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Quantitative Receptor Autoradiography: Application to the Study of Multiple Serotonin Receptors in Rat Cortex

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In recent years methods have been developed that allow the localization of receptors for drugs and neurotransmitters at the light- and electron-microscopic levels, using the technique of autoradiography (1). Two important developments in this field have been the availability of an in vitro method for the labeling of receptors for autoradiography (2) and the adaptation of this technique, thanks to the use of a ^{3}H-sensitive film, for quantitative microdensitometric determination of receptor densities (3-5).

Because of the use of in vitro conditions for labeling of receptors, allowing for the utilization of drugs with different affinities for specific receptor subtypes, and because of its high anatomical resolution, the quantitative autoradiography of receptors is a method especially well suited for the study of multiple receptors for a neurotransmitter (6).

In this paper I shall describe methods for the computer-assisted microdensitometric measurement of receptor concentrations and the application of this technology to the study of the laminar distribution of serotonin-1 (5HT-1) and serotonin-2 (5HT-2) (7-8) receptors in the rat cortex.

QUANTITATIVE RECEPTOR AUTORADIOGRAPHY

Labeling of receptors for autoradiography

The brains from adult male rats (Wistar, 250 g body weight) were used in all experiments described in this paper. Ten-micron slide-mounted sections were prepared as previously described (2). Serotonin receptors were labeled with ^{3}H-LSD as described by Meibach et al., (9) and Young and Kuhar (10). Serotonin-2 receptors were also labeled with ^{3}H-spiperone as described by Palacios et al., (11).

The different components of the binding of these ligands were analyzed by using drugs with different affinities for serotonin and dopamine receptors. Serotonin, spiperone, ketanserin and cinanserin were used in the study of 5HT receptors. Sulpiride and ADTN (2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene) were used to block dopamine receptors.

Generation and Standardization of Autoradiograms

Autoradiograms were generated by apposing the labeled tissue sections against a ^{3}H-sensitive film (^{3}H-ultrofilm, LKB, Sweden). Exposure time was one and two months. Standards containing different amounts of a ^{3}H-labeled, non-volatile substance were exposed along with the labeled sections. These standards were prepared with brain paste as described by Unnerstall et al. (12,13). Because of the different self-absorption for β-particles by white and gray matter (14), two different sets of standards were prepared by using regions of the brain enriched in gray or white matter. Figure 1 shows a plot of the densitometric readings of these two sets of standards, and demonstrates a more than 3-fold greater self-absorption for beta-particles by the white matter compared with the gray matter. Use of standards containing only white matter or mixtures of white and gray matter will result in overestimation of the receptor concentrations in gray matter areas. In this study gray matter standards were used in all experiments.

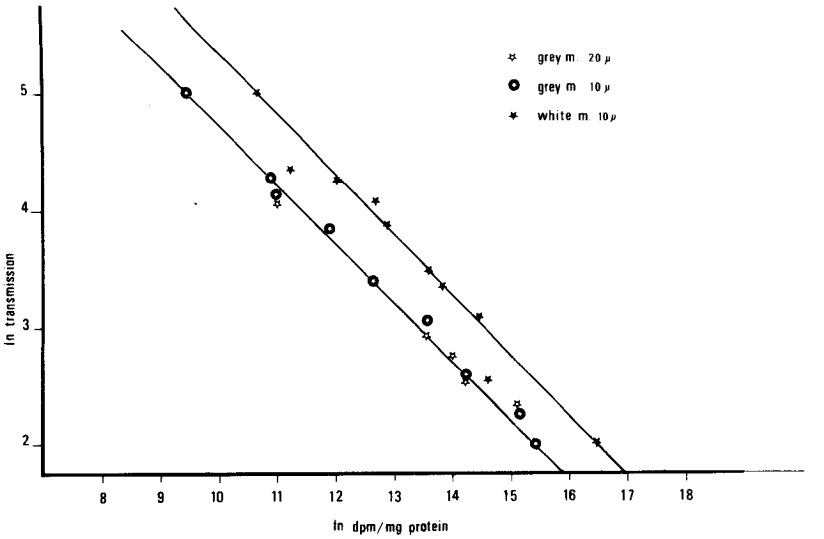

Fig. 1. Double logarithmic plot of transmission versus concentration of radioactivity in the standards, in dpm/mg of protein. Gray matter standards, cut at 10 or 20 µm thickness, produced the same standard curves. White matter standards produced a parallel standard curve, but displaced to the right, indicating that for the same amount of radioactivity these standards have a more than 3-fold lower optical density.

