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Serotonin_{2C} Receptor (5-HT_{2C}R) Localization in GABA Neurons of the Rat Medial Prefrontal Cortex: Implications for Understanding the Neurobiology of Addiction

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Abstract

Serotonin (5-HT) action via the 5-HT_{2C} receptor (5-HT_{2C}R) provides an important modulatory influence over neurons of the prefrontal cortex (PFC), which is critically involved in disorders of executive function including substance use disorders. In the present study, we investigated the distribution of the 5-HT_{2C}R in the rat prelimbic PFC (PrL), a subregion of the medial PFC (mPFC), using a polyclonal antibody raised against the 5-HT_{2C}R. The expression of 5-HT_{2C}R immunoreactivity (IR) was highest in the deep layers (layer V/VI) of the mPFC. The 5-HT_{2C}R-IR was typically most intense at the periphery of cell bodies and the initial segment of cell processes. Approximately 50% of the 5-HT_{2C}R-IR detected was found in GAD 67-positive neurons. Of the subtypes of γ -aminobutyric acid (GABA) interneurons identified by expression of several calcium-binding proteins, a significantly higher percentage of neurons expressing IR for parvalbumin also expressed 5-HT_{2C}R-IR than did the percentage of neurons expressing calbindin-IR or calretinin-IR that also expressed 5-HT_{2C}R-IR. Since parvalbumin is located in basket and chandelier GABA interneurons which project to cell body and initial axon segments of pyramidal cells, respectively, these results raise the possibility that the 5-HT_{2C}R in the mPFC acts via the parvalbumin-positive GABAergic interneurons to regulate the output of pyramidal cells in the rat mPFC.

Keywords

Immunohistochemistry; parvalbumin; calbindin; calretinin; prelimbic prefrontal cortex

Recent advances in understanding the neural circuitry and mechanisms underlying the vulnerability to drug abuse, the progression of drug use to addiction, and the triggers for relapse provide hope that new therapeutic approaches are forthcoming for this brain disorder (Kalivas and Volkow, 2005; Koob and Le, 2005; Hyman et al., 2006). A loss of the normal regulatory role for prefrontal cortex (PFC) over the mesoaccumbens dopamine (DA) circuit that is central to drug reward has been identified as a key component in addiction, and pharmacological approaches to reinstate normal PFC function may prove to be

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therapeutically useful (Kalivas and Volkow, 2005). The PFC is densely innervated by serotonin (5-HT) neurons of the midbrain (Van Bockstaele et al., 1993) and actions via the family of 5-HT₂ receptors (5-HT₂R) provides an important modulatory influence over PFC function (Pompeiano et al., 1994; Clemett et al., 2000; Lopez-Gimenez et al., 2001). Recent reports have outlined an inhibitory role for the PFC 5-HT₂C_R in mediating the behavioral effects of psychostimulants such as cocaine (Filip and Cunningham, 2003) and 3,4-methylenedioxymethamphetamine (MDMA; Ramos et al., 2005). While both transcript and protein for the 5-HT₂C_R have been localized in PFC (Pompeiano et al., 1994; Clemett et al., 2000; Lopez-Gimenez et al., 2001; Pandey et al., 2006), a better understanding of the mechanisms of action through which the PFC 5-HT₂C_R controls stimulant-induced behaviors is required, and a first step in this analysis is to gain a more complete understanding of the localization of the 5-HT₂C_R protein in PFC.

The rat PFC has been divided into medial, lateral and ventral regions (Dalley et al., 2004). The medial PFC (mPFC) in rat is suggested to share analogous function with the PFC in human and, as such, appears to be involved in working memory, attention, and reward (Tzschentke, 2000; Dalley et al., 2004). The rat mPFC consists of the anterior cingulate cortex (ACC), the prelimbic PFC (PrL), and the infralimbic PFC (IL; Dalley et al., 2004; Paxinos and Watson, 2005). The most prominent subcortical afferents to the mPFC arise in the mediodorsal nucleus of the thalamus (Fuster, 1997). The mPFC also receives input from the raphe nuclei, the ventral tegmental area (VTA), the hypothalamus as well as other neocortical regions, while efferent pathways leave the mPFC to reciprocally innervate practically every structure which sends fibers to this cortical region (Fuster, 1997). The two major cell types that exist in the mPFC are glutamate-containing pyramidal projection neurons and γ -aminobutyric acid (GABA) local circuit interneurons (Gabbott et al., 1997; Fuster, 1997). The GABA interneurons form numerous synapses on pyramidal projection neurons and play a crucial regulatory function over PFC pyramidal output (Eyles et al., 2002). Several subclasses of GABA interneurons exist in the mammalian cerebral cortex, which are distinguished by the expression of the defined calcium-binding proteins parvalbumin, calbindin, and calretinin (Andressen et al., 1993; Gabbott et al., 1997). Interneurons which express parvalbumin, calbindin, or calretinin have distinct morphological and neurochemical, electrophysiological characteristics (Andressen et al., 1993; Conde et al., 1994; Gabbott et al., 1997; Gonzalez-Burgos et al., 2005). As such, knowledge of the distribution of the 5-HT₂C_R among neuronal subpopulations in the mPFC may provide insight into the nature of 5-HT₂C_R control of cortical function at the cellular level.

