Reconsideration of 5-Hydroxytryptamine (5-HT)$_7$ Receptor Distribution Using [$^3$H]5-Carboxamidotryptamine and [$^3$H]8-Hydroxy-2-(di-n-propylamino)tetraline: Analysis in Brain of 5-HT$_{1A}$ Knockout and 5-HT$_{1A/1B}$ Double-Knockout Mice

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Abstract

The characterization and anatomical distribution of 5-hydroxytryptamine (5-HT)$_7$ receptor binding sites in brain tissue has been hampered by the lack of a specific radioligand. In the present autoradiographic study, we took advantage of 5-HT$_{1A}$ knockout and 5-HT$_{1A/1B}$ double-knockout mice to revisit the pharmacological characterization and anatomical localization of 5-HT$_7$ binding sites in mouse brain using [$^3$H]5-carboxamidotryptamine (5-CT) and [$^3$H]8-hydroxy-2-(di-n-propylamino)tetraline (8-OH-DPAT). The distribution pattern of [$^3$H]5-CT binding sites (2 nM) in the brain of mice lacking the 5-HT$_{1A/1B}$ receptor was scarce and confined to the septum, globus pallidus, thalamus, hypothalamus, amygdala, cortex, and substantia nigra. The low densities of [$^3$H]5-CT binding sites detected in septum, thalamus, hypothalamus, amygdala, cortex, and substantia nigra. The low densities of [$^3$H]5-CT binding sites detected in septum, thalamus, hypothalamus, amygdala, cortex, and substantia nigra of 5-HT$_{1A/1B}$ knockout mice were displaced by N-[-3-(2-dimethylamino)ethoxy-4-methoxy-phenyl]-2’-methyl-4’-(5-methyl-1,2,4-oxadiazol-3-yl)-(1,1’-biphenyl)-4-carboxamide hydrochloride (SB-216641) (1 M), demonstrating the 5-HT$_{1D}$ nature of these binding sites. In contrast to the low densities of [$^3$H]5-CT binding sites, high-to-moderate densities of [$^3$H]8-OH-DPAT binding sites (10 nM) were found throughout the brain of 5-HT$_{1A}$ and 5-HT$_{1A/1B}$ knockout mice (olfactory system, septum, thalamus, hypothalamus, amygdala, CA3 field of the hippocampus, cortical mantle, and central gray). These [$^3$H]8-OH-DPAT binding sites were displaced by 10 M SB-269970, risperidone, and methiothepin but not by pindolol, N-tert-butyl-3-[4-(2-methoxyphenyl)piperazin-1-yl]-2-phenylpropanamide (WAY-100135), or citalopram. We conclude that despite its high affinity for the 5-HT$_7$ receptor in tissue homogenates, [$^3$H]5-CT is not a good tracer for measuring 5-HT$_7$ receptor binding sites autoradiographically. Also, the lower affinity ligand [$^3$H]8-OH-DPAT is a much better tracer for autoradiographic studies at the 5-HT$_7$ receptor binding sites.

The 5-HT$_7$ receptor is the most recently identified member of the family of G protein-coupled 5-HT receptors (for review, see Vanhœnacker et al., 2000). The 5-HT$_7$ receptor has been cloned from rat (Lovenberg et al., 1993; Ruat et al., 1993; Shen et al., 1993), mouse (Plassat et al., 1993), human (Bard et al., 1993), and guinea pig (Tsou et al., 1994). The receptor is positively coupled to adenylyl cyclase, preferentially via Gs. It exhibits a high degree of interspecies homology (approximately 95%) but a low sequence homology with other 5-HT receptors (<40%). In rat and human, three different splice variants have been described to date, which are thought to have similar pharmacology and function (Heidmann et al., 1997; Krobert et al., 2001). The pharmacological profile of the 5-HT$_7$ receptor is consistent across all tested species and is characterized by high affinity for the 5-HT$_7$ agonists 5-CT, 5-HT, and 8-OH-DPAT; and the 5-HT$_2$ antagonists ritanserin, metergoline, mesulergine, and risperidone. Possible physiological functions for this receptor have been suggested, including relaxation in several vascular preparations (Bard et al., 1993; Shen et al., 1993) and circadian rhythm control via the suprachiasmatic nucleus of the hypothalamus (Lovenberg et al., 1993; Gannon, 2001). Because 8-OH-DPAT has been shown to exhibit a relatively high affinity for the 5-HT$_7$ receptor, multiple functions formerly

