (B) [^{3}H]PRZ binding in intact tissue sections through the medial thalamus of the rat. Sections were incubated with 0.5 nM of each ligand in the presence of varying concentrations of the displacing drugs (Tris buffer). After the final wash, sections were wiped from the slides and counted using standard scintillation counting techniques. Note the distinctly different pharmacological profiles of [^{3}H]WB and [^{3}H]PRZ, particularly the shallow displacements of [^{3}H]WB binding by the α_1-antagonist prazosin and the α_2-antagonist yohimbine. Similar profiles were obtained in the phosphate buffered system; however, drug potencies were shifted left by approximately an order of magnitude.

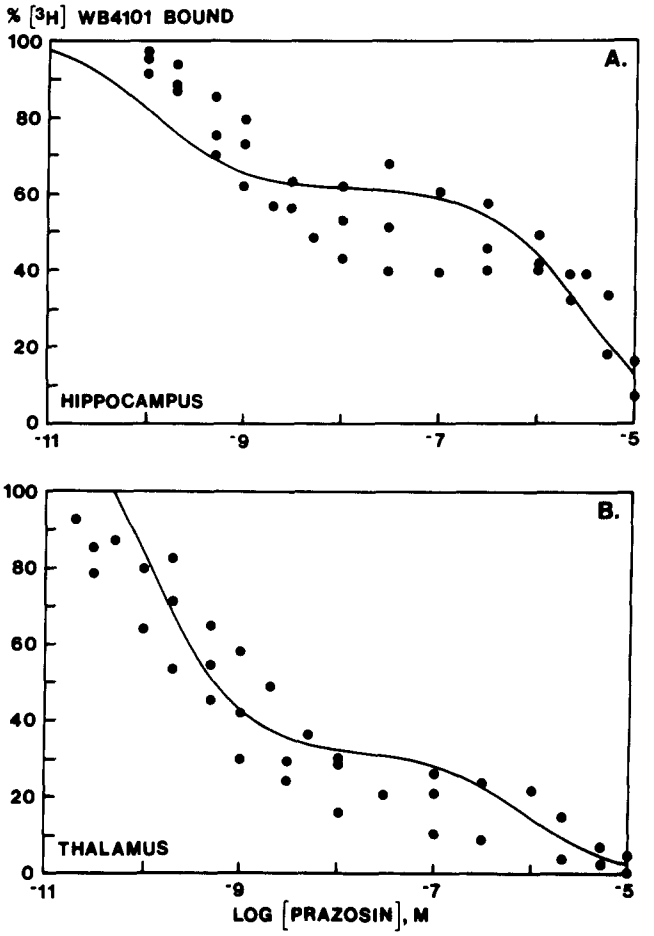


FIG. 6. Displacement of [^{3}H]WB binding by unlabeled prazosin in hippocampal and thalamic membranes. Membranes were labeled with 0.2 nM [^{3}H]WB in a 50 mM $Na^+ \cdot K^+ \cdot$phosphate buffer (pH 7.4 at 25°C). Prazosin displaced approximately 40% of the [^{3}H]WB bound in the hippocampus with high affinity and displaced approximately 75% of the [^{3}H]WB bound in the thalamus. Parameter estimates for the high and low affinity components of the prazosin displacements are given in Table 2. The points shown represent the data derived from three replicate experiments performed in parallel. The regression lines shown were derived from the parameter estimates obtained using the program SCAFIT which assumes independence of multiple binding sites [63]. While these data may indicate heterogeneity of [^{3}H]WB binding sites in these two reasons, the shape of the fits of the data could also suggest cooperativity. See text for details.

RESULTS

Saturations Experiments

[^{3}H]PRZ and [^{3}H]WB labeled sites in hippocampal and thalamic membranes with an extremely high affinity under the conditions utilized in these experiments (Table 1). The binding affinities for [^{3}H]PRZ and [^{3}H]WB were equivalent in the two regions analyzed. [^{3}H]PRZ labeled more than twice the number of binding sites in the thalamus as opposed to the hippocampus. On the other hand, [^{3}H]WB labeled approximately an equal number of binding sites in the two regions. In the hippocampus, an additional low-affinity/high capacity binding site was seen with both ligands. However, analysis using the program SCAFIT recognized this low-affinity site as non-specific binding since a one-site model provided the best fit of the data.

Competition Experiments

Displacements of [^{3}H]WB binding by unlabeled prazosin were biphasic with K_I's of 131 pM and 76.1 nM for the high- and low-affinity components respectively in the thalamus and 115 pM and 874 nM respectively in the hippocampus (Fig. 6 and Table 2). Prazosin displaced 75% of the [^{3}H]WB binding sites in the thalamus with high-affinity while displacing only 40% of the binding in the hippocampus. The K_I's for the high-affinity component of the prazosin displacements were 5 to 6 times higher than their respective K_D's in these regions.

WB4101 displacement of [^{3}H]PRZ binding also differed in the two regions studied (Table 2). In the thalamus, WB4101 displaced [^{3}H]PRZ binding with a K_I of 352 pM and a Hill co-efficient close to unity. The affinity of unlabeled WB4101 in displacing [^{3}H]PRZ was 5 times less than its K_D using the labeled compound in similar preparations. On the other hand, WB4101 displacement of [^{3}H]PRZ in the hippocampus was biphasic, with K_I's of 13.1 and 874 pM for the high- and low-affinity components respectively. Thus, when comparing the high-affinity components of WB4101 displacements

TABLE 1

[^{3}H]PRAZOSIN AND [^{3}H]WB4101 SATURATION PARAMETERS IN THALAMIC AND HIPPOCAMPAL MEMBRANES (MEAN ± SEM)

[^{3}H]PRAZOSIN	[^{3}H]WB4101
Thalamus	
K_D= 25.4 (23.1– 28.1) pM B_{max}=246.0 (234.0–258.0) fmol/mg protein	K_D= 74.4 (68.7– 96.2) pM B_{max}=180.6 (157.0–164.4) fmol/mg protein
Hippocampus	
K_D= 18.5 (16.4– 28.1) pM B_{max}=112.2 (106.2–118.2) fmol/mg protein	K_D= 59.9 (50.8– 73.1) pM B_{max}150.0 (135.6–164.4) fmol/mg protein

Incubations were performed in a final volume of 5.0 ml with a tissue concentration of 5.0 mg wet weight/ml for hippocampal membranes and a tissue concentration of 2.5 mg wet weight/ml for thalamic membranes. Parameter estimates were obtained using the program SCAFIT [63]. The data represents the compiled results of three experiments for each ligand/brain region.

