A NOVEL *IN VITRO* METHOD FOR THE LIGHT MICROSCOPIC AUTORADIOGRAPHIC LOCALIZATION OF DRUG AND NEUROTRANSMITTER RECEPTORS

by

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ABSTRACT

Neurotransmitters and many drugs active in the central nervous system express their actions through specific sites termed "receptors." These receptors have been well characterized in the past by physiological and biochemical techniques, such as binding assays.

The anatomical resolution and quantitative sensitivity of these binding studies was improved considerably by an *in vitro* light microscopic autoradiographic method described in this thesis. The method involved two steps. First, receptors were labeled in tissue sections that had been cut on a cryostat-microtome and thaw-mounted onto microscope slides. The labeling was accomplished by dipping the slides with the sections into buffers containing the tritiated compounds as well as other compounds of interest. The sections were then scraped off for counting in biochemical studies or dried for subsequent autoradiography.

The second step, autoradiography, employed flexible, emulsion-coated coverslips which were placed securely against the labeled tissues. After appropriate exposure durations, the autoradiograms were developed and the tissues fixed and stained. The distributions of silver grains representing the receptors were then examined under a light microscope.

This technique was successfully applied to the study of the distribution of opiate and opioid peptide receptors as well as benzodiazepine receptors in rat and human brain tissue. The opiate and opioid peptide receptor distributions were identical to those seen in *in vivo* studies. Benzodiazepine receptors were also seen to be distributed in a heterogeneous fashion. High concentrations were observed in the cerebral cortex, molecular layer of the cerebellum, parts of the limbic system, olfactory bulb, and hypothalamus, and substantiae gelatinosae of the spinal trigeminal nucleus and spinal cord. White matter areas showed negligible levels of receptor.

The technique presented here offers several advantages over those available previously. Access to the central nervous system is now assured since the blood-brain barrier is circumvented and metabolism is avoided by performing the binding under appropriate conditions. Good specific-to-nonspecific binding ratios are usually obtained by washing the sections after labeling. The binding in tissue samples at least 1,000 times smaller than in previous binding assays is now measurable. No longer is it necessary to inject animals with large amounts of radioactivity or be concerned with blood flow.

One can manipulate the binding media and also biochemically define the receptor which is then studied

autoradiographically. Different conditions and ligands may be studied in consecutive sections from the same animal. Finally, human post-mortem tissue is amenable to investigation radiohistochemically.

To Alice

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INTRODUCTION

The notion that compounds produce their effects on excitable tissues through specific sites termed "receptors" originated with the work of J.N. Langley a century ago (Langley 1878, 1905, 1906, 1909). During the succeeding 100 years much has been learned about the characteristics of the interactions between drugs and their receptors (see Goldstein et al., 1974; Cuatrecasas and Hollenberg, 1976; Yamamura et al., 1978; O'Brien, 1979). Until recently, most of this information has been gained through bioassay systems using various agonists and antagonists. In this way, a great deal of information was obtained on the structural, physiological and even kinetic aspects of many drug actions at their receptors (e.g., see Horn, 1975, for a review of this approach as applied to biogenic amines).

More recently, the ability to study membrane-bound receptors on whole cells, in membrane preparations, and in purified form has been facilitated by the production of high specific activity (greater than one curie per millimole) radiolabeled compounds which retain the characteristics of the corresponding unlabeled compound (Cuatrecasas 1971, 1972; Franklin and Potter, 1972; Meunier et al., 1972). Cuatrecasas pioneered this aspect of receptor investigation through his elegant studies of the insulin receptor (Cuatrecasas, 1971). He used ^{125}I-insulin at a

specific activity of 1.45 Ci/μmole to demonstrate specific binding to adipocytes by subtracting the amount of ^{125}I-insulin bound in the presence of high concentrations (40-80 μg/ml) of unlabeled insulin (termed "nonspecific binding") from the total ^{125}I-insulin bound in the absence of "cold" insulin. He then demonstrated several important criteria of specific receptor binding. First, whereas the non-specific binding was not saturable, the specific binding was. Furthermore, only the specific binding was time and temperature dependent. In addition, the amount of insulin bound correlated well with the biologic activity of insulin as evidenced by its ability to enhance conversion of glucose to carbon dioxide in adipocytes. Cuatrecasas arrived at a value for the dissociation constant (K_D) of the interaction, 5×10^{-11} M, which compared favorably with the concentration of insulin required to produce half-maximal stimulation of glucose oxidation, 6.1×10^{-11} M. Further evidence for the specificity of this interaction was that the unrelated peptides adrenocorticotropin hormone, growth hormone, prolactin, vasopressin, glucagon, and oxytocin failed to inhibit insulin binding at 40 μg/ml. Finally, the interaction involved no chemical change of insulin or its receptor.

Hence, Cuatrecasas (1971) demonstrated four major criteria of receptor binding: saturability, physiological

relevance, high affinity, and appropriate drug pharmacology. Three additional useful criteria were developed during investigations of the opioid receptor. These criteria were: agonist-antagonist interactions, stereospecificity, and physiologically relevant subcellular and regional distributions.

Three investigations nearly simultaneously demonstrated specific binding of opiates in nervous tissue (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973) following the guidelines outlined by Goldstein et al. (1971) . Agonists such as etorphine, levorphanol, and dihydromorphine and antagonists such as diprenorphine and naloxone displaced one another very effectively at low concentrations (ED_{50}'s of 10^{-8}-10^{-10}M) (Pert and Snyder, 1973; Simon et al., 1973; Pert et al., 1973). Parenthetically, the apparent greater potency of the opiate antagonists in vitro was a consequence of the preference of the receptors for the antagonist confirmation in the presence of sodium ions (Pert et al., 1973; Simon, 1975; Snyder, 1977). This property was useful in predicting relative agonist/antagonist activities of opioids. Another important criterion available for the assessment of the opiate receptor was stereospecificity. The analgesically inactive enantiomers of the opiates, such as dextrorphan, were much less potent in competing for binding sites than

their active counterparts (Pert and Snyder, 1973; Simon *et al.*, 1973). The third additional criterion was physiologically relevant subcellular and regional distribution.

The synapse was considered a likely site of opiate action. And, indeed, the opiate receptor was found in its greatest percentage in the microsomal "P_2" fraction which contains synaptosomes (Pert and Snyder, 1973; Terenius, 1973). When the P_2 fraction was further examined, Pert *et al.* (1974) found 89% of the opiate receptor associated with synaptosomal ghosts, damaged synaptosomes and membrane fragments. In a similar fashion, binding studies using tissue from discrete brain regions in human and primate brains revealed a distribution of opiate receptors unlike that for any known neurotransmitter (Hiller *et al.*, 1973; Kuhar, *et al.*, 1973). However, the regional distributions often correlated with known or suspected sites of opiate actions. For example, Wei *et al.* (1972, 1973) applied naloxone crystals to various brain regions in cats which were morphine-dependent. Withdrawal symptoms were especially prominent upon application of naloxone to the medial thalamus and medial aspects of the diencephalic-mesencephalic juncture, both areas of high opiate binding (Hiller *et al.*, 1973; Kuhar, *et al.*, 1973).

Therefore, using the above seven criteria, one can usually adequately characterize a given receptor. However, even the regional distribution studies of the receptor using homogenates do not provide very good anatomical resolution. One is limited by the amount of tissue one can examine accurately. Fortunately, several approaches exist which offer greater anatomical resolution in the analysis of a receptor. These include various electrophysiological, immunohistochemical, fluorescent probe, and autoradiographic techniques.

The electrophysiological approach basically entails measuring neuronal activity while applying a compound of interest. At the end of the experiment, a dye is deposited or a lesion made to mark the location of the recorded cell. In this fashion, excellent anatomical information may be obtained on the location of drug-responsive cells. For example, we (Bird and Kuhar, 1977; Young *et al.*, 1977) and others (Nicoll *et al.*, 1977; Zieglgänsberger and Tulloch, 1979) have managed to examine different regions of the rat central nervous system for opiate alkaloid and peptide responsive cells. We showed that in the upper pons, cells in the locus ceruleus and nucleus parabrachialis were much much more responsive than cells in the adjacent tissue. This corresponded well with autoradiographic studies in this region (Atweh and Kuhar, 1977b) described below.

Still further resolution may be obtained electrophysiologically. Frederickson et al. (1978) demonstrated that taurine, in contrast to gamma-aminobutyric acid (GABA), appeared to be more depressant when applied in the cerebellar Purkinje cell dendritic zone than when applied near the soma. Similarly, Zieglgänsberger et al. (1979) used double recording techniques with recessed iontophoresing barrels to investigate the excitatory effect of opioids on hippocampal pyramidal cells. They concluded that GABA-containing interneurons (probably basket cells) were being inhibited and releasing the pyramidal cells from their inhibition.

This approach may be quite fruitful but necessary cautions must be heeded (Bloom, 1974), especially during interpretation. The controls over current, and, to a certain extent, over leakage are maintained with good equipment (Salmoiraghi and Weight, 1967). One must be sure of the identity of the cell being tested, either by characteristic discharge patterns, by response to stimulation of specific antidromic or orthodromic projections, or by marking recording sites on or in cells with a dye which can be cytologically examined subsequently. The relative influence of pre- and post-synaptic receptors is a valid concern. Intracellular recording techniques in combination with agents to prevent synaptic transmission (e.g., Mg^{++}) can

shed light on this problem. Finally, the specificity of the agent being used must be examined. If the drug is given parenterally, systemic physiologic effects and side effects (lowered blood pressure, sedation) must be taken into account in the interpretation of the drugs' actions on a single neuron. If the agent is applied iontophoretically, then stereospecificity and antagonism may be used as criteria of cell reponse. Nevertheless, one must be concerned that two apparent antagonistic compounds are interacting indirectly through different receptors on the same or different cells or that a compound is working through interneurons (see above and Curtis, et al., 1971). The major drawback of this technique is the enormous time and effort which would be needed to map even the smallest areas of the brain. This technique is probably best utilized in conjunction with methods which give broader mappings of receptor densities. Another serious drawback is that this technique can not be applied to study humans.

Immunohistochemical studies on receptor localization have also been pursued. There are basically three approaches included here. Ligands which have some sort of tag may be used to bind and identify the receptor. Alternatively, the ligand may contain an immunological determinant which may be exploited by any of several antibody recognition techniques. Thirdly, one may use antibodies directed at the receptors themselves to localize them.

The first approach generally uses fluorescent, electron dense, or enzyme labeled ligands. (Radiolabeled ligands for autoradiography are discussed extensively below). Preliminary experiments are necessary to assure oneself that the tagged compound still recognizes the receptor, and with sufficient affinity to avoid diffusion from the receptor. This is especially important for electron microscopic studies.

Fluorescent probes to identify and localize neurotransmitter receptors have not been very successful to date. Initial studies of the beta-adrenergic receptor (Atlas and Levitzki, 1977; Atlas et al., 1977) actually demonstrated endogenous autofluorescent granules, probably lipofuchsin (Hess, 1979; Correa et al., 1980). Hazum et al. (1979) have recently demonstrated enkephalin receptors using fluorescent probes and image-intensifying equipment. These receptors, studied in tissue culture, were seen in patches using the fluorescent enkephalin derivative, Tyr-D-Ala-Gly-Phe-Leu-Lys-Rhodamine. The patches were not present at 4° C or in the presence of 10^{-6} M [D-Ala^2,Leu^5]enkephalin. Since the studies were performed in Tris-HCl buffer and physiological concentrations of sodium ion greatly reduced the clustering, the significance of the finding is unclear. In any case, it was also observed that the clusters were not internalized. Whether this technique will be applicable

to tissue sections *in vitro* is uncertain. The expense of the equipment is a drawback.

The electron dense and enzyme labeled methods have traditionally been utilized in conjunction with protein ligands, such as the neurotoxin, alpha-bungarotoxin (αBT). This ligand has the advantage of binding nearly irreversibly to an acetylcholine receptor-associated site. Hourani *et al*. (1974) used ferritin-αBT conjugates to label toxin binding sites in membranes of muscle cells grown in tissue culture. They determined that although the conjugates had decreased potency, they were still quite capable of blocking the effect of iontophoretically applied acetylcholine. Electron microscopy revealed small clusters of conjugates which were blocked by preincubation in D-tubocurare or αBT. An αBT-horseradish peroxidase conjugate technique has also been used to localize nicotinic receptors (Vogel *et al*, 1977). The investigators noted deposits using 3,3'-diaminobenzidine in the inner plexiform layer of the chicken retina in the bipolar ribbon synapses as well as amacrine synapses. The deposits were absent in samples preincubated in the presence of tubocurare. Similar studies were performed with the same conjugate in muscle tissue of human myasthenia gravis patients and rat (Engel *et al*., 1977) and other species (Lentz *et al*., 1977).

High resolution studies are possible using these techniques but will probably be limited to irreversibly, or nearly irreversibly, binding ligands of high molecular weight.

The second approach, immunohistochemical methods directed against the ligands themselves, have been popular for studying the nicotinic receptor, especially. Basically, antibodies are generated which are directed toward the ligand (presumably very tightly bound but it remains to be seen if an antibody itself could stabilize a diffusible ligand to its receptor). These antibodies may themselves be labeled (^{125}I, peroxidase or electron dense tags) or have labeled antibodies directed against the first antibodies. Another approach uses a sandwich technique with unlabeled antibodies (Mason _et al._, 1969). First, the receptor is labeled with the ligand and then bound by an antibody directed toward the ligand. Next, an antibody directed against the first antibody is added. After that, antibody against peroxidase, for example, raised in the same animal as the first one, is applied. Finally, peroxidase is added and the reaction products are formed. A modification of this technique combining the antiperoxidase antibody and peroxidase at the start was introduced by Sternberger (1972). These methods have been used extensively in studying various neurotoxins, such as alpha-bungarotoxin

(e.g., Daniels and Vogel, 1975; Bender et al., 1976) and tetanus toxin (Fedinec et al., 1970).

The final approach, for which there is little data at the present, involves using antibodies directed toward the receptors themselves. The most progress has been attained in obtaining antibodies against the nicotinic acetylcholine receptor.

Mixed populations of antibodies generated against the receptor from Torpedo californica have been used to show that antigenic determinants appear on both sides of the plasma membrane (Tarrab-Hazdai et al., 1978). Monoclonal antibodies against Torpedo (Moshly-Rosen et al., 1979; Gomez et al., 1979) and rat skeletal muscle (Miller and Hall, 1977) have also been produced against different parts of the receptor. One group (Yu et al., 1979) has used their monoclonal antibodies against Torpedo acetylcholine receptors to study immunohistochemically the distribution of receptors on rat myotubes. Young myotubes revealed numerous small, round patches while mature myotubes showed 2-3 ring-shaped patches in the central portion. Recent advances in purifying other receptors (Carson et al., 1977, Hurko, 1978; Yousufi et al., 1979; Gavish et al., 1979) promise to yield advances in detecting receptors immunohistochemically in the near future. A very interesting and, perhaps, related finding may offer another approach to detecting receptors directly by

immunohistochemical means. Antibodies generated against retinol-binding protein or insulin were then themselves used to generate a second set of antibodies. These latter antibodies were found to block ^{3}H-retinol uptake in rat intestinal epithelial cells or ^{125}I-insulin binding to its receptor (Sege and Peterson, 1978). Whether these second antibodies are "ligand-like" or anti-receptor in nature is unclear at present. Nevertheless, they may be useful for receptor localization.

The final method of neurotransmitter receptor localization is autoradiography. If a receptor can be tagged with a radiolabeled ligand, then it may be possible to place the tissue with the labeled receptor in proximity to emission-sensitive film or emulsion to record the disintegrations. Upon development of the autoradiogram, one has a record of the location of the receptors which may be examined microscopically. Initial studies were made of tritiated substances that were injected into the animal. The binding of the ligand was reversible and care was taken to avoid diffusion of the ligand from the receptor during processing for autoradiography. The frozen tissue containing the ligand-receptor complex was cut on a cryostat-microtome to yield thin sections which were mounted onto emulsion-coated slides (Appleton, 1964; Stumpf and Roth, 1964). A variation of this technique (Stumpf and Roth, 1966) designed for the

autoradiography of diffusible substances was first attempted on neurotransmitter receptors by Kuhar and Yamamura (1974, 1975, 1976) with ^{3}H-quinuclidinyl benzilate (QNB), a muscarinic receptor antagonist. Rats were injected intraveneously with ^{3}H-QNB and then sacrificed at a time when the bulk of the radioactivity was bound specifically to muscarinic receptors. Autoradiograms revealed heterogeneous distributions of receptors upon light-microscopic examination. Control animals pretreated with a different muscarinic antagonist, atropine, showed only a low, background level of grains.

Following this initial success with the muscarinic receptor, other receptors were examined by the same technique. These include the receptors for opiates using ^{3}H-diprenorphine and ^{3}H-etorphine (Pert *et al.*, 1976; Atweh and Kuhar, 1977a,b,c), for ^{3}H-reserpine (Murrin *et al.*, 1977), and for ^{3}H-spiroperidol (Klemm *et al.*, 1979; Murrin and Kuhar, 1979). These procedures are termed "*in vivo* labeling" or just "*in vivo*" autoradiography.

The principles of *in vivo* labeling autoradiography have been mentioned briefly above. Unfortunately, several important problems are encountered and, thus, limit the usefulness of *in vivo* labeling autoradiography. Perhaps foremost among these problems is the inability of many ligands of interest to gain access to the central nervous

system. Several factors, either acting alone or in combination, account for the failure of delivery. One factor is the blood-brain barrier which effectively excludes many compounds from the brain. Another factor often encountered, especially when the injected compound is a peptide, is metabolism. This aspect has two concerns, in addition. First, serum proteases and other enzymes may rapidly inactivate the compound. Second, metabolites may themselves compete for the same or different sites; thus complicating the interpretation of resultant autoradiograms.

A common problem with *in vivo* labeling autoradiography is that the signal-to-noise ratio or specific-to-nonspecific binding is often poor. In this case, it is difficult or impossible to pick out those significant sites of action. A corollary of this problem may be seen when highly lipophilic ligands are employed. They have a tendency to accumulate in white matter areas in brain nonspecifically, causing some confusion in interpretation.

A host of smaller problems may plague an *in vivo* study. One is the potential high cost of loading entire animals with the isotopically-labeled compounds. Another is the difficulty of studying more than one compound in the same animal to compare precisely their distributions. Although this is technically feasible through the use of different isotopes, all the above caveats are then at least doubled

for each study. Another potential concern, albeit hypothetical, is the distribution of blood flow. This should not be a concern because, after all, one is looking for receptors mediating the drug's effects; but, this may not necessarily be correct. For example, suppose one chooses to examine the receptor distribution one hour after injection because one area of interest has a good specific-tononspecific binding ratio. Another drug-responsive area may not yet have cleared its nonspecific binding to the same extent.

Finally, the greatest problem with *in vivo* labeling autoradiography is the impossibility of studying human tissue. Since several neurological and psychiatric diseases are believed to have receptor alterations, one would like to be able to label tissue receptors *in vitro* and perform autoradiography subsequently. Huntington's chorea, for example, has been reported to have increased GABA (Lloyd and Dreksler, 1979) and benzodiazepine (^{3}H-flunitrazepan) (Reisine *et al.*, 1979) binding and decreased muscarinic (^{3}H-QNB), serotonergic (^{3}H-serotonin and ^{3}H-lysergic acid diethylamide), beta-adrenergic (^{3}H-dihydroalprenolol) and ^{3}H-spiroperidol binding (Enna *et al.*, 1976; Reisine *et al.*, 1977) in several areas. In schizophrenia, ^{3}H-spiroperidol binding is increased in the human caudate and putamen (Crow *et al.*, 1978; Lee and Seeman, 1980;

Reisine *et al.*, 1980) while opiate (^{3}H-naloxone) binding is decreased in the caudate (Reisine, 1980). Autoradiographic studies would be useful in directing further studies on these and other diseases' underlying disorders.

In vitro labeling of receptors in tissues with subsequent autoradiography (*in vitro* labeling autoradiography) would circumvent many of the above problems. Midgley (1972, 1973) incubated slide mounted tissue sections (ovarian and testicular) with various ^{131}I- or ^{125}I-labeled gonadotropins ($10^{-10}M$) and then dipped the slides with the sections into a nuclear emulsion. This important work provided much new knowledge on these peptides' sites of action (Richards and Midgley, 1976). For example, in pseudopregnant rats, human chorionic gonadotropin bound to corpora lutea interstitial tissue, and outer granulosa cells whereas human follicle-stimulating hormone was restricted to granulosa cells of the large follicles. In addition, he showed that binding of hormones varied during the rat estrous cycle and at different times before and after delivery (Midgley, 1973). For controls, he added 500-3,000 fold excess of unlabeled hormone in addition to the labeled one. This technique has been applied with great advantage by endocrinologists recently and the refined technique has become very useful in elucidating the many interacting

hormonal influences on reproduction (Lee and Ryan, 1973; Zeleznik *et al.*, 1974; Dal Lago *et al.*, 1975; 1976, 1977; Holt *et al.*, 1976; Richards and Midgley, 1976; Bortolussi, *et al.*, 1977).

We were able to use this *in vitro* technique for the opiate receptor (Young and Kuhar, 1978) but considerable diffusion often remained and we considered the method too variable (see Methods; Young and Kuhar, 1979c). Of course, when one uses irreversibly binding ligands such as ^{125}I-alpha-bungarotoxin (Polz-Tejera, 1975), which labels cholinergic nicotinic receptors, or ^{3}H-propylbenzylylcholine mustard (Rotter *et al.*, 1977, 1979a,b,c,d), which labels cholinergic muscarinic receptors, diffusion no longer is a concern. However, we wished to develop a general method applicable to all reversibly binding and, hence, diffusible ligands.

