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Serotonin-2 Receptors in the CNS of Alcohol-Preferring and -Nonpreferring Rats

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McBRIDE, W. J., E. CHERNET, J. A. RABOLD, L. LUMENG AND T.-K LI. *Serotonin-2 receptors in the CNS of alcohol-preferring and -nonpreferring rats.* PHARMACOL BIOCHEM BEHAV **46**(3) 631–636, 1993.—The densities of serotonin-2 (5-HT_2) receptors, labelled with [^{3}H]ketanserin (Ket), were examined in the CNS of alcohol-naive, adult, male alcohol-preferring (P) and -nonpreferring (NP) rats using quantitative autoradiography. The densities of binding sites labelled with 2.0 nM [^{3}H]Ket were 15–25% lower ($p < 0.05$) in layer IV of the medial prefrontal, frontal, cingulate, parietal, and temporal cortices of the P line compared with the NP line. [^{3}H]Ket binding in the P rats was also 40–50% lower ($p < 0.05$) in the medial and lateral nucleus accumbens, olfactory tubercle, and caudate-putamen, and 20% lower ($p < 0.05$) in the claustrum, compared with the NP line. No differences in 2.0 nM [^{3}H]Ket binding were observed between the lines in the piriform cortex, hypothalamus, amygdala, hippocampus, ventral tegmental area, substantia nigra, central gray, and interpenduncular nucleus. Scatchard analysis of [^{3}H]Ket binding (0.5–6.0 nM), determined in layer IV of the frontal and parietal cortex and in the lateral nucleus accumbens, indicated lower B_{max} values without a change in K_d for the P compared to the NP rats. The results of this study indicate that there are fewer 5-HT_2 receptors in certain CNS regions of the P relative to the NP rats.

Alcohol-preferring rats Alcohol-nonpreferring rats Serotonin-2 receptors Autoradiography CNS Ketanserin binding

THE alcohol-preferring (P) and alcohol-nonpreferring (NP) lines of rats have been selectively bred for their disparate alcohol drinking characteristics (11). The P line will generally consume, in a free-choice situation, greater than 5 g ethanol/kg b.wt./day, while the NP rats drink less than 1 g ethanol/kg/day. The P line of rats has been well characterized and has satisfied criteria (4) for a suitable animal model of alcoholism. The P rats: a) orally self-administer ethanol under operant-responding conditions (18,25); b) drink sufficient amounts of ethanol to produce pharmacologically relevant blood alcohol concentrations (19); c) consume ethanol for its CNS positive-reinforcing actions (27); and d) develop tolerance (7) and dependence (28) under free-choice alcohol drinking conditions.

The possible involvement of the CNS serotonin (5-HT) systems in regulating alcohol drinking behavior of the P rats is indicated by neurochemical and neuropharmacological studies. The contents of 5-HT and/or its major metabolite 5-hydroxyindoleacetic acid (5-HIAA) are significantly lower in several CNS regions (e.g., nucleus accumbens, hippocampus, hypothalamus, etc.) of the alcohol-naive P rats compared with the NP rats (20,21). The lower levels of 5-HT and/or 5-HIAA in the P line may be due to lower 5-HT innervation, since there are fewer immunostained 5-HT fibers in several of the same brain regions of the P relative to the NP rats (32).

Neuropharmacological studies provide evidence that lower 5-HT innervation and/or function may be a key factor in promoting high alcohol intake of the P line of rats. The systemic administration of agents that can increase the synaptic levels of 5-HT (e.g., fluoxetine, a 5-HT uptake inhibitor; fenfluramine, a 5-HT releaser; and 5-hydroxytryptophan, a 5-HT precursor) can significantly reduce alcohol intake of the P rats (16,17). Furthermore, systemic administration of 5-HT_1 and 5-HT_2 agonists have also been shown to reduce alcohol drinking of the P line of rats (16,17).

