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SEROTONIN RECEPTORS IN HIPPOCAMPUS AND FRONTAL CORTEX

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The inhibition by various serotonin agonists and antagonists of the binding of 3 nM ^{3}H-d-LSD, 1.7 nM ^{3}H-serotonin and 0.22 nM ^{3}H-spiperone to homogenates of calf hippocampus and frontal cortex was studied. The 50% inhibitory concentrations (IC_{50}) for these drugs versus ^{3}H-d-LSD binding had similar values to and correlated with corresponding IC_{50} values versus ^{3}H-serotonin binding in the hippocampus, suggesting that ^{3}H-LSD and ^{3}H-serotonin label similar sites in this region. In the calf frontal cortex, serotonin revealed a biphasic inhibition against ^{3}H-d-LSD binding and the tryptamines inhibited over a concentration range of 10 000-fold. The IC_{50} values of various drugs versus ^{3}H-d-LSD binding correlated with the IC_{50} values versus ^{3}H-serotonin, but did not correlate with the IC_{50} values versus ^{3}H-spiperone. These data suggest that ^{3}H-d-LSD bound to more than one serotonin site in the calf frontal cortex and that ^{3}H-spiperone bound to a separate serotonergic site. Scatchard analyses of the binding for these three ^{3}H-ligands indicated that in the calf frontal cortex the density of ^{3}H-d-LSD sites was approximately equal to the sum of the densities for ^{3}H-serotonin (S-1 sites) and ^{3}H-spiperone (S-2 sites). Two weeks after serotonin-depleting radiofrequency heat lesions of the midbrain dorsal and median raphe nuclei in rats, both ^{3}H-serotonin and ^{3}H-LSD showed enhanced binding in the hippocampus. These data support previous suggestions that supersensitivity develops specifically in serotonin receptors following afferent denervation.

Spiperone Serotonin supersensitivity LSD Tryptamines Raphe lesions

1. Introduction

Although there are several reports describing the labeling of brain serotonin receptors by ^{3}H-serotonin or ^{3}H-d-LSD (Farrow and Van Vunakis, 1973; Bennett and Aghajanian, 1974; Fillion et al., 1976; Bennett and Snyder, 1976; Burt et al., 1976; Fillion et al., 1978; Whitaker and Seeman, 1978a,b; Fuxe et al., 1978; Nelson et al., 1978), it is still not clear whether both ligands label the same type of serotonin receptor. For example, the serotonin concentration for 50% inhibition (IC_{50} value) of ^{3}H-d-LSD binding is generally 35-1000 nM (Bennett and Aghajanian, 1974; Bennett and Snyder, 1976; Fillion et al., 1978; Whitaker and Seeman, 1978a) which is much higher than the serotonin IC_{50} value of 1-10 nM for the binding of ^{3}H-serotonin. If the sites labeled by ^{3}H-serotonin and ^{3}H-d-LSD are truly identical, the molar IC_{50} values for serotonin ought to be comparable to displace both tritiated ligands (cf. Titeler and Seeman, 1978).

This apparent inconsistency can be explained if these radioreceptor assays were not sufficiently selective. Alternatively, previous work used brain regions in which multiple receptor sites may have existed for serotonin and related compounds; these regions were: rat whole brain (Bennett and Aghajanian, 1974; Fillion et al., 1978), rat whole cortex (Bennett and Snyder, 1976) or calf caudate (Whitaker and Seeman, 1978a). Since these previously studied regions would be expected to harbor several types of receptors, it is

desirable to compare the binding of ^{3}H-serotonin and ^{3}H-d-LSD in brain regions containing predominantly serotonin sites, namely in the hippocampus and the frontal cortex (Azmitia, 1978a,b). A start in this derection was made by Burt et al. (1976) who found that the IC_{50} values for d-LSD and serotonin were 6 and 18 nM, respectively, on ^{3}H-d-LSD binding to homogenates of calf hippocampus. The present study extends the work of Burt et al. (1976) to other drugs which inhibit the binding of both ^{3}H-d-LSD and ^{3}H-serotonin to hippocampus and frontal cortex. The inhibitory effects of these drugs on ^{3}H-spiperone binding were also examined, since this ^{3}H-ligand can label serotonin receptors (Leysen et al., 1978b; Creese and Snyder, 1978). Furthermore, in order to assess indirectly the potential synaptic location of these binding sites, we have determined the binding capacity f these ^{3}H-ligands to brain tissues of rats previously lesioned in the midbrain raphe nuclei.

2. Materials and methods

2.1. *Preparation of calf tissue homogenates*

Fresh calf brains were obtained from the Canada Packers Hunisett plant (Toronto). The hippocampus and the frontal cortex (gently scraped off with a scalpel) were removed within 2 h after death and suspended in buffer at an approximate concentration of 50 mg wet weight/ml of buffer. Unless otherwise specified, the buffer used throughout contained 15 mM Tris-HCl (pH 7.4), 5 mM Na_2EDTA, 1.1 mM ascorbate and 12.5 μM pialamide. A preliminary crude homogenate of the suspension was made using a glass homogenizer with a teflon piston (0.13-0.18 mm clearance); this piston, rotating at 500 rpm, was passed up and down 20 times.

The suspension was then centrifuged at 39 000 × *g* for 15 min at 4°C; the supernatant was discarded and the pellets, resuspended in 8 vol of buffer, were briefly rehomogenized in the glass-teflon homogenizer. The homogenate was finally suspended at a concentration of 300 mg wet weight of the original tissue per 5 ml of buffer and stored at −20°C in 5 ml aliquots. Before using, samples were thawed, pre-incubated for 10 min at 37°C (Nelson et al., 1978) after which 15 ml of buffer were added. This final suspension was homogenized with a Polytron (Brinkmann Instrument Co.) at a setting of 7 using a PT-10 homogenizer probe in a 50 ml polycarbonate tube.

2.2. *Midbrain raphe lesions*

Electrothermal lesions of the dorsal and median raphe nuclei were made in male Wistar rats (200 g; High Oak Ranch, Ontario) as previously described (Coscina and Stancer, 1977). The stereotaxic coordinates were: AP: −0.35 mm; ML: 0 (midline); DV: −7.5 and −5.5 mm from dura; tooth bar setting at −2.5 mm; 55° for one min at both loci (see Coscina and Stancer, 1977 for details). The lesioned animals were killed 14 days later and the brain regions processed in the same way as those from the calf brain.

