

125I-Lysergic Acid Diethylamide Binds to a Novel Serotonergic Site on Rat Choroid Plexus Epithelial Cells

KEITH A. YAGALOFF AND PAUL R. HARTIG

Department of Biology, Johns Hopkins University, Baltimore, Maryland 21218

Abstract

125I-Lysergic acid diethylamide (125I-LSD) binds with high affinity to serotonergic sites on rat choroid plexus. These sites were localized to choroid plexus epithelial cells by use of a novel high resolution stripping film technique for light microscopic autoradiography. In membrane preparations from rat choroid plexus, the serotonergic site density was 3100 fmol/mg of protein, which is 10-fold higher than the density of any other serotonergic site in brain homogenates. The choroid plexus site exhibits a novel pharmacology that does not match the properties of 5-hydroxytryptamine-1a (5-HT1a), 5-HT1b, or 5-HT2 serotonergic sites. 125I-LSD binding to the choroid plexus site is potently inhibited by mianserin, serotonin, and (+)-LSD. Other serotonergic, dopaminergic, and adrenergic agonists and antagonists exhibit moderate to weak affinities for this site. The rat choroid plexus 125I-LSD binding site appears to represent a new type of serotonergic site which is located on non-neuronal cells in this tissue.

125I-Lysergic acid diethylamide (125I-LSD) binds to serotonin 5-hydroxytryptamine-2 (5-HT2) receptors in the mammalian brain (Engel et al., 1984; Kadan et al., 1984). In a recent autoradiographic study, Nakada et al. (1984) noted a high level of 125I-LSD binding in the lateral ventricles, which was displaced by ketanserin, a potent serotonin 5-HT2 ligand. Other investigators have described the binding of 125I-LSD and 5-HT to a particular ventricular structure, the choroid plexus (Diab et al., 1971; Meibach et al., 1980; Palacios et al., 1983), but these studies did not describe the pharmacological properties of the choroid plexus binding sites. We decided to investigate ventricular 125I-LSD-binding sites to determine their cellular location and binding properties. Our results show that binding sites for 125I-LSD are located on epithelial cells of the choroid plexus. These binding sites exhibit a unique serotonergic pharmacology which does not match the properties of either 5-HT2 or 5-HT2 sites in the brain. These sites are present at a density 10-fold higher than the density of any other serotonergic site in brain membrane homogenates. The choroid plexus 125I-LSD-binding site appears to represent a new type of mammalian brain serotonergic site. A preliminary report of this work has been published (Hartig and Yagaloff, 1985).

Materials and Methods

Autoradiography. Coronal sections (6 to 0 μm) of frozen adult rat brains were thaw-mounted onto subbed microscope slides, air-dried for 20 min, and stored at −20°C in desiccated microscope boxes. The mounted tissue sections were brought to room temperature and then labeled with 125I-LSD by a technique similar to the method of Nakada et al. (1984). Sections were incubated for 60 min at room temperature in 50 μM Tris-HCl buffer (pH 7.6) containing either 1.5 nM 125I-LSD (2000 Ci/mmol) or 1.5 nM 125I-LSD and 1 μM ketanserin, to determine nonspecific binding. 125I-LSD was synthesized by the method of Moretti-Rojas et al. (1983) and purified by the method of Hartig et al. (1985a). The labeled sections were washed four times for 20 min each in ice-cold 50 μM Tris-HCl (pH 7.6), dipped briefly in ice-cold distilled water, and blotted dry with cold air. Labeled slides were exposed either against Kodak AR-10 stripping film-coated microscope slides or against LKB Ultrofilm. The film was exposed for 24 hr in an x-ray cassette at 4°C, developed in a Kodak D19 developer for 2 min at 20°C, and processed, and air dried. Stripping film slides were prepared by floating precuts of stripping film (emulsion side up) onto distilled water, picking them up from below with subbed microscope slides, wrapping the film around the slide, and drying thoroughly. Slides with labeled sections were clamped tightly against stripping film slides (using binder clips) and placed in a light-tight, desiccated box for 4 days at 4°C. The film was developed for 2 min in a Kodak D19 developer at 20°C, washed 30 sec in water, fixed 12 min in Kodak fixer, rinsed, and air dried. Specific binding was typically 80% of the total 125I-LSD bound. Labeled sections were stained with Harris' hematoxylin and counterstained with eosin Y after the autoradiographic exposure.