Neurochemical evidence from ligand-binding studies indicates that there are differences in the densities of 5-HT_1 receptors between the P and NP lines of rats. Higher B_{max} values (45–50%) for 5-HT_1 binding sites have been found in synaptic

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membrane preparations from the cerebral cortex and hippocampus of the P line compared with the NP rats (30). In a subsequent study, it was determined that the densities of 5-HT_{1A} receptors were significantly greater in membrane preparations from the cerebral cortex and hippocampus of the P rats than of the NP rats (31). These findings suggest that an upregulation of 5-HT_{1A} receptors may have developed in the P rats as a compensatory mechanism to adapt to the lower 5-HT innervation and/or function. Differences in the densities of 5-HT_2 receptors in the CNS of P and NP rats have also been studied. Using [^{3}H]ketanserin binding to label 5-HT_2 sites in synaptic membrane preparations from the posterior portion of the cerebral cortex, no differences were found in K_d or B_{max} values between the P and NP rats (30). However, because of the limited nature of this study, the widespread differences in 5-HT contents and innervations between the P and NP lines, and the potential involvement of 5-HT_2 receptors in regulating alcohol intake of the P rats (16,17), an autoradiographic study was undertaken to examine in more detail the densities of 5-HT_2 receptors in several CNS regions of alcohol-naive P and NP rats.

METHOD

Alcohol-naive, adult, male P and NP rats, 90–120 days of age and weighing approximately 300–350g, were used for these studies. These lines of rats have been selectively bred for over 30 generations and their alcohol drinking characteristics are well established (12). Animals were housed individually in a temperature-controlled room with a 12L : 12D cycle (lights on at 0600) for at least 1 week prior to killing.

Experiment 1

Rats (N = 4 per line) were killed by decapitation; the brains were rapidly removed and placed in liquid N_2 for approximately 8 s. Frozen brains were then stored at −70°C until they were sectioned.

Frozen brains were placed in a cryostat (set at −20°C) for approximately 1 h prior to sectioning (Reichert-Jung Cryostat Microtome, Cambridge Instuments, Buffalo, NY). Sections (20 μm thick) were prepared and mounted on subbed slides (six sections/slide). A total of 72 sections from selected regions were prepared from each brain (36 sections for total binding and 36 adjacent sections for nonspecific binding). Mounted sections were stored at −70°C until brain sections from all P and NP rats had been prepared. Most sections were stored for 7 to 10 days prior to incubation with the radioactive ligand. An additional 36 sections were prepared for histological examination following standard staining procedures with cresyl violet.

The incubation conditions for labelling 5-HT_2 receptors for autoradiographic analysis were the same as those describe by Pazos et al. (24) except that nonspecific binding was determined in the presence of mianserin in place of methysergide. Briefly, the mounted sections were first preincubated for 15 min at room temperature in 0.17 M Tris-HCl buffer (pH 7.7); the mounted sections were then transferred to 0.17 M Tris-HCl buffer (pH 7.7) containing 2.0 nM [^{3}H]ketanserin (73.5 Ci/mmol; Amersham, Arlington Heights, IL) and incubated for 2 h at room temperature for total binding. Nonspecific binding was determined in the presence of 1.0 μM mianserin (Research Biochemical Incorporated, Natick, MA) under the same incubation conditions as used for total binding. Following this incubation, sections were transferred to ice-cold 0.17 M Tris-HCl (pH 7.7) buffer solutions and given two 10-min rinses to remove unbound [^{3}H]ketanserin. Sections were then briefly dipped in ice-cold distilled water to reduce salt deposits. Following the washing steps, sections were dried with a stream of cold, dry air. Sections (20 μM thick) from 10 frozen brain paste standards covering a wide range of radioactivity were also mounted on a subbed slide and dried under a stream of cold, dry air. Brain sections from P and NP rats along with brain paste standards were placed in standard X-ray cassettes (10 × 12 in.) and apposed to [^{3}H]ultrofilm (LKB, Mager Scientific, Dexter, MI). Each cassette contained one set of standards and sections, for total and nonspecific binding, from the same brain regions of P and NP rats. Cassettes were stored at 4°C for 30 days prior to developing and fixing the ultrofilm.