The purpose of the present study was to investigate the distribution of the 5-HT₂C_R protein in the mPFC and the localization of 5-HT₂C_R to subtypes of GABA neurons in the PrL area of the mPFC. The PrL was chosen as a representative area of the mPFC as the distribution of the subtypes of GABA neurons has been shown to be similar across the three subregions of the mPFC (Gabbott et al., 1997). To this end, we have employed double-label, fluorescence immunohistochemistry to simultaneously visualize the 5-HT₂C_R protein (Bubar et al., 2005; Bubar and Cunningham, 2007) in GABA neurons labeled with the synthetic enzyme glutamic acid decarboxylase (GAD 67), and/or the calcium-binding proteins parvalbumin, calbindin, and calretinin. Investigation of the 5-HT₂C_R localization in this population of neurons in the mPFC will provide insight into the molecular mechanisms underlying the function of the 5-HT₂C_R localized to PFC and its role in disorders of executive function, such as addiction.

Experimental Procedures

Animals

Naïve, male Sprague-Dawley rats (Harlan, Houston, TX, USA) weighing 250–300 g were used. The rats were housed four to a cage in standard plastic rodent cages in a colony room maintained at $21 \pm 2^\circ\text{C}$ and at 40–50% humidity under a 12-h light/dark cycle (06:00–18:00 h) for at least one week before experiments. Food and water were available *ad libitum*. All experiments were carried out in accordance with the *NIH Guide for the Care and Use of Laboratory Animals* (National Institute of Health, 1986) and with approval by the UTMB Institutional Animal Care and Use Committee.

Antibodies

For Western blot analyses, the affinity purified goat polyclonal anti-5-HT_{2C}R antibody [SR-2C (N-19): sc-15081, Lot No. L0904, Santa Cruz Biotechnology, Inc., CA] was used at a concentration of 1:500 at 4°C overnight. The monoclonal mouse anti-post-synaptic density-95 (PSD-95) antibody (MAB1598; Chemicon, Temecula, CA) was used at a concentration of 1:1000 at room temperature for 1 hr. The monoclonal mouse anti-syntaxin antibody (MAB336; Chemicon) was used at a concentration of 1:5000 at room temperature for 1 hr. Secondary antibodies utilized include horseradish peroxidase (HRP) conjugated sheep anti-mouse (1:2000–1:5000; AP300P; Chemicon.) and donkey anti-goat secondary antibody (1:2000; 6420-05; Southern Biotech, Birmingham, AL).

For immunohistochemical analyses, the following primary antibodies were used: anti-5-HT_{2C}R polyclonal antibody raised in goat (1:100 dilution, sc-15081, lot G102, Santa Cruz Biotechnology, Inc.) (Bubar et al., 2005); anti-glutamic decarboxylase 67 (GAD 67) polyclonal antibody raised in rabbit (1:150 dilution, sc-5602; Santa Cruz Biotechnology, Inc.); anti-parvalbumin monoclonal antibody raised in mouse (1:1000 dilution, P-3088, Sigma-Aldrich Corp.); anti-calbindin monoclonal antibody raised in mouse (1:1000 dilution, C-9848, Sigma-Aldrich Corp.); anti-calretinin polyclonal antibody raised in rabbit (1:3000 dilution, AB-5054, Chemicon International, Inc.). The following secondary antibodies were used: donkey anti-goat antibody conjugated to Alexa Fluor® 488 (1:500 dilution, A-11055, Invitrogen Corporation, Carlsbad, CA, USA); donkey anti-rabbit antibody conjugated to Alexa Fluor® 555 (1:2000 dilution, A-31572, Invitrogen Corporation); donkey anti-mouse antibody conjugated to Alexa Fluor® 555 (1:2000 dilution, A-31570, Invitrogen Corporation). All antibodies were diluted to the final concentration in blocking buffer (as above).

Western Blot Analyses

Subcellular Fractionation and Post-Synaptic Density Isolation—The post-synaptic density (PSD) was isolated according to a subcellular fractionation protocol slightly modified from published protocols (Phillips et al., 2001; Moron et al., 2007). In brief, naive rats (n=4) were anesthetized using chloral hydrate (800 mg/kg, IP) and PFC tissue was microdissected, pooled and homogenized in 0.32 M sucrose solution [0.1 mM CaCl₂] containing protease inhibitor cocktail (Sigma-Aldrich Corp., St. Louis, MO, Cat. No. P8340) and phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich Corp., Cat. No. P2850 and P5726, respectively). An aliquot (total homogenate fraction) was taken. The remaining homogenate sample was brought to a final concentration of 1.25 M sucrose solution by adding 2 M sucrose and 0.1 mM CaCl₂ and was transferred to a 25 mL ultracentrifuge tube, overlaid with 1 M sucrose and subsequently ultracentrifuged in a fixed angle rotor (50.2 Ti Rotor, Beckman) at 28,000 RPM for 3 hrs at 4°C . The synaptosomal fraction was collected at the interface of the 1.25 M and the 1 M sucrose phase and diluted in 15.6 mL hypotonic solution [0.1 mM CaCl₂, 1 M Tris pH 6 buffer, 10% Triton X-100 and protease and

phosphatase inhibitor cocktails], mixed for 20 min at 4°C and ultracentrifuged at 21,000 RPM for 20 min at 4°C. The supernatant was discarded. The pellet (containing the synaptic junction) was resuspended in 10 mL of buffer [20 mM Tris, 1% Triton X-100, pH 8], mixed for 20 min at 4°C and ultracentrifuged at 21,000 RPM for 20 min at 4°C. The supernatant (containing presynaptic active zone) was discarded and the pellet (containing the PSD) was resuspended in 10 mL pH 8 buffer, mixed for 20 min at 4°C and ultracentrifuged at 21,000 RPM for 20 min at 4°C. The resultant supernatant was discarded and the remaining pellet (PSD fraction) was resuspended in 1% SDS. Both aliquots (total homogenate and PSD) were frozen at -80°C until protein analysis was conducted. Total protein concentration was determined for each fraction using a BCA protein determination kit (Pierce, Rockford, IL). Equal amounts of protein (7 µg from each fraction; reduced with an appropriate volume of Laemmli sample buffer with DTT for 20 min at 70°C) were separated by SDS-PAGE using a 10% BisTris pre-cast gels (Novex, San Diego, CA).