TABLE 2

SUMMARY OF COMPETITION PARAMETERS FOR UNLABELED PRAZOSIN AND RS-WB4101 DISPLACING THE BINDING OF LABELED LIGAND IN THALAMIC AND HIPPOCAMPAL MEMBRANES

Thalamus	Hippocampus
Prazosin vs. [^{3}H]WB4101	
K_{A1}= 7.66 ± 1.34 × 10^9 M^{-1} (K_I=131 pM) % B_{max}=74.6	K_{A1}= 8.70 ± 4.26 × 10^9 M^{-1} (K_I=115 pM) %B_{max}=38.4
K_{A2}= 1.31 ± 0.86 × 10^6 M^{-1} (K_I= 76.3 nM) %B_{max}= 25.4	K_{A2}= 4.06 ± 3.07 × 10^5 M^{-1} (K_I=247 nM) %B_{max}=61.6
RS-W4101 vs. [^{3}H]Prazosin	
K_A= 2.84 ± 0.87 × 10^9 M^{-1} (K_I=352 pM) %B_{max}=100	K_{A1}= 7.58 ± 2.53 × 10^{10} M^{-1} (K_I= 13.1 pM) %B_{max}=75.7 K_{A2}= 1.14 ± 0.84 × 10^9 M^{-1} %B_{max}=24.3

Binding sites were labeled with 0.2 nM of [^{3}H]WB4101 or [^{3}H]prazosin. The K_A's represent the affinity constants for the unlabeled ligands while the % B_{max} represents the percentage of labeled binding sites displaced by the unlabeled drug with a given affinity. Parameter estimates were obtained from three experiments using the program SCAFIT [63]. Parameter estimates for the labeled ligands were obtained from the data shown in Table 1.

of [^{3}H]PRZ binding, WB4101 was over 20 times more potent in the hippocampus than in the thalamus.

WB4101 is a racemic mixture of two stereoisomers, and differences in the relative potencies of these two isomers have been noted in various peripheral tissues [26, 62, 87]. In order to determine whether the differences seen in the WB4101 displacements of [^{3}H]PRZ binding in the thalamus and hippocampus could be attributed to a similar phenomenon, the potencies of the S- and R-isomers of WB4101 were determined in the two tissues. The results are shown in Fig. 7. In both preparations, the S-isomer was more potent that the R-isomer as predicted by physiological studies [26, 62, 87]. However, both isomers were more potent in the hippocampus than in the thalamus. Based on K_I approximations derived from analysis using the program EBDA, the R-/S-potency ratio in the hippocampus was 62.6 (R-WB4101, K_I=1.09 nM; S-WB4101, K_I=17.4 pM). In the thalamus, on the other hand, the R-/S-potency ratio was 17.2 (R-WB4101, K_I=5.5 nM; S-WB4101, K_I=320 pM). S-WB4101 was 18.4 times more potent in the hippocampus than in the thalamus; R-WB4101 was only 5.0 times more potent in the hippocampus than in the thalamus.

Given the complexities seen in the interactions between prazosin and WB4101 revealed by these studies, it appeared that the α_1-receptor in these two brain regions may be biochemically different. In order to distinguish this possibility from the possibility of interacting binding sites, analysis of the dissociation of [^{3}H]PRZ and [^{3}H]WB from their binding sites in the presence of excess (10 μM) unlabeled prazosin or WB4101 was undertaken. The necessity for this analysis was particularly underscored by inspection of the prazosin displacement curves of [^{3}H]WB binding as fit by the program SCAFIT. While the curves that were obtained using a two-site model provided a statistically appropriate fit of the data (mean square=43.9, F(29,31)=47.89, $p<0.001$), significant runs were obtained and the plotted regression line visually appeared inappropriate (Fig. 6). Since SCAFIT assumes a model where binding sites are independent [63], it was necessary to test the possibility of cooperative binding sites.

No difference was seen in the rate of dissociation of [^{3}H]PRZ in either region in the presence of excess prazosin or WB4101 (K_{-1}=0.0123±0.0006 min^{-1}). Similarly, no difference was seen in the rate of dissociation of [^{3}H]WB in the thalamus in the presence of either prazosin or WB4101 (K^{-1}=0.030±0.004 min^{-1}, Fig. 8A). On the other hand, in the hippocampus, the rate of dissociation of [^{3}H]WB in the presence WB4101 or prazosin was dramatically different (+WB4101, K_{-1}=0.128 min^{-1}; +prazosin, K_{-1}=0.014 min^{-1}, Fig. 8B).

Based on the parameter estimates obtained in the homogenate binding assays, autoradiographic experiments were performed using the modified Krebs phosphate buffer and saturating concentrations of [^{3}H]PRZ and [^{3}H]WB in coronal sections through the medial thalamus of the rat. An example of the results that were obtained is shown in Fig. 9. Under these conditions, the distribution of [^{3}H]PRZ and [^{3}H]WB binding sites in the cortex, thalamus, amygdala and hypothalamus were similar. However, [^{3}H]WB labeled relatively more sites in the medial amygdala and ventromedial hypothalamus than [^{3}H]PRZ, and [^{3}H]WB binding in the hippocampus and dentate gyrus differed noticeably from [^{3}H]PRZ binding, as had been seen in the earlier experiments. As predicted, WB4101 was much less potent in displacing [^{3}H]PRZ binding in the thalamus and cortex than in the hippocampus, amygdala and hypothalamus. Similarly, prazosin significantly displaced [^{3}H]WB binding from the thalamus and neocortex, while it was less efficacious in displacing [^{3}H]WB binding in the limbic cortex, amygdala and hypothalamus. Further, as predicted from the kinetic experiments, prazosin actually increase the binding of [^{3}H]WB in the hippocampus and dentate gyrus by approximately 12–16% based on the increase in relative dpm's derived from standardized optical density data. Phentolamine uniformly displaced [^{3}H]PRZ binding in all regions analyzed. On the

