Regional Differences in Binding of [3H]LSD and [3H]5-HT in Calf Hippocampal Slices Revealed by Radioautography and Rapid Filtration Studies*

RICHARD C. MEIBACH, SHERYL G. BECK, SAUL MAAYANI and JACK PETER GREEN

Department of Pharmacology, Mount Sinai School of Medicine of the City University of New York, New York, NY 10029 (U.S.A.)

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Previous radioautographic experiments demonstrated that binding sites labeled by [3H]5-HT and [3H]LSD in rat brain were seen in all layers of CA1, CA4 and the dentate gyrus but not in fields CA2 and CA3 of the hippocampus. In an attempt to confirm this observation we performed binding assays on homogenates from selected areas of calf hippocampus since the small size of the rat hippocampus precluded using preparations from this animal for this purpose. Studies on homogenates from calf hippocampal regions, were done after we determined that the binding to slices in vitro was similar in the calf and rat. Binding of both [3H]5-HT and [3H]LSD by homogenates of CA1 and dentate gyrus, but not of CA3, was saturable. These studies show that the qualitative differences in binding site distribution within the calf hippocampus seen by radioautography reflect quantitative differences in the densities of binding sites revealed by the homogenate studies.

INTRODUCTION

The binding sites for 5-hydroxytryptamine (5-HT) in brain have been defined with either [3H]5-HT or [3H]lysergic acid diethylamide ([3H]LSD) as labeling ligands both in brain homogenates4,5,9,10,12,16,17 and in coronal slices from rat brain7,14,15. The regional distribution of the binding sites in homogenates labeled by [3H]5-HT and [3H]LSD was similar, and showed a heterogeneous distribution with highest densities in the hippocampus, striatum, and cortex5,10,18. Radioautography of rat brain coronal slices in vitro revealed additional sites of high-affinity binding (e.g. choroid plexus, septum, raphe, substantia nigra and interpeduncular nucleus) and furthermore, showed the discrete distribution of the binding sites within the brain regions7,14,15,22.

Notable was the hippocampus of the rat where sites shared by 5-HT and LSD were seen in all layers of fields CA1, CA4 and the dentate gyrus (DG), but not in areas CA2 and CA37,14,15. This striking difference warrants confirmation by binding studies in homogenates from these regions of the hippocampus. Since the rat provides insufficient tissue for this work, we did the experiments on homogenates of calf hippocampus. In parallel, we did radioautography with [3H]LSD and [3H]5-HT on calf hippocampal brain slices in vitro.

MATERIALS AND METHODS

Calf brains, that were stored on ice immediately after being removed, were obtained within 2–3 h of slaughter. After the hippocampi were removed, they were frozen in liquid freon to be cut by a cryostat, or used whole or dissected for high-affinity binding experiments in homogenates.

Dissection of calf hippocampus

The hippocampus was placed on a plexiglas plate over an ice bath. Coronal slices 10 mm thick were cut, transverse to the longitudinal axis, and placed with the cut side facing up and further dissected under a Nikon stereomicroscope. Four cuts were made...
to divide the hippocampus into 3 parts. The subiculum was removed from CA1 by a cut made at the hippocampal fissure and discarded. CA1 was separated from CA2 by a transverse cut made from the end of the superior blade of the DG. The DG was separated from CA4 by making a C-shaped cut from the end of the superior blade along the cell body layer of the granule cells. The resulting parts of the dissection were identified as CA1, CA3 (containing CA2 and CA4) and the DG.

Binding to calf hippocampal membrane fractions

Whole hippocampus or the dissected subfields were weighed and homogenized in 40 vols. of ice-cold 0.32 M sucrose (10 strokes in a Teflon pestle glass homogenizer and twice for 15 s in a Poltron, setting number 7). The homogenate was centrifuged for 10 min at 1000 g and the pellet discarded. The supernatant was centrifuged for 12 min at 40,000 g for 1 min. The pellet (P₂) was washed by suspension in 40 vols. of 50 mM Tris-maleate containing 0.1% ascorbate (pH 7.4 at 37 °C) followed by centrifugation at 40,000 g for 12 min. The washed P₂ was resuspended in 10 vols. of the same buffer and incubated for 10 min at 37 °C to reduce endogenous 5-HT. Pargyline (0.05 mM) was added to the final P₂ suspension and kept on ice until used. Protein was measured by the method of Lowry et al. with bovine serum albumin as standard.