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; 5-CT, 5-carboxamidotryptamine; 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)tetraline; SB-269970, N-[-3-(2-[(2,4-methylpiperidin-1-yl)ethyl]pyrrolidine-1-sulfonyl)-2-propylamino)tetraline: Analysis in Brain of 5-HT$_{1A}$ Knockout and 5-HT$_{1A/1B}$ Double-Knockout Mice
attributed to a 5-HT1A-like receptor (including adenylate cyclase stimulation in gastrointestinal, cardiovascular, and hypothalamic preparations) may be likely attributable to the 5-HT7 subtype. Atypical antipsychotics, such as clozapine and risperidone, and some antidepressants display high affinity for this receptor. The significance of 5-HT7 receptor blockade with regard to antipsychotic or antidepressant drug action remains to be elucidated (Eglen et al., 1997; Vanhoe nacker et al., 2000).

The specific distribution of 5-HT7 receptor mRNA in rat and guinea pig brain is well described (To et al., 1995; Gustafson et al., 1996; Mengod et al., 1996; Kinsey et al., 2001; Neumaier et al., 2001). However, the characterization and study of the anatomical distribution of 5-HT7 receptor binding sites in brain tissue have been hampered by the lack of a specific radioligand. Several attempts have been made using available radioligands in rat brain homogenates (Stowe and Barnes, 1998; Hemedah et al., 1999) or tissue sections (To et al., 1995; Waeger and Moskowitz, 1995; Gustafson et al., 1996; Mengod et al., 1996). The autoradiographic studies used a relatively none selective radioligand (i.e., [3H]5-CT) in the presence of drugs to mask other binding sites. Very low densities of 5-HT7 binding sites have been reported in these studies, whereas a relatively high abundance of 5-HT7 receptor mRNA or 5-HT7 immunoreactivity has been reported in rat brain (Gustafson et al., 1996; Mengod et al., 1996; Kinsey et al., 2001; Neumaier et al., 2001). The authors have concluded that the masking compounds added in these autoradiographic studies might have sufficient affinity for the 5-HT7 receptor to occupy part of the 5-HT7 binding sites at the concentration used, thus occluding full visualization of this receptor. An attempt to label 5-HT7 receptors in hamster brain has also been made using [3H]8-OH-DPAT in the presence of pindolol for 5-HT7 receptor occlusion (Duncan et al., 1999). However, no pharmacological characterization was presented in the latter study. Furthermore, the concentration of [3H]8-OH-DPAT (1.8 nM) used by Duncan and colleagues seems to be very low with respect to its moderate affinity for 5-HT7 receptor binding sites in recombinant cell lines or in rat brain tissue homogenates (Kd of 30–65 nM; Lovenberg et al., 1993; Eglen et al., 1997; Krobort et al., 2001).

In the present autoradiographic study, we took advantage of 5-HT1A knockout and 5-HT1A/1B knockout mouse lines to revisit the anatomical localization and pharmacological characterization of 5-HT7 binding sites in mouse brain using [3H]5-CT and [3H]8-OH-DPAT. We tested the hypothesis whether the 5-HT1A and 5-HT1B/1D masking compounds added in the previous [3H]5-CT autoradiographic studies (Waeger and Moskowitz, 1995; Gustafson et al., 1996; Mengod et al., 1996) truly occluded the 5-HT7 receptor binding sites at the concentration used. In mice lacking the 5-HT1A receptor, we performed concentration binding studies to determine the suitable concentration of [3H]8-OH-DPAT for labeling of 5-HT7 receptor binding sites. The 5-HT7 binding sites were characterized by measuring the potency of several compounds, including a selective 5-HT7 receptor antagonist (SB-269970; Hagan et al., 2000) to inhibit 5-HT7 binding sites. This study provides a detailed anatomical localization of 5-HT7 receptor binding sites throughout the brain of 5-HT1A and 5-HT1A/1B knockout mice and addresses the controversial issue of the atypical feature of [3H]5-CT binding sites observed in 5-HT1A receptor-containing regions (Waeger and Moskowitz, 1995; Castro et al., 1997).

**Experimental Procedures**

**Generation of 5-HT1A Knockout and 5-HT1A/1B Double-Knockout Mice**

5-HT1A receptor knockout mice had been generated as described previously (Ramboz et al., 1998). 5-HT1A/1B double-knockout mice were obtained by crossing homozygous 5-HT1A knockout mice with previously generated homozygous 5-HT1B receptor knockout mice (Saudou et al., 1994). This first cross led to offspring that were heterozygous for both the 5-HT1A and 5-HT1B mutant alleles. Subsequent breeding of the heterozygote mice led to the generation of double-knockout mice. Southern blot analysis of tail DNA was used to determine which mice were homozygous mutant for both receptors. All the experiments described in this study have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

**Tissue Preparation for Radioligand Autoradiography**

Knockout or wild-type mice were asphyxiated by carbon dioxide and decapitated (n = 3 in each group). Brains were immediately removed from the skull and rapidly frozen in dry ice. Frontal sections (20 µm in thickness) were cut using a cryostat-microtome (Microm HM505E; Microm International GmbH, Walldorf, Germany) and thaw-mounted on adhesive microscope slides (Superfrost+ Plus; VWR, West Chester, PA). The sections were kept at −70°C until use.