2.3. *Determination of noradrenaline, dopamine and serotonin*

The contents of endogenous noradrenaline, dopamine and serotonin in sham-treated and lesioned rat brains were measured using modifications of previously described methods (Davis et al., 1964; Barchas et al., 1972). Rat brain tissues were homogenized in 10 vol 80% ethanol, centrifuged, rehomogenized in 5 vol 80% ethanol, and recentrifuged. The pooled supernatants were passed through 10 × 0.3 cm glass columns containing Amberlite GC50 (bed height, 4 cm) at 0.6 ml/min. The columns were washed (3 × 3 ml deionized water), and the amines eluted in 2.5 ml 0.5 N acetic acid into tubes (25 μl/tube) containing 25 μl of 1% ascorbic acid and 1% EDTA solutions.

The fluorometric analyses of serotonin, noradrenaline and dopamine were modified from published procedures (Laverty and Taylor,

1968; Maickel et al., 1968; Shellenberger and Gordon, 1971). For serotonin analysis, 0.5 ml eluate was added to 100 µl 1% cysteine and 1.2 ml ophthaldehyde solution (5 mg/100 ml conc. HCl). The tubes were heated in boiling water for 15 min, cooled and fluorescence measured (activation 372 nm; emission 478 nm). For noradrenaline and dopamine analysis, 1 ml eluates (buffered with 1.5 ml 0.1 M phosphate-buffer, pH 7, containing EDTA) were adjusted to pH 6.5-7.0 with 0.4 ml 1 N NaOH. To begin oxidation, 0.2 ml 0.1 N iodine reagent was added; after 2 min 0.5 ml alkaline sulfite solution was added; after 2 min the samples were acidified with 0.4 ml glacial acetic acid, heated (boiling water bath) for 4 min. Noradrenaline fluorescence was measured after 30 min (activation 372 nm; emission 475 nm). Dopamine fluorescence was measured after returning the samples to the boiling water bath for another 40 min, cooling and waiting 60 min (activation 320 nm; emission 370 nm).

2.4. *Specific binding of ^{3}H-serotonin*

The [^{3}H]-serotonin binding assays were done using 12 × 75 mm glass test tubes, in which the following aliquots were placed (using Eppendorf Brinkmann pipettes with polypropylene tips): 0.1 ml of buffer (with or without 600 nM serotonin oxalate); 0.1 ml of buffer with different concentrations of competing drugs; 0.2 ml of [^{3}H]-serotonin (25.6 Ci/mmole; New England Nuclear Corp., Boston; final concentration of 1.7 nM); 0.2 ml of brain homogenate (0.2 mg protein per tube). Each determination was done in quintuplicate, with five tubes containing serotonin and five containing buffer. After the tubes were incubated for 30 min at room temperature (22°C), an aliquot of 0.5 ml was removed from each tube and filtered by vacuum, through a glass fibre filter (GF/B, Whatman, 24 mm diameter) using a Millipore stainless steel mesh support for the filter. The filter was then washed with 10 ml of buffer at room temperature. The wash was delivered by gravity from a syringe re-pipette in under 4 sec. The filter was placed into a liquid scintillation vial, 8 ml of Aquasol (New England Nuclear Corp.) was added, and the filters monitored for ^{3}H (after 6 h, during which time the filters became translucent) at 44% efficiency, using a Packard liquid sintillation spectrometer. Specific binding of ^{3}H-serotonin was defined as the total amount bound minus that bound in the presence of 100 nM non-radioactive serotonin. Since the addition of Ca^{2+} (cf. Bennett and Snyder, 1976; Nelson et al., 1978) did not affect the IC_{50} values of the various drugs tested on ^{3}H-serotonin in the present study, the same buffer (containing EDTA) was used for all three ^{3}H-ligands in these experiments.

2.5. *Specific binding of ^{3}H-d-LSD*

The method for measuring the binding of ^{3}H-d-LSD (10.7 Ci/mmole, Amersham Co.) was similar to that for ^{3}H-serotonin with the following differences. The final concentration of ^{3}H-d-LSD was 3 nM, and the specific binding of ^{3}H-d-LSD was defined as that amount of ^{3}H-d-LSD binding which was displaceable by 200 nM d-LSD. The concentrations of drugs which inhibited the specific binding of ^{3}H-d-LSD by 50% (i.e. the IC_{50} values) were the same whether or not 50 nM phentolamine was present in the assays on frontal cortex and hippocampus; 50 nM phentolamine had been used previously in calf caudate tissue to preclude the binding of ^{3}H-LSD to adrenergic sites (Whitaker and Seeman, 1978b).

2.6. *Specific binding of ^{3}H-spiperone*

The binding of ^{3}H-spiperone (23 Ci/mmole; New England Nuclear, Boston) to frontal cortex and hippocampus was done using concentrations between 0.05 and 3 nM for Scatchard analysis, and 0.22 nM for all the inhibition-type of experiments. Otherwise, the experimental conditions and procedures were the same as those for the radioreceptor assays using ^{3}H-serotonin or ^{3}H-d-LSD. The

definition of specific binding of ^{3}H-spiperone is given in the appropriate sections.

The specific binding of ^{3}H-serotonin, ^{3}H-LSD and ^{3}H-spiperone was always measured on tissue that had been kept frozen. Previous experience in this laboratory indicated that the data from such frozen tissue are very similar to that for fresh tissue, using these ^{3}H-ligands.

2.7. Drugs

All drugs were HCl salts, except serotonin creatinine sulfate, methysergide hydrogen maleate (Sandoz), bufotenin mono-oxalate hydrate and methiothepin maleate. We thank Farmitalia for metergoline, Organon Labs for mianserin, McNeil Laboratories for haloperidol, and the Health Protection Branch, Department of National Health and Welfare, Ottawa, Canada for dimethyltryptamine, psilocin, bufotenin and d-LSD.