With this in mind, we have developed an *in vitro* labeling autoradiographic technique which circumvents most of the problems mentioned above. In addition, several other advantages have become apparent. The method we have devised is a combination of previous techniques with some modifications. Basically, one labels a slide-mounted tissue section by incubating it in a solution containing the radiolabeled ligand and then washes to remove nonspecific binding (Young and Kuhar, 1979c). Then, in applying an elegant technique described by Roth *et al.* (1974), one places

an emulsion-coated, flexible coverslip against the tissue section to produce the autoradiogram (other methods have become available and are critically examined in the discussion).

This procedure ameliorates several of the problems associated with *in vivo* labeling autoradiography. Access to the central nervous system receptors is now assured since the blood-brain barrier is circumvented and metabolism is avoided by performing the binding at low temperatures or by adding appropriate enzymatic inhibitors to the binding media. Good specific-to-nonspecific binding ratios are usually obtained (often greater than 20:1) by washing the sections after labeling. No longer is it necessary to load entire animals with costly drugs or be concerned with blood flow.

This *in vitro* technique enables one to manipulate the binding media so that the effects of ions, nucleotides, or other compounds of interest on the labeling of the receptors may be studied in a regional or quantitative manner, for example. Because we can manipulate the binding media, we can biochemically define the receptor we are studying. In this manner, we obtain a clear, unambiguous understanding of just what a subsequent autoradiogram represents. Consecutive sections may be studied under different conditions and various ligands can be used in the same small regions of the

same animal. And, of course, human post-mortem tissue becomes amenable to investigation radiohistochemically.

METHODS

As mentioned above, the method we have developed involves labeling tissue sections mounted on slides and attaching flexible, emulsion-coated coverslips to these slides for the autoradiography. The overall procedure is shown schematically in Figure 1.

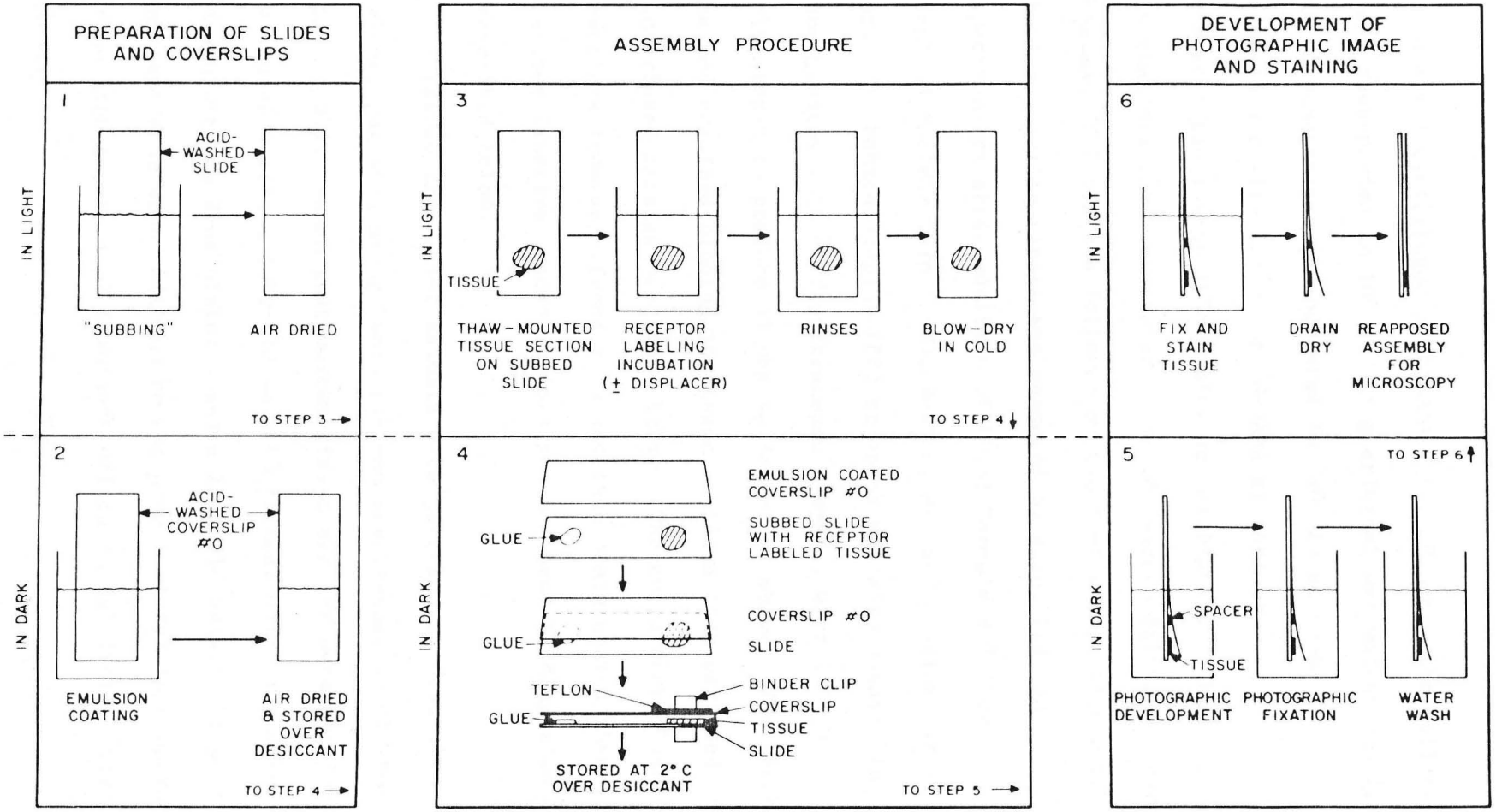
Tissue Preparation

We usually use male, 180-225 g, Sprague-Dawley rats (Madison, WI) which we sacrifice by decapitation. The brains are then rapidly removed and 1 to 3 mm slices of appropriate regions are frozen onto microtome chucks and stored in liquid nitrogen if not used immediately. In an attempt to improve the tissue morphology, we routinely perfuse the pentobarbital anesthetized rats with 100 to 200 ml of 0.1% formaldehyde (1 ml concentrated reagent per L of phosphate-buffered saline; Fisher, Fairlawn, NJ). The effects of fixatives are measured in three ways. The first is to compare the K_D's and B_{max}'s in unperfused and perfused animals in conventional binding assays. The opiate receptor was assayed with ^{3}H-D-alanine^2-methionine^5-enkephalinamide (^{3}H-DAMA; Amersham Corp., Arlington Heights, IL; 25 Ci/mmole) by the method of Simantov *et al.* (1978). Briefly, 1 g of brain was homogenized per 100 ml of 50 mM Tris-HCl, pH 7.7 at 0°C and centrifuged at 500 g for 10 min. The supernatant was incubated at 37° C for

Figure 1

Schematic illustration of *in vitro* labeling technique.

Adapted from Young and Kuhar (1979c) and Roth *et al*. (1974).


PREPARATION OF SLIDES AND COVERSLIPS
ASSEMBLY PROCEDURE
DEVELOPMENT OF PHOTOGRAPHIC IMAGE AND STAINING
IN LIGHT
IN DARK
1
ACID-WASHED SLIDE
"SUBBING"
AIR DRIED
TO STEP 3
2
ACID-WASHED COVERSLIP #0
EMULSION COATING
AIR DRIED & STORED OVER DESICCANT
TO STEP 4
3
TISSUE
THAW-MOUNTED TISSUE SECTION ON SUBBED SLIDE
RECEPTOR LABELING INCUBATION (± DISPLACER)
RINSES
BLOW-DRY IN COLD
TO STEP 4
4
EMULSION COATED COVERSLIP #0
GLUE
SUBBED SLIDE WITH RECEPTOR LABELED TISSUE
COVERSLIP #0
GLUE
SLIDE
TEFLON
BINDER CLIP
GLUE
COVERSLIP
TISSUE
SLIDE
STORED AT 2° C OVER DESICCANT
TO STEP 5
5
TO STEP 6
SPACER
TISSUE
PHOTOGRAPHIC DEVELOPMENT
PHOTOGRAPHIC FIXATION
WATER WASH
6
FIX AND STAIN TISSUE
DRAIN DRY
REAPPOSED ASSEMBLY FOR MICROSCOPY

40 min and centrifuged at 20,000 g for 20 min. The pellets were resuspended in 100 ml per g original wet weight and 1.9 ml of homogenate was incubated for 40 min at room temperature with 0.1 ml of ^{3}H-DAMA at various concentrations with 10^{-6}M naloxone for blanks. The incubations were terminated by rapid vacuum filtration over Whatman GF/B filters followed by two 5 ml washes with cold buffer. Radioactivity was measured by scintillation spectrometry after addition of 10 ml Formula 947 (New England Nuclear Corp (NEN), Boston, MA) and shaking for 1 hr. The benzodiazepine (BZ) receptor was also assayed in homogenates with ^{3}H-flunitrazepan (^{3}H-FLU) with 10^{-6} M clonazepam to produce blanks as described above. The second method examined binding in tissue sections from perfused and unperfused rats as described below. The third method used sections from unperfused rats and then preincubated them in various fixative concentrations and measured binding also as described below.

Tissues from other animals were perfused as described above; but when using human post-mortem tissue, we did not fix at all. Human post-mortem tissue samples were obtained at autopsy from a 50-y-old male (3 hr post-mortem; sudden death with no drug history) and a 22-y-old male (4 hr post-mortem; stab wound with no recent drug history) in cooperation with the Medical Examiners Office in Baltimore, Maryland.

In Vitro Tissue Section Labeling

The tissues were cut on a Harris cryostat microtome (Harris Manufacturing Co., N. Billerica, MA), thaw-mounted onto subbed slides (Corning, Corning, NY), and stored for 7-14 days at -20°C to permit adhesion of the tissues to the slides. In general, we used 10-14μ thick sections for the biochemical studies. Linearity with tissue thickness was examined. We examined the opiate receptor and benzodiazepine receptor (BZR) in these studies as they were well characterized in homogenate studies (Braestrup and Squires, 1977; Möhler and Okada, 1977; Childers et al., 1978; Goldstein, 1976; Kuhar, 1978; Simon and Hiller, 1978).

The slides with tissue sections were brought to room temperature individually immediately before incubation. Preincubations, incubations and washes were performed in 5 and 8 slot Coplin jars containing 25 and 50 ml of solution, respectively. We usually employed Tris-HCl (170 mM, pH 7.4-7.7) as the buffer depending on the binding optimum of the receptor under investigation. The concentration cited above for the buffers represents an osmolality of 320 mOsm. The same buffer was used, unless otherwise noted, in all steps: preincubation, incubation and washing. Occasionally, preincubation was necessary to remove a competing endogenous ligand from the tissue.

We usually chose ice-water temperatures initially in biochemical studies to minimize any possibility of metabolism or degradation of receptor or ligand. However, if satisfactory ratios were not obtained or binding equilibrium was reached only after several hours, we then employed higher incubation temperatures (e.g., 20° C). The time of incubation was the time at which equilibrium was reached.

Nonspecific binding was obtained by adding 1000-fold excess of a related compound. Of course, it is better not to use the same compound that is being studied so that isotopic dilution is not a concern.

The purpose of the wash was to reduce nonspecific binding to a greater degree than specific binding. The washing times were determined empirically, also. It was generally found that two short washes were better than one longer one. Occasionally, the specific binding had a short half-time of dissociation (e.g., ^{3}H-flunitrazepam) and only one short wash was used to avoid excessive loss of specific binding. We performed all our washes at ice-water temperatures. The nonspecific binding, because of its low affinity, generally dissociated quite rapidly even at this temperature.

As mentioned above, an important aspect of *in vitro* autoradiography is the ability to extensively characterize the receptor biochemically before beginning the auto-

radiographic study. For the biochemical studies, the tissues were wiped off the slides with Whatman GF/B filters, placed in a scintillation jars with 15 ml of 947 scintillation fluid, kept overnight at 4°C, and counted. We have listed in Table I some of the biochemical studies we performed to compare with previous in vitro membrane homogenate studies of receptors. Of great importance were the kinetics of the binding. We determined the association and dissociation rates as well as the dissociation constant (K_D). The number of receptors in the tissue (B_{max}) was also determined. In general, the B_{max} that we determined was slightly to several fold greater than that found in homogenate studies. This was probably due to the retention of receptors otherwise lost during the various isolation procedures involved in preparing a certain fraction for study in homogenates.

Of no less importance is the chemical specificity of the binding. The receptor should show the appropriate stereospecificity and only pharmacologically related compounds should displace the tritiated ligand (and they should show appropriate K_i's). Ion and nucleotide effects are important to check as well. Another important consideration is regional binding specificity. If the cerebellum, for example, contained few receptors in homogenate studies, it should show few in the section binding

TABLE I

Preliminary biochemical studies checklist

Dissociation rate

Association rate

Dissociation constant (K_D)

Receptor number (B_{max})

Stereospecificity

Pharmacological specificity (e.g., displacement by appropriate other ligands and effects of ions and nucleotides)

Regional Studies

Metabolic or degradative studies

---

also. Finally, we used thin-layer chromatography to assure ourselves that no degradation of the ligand was occurring. We have not yet observed any when all stages of labeling were kept at 0° C or 20° C and if appropriate precautions were taken. These precautions included: 1) using subdued light when studying light sensitive ligands (e.g., dihydromorphine), 2) including 0.001% (w/v final) ascorbic acid as an antioxidant when studying biogenic amines (e.g., serotonin and norepinephrine), and 3) protecting peptides (e.g., enkephalins and neurotensin) from degradation if the incubations were performed at 20° C by including 10^{-4}M bacitracin and/or .005% bovine serum.

The following radiolabeled ligands were used in these studies: ^{3}H-leucine-enkephalin (LE, 15.5 or 25 Ci/mmole), ^{3}H-dihydromorphine (DHM, 35.5 or 44 Ci/mmole), and ^{3}H-flunitrazepam (84.3 Ci/mmole) were purchased from New England Nuclear Corp. (Boston, MA). ^{3}H-Diprenorphine (DPN, 16 or 10 Ci/mmole) and ^{3}H-DAMA were purchased from Amersham Corp. Guanosine 5'-triphosphate (GTP, sodium salt) was obtained from Sigma Chemical Co. (St. Louis, MO). Dextrophan, levorphanol, naloxone, clonazepam, and diazepam were gifts from Dr. S. H. Snyder. The benzodiazepines Roll-6896 [(+) B10] and Roll-6893 [(-) B10] were gifts from Dr. H. Möhler.

Autoradiography.

After we had satisfied ourselves that we were labeling the opiate or benzodiazepine receptors, we then proceeded to the autoradiography. Instead of wiping off the sections for biochemical assay, we dipped the labeled sections on the slides very briefly (approximately 2s) into distilled H_2O and then placed them on a cold plate where cool, dry air was blown over the sections to dry them. The water dip hastened this drying procedure by removing some of the salts. In some experiments, the slides were placed on a hot plate (highest setting) to flash dry. In others, the sections were rapidly frozen on dry ice and then freeze-dried over-

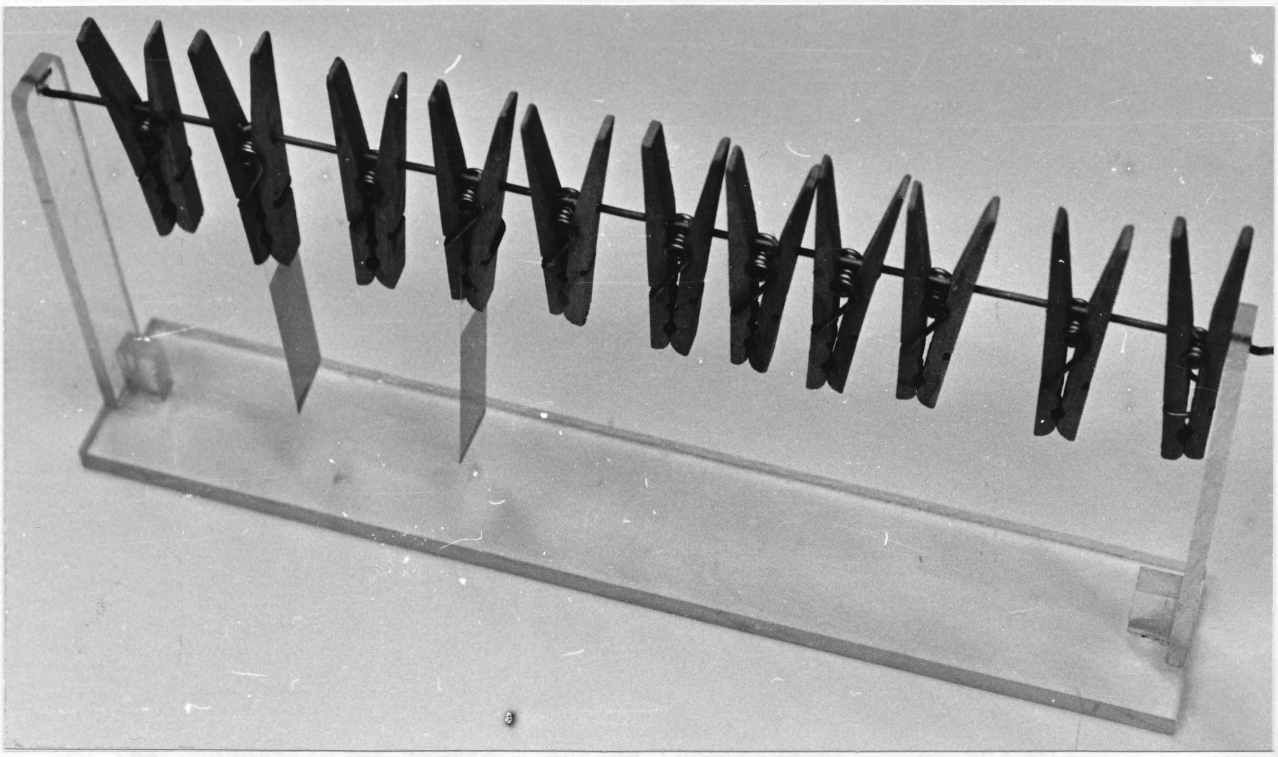
night. At this point, the slides were kept in small slide boxes at 4°C until ready for use. The boxes were closed with tape so that when they were removed from the refrigerator, moisture did not collect on the sections while the slides were warming to room temperature.

Several different approaches to the autoradiography were examined before deciding on the one outlined below which uses emulsion-coated coverslips. The slides with the ^{3}H-DPN labeled sections were dipped in NTB-3 nuclear emulsion (Kodak, Rochester, NY) at 42°C or 30°C after cooling from 42°C. Some sections were placed under AR10 stripping film which had been floating in 9:1 dilution of 20% (w/v) sucrose/0.01% potassium bromide solution. The film was lifted from the solution and wrapped around the slide. The stripping film was then blown dry or allowed to air dry.

The method of Roth et al (1974) was adapted for all other autoradiographic studies and will be described now. Acid-washed coverslips (25 x 77 mm, Corning #0, Corning, NY) were dipped into Kodak NTB-3 emulsion (Rochester, NY; diluted 1:1 with water. Air bubbles were allowed to escape to the surface), dried for 3 hours (Figure 2), and stored with dessicant in black, light-tight boxes for 24 hours to 7 days (Figure 3). The background was higher if older coverslips were used. The use of Kodak NTB2 and Ilford L4 (Ilford, England) nuclear emulsions was also examined.

Figure 2

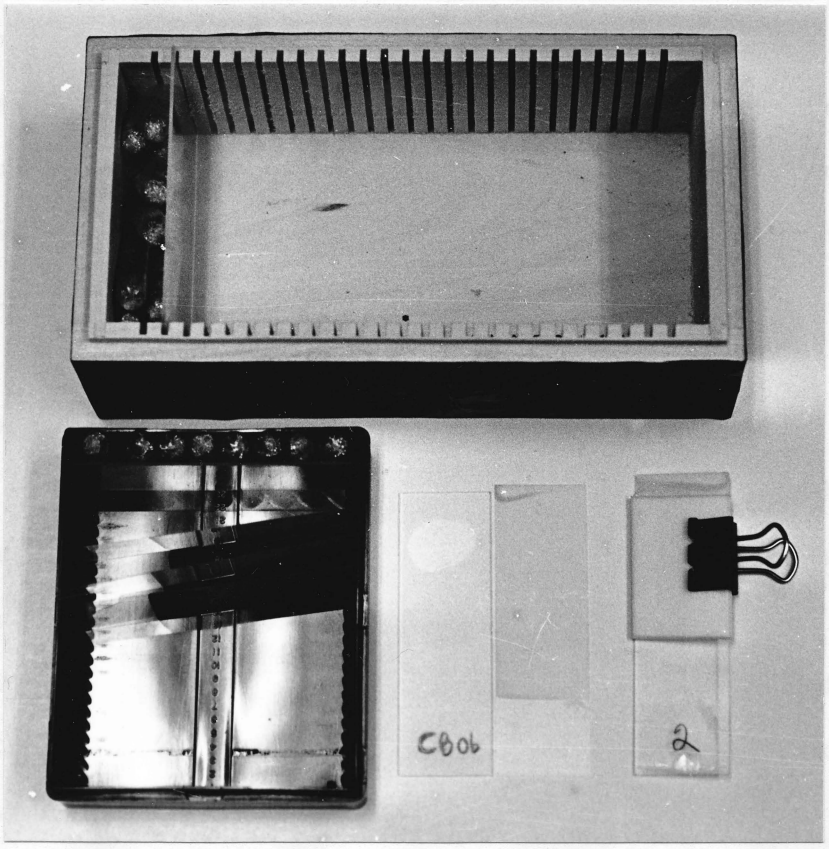
Rack with clothespins to hold emulsion-coated coverslips while drying.