The sectioning protocol for the regional distribution study included series of coronal sections covering the entire brain with an interspace of 200 µm. Adjacent sections at the level of septum, thalamus, and substantia nigra were used to generate concentration binding and inhibition curves.

**Receptor Autoradiography**

Sections were thawed and dried under a cold air stream and then preincubated for 3 × 10 min in 50 mM Tris buffer, pH 7.4, at ambient temperature by immersing the slides in the microsopic slides in a 400-m1 jar.

**[3H]5-CT Binding.** Sections were incubated (drop incubation 150 µl placed on each section) for 60 min at ambient temperature in medium containing 2 nM [3H]5-CT, 50 mM Tris pH 7.4, 0.5 mM EDTA, 10 mM MgSO4, 10 µM pargyline, and 0.1% acid ascorbic acid. SB-266641 (1 µM) or 10 µM SB-269970 was added to the incubation medium on adjacent sections.

**[3H]8-OH-DPAT Binding.** For concentration binding curves, [3H]8-OH-DPAT was used at 0.1, 1, 2, 4, 8, 10, 20, and 40 nM in a medium containing 50 mM Tris pH 7.4, 4 mM CaCl2, and 1 mM MgCl2. Sections were incubated for 60 min at ambient temperature. For inhibition and distribution studies, [3H]8-OH-DPAT was used at 10 nM. Inhibition of [3H]8-OH-DPAT binding by SB-269970, WAY-100135, pindolol, risperidone, methiothepin, citalopram, SB-216641, 5-HT, and 5-CT (using 10 concentrations per compound within a range of 10 pM to 100 µM) was performed at the level of the septum.

For both radioligands, nonspecific binding was measured in the presence of 10 µM 5-HT (inhibition study) or 10 µM SB-269970 (distribution study). After incubation, the excess radioligand was washed off by immersing the microscope slides in a jar (2 × 5 min for both radioligands) with the respective incubation buffer at 4°C, followed by a quick rinse in water and drying under a cold air stream. The sections and standard tritiated microsquares were placed in a light-tight cassette and covered with light-sensitive [3H]Hyperfilm. After a 4-week exposure ([3H]8-OH-DPAT) or a 6-week exposure ([3H]5-CT) they were developed manually in D-19 developer for 2 min and fixed with Readymatic for 3 min (Eastman Kodak, Rochester, NY).
Autoradiograms were digitized using a high-resolution scanner (Agfa Arcus II; AGFA Corp., Ridgefield Park, NJ) and Adobe Photoshop 5.0 (Adobe Systems, Mountain View, CA). Autoradiograms were quantified using Image Gauge version 3.12 (Fujifilm, Tokyo, Japan). Optical densities in the anatomical regions of interest were transformed into levels of bound radioactivity after calibration of the image analyzer with gray values generated by the coexposed standards. Radioligand binding signal in the absence and presence of competitors was expressed in femtomoles per milligram of protein. Nonspecific ligand binding was determined in adjacent sections incubated with 10 μM 5-HT or 10 μM SB-269970.

Ligand concentration binding curves and sigmoidal inhibition curves were generated and fitted by nonlinear regression analysis using GraphPad Prism software (GraphPad Software, San Diego, CA). The $B_{\text{max}}$, $K_D$, and $pIC_{50}$ (–log IC50) values were derived from the curve calculation. The IC50 is the concentration producing 50% inhibition of specific radioligand binding.

**Materials**

[3H]8-OH-DPAT (135 Ci/mmol) and [3H]5-CT (27 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). Hyperfilm-3H and 3H standard strips were obtained from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). WAY-100135, pindolol, risperidone, methiothepin, and SB-264461 were from Tocris Cookson (Ellisville, MO); and SB-269970, 5-HT, and 5-CT were from Sigma-Aldrich (St. Louis, MO). Citalopram was synthesized at the Johnson & Johnson Pharmaceutical Research & Development LLC.