3. Results

3.1. Calf hippocampus

Figs. 1 and 2 indicate that the inhibitory potencies (i.e. IC_{50} values) of various drugs against the binding of ^{3}H-d-LSD in the calf hippocampus were similar to those against the binding of ^{3}H-serotonin. As illustrated in fig. 1, the inhibitory actions of the drugs spanned approximately two orders of magnitude, indicating that the Hill coefficients were close to unity (0.94-0.98); the Hill coefficient for haloperidol's inhibition of ^{3}H-d-LSD binding (data not in figs. 1 and 2) was 0.95.

The dissociation constant (K_D) and the density of binding sites (B_{max}) for each ^{3}H-ligand were measured by Scatchard analysis using a range of ^{3}H-ligand concentrations (fig. 3). The data were analyzed by both non-linear regression and graphical procedures (Rosenthal, 1967). The binding of ^{3}H-sero-

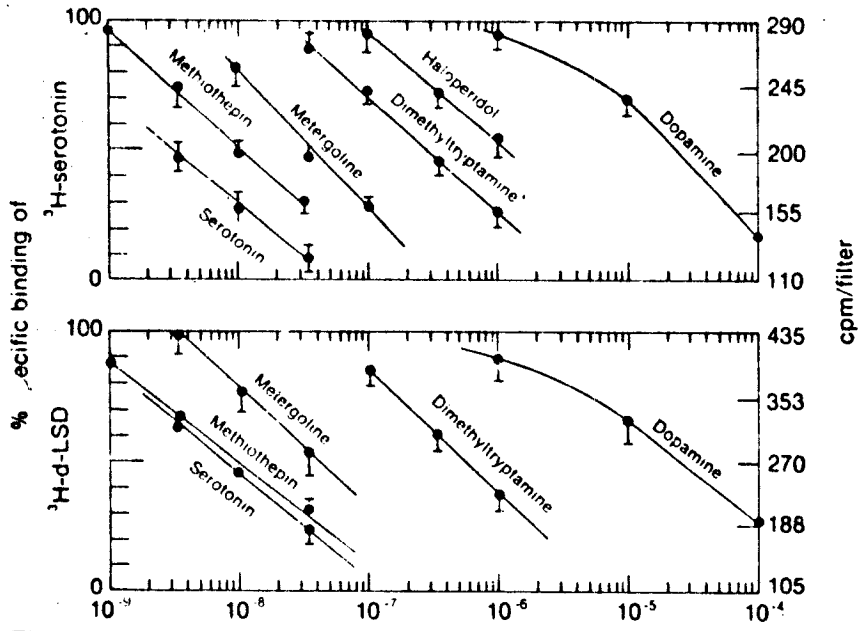

Fig. 1. Calf hippocampus. Examples of the competitive inhibition by various drugs on the specific binding of ^{3}H-serotonin (top) and of ^{3}H-d-LSD (bottom). Specific binding of ^{3}H-serotonin was defined as that displaceable by 100 nM serotonin, while that for ^{3}H-d-LSD was defined as that displaceable by 200 nM d-LSD. Points indicate mean: vertical bars indicate S.E.M. of 3 to 6 experiments. *Abscissa:* mol/l.

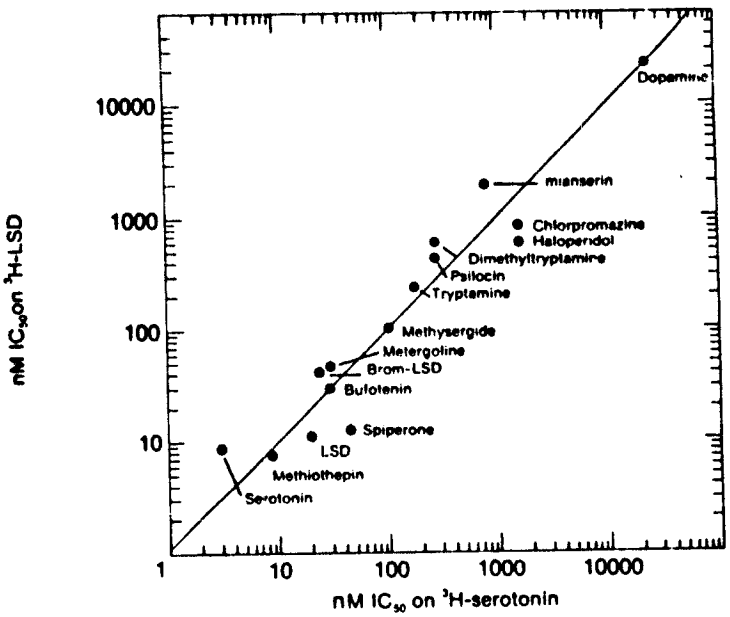

Fig. 2. Calf hippocampus. Correlation between the IC_{50} values for various drugs which inhibited the binding of ^{3}H-d-LSD and of ^{3}H-serotonin to homogenates of the calf hippocampus (some data taken from fig. 1). The correlation coefficient was 0.95.

tonin revealed a single set of sites with a K_D of 0.9 nM and a B_{max} of 85 ± 5 fmol/mg protein. The binding of ^{3}H-d-LSD revealed two sets of sites, the high-affinity sites having a K_D of 0.7 nM and a B_{max} of 60 ± 9 fmol/mg protein, while the low-affinity sites had a K_D of 10 nM and a B_{max} of 260 fmol/mg protein (see fig. 3 legend for additional details).

Since ^{3}H-spiperone can label serotoninergic sites (Leysen et al., 1978b), the binding of this ^{3}H-ligand was also measured (fig. 3). Defining specific binding of ^{3}H-spiperone as that which was displaceable by 100 nM spiperone (Hartley and Seeman, 1978; Seeman et al., 1979), this ^{3}H-ligand revealed two sets of binding sites: high-affinity binding sites with a K_D of 0.4 nM and a B_{max} of 52 fmol/mg protein, and low-affinity sites with a K_D of 4 nM and a B_{max} of 350 fmol/mg protein. As Leysen et al. (1978a) have pointed out, each ^{3}H-ligand, particularly ^{3}H-spiperone, may also have 'saturable, non-specific binding sites'. These sites remain after heating the tissue. As indicated in fig. 3, boiling calf hippocampus homogenate (in water for 1 h) only removed the high-affinity binding sites for ^{3}H-spiperone, indicating that the low-affinity sites were probably non-specific binding sites.