Western Blot Assay—Following gel electrophoresis, proteins were transferred to a PVDF membrane (BIORAD, Hercules, CA) via semi-dry electroblotting (Alltech, Deerfield, IL) for 2 hr at 18V. The membrane was blocked with 5% milk for 2 hr, followed by incubation with the primary antibody for 1 hr-overnight. Membranes were rinsed 3 × 20 min in TBS + 1% Tween (TBS-T), and incubated with the secondary antibody for 45 min. Membranes were rinsed again 3 × 10 min in TBS-T prior to incubation with the Enhanced Chemiluminescence Plus (ECL) Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ) for 5 min. The membrane was exposed to film [Midwest Scientific Bplus Ref EBA45 (8×10 in)] for 1 sec to 10 min, depending upon the antibody being detected. The membrane was probed with all three antibodies; antibodies were stripped from the membrane via incubation in Re-blot Plus Mild (Chemicon) for 7–15 min prior to re-probing with each additional antibody according to the procedures described above. For peptide neutralization studies, the anti-5-HT_{2C}R antibody was combined with a 4-fold excess of its corresponding blocking peptide (sc-15081 P; Santa Cruz Biotechnology) in a final volume of 500 µL TBS at 4°C overnight. Following blocking, the mixture was diluted in 1% Milk TBS to proceed with the western blot assay.

Immunohistochemical Analyses

Perfusion and sectioning—Rats (n = 4) were anaesthetized with sodium pentobarbital (100 mg/kg) and transcardially perfused with 10 mmol/L phosphate buffered saline (PBS, pH 7.2) followed by 3% paraformaldehyde in 10 mmol/L PBS (pH 7.2). Brains were removed, blocked at mid-pons, and postfixed in 3% paraformaldehyde of PBS for 2 h at room temperature. Brains were then cryoprotected in 30% sucrose for 48 h at 4°C, rapidly frozen on crushed dry ice, and stored at -80°C until sectioning. Coronal sections (20 µm) containing the mPFC (-2.7 through -3.7 mm from Bregma) were taken from all brains using a cryostat at -20°C (Leica CM 1850, Leica Microsystems Nussloch GmbH, Nussloch, Germany) according to the atlas of Paxinos and Watson (Paxinos and Watson, 2005). For one rat, thicker sections (40 µm) adjacent to 20 µm sections were taken for the cresyl violet staining to determine the layers of PrL.

Immunohistochemistry—Immunohistochemistry was performed on free-floating brain sections (20 µm) according to a previously published protocol (Bubar et al., 2005; Bubar and Cunningham, 2007). Briefly, brain sections were washed (5 × 10 min) with 10 mmol/L PBS (pH 7.2) using an orbital shaker and incubated for 60 min in blocking buffer (PBS supplemented with 1.5 % normal donkey serum, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at room temperature under continuous agitation on an orbital shaker. The blocking buffer was aspirated, and the sections were incubated with the appropriate primary antibody. Incubation was carried out for 44 hours at 4°C, under

continuous agitation on an orbital shaker. After six washes (6 min each wash) in 10 mmol/L PBS (pH 7.2), the sections were protected from light and further incubated for 60 min at room temperature with the appropriate secondary antibody. Sections were washed with PBS (3 × 10 min) and mounted using a 0.1% Drefit® solution onto gelatin chromium-coated slides. The slides were then coverslipped using Vectashield® fluorescent mounting medium with DAPI (4', 6-diamidino-2-phenylindole; Vector Laboratories, Ltd., Burlingame, CA, USA) to label nuclei, and stored protected from light at 4 C until viewing.

Nissl staining—Brain sections (40 µm) adjacent to the sections processed for the 5-HT_{2C}R immunohistochemistry in one rat were processed for Nissl staining to identify basic neuronal structures. Slides with the 40 µm brain sections were immersed for 2 min in each of the following: 95% alcohol, 70% alcohol, and distilled water. Sections were stained in 0.5% cresyl violet for 2–3 min, differentiated in water for 0.5 min and then dehydrated for 2 min in each of the following: 70% alcohol, 95% alcohol, 100% alcohol, placed in Histoclear (National Diagnostics, Atlanta, GA, USA) for 10 min and cover-slipped with Permount (Fisher Scientific, Fair Lawn, NJ, USA). The slides were cleaned and viewed after they were dry.

Image analysis—Digital images were captured from brain sections using an Olympus BX51 fluorescent microscope (Olympus America Inc., Melville, NY, USA) equipped with a Hamamatsu camera (Model C4742-95, Hamamatsu Corp., Bridgewater, NJ, USA) interfaced to a personal computer with SimplePCI software (version 5.1, Compix Inc., Cranberry Township, PA, USA). A 4x, 10x, 20x, or 40x objective was used to capture all photographs for final magnification of 40x, 100x, 200x, or 400x, respectively. Photomicrographs (at a magnification of 40x) were taken of areas which were 2130 µm × 1700 µm and included layers I to VI of the PrL (see box in Figure 1). These photomicrographs were used to quantify the expression of 5-HT_{2C}R-immunoreactive (IR) cells in superficial (layers I and II/III) and deep layers (layers V and VI) of the PrL and their colocalization with GAD 67-, parvalbumin-, calbindin- or calretinin-IR. Three coronal sections including the mPFC were taken from each rat at +3.2 mm anterior-posterior from bregma. Photomicrographs at a magnification of 100x were taken from layers I–VI of the PrL and used to show location of 5-HT_{2C}R-IR. Photomicrographs with magnification of 200x were taken from layer V of the PrL and used to show the colocalization of the 5-HT_{2C}R with either GAD 67, parvalbumin, calbindin or calretinin. Photomicrographs with magnification of 400x were taken from layer V of the PrL and used to show subcellular staining pattern of the 5-HT_{2C}R-, GAD 67-, parvalbumin-, calbindin-, and calretinin-IR. Green fluorescence emitted by the Alexa Fluor® 488 antibodies was visualized using a yellow GFP filter set (#41017; Chroma Technology Corporation, Rockingham, VT, USA), while the red fluorescence emitted by the Alexa Fluor® 555 antibodies was visualized using a narrow band green excitation filter set (U-MNG2, Olympus). In addition, DAPI staining was visualized using a blue GFP II filter set (#31041, Chroma Technology Corporation). For each section, up to three images were captured of the same viewing area, one for each filter set detecting immunoreactivity (IR) for each antibody and DAPI, and then resultant images were overlaid.