**Results**

**[3H]5-CT Autoradiography in Wild-Type and 5-HT1A/1B Double-Knockout Mice**

**Wild-Type Mice.** Mouse brain slices were incubated with 2 nM [3H]5-CT, a concentration that is just above saturation for most brain regions examined (data not shown). At this concentration of [3H]5-CT, strong labeling was observed in septum (Fig. 1a, a'), hippocampus, hypothalamus, and thalamus at rostro (b and b') and caudal levels (c and c') visualized by receptor autoradiography (frontal sections). Cpu, caudate putamen; Cx, cortex; GP, globus pallidus; Hip, hippocampal formation; Hyp, hypothalamus; S, septum; Tha, thalamus. Scale bar, 2.2 mm.

Autoradiograms were digitized using a high-resolution scanner (Agfa Arcus II; AGFA Corp., Ridgefield Park, NJ) and Adobe Photoshop 5.0 (Adobe Systems, Mountain View, CA). Autoradiograms were quantified using Image Gauge version 3.12 (Fujifilm, Tokyo, Japan). Optical densities in the anatomical regions of interest were transformed into levels of bound radioactivity after calibration of the image analyzer with gray values generated by the coexposed standards. Radioligand binding signal in the absence and presence of competitors was expressed in femtomoles per milligram of protein. Nonspecific ligand binding was determined in adjacent sections incubated with 10 μM 5-HT or 10 μM SB-269970.

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not shown), and central gray (data not shown). A nearly identical pattern of labeling was observed in the presence of a saturating concentration (10 μM) of the selective 5-HT$_7$ receptor antagonist SB-269970 (Fig. 1, a’–c’). Under these conditions (in the presence of 10 μM SB-269970) [3H]5-CT binding densities were slightly decreased in septum (percentage of decrease: mean ± S.D., 14.1 ± 4.1%; Fig. 1a’), cortex (percentage of decrease: mean ± S.D., 4.8 ± 1.1%; Fig. 1, a’–c’), thalamus (percentage of decrease: mean ± S.D., 15.2 ± 3.9%; Fig. 1, b’ and c’), hypothalamus (percentage of decrease: mean ± S.D., 14.5 ± 3.9; Fig. 1, b’ and c’), and amygdala (percentage of decrease: mean ± S.D., 12.7 ± 2.4%; data not shown).

5-HT$_{1A/1B}$ Knockout Mice. When the same procedure is used in the brain of the double-knockout (5-HT$_{1A/1B}$) mice, a different pattern emerges. The distribution pattern of [3H]5-CT binding sites throughout the brain of 5-HT$_{1A/1B}$ knockout mice was scarce and confined to septum (mean ± S.D., 11.8 ± 2.0 fmol/mg of protein; Fig. 2a), cortex (mean ± S.D., 4.7 ± 0.5 fmol/mg of protein; Fig. 2, a–c), globus pallidus (mean ± S.D., 16.6 ± 1.8 fmol/mg of protein; Fig. 2a), thalamus (mean ± S.D., 12.7 ± 5.3 fmol/mg of protein; Fig. 2b), hypothalamus (mean ± S.D., 10.5 ± 1.9 fmol/mg of protein; data not shown), amygdala (mean ± S.D., 10.1 ± 1.5 fmol/mg of protein; Fig. 2b), and substantia nigra (mean ± S.D., 18.8 ± 2.5 fmol/mg of protein; Fig. 2c). In the presence of 10 μM SB-269970, all the [3H]5-CT receptor binding was displaced in septum, cortex, hypothalamus, amygdala, and thalamus (Fig. 2, a’–c’) but not in globus pallidus or substantia nigra (Fig. 2, a’ and c’). These residual binding sites in the globus pallidus and substantia nigra of 5-HT$_{1A/1B}$ knockout were, however, displaced by the 5-HT$_{2D}$-selective ligand SB-216641 (1 μM) (Fig. 2, a” and c”). We had previously determined that 1 μM SB-216641 was sufficient to occlude all the [3H]GR125743 binding to 5-HT$_{1D}$ receptor sites in substantia nigra and globus pallidus (data not shown). SB-216641 does not show any affinity ($K_i > 10$ μM) for the recombinant h5-HT$_7$ receptor (Price et al., 1997).