Quantitative autoradiography was carried out with an AIC micro-image analysis system (Analytical Imaging Concepts Inc., Atlanta, GA). Three to six bilateral measurements were usually made for the majority of nuclei and subregions to obtain total and nonspecific binding values. Values for specific binding were determined by subtracting nonspecific from total binding for adjacent sections. The mean of these three to six values for specific binding in each nucleus or subregion was determined and used to represent the value obtained from an individual animal. These mean values for equivalent areas in different animals were grouped according to rat line and statistically analyzed. Brain areas were identified using the rat brain atlas of Paxinos and Watson (23).

Experiment 2

Rats (N = 5 per line) were killed by decapitation and their brains frozen, sectioned, and processed as in Experiment 1 except for the following conditions: a) 10 sections from each rat were obtained between AP +2.20 mm and +0.70 mm from bregma; b) individual sections from each rat were incubated with 0.5, 1.0, 2.0, 4.0, or 6.0 nM [^{3}H]ketanserin in 0.17 M Tris-HCl buffer (pH 7.7) with or without 1.0 μM mianserin; and c) sections were apposed to the ultrofilm for 14 days at 4°C. These changes in the procedure were made to allow Scatchard analysis of [^{3}H]ketanserin in selected regions for individual rats. Shorter exposure times were used, since the total amount of binding at the 4.0 and 6.0 nM concentrations was estimated to be too high to give reliable densitometry readings with a 30-day exposure.

RESULTS

In Experiment 1, a single concentration (2.0 nM) of [^{3}H]ketanserin was used to determine differences between the P and NP lines of rats. Within the cerebral cortex, layer IV had the highest density of [^{3}H]ketanserin binding sites. The densities of [^{3}H]ketanserin-labelled sites were 15% to 25% lower in layer IV of the medial prefrontal, frontal, cingulate, parietal, and temporal cortices of the P line compared with the NP line (Fig. 1). Of the six regions measured within the cerebral cortex, only the occipital cortex did not show a difference between the P and NP lines in the density of [^{3}H]ketanserin binding (Fig. 1).

The densities of [^{3}H]ketanserin-labelled sites were also significantly lower in several limbic and basal ganglia regions of the P relative to the NP rats (Fig. 2). The values for [^{3}H]ketanserin binding were 40–50% lower in the medial and lateral nucleus accumbens, olfactory tubercle, and caudate-putamen of the P rats. In the case of the claustrum, the difference between the rat lines was approximately 20%.

The densities of [^{3}H]ketanserin sites were sufficiently high

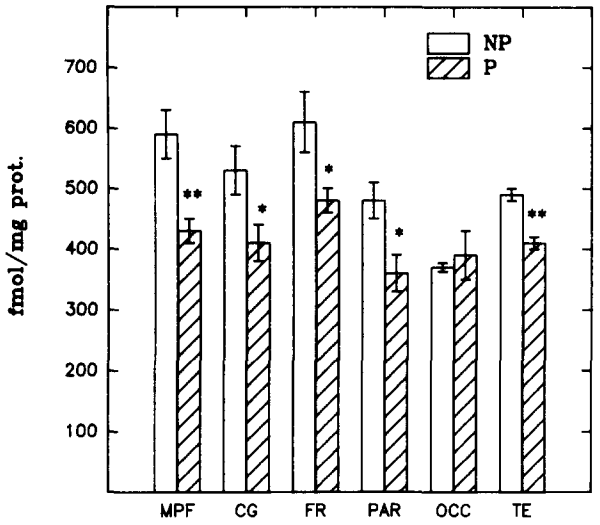

FIG. 1. Densities of 5-HT$_2$ recognition sites labelled with 2.0 nM [^{3}H]ketanserin in layer IV of subregions of the cerebral cortex of P and NP rats. Data represent the mean ± SEM of four animals/line for each of the subregions. Statistical significance was determined with the two-tailed Student's t-test. *$p < 0.05$ and **$p < 0.025$. Abbreviations used: MPF, medial prefrontal cortex; CG, cingulate cortex; FR, frontal cortex; PAR, parietal cortex; OCC, occipital cortex; TE, temporal cortex.