Computer-assisted quantification of the autoradiograms

Autoradiograms were analyzed using a computerized image-analysis system developed in our laboratories (Schweizer et al.). A television camera coupled to a microscope is used to digitalize the autoradiographic image into a 256x256 matrix of digital numbers. The areas of interest are outlined in the image displayed in a television monitor using a light-sensitive pen. The mean density of the area is calculated by a microprocessor that is programed to transform it into a receptor density. For this the quantification process is initiated by the reading of the standard curve. This generates a factor for the transformation of optical density into radioactivity concentration in dpm/mg of protein. Taking into account the specific activity of the ligand used in the experiment, the optical densities are converted to receptor concentrations, in fmol/mg of protein.

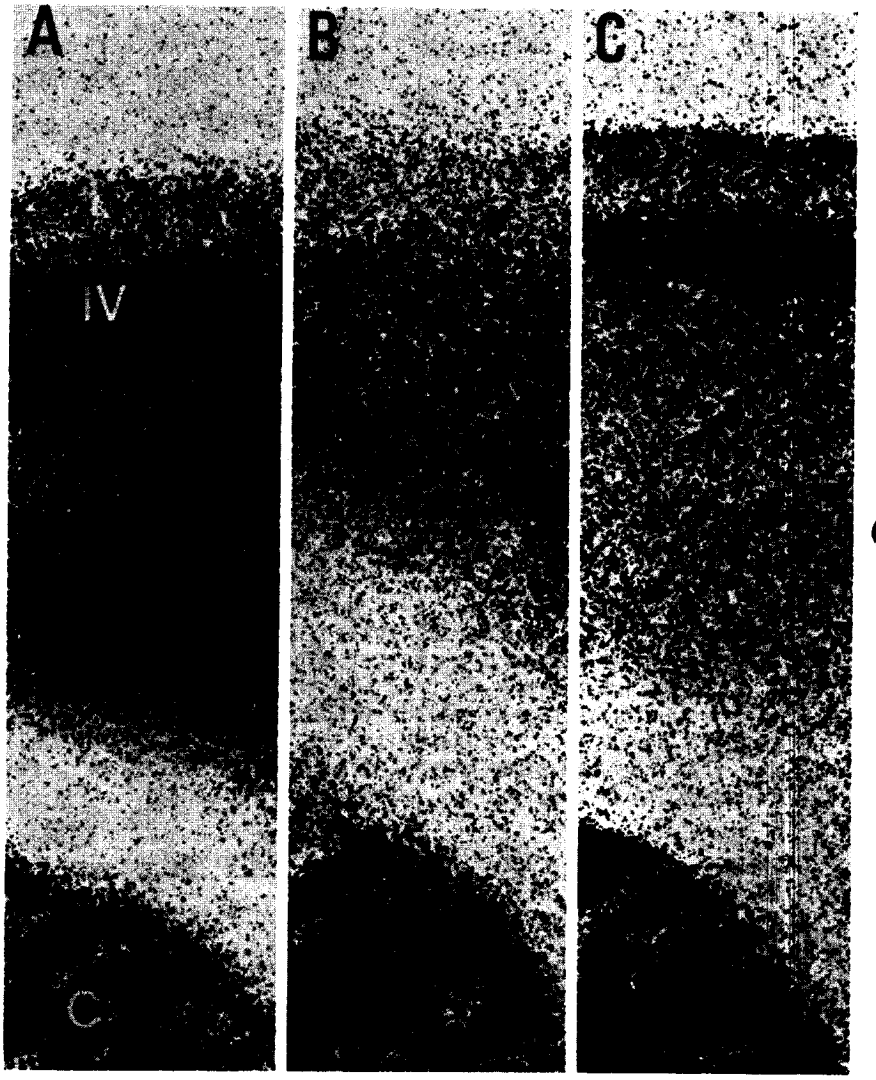

Fig. 2. Autoradiographic localization of ^{3}H-LSD binding sites in the rat cortex. A is a photomicrograph of the autoradiogram from a tissue section incubated with 5nM ^{3}H-LSD. Note a high density of autoradiographic grains in lamina IV (IV), in the layers below this one and in the nucleus caudate-putamen (C). B shows a section, consecutive to that in A, incubated with 0.1 μM ketanserin to block 5HT-2 receptors. Note the reduction of autoradiographic grains, particularly in laminae I (I) and IV. The section shown in C was incubated with 30 nM serotonin to block 5HT-1 receptors. A considerable decrease in the binding of ^{3}H-LSD to the deepest cortical layers can be observed.

LAMINAR DISTRIBUTION OF SEROTONIN-1 AND SEROTONIN-2 RECEPTORS IN THE RAT CORTEX

As an example of the application of quantitative autoradiographic methods to the study of multiple neurotransmitter receptors, I shall describe the localization of 5HT-receptors in the rat frontal cortex.

Tritiated LSD and ^{3}H-spiperone were used as ligands for these receptors. Binding of ^{3}H-LSD was observed in all areas and layers of the rat cortex, but varied in the different areas and laminae.

Figure 2A shows the distribution of ^{3}H-LSD binding sites in the frontal cortex of the rat. Binding was elevated in laminae IV, V and VI, while the density of autoradiographic grains was somewhat lower in laminae I, II and III.

A section consecutive to the one shown in Fig. 2A, incubated with ^{3}H-LSD in the presence of 0.1 μM ketanserin, a specific blocker of 5HT-2 receptors (15), is shown in Fig. 2B. Binding of ^{3}H-LSD was decreased in all layers of the cortex but particularly in the laminae I, II, III and IV. Co-incubation of ^{3}H-LSD with 30 nM serotonin, a concentration that will block preferentially binding to 5HT-1 receptors, resulted in the inhibition of ^{3}H-LSD binding to sites localized mainly in the deep laminae V and VI.