[3H]8-OH-DPAT Concentration Binding Study in Wild-Type, 5-HT$_{1A}$ Knockout, and 5-HT$_{1A/1B}$ Double-Knockout Mice

To determine the suitable concentration of [3H]8-OH-DPAT for 5-HT$_7$ receptor labeling in 5-HT$_{1A}$ knockout or 5-HT$_{1A/1B}$ knockout mice, concentration binding curves were performed in brain sections at the level of the septum. Nonspecific binding measured in the presence of 10 μM 5-HT increased linearly with the radioligand concentration (data not shown). At a low concentration of [3H]8-OH-DPAT (1 nM), moderate densities were measured in wild-type animals (Fig. 3a) but not in 5-HT$_{1A}$ knockout mice (Fig. 3b) or 5-HT$_{1A/1B}$ double-knockout mice (Fig. 3b’). At 2 nM, high densities were measured in wild-type mice (Fig. 3c), whereas very low densities were measured in 5-HT$_{1A}$ knockout mouse (Fig. 3d) or 5-HT$_{1A/1B}$ double-knockout mice (Fig. 3d’). Consistently, lower $K_i$ values (1.30 nM, 95% confidence interval 0.35–2.25 nM) were derived in septum of wild-type compared with knockout animals (5-HT$_{1A}$ knockout 20 nM, 95% confidence interval 14–27 nM; 5-HT$_{1A/1B}$ knockout 21 nM, 95% confidence interval 11–31 nM). At 10 nM [3H]8-OH-DPAT high densities were detectable in both wild-type (Fig. 3e) and 5-HT$_{1A}$ knockout mouse septum (Fig. 3f) or 5-HT$_{1A/1B}$ double-knockout mouse (Fig. 3f’). The level of [3H]8-OH-DPAT binding site densities measured in the septum of knockout animals reached approximately 60% of [3H]8-OH-DPAT binding sites in septum of wild-type mice (wild-type: $B_{max}$ of 688 fmol/mg of protein, 95% confidence interval 577–792 fmol/mg of protein; 5-HT$_{1A}$ knockout: $B_{max}$ of 407 fmol/mg of protein, 95% confidence interval 318–498 fmol/mg of protein; and 5-HT$_{1A/1B}$ knockout: $B_{max}$ of 370 fmol/mg of protein, 95% confidence interval 250–481 fmol/mg of protein). Curve fitting for two-site binding analysis in wild-type mice was not possible (not enough points were included in this analysis).

Fig. 3. [3H]8-OH-DPAT concentration binding study (1 nM, a, b, and b’; 2 nM, c, d, and d’; and 10 nM, e, f, and f’) in wild-type (a, c, and e) 5-HT$_{1A}$ knockout (b, d, and d’) and 5-HT$_{1A/1B}$ double-knockout (b’, d’, and f’) mice visualized by receptor autoradiography (frontal sections at the septum level). Note the absence of binding sites in 5-HT$_{1A}$ knockout and 5-HT$_{1A/1B}$ double-knockout mice at 1 nM [3H]8-OH-DPAT (b and b’). Cx, cortex; S, septum. Scale bar, 2.0 mm.
Pharmacological Characterization of $[^3H]$8-OH-DPAT Binding in Wild-Type, 5-HT$_{1A}$ Knockout, and 5-HT$_{1A/1B}$ Double-Knockout Mice

Pharmacological characterization was performed by measuring the potency of several compounds (SB-269970, WAY-100135, pindolol, citalopram, SB-216641, risperidone, methiothepin, 5-HT, and 5-CT) to inhibit specific $[^3H]$8-OH-DPAT binding (10 nM). Representative autoradiograms for the inhibition of $[^3H]$8-OH-DPAT binding sites in septum and cortex by SB-269970, pindolol, and WAY-100135 are shown in Fig. 4. In the absence of cold compounds $[^3H]$8-OH-DPAT binding sites densities were more abundant in wild-type septum and cortex (Fig. 4a) compared with the 5-HT$_{1A}$ and 5-HT$_{1A/1B}$ knockout mice (Fig. 4, b and c). The selective 5-HT$_7$ antagonist SB-269970 (10 $\mu$M) displaced part of the $[^3H]$8-OH-DPAT binding site densities in wild-type mouse septum (Fig. 4d) and all the $[^3H]$8-OH-DPAT binding site densities in knockout mice but not in wild-type mice (data not shown). The selective 5-HT$_{1A}$ agonist pindolol (10 $\mu$M) or antagonist WAY-100135 (10 $\mu$M) displaced part of the $[^3H]$8-OH-DPAT binding site densities in wild-type mice but did not displace any $[^3H]$8-OH-DPAT binding site densities in knockout mice (Fig. 4, g–l). Citalopram (10 $\mu$M) did not displace any $[^3H]$8-OH-DPAT binding site densities in knockout or wild-type (data not shown). Similarly, the 5-HT$_{1D}$ antagonist SB-216641 (10 $\mu$M) did not displace any $[^3H]$8-OH-DPAT binding site densities in knockout or wild-type (data not shown). In both wild-type and knockout mice, 10 $\mu$M of 5-HT or 5-CT completely occluded $[^3H]$8-OH-DPAT binding sites (data not shown). Inhibition curves for all the tested compounds were generated from measurement in septum and the pIC$_{50}$ values are listed in Table 1.