For saturation experiments, 0.3–0.5 mg protein of P₂ suspension were incubated with 100 μl of varying concentrations of [³H]LSD with or without 0.01 mM 5-HT in 25 ml Tris-maleate buffer, 50 mM, pH 7.4 at 23 °C, containing 0.1% ascorbate and 0.05 mM pargyline. Incubations were carried out at 23 °C for 90 min. After incubation, the sections were washed in 3 changes of ice-cold 50 mM Tris-maleate (pH 7.4 at 0 °C) for 10 min each. While still wet, the sections were removed from the glass slides with Whatman GF/C glass fiber filters held by a hemostat. The filter paper was put in a scintillation vial with 10 ml of Formula 963 for counting in the Beckman LS9000 scintillation spectrometer. Background values, measured by wiping the back of the slide with filter paper, were negligible. Quin-tuplicate determinations were made at each concentration of radioactive ligand.

Hippocampal slices were labeled with [³H]5-HT as described for [³H]LSD. However, non-specific binding was defined by displacement of [³H]5-HT by addition of 1 μM LSD to the incubation baths after the initial 90 min. incubation. The sections were incubated for an additional 15 min, washed with 3 changes of ice-cold buffer for 3 min each, then wiped from the slides with Whatman GF/C filters and counted as described above. All experiments were conducted in quintuplicates.

To determine the time to attain equilibrium, the sections or samples were incubated for 10, 30, 60, 90 or 120 min in [³H]LSD or [³H]5-HT in the absence or presence of 1 μM 5-HT or 1 μM LSD, respectively. Samples or sections were washed, filtered and counted as described above. To determine the reversibility of the binding, the samples or sections were incubated for 90 min with [³H]LSD or [³H]5-HT; 0.01 mM 5-HT or LSD, respectively, was added to...
the incubation medium and the samples further incubated for 5, 10, 20, 30, 40, 60, 90 or 120 min. The samples were washed, filtered and counted as described. The sections were rinsed, wiped from the slides with filter paper and counted as described.

Sensitivity of binding sites to denaturation was checked by placing the sections or samples in boiling water bath for 5 min. The samples or sections were incubated for 90 min in [3H]LSD or [3H]5-HT with or without 1 μM 5-HT or 1 μM LSD, respectively. The samples or sections were rinsed, filtered or wiped from the slides and counted as described.

Radioautography

For visualization of [3H]LSD sites sections were incubated for 90 min at room temperature in 5.5-8 nM [3H]LSD, and alternate sections were incubated in [3H]LSD plus 0.01 μM of 5-HT. For [3H]5-HT sites sections were incubated for 90 min at room temperature in 3-6 nM of [3H]5-HT. After the initial incubation 0.01 mM LSD was added to the medium containing alternate sections and the sections were incubated for an additional 15 min. The difference in the protocol is based on results from experiments on kinetics of binding (see Results). The sections were washed 3 times in ice-cold medium for 10 min each for the sections incubated in [3H]LSD, or 3 min each for the sections incubated in [3H]5-HT. All sections were processed for radioautography by a modification of the method described15. After incubation and washing, the sections were dried at room temperature. Emulsion-coated (Kodak NTB2 diluted 1:1 with distilled water) coverslips or slides were opposed to the sections. Sections were allowed to develop for 4-6 weeks at 4 °C. The coverslips or coverslides were developed in D-19. The sections were fixed in Carnoy's fixative and stained in 1% Pyronin Y. Examination and photography of reduced silver grains was made with a Wild M-400 Photomacroskop under dark field illumination. Quantification of reduced silver grains was obtained at a magnification of 400X with a Zeiss research microscope.

D-[2(N)-3H]lysergic acid diethylamide (13.3 Ci/mmol) was obtained from Amersham and [3H]5-HT (29.2 Ci/mmol) from New England Nuclear. Unlabeled LSD as the tartrate salt was obtained from NIDA and unlabeled 5-HT oxalate was obtained from Sigma. Purity of the labeled compounds was tested by thin-layer chromatography (for [3H]LSD, chloroform: methanol: concentrated ammonia 90:5:1 on Eastman 13181 silica gel plates; for [3H]5-HT n-butanol:glacial acetic acid:water 25:4:10 on Whatman No.1 chromatography paper).