The emulsion-coated coverslips were attached to the slides with the tissue sections in the presence of a Whatman #2 safelight (present during all procedures with the emulsion). This was accomplished by placing a drop of glue (Super Glue #3, Loctite Corp., Cleveland, OH) on the end of the slide away from the tissue section. After the glue set (25 sec), squares of teflon (1/8" thick) were put on top of the coverslips and the assemblies were held together with #20 binder clips (Figures 1 and 3). The assemblies were placed in a wooden slide box containing dessicant and wrapped with a light opaque tape (Figure 3). The box itself was stored in a Tupperware bread box (Dart Industries; Orlando, Florida) with more dessicant at 2-4° C for varying exposure times. As a rule of thumb, we allowed 6-8 weeks of exposure for 1500 dpm/mg tissue and extrapolated from that value for other cases. The autoradiography was examined for linearity with tissue radioactivity as well as for linearity with time. The effect of tissue thickness was also examined. The production of spurious grains (positive chemography) was assessed in the sections incubated in the presence of excess ligand. Negative chemography was assessed by using emulsion-coated coverslips which had been exposed to light briefly. This was accomplished by placing the coverslips

Figure 3

Items used in *in vitro* labeling autoradiography. A plastic slide box with Drierite capsules and several emulsion-coated coverslips is shown at the bottom left. A slide with a tissue section and a completed assembly with teflon square and binder clip are shown at the bottom right. At the top of the picture is a wooden slide box with Drierite capsules in which the assemblies are stored during exposure.



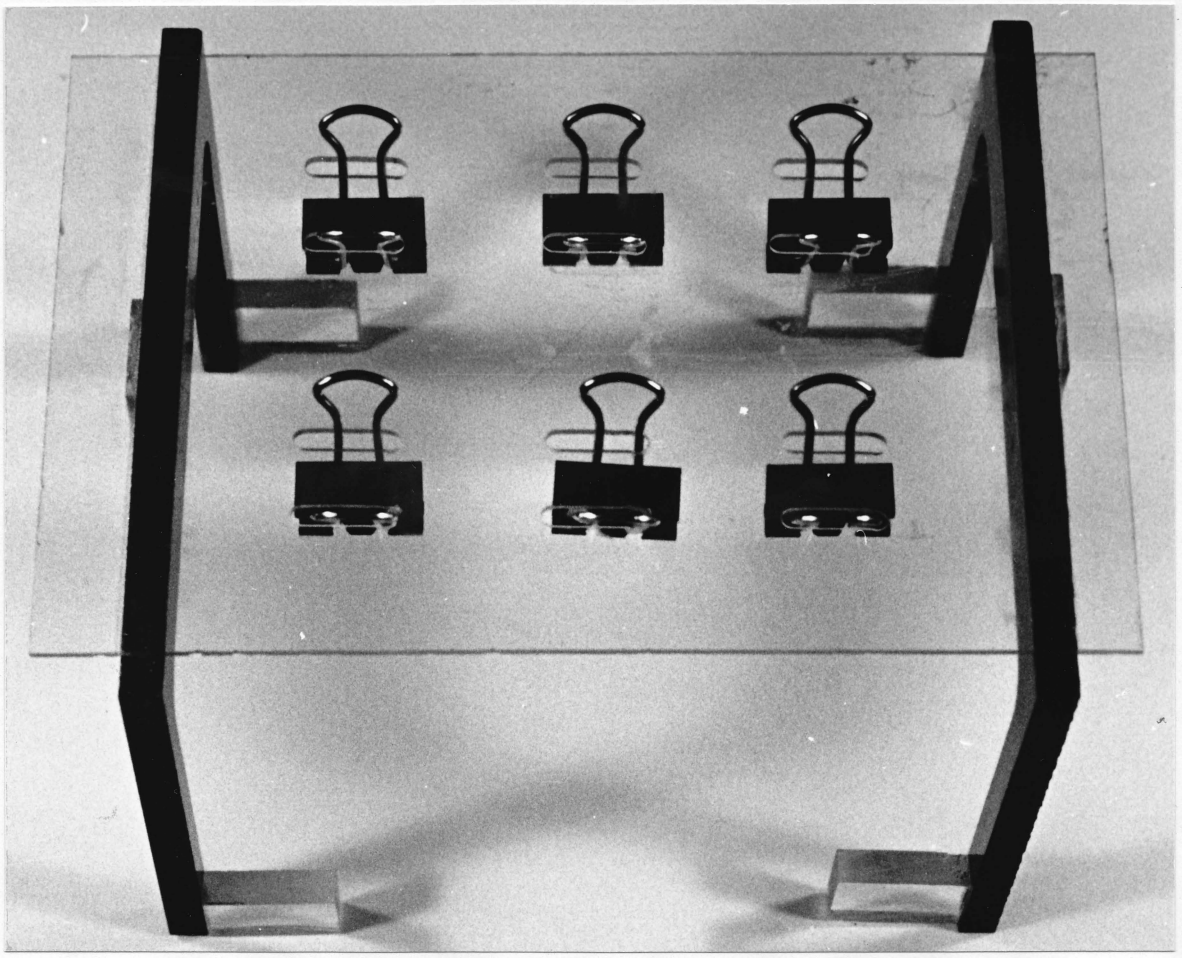
40 cm below a Durst enlarger with a 135 mm lens (f22) and illuminating for 5 s.

After exposure, the binder clips were removed and the coverslips gently bent away from the tissue sections with a round spacer. The emulsion was developed in Dektol (Kodak; 1:1 with water) for 2 min at 27°C, placed in Kodak Liquid Hardener (1:13 with water) for 15 sec, fixed in Kodak Rapid Fix for 3 min, and rinsed in distilled water for 20 min. Different development times were examined, also. The tissues were then fixed in Carnoy's solution and stained with pyronine Y (see Table II). A device for holding 12 asemblies for development, fixation and staining is shown in Figure 4. The tissue sections were dried for several hours at 40° C and then the spacers were removed and the coverslips reapposed with Permount (Fisher; Fair Lawn, NJ).

When the Ilford L4 emulsions were used, a different development system was used. Stock D170 (25 g Na_2SO_3 and 1 g KBr per 200 ml distilled water) was diluted 4:1 with water and 0.45 g of 2,4-diaminophenol dihydrochloride was added. The autoradiograms (2:1 and 1:1 coatings of emulsions) were then developed in this solution for 7 min at 18°C. This was followed by a quick water rinse, 24% sodium thiosulfate for 5 min, a second water rinse, and then 2 min

Figure 4

A plexiglass rack which can hold 12 back-to-back assemblies for sequential development of the autoradiograms and fixing and staining of the tissues.



in water. The assemblies were processed routinely after this point.

Several different microscopes were used to evaluate the autoradiograms. We used a Zeiss Standard microscope (Carl Zeiss; West Germany) with an oil immersion 40 x or 100 x objective in conjunction with a calibrated eye-piece grid for counting grains. A Leitz Ortholux microscope equipped with a Hinsch-Goldman box (Bunton Instrument Co., Inc.; Rockville, MD) or an Olympus Stereoscope with a JM brightfield/darkfield illuminator (Olympus Optical Co., Ltd.; New York, NY) was used to obtain lower-power darkfield micrographs.

TABLE II

Steps for fixing and staining of tissues

1. 10-15 min Carnoy's Fixative (ethanol: chloroform: glacial acetic acid/6:3:1).
2. 10s H_2O
3. 45-60s buffer (mix 0.2 M Na_2HPO_4 and 0.1M citric acid to pH 5.3).
4. 30-60s 1% Pyronine Y (in above buffer).
5. Several H_2O rinses.
6. Dry on warm surface.

RESULTS

The results section is composed of two parts. The first one details our biochemical studies on the properties of the receptors in our slide-mounted tissue sections. These studies were necessary to characterize the receptors so that subsequent autoradiograms would be fully interpretable, at least from the standpoint of defining what the silver grains represented.

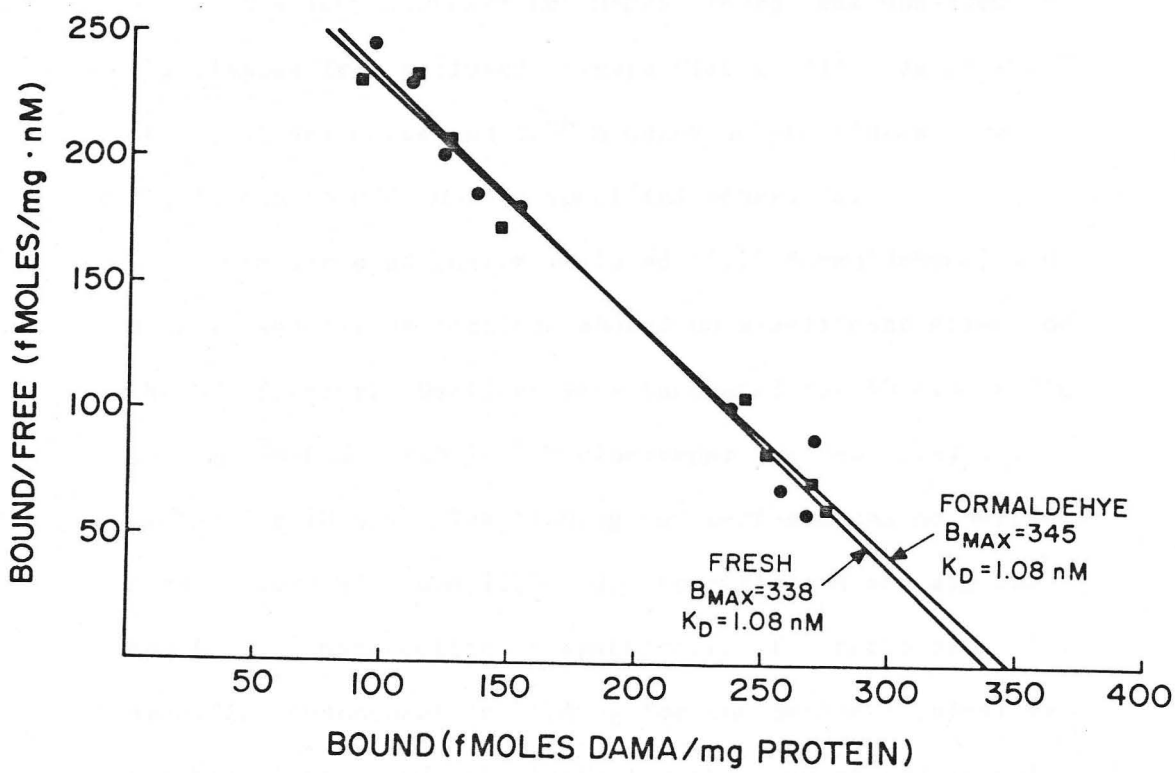
The second part describes our autoradiographic results. The method and the properties of the nuclear emulsion are explored with the purpose of developing a quantitative and sensitive technique. Applications of the technique to the opiate and benzodiazepine receptors are then detailed.

Initial biochemical studies.

We perfused our animals routinely with 0.1% formaldehyde to improve slightly the tissue histology. Three rats were perfused with 0.1% formaldehyde as described in Methods. The brains from these animals (minus cerebella) were homogenized and crude membranes utilized in binding assays with ^{3}H-DAMA according to Simantov *et al*. (1978). The results of these studies were compared with the results of binding assays from nonperfused animals (Fig. 5) and revealed essentially identical K_D and B_{max} values. Similar binding studies on the benzodiazepine receptor after perfusion with 2% paraformaldehyde and 0.1% glutaraldehyde also showed no effect on K_D or B_{max}.

Figure 5

Effects of formaldehyde on ^{3}H-opioid receptor binding. Three rats (200 g) were each perfused with 100 ml of ice cold PBS, pH 7.4 at 0°C, containing 0.1% formaldehyde (v/v) (■). Three brains were not perfused and served as controls (●). Crude membrane preparations were prepared from brains (minus cerebella) and binding performed with ^{3}H-DAMA as described by Simantov et al. (1978). The K_Ds and B_{max}s were not significantly changed. This was demonstrated in an additional experiment.



The following studies were performd after placing tissue sections on slides as described in Methods. Coronal sections of forebrain from nonperfused animals were preincubated with various concentrations of formaldehyde, rinsed briefly, and assayed in section binding studies. Fixation with 0.1 or 0.5% formaldehyde did not change the specific binding compared to unfixed tissues. However, fixation with 2% formaldehyde caused a significant loss of ^{3}H-DPN binding (Table III). In other experiments, some animals were prefused with 0.1% formaldehyde and sections of tissue were placed on slides for binding and compared to sections of tissue from animals that were perfused with PBS only. A slight increase in ^{3}H-DPN binding was observed in the tissues from perfused animals (Table III). As noted above, blanks contained 10^{-6} M naloxone and rinses were for 10 min at 0°C, unless specified otherwise.

A similar study using perfused (0.1% formaldehyde) and nonperfused tissue sections showed no significant effect on the BZ receptor. Sections were incubated for 40 min at 0°C in 1 nM ^{3}H-FLU (with 10^{-6} M clonazepam for controls) and washed for 10 min. The binding for perfused and nonperfused tissues were 9209 and 11,300 dpm specific and 488 and 662 dpm (n = 3) nonspecific, respectively. The ratio of specific-to-nonspecific binding for the perfused animal was slightly better than the ratio for the nonperfused animal.

TABLE III

EFFECTS OF FORMALDEHYDE ON ^{3}H-DIPRENORPHINE BINDING

For (A), male rats (200 g) were perfused with 200 ml ice-cold PBS, pH 7.4 at 4°C. Fourteen μ sections (A7000μ, Konig and Klippel, 1963) were thaw-mounted onto slides and preincubated for 10 min at 25°C in 0.17 M Tris-HCl, pH 7.4 containing various concentrations of formaldehyde. Binding of ^{3}H-DPN (2.33 nM; plus 10^{-6}M naloxone for blanks) was performed for 20 min at 25°C in Tris-HCl, pH 7.4, and rinsed as described in the text.

For (B), male rats (200 g) were perfused with 200 ml ice-cold PBS, pH 7.4 at 4°C with or without formaldehyde. Binding (without preincubation) was performed as described in (A). Data represent the means ± S.D of 3 determinations.

Formaldehyde concentration (%v/v)	^{3}H-DPN bound (fmol/mg tissue wet weight)	
	Total	Specific
(A) Preincubation of mounted tissue sections with formaldehyde:		
0	49.2 ± 8.4	43.7 ± 7.6
0.1	45.5 ± 2.7	40.1 ± 3.3
0.5	59.1 ± 8.3	52.1 ± 8.8
2.0	24.8 ± 1.0§	17.5 ± 1.8§

B) Perfusion of animals with PBS containing formaldehyde:

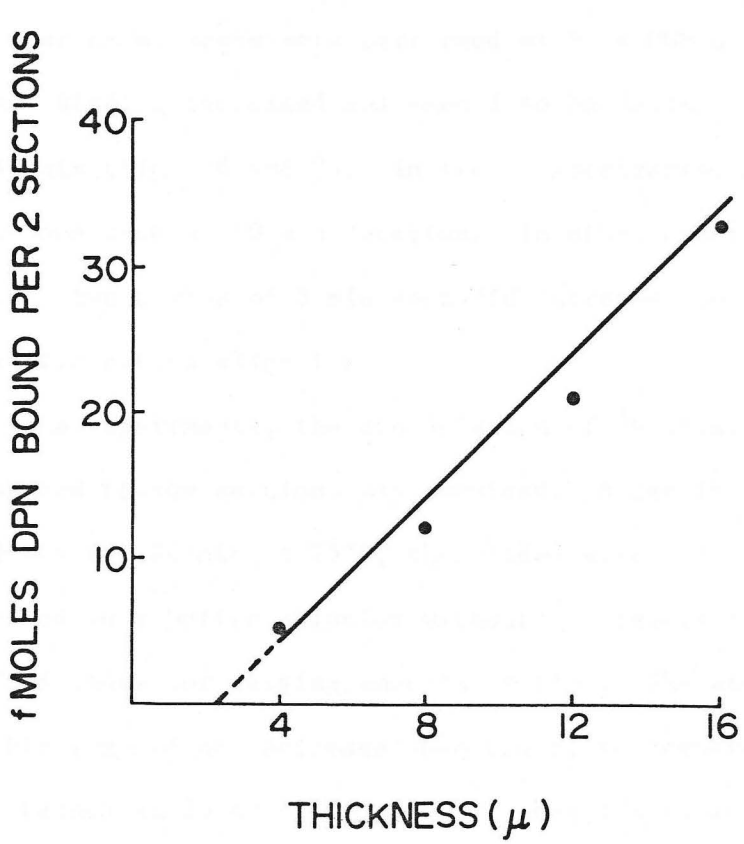
0	69.5 ± 7.0	59.1 ± 1.1
0.1	80.8 ± 17.0	63.7 ± 24.1

§2.0% formaldehyde differs from 0% < 0.05 by paired t-test.

Four, 8, 12 and 16μ sections were cut from tissues perfused with 0.1% formaldehyde as described in Methods. The mounted sections were incubated with ^{3}H-DPN for 20 min at 25°C in Tris buffer. ^{3}H-DPN binding increased linearly with section thickness (Fig. 6). Interestingly, the extrapolation of the binding curve toward the zero thickness axis suggested that sections between 0 and 2μ would show reduced or no binding. This indicated that a certain portion of the mounted sections was either inaccessible to binding, had damaged receptors or was not removed from the slide for scintillation counting. When ^{3}H-FLU (1 nM) was used, the binding was also linear with tissue thickness (r^2 = 0.99) and, again, extrapolation revealed 1.6μ of tissue was noncontributory.

Figure 6

The binding of ^{3}H-DPN to sections of various thicknesses. Rats were perfused with 100 ml of ice-cold PBS, pH 7.4, containing 0.1% formaldehyde (v/v). Frozen sections were cut at various thicknesses and incubated in 1.11 nM ^{3}H-DPN for 20 min at 25°C and rinsed as described in the text. The blanks contained ^{3}H-DPN plus 10^{-6} M naloxone. The rinse consisted of two 5-min washes at 0°C. The extrapolated line intersects the abscissa at a thickness of 2.27 μ. This experiment was repeated one other time with ^{3}H-DPN and once with ^{3}H-FLU.



Opioid Biochemistry

Mounted coronal sections from rat forebrain were incubated with ^{3}H-DHM, ^{3}H-DAMA and ^{3}H-DPN for differing times. Following the incubations, the sections were rinsed in buffer for a total of 10 min at 0° C. ^{3}H-DPN binding increased over time and seemed to saturate at 10-20 min (Fig. 7). In these experiments, after the binding incubations, there were two washes of 5 min each rather than one 10 min wash. In general, this seemed to result in higher specific to non-specific ratios for all ligands.

Similar experiments were performed with ^{3}H-DHM and ^{3}H-DAMA. Binding increased and seemed to be maximal at about 40 min (Figs. 8 and 9). In these experiments, there was only one wash of 10 min duration. In other experiments not shown, two washes of 5 min each did increase specific to non-specific ratios slightly.

In some experiments, the dissociation of ^{3}H-ligands from mounted tissue sections was examined. After incubation with ^{3}H-DPN for 20 min at 25°C, the slides were transferred to a buffer solution without ^{3}H-ligands and maintained there for varying amounts of time. The specific ^{3}H-DPN binding did not decrease when the rinse temperature was maintained at 25 or 0°C. However, when the rinse temperature was raised to 42°C, there was a rapid loss of

Figure 7

The association of ^{3}H-DPN to rat tissue sections. Ten μ formaldehyde-treated sections were prepared, incubated in the presence of 1.51 nM ^{3}H-DPN for various times, and washed as described in Figure 6 and in the text. The results of this experiment were reproduced twice.

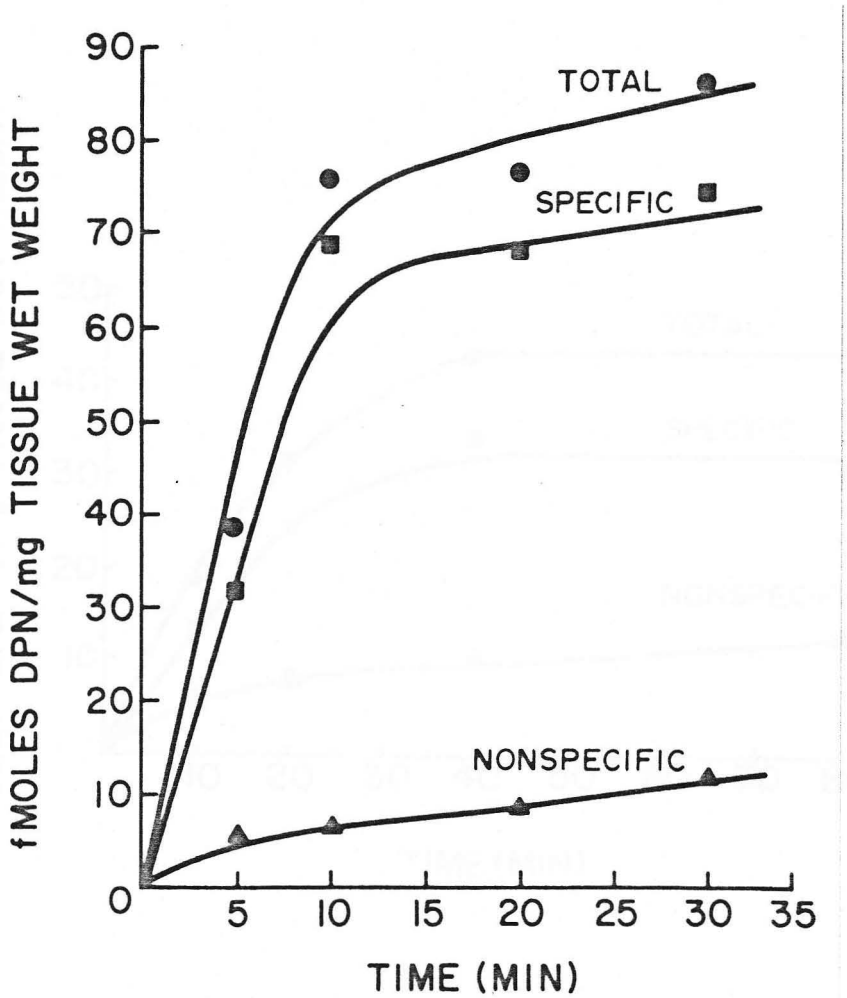


Figure 8

The association of ^{3}H-DAMA to rat tissue sections. Sections were incubated in 6.29 nM ^{3}H-DAMA for various lengths of time at 0°C and washed by two 5 min rinses in buffer alone and further processed as described in the text and Figure 6.