Homogeneous-binding assays. The choroid plexus was dissected from the lateral and third ventricles of 40 adult rat brains (Pel Freezer). From each rat brain we obtained approximately 1.3 mg (wet weight) of choroid plexus tissue. The combined tissue was homogenized in 2 ml of ice cold 0.32 M sucrose (eight strokes in a Teflon pestle tissue homogenizer rotating at 500 rpm) and centrifuged for 15 min with 750 × g. The supernatant was centrifuged again at 750 × g for 15 min. The resulting supernatant was centrifuged and centrifuged 20 min at 35,000 × g (4°C). The pellet was centrifuged 20 min at 35,000 × g (4°C). The resulting pellet was centrifuged by homogenization in 2 ml of 50 μM Tris-HCl (pH 7.6) and stored under liquid nitrogen until use. Membrane preparations of choroid plexus from adult mouse, pig, rabbit, and cow brains were prepared as described for rat brain using choroid plexuses dissected from 2 to 25 brains and homogenized in an appropriate volume of 0.32 M sucrose. Protein concentrations were determined by the method of Lowry et al. (1951). A buffer of 50 μM Tris-HCl, pH 7.6 (at 23°C), and a tissue concentration of 0.18 mg of protein/ml were used for the determination of the 125I-LSD dissociation constant (Scatchard analysis). The 125I-LSD concentration was separated measured for each dilution used in the Scatchard analysis. This avoids errors which can arise from adsorption of the radioligand during dilutions and transfers (Hartig et al., 1985a). Nonspecific binding was defined by 1 μM ketanserin in the Scatchard experiments and all other binding studies. Sample incubation, filtration, and counting conditions were the same as previously described for 125I-LSD binding to rat frontal cortex membranes (Kadan et al., 1984).

In the competition (IC50) experiments, binding equilibrium was reached...
during the 15-min incubation at 37°C, at which time less than 10% of the added radioligand was bound. Specific binding was consistently 85 to 90% of total binding at the tissue concentration used (0.18 mg of protein/ml), with the radioligand concentration near 3.5 nM. The $^{125}$I-LSD concentration was between 3 and 4 nM for all competition studies. A buffer containing 50 mM Tris, 10 μM pargyline, 5 mM EDTA, 1 mM sodium ascorbate, pH 7.6, was used for all assays. Only small changes in total and specific binding were seen with this buffer as compared to 50 mM Tris-HCl alone. Total binding was typically 40,000 dpm in each 30 μl sample used for these experiments. Thirty microliters represents the lower limit of final sample volume that can be accurately handled in these binding experiments. Lower tissue concentrations can be used, but the ratio of specific to nonspecific binding decreases due to an increased contribution from radioligand binding to the glass fiber filters. (+)-LSD and (-)-LSD for these studies were obtained from the National Institute on Drug Abuse.