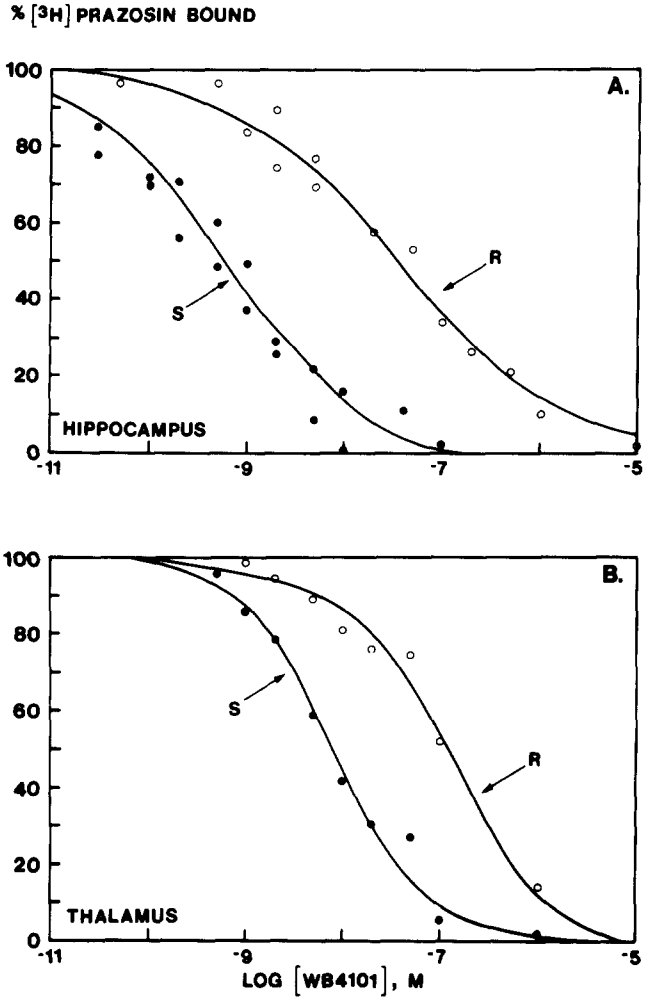


FIG. 7. Displacement of [^{3}H]PRZ binding in hippocampal and thalamic membranes by the S- and R-isomers of WB4101. Membranes were labeled with 0.2 nM [^{3}H]PRZ in phosphate buffer. In both preparations, the S-isomer was more potent than the R-isomer. However, both isomers were more potent in the hippocampal as opposed to the thalamic preparation. Based on K_I approximations derived from analysis using the program EBDA [55], the R-/S-potency ratio in the hippocampus was 62.6 (R-WB4101, K_I=1.09 nM; S-WB4101, K_I=17.4 pM). In the thalamus, on the other hand, the R-/S-potency ratio was 17.2 (R-WB4101, K_I5.5 nM; S-WB4101, K_I=320 pM). S-WB4101 was 18.4 times more potent in the hippocampus than in the thalamus; R-WB4101 was only 5.0 times more potent in the hippocampus than in the thalamus. The data shown is derived from a representative experiment. These results were replicated. See text for details.

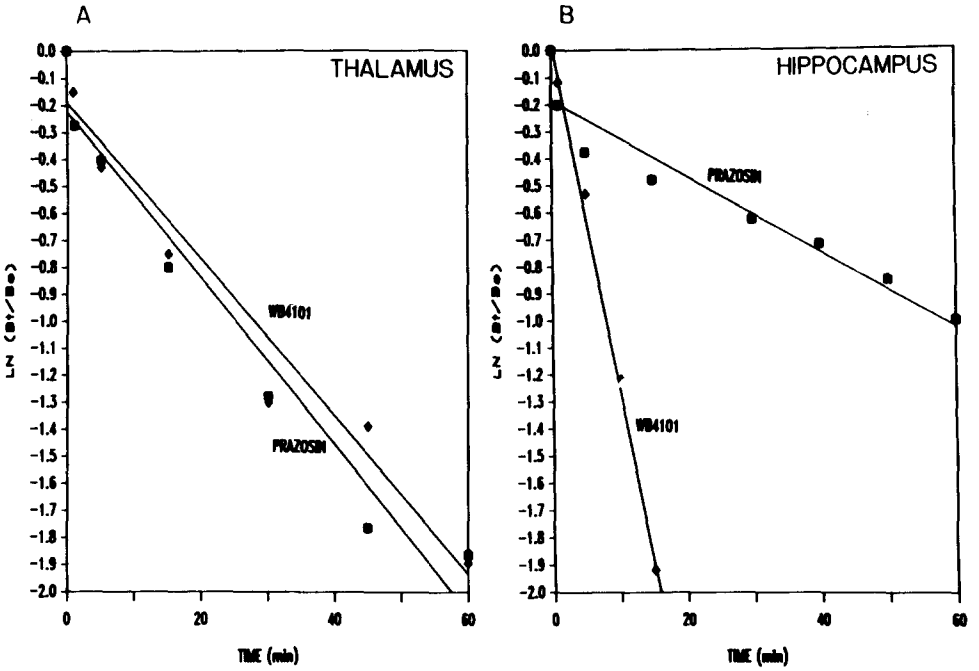


FIG. 8. Dissociation of [^{3}H]WB binding in thalamic and hippocampal membranes in the presence of excess prazosin or WB4101. Membranes were incubated to equilibrium with 0.2 nM [^{3}H]WB (60 min). At that time, 10 μM prazosin or WB4101 was added to the incubation. In the thalamus, no difference was seen in the rate of dissociation of [^{3}H]WB in the presence of excess prazosin or WB4101 (K_{-1}=0.03 min^{-1}). On the other hand, the rates of dissociation of [^{3}H]WB in the hippocampal preparation were distinctly different in the presence of excess WB4101 or prazosin (+WB4101, K_{-1}=0.128 min^{-1}; +prazosin, K_{-1}=0.014 min^{-1}). Both dissociation constants were also different from the constant obtained in the thalamic preparation. In parallel experiments in which the rate of dissociation of [^{3}H]PRZ was determined under the same conditions, no differences in the K_{-1}'s were seen. The data shown are from a representative experiment. These results, when coupled with the results obtained from the equilibrium binding assays, further support the hypothesis that the α_1-receptor complexes are different in these two brain regions. Further, at least in the hippocampus, these data indicate that the prazosin and WB4101 binding sites are cooperatively linked. See text for details.

other hand, even 10 μM phentolamine could not displace all of the [^{3}H]WB binding in areas such as the molecular layer of the dentate gyrus, CA1 area of the hippocampus and medial nucleus of the amygdala. These data are summarized in Table 3.

DISCUSSION

Certain reports have suggested that multiple α_1 receptor sites exist [16,52]. While it may be tempting to extrapolate this hypothesis to the data presented in this report, this concept could not explain the complexities observed in the interactions of prazosin and WB4101. Rather, these data suggest that [^{3}H]PRZ and [^{3}H]WB bind to two different sites that may be associated with an α_1-receptor/effector complex. This suggestion is supported by the binding data which indicates that the equilibrium kinetic and inhibition constants differ for these two compounds in the regions that were studied while the autoradiographic data revealed a similar distribution of binding sites in many of the regions that were looked at. This anomaly is especially apparent in the thalamus, where the distribution of [^{3}H]PRZ and [^{3}H]WB binding sites are identical while the K_D's and K_I's of these compounds are five- to six-fold different.

Other reports have suggested that the regional differences in the relative potencies of the stereoisomers of WB4101 represent a differential action at α_1- and α_2-receptors, with the lower potency of S-WB4101 representing an action at α_2-receptors [62,87]. This hypothesis is not supported by the present data. The autoradiographic data presented in this report demonstrates marked differences in the distribution of α_1 and α_2-binding sites in the regions that were studied. Further, in parallel experiments, the α_2-antagonist raûwolscine at a concentration of 100 μM displaced a significant amount of [^{3}H]WB in the thalamus (data not shown). This is just the opposite from what would be predicted based on the potencies of WB4101 in these regions. While [^{3}H]rauwolscine binding sites have a similar distribution to [^{3}H]WB sites in the hippocampus and dentate gyrus, this similarity may be more representative of the vagaries of [^{3}H]rauwolscine binding rather than binding to α_2-receptors (J. Unnerstall, manuscript in preparation).