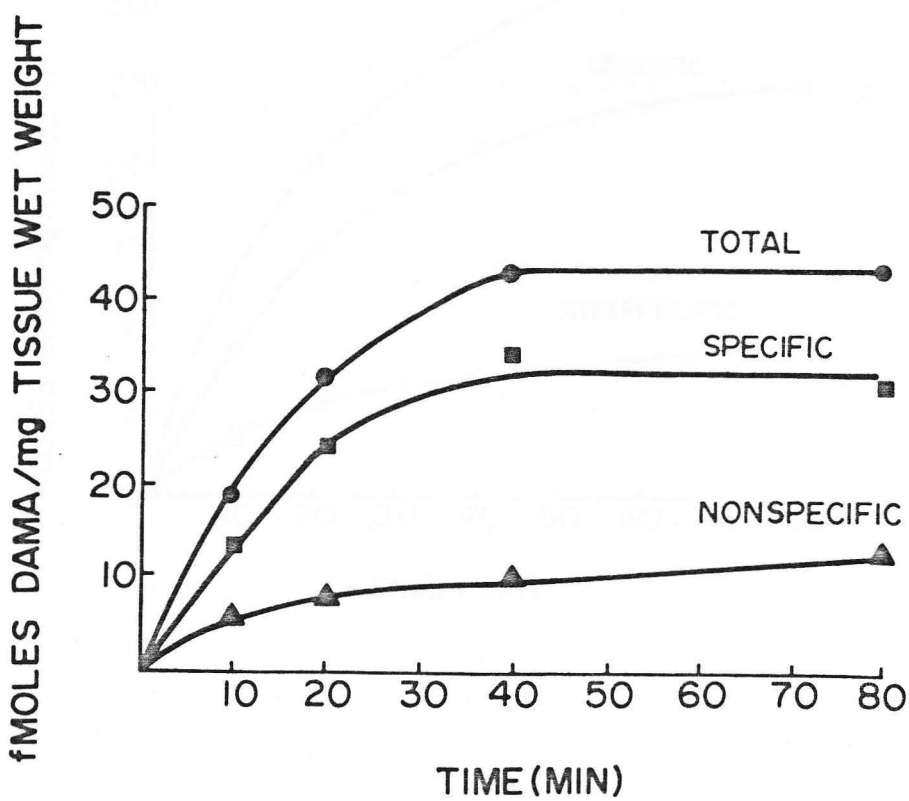
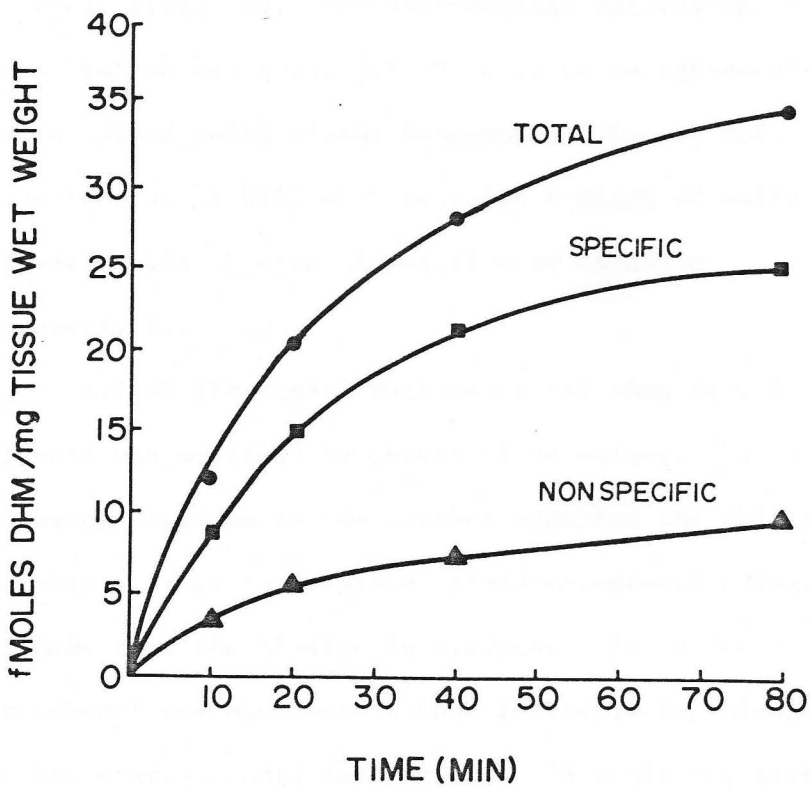


Figure 9

The association of ^{3}H-DHM to rat tissue sections. Sections were incubated in 4.95 nM ^{3}H-DHM for various lengths of time and processed as described in Figure 6 and the text.



^{3}H-DPN (Fig. 10). Similar results were obtained for the binding of ^{3}H-LE. Thus, washing the mounted tissue sections to remove non-specific opiate binding did not have a significant effect on the specific binding when performed at low temperatures.

When mounted sections were incubated with increasing concentrations of ^{3}H-DPN, the binding was found to be saturable (Fig. 11). The half-maximal saturating concentration was about 0.4 nM, a value in agreement with that obtained using tissue homogenates (Kuhar, data unpublished). A Hill plot revealed a slope of unity suggesting the absence of positive or negative cooperativity.

In all of the experiments described thus far, 10^{-6} M naloxone was utilized to obtain blank values. In the following experiments, we further examined the ability of a variety of drugs to displace opioid receptor binding. Table IV shows that the binding is stereospecific since levorphanol was much more potent in displacing binding than was its stereoisomer, dextrorphan. In addition, atropine, chlorpromazine, WB4104, propranolol, clonidine, BOL-148 and Δ^9-THC at 10^{-6} M had no effect on ^{3}H-DPN binding.

Figure 10

The dissociation of ^{3}H-DPN from rat tissue sections. Fourteen μ formaldehyde-treated sections were prepared and incubated in ^{3}H-DPN for 20 min at 25°C as described in the text. Specific binding was determined after washing for various times in 0.17 M Tris-HCl, pH 7.4 at 25°C, either with (▲) or without (■) a preliminary wash of 20 min at 4° C. Specific binding was also determined after washing for various times at 42°C after a 20 min wash at 0°C (●). ^{3}H-DPN dissociation was negligible at 0°C for 2 h (data not shown). Several other experiments showed similar data.

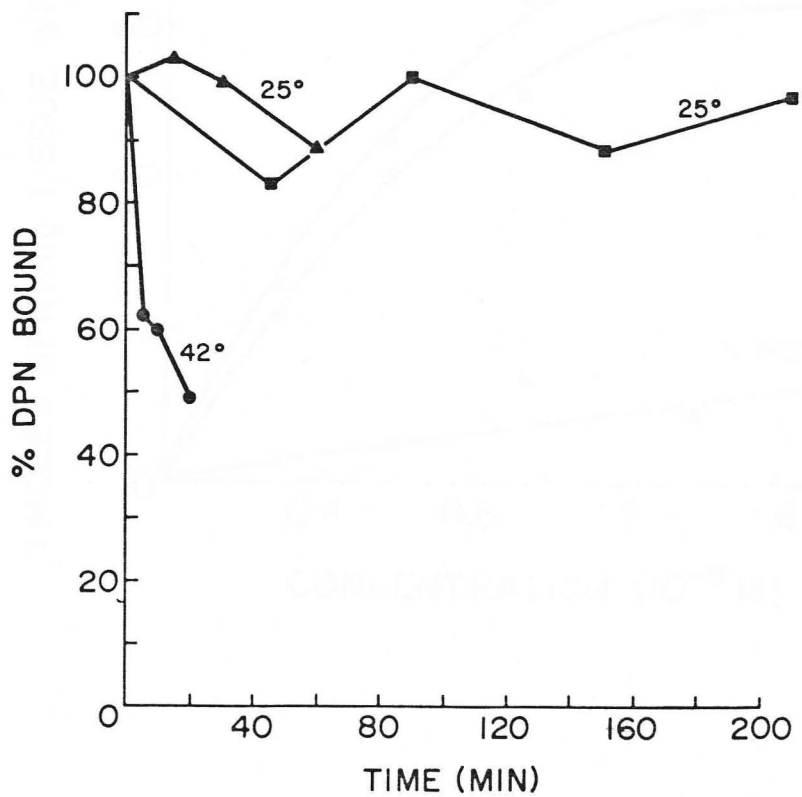


Figure 11

The saturation of ^{3}H-DPN binding. Fourteen μ formaldehyde-treated sections were prepared, incubated in various concentrations of ^{3}H-DPN for 20 min at 25°C, and washed as described in the text. Specific binding exhibited a K_D of approximately 0.4 nM. This experiment was reproduced two additional times.

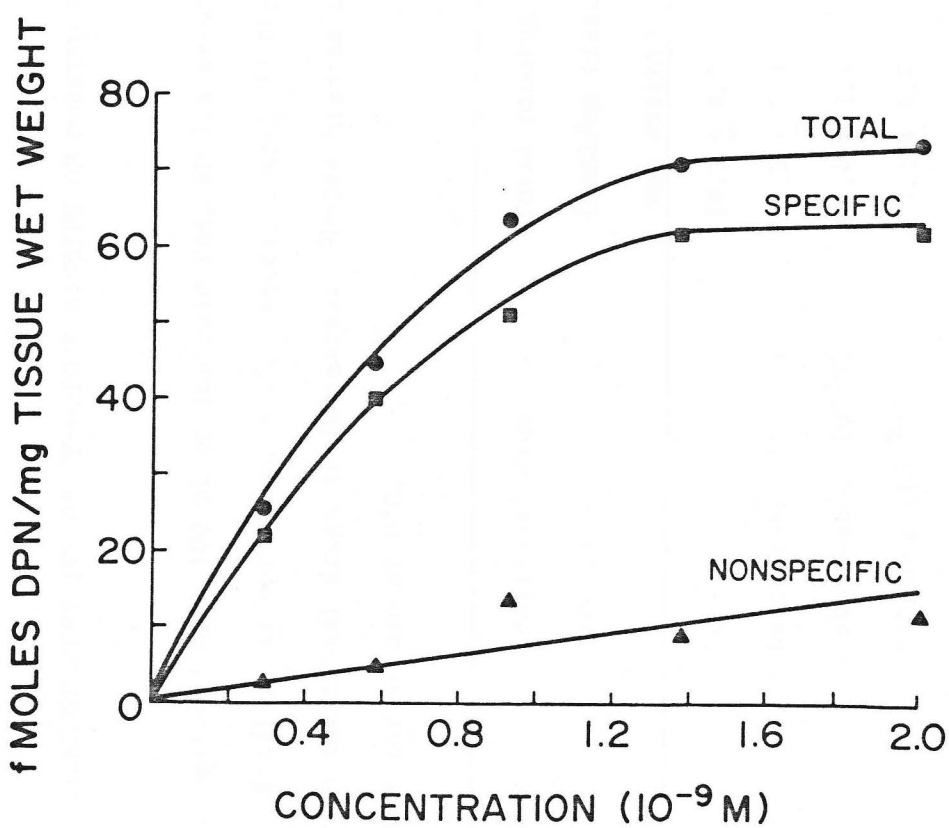


TABLE IV

STEREOSPECIFICITY OF ^{3}H-OPIOID BINDING ON MOUNTED TISSUE SECTIONS

Male rats were perfused with 100 ml of ice-cold PBS, pH 7.4 at 4°C containing 0.1% formaldehyde. Binding was performed on 10μ sections with ^{3}H-DPN or ^{3}H-DAMA as described in the text with additional drugs as indicated. ^{3}H-DPN binding proceeded for 20 min at 25°C and ^{3}H-DAMA for 40 min at 0°C.

Tritiated ligand (conc.)	Additional drug (conc.)	Total binding (fmol/mg tissue wet weight)	% of binding without drug
DPN (0.86 nM)	None	29.3 ± 4.5	100
DPN (0.86 nM)	Dextrorphan (10^{-7}M)	27.2 ± 1.7	93
DPN (0.86 nM)	Levorphanol (10^{-7}M)	4.4 ± 1.1	15
DPN (0.86 nM)	Naloxone (10^{-7}M)	7.3 ± 0.8	25

DPN (1.22 nM)	None	37.9 ± 3.7	100
DPN (1.22 nM)	Dextrorphan (10^{-6}M)	25.3 ± 3.5	67
DPN (1.22 nM)	Levorphanol (10^{-6}M)	4.8 ± 1.4	13
DPN (1.22 nM)	Naloxone (10^{-6}M)	8.6 ± 3.7	23
DAMA (3.97 nM)	None	20.0 ± 0.7	100
DAMA (3.97 nM)	Dextrorphan (10^{-7}M)	20.0 ± 0.9	100
DAMA (3.97 nM)	Levorphanol (10^{-7}M)	6.5 ± 1.1	33
DAMA (3.97 nM)	Naloxone (10^{-7}M)	8.1 ± 1.1	41
DAMA (3.80 nM)	None	20.2 ± 1.8	100
DAMA (3.80 nM)	Dextrorphan (10^{-6}M)	17.0 ± 0.1	84
DAMA (3.80 nM)	Levorphanol (10^{-6}M)	2.7 ± 0.4	13
DAMA (3.80 nM)	Naloxone (10^{-6}M)	3.2 ± 0.3	16

Since opiate receptor binding shows marked sensitivity to sodium, we tested the effects of high sodium concentrations (100 mM) on the binding of ^{3}H-LE, ^{3}H-DAMA and ^{3}H-DHM to mounted tissue sections. In all cases, we observed the depression of binding which had previously been reported using homogenates (Table V). A similar depression of agonist binding with 5 x 10^{-5} M GTP was observed as noted previously in membranes (Blume, 1978; Childers and Snyder, 1978). Sodium and GTP together eliminated opiate agonist binding.

In the experiments with the peptide binding, we were concerned that there might be some metabolism of the ligands. Accordingly, we extracted the radioactivity from the tissue sections and chromatographed the samples using ethyl acetate:pyridine:water:acetic acid (65:18:9:4). We never observed any metabolite with LE or diprenorphine under the conditions of the experiments. ^{3}H-DHM binding was performed in subdued light because of the reported photosensitivity of the compound.

When sections of cerebellum were placed on the slides, there was much less stereospecific binding as compared to frontal brain sections which is in agreement with biochemical assays using homogenates (Childers _et al._, 1978). ^{3}H-DPN binding revealed 2404 dpm/mg in

TABLE V

THE EFFECT OF SODIUM ION ON ^{3}H-OPIOID BINDING

Male rats were perfused with 100 ml of ice-cold PBS, pH 7.4. Ten μ sections were preincubated for 30 min in 60 mM Tris-HCl, pH 7.4, at 9°C containing either 100 mM NaCl or KCl. Incubations with tritiated ligands in the same buffered salt solutions were performed for 40 min at 0°C and then washed for 10 min at 0°C in the same buffered solutions as described in the text.

Tritiated ligand	Specific binding (fmol/mg tissue wet weight)	
	KCl	NaCl
LE (3.17 nM)	17.4 ± 1.0	8.3 ± 1.8*
DAMA (4.08 nM)	14.2 ± 0.9	5.2 ± 1.4*
DHM (3.60 nM)	13.1 ± 2.4	5.4 ± 1.7*

*NaCl-treated differ from KCl-treated $p < 0.001$ by t statistic for two means.

striatal/cortical and 265 dpm/mg in cerebellar sections. When mounted tissue sections were preincubated with Tris-HCl buffer for 30 min at 25°C or for 5 min with 5 x 10^{-5} M GTP and 100 mM NaCl with 15 min additional wash at 25°C (prior to incubation with ^{3}H-opioids), there was no change in total binding compared to nonpreincubated tissue sections [however, this preincubation is necessary for autoradiography as discussed in Young and Kuhar (1979)].

Benzodiazepine Biochemistry

Before autoradiographic studies were performed, we also critically examined biochemically the binding characteristics of ^{3}H-FLU to the slide-mounted tissue sections. As mentioned above, we routinely employed a 0.1% formaldehyde fixation which improved tissue morphology (Young and Kuhar, 1979c) but did not alter the K_D or B_{max} of BZR either in rat tissue homogenates or slide-mounted tissue sections. We also found that preincubation for up to 30 min in buffer at 25°C did not significantly improve ^{3}H-FLU binding in subsequent incubations. Thus, no preincubation was used.

The dissociation of ^{3}H-FLU from the slide-mounted tissue sections was examined. Coronal sections of rat forebrain (10μ) containing caudate nucleus and septum were incubated with 1 nM ^{3}H-FLU for 40 min at ice-water temperatures. The tissue sections were then transferred to

buffer for various lengths of time. Following the wash, the radioactivity was measured in the sections as described above. ^{3}H-FLU dissociated from the receptor with half-times of 19 min at 0°C and 5.8 min at 22° C (Figure 12). The best ratios of specific to nonspecific binding (20:1) were observed after about 10 min of wash and this length of wash was used routinely for the following biochemical experiments.

When the association of ^{3}H-FLU to receptors was examined, we observed that binding reached equilibrium by about 20 min (Figure 13). Thus, 40 min incubations were routinely used for subsequent experiments to insure equilibrium labeling.

The kinetics of binding of ^{3}H-FLU in these preparations revealed a K_D of 2.79 $\pm$ 0.65 nM (mean $\pm$ s.d., n = 4) and a B_{max} of 210 $\pm$ 36 fmol/mg tissue wet weight (n = 4) by Scatchard analysis (a typical experiment is shown in Figure 14). Hill plots of these data revealed Hill coefficients of unity.

Pharmacological specificity studies were also performed using these thaw-mounted tissue sections. The previously reported stereospecificity of the binding was reproduced

Figure 12

The dissociation of ^{3}H-FLU from tissue sections. The tissues were incubated in 1 nM ^{3}H-FLU in 0.17 M Tris-HCl, pH 7.4. with or without 10^{-6} M clonazepam for 40 min at 0°C. The sections were then rinsed for the indicated times, wiped off and counted as described in the text (□ ■, total;△ ,▲ , specific; 0,●. nonspecific). This experiment was repeated three times.

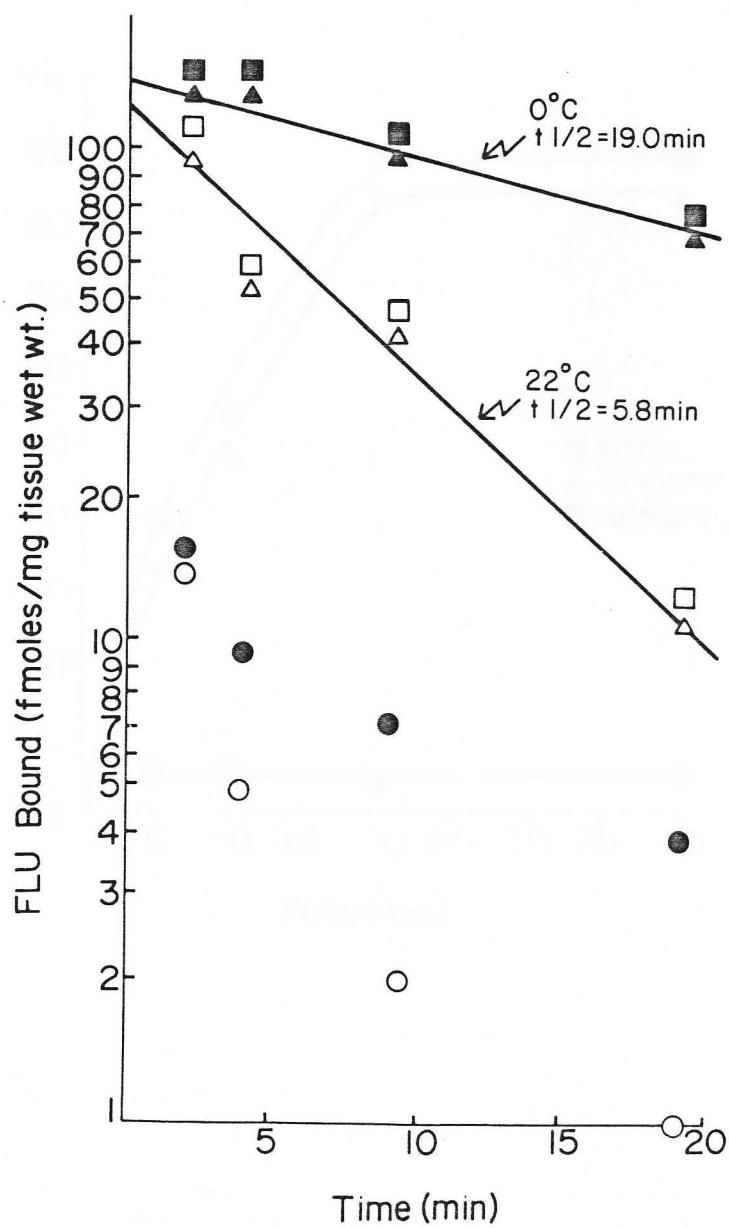


Figure 13

The association of ^{3}H-FLU to tissue sections. The tissues were incubated in 1 nM ^{3}H-FLU with or without 10^{-6} M clonazepam at 0°C for the indicated times, washed for 10 min and processed as described in Figure 12 and the text. This experiment was repeated twice.