Results

 Autoradiography. The binding of $^{125}$I-LSD to a coronal section from adult rat brain is shown in Figure 1. In agreement with previous studies (Engel et al., 1984; Nakada et al., 1984), this Ultrofilm autoradiograph shows a high density of $^{125}$I-LSD binding in layer IV of the cortex, in the claustrum, and in the striatum, which is reduced to background levels by inclusion of 1 μM ketanserin during the labeling. Especially high levels of $^{125}$I-LSD binding are seen (Fig. 1) in the choroid plexus of the lateral and third ventricles. The density of $^{125}$I-LSD labeling in choroid plexus exceeds the labeling density observed in any other region of the rat brain. Although it was possible to localize $^{125}$I-LSD binding to the choroid plexus using Ultrofilm, the relatively low resolution of this film prevented us from determining the cellular location of binding sites within the choroid plexus. In order to obtain higher resolution autoradiographs, we used Kodak AR.10 stripping film. The grain size of this stripping film is approximately 6 times smaller than that of Ultrofilm $^{3}$H, which results in greater resolution with a proportionate decrease in sensitivity. In addition, the film is of consistently uniform thickness, is easily manipulated, and has a longer shelf life than nuclear emulsions (NTB2, NTB3) which are commonly used for high resolution autoradiographs. The stripping film autoradiograph reveals (Fig. 2) that $^{125}$I-LSD-binding sites are located on epithelial cells distributed throughout the choroid plexus. These epithelial cells form a monolayer of cuboidal cells in direct contact with the cerebrospinal fluid (Tennyson and Pappas, 1968). Within the ventricular region, $^{125}$I-LSD labeling is found exclusively on the choroid plexus. Ependymal cells which line the walls of the ventricles are not labeled by $^{125}$I-LSD.

 Homogenate binding assays. We dissected the choroid plexus from adult rat brains, prepared a membrane homogenate, and examined the pharmacological properties of the $^{125}$I-LSD-binding site. The binding of $^{125}$I-LSD to the choroid plexus homogenate (Fig. 3) yields a linear Scatchard plot (Hill slope = 0.98) and an average dissociation constant (Kₐ) of 3.4 ± 0.1 nM (mean ± SEM). In contrast, $^{125}$I-LSD has been shown to bind to 5-HT₂ receptors with a Kₐ of 1 to 1.5 nM (Engel et al., 1984; Kadan et al., 1984; Hartig et al., 1985a) to dopamine D₂ sites with a Kₐ of 9.1 nM (Hartig et al., 1985b), to 5-HT₃ sites with an apparent Kₐ of 30 nM (Engel et al., 1984), and very weakly, if at all, to 5-HT₁, histamine H₁, or adrenergic sites (Engel et al., 1984). The site density (B Max) for $^{125}$I-LSD binding to the choroid plexus homogenate is approximately 3100 fmol/mg of pro-

Figure 1. Autoradiograph of $^{125}$I-LSD binding to a coronal section from adult rat brain. The image is reversed such that areas with a high density of labeling appear as white grains on a dark background. The section was labeled with 1.5 nM $^{125}$I-LSD and exposed against Ultrofilm for 24 hr. High levels of labeling are seen in the choroid plexus of the lateral and third ventricles (arrows), in layer IV of the cortex, in the claustrum, and in the striatum.
Figure 2. High resolution autoradiograph of \(^{125}\text{I}\)-LSD labeling of rat lateral ventricle. A, Photomicrograph of a rat lateral ventricle stained with hematoxylin and eosin Y after autoradiography. B, Autoradiograph of \(^{125}\text{I}\)-LSD binding to this same section. Regions of high density labeling appear as white grains on a black background. The section was labeled with 1.5 nM \(^{125}\text{I}\)-LSD and exposed against AR.10 stripping film for 4 days. The bar represents 200 μm. CC, corpus callosum; CN, caudate nucleus; CP, choroid plexus; LS, lateral septal nucleus; V, lateral ventricle.

Figure 3. Binding of \(^{125}\text{I}\)-LSD to rat choroid plexus membrane preparations. Total (○) and nonspecific (■) binding were determined in the absence or presence of 1 μM ketanserin, respectively. Data are shown as a saturation plot and the corresponding Scatchard plot (inset) from a single representative experiment. Data points represent mean ± SD values from triplicate assays at each concentration. The \(K_d\) value is 3.45 nM and the \(B_{max}\) is 3117 fmol/mg of protein in this single experiment.