It is apparent that 5HT-1 and 5HT-2 receptors have a differential distribution in the frontal cortex; 5HT-1 receptors are concentrated in laminae V and VI and 5HT-2 receptors in laminae I through IV.

These results were confirmed by using ^{3}H-spiperone, a neuroleptic that binds to 5HT-2 receptors in the cerebral cortex (7,8,11). Fig. 3A shows the localization of ^{3}H-spiperone binding sites in the rat cerebral cortex and demonstrates an enrichment of sites in the laminae I and IV and lower densities in the other laminae. The addition of 1 μM concentration of the neuroleptic sulpiride, that specifically blocks dopamine receptors (8), to the incubation medium resulted in a complete blockade of the binding of ^{3}H-spipe-

rone to the nucleus caudate-putamen (Fig. 3B), without affecting the binding in the cortex. On the other hand, 0.1 μM ketanserin completely inhibited the binding of ^{3}H-spiperone to cortical sites without affecting binding to the sites in the caudate-putamen. These results demonstrate again the presence of 5HT-2 receptor sites in the more external cortical layers. Similar results were obtained in these and previous experiments (11) using other displacing agents such as ADTN, cinanserin and 5HT.

A further confirmation of the laminar distribution of 5HT-receptors in the rat cortex has been provided by the autoradiographic localization of ^{3}H-5HT binding sites. Rainbow et al., (3) have shown that the binding sites for ^{3}H-5HT, under conditions in which 5HT-1 receptors are preferentially labeled, are concentrated in the deepest cortical laminae IV through VI.

Finally, in order to obtain a quantitative estimation of the distribution of 5HT receptors in the rat cortex, complete displacement curves were constructed using consecutive saggital sections and increasing concentrations of different displacers, such as 5HT, ketanserin, cinanserin and spiperone. As an example, Fig. 4 shows the results obtained using ^{3}H LSD as ligand and 5HT as displacer. When the mean receptor density of a cross section of the cortex ("Total cortex", Fig. 4A) was measured, it was observed that 5HT displaced ^{3}H-LSD binding in a biphasic manner. Fifty to 60% of the binding sites were sensitive to low 5HT concentrations (IC_{50} approximately 2-3 nM) while the remaining ^{3}H-LSD sites required higher 5HT concentrations (IC_{50} around 0.3-1 μM) to be blocked.

