Characterization of Multiple $[^3H]5$-Hydroxytryptamine Binding Sites in Rat Spinal Cord Tissue

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Abstract: High-affinity $[^3H]5$-hydroxytryptamine ($[^3H]5$-HT) binding in the rat spinal cord is similar to that demonstrated in the frontal cortex. $[^3H]5$-HT binds with nearly the same affinity to sites in both tissues. Furthermore, similar patterns of displacement of $[^3H]5$-HT were seen in both tissues, with either spiperone or LSD as the unlabeled ligand. This high-affinity binding appears to be to multiple sites, since displacement studies using 2 nM $[^3H]5$-HT result in Hill coefficients less than unity for spiperone, LSD, and quipazine (Hill coefficients ($n_H$): 0.44, 0.39, 0.40, respectively). These sites apparently have an equal affinity for $[^3H]5$-HT, since unlabeled 5-HT did not discriminate between them. Thus, the high-affinity $[^3H]5$-HT binding in the spinal cord may be analogous to that observed in the frontal cortex, where two populations of sites have previously been described (5-HT$_{1A}$, 5-HT$_{1B}$). In addition to the multiple high-affinity spinal cord binding sites, a low-affinity $[^3H]5$-HT binding component was also identified. A curvilinear Scatchard plot results from saturation studies using $[^3H]5$-HT (0.5–100 nM) in the spinal cord. The plot can be resolved into sites having apparent dissociation constants of 1.4 nM and 57.8 nM for the high- and low-affinity components, respectively. Additional support for a change in affinity characteristics at higher radioligand concentrations comes from the displacement of 30 nM $[^3H]5$-HT by the unlabeled ligand. A nonparallel shift in the dissociation curve was seen, resulting in a Hill coefficient less than unity (0.32). None of the specifically bound $[^3H]5$-HT in the spinal cord is associated with the 5-HT uptake carrier, since fluoxetine, an inhibitor of 5-HT uptake, does not alter binding characteristics. In addition, a 5-HT binding site analogous to the site designated 5-HT$_2$ was not apparent in the spinal cord. Ketanserin and cyproheptadine, drugs that are highly selective for 5-HT$_2$ sites, did not displace $[^3H]5$-HT from spinal tissue, and $[^3H]$spiperone, a radioligand that binds with high affinity to 5-HT$_2$ sites, did not exhibit saturable binding in the tissue. Thus, the 5-HT$_2$ binding site reported in other regions of the central nervous system, and the serotonin uptake carrier do not appear to contribute to the multiple binding sites demonstrated in the spinal cord. Key Words: Spinal cord—$[^3H]5$-hydroxytryptamine binding—Spiperone—LSD—Fluoxetine—Ketanserin—Quipazine.

The existence of multiple serotonin (5-HT) receptors has been suggested by several investigators, who used electrophysiological (Roberts and Straughn, 1967; Aghajanian and wang, 1978; McCall and Aghajanian, 1979) or receptor binding techniques (Peroutka and Snyder, 1979; Pedigo et al., 1981). (For a review of multiple receptors, see Snyder and Goodman, 1980; Langer, 1981.) However, these studies have dealt exclusively with brain tissues, and while others have suggested the existence of a multiple 5-HT receptor system in the periphery (Gaddum and Picarelli, 1957), the possibility that there is a similar system in the spinal cord has not been examined.

The experiments described in this paper were undertaken to characterize 5-HT binding in the spinal cord, and to compare the data obtained with those previously reported for frontal cortex tissue (Peroutka and Snyder, 1979; Pedigo et al., 1981). Kinetic analysis of data generated from ligand binding studies was used both to determine the existence of multiple 5-HT binding sites and to

Abbreviations used: 5-HT, 5-Hydroxytryptamine (serotonin); LSD, Lysergic acid diethylamide.
characterize these sites partially with respect to drug affinities.