TABLE 1

DENSITIES OF 5-HT$_2$ SITES LABELLED WITH [^{3}H]KETANSERIN IN SEVERAL CNS REGIONS OF P AND NP RATS

Region	fmol/mg Protein	
	NP	P
Piriform, cortex,		
Anterior	434 ± 45	345 ± 17
Hypothalamus		
Lateral	55 ± 7	48 ± 18
Posterior	134 ± 41	130 ± 28
Basolateral amygdaloid nu.	122 ± 20	107 ± 10
Hippocampus		
Dorsal area	67 ± 6	76 ± 7
Ventral area	106 ± 2	155 ± 20
Ventral dentate gyrus	358 ± 14	427 ± 33
Ventral tegmental area	83 ± 24	108 ± 27
Substantia nigra		
Pars reticulata	70 ± 22	96 ± 6
Pars compacta	86 ± 9	95 ± 9
Central gray	56 ± 11	87 ± 16
Dorsal central gray	44 ± 13	45 ± 17
Interpeduncular nu.	105 ± 7	105 ± 27

Data are the mean ± SEM of four animals each. There were no statistically significant differences between the P and NP lines for any of these CNS regions.

in several other CNS regions to do reliable comparative quantitative autoradiographic analysis of the P and NP rat (Table 1). No differences in [^{3}H]ketanserin binding were observed between the lines in any of these regions, which included the anterior piriform cortex, lateral and posterior hypothalamus, basolateral amygdala, hippocampus, ventral tegmental area, substantia nigra, central gray, and interpeduncular nucleus. For many regions (e.g., other layers of the cerebral cortex), the densities of [^{3}H]ketanserin were too low to obtain reliable values for quantitative comparisons between the lines.

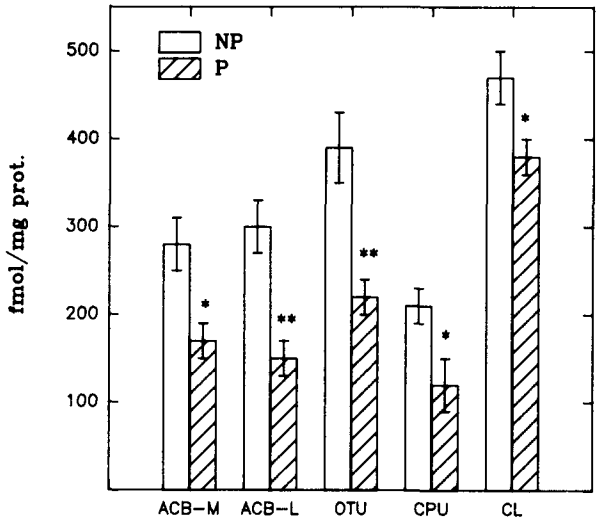

FIG. 2. Densities of 5-HT$_2$ recognition sites labelled with 2.0 nM [^{3}H]ketanserin in limbic and basal ganglia regions of P and NP rats. Data represent mean ± SEM of four animals/line for each of the regions. Statistical significance was determined with the two-tailed Student's t-test. *$p < 0.05$ and **$p < 0.025$. Abbreviations used: ACB-M, -L, nucleus accumbens-medial, -lateral; OTU, olfactory tubercles; CPU, caudate-putamen; CL, claustrum.