The quantitative analysis of the separate laminae (Fig. 4B) confirmed the qualitative results discussed above. Biphasic displacement curves were found in all the cortical layers, with the exception of lamina I that appears to contain mainly 5HT-2 receptors. Lamina IV was also rich in 5HT-2 receptors although about 30% of the sites were of the 5HT-1 type. Laminae V and VI were enriched in 5HT-1 sites, although 5HT-2 sites were also present in variable proportion (40 and 25% of the total, respectively).

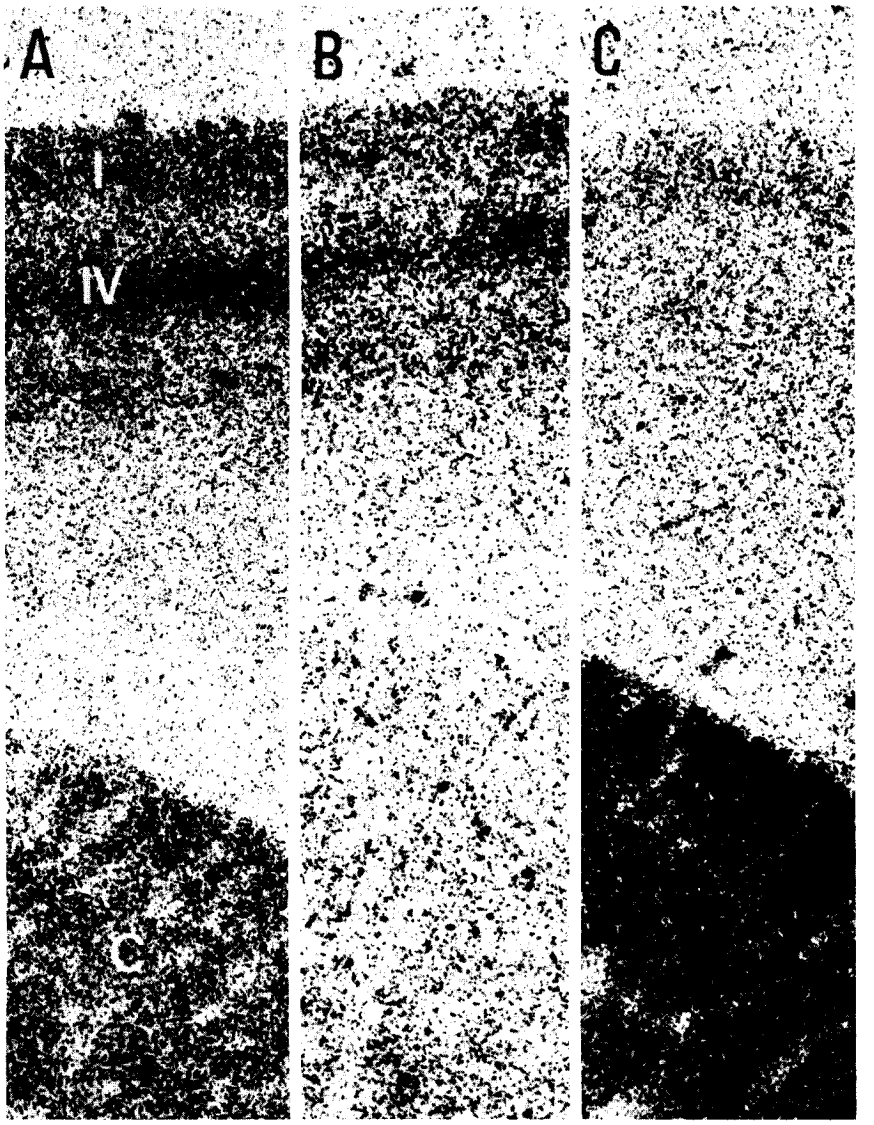

Fig. 3. Autoradiographic localization of ^{3}H-spiperone binding sites in the rat cortex. A shows the binding of 0.4 nM ^{3}H-spiperone to cortical layers (I and IV) and to sites in the nucleus caudate-putamen (C). The effects of co-incubation with 1 μM sulpiride are shown in B. Note the inhibition of the binding of ^{3}H-spiperone to sites in the caudate, without effects on the cortical binding. C shows the effects of ketanserin (0.1 μM) on the binding of ^{3}H-spiperone. The cortical sites were blocked while the sites in the caudate were not affected.