MATERIALS AND METHODS

Tissue preparation

Tissues were prepared according to the method of Peroutka and Snyder (1979). Briefly, frontal cortex and/or spinal cord tissues were removed from adult male Sprague-Dawley rats (225–250 g, Hilltop Laboratories, Scottsdale, PA) shortly after decapitation. Tissues were homogenized in a Teflon/glass homogenizer, in 10 volumes of 0.32 M sucrose, then centrifuged at 700 × g for 10 min in a Sorval RC-2B centrifuge. The resulting supernatant was decanted and centrifuged once more at 40,000 × g for 12.5 min to obtain a crude synaptosomal pellet. The synaptosomes were then lysed by resuspension of the pellet in 10 volumes of 50 mM Tris-HCl (pH 7.5) by means of a Brinkman Polytron (setting 5 for 10 s). Following a 10-min incubation at 37°C, the tissue was centrifuged (40,000 × g, 12.5 min) and the synaptosomal membrane fragments were resuspended (as above) in an appropriate volume of assay buffer (50 mM Tris-HCl containing 10 μM pargyline, 4 mM CaCl₂, and 0.1% ascorbic acid, pH 7.7) to give tissue concentrations of either 10 mg/ml ([³H]spiperone assays) or 20 mg/ml ([²H]5-HT assays). This suspension was incubated at 37°C for 15 min and stored on ice (up to 2 h) until it was used for the binding reaction.

Receptor binding assays

Incubation tubes contained 800 μl of tissue suspension, 100 μl of a [³H] ligand at the appropriate concentration, and either 100 μl of buffer or 100 μl of the appropriate concentration of competing drug. Binding reactions were performed at 37°C for 10 min ([³H]5-HT, frontal cortex), 15 min ([³H]spiperone, either frontal cortex or spinal cord), or 20 min ([²H]5-HT, spinal cord). All tubes were run in triplicate, with each experiment being run a minimum of three times. Reactions were terminated by vacuum filtration of the contents of the incubation tubes on Whatman GF/B filters. The filters were rinsed with three 5-ml aliquots of assay buffer, and the amount of radioactivity trapped on the filters was determined by liquid scintillation spectrophotometry following a 5-h extraction in scintillation cocktail.

Preliminary experiments were conducted to verify that when either frontal cortex or spinal cord tissue was used, proper receptor binding criteria were met under the conditions chosen for the assays. Saturable [³H] ligand binding was shown to be optimal at the pH (7.7) and temperature (37°C) maintained during the assay. Binding was also shown to be dependent on the tissue concentration chosen for the assay, and to be at equilibrium at the time chosen for termination of the reactions. To assay for metabolism of the ligand, the supernatant resulting from the centrifugation of the effluent collected during the rinsing of the filters was reincubated with additional aliquots of tissue suspension. No decrease in the amount of radioligand binding was detected, nor were any metabolites of the radioligand detected (by HPLC—Yaksh and Tyce, 1979) in the resulting effluent.

The ratio of specific binding, defined as total binding less binding in the presence of 1 μM 5-HT, to total binding was higher in the frontal cortex than in the spinal cord.

Values were consistently 60–70% and 35–40%, respectively, when the radioligands were purified prior to use by high performance liquid chromatography (Yaksh and Tyce, 1979). No differences in the ratio of specific to nonspecific binding were observed with increasing concentrations of unlabeled ligand up to 10⁻³ M, the concentration used in saturation studies. Kinetic parameters from saturation studies using spinal cord tissue were determined by a nonlinear least-squares regression analysis (NLIN—Barr et al., 1976) of Scatchard plot data (Scatchard, 1949), according to the following equation, which describes the amount bound (B) at a given free ligand concentration [L], using a 2-site model:

\[ B = \left( \frac{B_{max1}}{K_{d1} + [L]} \right) + \left( \frac{B_{max2}}{K_{d2} + [L]} \right) \]

Hill coefficients were calculated from displacement studies by the method described by Wetland and Molinoff (1981). The [³H] ligands were purchased from New England Nuclear (Boston, MA), 5-HT from Sigma (St. Louis, MO). LSD (lysergic acid diethylamide) was obtained from NIDA, and all other drugs were graciously supplied by the following companies: Janssen Pharmaceuticals, Beerse, Belgium (spiperone, ketanserin); Eli Lilly & Co., Indianapolis, IN (fluoxetine); Merck, Sharp and Dohme, Rahway, NJ (cyproheptadine); Miles Laboratories, Elkhart, IN (quipazine). All drugs were diluted with assay buffer to their appropriate concentrations; [³H]spiperone and [²H]5-HT were diluted with distilled water.