Anatomical Localization of $[^3H]$8-OH-DPAT Binding Sites in Wild-Type, 5-HT$_{1A}$ Knockout, and 5-HT$_{1A/1B}$ Double-Knockout Mice

 Autoradiograms of $[^3H]$8-OH-DPAT receptor binding (10 nM) in wild-type and 5-HT$_{1A}$ knockout, and 5-HT$_{1A/1B}$ double-knockout mice are summarized in Table 2. The distribution patterns of $[^3H]$8-OH-DPAT binding sites in 5-HT$_{1A}$ and 5-HT$_{1A/1B}$ knockout mouse brains were identical (Table 2). In wild-type mice, high densities of $[^3H]$8-OH-DPAT binding sites were observed in olfactory system, septum, bed nucleus of the stria terminalis, amygdala, hypothalamus, hippocampal formation, central gray, raphe nuclei, and throughout the cortical mantle (Fig. 5, a–f). SB-269970 (10 $\mu$M) displaced part of $[^3H]$8-OH-DPAT binding sites in wild-type olfactory system (Fig. 5a), septum (Fig. 5b), bed nucleus of stria terminalis (Fig. 5b), amygdala (Fig. 5c), hypothalamus (Fig. 5, c’–e’), thalamus (Fig. 5, c’ and d’), and central gray (Fig. 5f).

 In 5-HT$_{1A}$ knockout mouse brain high densities of $[^3H]$8-OH-DPAT binding sites were measured in olfactory system (Fig. 5g), septum (Fig. 5h), bed nucleus of the stria terminalis (Fig. 5, h and i), thalamus (Fig. 5, i and j), amygdala (Fig. 5, i and j), hypothalamus (Fig. 5, h–k), and entorhinal cortex (Fig. 5, j–l). Moderate densities were measured in CA3 field of the hippocampal formation (Fig. 5, j and k), central gray (Fig. 5l), and throughout the cortical mantle. Low densities were observed in cerebellum (data not shown). All the $[^3H]$8-
Potency of compounds (pIC_{50} values, 95% confidence interval in parentheses) for inhibition of [3H]8-OH-DPAT binding sites in septum of wild-type (1.5 nM) or knockout mice (10 nM) measured using quantitative receptor autoradiography (n = 3)

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<tr>
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<td>6.6 (7.2–6.4)</td>
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<tr>
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<td>6.7 (7.1–7.4)</td>
<td>N.D.</td>
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<tr>
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<td>&lt;5</td>
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<tr>
<td>SB-216641</td>
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N.D., not determined; WT, wild-type; KO, knockout.

OH-DPAT binding sites were displaced by 10 µM SB-269970 in 5-HT_{1A} knockout mice (Fig. 5, g–i).

**Discussion**

In the present autoradiographic study, we took advantage of selective pharmacological tools, 5-HT_{1A} knockout and 5-HT_{1A/B} double-knockout mice to revisit the pharmacological characterization and anatomical localization of 5-HT_{7} binding sites in mouse brain using [3H]5-CT and [3H]8-OH-DPAT. For the first time, the brain distribution of 5-HT_{7} receptor binding sites was studied in the absence of masking compounds.