Table I summarizes the results of competition binding studies (IC\(_{50}\) assays) using various ligands for serotonergic, dopaminergic, and adrenergic receptor sites. Serotonin and the serotonergic antagonists mianserin, (+)-LSD, and ketanserin are all potent inhibitors of \(^{125}\text{I}\)-LSD binding. Serotonin is over 200-fold more potent than the other monoaminergic agonists we tested. (+)-LSD, with a \(K_I\) of >5 μM, is at least 1000 times less potent than (+)-LSD in displacing \(^{125}\text{I}\)-LSD binding, demonstrating the stereospecificity of binding at this site. Dopamine and the dopamine antagonist haloperidol are both weak displacers of \(^{125}\text{I}\)-LSD binding. The α- and β-adrenergic antagonists phentolamine and propranolol both show weak displacement of \(^{125}\text{I}\)-LSD as does the adrenergic agonist, epinephrine. Chlorpheniramine, a histamine H1 antagonist, is a very weak inhibitor of \(^{125}\text{I}\)-LSD binding, as is the selective 5-HT\(_1\) agonist, 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino)tetralin).

Addition of 1 mM sodium ascorbate, 5 mM EDTA, and 10 μM pargyline to the incubation mixture had little effect on the total or specific binding of \(^{125}\text{I}\)-LSD. However, the addition of ascorbate in the absence of EDTA caused a large decrease (50%) in specific \(^{125}\text{I}\)-LSD binding. This may be due to ascorbate-catalyzed lipid peroxidation, as was shown to occur in several receptor systems (Haikkila et al., 1982; Haikkila, 1983; Muakkassah-Kelly et al., 1983).
Rat choroid plexus possesses a novel serotonergic $^{125}$I-LSD-binding site. This site exhibits a high affinity for serotonin and for several serotonergic antagonists but a weak affinity for adrenergic and dopaminergic ligands. The ligand affinity profile of the choroid plexus site is unique and its site density ($B_{max}$ value) of 3100 fmol/mg of protein is more than 10-fold higher than the density of any other serotonergic site in brain homogenates. A high affinity $^{125}$I-LSD-binding site was also detected in homogenates from mouse, pig, rabbit, and cow choroid plexus, but the apparent site density and ratio of specific to nonspecific binding was much higher in the rat brain.

The serotonergic choroid plexus site does not resemble any of the three serotonergic sites previously described in the mammalian brain: 5-HT$_1A$, 5-HT$_1B$, or 5-HT$_2$. GTP and divalent cations modulate agonist affinity for the 5-HT$_1A$ (Mallet and Hamon, 1982; Hamon et al., 1983; Sills et al., 1984a) and 5-HT$_2$ sites (Battaglia et al., 1984) in a manner characteristic of adenylyl cyclase-linked receptors. We did not detect significant GTP modulation of agonist binding to rat choroid plexus sites under conditions that cause large effects on cerebral 5-HT$_2$, citalopram.

In comparison to the 5-HT$_1A$ site labeled by $^{125}$I-LSD in rat frontal cortex (Kadan et al., 1984), the choroid plexus site exhibits a 37-fold higher affinity for serotonin and a 7-fold lower affinity for the antagonists ketanserin and cinanserin. One serotonergic antagonist, mianserin, is much more potent at the choroid plexus cite than at the 5-HT$_1A$ site. In addition, the affinity of $^{125}$I-LSD for the choroid plexus site is 2- to 3-fold lower than its affinity for serotonin 5-HT$_2$ sites (Engel et al., 1983; Kadan et al., 1984; Hartig et al., 1985b).

The choroid plexus site also differs significantly from the 5-HT$_1A$ site. Cinanserin and ketanserin are weak inhibitors ($K_i = 1$ and 4$ \mu$M, respectively) of 5-HT$_1A$ binding in the hippocampus (Gozlan et al., 1983), whereas these same compounds are relatively potent inhibitors of $^{125}$I-LSD binding to the choroid plexus site ($K_i = 14$ and 28$ \mu$M, respectively). Furthermore, the selective 5-HT$_1A$ agonist 8-OH-DPAT (Medeiros and Frizzardi, 1983) has a weak affinity for the choroid plexus site ($K_i = 7.7 \mu$M), and the ligand affinity profile for $^{125}$I-serotonin binding to the 5-HT$_1A$ site (Pedigo et al., 1981; Sills et al., 1984b) differs significantly from that of the choroid plexus site. The choroid plexus site also differs from the 5-HT$_1B$ site. The 5-HT$_1B$ site exhibits a weak affinity for most serotonergic antagonists (Pedigo et al., 1981; Fardin et al., 1984; Sills et al., 1984b), whereas the choroid plexus site exhibits a strong to moderate affinity for all serotonergic antagonists we tested.