In the second experiment, binding at several concentrations of [^{3}H]ketanserin was measured to determine if the lower densities of binding sites observed in the P line were due to differences in affinities (K_d) and/or number of sites (B_{max}). Scatchard analysis of three regions of the forebrain indicated a difference in B_{max} but not K_d values between the P and NP lines (Figs. 3–5). Lower B_{max} values were found for layer IV of the parietal (Fig. 3) and frontal (Fig. 4) cortices, and for the lateral nucleus accumbens (Fig. 5) of the P compared with the NP line. Because of limitations of the autoradiographic technique and the regions sectioned, it was not possible to obtain sufficient values for Scatchard plots of all the CNS areas analyzed in the anterior portion of the brain. Total binding (specific plus nonspecific) at the 4.0 and 6.0 nM concentrations in the olfactory tubercle, caudate-putamen, and claustrum exceeded the linear range of the image analyzer and prevented accurate measurements of these values. A combination of too high total binding at the 6.0 nM concentration and inability to obtain sections having a consistent nucleus accumbens throughout all the [^{3}H]ketanserin concentrations prevented Scatchard analysis of the medial nucleus accumbens. Layer IV of the medial prefrontal cortex and cingulate cortex areas was not sufficiently uniform throughout the sections to obtain values at all the [^{3}H]ketanserin concentrations. The temporal cortex (layer IV) is located posterior to the sections prepared for Scatchard analysis.

DISCUSSION

The data obtained with [^{3}H]ketanserin binding suggest that there are fewer 5-HT$_2$ receptors in certain CNS regions of the

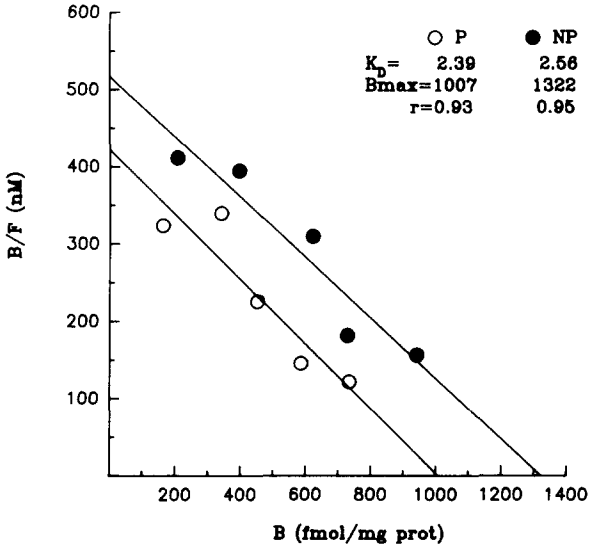

FIG. 3. Scatchard analysis of [^{3}H]ketanserin binding (0.5–6.0 nM) in layer IV of the parietal cortex of P and NP rats. Data are the composite of the mean values obtained at each concentration for five animals/line. $K_d$and B_{max} values were determined for individual animals and statistically analyzed: 2.7 ± 0.4 vs. 2.8 ± 0.6 nM for P vs. NP, $p < 0.9$; and 1080 ± 100 vs. 1380 ± 220 fmol/mg prot. for P vs. NP, $p < 0.02$.

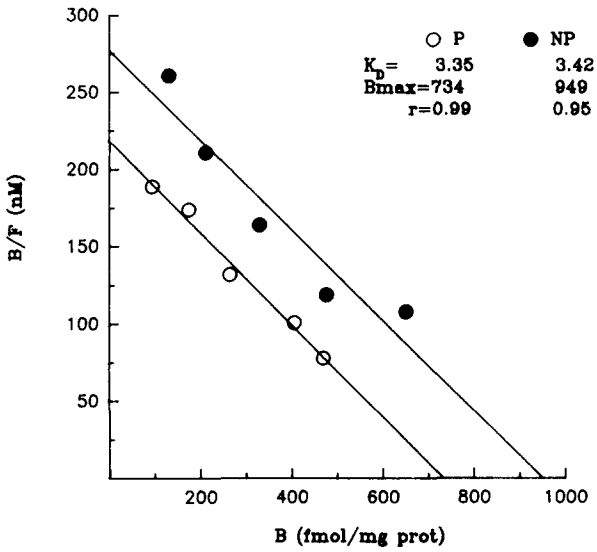

FIG. 5. Scatchard analysis of [^{3}H]ketanserin binding (0.5–6.0 nM) in the lateral nucleus accumbens of P and NP rats. Data are the composite of the mean values obtained at each concentration for five animals/line. K_d and B_{max} values were determined for individual animals and statistically analyzed: 3.5 ± 1.0 vs. 3.4 ± 0.9 nM for P vs. NP, $p < 0.9$; and 750 ± 120 vs. 930 ± 200 fmol/mg prot. for P vs. NP, $p < 0.1$.