Thus, a reasonable hypothesis could predict that prazosin and WB4101 act at different sites within an α_1-receptor/effector complex. Further, differences in the relative potencies and efficacies of these compounds in various regions suggest differences in the coupling efficiencies of these components and differences in the relative proportion

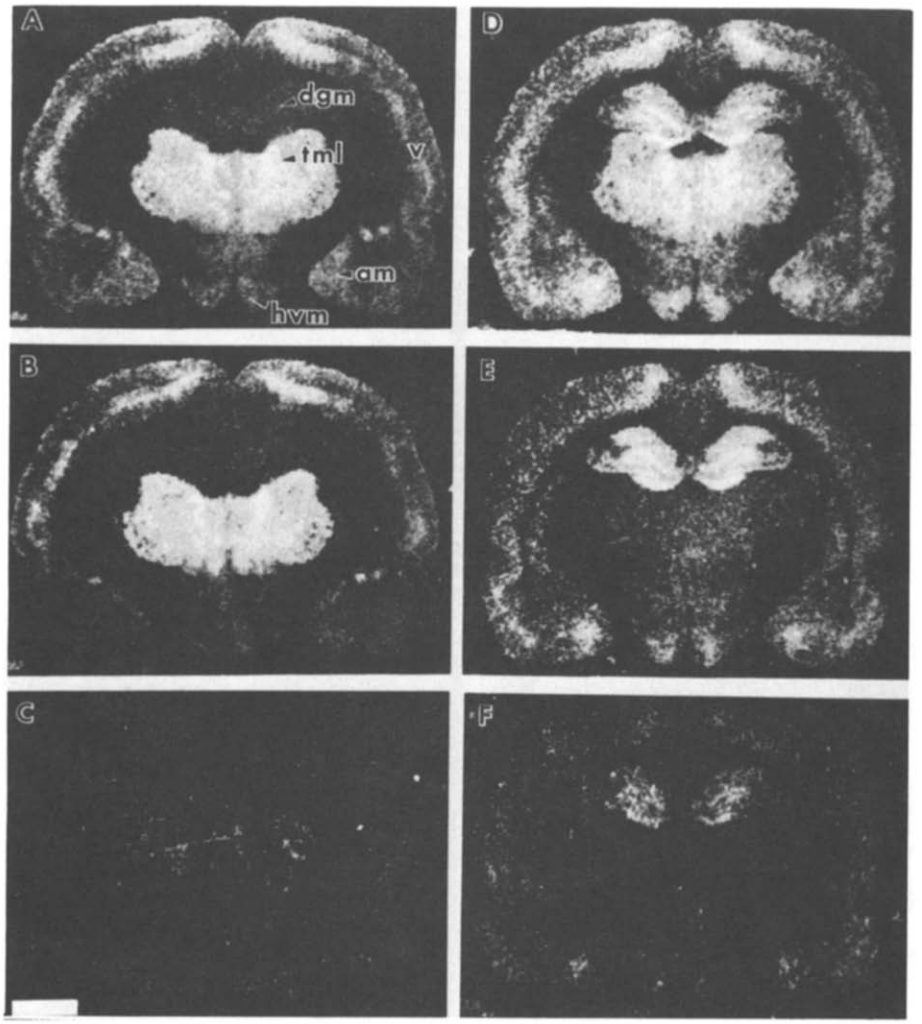


FIG. 9. Autoradiographic analysis of [^{3}H]PRZ (A, B, C) and [^{3}H]WB (D, E, F) binding sites in coronal sections through the medial thalamus of the rat. The autoradiograms shown are computer-digitized reverse gray scale (darkfield) images generated from images produced on [^{3}H]Ultrofilm. Thus, bright areas represent regions of high binding site density. The LAI Image Analysis System was utilized in the analysis of these images. A. Total (2.0 nM) [^{3}H]PRZ binding; B. 2.0 nM [^{3}H]PRZ in the presence of 10 nM unlabeled WB4101; C. 2.0 nM [^{3}H]PRZ in the presence of 10 μM phentolamine; D. Total (2.0 nM) [^{3}H]WB binding; D. 2.0 nM [^{3}H]WB in the presence of 30 nM prazosin; E. 2.0 nM [^{3}H]WB in the presence of 10 μM phentolamine. Assuming a single class of binding sites (based on the parameter estimates for the high-affinity components of prazosin and WB4101 binding obtained homogenate and tissue section assays) prazosin and WB4101 should have displaced nearly 90% of the total binding of the respective ligands, while phentolamine should have completely displaced [^{3}H]PRZ and [^{3}H]WB total binding. Based on these results, this clearly is not the case. These results indicate a marked regional heterogeneity of α_1-receptor complexes. Further, [^{3}H]WB may bind to a site not even associated with an α_1-receptor in regions such as the molecular layer of the dentate gyrus or the medial nucleus of the amygdala. See Table 3 for a summary of the regional differences in the displacements of [^{3}H]PRZ and [^{3}H]WB binding. Bar=2000 μm. Abbreviations: am—medial nucleus of the amygdala; dgm—molecular layer of the dentate gyrus; hvm—ventromedial nucleus of the hypothalamus; tml—mediolateral nucleus of the thalamus; v—lamina V of the cortex.

TABLE 3

AUTORADIOGRAPHIC ANALYSIS OF [^{3}H]PRAZOSIN AND [^{3}H]WB4101 DISPLACEMENTS IN SEVERAL REGIONS OF THE RAT CNS BY UNLABELED PRAZOSIN AND WB4101

[^{3}H]Prazosin		[^{3}H]WB4101	
Region	WB/PHENT × 100	Region	PRZ/PHENT × 100
	Low Displacement		
tvm	19.2	dgm	0
tl	21.1	CA1	0
tml	23.8		
	Moderate Displacements		
ctx	35.1	hd	31.8
tm	40.1	am	33.2
dgm	41.2	hvm	34.3
CA1	43.7	ctx	45.3
hd	52.7		
hvm	57.4		
	Large Displacements		
am	70.2	tm	73.7
		tl	74.8
		tvm	78.8
		tml	80.7

The numbers represent the percent of Phentolamine-displaceable [^{3}H]Prazosin binding displaced by 10 nM RS-WB4101 or [^{3}H]WB4101 binding displaced by 30 nM prazosin (n=3).

The concentrations of displacers used was based on the assumption of a single class of high-affinity binding sites for both ligands. Based on the parameter estimates given in Tables 1 and 2, approximately 89.8% of the [^{3}H]PRZ binding and 89.2% of the [^{3}H]WB binding should have been displaced under these conditions if the above assumption was true. Abbreviations: am—medial nucleus of the amygdala; CA1—CA1 field of the hippocampus; ctx—cingulate cortex; dgm—molecular layer of the dentate gyrus; hd—dorsomedial hypothalamus; hvm—ventromedial hypothalamus; tl—dorsolateral thalamus; tm—dorsomedial thalamus. tml—mediolateral thalamus; tvm—ventromedial thalamus. Anatomical regions were defined using the atlas of Paxinos and Watson [75]. Optical densities were calibrated by the use of standards exposed along with the experimental tissue and relative concentrations of radioactivity were regionally compared. The LAI Image Analysis System was used to analyze the data.

of these different complexes in the various regions. Since prazosin has been shown to be a potent antagonist of α_1-mediated adrenergic responses in both the CNS and the periphery [13,57, 58, 83], it could further be proposed that prazosin acts directly at the α_1-receptor. On the other hand, WB4101 might act at some more universal regulatory or effector component since [^{3}H]WB binding is not completely displaced by phentolamine in every region studied. This hypothesis would require further testing in physiological models and molecular characterizations of the α_1-adrenergic receptor. Thus, while radioligand binding and autoradiographic studies can provide important answers to questions concerning the functional and biochemical nature of the interactions of neurotransmitters and drugs at their effector sites, reasonable answers to molecular questions, such as those presented here, may only come about through the use of molecular probes such as monoclonal antibodies directed toward the binding site or receptor complex. The use of such probes is beginning to be explored with the α-adrenergic receptor [21].