The rat choroid plexus $^{125}$I-LSD-binding site was localized to the epithelial cell layer by high resolution light microscopic autoradiography using a stripping film technique. The sites are distributed throughout the choroid plexus epithelial cell layer but are absent from ependymal cells which line the ventricles. Various serotonergic sites have also been found on other non-neuronal cells. $^{3}$H-Serotonin binding sites have been described on crude glial cell membrane fractions (Fillon et al., 1980), on C6 glioma cells (Whitaker-Azmitia and Azmitia, 1984), and on astrocytes in primary cultures (Hertz et al., 1979; Whitaker-Azmitia and Azmitia, 1984). In our own laboratory we have found serotonergic $^{3}$H-LSD-binding sites on astrocyte-enriched fractions isolated from adult rat brains (Gal and Hartig, 1982). These studies demonstrate that serotonergic sites are found on a variety of non-neuronal cells and emphasize the uncertainty that currently exists regarding the cellular location of serotonergic sites in the mammalian brain.

Following completion of this work, we learned of a paper by Palacios and co-workers (Pazos et al., 1984) which describes the properties of a new serotonergic site (termed 5-HT$_{ch}$) labeled by $^{3}$H-mesulergine and $^{3}$H-serotonin in pig choroid plexus. The serotonergic site they characterized in pig choroid plexus is present at a much lower density than is the rat choroid plexus binding site. Although there is a clear similarity between the 5-HT$_{ch}$ site defined by $^{3}$H-serotonin and the $^{125}$I-LSD-binding site in choroid plexus, there are also differences which may be important. First, the number of choroid plexus 5-HT$_{ch}$ sites per mg of protein is lower than the number of 5-HT$_1A$ sites per mg of protein. Second, the dissociation constant ($K_d$) for $^{3}$H-serotonin binding to the choroid plexus 5-HT$_{ch}$ site is 6.5 nm (Pazos et al., 1984), whereas the $K_d$ value for serotonin competition at $^{3}$H-mesulergine, and $^{125}$I-LSD sites is 20.
to 30 nm (Pazos et al., 1984; this paper), Finally, various compounds exhibit similar but not identical \( K_v \) values in displacing \(^{125}\text{I}-\text{LSD} \), \(^{3}H\)-mesulergine, or \(^{3}H\)-serotonin from choroid plexus binding sites. For example, cinanserin is a much weaker inhibitor of both \(^{3}H\)-serotonin and \(^{3}H\)-mesulergine binding (\( K_v = 1585 \) and 200 nm, respectively (Pazos et al., 1984)) than \(^{125}\text{I}-\text{LSD} \) binding to choroid plexus (\( K_v = 74 \) nm; Table I). These differences do not appear to be species effects since the binding properties in rat (Pazos and Palacios, 1985) and in pig (Pazos et al., 1984) are quite similar.

The physiological function of the choroid plexus serotonergic site has not been identified; thus, it cannot yet be classified as a receptor site. The choroid plexus is a richly vascularized structure which acts as a major, but not the only, site of cerebrospinal fluid (CSF) production. The epithelial cell serotonergic site in this tissue is well situated to modulate some aspect of CSF production or composition. In a recent ventriculocisternal perfusion study, serotonin was shown to be the most effective monoamine modulator of CSF, causing a 40% reduction in the rate of CSF production when the serotonin precursor 5-hydroxytryptophan was injected intravenously (Maeda, 1983).