P compared with the NP rat (Figs. 1–5). Since there is evidence for fewer immunostained 5-HT fibers in some CNS regions of the P relative to the NP line (32), one possible explanation may be that the 5-HT$_2$ sites are present presynaptically on 5-HT terminals, and the lower 5-HT$_2$ receptor densities reflect the presence of fewer 5-HT terminals in the P than in the NP

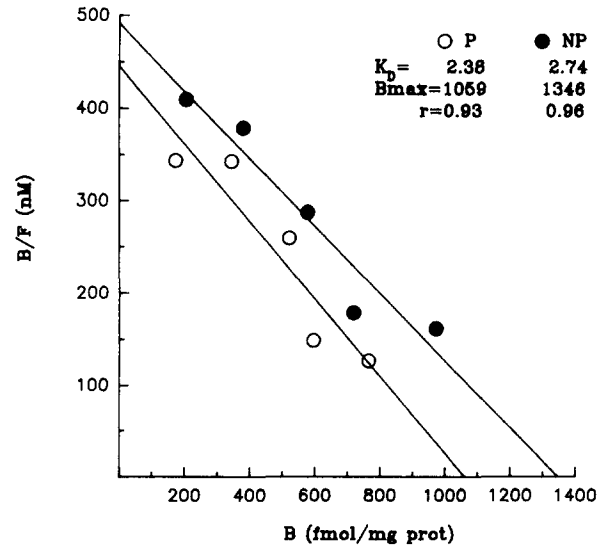

FIG. 4. Scatchard analysis of [^{3}H]ketanserin binding (0.5–6.0 nM) in layer IV of the frontal cortex of P and NP rats. Data are the composite of the mean values obtained at each concentration for five animals/line. K_d and B_{max} values were determined for individual animals and statistically analyzed: 3.1 ± 0.3 vs. 2.9 ± 0.3 nM for P vs. NP, $p < 0.4$; and 1230 ± 90 vs. 1390 ± 120 fmol/mg prot. for P vs. NP, $p < 0.07$.

rat. It is also possible that the lower 5-HT$_2$ values in the P rat could be due to postsynaptic differences between the lines. The relatively lower postsynaptic densities in the P rats might be due to: a) the loss of neuronal populations that have 5-HT$_2$ receptors; b) lack of development of neuronal populations having 5-HT$_2$ receptors; and/or c) decreased formation of 5-HT$_2$ sites in certain neurons.

If 5-HT$_2$ binding sites are located presynaptically on 5-HT terminals, their function is unknown, since evidence thus far does not indicate an autoreceptor role for 5-HT$_2$ receptors (13). Furthermore, selective lesioning of serotonergic fibers does not alter the densities of [^{3}H]ketanserin binding sites, suggesting a postsynaptic localization of 5-HT$_2$ receptors (6). Therefore, a more likely explanation for the differences in the densities of 5-HT$_2$ sites would be lower populations of neurons having 5-HT$_2$ receptors and/or decreased formation of 5-HT$_2$ receptors in certain neurons in the P line.

Since the P rats, compared with the NP rats, have decreased 5-HT innervation in certain forebrain regions (32) and fewer 5-HT neurons in the raphe nuclei (33), which may be due to developmental differences between the lines (22), it is possible that neurons that do not receive their normal 5-HT inputs do not develop properly (29). Since some of these neurons would likely express 5-HT$_2$ receptors, then the lower densities of 5-HT$_2$ sites observed in certain CNS regions of the P rat, compared with the NP rat, could possibly reflect the lack of development of certain neuronal populations.