RESULTS

[³H]Serotonin binding studies

Scatchard analysis of saturation data (1–40 nM [³H]5-HT) obtained from frontal cortex tissue yielded apparent Kᵦ and Bᵦ values of 4.4 nM and 14.9 pmol/g tissue, respectively. These results agree with values reported previously by Peroutka and Snyder (1979) (Kᵦ = 4.6 nM; Bᵦ = 9.2 pmol/g tissue) and Pedigo et al. (1981) (Kᵦ = 2.17 nM; Bᵦ = 8.27 pmol/g tissue). However, in contrast to the data obtained with frontal cortex tissue, a curvilinear Scatchard plot was obtained from analysis of saturation experiments (0.5–100 nM [³H]5-HT) with spinal cord tissue (Fig. 1). Nonlinear regression analysis of the data, assuming a 2-site model (see Materials and Methods), resulted in apparent Kᵦ values of 1.4 and 57.8 nM for the high- and low-affinity sites. The apparent Bᵦ values for the high- and low-affinity sites were calculated to be 0.7 and 13.1 pmol/g tissue, respectively. Alternative explanations of the change in affinity characteristics of [³H]5-HT binding (reflected in the curvilinear nature of Scatchard plot) might be the existence of negative cooperativity among a single population of binding sites or nonspecific interactions with membrane macromolecules that become more apparent at the higher radioligand concentrations.

To verify further the multiplicity of affinities describing [³H]5-HT binding in the spinal cord, displacement studies were performed using 2 and 30 nM radioligand concentrations. On the basis of the
above saturation experiments, it was expected that at a 2 nM radioligand concentration, high-affinity binding should predominate. In contrast, with 30 nM [3H]5-HT a sufficiently high concentration of ligand should have been achieved for low-affinity binding to be exhibited. (It should be pointed out, however, that the concentration required to elicit detectable low-affinity binding is only 15 times higher than the concentration used for preferential exhibition of high-affinity binding; therefore, even with 2 nM [3H]5-HT, some low-affinity binding should have occurred.)

A steep displacement curve was generated when 2 nM [3H]5-HT was displaced from spinal cord tissue by increasing concentrations of 5-HT (Fig. 2). Hill analysis of these data yielded a coefficient of 0.84, suggesting that the radioligand was being displaced from a population of sites with a single affinity for 5-HT. In contrast, when 30 nM [3H]5-HT was used, the resulting displacement curve had a more gradual slope. The Hill coefficient calculated from these data was 0.32, suggesting that either the radioligand was being displaced from multiple populations of binding sites or that negative cooperativity was more apparent at the higher radioligand concentration, and that the changing affinity of 5-HT binding sites was reflected in the nonparallel shift of the displacement curve.

The potencies of 5-HT, LSD, and spiperone in displacing the radioligand from the sites preferentially labeled by 2 nM [3H]5-HT are similar to those reported for the displacement of [3H]5-HT from the high-affinity [3H]5-HT binding site in the frontal cortex, designated 5-HT₁, by Peroutka and Snyder.
(1979) (Table 1; Fig. 3, 5-HT in comparison with spiperone only). Serotonin readily interacts with the spinal cord sites, resulting in an IC₅₀ value in the nanomolar range. The sites, however, display a weak affinity for spiperone, as indicated by an IC₅₀ value in the micromolar range, and LSD binding is characterized by an intermediate affinity. Quipazine, reported to be a presynaptic 5-HT receptor antagonist (Martin and Sanders-Bush, 1982), also displaced [³H]5-HT from spinal cord sites. Unlike the case for 5-HT, which yielded a Hill coefficient near unity, the displacement of [³H]5-HT by all these drugs yielded Hill coefficients less than unity (Table 1), suggesting that the radioligand was displaced from multiple sites. These results are consistent with those of Pedigo et al. (1981), who demonstrated that the high-affinity [³H]5-HT binding in the frontal cortex was to multiple sites (designated 5-HT₁A and 5-HT₁H) which were discriminated by spiperone.