To investigate distribution of the 5-HT_{2C}R-IR and its colocalization with GAD 67-IR and parvalbumin-, calbindin- and calretinin-IR in the PrL, the images were analyzed using a program written in MATLAB (The MathWorks, Inc., Natick, MA, USA) by Dr. Gilbert R. Hillman (Hillman and Johnston, 1983; Sepulveda et al., 2006). Red and green channels of the image data were used separately for analysis. Correction for variation in background staining was accomplished by comparing the intensity of each pixel to that of a local 50×50 pixel region. Pixels with intensity higher than background by a selectable number of gray levels were counted as labeled. This intensity difference between pixels and background

around pixels used to identify a cell was chosen according to the type of staining. A contiguous cluster of labeled pixels was considered to be a cell when the diameter was roughly equal to or larger than 10 μm .

Colocalization of two labels was determined by approximate coincidence of red and green labeling. This condition was determined by dilating both the red and the green images using a 3 $\times$ 3 structuring element, and then locating pixels that were labeled in both images after dilation. Each contiguous cluster of colocalized pixels was counted as a single cell. The number of cells exhibiting 5-HT_{2C}R-IR and colocalization of 5-HT_{2C}R-IR with GAD 67-IR or IR of one of the calcium-binding proteins were counted using the 40x magnification photomicrographs. Brain sections stained with cresyl violet were used to identify layers of the PrL. The cell numbers for each rat brain were calculated from the average of the numbers from three sections (around +3.2 mm prior to bregma) for a given rat.

A one-way ANOVA was used to analyze the differences in the percentage of cells labeled for 5-HT_{2C}R-IR which also contained parvalbumin-, calbindin- or calretinin-IR. A significant F value was followed by a comparison between specific groups using the Student Newman-Keuls procedure (SNK). All statistical analyses were conducted with the experimentwise error rate (α) at 0.05. Subsequent to analysis, minor adjustments to the brightness and contrast of some images were made during preparation for publication.

Results

5-HT_{2C}R Antibody Validation

A previously published study has demonstrated that the anti-5-HT_{2C}R antibody employed here (sc-15081) binds specifically to the 5-HT_{2C}R and does not cross react with the 5-HT_{2A}R in immunohistochemical analyses (Bubar et al., 2005; Bubar and Cunningham, 2007). In brief, robust and reproducible immunostaining was observed in mouse and rat brain consistent with findings with a home-generated 5-HT_{2C}R antibody (Clemett et al., 2000). The 5-HT_{2C}R-IR was also eliminated in transgenic mice lacking the 5-HT_{2C}R gene relative to wild type mice and did not stain native CHO cells (which do not express the 5-HT_{2C}R protein) or CHO cells that expressed the closely-related 5-HT_{2A}R (Bubar et al., 2005). Primary antibody only and secondary antibody only controls contained a complete absence of specific labeling of cells by either antibody alone in the current (data not shown) and previously published studies (Bubar et al., 2005; Bubar and Cunningham, 2007), suggesting that the observed IR was specific to the combination of 5-HT_{2C}R specific primary and secondary antibodies. These studies suggest that expression of the 5-HT_{2C}R protein can be readily detected using this antibody.

In the present study, we conducted additional validation of the anti-5-HT_{2C}R antibody through Western blot analyses of PFC tissue. Since the 5-HT_{2C}R is thought to be localized post-synaptically (Clemett et al., 2000; Lopez-Gimenez et al., 2001), we utilized a subcellular fractionation protocol (Phillips et al., 2001; Moron et al., 2007) to isolate the PSD fraction of the PFC, an electron dense structure where proteins that are involved in receiving and transducing synaptic signals are highly concentrated (e.g., membrane-bound receptors). Isolation of the PSD was confirmed via Western blot analysis showing that post-synaptic density-95 (PSD-95), a PSD marker (Banker et al., 1974; Hunt et al., 1996), was highly abundant in the PSD fraction (Figure 1). In contrast, syntaxin, a pre-synaptic marker (Bennett et al., 1992), while present in the total homogenate, was absent in the PSD fraction (Figure 1). In agreement with evidence of post-synaptic localization of the 5-HT_{2C}R (Clemett et al., 2000; Lopez-Gimenez et al., 2001), we observed an enrichment of the 5-HT_{2C}R in the PSD fraction (Figure 1). Specificity of the 5-HT_{2C}R signal was confirmed via peptide neutralization of the anti-5-HT_{2C}R. Pre-incubation of the anti-5-HT_{2C}R antibody

with a 4-fold excess of the peptide used to stimulate antibody production eliminated the 55 kDa band in the PSD fraction. (Figure 1). These results confirm that the anti-5-HT_{2C}R antibody detects a protein localized to the post-synaptic density of the PFC with the size and immunoreactive characteristics expected for a selective 5-HT_{2C}R antibody. Thus, these data support the use of this antibody as a tool to examine the localization of the 5-HT_{2C}R in the PFC.