The density of 5-HT$_2$ receptors in the CNS does not respond in the usual predictable manner to in vivo manipulations. Following destruction of serotonergic innervations, there is no evidence for denervation supersensitivity of 5-HT$_2$ receptors (1,26). In the P rat, there is evidence for denervation supersensitivity of 5-HT$_{1A}$ receptors in the cerebral cortex and hippocampus (31). Whereas the densities of 5-HT$_1$ receptors appear to be influenced by chronic elevations or reductions in

synaptic 5-HT levels, the densities of 5-HT$_2$ receptors are not. Chronic treatment with 5-HT reuptake blockers does not reduce 5-HT$_2$ sites (5). Paradoxically, chronic treatment with 5-HT$_2$ antagonists downregulates 5-HT$_2$ receptor sites (2,3, 10). Therefore, another possible explanation for the lower densities of 5-HT$_2$ sites in certain CNS regions of the P rat may be due to the presence of an endogenous antagonist for the 5-HT$_2$ receptor. Furthermore, the effects of this antagonist on the downregulation of the 5-HT$_2$ receptor may be exacerbated by decreased 5-HT innervation.

Within the cerebral cortex of the P rat, regions that showed lower densities of 5-HT$_2$ sites (Figs. 1, 3, and 4) also had decreased immunostained 5-HT fibers (32) as well as lower contents of 5-HT (20,21). In addition, these same regions of the P rat had higher densities of 5-HT$_{1A}$ receptors (14,16,31). A similar relationship between lower densities of 5-HT$_2$ receptors (Figs. 2 and 5) and lower 5-HT content (21) and innervation (32) was also observed for the nucleus accumbens of the P rat. While data for 5-HT-immunostained fibers are not available for the olfactory tubercle and caudate putamen of the P rat, data for 5-HT content (15,20) support a relationship between lower 5-HT$_2$ receptors and lower 5-HT innervation. Therefore, it appears that in these regions, 5-HT innervation may be influencing the number of 5-HT$_2$ receptors. This is likely to be a result of a developmental effect rather than a denervation action, since the densities of 5-HT$_2$ receptors are not altered by 5-HT lesions (1,6,26). However, the nature of this developmental effect is not known at this time. Moreover, this effect is not uniform throughout all CNS regions. For example, the P rats, compared with the NP rats, exhibited lower contents of 5-HT in the whole hippocampus (20), decreased 5-HT innervation in the middle and ventral dentate of the ventral hippocampus (32), and upregulation of 5-HT$_{1A}$ receptors in the whole (31) or ventral hippocampus (16), but do not show lower densities of 5-HT$_2$ receptors in any region of the hippocampus examined (Table 1). The reasons for the lack of a relationship between lower 5-HT$_2$ receptors and decreased 5-HT innervation in the hippocampus are unknown at this time.

Korpi et al. (9), using [^{3}H]ketanserin binding to membranes from the hippocampus, frontal cortex, and brain stem, found no differences in densities of 5-HT$_2$ sites between the alcohol-preferring AA rats and the alcohol-avoiding ANA rats. These results are in agreement with the present findings for the hippocampus (Table 1) and with the data indicating no differences between the P and NP lines in several brain stem regions, such as the ventral tegmental area, substantia nigra, central gray, and interpeduncular nucleus (Table 1). However, in the present study, the density of 5-HT$_2$ sites in layer IV of the frontal cortex is approximately 20% lower in the P than in the NP rat (Figs. 1 and 4), while no difference was observed between the AA and ANA rats for [^{3}H]ketanserin binding using membranes from the frontal cortex (9). If any small differences in the densities of 5-HT$_2$ receptors were present in layer IV of the frontal cortex between the AA and ANA rats, it is unlikely that these differences could be detected using membrane preparations from the entire frontal cortical area.

The relative distribution of 5-HT$_2$ sites labelled with [^{3}H]ketanserin observed in the present study in the CNS of the P and NP rats resembles closely the results published for [^{3}H]ketanserin in the CNS of Wistar rats (24). Although it is not possible to establish if the difference in B_{max} values between the lines is due to higher levels in the NP rats, lower quantities in the P line, or a combination of both, the data do supply additional evidence suggesting a role of the serotonergic system, and possibly 5-HT$_2$ receptors, being involved in regulating alcohol drinking behavior of the P rats.

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