3.2. *Calf frontal cortex*

The results in fig. 4 show data for the inhibition by various drugs on the binding of ^{3}H-serotonin, ^{3}H-d-LSD and ^{3}H-spiperone to homogenates of the calf frontal cortex. The inhibition by serotonin and other tryptamines (e.g. dimethyltryptamine) versus ^{3}H-d-LSD occurred over 4 orders of magnitude of concentration, suggesting that the inhibition was occurring at multiple binding sites for ^{3}H-d-LSD (cf. Titeler et al., 1978); in fact, the shallow slope for the inhibition by serotonin against ^{3}H-d-LSD clearly occurred in two phases, the transition of which was at 100 nM serotonin (fig. 4B).

The inhibition by the various drugs versus ^{3}H-spiperone (fig. 4C) also suggested that ^{3}H-

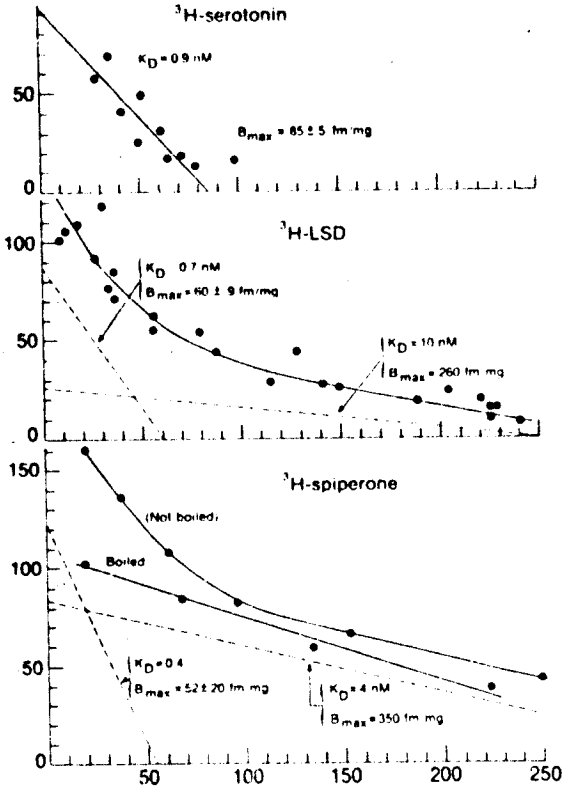

Fig. 3. Calf hippocampus. Measurement of the dissociation constants (K_D) and the density of binding sites (B_{max}) for ^{3}H-serotonin (top; correlation coefficient = 0.85), ^{3}H-d-LSD (middle) and ^{3}H-spiperone (bottom); specific binding was defined as that displaceable by 100 nM serotonin, 200 nM d-LSD and 100 nM spiperone, respectively. The data for ^{3}H-serotonin were from a representative experiment. For three experiments using ^{3}H-serotonin, the K_D was 0.9 ± 0.1 nM (mean ± S.E.M.), and the B_{max} was 85 ± 5 fmol/mg protein. The data for ^{3}H-LSD represent a superposition of data pooled from 3 separate experiments. The ^{3}H-LSD data were resolved into two components of binding using nonlinear regression analysis (interrupted lines); the K_D values for the high- and low-affinity components of ^{3}H-LSD binding were 0.7 ± 0.2 nM and 10 ± 3 nM, respectively, while the densities of binding sites (B_{max} values) were 60 ± 9 fmol/mg protein and 260 fmol/mg protein, respectively. The data for ^{3}H-spiperone were from a representative experiment. The binding of ^{3}H-spiperone was in each experiment resolved into 2 components of binding using graphical procedures (Rosenthal, 1967); the high- and low-affinity components of binding revealed K_D values of 0.4 ± 0.1 nM and 4 ± 1.5 nM, respectively, with corresponding B_{max} values of 52 ± 20 fmol/mg protein and 350 ± 120 fmol/mg protein. The high-affinity binding sites for all three ^{3}H-ligands were heat-labile (boiled tissue data for ^{3}H-spiperone are shown at bottom). *Ordinate:* bound/free, (fmol/mg)/(nmol/l). *Abscissa:* bound, fmol/mg protein.

spiperone was binding to multiple sites since the drugs inhibited over 3 or 4 orders of magnitude of drug concentration. An important observation was the fact that LSD, methiothepin and methysergide all revealed discontinuities at 50% of total ^{3}H-spiperone binding (fig. 4C). LSD, for example, inhibited 50% of the total binding of ^{3}H-spiperone over 2 orders of magnitude of concentration and gave no further competition between 1 and 10 μM d-LSD. The data in fig. 4C also show that serotonin was considerably more effective than dopamine in inhibiting the binding of ^{3}H-spiperone. This agrees with the work of Leysen et al. (1978b) who demonstrated that ^{3}H-spiperone binds to serotonergic receptors in the rat frontal cortex. All these observations suggested that 1-10 μM d-LSD would serve as an appropriate baseline for defining the specific binding of ^{3}H-spiperone to the frontal cortex serotonergic sites.

More data and relations between the IC_{50} (or IC_{25}) values for these ^{3}H-ligands are shown in fig. 5. There was a good correlation between the IC values for ^{3}H-d-LSD and ^{3}H-serotonin (particularly using the IC_{25} values for ^{3}H-d-LSD). Fig. 5C indicates that there was no relation whatever between the IC values for ^{3}H-spiperone and those for either ^{3}H-LSD or ^{3}H-serotonin.