5-HT_{2C}R-IR distribution and staining pattern in the mPFC

Immunoreactivity for the 5-HT_{2C}R was observed in all subregions of the rat mPFC including the anterior cingulate cortex (ACC), the prelimbic PFC (PrL), and the infralimbic PFC (IL), and displayed similar laminar distribution in each of the three subregions of the rat mPFC (data not shown). The expression of the 5-HT_{2C}R-IR was further analyzed in superficial and deep layers of the PrL (see box in Figure 2). In the area of the PrL analyzed (see box in Figure 2), 85% percent of all 5-HT_{2C}R-IR cells detected were located in the deep layers (layers V–VI) while only 15% were found in the superficial layers (layers I–III; Figure 3). The expression of the 5-HT_{2C}R was typically most intense along the periphery of cell bodies and processes proximal to the cell bodies (Figure 4A). The neurons expressing the 5-HT_{2C}R-IR typically appeared round-or fusiform-shaped (Figures 4A, 5A).

5-HT_{2C}R/GAD 67 immunofluorescence

The anti-GAD 67 antibody employed here (sc-5602) has been utilized in several prior studies for immunohistochemical (Majak and Pitkanen, 2003; Akema et al., 2005; Falk et al., 2006) and Western blot analyses (Akema et al., 2005; Sanchez et al., 2006). One study (Akema et al., 2005) revealed that this anti-GAD 67 antibody can detect similar numbers of cells in the medial preoptic area as detected using an anti-GABA antibody. Furthermore, they demonstrate that this antibody can detect a lipopolysaccharide (LPS)-induced increase in the number of GAD 67-expressing cells using either Western blot or immunohistochemical analysis, which also paralleled the increase in GABA-positive cells induced by LPS. These results suggest that this GAD 67 antibody can be utilized to detect GABA-synthesizing cells in the brain. In addition, validation studies conducted by Santa Cruz Biotechnology revealed that the sc-5602 antibody detects the decrease in GAD 67 protein expression induced by GAD-67 siRNA transfection in HeLa cells (C. Maraviglia, Santa Cruz Biotechnology, personal communication). In the current study, primary antibody only and secondary only controls contained a complete absence of specific labeling of cells by the GAD 67 antibody alone, suggesting that the observed IR was specific to the combination of GAD 67-specific primary and secondary antibodies (data not shown).

Sections containing the mPFC were simultaneously processed with the 5-HT_{2C}R and GAD 67 antibodies using the double-label immunofluorescence protocol. GAD 67-positive neurons were observed in cortical layers II–VI (data not shown). In general, GAD 67-IR was distributed evenly throughout the soma, with occasional extension into the proximal processes (Figure 4B, 5B). GAD 67-IR was detected in a subset of 5-HT_{2C}R-IR cells in the mPFC; an example of a section of the PrL stained simultaneously for GAD 67- and 5-HT_{2C}R-IR is shown in Figure 5. In the area of the PrL in which expression of the 5-HT_{2C}R-IR was quantified (see box in Figure 2), $50.4 \pm 4.77\%$ of the total detected cells that were stained for the 5-HT_{2C}R-IR also expressed GAD 67-IR. The percentage of neurons that expressed both 5-HT_{2C}R-IR and GAD 67-IR relative to all detected neurons expressing 5-HT_{2C}R-IR in layers V–VI ($54.38\% \pm 6.28\%$) was significantly higher than that seen in layers I–III ($19.88\% \pm 4.38\%$) ($p < 0.05$; data not shown).

Colocalization of 5-HT_{2C}R and calcium-binding proteins

Double-label immunofluorescence experiments using antibodies raised against the 5-HT_{2C}R and the calcium-binding proteins (parvalbumin, calbindin, and calretinin; Gritti et al., 2003; Liu et al., 2005) allowed us to determine whether the 5-HT_{2C}R was preferentially expressed in particular subtypes of GABAergic interneurons. The antibodies to the calcium-binding proteins diffusely stained soma and frequently stained processes of neurons (Figures 4C–E, 6B, 6E, 6H). In both layers I–III and layers V–VI of the PrL, the number of calbindin-IR and calretinin-IR cells was significantly lower than that of parvalbumin-IR cells (Figure 7).

Examples of sections of the PrL stained for parvalbumin-IR, calbindin-IR, or calretinin-IR in combination with the 5-HT_{2C}R-IR are shown in Figure 6C, 6F and 6I, respectively. The frequency of 5-HT_{2C}R-IR colocalization with the IR for each calcium-binding protein differed significantly in the deep layers ($F_{3,8} = 13.69$, $p < 0.0058$), but not the superficial layers ($F_{3,8} = 0.12$, $p < 0.8905$) of the PrL. In deep layers (V–VI), 25.4% of parvalbumin-IR neurons detected also displayed 5-HT_{2C}R-IR, while only 8.1% of calbindin- and 2.5% of calretinin-IR neurons contained 5-HT_{2C}R-IR (Figure 8). Thus, a significantly higher percentage of neurons with parvalbumin-IR showed colocalization with 5-HT_{2C}R-IR in comparison to calbindin-IR or calretinin-IR ($p < 0.05$, Figure 8).

Discussion

The present study reveals that the 5-HT_{2C}R is primarily expressed in the deep layers of the rat mPFC. About 50% of neurons expressing 5-HT_{2C}R-IR in the PrL region of the mPFC expressed GAD 67-IR. The 5-HT_{2C}R-IR was also found to be differentially distributed in subtypes of GABA interneurons, as defined by the presence of specific calcium-binding proteins. Compared to calbindin- and calretinin-IR interneurons, a much higher percentage of parvalbumin-IR neurons were found to be co-labeled for 5-HT_{2C}R-IR, suggesting that the 5-HT_{2C}R may exhibit its greatest influence upon this subtype of GABA interneuron in the mPFC.