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to Susan Goehring, Theresa Kopajtic and Naomi Taylor for their technical assistance, Roberta Proctor for her photographic and artistic assistance and Darlene Weimer, Mary Flutka and Sue Wrightson for their secretarial assistance. We would also like to thank Dr. James J. Valdes of the Department of Toxicology, Aberdeen Proving Grounds, Aberdeen, MD, for his assistance with some of the biochemical assays. Prazosin was a gift from Pfizer, Inc., Groton, CN. Phentolamine (Regitine-HCl_R) was a gift from Ciba-Geigy Corp., Summit, NJ. RS-WB4101 was obtained from Dr. Solomon H. Snyder (Department of Neuroscience, The Johns Hopkins University School of Medicine) while the stereoisomers of WB4101 were purified by and obtained from Dr. Wendell H. Nelson (Department of Medicinal Chemistry, University of Washington, Seattle, WA) through Dr. Robert R. Ruffolo, Jr. (Lilly Research Laboratories, Indianapolis, IN). Finally, we would like to thank Dr. Roger Summers (University of Melbourne, Australia) for his help and thoughtful ideas and Dr. Michael J. Kuhar, in whose laboratory this work was done, for his support, encouragement and advice. This work was supported by grants DA00266, MH00053 and a grant from the McKnight Foundation to M.J.K. and a National Science Foundation Predoctoral Fellowship to J.R.U.

REFERENCES

1. Aghajanian, G. K. Tolerance of locus coeruleus neurons to morphine and suppression of withdrawal response by clonidine. *Nature* **276**: 186–188, 1978.
2. Armstrong, D. M., C. A. Ross, T.-J. Joh, V. M. Pickel and D. J. Reis. Distribution of dopamine-, noradrenaline- and adrenaline-containing cell bodies in the rat medulla oblongata demonstrated by the immunocytochemical localization of catecholamine biosynthetic enzymes. *J Comp Neurol* **212**: 173–187, 1982.
3. Barnes, P. J., C. B. Basbaum, J. A. Nadel and J. M. Roberts. Pulmonary α-adrenoceptors: Autoradiographic localization using [^{3}H]prazosin. *Eur J Pharmacol* **88**: 57–62, 1983.
4. Battaglia, G., M. Shannon, B. Borgunvaad and M. Titler. Properties of [^{3}H]prazosin-labeled α_1-adrenergic receptors in rat brain and porcine neurointermediate lobe tissue. *J Neurochem* **41**: 538–54, 1983.
5. Bednarczyk, B. and J. Vetulani. Antagonism of clonidine to shaking behavior in morphine abstinence syndrome and to head twitches produced by serotonergic agents in the rat. *Pol J Pharmacol* **30**: 307, 1978.
6. Bentley, G. A., I. W. Copeland and J. Starr. The actions of some α-adrenoceptor agonists and antagonist in an antinociceptive test in mice. *Clin Exp Pharmacol Physiol* **4**: 405, 1977.
7. Bjorkland, A. and G. Skagsberg. Evidence for a major spinal cord projection from the diencephalic A11 dopamine cell group in the rat using transmitter-specific fluorescent retrograde tracing. *Brain Res* **177**: 170–175, 1979.
8. Blessing, W. W. and J. P. Chalmers. The projection of catecholamine (presumably dopamine)-containing neurons from hypothalamus to spinal cord. *Neurosci Lett* **11**: 35–40, 1979.

9. Blessing, W. W. and D. J. Reis. Inhibitory cardiovascular function of neurons in the caudal ventrolateral medulla of the rabbit: Relationship to the area containing noradrenergic neurons. *Brain Res* **253:** 161–171, 1982.
10. Blessing, W. W., J. B. Furness, M. Costa, M. J. West and J. P. Chalmers. Projection of ventrolateral medullary (A1) catecholamine neurons toward nucleus tractus solitarii. *Cell Tissue Res* **220:** 27–40, 1981.
11. Blessing, W. W., M. J. West and J. Chalmers. Hypertension, bradycardia and pulmonary edema in the conscious rabbit after brainstem lesions coinciding with the A1 group of catecholamine neurons. *Circ Res* **49:** 949–958, 1981.
12. Block, R., J. Feldman, P. Bousquet and J. C. Schwartz. Relationship between the ventromedullary clonidine-sensitive area and the posterior hypothalamus. *Eur J Pharmacol* **45:** 55–60, 1977.
13. Cavero, I. and A. G. Roach. The pharmacology of prazosin, a novel antihypertensive agent. *Life Sci* **27:** 1525–1540, 1980.
14. Cederbaum, J. M. and G. K. Aghajanian. Afferent projections to the rat locus coeruleus as determined by a retrograde-tracing technique. *J Comp Neurol* **178:** 1–16, 1978.
15. Chiba, T. and M. Kato. Synaptic structure and quantification of catecholaminergic axons in the nucleus tractus solitarius of the rat: Possible modulatory roles of catecholamines in baroreceptor reflexes. *Brain Res* **151:** 323–338, 1978.
16. Coates, J., U. Jahn and D. F. Weetman. The existence of a new subtype of α-adrenoceptor on the rat anocoecygeus is revealed by Sgd 101/75 and phenoxybenzamine. *Br J Pharmacol* **75:** 549–552, 1982.
17. Dahlstrom, A. and K. Fuxe. Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brainstem neurons. *Acta Physiol Scand* **62:** Suppl 232, 1–55, 1964.
18. Dashwood, M. R. Central and peripheral prazosin binding: An *in vitro* autoradiographic study in the rat. *Eur J Pharmacol* **86:** 51–58, 1983.
19. Dashwood, M. R. and J. Bagnall. An autoradiographic demonstration of prazosin binding to arterial vessels in the rat. *Eur J Pharmacol* **78:** 121–123, 1982.
20. Day, T. A., W. W. Blessing and J. D. Willoughby. Noradrenergic and dopaminergic projections to the medial preoptic area of the rat: A combined horseradish peroxidase/catecholamine fluorescence study. *Brain Res* **193:** 543–548, 1980.
21. Dausse, J.-P. and L. Diop. Monoclonal antibodies to rat brain α-adrenoceptors. *Eur J Pharmacol* **95:** 135–137, 1983.
22. Dennis, S. G., R. Melzack, S. Gutman and F. Boucher. Pain modulation by adrenergic agents and morphine as measured by three pain tests. *Life Sci* **26:** 1247–1259, 1980.
23. Drew, G. M. Evidence in favor of a selective α_1-adrenoceptor blocking action of WB4101 *in vivo*. *Naunyn Schmiedebergs Arch Pharmacol* **319:** 222–225, 1982.

23a Fallon, J. H., D. A. Koziell and R. Y. Moore. Catecholamine innervation of the basal forebrain. II. Amygdala, suprahinal cortex and entorhinal cortex. *J Comp Neurol* **180:** 509–532, 1978.