RESULTS

Saturation experiments on homogenates and slices

The amount of time needed to reach maximal binding (90 min) appeared to be the same for both [3H]5-HT and [3H]LSD. The time course of dissociation of [3H]5-HT and [3H]LSD binding in calf hippocampal slices was very different. The dissociation rate of bound [3H]5-HT was much faster than for [3H]LSD with a calculated t1/2<1 min as compared to a t1/2=10 min for [3H]LSD. Therefore, to define nonspecific binding of [3H]5-HT sites in the coronal slices, displacement by 1 μM LSD rather than competition was used, as described in Methods.

Saturation experiments for [3H]5-HT and [3H]LSD were carried out on homogenates and whole tissue slices from the hippocampus. The binding of [3H]LSD and [3H]5-HT was saturable both in homogenates from whole hippocampus and in slices. The values of Kd and Bmax of the specific binding were ob-

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Sensitivity of [3H]LSD and [3H]5-HT binding sites in calf hippocampus preparations to heat</th>
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<tbody>
<tr>
<td><strong>Preparation</strong></td>
<td><strong>[3H]LSD binding</strong></td>
</tr>
<tr>
<td></td>
<td><strong>No 5-HT</strong></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Slices*</td>
<td>195 ± 29</td>
</tr>
<tr>
<td>heated</td>
<td>48 ± 10</td>
</tr>
<tr>
<td>P2**</td>
<td>145 ± 3</td>
</tr>
<tr>
<td>unheated</td>
<td>111 ± 59</td>
</tr>
<tr>
<td>heated</td>
<td></td>
</tr>
</tbody>
</table>

* 5.1 nM [3H]LSD.  ** 7.7 nM [3H]LSD.  *** 6.5 nM [3H]5-HT.  **** 4.7 nM [3H]5-HT.
Fig. 1. A: photomicrograph of a cresyl-violet stained section of a calf hippocampus with the subfields of the hippocampus labeled. B: dark-field photomicrograph of the radioautographic localization of [3H]5-HT binding in a coronal section of calf hippocampus. Sections were incubated in 3 nM [3H]5-HT for 90 min at 25 °C, then washed in 3 changes of cold buffer. Reduced silver grains are present only in areas CA1 and DG.
tained from computer fits to a hyperbolic function. At the concentrations used, both [3H]5-HT and [3H]LSD showed similar affinities in homogenates and slices, i.e. \( K_d = 1.0-2.1 \text{ nM} \). The calculated \( B_{\text{max}} \) for [3H]LSD and [3H]5-HT sites was also similar in both preparations, i.e., 67-131 fmol/mg protein.

After homogenates and slices were heated the total amount of [3H]LSD or [3H]5-HT bound was reduced to that of the non-specific binding in the presence of excess 5-HT or LSD (Table I), implying the absence of specific binding in heat-denatured tissue.

**Radioautography**

The distribution of reduced silver grains in the coronal brain sections treated with [3H]5-HT was heterogeneous (Fig. 1). A very similar pattern was seen with [3H]LSD. The DG and field CA1 were labeled; no reduced silver grains could be found in fields CA2, CA3 or CA4 (Fig. 1). Grain counting confirmed this observation. The largest number of grains were found in the DG and CA1, whereas the number of grains in areas CA3 and CA4 was closer to background values (Table II). Competition by 1 μM 5-HT for [3H]LSD sites or displacement of [3H]5-HT sites by 1 μM LSD eliminated the reduced silver grains from all areas of the hippocampal coronal sections.

**[3H]5-HT and [3H]LSD binding sites in homogenates of dissected calf hippocampus**

To probe further the distribution of binding sites in the calf hippocampus, experiments were carried out on homogenates of CA1, DG and CA3. Saturation experiments were carried out for both [3H]5-HT and [3H]LSD binding sites. The binding was saturable in CA1 and DG. The \( B_{\text{max}} \) and \( K_d \) values for CA1 and DG were obtained from the hyperbolic fit of the data, and are shown in Table III. The \( K_d \) and \( B_{\text{max}} \) for [3H]LSD were similar in both CA1 and DG, as were the \( K_d \) and \( B_{\text{max}} \) for [3H]5-HT. However, the \( K_d \) and \( B_{\text{max}} \) were not the same for [3H]LSD and [3H]5-HT.

The \( B_{\text{max}} \) estimate when [3H]LSD was used to label the sites was larger than when [3H]5-HT was used as the labeling ligand. The affinity of [3H]5-HT appeared to be higher than that of [3H]LSD sites. The amount of specific binding in area CA3 was negligible in 4 of 6 experiments and therefore it was not possible to obtain estimates of the \( K_d \) and \( B_{\text{max}} \) in two experiments the binding of [3H]5-HT in CA3 appeared saturable, but the \( B_{\text{max}} \) (38 fmol/mg protein) was much less than in area CA1 or the DG (65 and 76 fmol/mg protein, respectively). Overall, the density of binding sites for both ligands in area CA3 was very low or negligible as compared with that in CA1 or the DG, in confirmation of the results obtained from radioautography.