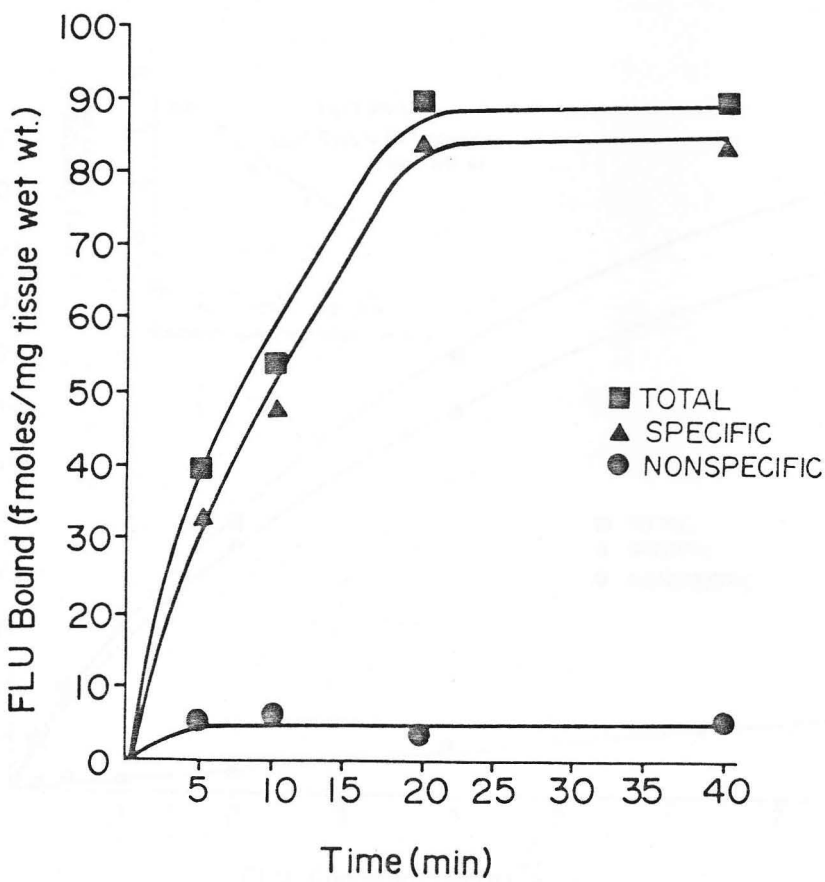
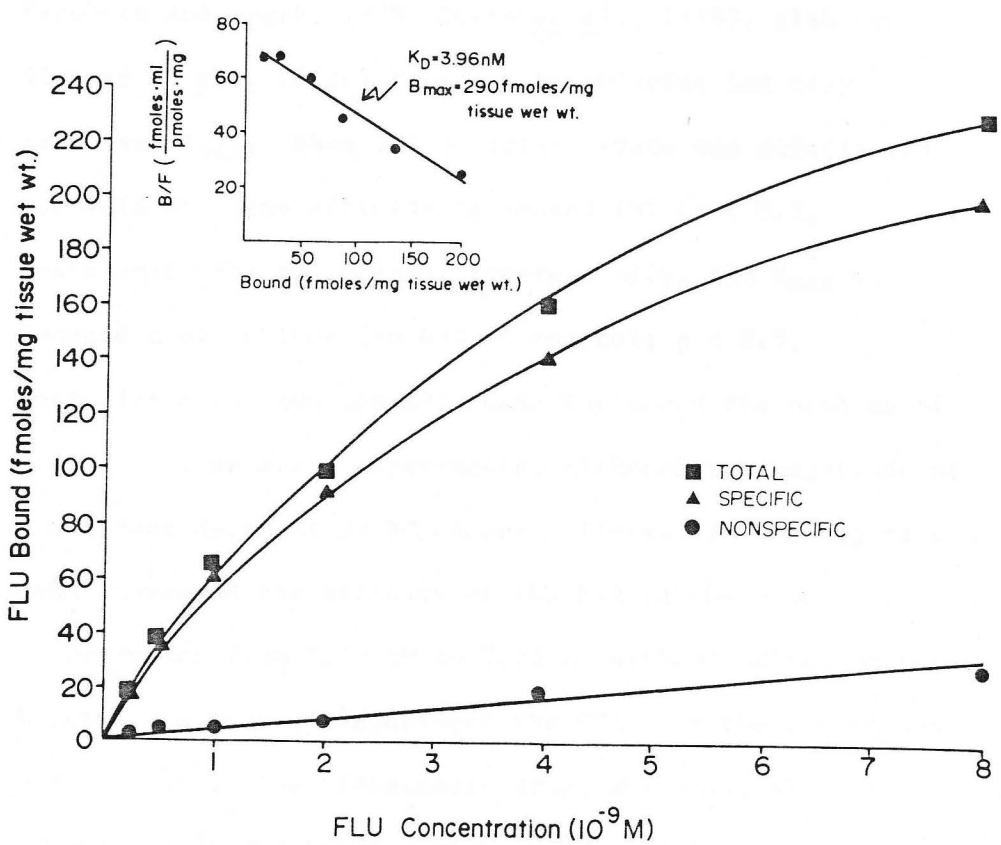


Figure 14

Saturation of ^{3}H-FLU binding in tissue sections. Tissues were incubated in varying concentrations of ^{3}H-FLU for 40 min at 0°C and processed as described in Figure 13 and the text. This experiment was repeated three times.



here. The clinically active BZ, (+)B10, inhibited ^{3}H-FLU binding with an IC_{50} of 8 nM. The inactive stereoisomer, (-)B10, was 15-fold less potent. In addition, numerous drugs at 10^{-6}M, such as levorphanol, norepinephrine, spiroperidol, atropine, bufotenine, triprolidine, glutamate, and kainate had no effect on ^{3}H-FLU binding.

Chloride ion and GABA have been reported to stimulate benzodiazepine receptor binding in homogenates by increasing the affinity (Tallman et al., 1978; Martin and Candy, 1978; Karobath and Sperk, 1979; Costa et al., 1979), although Klepner et al., (1980) report that chloride ion only increased B_{max}. When 309 mM Tris-citrate was substituted for Tris-HCl, the affinity decreased 19% ($p < 0.5$, t-statistic for two means). Interestingly, the B_{max} was reduced even further (to 68% of control; $p < 0.5$, t-statistic for two means). GABA increased the binding of BZR in four separate experiments, although the magnitude of the effect depended on BZ concentrations. In one experiment GABA increased the affinity of the BZR in the absence of chloride ion from 2.79 nM to 2.23 nM without affecting total binding. In another experiment the EC_{50} for the effect was 4.3×10^{-6} M. The GABAmimetic drug, muscimol, also stimulated ^{3}H-FLU binding with an ED_{50} of 1.2×10^{-6} M. The effect of GABA was blocked by (+)bicuculline with an IC_{50} of 12.5×10^{-6} M. Thus, even very subtle

ionic and pharmacological properties of the BZR were maintained and evident in these slide-mounted tissues sections prepared for autoradiographic studies.

Autoradiography

Preliminary Studies

We examined several autoradiographic procedures preliminarily before selecting the emulsion-coated coverslip technique. Various attempts to dip ^{3}H-DPN labeled sections into NTB3 emulsion at 30° or 42°C produced excessive diffusion and various artifacts in the emulsion. Although a crude regional distribution was retained, the crisp boundaries of patches in the rat striatum for instance, were obliterated.

We tried using Kodak AR10 stripping film which permits one to apply a 20μ thick nuclear emulsion over the tissue section. This method also produced variable results, probably because the water was retained in the tissue and film causing diffusion.

The conditions under which the method of Roth *et al.* (1974) was linear were examined. This was done in order to determine in what ranges of tissue radioactivity and times of exposure were the autoradiograms quantitative. In addition, the effect of tissue thickness on self-absorption of radiation was studied.

As can be seen in Fig 15, grain density over the cortex produced in the autoradiograms was linear with tissue to approximately 20,000 dpm/mg tissue wet weight. After this amount, the grain density seemed to flatten out indicating that the emulsion was being saturated by emissions. The emulsion also responded linearly to this amount of radioactivity (20,000 dpm/mg) for at least 6 mo (Fig 16). In addition, the grain density off the tissue (background) also increased with time but at a very slow rate (Fig. 16).

The β-emissions seemed to reach maximum efficiency of transmission by a tissue thickness of 8μ . (Fig. 17). This is in agreement with the tissue absorption observed by Falk and King (1963) who observed maximum efficiency at 6μ or more.

Different emulsions and emulsion development times were examined. We routinely used Kodak NTB3 diluted 1:1 with distilled water and developed in Dektol for 2 min as described in METHODS. In one experiment, this resulted in 74.5 grains/1000μ^2 over the molecular layer of the cerebellum after incubation in 1 nM ^{3}H-FLU and exposure for 2 weeks. The grain count off the tissue was 2.8 grains/1000μ^2. If NTB2 (1:1 with water) was used, the corresponding counts were 118.3 and 6.7. Similarly when Ilford emulsion (1:1 and 2:1 with water) was used, the counts were 71.8 and 115.0 over the tissue and 2.2 and 3.4 off the tissue for the two dilutions, respectively. The

Figure 15

Emulsion response as a function of tissue radioactivity. Coronal tissue sections (10μ) through the caudate-putamen were incubated in 0.10, 0.51, 1.03 and 5.13 nM ^{3}H-FLU for 40 min at 0°C, washed with two 5-min rinses and either wiped off and counted or processed for autoradiography. After a 2 week exposure, grains were counted in lamina V of the dorsomedial cortex.

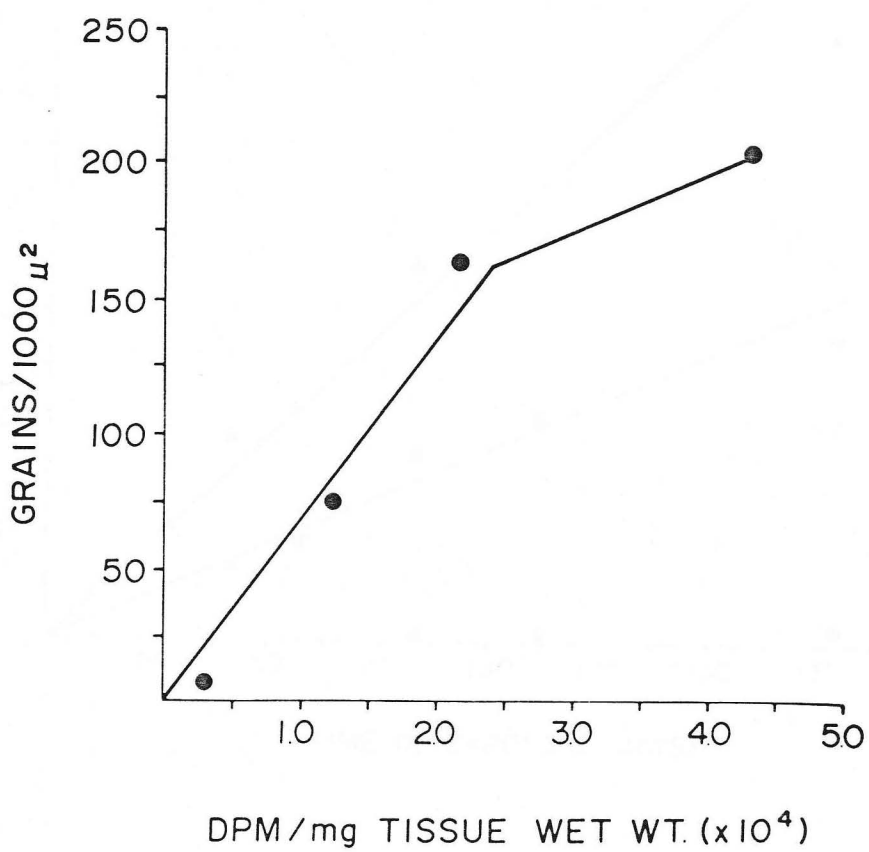


Figure 16

Emulsion response as a function of time of exposure. Cerebellar tissue sections (4μ) were incubated in 0.97 nM ^{3}H-FLU for 40 min at 0°C and processed for autoradiography as described in Figure 15 and text. Grains were counted after various exposure durations in the molecular layer (▲), granule cell layer (■), and off the tissue (background,●).

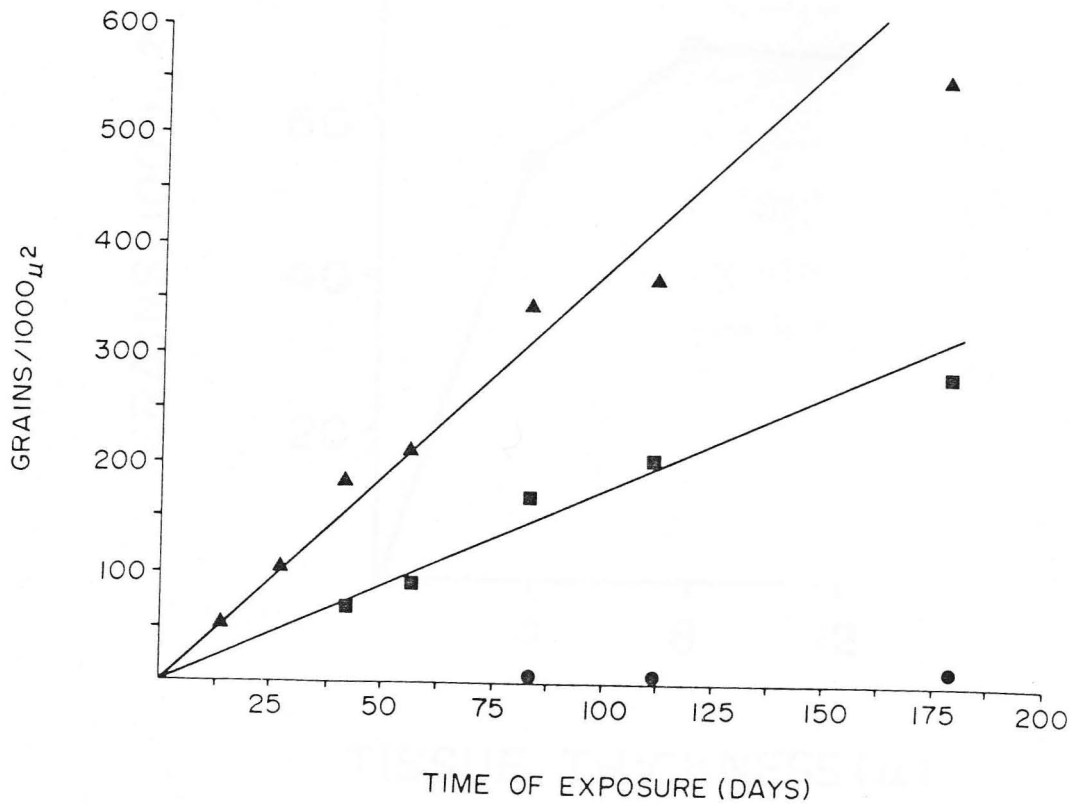
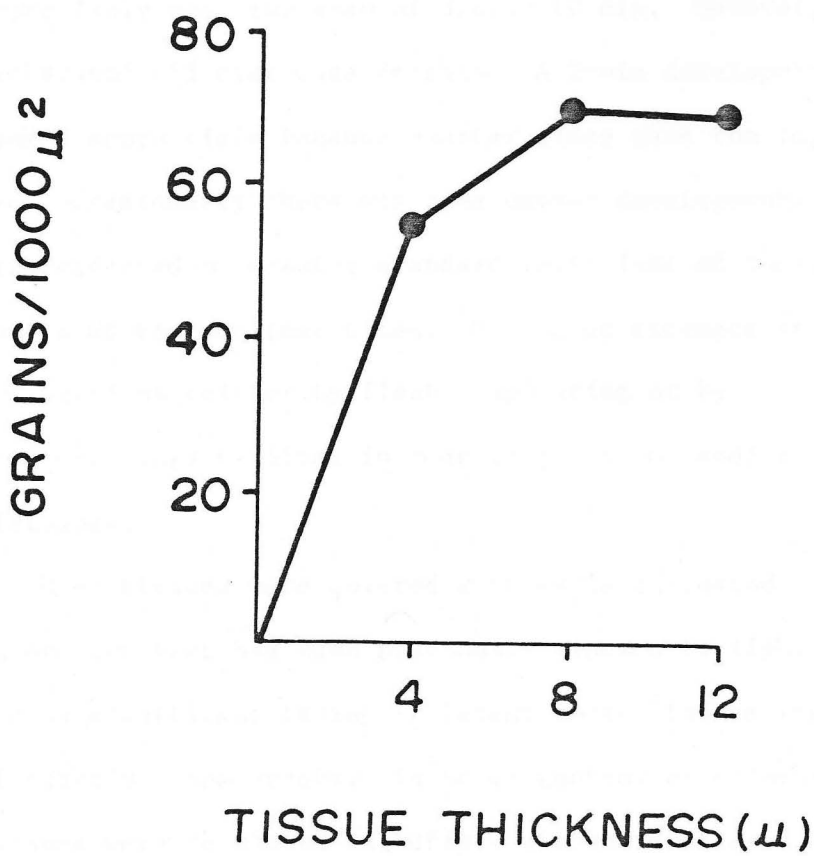


Figure 17

Emulsion response as a function of tissue thickness. Cerebellar tissue sections (4, 8, 12μ) were incubated in 0.97 nM ^{3}H-FLU and processed as described in Figure 15 and text. Grains were counted in the molecular layer after a 2 week exposure.



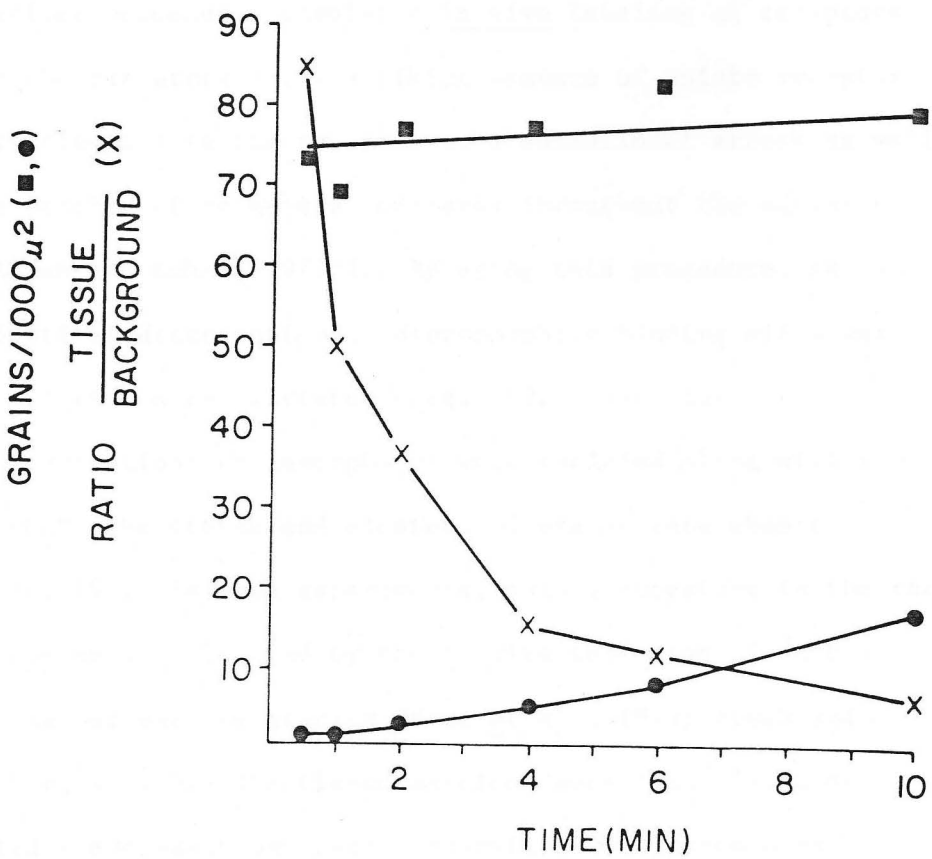
NTB2 and L4 (2:1) emulsions were approximately equal in sensitivity but the NTB2 had a higher background. When expressed as a ratio of counts over tissue to off tissue, the Ilford emulsions were 50% better than the Kodak NTB3 emulsions and the NTB3 was approximately 50% better than the NTB2 emulsion. Unfortunately, the difficulty in obtaining Ilford emulsions dictated against their use.

The effect of different development times is plotted in Figure 18. The net grain count over tissue did not rise appreciably over the span of 30s to 10 min. However, the background did rise considerably. A 2-min development time seemed appropriate because shorter times gave the impression that occasionally there was some uneven development. This was evidenced by greater standard deviations of the grain counts at the earliest times. Our other attempts at drying the sections (either by flash evaporating or by freeze-drying) resulted in poor localization and/or histology.

When tissues were covered with emulsion-coated coverslips that had been previously exposed to light, there was no significant fading of latent images indicating a lack of negative chemography. In other control experiments, tissues were incubated in buffers without tritiated ligands. Upon development, there was no artifactual production of autoradiographic grains (except occasionally over ependymal

Figure 18

The effect of different development times on the grain counts. Six μ cerebellar tissue sections were incubated in 1 nM ^{3}H-FLU and processed as described in Figure 15 and text except that the time in the Dektol varied. Grains were counted in the molecular layer minus off-tissue background (■) and off the tissue (●). Additionally, the ratios of net counts over tissue to off tissue were plotted (X).



cells) indicating an absence of positive chemography. The ^{3}H-opioid and ^{3}H-FLU autoradiograms showed levels of grains in white matter which were the same as seen in sections produced as blanks (excessive cold ligand present during the incubation).