Drugs which previously have been reported to show a preference for the 5-HT₂ site were also used in displacement studies. Ketanserin, a 5-HT₄-selective ligand (Leysen et al., 1982), and cyproheptadine, a drug showing a 500-fold greater preference for 5-HT₂ sites than 5-HT₁ (Williams and Martin, 1982), did not displace specifically bound radioligand from spinal cord tissue even when used in concentrations up to 1 μM (Table 1). These results suggest that a binding site for the radioligand analogous to the 5-HT₂ site described in other tissue (Peroutka and Snyder, 1979; Leysen et al., 1982) is absent in the spinal cord, as has been previously demonstrated by others (Blackshear et al., 1981; Leysen et al., 1982).

**[³H]Spiperone binding studies**

Saturation studies with a radioligand previously used to label 5-HT₂ sites in the frontal cortex (Peroutka and Snyder, 1979) were performed to verify the apparent absence of a 5-HT₁ site in the spinal cord.

[³H]Spiperone binding in the frontal cortex was shown to be both saturable and dissociable (Fig. 4, *left*) and yielded kinetic characteristics similar to those previously reported for this site (Peroutka and Snyder, 1979). However, [³H]spiperone binding increased linearly with increasing radioligand concentrations in the cord (Fig. 4, *right*), suggesting that the radioligand interacts with spinal cord sites only in a nonspecific manner.

Rapid metabolism of a spinal cord binding site could not account for our inability to detect specific [³H]spiperone binding, since the addition of spinal cord tissue to an incubation tube containing frontal cortex tissue did not decrease the amount of radioligand bound.

![FIG. 3](image_url) Displacement of 2 nM [³H]5-HT by 5-HT and spiperone in the spinal cord. Data presented are mean ± SEM of five experiments for each displacing ligand. B, Specifically bound [³H]5-HT. B₀, [³H]5-HT specifically bound in absence of displacing ligand.

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**TABLE 1. Effects of various drugs on the specific binding of [³H]5-HT to rat spinal cord tissue**

<table>
<thead>
<tr>
<th>Drugs (n)</th>
<th>IC₅₀ (nM)</th>
<th>nH</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT (5)</td>
<td>5.1 ± 1.48</td>
<td>0.84 ± 0.08</td>
</tr>
<tr>
<td>Spiperone (3)</td>
<td>1987 ± 865</td>
<td>0.44 ± 0.09</td>
</tr>
<tr>
<td>LSD (5)</td>
<td>25.4 ± 14.8</td>
<td>0.39 ± 0.08</td>
</tr>
<tr>
<td>Quipazine (5)</td>
<td>1150 ± 841</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>Ketanserin (3)</td>
<td>N.S.D.</td>
<td>—</td>
</tr>
<tr>
<td>Cyproheptadine (3)</td>
<td>N.S.D.</td>
<td>—</td>
</tr>
</tbody>
</table>

The concentration producing a 50% reduction in specifically bound [³H]5-HT (IC₅₀) was calculated by linear regression analysis of logit-log inhibition plots. Each value is the mean ± SEM of (n) separate experiments, using the averages of triplicate values. N.S.D. = No significant displacement seen at 10⁻⁶ M drug concentration.