**Autoradiographic Analysis of [3H]5-CT Binding Sites in Brain of 5-HT_{1A/B} Double-Knockout Mice.** In addition to the 5-HT_{7} receptor, a number of other 5-HT receptor subtypes have been reported to bind 5-CT with nanomolar affinity (Hoyer et al., 1994; Saudou and Hen, 1994). These include 5-HT_{1A} (Bodeke et al., 1992), 5-HT_{1B} (Voigt et al., 1991), 5-HT_{1D} (Bach et al., 1993), and 5-HT_{5A/B} receptors (Erlander et al., 1993). With the emergence of a selective 5-HT_{7} receptor antagonist (SB-269970; Hagan et al., 2000; Lovell et al., 2000) it has now become possible to specifically displace the 5-HT_{7} receptor component of [3H]5-CT binding. Given the high density of 5-HT_{7}, mRNA and 5-HT_{7} immunoactivity in septum, thalamus, and hypothalamus, we expected to see robust [3H]5-CT binding in these regions. We found that the pattern of [3H]5-CT labeling in the absence of cold compound in wild-type mice was nearly identical to that observed in the presence of a saturating concentration of the selective 5-HT_{7} receptor antagonist SB-269970 (Fig. 1). Slight decrease in [3H]5-CT density was observed in septum, cortex, amygdala, thalamus, and hypothalamus (P. Bonaventure, unpublished data). To clarify this unexpected observation we studied the autoradiographic distribution of [3H]5-CT binding sites in the brain of mice lacking the 5-HT_{1A} and 5-HT_{1B} receptors. Very low densities of [3H]5-CT binding sites were observed in restricted brain regions (septum, cortex, amygdala, thalamus, hypothalamus, globus pallidus, and substantia nigra) of 5-HT_{1A/B} double-knockout mice (Fig. 2). In the globus pallidus and substantia nigra, [3H]5-CT binding sites resistant to the selective 5-HT_{7} antagonist SB-269970 were observed. As mentioned, [3H]5-CT also binds to 5-HT_{1D} and 5-HT_{5} receptors. 5-HT_{5} receptor binding sites have been described to be restricted to the habenula (Waeb et al., 1998). We did not observe any [3H]5-CT binding sites in the habenula of 5-HT_{1A/B} knockout mice. This could be explained by the low concentration of [3H]5-CT used in this study compared with Waeb and colleagues’ study (2 versus 5 nM). To investigate the 5-HT_{5D} receptor component in the binding of [3H]5-CT in 5-HT_{1A/B} double-knockout mice, we performed an experiment using [3H]GR125743, a selective 5-HT_{5D} radioligand (P. Bonaventure, unpublished data). The distribution pattern of [3H]GR125743 binding sites throughout the brain of 5-HT_{1A/B} double-knockout mice was restricted to the globus pallidus and substantia nigra. The low densities of [3H]GR125743 detected in the globus pallidus and substantia nigra were fully displaced by 1 µM SB-216641, the 5-HT_{1D} antagonist. The [3H]5-CT binding sites resistant to SB-269970 observed in the globus pallidus and substantia nigra of 5-HT_{1A/B} knockout mice were also displaced by 1 µM of SB-216641, demonstrating the 5-HT_{1D} nature of these binding sites. In summary, low densities of [3H]5-CT binding sites corresponding to the 5-HT_{5} receptor were observed in septum cortex, amygdala, thalamus, and hypothalamus of 5-HT_{1A/B} double-knockout mice. Our results demonstrate that despite its high affinity for the 5-HT_{7} receptors in tissue homogenates (To et al., 1995; Stowe and Barnes, 1998; Thomas et al., 2000) or for the recombinant receptor heterologously expressed in mammalian cells (Bard et al., 1993; Plassat et al., 1993; Shen et al., 1993), [3H]5-CT is not a good tracer for measuring 5-HT_{7} binding sites autoradiographically.

In several autoradiographic studies (To et al., 1995; Waeb and Moskowitz, 1995; Gustafson et al., 1996; Mengod et al., 1996) using [3H]5-CT in the presence of masking agents, the authors have concluded that the low densities of [3H]5-CT observed under these conditions might be explained by the occupancy of the 5-HT_{7} receptor by the masking agents. In Waeb and Moskowitz’s (1995) study the masking of 5-HT_{5A} binding sites by 100 nM 8-OH-DPAT is likely to block a large fraction of the 5-HT_{7} receptor binding sites because of the affinity of this compound for the 5-HT_{7} receptor (Lovenberg et al., 1993; Plassat et al., 1993; Ruat et al., 1993; Shen et al., 1993). In Gustafson et al. (1996), the concentration of pindolol (160 nM) and P-aminophenylethyl-m-trifluoromethylphenyl piperazine (30 nM, LY-165163) used for blocking 5-HT_{1A} and 5-HT_{1B} receptor binding sites probably does not interfere with 5-HT_{7} receptor binding sites. The distribution of [3H]5-CT binding sites in the absence of masking compounds in the brain of 5-HT_{1A/B} double-knockout mice observed in the present study suggest that the 5-HT_{1A/B} receptor subtypes occlusion was not the critical issue for the low densities of 5-HT_{7} binding sites labeled by [3H]5-CT. Several hypotheses could explain the low densities of [3H]5-CT binding observed in the brains of 5-HT_{1A/B} knockout mice. Recently, two independent groups (Markstein et al., 1999; Thomas et al., 1999) demonstrated an interaction of 5-HT_{1A} and 5-HT_{7} receptors in hippocampus. The 5-HT_{7} receptor-mediated stimulation of adenyl cyclase activity by 5-CT seems to be augmented by a mechanism involving 5-HT_{1A} receptor activation. Therefore, the presence of the 5-HT_{1A} receptor may be required for the binding of [3H]5-CT to the 5-HT_{7} receptor in tissue section. One alternative explanation may be the binding conditions used in this study. Agonist radioligands, which only recognize the high-affinity state of the receptor are also more sensitive to differing salt conditions than antagonists. We repeated the different protocols described in the literature (different incubation buff-
ers), and no differences were observed in $[^3$H]5-CT binding densities. Similarly, no differences were observed with a longer preincubation time (1 h; P. Bonaventure, unpublished data). We did not study the influence of temperature on $[^3$H]5-CT binding.