Opioid Autoradiography

Coronal sections from frontal rat brain were incubated with various ^{3}H-opioids. In these experiments, we sought to label tissues with ^{3}H-DPN and compare the distribution obtained by this present method with that obtained by our earlier procedures involving in vivo labeling of receptors. In the rat striatum, a striking feature of opiate receptor distribution is the presence of a subcallosal streak as well as patches of receptors scattered throughout the striatum (Atweh and Kuhar, 1977c). By using this procedure, an identical distribution of diprenorphine binding sites was found in the rat striatum (Fig. 19). When high concentrations of levorphanol were included along with the ^{3}H-DPN, the streak and clusters of grains were absent (Fig. 19). In some experiments, opioid receptors in the rat striatum were labeled by the in vivo injection of ^{3}H-DPN as in our earlier studies (Pert et al., 1975; Atweh and Kuhar, 1977c). The tissue sections were then placed on slides and emulsion-coated coverslips were apposed as described above, rather than thaw-mounting the sections onto emulsion-coated slides as was done in the

Figure 19

Bright- and darkfield photomicrographs of autoradiograms of ^{3}H-DPN binding in rat tissue sections. Four μ, formaldehyde-treated sections were prepared, incubated in 4.22 nM ^{3}H-DPN for 10 min at 25°C and washed for 10 min at 0°C as described in the text. The adjacent section shown in the darkfield on the right had 10^{-6} M levorphanol present during incubation. Emulsion-coated coverslips were apposed for 4 weeks and then processes as in Figure 1 and as described in the text. Note the dense streak of grains below the corpus callosum (CC) and clusters of grains in the caudate-putamen (cp) of the darkfield photomicrograph on the left. This view is from level A7470μ of Konig and Klippel (1963). The levorphanol eliminated most specific binding as demonstrated in the darkfield photomicrograph on the right. The results were reproduced in 3 separate experiments. Bar equals 50μ.

cc
cp
cc
cp
cc
cp

earlier studies. Again, we found a distribution of receptors identical to that found in our earlier procedures and to that presented above by *in vitro* labeling.

As it is not possible to examine the distribution of most opioid peptides by *in vivo* procedures because they do not cross the blood-brain barrier or are metabolized before reaching the brain, we utilized mounted consecutive tissue sections incubated with ^{3}H-DAMA and ^{3}H-DHM (agonists) and with ^{3}H-DPN (antagonist) for autoradiography. In these experiments, the typical opiate receptor distribution in the rat striatum was observed (Fig. 20). In addition, the grain distributions were identical for the three ligands. When the ^{3}H-ligands were incubated with 10^{-6} M naloxone, the typical patchy distribution of binding sites in the rat striatum was blocked.

The *in vivo* technique also suffered from edge artifacts - the presence of grains along the edge of tissues. This limited to a small extent the analysis that could be performed in these regions. The *in vitro* technique appears to be relatively free of this artifact and we can now state that there is ^{3}H-opioid binding in layer I of the rat cerebral cortex and in the pyriform cortex, for example (Fig. 21).

Figure 20

Bright- and darkfield photomicrographs of ^{3}H-opioid and opiate binding in consecutive tissue sections from rat. Details of procedure and abbreviations as in Figure 19. The brightfield (B) shows part of the rat brain from level A8000μ (König and Klippel, 1963). Consecutive sections were incubated with ^{3}H-DAMA (A; 4.3 nM, 53 min at 0°C), ^{3}H-DPN (C; 1.1 nM, 20 min at 25°C), and ^{3}H-DHM (D; 4.0 nM, 53 min at 0°C). Notice the nearly identical patterns of grains in the three darkfield photomicrographs. Sections incubated in the presence of 10^{-6} M naloxone revealed negligible binding. This experiment was repeated once more. Bar equals 50μ .

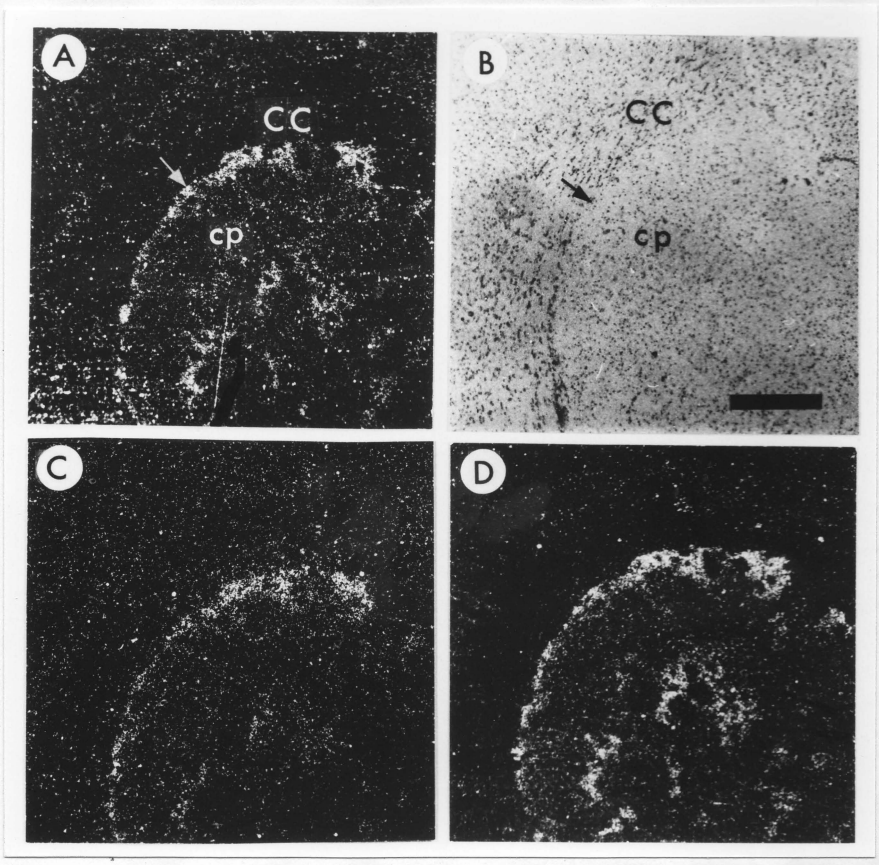

A
CC
cp
B
CC
cp
C
D

Figure 21

Darkfield photomicrograph of ^{3}H-DHM binding in rat. This coronal hemisection through a rat brain (A8600μ, Konig and Klippel, 1963) was incubated with ^{3}H-DHM (4.0 nM for 53 min at 0°C) and processed as described in the text and Figure 19. The subcallosal streak (long arrows) and clusters in the caudate-putamen (cp) are similar to those seen in Figures 19 and 20. An absence of edge artifact with this technique and the use of a "brain paste" to secure the tissue onto the microtome chuck lends authenticity to the increased binding seen in the pyriform cortex (double arrows) and layer I of the cerebral cortex (single arrows). Binding is also elevated in layer IV and areas 6 and 24 of the cortex (Krieg, 1946a,b) as well as the claustrum (CL) and olfactory tubercle (TULP). Long arrows are over the "brain paste". Bar equals 100μ.

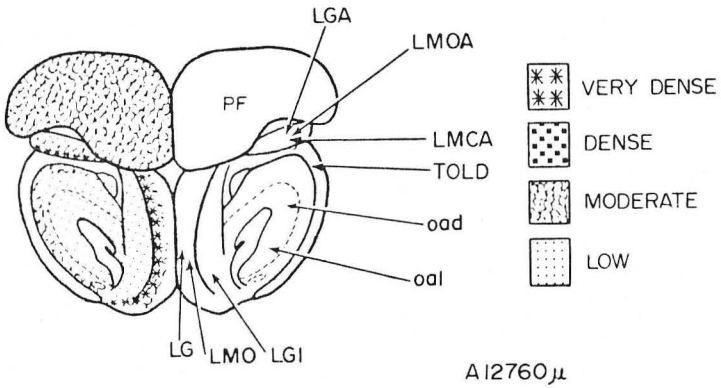
While the bulk of our studies involved these coronal sections of rat frontal cortex because of the rather striking distribution of receptors there, we also examined ^{3}H-DPN binding and autoradiography in other regions of rat brain. The distribution obtained by the present procedure was always the same as that found in earlier studies (Atweh and Kuhar, 1977a,b,c).

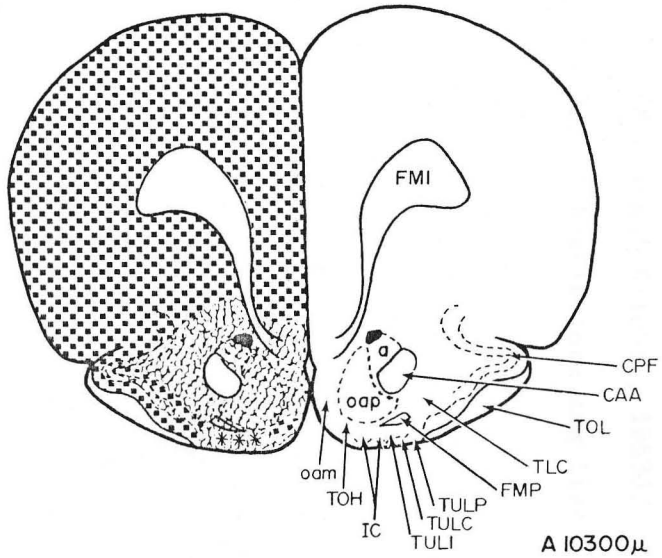
Benzodiazepine Autoradiography

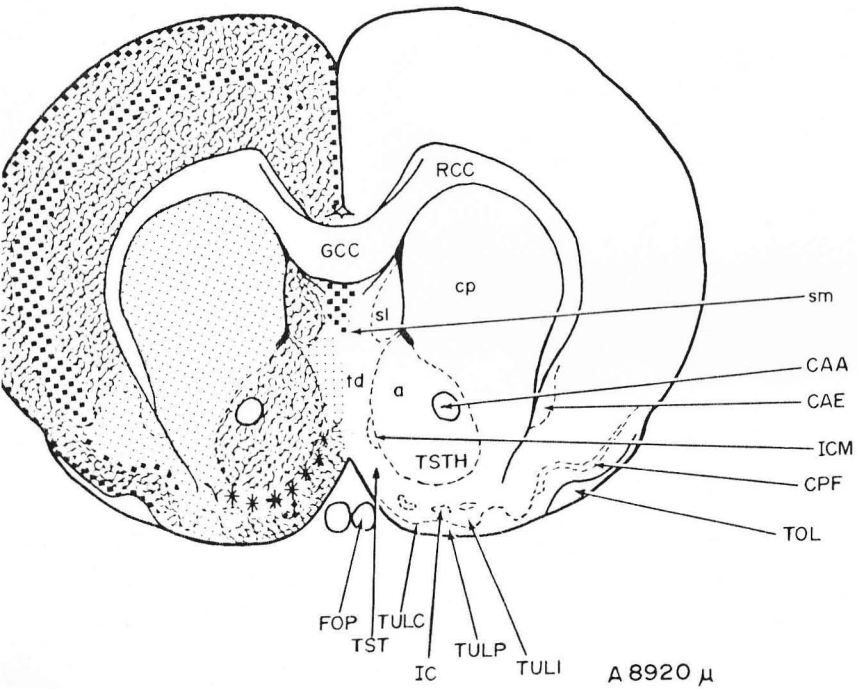
The radiohistochemical localization of benzodiazepine receptors (BZR) with ^{3}H-FLU varied dramatically in the rat brain. Some of the highest densities were observed in the rhinencephalon. In the olfactory part, the molecular layer of the olfactory bulb as well as the mitral cell layer of the accessory olfactory bulb revealed very high levels of BZR. Dense levels were observed in the insulae Calleja and the nucleus olfactorious while relatively few receptors were present in the rest of the olfactory lobe (Figure 22). In the limbic lobe, the greatest densities were seen in the region of the rostral median forebrain bundle (FMP; Figure 22). High densities were also seen in the hippocampus, dentate gyrus, pyriform cortex, nucleus septi medialis, and nuclei amygdaloideus centralis, medialis and lateralis (Figures 22, 23 and 25). The rest of the telencephalon contained only low to moderate densities of BZR with a few exceptions. The frontal cortex at the level of the forceps minor as well as lamina IV at most levels had dense levels.

Figure 22

Schematic mapping of BZR in rat brain, A6360 to 12,760μ (König and Klippel, 1963). Tissues were incubated in 1 nM ^{3}H-FLU for 40 min at 0°C, washed for 2 min and processed as described in the text. Exposure duration was 2 weeks. Five density levels are represented: very dense > 200 grains per 1000μ ^{2}; dense 170 to 200; moderate, 100 to 170; low, 10 to 100; and background <10. Abbreviations are listed in the appendix.


LGA
LMOA
PF
LMCA
TOLD
oad
oal
LG
LMO
LGI
VERY DENSE
DENSE
MODERATE
LOW
A 12760 μ


FMI
a
oap
CPF
CAA
TOL
TLC
FMP
oam
TOH
IC
TULP
TULC
TULI
A 10300 μ


RCC
GCC
cp
sm
sl
td
a
CAA
CAE
TSTH
ICM
CPF
TOL
FOP
TST
TULC
IC
TULP
TULI
A 8920 μ

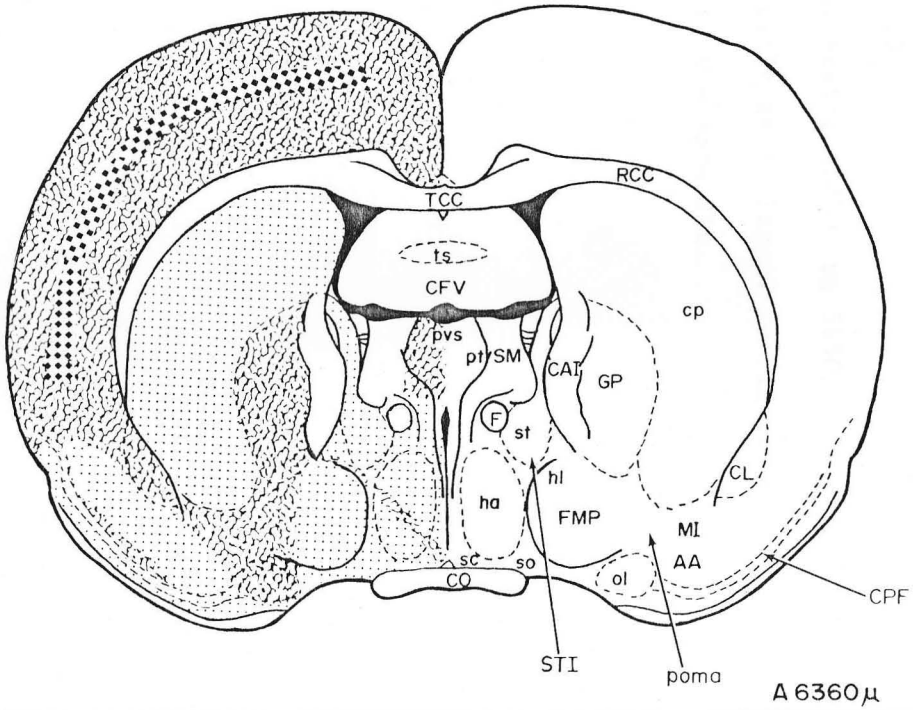
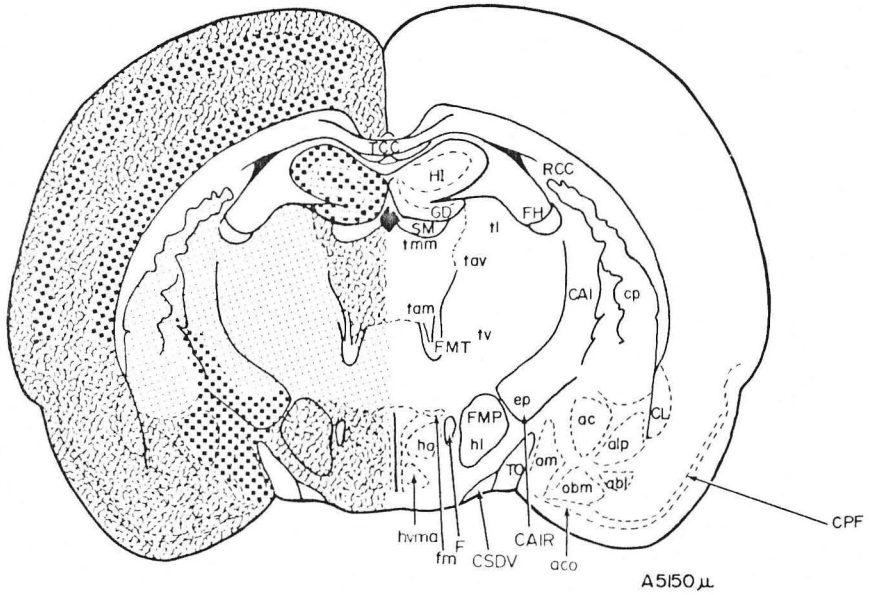
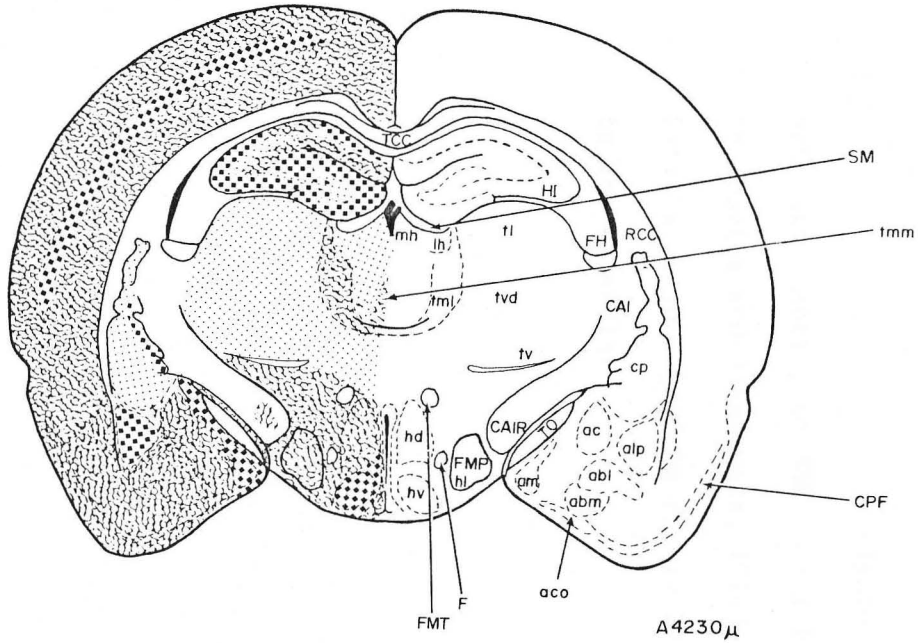

TCC
RCC
ts
CFV
cp
pvs
pt
SM
CAI
GP
F
st
hl
ha
FMP
CL
MI
AA
sc
so
CO
ol
CPF
STI
poma
A 6360 μ

Figure 23

Schematic mapping of BZR in rat brain, A1270 to 5150μ (König and Klippel, 1963). Details as in legend to Figure 22. Abbreviations are listed in the appendix.