24. Fielding, S. J., M. Wilker, M. Hynes, M. Szewczak, W. Novick and H. Lal. A comparison of clonidine with morphine for antinociceptive and antiwithdrawal actions. *J Pharmacol Exp Ther* **207:** 899–905, 1978.
25. Franz, D. N., B. D. Hare and K. L. McCloskey. Spinal sympathetic neurons: Possible sites of opiate-withdrawal suppression by clonidine. *Science* **215:** 1643–1645, 1982.
26. Fuder, H., W. L. Nelson, D. D. Miller and P. N. Patil. Alpha adrenoreceptors of rabbit aorta and stomach fundus. *J Pharmacol Exp Ther* **217:** 1–9, 1981.
27. Gold, M. S., D. E. Redmond, Jr. and H. D. Kleber. Clonidine blocks acute opiate withdrawal symptoms. *Lancet* **2:** 599–601, 1978.
28. Gold, M. S., A. L. C. Potash, I. Extein and H. D. Kleber. Clonidine in acute opiate withdrawal. *N Engl J Med* **302:** 1421–1422, 1980.
29. Gold, M. S., A. C. Pottash, D. R. Sweeney and H. D. Kleber. Opiate withdrawal using clonidine: A safe, effective and rapid non-opiate treatment. *JAMA* **243:** 343–346, 1980.
30. Goodman, R. R., S. H. Snyder, M. J. Kuhar and W. S. Young, III. Differentiation of delta and mu opiate receptor localizations by light microscopic autoradiography. *Proc Natl Acad Sci USA* **77:** 6239–6243, 1980.
31. Greengrass, P. and R. Bremner. Binding characteristics of [^{3}H]prazosin to rat brain α-adrenergic receptors. *Eur J Pharmacol* **55:** 323–326, 1979.
32. Hoehn-Saric, R., A. F. Merchant, M. L. Keyser and V. K. Smith. Effects of clonidine on anxiety disorders. *Arch Gen Psychiatry* **38:** 1278–1282, 1981.
33. Hoffman, B. B. and R. J. Lefkowitz. [^{3}H]WB4101—Caution about its role as an alpha-adrenergic subtype selective radioligand. *Biochem Pharmacol* **29:** 1537–1541, 1980.
34. Hokfelt, T., K. Fuxe, M. Goldstein and O. Johansson. Immunohistochemical evidence for the existence of adrenaline neurons in the rat brain. *Brain Res* **66:** 235–251, 1974.
35. Hopkins, D. A. and G. Holstege. Amygdaloid projections to the mesencephalon, pons and medulla oblongata in the cat. *Exp Brain Res* **32:** 529–547, 1978.
36. Hornung, R., P. Presek and H. Glossman. Alpha adrenoceptors in rat brain: Direct identification with prazosin. *Naunyn Schmiedebergs Arch Pharmacol* **308:** 223–230, 1979.
37. Jacobowitz, D. M. and M. Palkovits. Topographic atlas of catecholamine and acetylcholinesterase-containing neurons in the rat brain. I. Forebrain (telencephalon, diencephalon). *J Comp Neurol* **157:** 13–28, 1974.
38. Jones, B. E. and R. Y. Moore. Ascending projections of the locus coeruleus in the rat. II. Autoradiographic study. *Brain Res* **127:** 23–53, 1977.
39. Ko, G. N., J. D. Elsworth, R. H. Roth, B. G. Rifkin, H. Leigh and D. E. Redmond, Jr. Panic-induced elevation of plasma MHPG levels in phobic-anxious patients. *Arch Gen Psychiatry* **40:** 425–430, 1983.
40. Krettek, J. E. and J. L. Price. Amygdaloid projections to subcortical structures within the basal forebrain and brainstem in the rat and cat. *J Comp Neurol* **178:** 225–254, 1978.
41. Kruse, H., R. W. Dunn, K. L. Theurer, W. J. Novick and G. T. Shearman. Attenuation of conflict-induced suppression by clonidine: Indication of anxiolytic activity. *Drug Dev Res* **1:** 137–143, 1981.
42. Leckman, J. F., J. W. Maas, D. E. Redmond, Jr. and G. R. Heringer. Effects of oral clonidine on plasma 3-methoxy-4-hydroxy-phenethyleneglycol (MHPG) in man: Preliminary report. *Life Sci* **26:** 2179–2185, 1980.
43. Levitt, P. and R. Y. Moore. Origin and organization of brainstem catecholamine innervation in the rat. *J Comp Neurol* **186:** 505–528, 1979.
44. Lindvall, O. and A. Bjorklund. The organization of the ascending catecholamine neuron systems in the rat brain as revealed by the glyoxylic acid fluorescence method. *Acta Physiol Scand* Suppl **412:** 1–48, 1974.
45. Lipman, J. J. and P. S. J. Spencer. Clonidine and opiate withdrawal. *Lancet* **9:** 521, 1978.
46. Loewy, A. D., S. McKeller and C. B. Saper. Direct projections from the A5 catecholamine cell group to the intermediolateral cell column. *Brain Res* **174:** 309–314, 1979.
47. Loewy, A. D., J. H. Wallach and S. McKeller. Efferent connections of the ventral medulla oblongata in the rat. *Brain Res Rev* **3:** 63–80, 1981.
48. Loizou, L. A. Projections of the nucleus locus coeruleus in the albino rat. *Brain Res* **15:** 563–566, 1969.
49. Lyon, T. F. and W. C. Randall. Multiple central WB4101 binding sites and the selectivity of prazosin. *Life Sci* **26:** 1121–1129, 1980.
50. Massingham, R., M. L. Dubocovich, N. B. Shepperson and S. Z. Langer. *In vivo* selectivity of prazosin but not of WB4101 for postsynaptic alpha-1 adrenoceptors. *J Pharmacol Exp Ther* **217:** 467–474, 1981.