* IC₅₀ versus 2 nM [³H]5-HT.
Effect of fluoxetine, an inhibitor of the serotonin neuronal membrane transport process, on ligand binding in the spinal cord

The apparent $K_D$ of the low-affinity 5-HT binding component in the spinal cord (57 nM) was in the same range as the $K_m$ values calculated previously for the 5-HT uptake carrier (50 nM—Azzaro and Smith, 1977; 110 nM—Ahtee et al., 1981). Therefore, the effect of a 5-HT uptake inhibitor on the spinal cord binding of 30 nM $[^3H]5$-HT was examined to determine if the uptake site contributes to ligand binding in the spinal cord. Fluoxetine, when used over a wide concentration range ($10^{-10}$ to $10^{-5}$ M), had no effect on the amount of specifically bound radioligand. In addition, Hill coefficients resulting from the displacement of the radioligand by unlabeled 5-HT were the same in either the presence or the absence of the inhibitor (Fig. 5). These results are consistent with those of others who were unable to demonstrate an effect of fluoxetine on the binding of $[^3H]5$-HT to brain tissues (Bennett and Snyder, 1976; Savage et al., 1980).

**DISCUSSION**

High-affinity ($K_D$, 1.4 nM from Scatchard analysis—Fig. 1) $[^3H]5$-HT binding in the spinal cord resembles that observed in the frontal cortex. Similar $K_D$ and $B_{max}$ values describe this binding in both tissues. In addition, competing ligands exhibit similar characteristics in their displacement of 2 nM $[^3H]5$-HT ($IC_{50}$ values, Hill coefficients) from both frontal cortex and spinal cord tissue.

The high-affinity binding components of both the spinal cord and frontal cortex can apparently be subdivided into two populations of sites. Although 5-HT interacts at these sites with an equal affinity, they can apparently be differentiated by using other competing ligands which possess dissimilar potencies for displacing $[^3H]5$-HT from each of the subpopulations of sites. For example, Pedigo et al. (1981), using frontal cortex tissue, also reported low Hill coefficients for the displacement of $[^3H]5$-HT by spiperone. These investigators designated the high-affinity 5-HT binding sites differentiated by

![Modified Hill plot of the data resulting from the displacement of 30 nM $[^3H]5$-HT by unlabeled 5-HT in both the presence and absence of fluoxetine.](image)

![Comparison of $[^3H]spiperone binding in the cortex (left) and spinal cord (right). Data presented are representative of three separate experiments. Counting efficiencies ranged from 37 to 45%.](image)
spiperone 5-HT\textsubscript{A} and 5-HT\textsubscript{B}. Peroutka and Snyder (1979) may also have discriminated these multiple high-affinity \([\text{\textsuperscript{3}H}]\text{-5-HT}\) binding sites in rat frontal cortex, obtaining a Hill coefficient near unity for 5-HT, yet a low Hill coefficient describing the displacement of \([\text{\textsuperscript{3}H}]\text{-5-HT}\) by spiperone.

Thus, it appears that in the spinal cord, as has been suggested for brain tissues, certain drugs (spiperone, LSD, quipazine) may be capable of discriminating between subpopulations of high-affinity \([\text{\textsuperscript{3}H}]\text{-5-HT}\) binding sites (see \(n_H\) values in Table 1). In fact, because of similarities in \([\text{\textsuperscript{3}H}]\text{-5-HT}\) binding in frontal cortex and spinal cord tissues, these spinal cord sites may be analogous to the 5-HT\textsubscript{A} and 5-HT\textsubscript{B} sites in rat brain. In this regard, it would be valuable to examine the effects on high-affinity \([\text{\textsuperscript{3}H}]\text{-5-HT}\) binding in the spinal cord of the tryptamine and 5-HT analogs that have been shown to discriminate between 5-HT\textsubscript{A} and 5-HT\textsubscript{B} sites (Smit et al., 1981; Nelson et al., 1982).