TCC
HI
RCC
GD
SM
FH
tmm
tl
tav
CAI
cp
tam
tv
FMT
FMP
ep
ac
CL
hg
hl
alp
TO
am
abm
abl
hvma
fm
F
CSDV
CAIR
aco
CPF
A5150μ


SM
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TCC
HI
mh
lh
tl
FH
RCC
tml
tvd
CAI
tv
cp
hd
CAIR
ac
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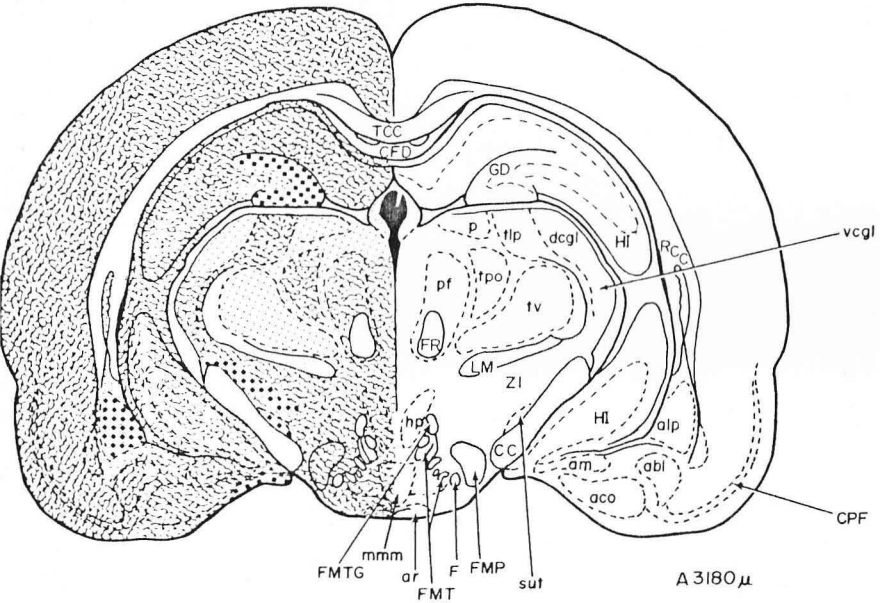
hv
abm
CPF
FMT
F
aco
A4230μ


TCC
CFD
GD
vcgl
p
tlp
dcgl
HI
RCC
pf
tpo
tv
FR
LM
ZI
hp
HI
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mmm
FMTG
ar
F
FMP
FMT
sut
A3180μ

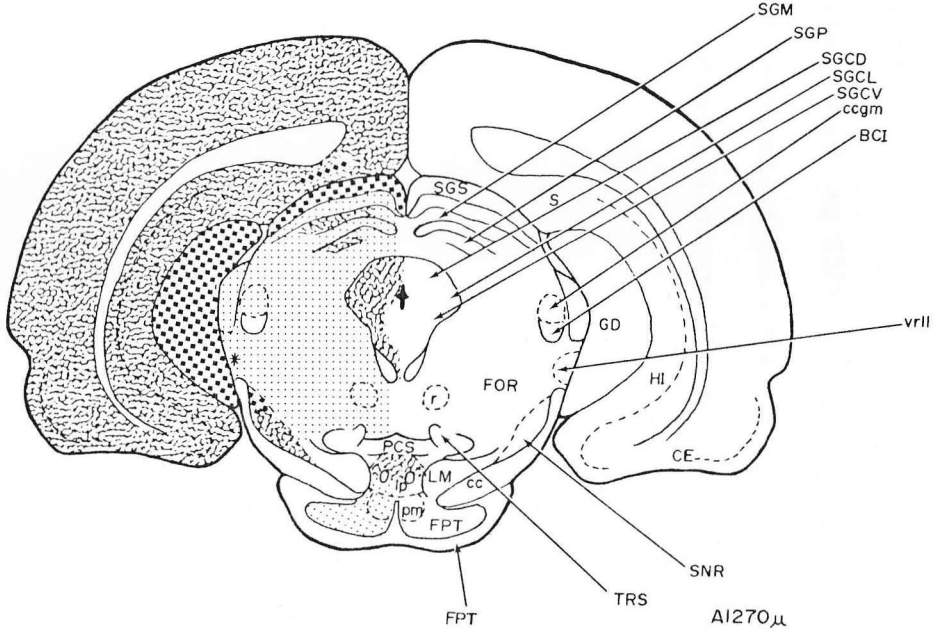
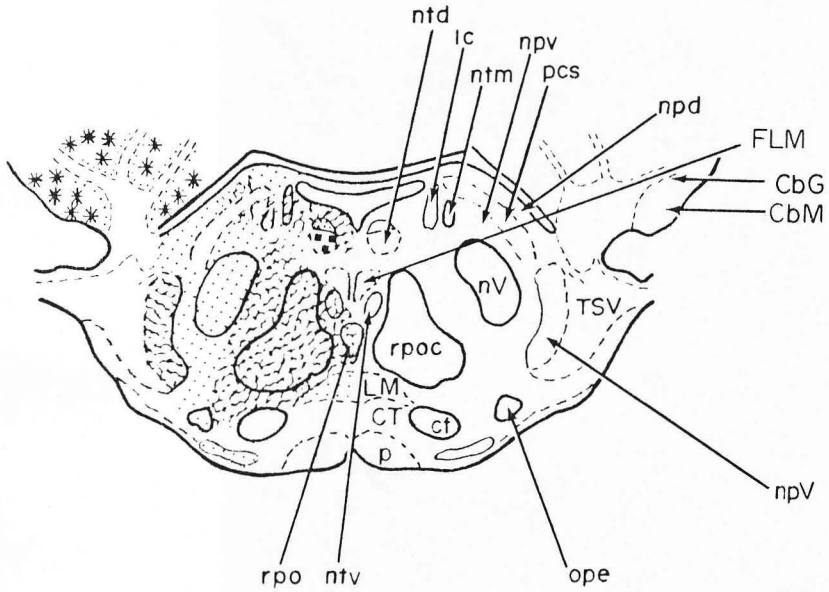
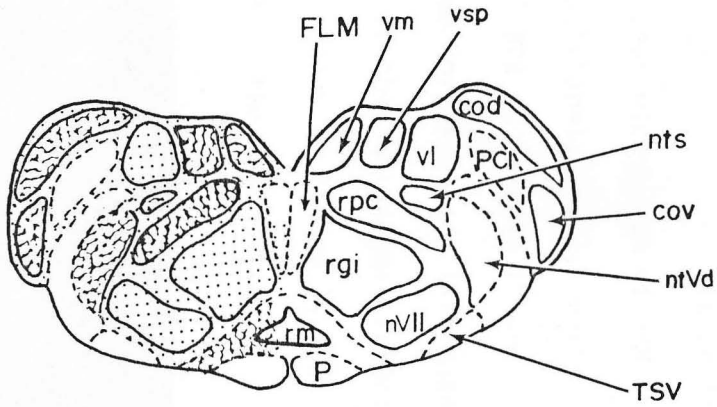

SGM
SGP
SGCD
SGCL
SGCV
ccgm
BCI
SGS
S
GD
vrll
FOR
HI
CE
PCS
LM
cc
pm
FPT
SNR
TRS
FPT
A1270μ

Figure 24

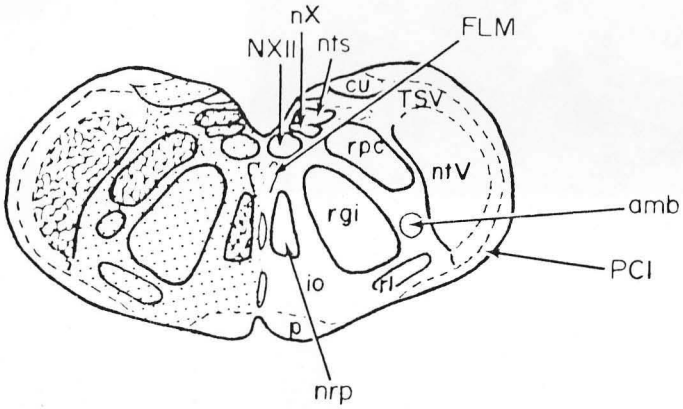
Schematic mapping of BZR in rat brain, P8000 to 2300μ (Palkovits and Jacobowitz, 1974) and cervical spinal cord. Details as in legend to Figure 22. Abbreviations are listed in the appendix.



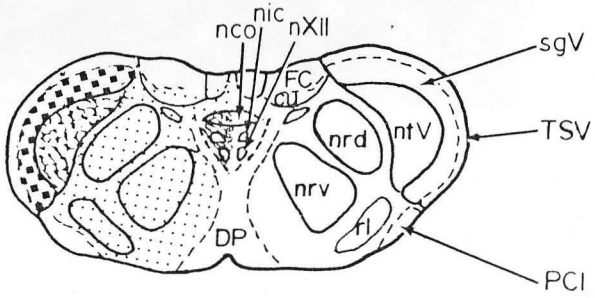
P2300μ



P4500μ



P6500μ



P8000μ

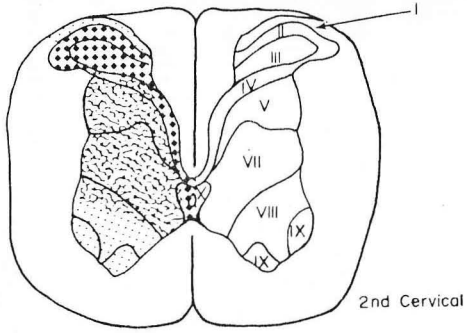
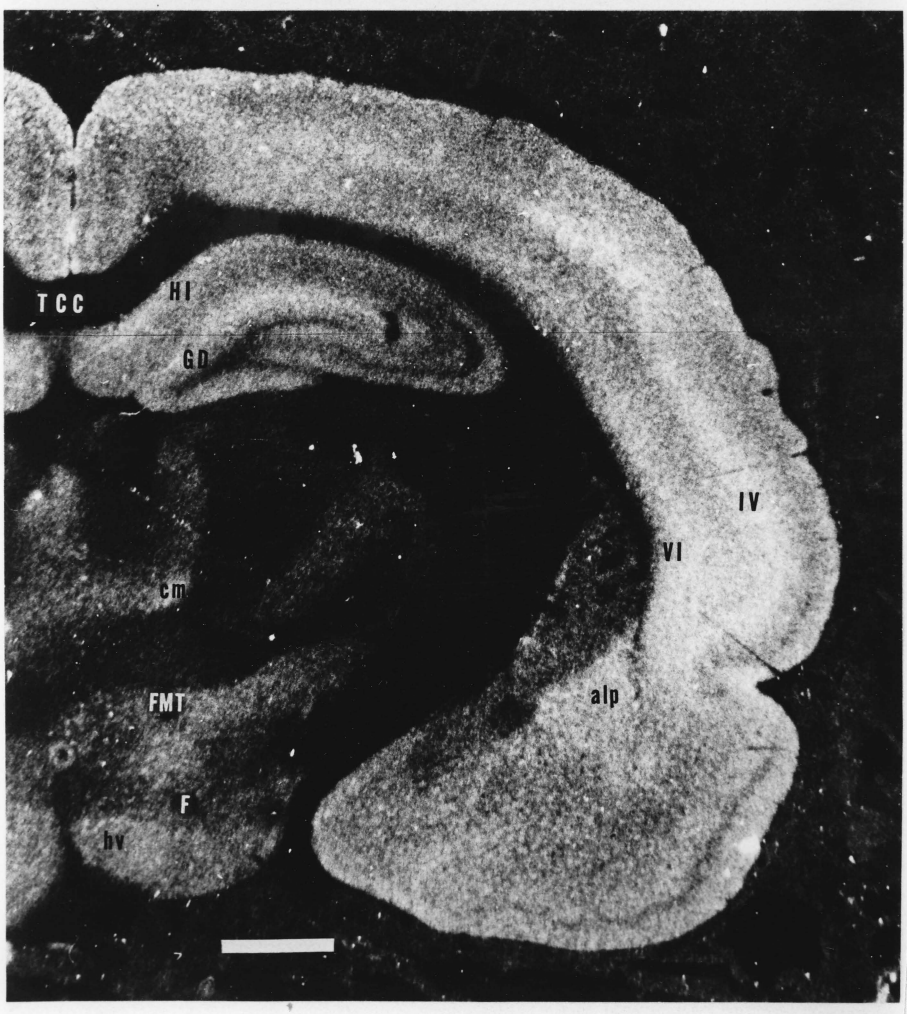


Figure 25

Darkfield photomicrograph of BZR in rat brain. A3990μ (König and Klippel, 1963). Notice the particularly high amounts of BZR in laminae IV and V of the cortex as well as the nucleus amygdaloideus lateralis, pars posterior (alp) and parts of the hippocampal formation (GD and HI). Details are as in legend to Figure 22. Bar = 1000μ. Abbreviations are listed in the appendix.



Laminae I and VI were occasionally elevated (Figures 22, 23, 25 and 26). Of the basal ganglia structures, only the nucleus entopeduncularis had dense amounts of BZR (Figures 22 and 23). The diencephalon exhibited several areas of dense BZR concentrations. These areas included the nucleus preopticus lateralis, nucleus ventromedialis hypothalamus, nucleus mamillaris lateralis, nucleus periventricularis rotundocellularis, nucleus subthalamicus and zona incerta (Figures 22, 23 and 25). Again, the bulk of this part of the rat forebrain contained relatively sparse amounts of BZR.

The hindbrain had even lower levels of BZR with several exceptions. One of the highest densities in the brain was found in the nucleus ventralis rostralis lemnisci lateralis (Figures 23 and 26). The inferior colliculus also showed a very high density of BZR. In the mesencephalon, high levels were seen in the stratum griseum superficiale colliculi superioris and the substantia nigra, pars lateralis (Figures 23 and 26).

In the rest of the rat brain, the molecular layer of the cerebellum had uniformly very high levels of BZR. Dense amounts were seen in the nucleus dentatus of the cerebellum, nuclei tegmenti dorsalis and ventralis of Gudden, substantia gelatinosa trigemini and laminae II, III, IV and X of the spinal cord (Figures 24 and 27). The inner plexiform layer

Figure 26

Darkfield photomicrograph of BZR in rat brain, A1270 μ (König and Klippel, 1963). Highest levels of BZR are in the gyrus dentatus (GD), substantia nigra, pars lateralis (SNL), cortex and superficial layer of the superior colliculus (SGS). Details as in legend to Figure 22. Bar = 1000 μ. Abbreviations are listed in the appendix.

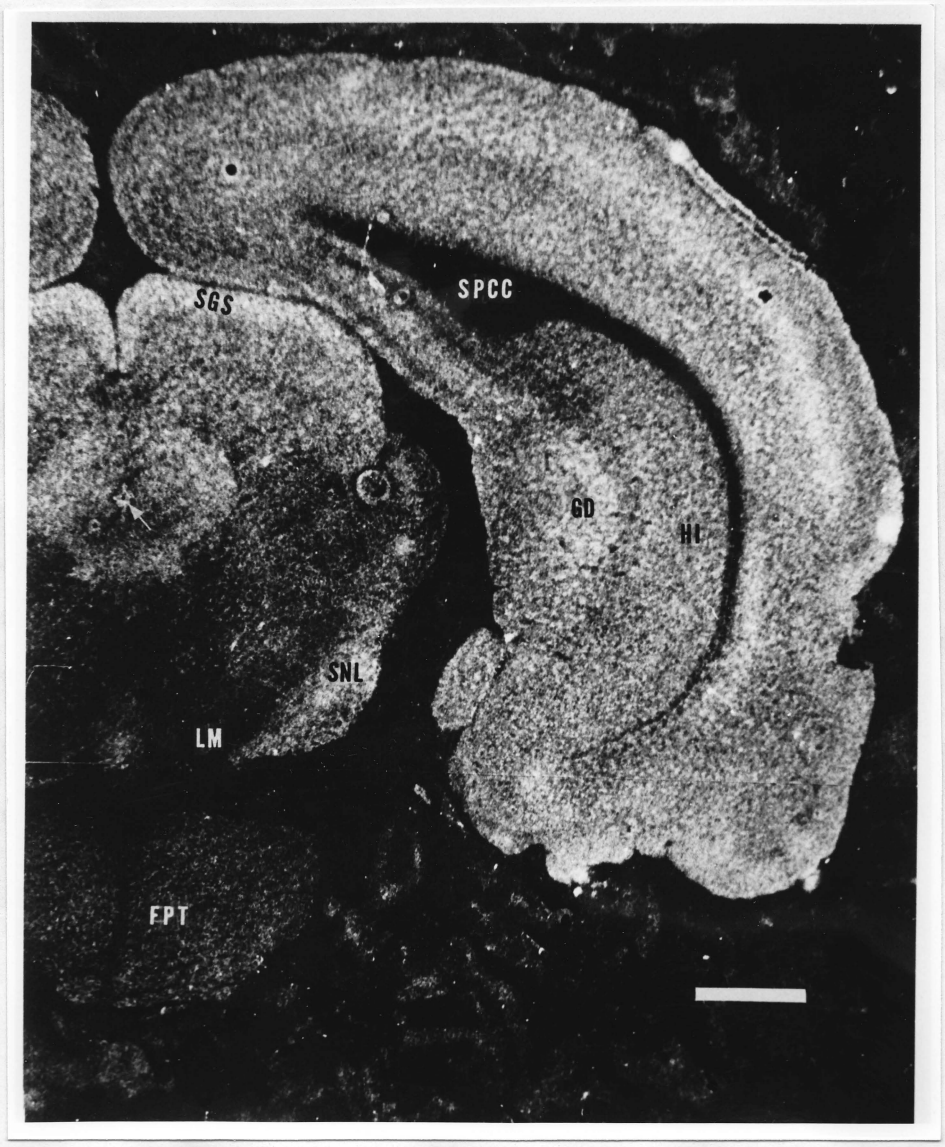
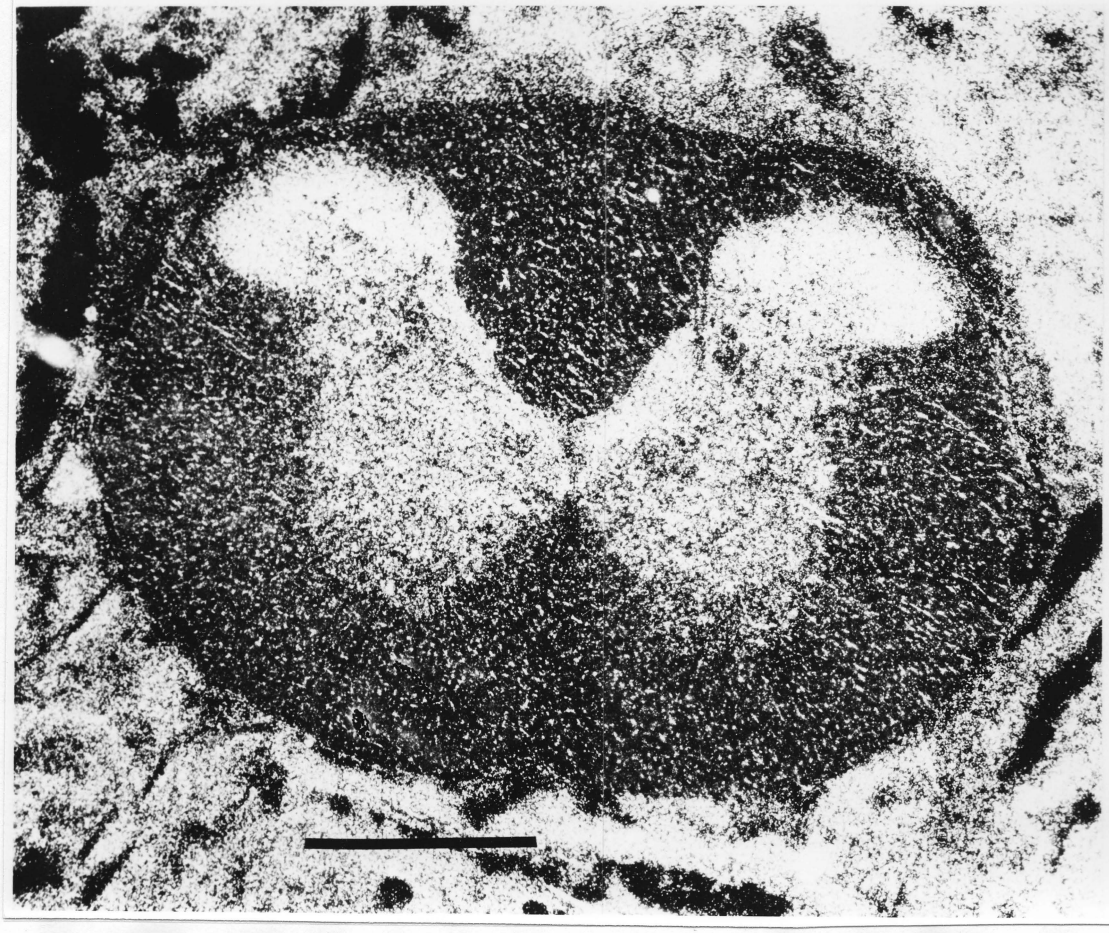


Figure 27

Darkfield photomicrograph of BZR in upper cervical spinal cord of rat. Details as in legend to Figure 22. The binding outside the spinal cord is in the mounting media, brain paste. Bar = 1000 μ.



of the retina was also labeled.

In human tissues, we examined the autoradiographic distribution of BZ receptors in cerebellar and cerebral cortical areas including the superior frontal, the precentral and the calcarine areas of the cortex. In all of these areas there was a striking absence of BZ receptors in the subcortical white matter. In the cerebellum, there was a high density of receptors in association with both the granule cell layer and the molecular layer. There was a slight reduction in receptor density in the Purkinje cell layer (Figure 28). In the cerebral cortical areas, there were variations in autoradiographic grain densities. The receptor density in the calcarine cortex, for example, was highest in Brodmann (1905) layers III, IVA and IVC (Figure 29). This striking variation emphasizes the value of this technique in the study of the neuroanatomical aspects of receptor localization.

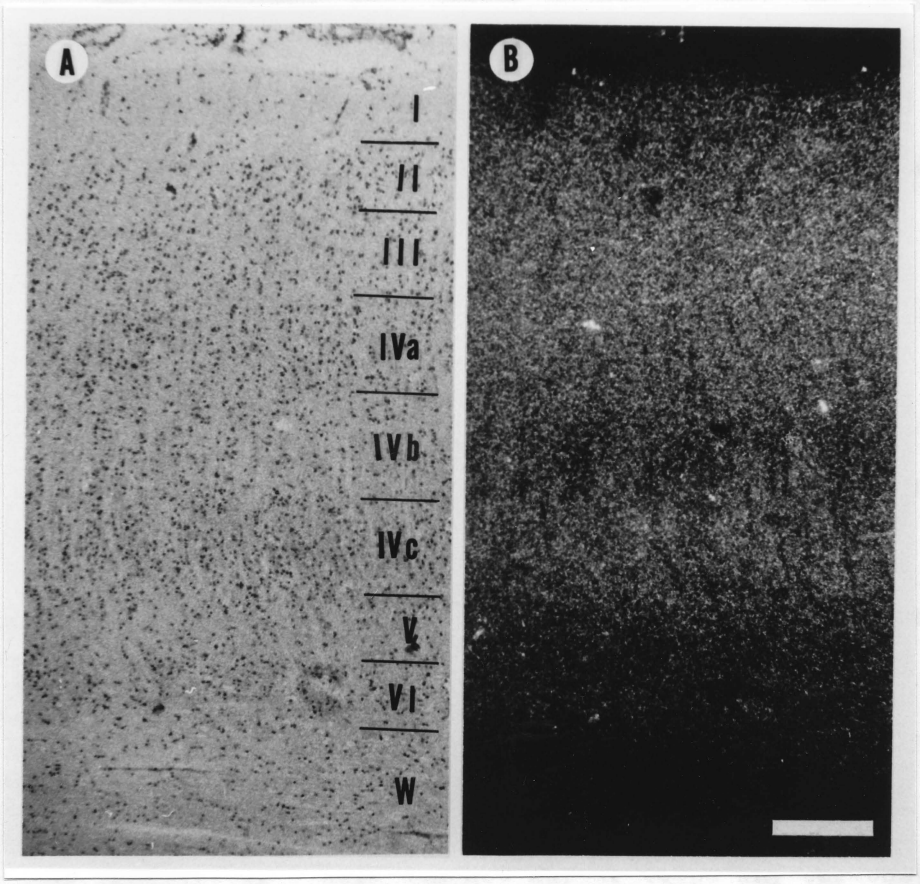
Figure 28

Benzodiazepine receptor in human cerebellum. The darkfield photomicrograph in A shows the autoradiographic grain distribution over the tissue in B. Note the high density of receptors in the molecular layer (M) and in the granule cell layer (G), the reduced density in the Purkinje cell layer (indicated by arrows), and the absence of receptors in the white matter (W). The darkfield photomicrograph in C shows the blockade of receptor binding in an adjacent 4μ section (brightfield not shown) due to the addition of 10^{-6} M clonazepam. The conditions for labeling the receptors are outlined in the text and Figure 22. Sections were incubated with 1 nM ^{3}H-flunitrazepam in Tris-HCl, (pH 7.4, 0.17 M) for 40 min at 0°C in the same buffer. The emulsion was exposed for 2 weeks at 2-4°C. In tissues from two humans, actual grain densities (grains per $1000\mu^{2}$ ± s.d., n = 6) for the cerebellar layers were as follows: molecular, 73 ± 9 and 79 ± 3; Purkinje cell, 67 ± 8 and 60 ± 18; granule cell, 77 ± 3 and 73 ± 8; and white matter 13 ± 7 and 8 ± 3. The corresponding values for rat cerebellum were 84 ± 17, 53 ± 5, 35 ± 4 and 6 ± 1. Grain densities in the presence of 10^{-6} M clonazepam were 5-11 grains per $1000\mu^{2}$. The grain densities were counted in at least two preparations in six areas. Scale bar = 500μ.