51. McCleary, P. E. and D. J. Leander. Clonidine analgesia and suppression of operant responding: Dissociation of mechanism. *Eur J Pharmacol* **69:** 63–69, 1981.
52. McGrath, J. C. Evidence for more than one type of postjunctional alpha-adrenoceptor. *Biochem Pharmacol* **31:** 467–484, 1982.
53. McKellar, S. and A. D. Loewy. Efferent projections of the A1 catecholamine cell group in the rat: An autoradiographic study. *Brain Res* **241:** 11–29, 1982.
54. McNeill, T. H. and J. R. Sladek, Jr. Simultaneous monoamine histofluorescence and neuropeptide immunocytochemistry: II. Correlative distribution of catecholamine varicosities and magnocellular neurosecretory neurons in the rat supraoptic and paraventricular nuclei. *J Comp Neurol* **193:** 1023–1033, 1980.
55. McPherson, G. A. A practical computer-based approach to the analysis of radioligand binding experiments. *Comput Prog Biomed* **17:** 107–114, 1983.
56. McPherson, G. A. and R. J. Summers. [^{3}H]Clonidine binding and [^{3}H]prazosin binding to α-adrenoceptors from regions of the rat kidney. *J Pharm Pharmacol* **33:** 189–191, 1981.
57. Menkes, D. B. and G. K. Aghajanian. α_1-Adrenoceptor-mediated responses in the lateral geniculate nucleus are enhanced by chronic antidepressant treatment. *Eur J Pharmacol* **74:** 27–35, 1981.
58. Menkes, D. B., J. M. Baraban and G. K. Aghajanian. Prazosin selectively antagonizes neuronal responses mediated by α_1-adrenoceptors in brain. *Naunyn Schmiedebergs Arch Pharmacol* **317:** 273–275, 1981.
59. Miach, P. J., J.-P. Dausse, A. Cardot and P. Meyer. [^{3}H]Prazosin binds specifically to 'α_1'-adrenoceptors in rat brain. *Naunyn Schmiedebergs Arch Pharmacol* **312:** 23–26, 1980.
60. Moore, R. Y. Catecholamine innervation of the basal forebrain. I. The septal area. *J Comp Neurol* **177:** 665–684, 1978.
61. Moore, S. D. and P. G. Guyenet. An electrophysiological study of the forebrain projection of nucleus commissuralis: Preliminary identification of presumed A2 catecholaminergic neurons. *Brain Res* **263:** 211–222, 1983.
62. Mottram, D. R. Differential blockade of pre- and postsynaptic α-adrenoceptors by the 2-R and 2-S enantiomers of WB4101. *J Pharm Pharmacol* **31:** 767–771, 1981.
63. Munson, P. J. and D. Rodbard. A versatile computerized approach for the characterization of ligand binding systems. *Anal Biochem* **197:** 220–239, 1980.
64. Nauta, W. J. H. and V. B. Domesick. Neural associations of the limbic system. In: *The Neural Basis of Behavior*, edited by A. L. Beckman. New York: Spectrum Publications, 1982, pp. 175–206.
65. O'Donohue, T. L. and D. M. Dorsa. The opiomelanotropinergic neuronal and endocrine systems. *Peptides* **3:** 353–395, 1982.
66. Olschowka, J. A., T. L. O'Donohue, G. P. Mueller and D. M. Jacobowitz. The distribution of corticotropin releasing factor-like immunoreactive neurons in rat brain. *Peptides* **3:** 995–1015, 1982.
67. Otterson, O. P. Afferent connections to the amygdaloid complex of the rat with some observations in the cat. III. Afferents from the lower brain stem. *J Comp Neurol* **202:** 335–356, 1981.
68. Paalzow, L. Analgesia produced by clonidine in mice and rats. *J Pharm Pharmacol* **26:** 361–363, 1974.
69. Paalzow, G. Development of tolerance to the analgesic effect of clonidine in rats: Cross-tolerance to morphine. *Naunyn Schmiedebergs Arch Pharmacol* **304:** 1–4, 1978.
70. Paalzow, G. and L. Paalzow. Clonidine antinonciceptive activity: Effects of drugs influencing central monoaminergic and cholinergic mechanisms in the rat. *Naunyn Schmiedebergs Arch Pharmacol* **292:** 119–126, 1976.
71. Paalzow, G. and L. Paalzow. Separate noradrenergic receptors could mediate clonidine-induced antinociception. *J Pharmacol Exp Ther* **223:** 795–800, 1992.
72. Palkovits, M. and D. M. Jacobowitz. Topographic atlas of catecholamine and acetylcholinesterase-containing neurons in rat brain. II. Hindbrain (mesencephalon, rhombencephalon). *J Comp Neurol* **157:** 29–42, 1974.
73. Palkovits, M., E. Mezey and L. Zaborszky. Neuroanatomical evidences for direct neural connections between the brainstem barareceptor centers and the forebrain areas involved in the neural regulation of the blood pressure. In: *Nervous System and Hypertension*, edited by P. Meyer and H. Schmitt. New York: Wiley, 1979, pp. 18–30.
74. Palkovits, M. and L. Zaborszky. Neuroanatomy of central cardiovascular control. Nucleus tractus solitarii: Afferent and efferent neuronal connections in relation to the baroreceptor reflex arc. *Prog Brain Res* **47:** 9–34, 1977.
75. Paxinos, G. and C. Watson. *The Rat Brain in Stereotaxic Coordinates*. New York: Academic Press, 1982.
76. Pickel, V. M., M. Segal and F. E. Bloom. A radioautographic study of the efferent pathways of the nucleus locus coeruleus. *J Comp Neurol* **155:** 15–42, 1974.
77. Rainbow, T. C. and A. Biegon. Quantitative autoradiography of [^{3}H]prazosin binding sites in rat forebrain. *Eur J Pharmacol*, in press, 1984.
78. Reddy, S. V. R., J. L. Maderdrut and T. L. Yaksh. Spinal cord pharmacology of adrenergic agonist-mediated antinociception. *J Pharmacol Exp Ther* **213:** 525–533, 1980.
79. Redmond, D. E., Jr. and Y. H. Huang. New evidence for a locus coeruleus-norepinephrine connection with anxiety. *Life Sci* **25:** 2149–2162, 1979.
80. Rehavi, M., B. Yavetz, O. Ramot and M. Sokolovsky. Regional heterogeneity of two high affinity binding sites for [^{3}H]WB-4101 in mouse brain. *Life Sci* **26:** 615–621, 1980.
81. Ricardo, J. A. and E. T. Koh. Anatomical evidence of direct projections from the nucleus of the solitary tract to the hypothalamus, amygdala and other forebrain structures in the rat. *Brain Res* **153:** 1–26, 1978.
82. Ritchie, T. C., K. N. Westlund, R. M. Bowker, J. D. Coulter and R. B. Leonard. The relationship of the medullary catecholamine containing neurons to the vagal motor nuclei. *Neuroscience* **7:** 1471–1482, 1982.
83. Rogawski, M. A. and G. K. Aghajanian. Activation of lateral geniculate neurons by norepinephrine: Mediation by an α-adrenergic receptor. *Brain Res* **182:** 345–359, 1980.
84. Ross, C. A., D. M. Armstrong, D. A. Ruggiero, V. M. Pickel, T.-H. Joh and D. J. Reis. Adrenaline neurons in the rostral ventrolateral medulla innervate thoracic spinal cord: A combined immunocytochemical and retrograde transport demonstration. *Neurosci Lett* **25:** 257–262, 1981.
85. Ross, C. A., D. A. Ruggiero, T.-H. Joh, D. H. Park and D. J. Reis. Adrenaline synthesizing neurons in the rostral ventrolateral medulla: A possible role in tonic vasomotor control. *Brain Res* **273:** 356–361, 1983.
86. Ross, C. A., D. A. Ruggiero and D. J. Reis. Afferent projections to cardiovascular portions of the nucleus tractus solitarius in the rat. *Brain Res* **223:** 402–408, 1981.
87. Ruffolo, R. R., Jr., W. L. Nelson and E. L. Yaden. Blockade of postjunctional vascular alpha 1- and alpha 2-adrenoceptors in pithed rat by the enantiomers of WB4101. *Naunyn Schmiedebergs Arch Pharmacol* **322:** 93–97, 1983.
88. Saavedra, J. M., M. Palkovits, M. J. Brownstein and J. Axelrod. Localization of phenylethanolamine-N-methyltransferase in the rat brain nuclei. *Nature* **248:** 695–696, 1974.
89. Saper, C. B. Anatomical substrates for the hypothalamic control of the autonomic nervous system. In: *Integrative Functions of the Autonomic Nervous System*, edited by C. M. Brooks, K. Koizumi and A. Sato. Tokyo: University of Tokyo Press, 1979, pp. 333–341.
90. Saper, C. B. Convergence of autonomic and limbic connections in the insular cortex of the rat. *J Comp Neurol* **210:** 163–173, 1982.
91. Saper, C. B. and D. Levisohn. Afferent connections of the median preoptic nucleus in the rat: Anatomical evidence for a cardiovascular integrative mechanism in the anteroventral third ventricular (AV3V) region. *Brain Res* **228:** 21–31, 1983.
92. Saper, C. B. and A. D. Loewy. Efferent connections of the parabrachial nucleus of the rat. *Brain Res* **197:** 291–317, 1980.