The K_D and B_{max} values for the binding of ^{3}H-serotonin, ^{3}H-d-LSD and ^{3}H-spiperone to the calf frontal cortex are shown in fig. 6. The binding of ^{3}H-d-LSD (fig. 6A) revealed a K_D of 2.8 nM and a B_{max} of 160 fmol/mg protein (defining specific binding as that displaceable by 1 μM d-LSD); as shown in fig. 6A, these sites were completely heat-labile (1 h in boiling water). The binding of ^{3}H-serotonin (fig. 6B) revealed a K_D of 0.7 nM and a B_{max} of 68 fmol/mg protein. The bind-

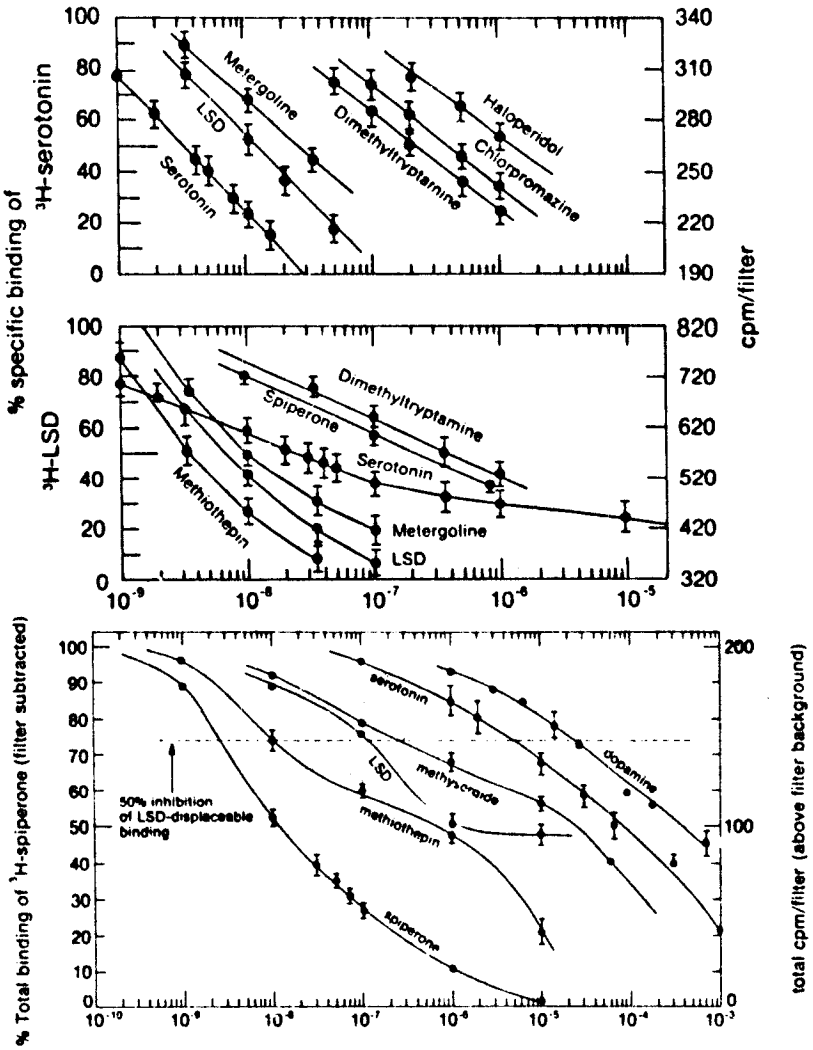

Fig. 4. Calf frontal cortex. Competition of various drugs for 1.7 nM ^{3}H-serotonin (top), 3 nM ^{3}H-d-LSD (middle) and 0.22 nM ^{3}H-spiperone (bottom). In order to calculate the IC_{50} values, 10 μM d-LSD was used to define the specific binding of ^{3}H-spiperone to the serotonergic sites. Note that serotonin was more effective than dopamine, ›ncurring with the data of Leysen et al. (1978b) on rat frontal cortex, and that d-LSD inhibited ^{3}H-spiperone .nding over 2 orders of magnitude of concentration with no further inhibition between 1 and 10 μM d-LSD. The Hill coefficient for spiperone inhibiting ^{3}H-5HT binding (da'a not shown) was 0.85. Vertical markers indicate S.E.M. of 4-6 experiments per point. Points without bars indicate averages of only two experiments. All determinations were in quadruplicate. *Abscissae:* mol/l.

ing of ^{3}H-spiperone (fig. 6C) indicated two sites, the high-affinity sites having a K_D of 0.57 nM and a B_{max} of 75 fmol/mg protein; these sites were heat-labile. The low affinity sites were heat-stable (fig. 6C) and had a K_D of 3.4 nM and a B_{max} of 290 fmol/mg protein. As mentioned in connection with the data in fig. 4, an appropriate baseline for defining the specific binding of ^{3}H-spiperone was 1-10 μM d-LSD. Using such a baseline,

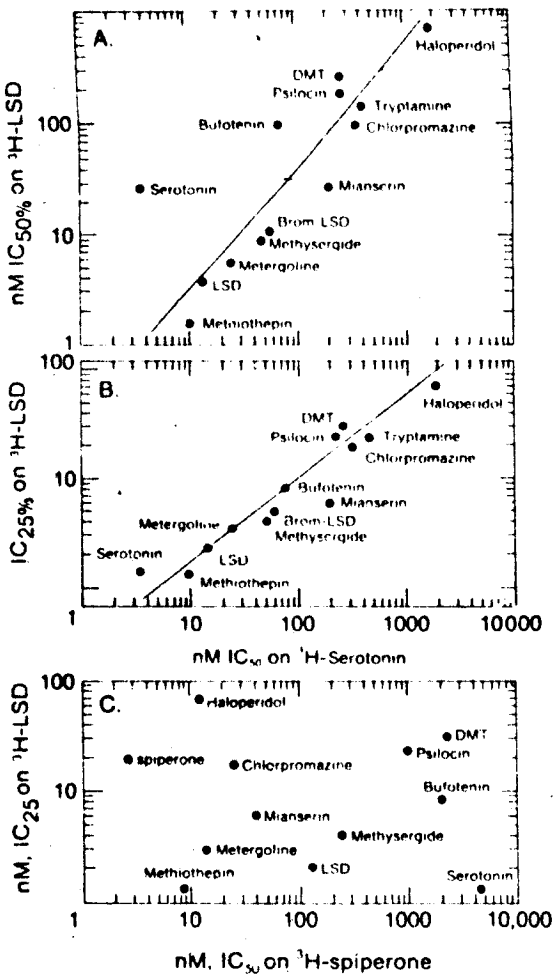

Fig. 5. Calf frontal cortex. The concentrations of various drugs (IC values) which inhibited ^{3}H-serotonin binding correlated with those which inhibited the binding of ^{3}H-d-LSD. There was a better correlation using the IC_{25} values for ^{3}H-LSD (fig. 5B) (correlation coefficient = 0.90) than those using IC_{50} values for ^{3}H-d-LSD (fig. 5A) (correlation coefficient = 81). There was no correlation between the IC alues on ^{3}H-d-LSD and those on the binding of ^{3}H-spiperone (fig. 5C), presumably because ^{3}H-d-LSD and ^{3}H-spiperone were not labeling the same serotonin receptor sites. DMT indicates dimethyltryptamine.