The 5-HT_{2C}R antibody employed here preferentially labeled cells in the deep layers (layers V/VI) compared to superficial layers (layers I/II/III) of the rat mPFC. These data are in keeping with previous *in situ* hybridization studies which demonstrated a distribution of the 5-HT_{2C}R mRNA restricted to layer V of PFC in rats (Pompeiano et al., 1994), monkeys (Lopez-Gimenez et al., 2001) and humans (Pasqualetti et al., 1999). The 5-HT_{2C}R-IR was most intense in the periphery of cell bodies and processes proximal to cell bodies in primarily round- or fusiform-shaped cells in the rat mPFC. The present study also demonstrated that 50% of the 5-HT_{2C}R-IR in the mPFC is colocalized with GAD 67-IR, a marker of GABA interneurons.

The expression of 5-HT_{2C}R-IR in mPFC GABA interneurons coincides with previous data demonstrating that 5-HT_{2C}R mRNA is present in a subpopulation of PFC GABA interneurons (Vysokanov et al., 1998) and that serotonergic axons in the monkey PFC predominantly synapse on interneurons (Smiley and Goldman-Rakic, 1996). Studies have also shown that the local administration of the 5-HT_{2A/2C}R agonist 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) within the mPFC increased extracellular GABA levels in this area (Abi-Saab et al., 1999), while systemic DOI induced c-Fos expression in GAD 67-positive mPFC interneurons (Abi-Saab et al., 1999). These c-Fos proteins were not expressed in 5-HT_{2A}R positive cortical neurons (Mackowiak et al., 1999), suggesting that the activation of PFC GABA interneurons by DOI is mediated by the 5-HT_{2C}R, not the 5-HT_{2A}R. Thus, there are a variety of data within the literature supporting the presence of 5-HT_{2C}R within PFC GABA interneurons. In the present study, however, only about half of the neurons displaying 5-HT_{2C}R-IR also possessed GAD 67-IR, suggesting either that the

detection of GAD 67-IR was limited by the sensitivity of the assay conditions, or that the remaining 5-HT_{2C}R-IR identified in the mPFC is localized to other cell types. Although several studies have indicated the absence of labeling for 5-HT_{2C}R mRNA in pyramidal-shaped cells in both human and monkey PFC (Pasqualetti et al., 1999; Lopez-Gimenez et al., 2001) and few pyramidal-shaped 5-HT_{2C}R-IR cells were observed in the present study, other studies using single cell RT-PCR have demonstrated that ~25 – 50% of pyramidal neurons in the PFC contain mRNA for the 5-HT_{2C}R (Vysokanov et al., 1998; Carr et al., 2002). Thus, it is probable that the remaining mPFC 5-HT_{2C}R-IR cells are pyramidal glutamate neurons (Figure 9), although additional co-labeling studies for 5-HT_{2C}R and a glutamate neuron-specific marker are necessary to confirm this hypothesis.

The subclasses of GABA interneurons, identified by the expression of the calcium-binding proteins parvalbumin, calbindin, and calretinin, have distinct morphological, neurochemical and electrophysiological characteristics (Andressen et al., 1993; Conde et al., 1994; Gabbott et al., 1997; Gonzalez-Burgos et al., 2005). The results of the present study revealed that the subtypes of PFC GABA neurons did not express 5-HT_{2C}R-IR equivalently. Only a small proportion of calbindin-positive interneurons (8.1%) or calretinin-positive neurons (2.5%) were found to contain 5-HT_{2C}R-IR. These findings suggest limited 5-HT_{2C}R control of calbindin-labeled (double-bouquet, neurogliaform, and Martinotti) neurons that innervate the distal dendrites of pyramidal cells (Conde et al., 1994; Gabbott et al., 1997), or calretinin-positive interneurons (double-bouquet and Cajal-Retzius cells) whose terminals selectively innervate other GABA interneurons or the distal dendrites of pyramidal cells (Conde et al., 1994; Gabbott et al., 1997).

The 5-HT_{2C}R-IR in the PrL was localized primarily to parvalbumin-positive interneurons, which include basket and chandelier neurons that innervate the cell body and the initial axon segment, respectively, of pyramidal neurons (Conde et al., 1994; Gabbott et al., 1997). Thus, the inhibitory synapses formed by parvalbumin-expressing basket and chandelier neurons are proximal to the initial axon segment of pyramidal cells, the site at which action potentials are generated. As such, a primary function of parvalbumin-positive GABA neurons is to modulate the efferent signaling of pyramidal neurons (Miles et al., 1996; Markram et al., 2004; Lewis et al., 2005). Since the 5-HT_{2C}R-IR was predominantly colocalized with parvalbumin-IR, the 5-HT_{2C}R-mediated influence on GABA interneurons would be expected to primarily target the basket and chandelier cells which provide the strongest inhibitory effects on the output of cortical pyramidal cells, compared to interneurons that innervate distal dendrites of the pyramidal cells, which likely function to regulate incoming afferent signals to the pyramidal neurons (Miles et al., 1996).