93. Saper, C. B., D. J. Reis and T.-J. Joh. Medullary catecholamine inputs to the anteroventral third ventricular cardiovascular regulatory region in the rat. *Neurosci Lett* **42:** 285–291, 1983.
94. Sawchenko, P. E. and L. W. Swanson. The organization of noradrenergic pathways from the brainstem to the paraventricular and supraoptic nuclei in the rat. *Brain Res Rev* **4:** 275–325, 1982.
95. Sawchenko, P. E. and L. W. Swanson. Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat. *J Comp Neurol* **205:** 260–272, 1982.
96. Schmitt, H., L.-D. LeDourec and N. Petllot. Antinociceptive effects of some α-sympathomimetic agents. *Neuropharmacology* **13:** 289–293, 1974.
97. Schmitz, J. M., R. M. Graham, A. Sagalowsky and W. A. Pettinger. Renal alpha-1 and alpha-2 adrenergic receptors: Biomedical and pharmacologica correlations. *J Pharmacol Exp Ther* **219:** 400–406, 1981.
98. Schwaber, J. S., B. S. Kapp, G. A. Higgins and P. R. Rapp. Amygdaloid and basal forebrain direct connections with the nucleus of the solitary tract and the dorsal motor nucleus. *J Neurosci* **2:** 1424–1438, 1982.
99. Sofroniew, M. V. Direct reciprocal connections between the bed nucleus of the stria terminalis and dorsomedial medulla oblongata: Evidence from immunohistochemical detection of tracer proteins. *J Comp Neurol* **213:** 399–405, 1983.
100. Sparber, S. B. and D. R. Meyer. Clonidine antagonizes naloxone-induced suppression of conditioned behavior and body weight loss in morphine-dependent rats. *Pharmacol Biochem Behav* **9:** 319–325, 1978.
101. Spaulding, T. C., S. Fielding, J. J. Venafro and H. Lal. Antinociceptive activity of clonidine and its potentiation of morphine analgesia. *Eur J Pharmacol* **58:** 19–25, 1979.
102. Spyer, K. M. Central nervous integration of cardiovascular control. *J Exp Biol* **100:** 109–128, 1982.
103. Summers, R. J., B. Jarrott and W. J. Louis. Selectivity of a series of clonidine-like drugs for α_1- and α_2-adrenoceptors in rat brain. *Neurosci Lett* **20:** 347–350, 1980.
104. Svensson, T. H. and U. Strombom. Discontinuation of chronic clonidine treatment: Evidence for facilitated brain noradrenergic transmission. *Naunyn Schmiedebergs Arch Pharmacol* **299:** 83–87, 1977.
105. Swanson, L. W. and B. K. Hartman. The central adrenergic system. An immunofluorescence study of the location of cell bodies and their efferent connections in the rat utilizing dopamine-β-hydroxylase as a marker. *J Comp Neurol* **163:** 467–506, 1975.
106. Swanson, L. W., P. E. Sawchenko, J. Rivier and W. W. Vale. Organization of ovine corticotropin releasing factor immunoreactive cells and fibers in the rat brain: An immunohistochemical study. *Neuroendocrinology* **36:** 165–186, 1983.
107. Takahashi, Y., K. Satoh, T. Sakmot, M. Tohyama and N. Shimizu. A major source of catecholamine terminals in the nucleus tractus solitarii. *Brain Res* **172:** 372–377, 1979.
108. Torvik, A. Afferent connections to the sensory trigeminal nuclei, the nucleus of the solitary tract and adjacent structures. *J Comp Neurol* **106:** 51–142, 1956.
109. Tseng, L.-F., H. H. Loh and E. L. Wei. Effects of clonidine on morphine withdrawal signs in the rat. *Eur J Pharmacol* **30:** 93–99, 1975.
110. Uhde, T. W., R. W. Post, L. Siever, M. S. Buschbaum, D. C. Jimerson, E. K. Silberman, D. L. Murphy and W. E. Bunney. Clonidine: Effects on mood, anxiety and pain. *Psychopharmacol Bull* **17:** 125–126, 1981.
111. Ungerstedt, U. Stereotaxic mapping of the monoamine pathways in the rat brain. *Acta Physiol Scand* Suppl **367:** 1–48, 1971.
112. Unnerstall, J. R., T. A. Kopajtic and M. J. Kuhar. Distribution of α_2 agonist binding sites in the rat and human central nervous system: Analysis of some functional, anatomic correlates of the pharmacologic effects of clonidine and related adrenergic agents. *Brain Res Rev* **7:** 69–101, 1984.
113. Unnerstall, J. R., D. L. Niehoff, M. J. Kuhar and J. M. Palacios. Quantitative receptor autoradiography using [^{3}H]Ultrofilm: Application to multiple benzodiazepine receptors. *J Neurosci Methods* **6:** 59–73, 1982.
114. Unnerstall, J. R., L. M. Orensanz, I. Fernadez and M. J. Kuhar. On the selectivity of WB4101 as an α_1 ligand: An autoradiographic study. *Neurosci Lett* Suppl **10:** S494, 1982.
115. U'Prichard, D. C., D. A. Greenberg and S. H. Snyder. Binding characteristics of a radiolabeled agonist and antagonist at central nervous system α-noradrenergic receptors. *Mol Pharmacol* **13:** 454–473, 1977.
116. Washton, A. M. and R. B. Resnick. Clonidine for opiate detoxification: Outpatient clinical trials. *Am J Psychiatry* **137:** 1121–1122, 1980.
117. Weiss, J. M., W. H. Bailey, P. A. Goodman, L. J. Hoffman, M. J. Ambrose, S. Salman and J. H. Charry. A model for neurochemical study of depression. In: *Behavioral Models and the Analysis of Drug Action*, edited by A. Levy and M. Y. Spiegelstein. New York: Elsevier/North Holland Biomedical Press, 1982, pp. 195–223.
118. Westlund, K. N., R. M. Bowker, M. G. Ziegler and J. D. Coulter. Noradrenergic projections to the spinal cord of the rat. *Brain Res* **263:** 15–32, 1983.
119. Young, W. S., III and M. J. Kuhar. A new method for receptor autoradiography: [^{3}H]-opioid receptors in rat brain. *Brain Res* **179:** 255–270, 1979.
120. Young, W. S., III and M. J. Kuhar. Noradrenergic α_1 and α_2 receptors: Autoradiographic localization. *Eur J Pharmacol* **59:** 317–319, 1979.
121. Young, W. S., III and M. J. Kuhar. Noradrenergic α_1 and α_2 receptors: light microscopic autoradiographic localization. *Proc Natl Acad Sci USA* **77:** 1696–1700, 1980.