the specific binding of ^{3}H-spiperone to the calf frontal cortex revealed a single set of sites with a K_D of 0.34 nM and B_{max} of 53 fmol/mg protein (fig. 6D).

3.3. Effects of midbrain raphe lesions on ^{3}H-ligand binding

Forebrain tissue from raphe-lesioned and sham-operated rats was measured for its endogenous content of noradrenaline, dopamine and serotonin. Four pooled sets of 5 forebrains in each pool were measured from both groups as this same pooling was used to permit radioreceptor assays. Excluding the regions which had been removed for radioreceptor assay (frontal cortex, hypothalamus, hippocampus and striatum), raphe lesions resulted in an 82% decrease of serotonin (from 444 ± 21 ng/g to 79 ± 8 ng/g; $P < 0.001$, using Student's t-test), an insignificant increase in dopamine content (6%, from 560 ng/g to 594 ng/g), and a small but significant decrease in noradrenaline content (from 303 ± 3 ng/g down to 256 ± 8 ng/g). Previous experience in this laboratory (D.C.) indicated that raphe lesions were always equally effective in different forebrain regions.

As shown in figs. 7 and 8, raphe lesions resulted in an apparently increased density of ^{3}H-d-LSD sites in the hippocampus (fig. 7) (from a control value of 135 ± 7 fmol/mg protein to 163 ± 7 fmol/mg protein) and a slight but insignificant increase in the rat frontal cortex (fig. 8). The raphe lesions also increased the specific binding of ^{3}H-serotonin in the rat hippocampus (fig. 7) but not in the frontal cortex (fig. 8). The lesions had no effect on the binding of ^{3}H-spiperone (fig. 7).

4. Discussion

The results in figs. 1 and 2 suggest that ^{3}H-d-LSD and ^{3}H-serotonin bind to similar sites in the calf hippocampus since the IC_{50} values for the two ^{3}H-ligands were similar (fig. 2). The densities of the high-affinity sites for these two ^{3}H-ligands were also approximately similar (fig. 2; 85 ± 5 fmol/mg protein for ^{3}H-serotonin; 60 ± 9 fmol/mg protein for ^{3}H-LSD).

The data in the calf frontal cortex (figs.