**DISCUSSION**

The binding characteristics, i.e. the \( K_d \) and \( B_{\text{max}} \) of sites labeled by [3H]5-HT and [3H]LSD both in homogenates and in coronal slices from whole calf hippocampus, were very similar and in agreement with values previously reported for rat brain homogenates\(^{6,9,10,12,16,17}\) and coronal slices from rat brain\(^{7,14,15}\). The sites labeled in the coronal slices were destroyed by heat. The binding of both tritiated ligands was reversible. It can be concluded that both

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**TABLE III**

Binding parameters of [3H]LSD and [3H]5-HT in dissected regions of the P3 fraction of calf hippocampus.

<table>
<thead>
<tr>
<th>Region</th>
<th>[3H]LSD</th>
<th>[3H]5-HT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_d ) (nM)</td>
<td>( B_{\text{max}} ) (fmol/mg prot)</td>
</tr>
<tr>
<td>CA1</td>
<td>2.8 ± 0.9</td>
<td>138 ± 38</td>
</tr>
<tr>
<td>DG</td>
<td>6.2 ± 4.1</td>
<td>190 ± 40</td>
</tr>
<tr>
<td>CA3</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. of 3 experiments.
[3H]LSD and [3H]5-HT are binding to specific high-affinity binding sites in coronal slices, as in homogenates. The overall binding characteristics would suggest that the sites labeled by these ligands are very similar or even identical, but the regional differences (Table III) discussed below preclude such a conclusion.

Radioautographs showed an uneven distribution of reduced silver grains within the calf hippocampal coronal sections. The label was evenly distributed in all layers of DG and area CA1 and the grain density in the two areas was very similar; no silver grains were present in areas CA2, CA3, and CA4. This heterogeneous distribution of the binding sites was confirmed by high-affinity binding experiments in homogenates from dissected regions of the hippocampus, areas CA1, CA3 and the DG. The Kd of the sites labeled by [3H]5-HT and sensitive to 0.01 mM LSD was approximately two-fold lower in areas CA1 and DG than for the sites labeled by [3H]LSD and sensitive to 0.01 mM 5-HT. Also, the number of sites labeled by [3H]5-HT was approximately half the number of [3H]LSD-labeled sites in both DG and CA1. The binding of both [3H]5-HT and [3H]LSD in area CA3 was too low and inconsistent to estimate the Kd and Bmax. Consonant with the radioautograms, the results from the homogenate experiments showed no detectable binding sites in CA3 in contrast with CA1 and DG (Table III). Therefore, it can be concluded that the qualitative differences in grain densities visualized by radioautography within the hippocampus are representative of absolute differences in binding site densities as quantified by homogenate studies.

Like the calf hippocampus, the rat hippocampus14,15 showed a high density of binding sites, labeled by [3H]LSD and [3H]5-HT, in CA1 and the DG, and negligible binding in CA2 and CA3. But in the rat a high density of binding sites was also apparent in CA4, whereas in calf no binding was seen in CA4. In contrast, in human hippocampal tissue, the distribution of 5-HT binding sites labeled by [3H]5-HT appears to be uniform in areas CA1, CA3 and CA4, with a higher density of sites in the DG. Clearly, the most apparent difference in the distribution of the binding sites among species is in areas CA3 and CA4.

At least in rat there is no correlation between the distribution of 5-HT binding sites and the distributions of 5-HT or its synthesizing enzyme19, or the distribution of 5-HT containing nerve terminals as identified by autoradiography1,3 and immunohistochemistry11, and by 5-HT uptake2.

The binding sites may relate to physiological and pharmacological effects of 5-HT and LSD. In the rat isolated hippocampal slice21, the 5-HT-stimulated increase in extracellular K+ concentration was elicited from CA1 and DG but not from CA3 (although electrically evoked increase in extracellular K+ occurred equally from both CA1 and CA3), perhaps consonant with our finding of dense labeling in CA1 and DG and negligible binding in CA3. Intracellular recordings showed that all 3 regions were hyperpolarized by 5-HT, but the accompanying decrease in input resistance was much smaller in CA3 than in CA1 or DG20.

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