In addition to the demonstration that $[^3$H]5-CT labels only a small fraction of 5-HT 7 receptor binding sites in tissue sections, our study shows that no binding sites remain in the brain of 5-HT1A/1B knockout mice in the presence of SB-279970 and SB-216641 for 5-HT 7 receptor and 5-HT1D receptor binding site blockade, respectively. This observation sheds light on the controversial issue of the atypical feature of $[^3$H]5-CT binding sites observed in 5-HT 1A receptor-containing regions (Waeber and Moskowitz, 1995; Castro et al., 1997). These sites, described by Castro and colleagues as a new serotonergic recognition site, most likely correspond to the “atypical” 5-HT 1A binding sites described by Waeber and Moskowitz (1995).

**Autoradiographic Analysis of $[^3$H]8-OH-DPAT Binding Sites in Brain of 5-HT 1A Knockout and 5-HT 1A/1B Double-Knockout Mice.** We found that $[^3$H]5-CT is not a good tracer for autoradiographic studies. So we examined another ligand, $[^3$H]8-OH-DPAT, a 5-HT 1A receptor agonist that has been shown to exhibit moderate affinity for the recombinant 5-HT 7 receptor (Bard et al., 1993; Lovenberg et al., 1993; Plassat et al., 1993). An attempt to label 5-HT 7 receptors in hamster brain has been previously reported using 1.8 nM $[^3$H]8-OH-DPAT in the presence of 500 nM pindolol for 5-HT 1A receptor occlusion (Duncan et al., 1999). It is likely that the binding sites reported in Duncan et al. (1999) correspond to unmasked 5-HT 1A receptor binding sites. Our results show that a low concentration (2 nM; Fig. 3) of $[^3$H]8-OH-DPAT labeled a small fraction of 5-HT 7 receptor binding sites. In the present study $[^3$H]8-OH-DPAT (10 nM) was found to be a suitable tracer for autoradiographic studies of the 5-HT 7 receptor binding sites in the brain of 5-HT 1A knockout and 5-HT 1A/1B double-knockout mice. The pharmacological characterization of these $[^3$H]8-OH-DPAT binding sites demonstrates the 5-HT 7 nature of these binding sites. $[^3$H]8-OH-DPAT has been shown to label the 5-HT uptake recognition site rat striatum (Alexander and Wood, 1988). In the present study, citalopram (up to 10 μM), which displays high affinity for the 5-HT uptake sites (Hyttel, 1977), did not displace any binding sites in the septum, cortex, or striatum of 5-HT 1A knockout or 5-HT 1A/1B double-knockout mice.

Using $[^3$H]8-OH-DPAT, we have studied the brain distribution of 5-HT 7 receptor binding sites with an increased degree of specificity compared with previous studies (Wae-
The anatomical distribution of the \(^{3}H\)8-OH-DPAT binding sites observed in knockout mice matched the distribution of 5-HT\(_7\) receptor mRNA and 5-HT\(_7\) receptor immunoreactivity reported in the literature. Within the hippocampal formation, the distribution described in this study, i.e., strong labeling in CA3 and low densities in CA1, parallels the findings described by Neumaier et al. (1996). The anatomical distribution of the \(^{3}H\)8-OH-DPAT binding sites observed throughout the hypothalamus (including the suprachiasmatic nucleus), suggesting a role for 5-HT\(_7\) in the control of neuroendocrine and homeostatic functions.

### Conclusions

The results presented in this study show that despite its high affinity for 5-HT\(_7\) receptor in tissue homogenates, \(^{3}H\)5-HT is not a good tracer for measuring 5-HT\(_7\) receptor binding sites autoradiographically. The lower affinity ligand \(^{3}H\)8-OH-DPAT is a much better tracer for autoradiographic studies at the 5-HT\(_7\) receptor binding sites. Using \(^{3}H\)8-OH-DPAT, high-to-moderate densities of 5-HT\(_7\) receptor binding...
sites were found throughout the brains of mice lacking the 5-HT$_{1A}$ receptor.

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References


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