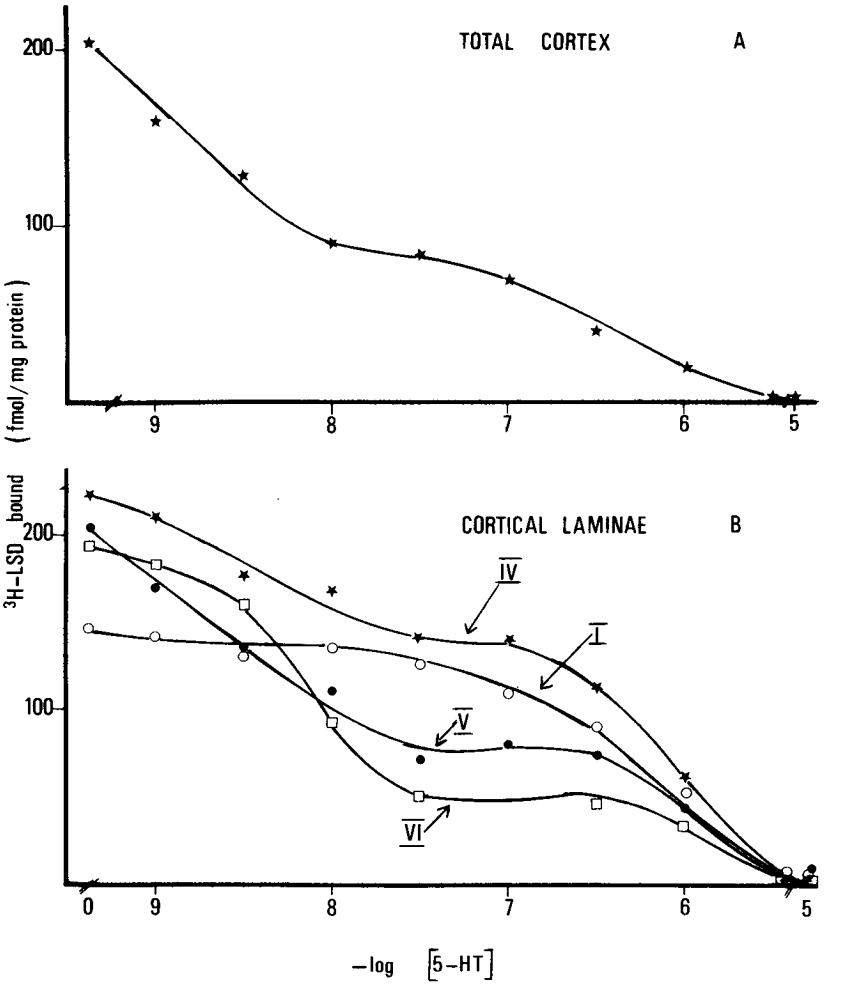

Fig. 4. Displacement curves of 5 nM ^{3}H-LSD binding to the rat cortex by 5-HT as determined microdensitometrically. In A the mean density of the cortex was measured. B shows the displacement curves for individual laminae (I, IV, V and VI). Points are the means of 9 measurements, three for each of three animals. The standard deviation was always less than 5% of the mean value.

In conclusion, by using quantitative, computer-assisted, autoradiographic techniques it was possible to demonstrate the differential laminar distribution of 5HT receptor subtypes in the rat cortex. The pharmacological characteristics and the relative proportions of these receptors were also analyzed with high anatomical resolution.

Because of the different afferent and efferent connections of the cortical laminae one could suggest that activation or blockade of one or the other 5-HT receptor subtype will influence brain function in a differential way.

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