The low-affinity \([\text{\textsuperscript{3}H}]\text{-5-HT}\) binding component in the spinal cord has been more difficult to characterize. Although two approaches have been used in its identification—saturation studies (0.5–100 nM \([\text{\textsuperscript{3}H}]\text{-5-HT}\)) and displacement studies (2 and 30 nM \([\text{\textsuperscript{3}H}]\text{-5-HT}\))—neither experiment rules out the possibility that negative cooperativity or nonspecific membrane interactions result in the curvilinear Scatchard plot (Fig. 1) and the nonparallel shift in the displacement curves (Fig. 2). On the other hand, if only sites with a single affinity were present, then a linear Scatchard plot (see Pedigo et al., 1981) and a parallel shift in the displacement curve (with no change in Hill coefficient) would have resulted. In addition, certain artificial conditions contributing to apparent multiple affinities can apparently be ruled out. When HPLC techniques were used to analyze filtrate fractions and to purify the isotope prior to its use, no change in the radioligand (metabolism or self-aggregation) or \(\text{\textsuperscript{3}H}\)-impurities could be detected. Furthermore, the use of membrane fractions and the filtration technique precludes the possibility of a mobile receptor with varying affinities, contributing to the \([\text{\textsuperscript{3}H}]\text{-5-HT}\) binding examined in this study. Finally, it is unlikely that the radioligand and the competing drug have different affinities for the binding site, since unlabeled 5-HT was used in these studies.

Although the significance of the low-affinity \([\text{\textsuperscript{3}H}]\text{-5-HT}\) binding remains to be determined, it can be concluded however, that none of the spinal cord \([\text{\textsuperscript{3}H}]\text{-5-HT}\) binding is associated with either the 5-HT uptake carrier site, or the 5-HT\textsubscript{2} binding site. A spinal cord site analogous to the high-affinity \([\text{\textsuperscript{3}H}]\text{spiperone site in the frontal cortex (5-HT\textsubscript{A}) could not be detected. \([\text{\textsuperscript{3}H}]\text{spiperone binding in the spinal cord was characteristic of nonspecific interactions, increasing linearly with increasing radioligand concentrations. Also, ketanserin and cyproheptadine, two drugs that are highly selective for the 5-HT\textsubscript{A} site (Leysen et al., 1982; Williams and Martin, 1982), were unable to displace specifically bound \([\text{\textsuperscript{3}H}]\text{-5-HT}\) from spinal cord tissue, further suggesting a lack of 5-HT\textsubscript{A} receptors in this tissue. These results are in agreement with the work of other investigators who failed to demonstrate specific \([\text{\textsuperscript{3}H}]\text{ketanserin (Leysen et al., 1982 and \([\text{\textsuperscript{3}H}]\text{spiperone (Blackshear et al., 1981) binding in the spinal cord.}

In addition, fluoxetine, a specific 5-HT uptake inhibitor, had no effect on spinal \([\text{\textsuperscript{3}H}]\text{-5-HT}\) binding, in agreement with what had been shown previously for brain tissues (Bennett and Snyder, 1979; Savage et al., 1980). The drug, when used in concentrations up to 200 times greater than its reported \(K_i\) for inhibiting uptake (Wong et al., 1974), did not displace specifically bound radioligand. Moreover, Hill coefficients obtained from an analysis of the ability of unlabeled 5-HT to displace 30 nM \([\text{\textsuperscript{3}H}]\text{-5-HT}\), in the presence or absence of the inhibitor, did not differ. The exact mechanism of action of fluoxetine has not been described; possibly, the inhibitor acts at a site adjacent to the carrier, as has been proposed for one 5-HT uptake inhibitor (Ahtee et al., 1981), but this does not seem likely. Fluoxetine has been shown to be a competitive inhibitor of 5-HT uptake (Wong et al., 1974), and therefore the drug is more likely to act at the same carrier site as the substrate. However, even if fluoxetine were to bind to a site adjacent to the uptake carrier, a displacement of \([\text{\textsuperscript{3}H}]\text{-5-HT}\) from the site would still be expected, as the inhibitor allosterically interferes with the uptake process.

The physiological significance of the multiple 5-HT binding sites in the frontal cortex and the spinal cord remains unresolved. However, with the recent demonstration of 5-HT autoreceptors (Farnebo and Hamberger, 1974; Cerrito and Raiteri, 1979; Gothert and Weinheimer, 1979), the possibility of presynaptic modulation of 5-HT release offers one potential role for these binding sites. This aspect of serotonergic neuronal function is currently under investigation in this laboratory.

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