The choroid plexus serotonergic site may act as a receptor site for serotonergic modulation of CSF production. It is also possible that the choroid plexus serotonergic site serves as a transport site for serotonin or a closely related monamine. Two studies (Tochino and Schanker, 1965; Lindvall et al., 1980) have described a choroid plexus active transport system for serotonin, whereas another study (Chan-Palay, 1976) failed to find serotonin uptake by this structure. If the choroid plexus serotonergic site is a receptor transport carrier, its binding properties differ markedly from those of other serotonin uptake systems (Whipple et al., 1983; Wong et al., 1983).

It is not clear whether the choroid plexus serotonergic site is activated by serotonin released locally within the choroid plexus or by serotonin released from a remote site. Extensive immunochemical studies with serotonin antibodies (Lidov et al., 1980; Steinbusch, 1981) and histochemical fluorescence studies (Aghajanian and Gallager, 1975) failed to note any serotonergic innervation of the choroid plexus. Recently, however, in ovo serotonergic innervation of rat choroid plexus was detected when rats were pretreated with reserpine and 5-hydroxytryptophan (Napoleone et al., 1982), and low levels of serotonin were detected in the choroid plexus by a sensitive enzymatic assay (Moakowitz et al., 1979). The in ovo serotonergic innervation of choroid plexus occurs mostly on the walls of blood vessels in the choroid plexus with one report suggesting that a few indoleaminergic fibers contact the epithelial cells (Napoleone et al., 1982), whereas another study (Nakamura and Moriyasu, 1978) found no nerve endings in direct contact with epithelial cells. Another possibility is that serotonin reaches the choroid plexus following release from rich presynaptic stores in the ventricles. A dense network of serotonergic fibers is present on the surface of ependymal cells which line the walls of the ventricles (Aghajanian and Gallager, 1975). These supraneural fibers contain numerous varicosities with small and large vesicles in which serotonin is stored (Richards and Gelpen, 1982), as well as an active serotonin uptake system which is stimulated by electrical excitation (Chan-Palay, 1976) of the corresponding cell bodies in the raphe nuclei (Aghajanian and Gallager, 1975). Interestingly, the supraneural serotonergic fibers do not form recognizable synapses with the ependymal cells (Richards and Gelpen, 1982), but they are situated in contact with CSF fluid which may serve to receive and distribute serotonin released by these fibers. The target sites do not appear to be ependymal cells since these cells are devoid of serotonergic binding sites (Meibach et al., 1980; see Fig. 2). Suspended in the CSF, however, is the choroid plexus which is richly endowed with serotonergic binding sites. It is tempting to speculate that serotonin serves a hormonal or neuromodulatory role in this system in which it is released into the CSF under control of the raphe nuclei, diffuses through the CSF, and activates receptor sites on the choroid plexus which control some aspect of CSF production.

Examples of similar action-at-a-distance by serotonin (neuromodulatory or hormone-like action) have been observed in other systems (for reviews see Kupfermann, 1979; Aghajanian, 1981; Bloom, 1981; Gerfen, 1981; Shain and Carpenter, 1981).

The current study extends our understanding of the choroid plexus serotonergic site in several new directions. We have localized this site to the epithelial cell layer of choroid plexus. The utility of \(^{125}\text{I}-\text{LSD} \) as a marker for choroid plexus sites has been demonstrated and the high sensitivity of this ligand has made it possible to study the pharmacological properties of this site in membrane homogenates from rat choroid plexus, where the amount of available tissue is extremely limited. The choroid plexus should provide an excellent source for future studies on the cell biology of this serotonergic site because it is a relatively simple tissue which contains only a few different cell types and it can be maintained in culture (Crock et al., 1981). Since the choroid plexus serotonergic site is present at very high densities, this tissue should also be useful for biochemical studies. With the localization of this novel serotonergic site to choroid plexus epithelial cells, we can focus on these cells in an effort to understand this intriguing ventricular serotonergic system.

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