Activation of the 5-HT_{2C}R depolarizes neuronal membranes and increases the firing rate of neurons (Stanford et al., 2005). Thus, endogenous or exogenous stimulation of the 5-HT_{2C}R localized to parvalbumin-positive interneurons in the mPFC would likely induce GABA release, which would in turn act to inhibit output of the pyramidal cells (Figure 9; Gabbott et al., 1997; Gonzalez-Burgos et al., 2005). The localization of 5-HT_{2C}R- to the deep layers of the rat mPFC (layers V–VI), suggests that the 5-HT_{2C}R acts to modulate the output of neurons in these layers. The pyramidal efferent neurons in layers V–VI primarily project to subcortical regions, including the VTA and nucleus accumbens (NAc), the origin and terminal regions of the DA mesoaccumbens pathway (Gabbott et al., 2005). Here, pyramidal neurons function to regulate neurotransmission within the pathway. Thus, release of GABA via activation of the 5-HT_{2C}R on parvalbumin-positive mPFC GABA interneurons would be expected to reduce excitatory output from the mPFC to the mesoaccumbens pathway, enabling mPFC 5-HT_{2C}R to indirectly regulate glutamate and DA levels in the NAc (Figure 9). In support of this concept, iontophoretic application of the 5-HT_{2C}R agonist mCPP suppressed spontaneous and/or glutamate-activated firing of PFC neurons (Bergqvist et al.,

1999), while electrical stimulation of the rat mPFC enhanced glutamate levels in the VTA (Rossetti et al., 1998) and NAc (You et al., 1998) as well as DA release in the NAc (You et al., 1998); but see (Jackson et al., 2001). Taken in conjunction with the observation that activation of mPFC GABA receptors inhibited both glutamate release in the VTA and NAc and DA release in the NAc (Karreman and Moghaddam, 1996; Harte and O'Connor, 2005), these data suggest that stimulation of mPFC 5-HT_{2C}R on parvalbumin-positive GABA interneurons would function to reduce excitatory glutamate output as well as subsequent DA neurotransmission within the mesoaccumbens pathway (Figure 9).

The mesoaccumbens pathway is thought to be integral in psychological processes including motivation, reward and mood (Salamone, 1996; Pierce and Kumaresan, 2006; Nestler and Carlezon, Jr., 2006), and is an important site for the actions of psychostimulants such as cocaine (Filip and Cunningham, 2003; Pierce and Kumaresan, 2006). Thus, alterations in accumbal DA and/or glutamate levels consequent to stimulation of mPFC 5-HT_{2C}R may be one mechanism by which the 5-HT_{2C}R modulates the neurochemical and behavioral effects of psychostimulants. Intra-mPFC infusion of 5-HT_{2C}R agonists have been shown to block the hypermotive and discriminative stimulus effects of cocaine (Filip and Cunningham, 2003), and block the expression of sensitization (the progressive enhancement of the hypermotive effects of a drug following repeated drug exposure) to 3,4-methylenedioxymethamphetamine (MDMA; Ramos et al., 2005). Intra-mPFC infusion of a 5-HT_{2C}R antagonist, on the other hand, enhanced cocaine-induced hyperactivity and increased recognition of the stimulus effects of cocaine (Filip and Cunningham, 2003) in the absence of overt effects of intra-mPFC 5-HT_{2C}R ligands alone. Thus, these results are in keeping with the model that activation of the 5-HT_{2C}R on mPFC GABA interneurons inhibits output of the mPFC to ultimately reduce the behavioral effects of psychostimulants (Figure 9). However, should the 5-HT_{2C}R also be localized directly on pyramidal output neurons (Figure 9), stimulation of these 5-HT_{2C}R would be expected to enhance pyramidal neuron activity, thereby opposing the effect of 5-HT_{2C}R on GABA interneurons.

In summary, our results demonstrate a higher percentage of parvalbumin-positive neurons with 5-HT_{2C}R-IR than the calbindin- and calretinin-positive neurons, suggesting that the activation of 5-HT_{2C}R in the mPFC may reduce the output of the pyramidal neurons by increasing the excitability of the inhibitory parvalbumin-positive GABA neurons. This new knowledge of the distribution of 5-HT_{2C}R among the subclasses of mPFC interneurons provides insight into the mechanisms by which the mPFC 5-HT_{2C}R modulates cortical output.

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Abbreviations

5-HT	5-hydroxytryptamine, serotonin
5-HT _{2C} R	serotonin _{2C} receptor
ACC	anterior cingulate cortex

DA	dopamine
DOI	1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane
GABA	γ -aminobutyric acid
GAD 67	glutamate decarboxylase, isoform 67
IL	infralimbic prefrontal cortex
IR	immunoreactivity
MDMA	3,4-methylenedioxy-methamphetamine
mPFC	medial prefrontal cortex
NAc	nucleus accumbens
PFC	prefrontal cortex
PrL	prelimbic prefrontal cortex
PSD	post-synaptic density
PSD-95	post-synaptic density-95
VTA	ventral tegmental area

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Figure 1. Western blot analysis demonstrating detection of the 5-HT_{2c}R in the PSD of the PFC Total homogenate (Total) and PSD-associated proteins (PSD) from the PFC were analyzed by Western blot using antibodies against PSD-95 (a post-synaptic density marker), syntaxin (a pre-synaptic marker) and the 5-HT_{2c}R. In addition, peptide neutralization of the 5-HT_{2c}R antibody with its appropriate blocking peptide eliminated the anti-5-HT_{2c}R antibody-associated band. Data presented are from a single blot that was stripped and re-probed. Location of the molecular weight markers are shown to the left of the blots.

Figure 2. Schematic drawing of representative section used for the quantification of IR cells in the PrL

The drawing (bregma 3.24 mm) was adapted from Paxinos and Watson (2004). The box shows the area in which photomicrographs at a magnification of 40x were taken to quantify the numbers of 5-HT_{2C}R-IR cells in superficial (layers I and II/III) and deep layers (layers V and VI) of the PrL and their colocalization with GAD 67-, parvalbumin-, calbindin- or calretinin-IR. ACC: anterior cingulate cortex; PrL: prelimbic cortex; IL: infralimbic cortex.