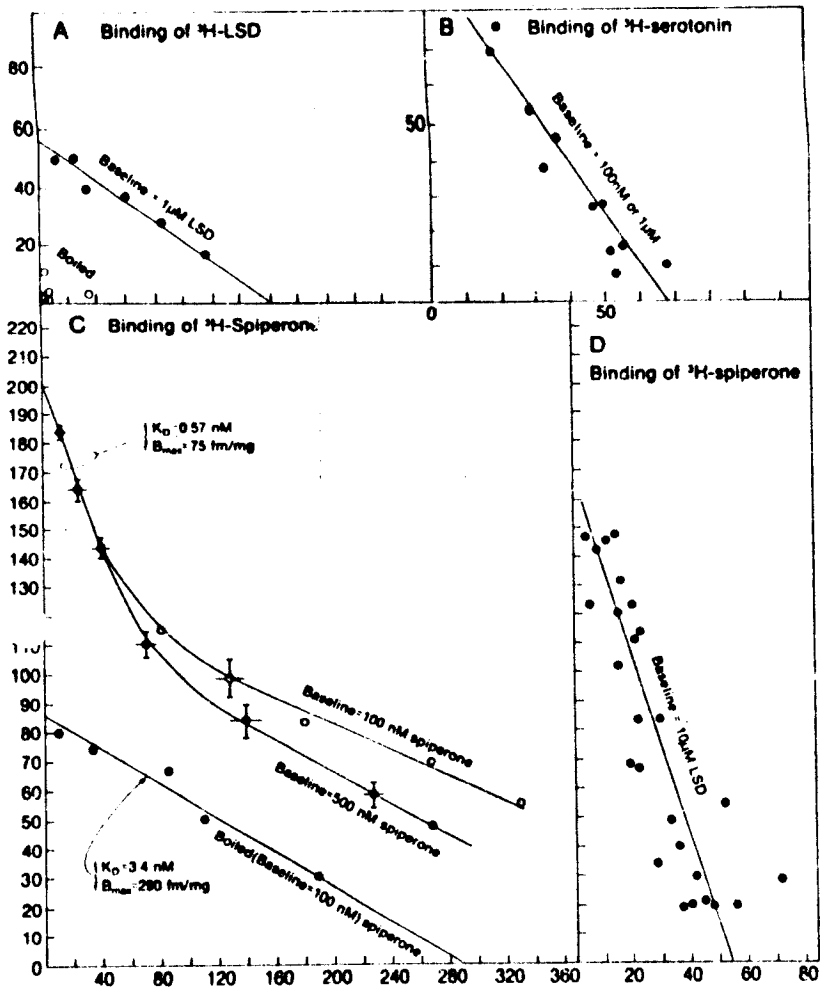

Fig. 6. Calf frontal cortex. K_D and B_{max} parameters for the binding of ^{3}H-d-LSD (fig. 6A). ^{3}H-serotonin (fig. 6B) and ^{3}H-spiperone (figs. 6C and 6D), using different baselines (as indicated) to define specific binding of each ^{3}H-ligand. (A) The binding of ^{3}H-LSD (regression coefficient was 0.96) revealed a K_D of 2.8 ± 0.3 nM (mean ± S.E.M. from 3 experiments, only 1 representative experiment of which is shown above) and a B_{max} of 160 ± 20 fmol/mg protein. The high affinity binding sites for ^{3}H-LSD were heat-labile, as shown by the points indicated by 'boiled' (1 h in boiling water bath). (B) The binding of ^{3}H-serotonin (defined as that inhibited by 100 nM serotonin) revealed a K_D of 0.7 ± 0.2 nM (mean ± S.E.M. from 3 experiments, only 1 representative experiment of which is shown above, and which had a regression coefficient of 0.92) and B_{max} of 68 ± 15 fmol/mg protein. (C) The binding of ^{3}H-spiperone to calf frontal cortex was biphasic and the two components were resolved by nonlinear regression analysis. The data shown here are a composite from 3 experiments. The K_D values for the high- and low-affinity components were 0.57 ± 0.10 nM and 3.4 ± 0.5 nM (mean ± S.E.M.), respectively. The corresponding B_{max} values were 75 ± 10 fmol/mg protein and 290 ± 30 fmol/mg protein. The high-affinity binding sites were heat-labile (data indicated by 'boiled'). (D) The binding of ^{3}H-spiperone, with specific binding defined as that inhibited by the addition of 10 μM LSD, revealed a K_D of 0.34 ± 0.09 nM and a B_{max} of 53 fmol/mg protein; the results shown above are a superposition of data (regression coefficient of 0.90) from 3 experiments, from which the K_D and B_{max} values were obtained. The density of high-affinity binding sites for ^{3}H-spiperone, as defined by using either excess spiperone (fig. 6C) or excess d-LSD (fig. 6D) were approximately similar (53 to 75 fmol/mg protein). *Ordinates:* B/F, (fmol/mg)/(nmol/l). *Abscissae:* bound, fmol/mg protein.

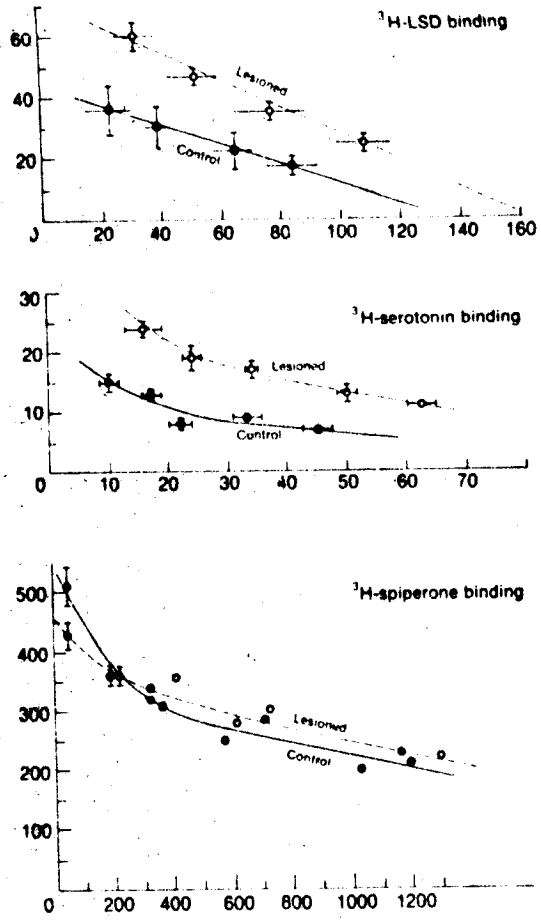

Fig. 7. Rat hippocampus. The specific binding of ^{3}H-d-LSD and ^{3}H-serotonin to rat hippocampus 2 weeks after making electrolytic lesions of the raphe nucleus. The correlation coefficient (r) for the control ^{3}H-LSD data was 0.97 while that for the binding of ^{3}H-LSD to the lesioned tissue had an r of 0.94. For ^{3}H-LSD binding the intercept on the abscissa (B_{max}) was 163 ± 7 fmol/mg protein (mean ± S.E.M.) and differed significantly ($P < 0.01$) from the control value of 135 ± 7 fmol/mg protein. No statistical analysis was done on the data for ^{3}H-serotonin binding, since there appeared to be more than one component of binding and the data were insufficient to resolve into two sets of binding sites. ^{3}H-Spiperone binding (defined as that displaceable by 100 nM spiperone) was unaffected by the lesions. Vertical bars indicate S.E.M., and horizontal bars indicate the range of values grouped. Each point represents the mean of 5 assays of 4 independent pools of tissues, each pool of tissue coming from 3 rats. *Ordinates:* bound/free, (fmol/mg)/(nmol)/l). *Abscissae:* bound, fmol/mg protein.

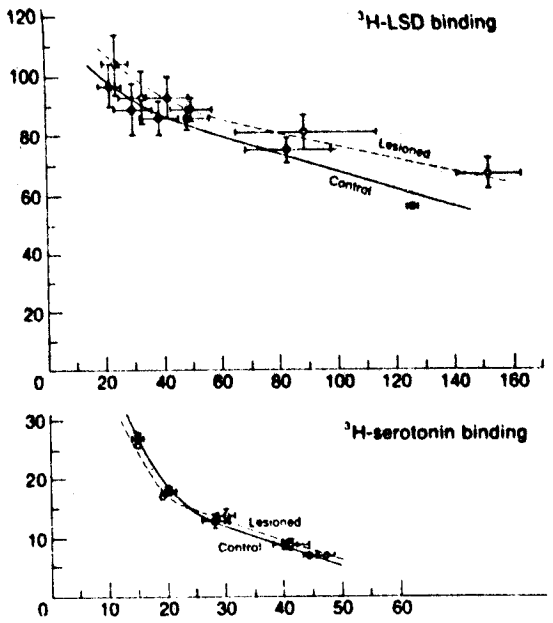

Fig. 8. Rat frontal cortex. Raphe nucleus lesions had no effect on the specific binding of ^{3}H-d-LSD or ^{3}H-serotonin to rat frontal cortex homogenates. Other details were as in fig. 7. *Ordinates:* bound/free, (fmol/mg)/(nmol/l). *Abscissae:* bound, fmol/mg protein.