A
B
M
W
C

Figure 29

Benzodiazepine receptor in human calcarine cortex. The darkfield photomicrograph in B shows the autoradiographic grain distribution over the tissue in A where the cortical layers (I-VI) and the subcortical white matter (W) are labeled. The receptor density varied in the different layers (for example, elevated in Brodmann (1905) layers III,IVA and IVC) and was relatively negligible in the white matter. Adjacent sections labeled in the presence of 10^{-6} M clonazepam (not shown) displayed a loss of autoradiographic grains with a resultant grain density similar to that shown in B for white matter. Experimental details are given in the text and in the legend to Figure 28. Scale bar = 250μ .


A
I
II
III
IVa
IVb
IVc
V
VI
W
B

DISCUSSION

One of the great advantages of *in vitro* labeling autoradiography is the ability to fully characterize a receptor being labeled in the tissue sections. This enables one to understand just what the silver grains in a subsequent autoradiogram represent. This biochemical analysis of binding sites for ^{3}H-opiates, -opioids, and -benzodiazepines is discussed below followed by a discussion of the autoradiographic results and *in vitro* labeling autoradiography itself.

Preliminary Biochemical Studies

The opioid receptors in our slide-mounted tissue sections (formaldehyde fixed) had all of the characteristics found in other *in vitro* binding studies with homogenates (Childers *et al.*, 1978; Goldstein, 1976; Kuhar, 1978; Simon and Hiller, 1978). Fixation with low concentrations of formaldehyde improved tissue morphology and did not result in a loss of receptors or a significant change in binding characteristics. There did not appear to be any serious problem of accessibility of ligand to receptors in the sections since the B_{max} was similar to that observed using homogenates and since the binding appeared to be linear with section thickness. All of the ligands studied here appear to reach near maximal association to receptors at 20-40 min of incubation. The fact that there is no significant loss of

^{3}H-DPN binding at 0 or 15°C for the longest time measured, 210 min, indicates that the binding is quite tight and that the problem of diffusion of ligand from receptor during subsequent steps is minimal. This slow dissociation rate as well as the saturation data agree with findings using homogenates of brain tissue (Simantov et al., 1978). The drug specificity, stereospecificity, sodium effect, and lack of metabolism of drugs in our tissue sections is also quite similar to that observed in studies with homogenates (Childers et al., 1978; Goldstein, 1976; Kuhar, 1978; Simon and Hiller, 1978).

A biochemical analysis of the binding of ^{3}H-FLU to our slide-mounted tissue sections revealed that the binding had the characteristics of the benzodiazepine receptor. The binding was saturable with kinetic constants similar to those obtained in other in vitro studies using homogenates (Squires and Braestrup, 1977; Möhler and Okada, 1977). The pharmacological specificity of the binding was also in agreement with these other studies (Braestrup and Squires, 1978; Möhler and Okada, 1978; Tallman et al., 1978; Martin and Candy, 1978; Costa et al., 1979, Klepner et al., 1980). The high degree of specific binding compared to nonspecific binding was especially advantageous because the autoradiograms represented nearly all specific binding.

Autoradiography

We used the method of Roth *et al.* (1974) for our autoradiographic procedure to avoid diffusion of the ligand from the receptor. Theoretically and practically, no diffusion can occur after this point because the emulsion on the coverslip is placed in contact with the tissue when both are dry. It should be mentioned that other techniques are possible and may serve as well as ours in avoiding diffusion in practice. For example, loops of wet or dried emulsion may be applied to the tissue (Baughman and Bader, 1977). Yet another way to produce autoradiograms is to dip the receptor-labeled slide-mounted tissue sections into molten emulsion after the slides have been coated with some material to prevent contact of the emulsion with the receptor-labeled tissue. For example, coating the tissue with 1-2% solution of celluloid in isoamyl acetate before emulsion dipping seems to produce good autoradiograms, at least in the few cases we have tested. But we feel that the potential for significant diffusion of the ligand during the first dipping is great and caution is required.

Opiate and Opioid Receptors

The autoradiographic distribution of ^{3}H-DPN binding sites obtained by this modified procedure was identical to that found in our earlier studies using *in vivo* labeling (Atweh and Kuhar, 1977a,b,c). Also, we were able to

successfully produce autoradiographs utilizing ^{3}H-DHM and ^{3}H-DAMA, ligands that are not amenable to *in vivo* labeling, either because their dissociation rates are faster than that of diprenorphine or because the intact peptide does not reach the receptors in the brain. In these studies, we did not rigorously address the issues of whether the high and low affinity binding sites have the same anatomical distribution or whether peptide and drug binding sites and agonist and antagonist binding sites are precisely the same in all areas. Preliminary studies using ^{125}I-labeled opioid peptides have revealed two opioid receptors anatomically (Goodman, *et al.*, in preparation) which seem to correspond to the notion of μ and δ receptors (Martin *et al.*, 1976; Lord *et al.*, 1977; Chang and Cuatrecasas, 1979; Chang *et al.*, 1979).

Benzodiazepine Receptors

We observed a wide variation in BZR density throughout the various regions of rat brain (Young and Kuhar, 1979a, 1980c). Because we were limited to light microscopic studies, our data do not reveal whether the receptor was on neurons or glia. However, the bulk of the evidence appears to favor a neuronal localization (Young and Kuhar, 1979a, 1980c). Recent electron microscopic autoradiographic studies utilizing a photo-affinity label for BZR in brain suggest a neuronal and possibly nerve terminal localization of BZR

(Battersby et al., 1979). Thus, the methodology exists for not only a rapid, light microscopic regional examination of the brain but also for ultrastructural studies to more precisely define the location of BZR.

We have observed species differences in BZR distributions (Young and Kuhar, 1979a). For example, in the human cerebellum, high densities of BZR were found both in the molecular and granule cell layers. However, in the rat, the bulk of the receptor was in the molecular layer. Thus, the results from animal studies should be extended to human only with caution.

A number of studies have suggested some interaction or relationship between GABA and BZ receptors (Haefely, 1978; Karobath and Sperk, 1979; Tallman et al., 1978; Martin and Candy, 1978; Costa et al., 1975). It is interesting to note that the distribution of BZR and high affinity GABA receptors are often dissimilar (Young and Kuhar, 1979a, 1980; Palacios et al., 1980). In fact, regional stimulation of ^{3}H-FLU binding by 2×10^{-4} M GABA in autoradiograms does not correspond to the regional distribution of high affinity GABA receptors (Young and Kuhar, 1979a; Palacios et al., 1980).

There have been many physiological, clinical and pharmacological studies of the effects of benzodiazepine administration (for reviews, see Greenblatt and Shader, 1974a,b; Haefely, 1978; Randall et al., 1974; Costa et al.,

1975, Tallman et al., 1980). In this study, we observed high densities of BZR in anatomical areas involved with these physiological functions altered by BZ administration. One must be very cautious in assuming that there is an actual connection between these highly labeled areas and clinical drug effects, and that only the highly labeled (rather than low density) areas are critical for clinical effects. Species differences are likely to be significant, as well. However, some relationships between areas with high BZR levels and drug effect are striking. For example, anxiety is a complex function thought to be involved with the limbic system which has been implicated as the anatomical "seat" of emotion and its physiological, behavioral and endocrinological sequelae. The circuitry is complex and involves the hippocampus, amygdala, hypothalamus and some associated areas (Papez, 1937; Zeman and Innes, 1963). It has been speculated that the antianxiety action of BZ's is due to suppression of the limbic system. Spontaneous and evoked activity of the hippocampus, amygdala and related structures are profoundly depressed by BZ administration (Schallek and Kuehn, 1960; Chou and Wang, 1977; Matthews and Connor, 1977; Robinson and Wang, 1979; Greenblatt and Shader, 1974a). In our study, we saw high densities of BZR in much of the limbic system, including parts of the amygdaloid complex, hippocampal formation, pyriform cortex, nuclei septi medialis and

mamallaris, and hypothalamus. Our results suggest more specifically which parts of the limbic system might be important for BZ effects. For example, only certain amygdaloid nuclei (centralis, medialis and lateralis) contain high levels. Lippa and his coworkers (1979) have provided evidence for the frontal cortex's role in conflict behavior and have shown that neurons in the cortex are inhibited by flurazepam. The responsive cells in that study fall in the areas where we observed high levels of BZR (cortex at level of forceps minor and what appears to be lamina IV of cortex).

The BZ's are employed extensively as anticonvulsants. They have been shown to prevent the spread of seizures from the cortex, thalamus, and limbic structures (Chusid and Kopeloff, 1962; Guerrero-Figueroa _et al._, 1969a,b; Ben-Ari, 1979). The high density and widespread distribution of BZR in these areas suggests that this could be a direct rather than an indirect effect of BZ administration. In fact, rapid increases in cortical BZR are seen in the rat after experimental seizures (Paul and Skolnick, 1978).

Cerebellar Purkinje cell activity is involved in motor coordination and muscle tone. BZ's have been shown to reduce Purkinje cell firing (Pieri and Haefely, 1976; Montarolo _et al._, 1979) and high doses of BZ have been shown to produce ataxia and incoordination. These observations are in accord

with the high levels of BZR in the molecular layer of the cerebellum which contains the dendrites of the Purkinje cells. Muscle tone is also thought to be controlled, in part, through polysynaptic reflexes in the reticular formation; we saw low to moderate levels in this area.

Other effects of BZ administration have been described. They have been shown to stimulate the appetite (Dantzer, 1978). Corresponding to this, there were high levels of BZR in the ventromedial nucleus of the hypothalamus, an area associated with satiety. Although conflicting roles for BZ's in analgesia have been reported (Weiss, 1969; Lineberry and Kalics, 1978), it is interesting that we found high densities of BZR in many areas of the brain associated with pain, including the substantiae gelatinosae of the spinal cord and spinal trigeminal nucleus and, to a lesser extent, in the intralaminar nuclei and periadqueductal grey. BZ's are notably without extrapyramidal side effects and there were low levels of BZR in most of the basal ganglia. However, significant BZR levels were observed in the zona incerta, nucleus entopeduncularis, nucleus subthalamicus and substantia nigra, pars lateralis. Lesions of the nucleus subthalamicus in man cause hemiballismus and it would be interesting to learn what effect, if any, BZ's have on this disease. In this regard, a report by Delgado (1973) showed that body-jerking elicited by electrical stimulation of the

adjacent Forel's Field (H_2) in the monkey was inhibited by chlordiazepoxide.

Thus, one can make a number of interesting associations between anatomical distributions of BZR and the physiological effects of these drugs. However, still other areas have high levels of BZR and we are currently unaware of their significance. For example, what role do the BZR serve in the nucleus ventralis rostralis lemnisci lateralis or in parts of the optic system (inner plexiform layer of the retina and superior colliculus)? Hopefully the results of this study will provide some guidance in the further exploration of the actions of benzodiazepines in brain.

In Vitro Labeling Autoradiography

The ability to examine the distribution of receptors at the light microscopic level by the present approach has many advantages over earlier studies where receptors were labeled by *in vivo* injection. Several different receptors can be examined in the same small tissue area. For example, one can examine the distribution of ^{3}H-DHM, ^{3}H-DAMA and ^{3}H-DPN binding in adjacent 4 μ sections. This method is certainly applicable to a wide variety of other labeled compounds. We have successfully produced autoradiograms for α_1-, α_2-, and β-adrenergic (Young and Kuhar, 1979d, 1980a; Palacios and Kuhar, 1980), serotonergic (Young and Kuhar, 1980d), neurotensinergic (Young and Kuhar, 1979b), H_1-histaminergic

(Palacios et al., 1979) and GABAergic Palacios et al., 1980) receptors in both animals and human tissue.

After in vivo administration of these compounds, the ratio of specific to nonspecific binding is poor. But by this in vitro technique, one can wash away nonspecific binding more readily than specific binding and greatly enhance the ratio. And we have shown the distribution of peptide binding sites. Attempts to obtain the autoradiographic distribution of peptide binding sites by intravenous administration have failed because of the rapid metabolism. Another advantage of in vitro labeling is that one can assess the pharmacology of the receptor system and choose the best binding conditions and also more readily manipulate the binding media. For example, Wamsley et al. (1980) recently have been able to determine autoradiographically that the muscarinic cholinergic high and low affinity sites have different regional distributions. One can look at the effect of various ions and nucleotides on agonist binding sites in adjacent sections. Such an experiment is not possible when one labels tissue by the in vivo method. It may also be that this technique is more physiologically sound in reproducing the synaptic environment. Yet another advantage is the ability to perform light microscopic autoradiographic studies of receptors in postmortem tissue, including that from human. We have done

these studies with ^{3}H-opioids and with ^{3}H-benzodiazepines. Thus, this flexible approach enables one to label tissue <u>in vitro</u> with various receptor ligands and has many advantages over methods that have been utilized to date.

APPENDIX

Abbreviations

AA	Area amygdala anterior
BCI	Brachium colliculi inferioris
CA	Commissura anterior
CAE	Capsula externa
CAI	Capsula interna
CAIR	Capsula interna, pars retrolenticularis
CbG	Lamina granulosum cerebelli
CbM	Lamina molecularis cerebelli
CC	Crus cerebri
CE	Cortex entorhinalis
CFD	Commissura fornicus dorsalis
CFV	Commissura fornicus ventralis
CL	Claustrum
CO	Chiasma opticum
CPF	Cortex piriformis
CSDV	Commissura supraoptica dorsalis, pars ventralis
CT	Corpus trapezoideum
DP	Decussatio pyramidis
F	Columna fornicis
FC	Fasciculus cuneatus
FH	Fimbria hippocampi
FLM	Fasciculus longitudinalis medialis
FMI	Forceps minor
FMP	Fasciculus medialis prosencephali

FMT	Fasciculus mamillothalamicus
FMTG	Fasciculus mamillotegmentalis
FOP	Fasciculus opticus
FOR	Formatio reticularis
FPT	Fibrae pontis transversae
FR	Fasciculus retroflexus
GCC	Genu corporis callosi
GD	Gyrus dentatus
GP	Globus pallidus
H	Habenula
HI	Hippocampus
IC	Insulae Calleja
ICM	Insula Calleja magna
LCMA	Lamina cellularum mitralium bulbi olfactorii accessorii
LG	Lamina glomerulosa bulbi olfactorii
LGA	Lamina glomerulosa bulbi olfactorii accessorii
LGI	Lamina granularis interna bulbi olfactorii
LM	Lemniscus medialis
LMO	Lamina molecularis bulbi olfactorii
LMOA	Lamina molecularis bulbi olfactorii accessorii
MI	Massa intercalata
P	Tractus corticospinalis
PCI	Pedunculus cerebellaris inferioris
PCS	Pedunculus cerebellaris superioris

PF	Polus frontalis
RCC	Radiatio corporis callosi
S	Subiculum
SGCD	Substantia grisea centralis, pars dorsalis
SGCL	Substantia griseum centralis, pars lateralis
SGM	Stratum griseum mediale colliculi superioris
SGP	Stratum griseum profundum colliculi superioris
SGPV	Substantia grisea periventricularis
SGS	Stratum griseum superficiale colliculi superioris
SM	Stria medullaris thalami
SNL	Substantia nigra, pars lateralis
SNR	Substantia nigra, zona reticulata
SPCC	Splenium corporis callosi
STI	Stria terminalis, pars infracommissuralis
TCC	Truncus corporis callosi
TO	Tractus opticus
TOH	Tractus olfactohypothalamicus
TOL	Tractus olfactorius lateralis
TOLD	Tractus olfactorius lateralis, pars dorsalis
TRS	Tractus rubrospinalis
TST	Tractus septotubercularis et tuberculoseptalis
TSTH	Tractus striohypothalamicus
TSV	Tractus spinalis nervi trigemini

TULC	Tuberculum olfactorium, pars corticalis, lamina pyramidalis
TULI	Tuberculum olfactorium, pars interna, lamina polymorphica
TULP	Tuberculum olfactorium, pars corticalis, lamina plexiformus
ZI	Zona incerta
a	Nucleus accumbens
abl	Nucleus amygdaloideus basalis, pars lateralis
abm	Nucleus amygdaloideus basalis, pars medialis
ac	Nucleus amygdaloideus centralis
aco	Nucleus amygdaloideus corticalis
alp	Nucleus amygdaloideus lateralis, pars posterior
am	Nucleus amygdaloideus medialis
amb	Nucleus ambiguus
ar	Nucleus arcuatus
ccgm	Nucleus centralis corporis geniculati medialis
cm	Nucleus centre median
cod	Nucleus cochlearis dorsalis
cov	Nucleus cochlearis ventralis
cp	Nucleus caudatus putamen
ct	Nucleus corporis trapezoidei
cu	Nucleus cuneatus
dcgl	Nucleus dorsalis corporis geniculati lateralis
ep	Nucleus entopeduncularis

fm	Nucleus paraventricularis, pars magnocellularis
ha	Nucleus anterior (hypothalami)
hd	Nucleus dorsomedialis (hypothalami)
hl	Nucleus lateralis (hypothalami)
hp	Nucleus posterior (hypothalami)
hv	Nucleus ventromedialis (hypothalami)
hvma	Nucleus ventromedialis (hypothalami), pars anterior
io	Nucleus olivaris inferior
ip	Nucleus interpeduncularis
lc	Locus coeruleus
lh	Nucleus habenulae lateralis
mh	Nucleus medialis habenulae
mmm	Nucleus mamillaris medialis, pars medialis
nco	Nucleus commissuralis
nic	Nucleus intercalatus
npd	Nucleus parabrachialis
npv	Nucleus parabachialis
npV	Nucleus principalis nervi trigemini
nrd	Nucleus reticularis medullae oblongatae, pars dorsalis
nrp	Nucleus reticularis
nrv	Nucleus reticularis medullae oblongatae, pars ventralis
ntd	Nucleus tegmenti dorsalis Gudden

ntm	Nucleus tractus mesencephali
nts	Nucleus tractus solitarii
ntv	Nucleus tegmenti ventralis Gudden
ntV	Nucleus tractus spinalis nervi trigemini
ntVd	Nucleus tractus spinalis nervi tegemini pars dorsomedialis
nV	Nucleus originis nervi trigemini
nVII	Nucleus originis nervi facialis
nX	Nucleus originis dorsalis vagi
nXII	Nucleus originis nervi hypoglossi
oad	Nucleus olfactorius anterior, pars dorsalis
oal	Nucleus olfactorius anterior, pars lateralis
oam	Nucleus olfactorius anterior, pars medialis
oap	Nucleus olfactorius anterior, pars posterior
ol	Nucleus tractus olfactorii lateralis
ope	Nucleus preolivaris externus
p	Nucleus pretectalis
pf	Nucleus parafascicularis
pm	Nuclei pontis, pars medialis
poma	Nucleus preopticus magnocellularis
pt	Nucleus paratenialis
pvs	Nucleus periventricularis stellatocellularis
r	Nucleus ruber
rgi	Nucleus reticularis gigantocellularis
rl	Nucleus reticularis lateralis

rm	Nucleus raphe magnus
rpc	Nucleus reticularis parvocellularis
rpo	Nucleus raphe pontis
rpoc	Nucleus reticularis pontis caudalis
sc	Nucleus suprachiasmaticus
sgV	Substantia gelatinosa trigemini
sl	Nucleus septi lateralis
sm	Nucleus septi medialis
so	Nucleus supraopticus
st	Nucleus interstitialis striae terminalis
sut	Nucleus subthalamicus
tam	Nucleus anterior medialis thalami
tav	Nucleus anterior ventralis thalami
td	Nucleus tractus diagonalis (Broca)
tl	Nucleus lateralis thalami
tlp	Nucleus lateralis thalami, pars posterior
tml	Nucleus medialis thalami, pars lateralis
tmm	Nucleus medialis thalami, pars medialis
tpo	Nucleus posterior thalami
ts	Nucleus triangularis septi
tv	Nucleus ventralis thalami
tvd	Nucleus ventralis thalami, pars dorsomedialis
vcgl	Nucleus ventralis corporis geniculati lateralis
vl	Nucleus vestibularis lateralis

vm Nucleus vestibularis medialis

vrll Nucleus ventralis rostralis lemnisci lateralis

vsp Nucleus vestibularis spinalis

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