Figure 3. Stratification and segregation of immunoreactive profiles for the 5-HT_{2C}R-IR in the PrL

Expression of 5-HT_{2C}R-IR (**green, A**) was segregated into specific cortical layers shown by comparison with neighboring sections (**B**) stained with cresyl violet, to aid in identifying the cortical layers (I–VI). Note that most 5-HT_{2C}R-IR cells are located in layer V–VI. A few cells are also stained in layers I and II/III. (**C**) is a schematic drawing adapted from Paxinos and Watson (2004) corresponding to coronal sections (Bregma 3.24) from which images in **A** and **B** were captured. Scale bar: 200 μ m. ACC: anterior cingulate cortex; PrL: prelimbic cortex; IL: infralimbic cortex.

Figure 4. Photomicrographs of immunostained neurons in the deep layers of the PrL illustrate the subcellular distribution patterns of IR associated with each of the immunoreagents used in this study

The 5-HT_{2C}R-IR (**green, A**) was typically most intense at the periphery of the cell bodies, with some extension into the proximal dendrites; DAPI stained nuclei are also shown (**blue**). The antisera against GAD 67 (**red, B**) produced a relatively homogeneous staining of the cell soma and occasionally weakly labeled the proximal dendrites. The antisera against parvalbumin (**red, C**), calbindin (**red, D**) and calretinin (**red, E**) produced a diffuse staining that labeled the soma and proximal and distal neurites. Scale bar, 20 μ m.

Figure 5. Colocalization of GAD 67 and 5-HT_{2C}R in layer IV of the PrL
 Photomicrographs of double-label immunofluorescent staining for the 5-HT_{2C}R (**green, A**), and GAD 67 (**red, B**). Figure **C** displays the overlay of images in A and B to demonstrate colocalization. Approximately 50% of 5-HT_{2C}R-positive cells in the field also contain GAD 67-IR. Examples of cells that express immunoreactivity for GAD 67 and 5-HT_{2C}R are shown by arrows. **D**. Schematic drawing adapted from Paxinos and Watson (2004) corresponding to coronal sections (bregma 3.24 mm) from which images in A–C were captured. Scale bar: 50 μm. ACC: anterior cingulate cortex; PrL: prelimbic cortex; IL: infralimbic cortex.

Figure 6. Colocalization of calcium-binding proteins and 5-HT_{2C}R in layer IV of the PrL

In all cases, the 5-HT_{2C}R-IR is demonstrated by Alexa Fluor® 488 (**green, A, D, G**), and parvalbumin-, calbindin-, and calretinin are demonstrated by Alexa Fluor® 555 staining (**red, B, E, H**, respectively). Figures **C, F and I** display the overlay of images in **A+B, D+E** and **G+H**, respectively. Of the three classes of interneurons, double-labeled parvalbumin cells tended to express the greatest degree of 5-HT_{2C}R-IR. Arrows show the cells with both parvalbumin-IR and 5-HT_{2C}R-IR. All images were taken from the same area as depicted in Figure 5D. Scale bar: 100 μ m.

Figure 7. Number of parvalbumin-, calbindin-, and calretinin-positive cells in the rat PrL
The average number of parvalbumin-, calbindin-, and calretinin-IR cells detected ($\pm$ S.E.M.) in the PrL are presented ($n = 3$ rats). The number of calretinin- or calbindin-positive cells detected is significantly lower ($p < 0.05$) than the number of parvalbumin-positive cells detected in both the superficial layers (layers I-III; **A**) and the deep layers (layers V/VI; **B**) of the PrL.

Figure 8. Number of parvalbumin-, calbindin-, and calretinin-positive cells in the rat PrL that co-express 5-HT_{2C}R-IR

Interneuron populations were defined by IR staining for parvalbumin, calbindin, or calretinin. The average of the percentage of total cells detected for each interneuron subpopulation that also contained 5-HT_{2C}R-IR ($\pm$ S.E.M.) is presented ($n = 3$ rats). The percentage of calretinin or calbindin-positive cells which express 5-HT_{2C}R-IR is significantly lower ($p < 0.05$) than the number of parvalbumin-positive cells in the deep layers (layers V/VI; **B**) but not the superficial layers (layers I-III; **A**) of the PrL.

Figure 9. Schematic representation of the sites of action for the PrL 5-HT_{2c}R to modulate PFC output to the VTA and NAc

The projection from 5-HT neurons in the raphe nuclei (Raphe) to the prefrontal cortex (PFC) is represented by the solid light blue line. The 5-HT_{2c}R located in the PFC are represented by light blue hexagons. The GABA interneurons that express parvalbumin (GABA/PV; red), calbindin (GABA/CB; green) or calretinin (GABA/CR; yellow) are also illustrated. Pyramidal glutamate (Pyr) projections from the PFC to the VTA and NAc are represented by solid purple lines. Dopamine projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) is represented by a solid dark blue line. The 5-HT_{2c}R is expressed in a greater proportion of parvalbumin-containing GABA interneurons and

therefore the 5-HT_{2C}R has its greatest influence (denoted by thick red lines) upon this subtype of GABA neuron compared to GABA neurons that possess calbindin or calretinin, where the 5-HT_{2C}R A exerts minor influence upon activity of these subtypes of GABA neurons (denoted by dotted green and yellow lines, respectively). Stimulation of mPFC 5-HT_{2C}R on parvalbumin-positive GABA interneurons would function to reduce excitatory glutamate output as well as subsequent DA neurotransmission within the mesoaccumbens pathway (VTA and NAc). Although not assessed in this study, the 5-HT_{2C}R may also be expressed in pyramidal neurons (blue hexagon with question mark).