4-6) gave additional clues as to the possible inter-relations between the sites for the three ^{3}H-ligands studied. The biphasic nature of the serotonin ^{3}H-d-LSD inhibition (fig. 4B) and the shallow slopes spanning 4 orders of magnitude of concentration of other agonists such as dimethyltryptamine on ^{3}H-LSD indicated that ^{3}H-d-LSD might be labeling more than one site. The IC_{25} values for ^{3}H-LSD correlated with the IC_{50} values for ^{3}H-serotonin (fig. 5B); this correlation was better than that between the two sets of IC_{50} values (fig. 5A), an observation consistent with the possibility that ^{3}H-d-LSD binds to more than one site.

Scatchard analysis of the binding of ^{3}H-d-LSD to the calf frontal cortex (fig. 6A), however, revealed a single set of binding sites for ^{3}H-d-LSD with a K_D of 2.8 nM. If, therefore, ^{3}H-d-LSD binds to more than one site, as suggested by the data in figs. 4B, 5A and 5B, it does so with similar affinities to those sites.

The data in fig. 4C indicated that ^{3}H-spiperone also bound to serotonergic sites in the calf frontal cortex, based on the following observations. (1) Serotonin was the most effective neurotransmitter tested. The IC_{50} for serotonin was 4.5 μM, which was much lower than 23 μM for dopamine or 100 μM for adrenaline and noradrenaline. This finding is in agreement with the work of Leysen et al. (1978b) on rat frontal cortex. (2) The three serotonin antagonists, d-LSD, methiothepin and methysergide, all inhibited the binding of ^{3}H-spiperone to approximately the same level of 50% of total binding (see fig. 4C); concentrations of methiothepin and methysergide higher than 10^{-6} M and 10^{-5} M, respectively, further inhibited ^{3}H-spiperone binding, however, in what appeared to be a separate (and low-affinity) component of inhibition. Thus, a concentration of 1 μM of either d-LSD, methiothepin or methysergide served to define the high-affinity (or 'specific') binding sites for ^{3}H-spiperone to frontal cortex. (3) A further indication that ^{3}H-spiperone was binding to serotonergic sites was that there was a qualitative relation between the IC_{50} values for serotonin antagonists and their in vivo potencies to antagonize tryptamine-induced clonic seizures in rats (data from Leysen et al., 1978b). For example, the ED_{50} values to antagonize seizures were between 2 and 2.48 μmol/kg for mianserin, chlorpromazine and haloperidol; the IC_{50} values for these three drugs on ^{3}H-spiperone were between 12 and 38 nM. Methiothepin and spiperone, which were much more potent in antagonizing the tryptamine-induced seizures (ED_{50} values of 0.2 μmol/kg), were much more potent in inhibiting the binding of ^{3}H-spiperone (IC_{50} values of 9 and 2.5 nM, respectively). (4) The binding of ^{3}H-spiperone to calf frontal cortex was also tested in the presence of different concentrations of serotonin (data not presented here) and the inhibition was found to be of the competitive type. These criteria were used by Leysen et al. (1978b) for defining serotonergic binding sites for ^{3}H-spiperone. Finally, although both ^{3}H-d-LSD and ^{3}H-spiperone bound to serotonergic sites, there was no correlation between the IC values for these two ^{3}H-ligands (fig. 5C). This absence of a correlation suggests that ^{3}H-d-LSD and ^{3}H-spiperone label different types of serotonergic sites. The density of ^{3}H-d-LSD sites (160 fmol/mg protein) was higher than the density of high-affinity sites for ^{3}H-spiperone (75 fmol/mg protein) or the density of ^{3}H-serotonin sites (68 fmol/mg protein) but happened to be approximately equal to the sum of the site densities for the latter two ^{3}H-ligands (fig. 6). This same type of observation has very recently been made by Peroutka and Snyder (1979) in their studies on the rat brain.

It is known that animals can be made behaviourally supersensitive to serotonin-like drugs by lesions of the serotonin neurones (Stewart et al., 1976); some neurones also become iontophoretically more sensitive to serotonin (particularly in the amygdala and the lateral geniculate) if the depletion of serotonin is sufficient (De Montigny et al., 1979).

Early work had not found any effect of serotonergic lesions on the binding of ^{3}H-d-LSD or ^{3}H-serotonin (Bennett and Aghajanian, 1974; Bennett and Snyder, 1976) possibly because these early studies may have been detecting low-affinity binding (i.e. the serotonin IC_{50} values versus ^{3}H-d-LSD were 200-1000 nM).

The present data (fig. 7), however, indicate that raphe lesions which produced marked depletion of endogenous serotonin resulted in elevations in the densities of ^{3}H-d-LSD and ^{3}H-serotonin binding sites. These data agree better with more recent work on this point. For example, Nelson et al. (1978, 1979) found that the density of ^{3}H-serotonin sites increased by 39 to 48% in the hippocampus after intracerebral injection of 5,7-dihydroxytryptamine which lowered endogenous serotonin concentration by 80%. Also, Fuxe et al. (1978) have observed a 58% increase in the density of ^{3}H-d-LSD sites in the cerebral cortex following 5,7-dihydroxytryptamine lesions. Overall, therefore, the present data

(fig. 7) together with those of Nelson et al. and Fuxe et al. are consistent with the conclusion that ^{3}H-d-LSD and ^{3}H-serotonin predominantly label postsynaptic sites. Compatible with this is the work of Coyle et al. (1978) and Fillion et al. (1979) who both found that lesions of postsynaptic neurones using kainic acid reduced the density of ^{3}H-serotonin binding sites.

The serotonin sites labeled by ^{3}H-spiperone are presumably also postsynaptic since unilateral lesions by 5,7-dihydroxytryptamine elevate the amount of ^{3}H-spiperone binding (Azmitia, 1978a). The present study (fig. 7, bottom), however, did not detect a significant difference in the binding of ^{3}H-spiperone in the deafferented tissue.

Future work in this area ought to compare the serotonin receptors between rat and calf. Future work using lesion-type of experiments in rats should also distinguish between high-affinity (heat-labile) sites from low-affinity (heat-stable) binding sites (cf. Haigler and Aghajanian, 1977).

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