Pyramidal Neurons in Rat Prefrontal Cortex Projecting to Ventral Tegmental Area and Dorsal Raphe Nucleus Express 5-HT$_{2A}$ Receptors

The prefrontal cortex (PFC) is involved in higher brain functions altered in schizophrenia. Classical antipsychotics modulate corticolimbic circuits mainly through subcortical D2 receptor blockade, whereas second generation (atypical) antipsychotics preferentially target cortical 5-HT receptors. Anatomical and functional evidence supports a PFC-based control of the brainstem monoaminergic nuclei. Using a combination of retrograde tracing experiments and in situ hybridization we report that a substantial proportion of PFC pyramidal neurons projecting to the dorsal raphe (DR) and/or ventral tegmental area (VTA) express 5-HT$_{2A}$ receptors. Cholera toxin B application into the DR and the VTA retrogradely labeled projection neurons in the medial PFC (mPFC) and in orbitofrontal cortex (OFC). In situ hybridization of 5-HT$_{2A}$ receptor mRNA in the same tissue sections labeled a large neuronal population in mPFC and OFC. The percentage of DR-projecting neurons expressing 5-HT$_{2A}$ receptor mRNA was ~60% in mPFC and ~75% in OFC (n = 3). Equivalent values for VTA-projecting neurons were ~55% in both mPFC and ventral OFC. Thus, 5-HT$_{2A}$ receptor activation/blockade in PFC may have downstream effects on dopaminergic and serotonergic systems via direct descending pathways. Atypical antipsychotics may distally modulate monoaminergic pathways through PFC 5-HT$_{2A}$ receptor blockade, presumably decreasing the activity of neurons receiving direct cortical inputs.

Keywords: antipsychotics, dopamine, pyramidal neurons, schizophrenia, serotonin receptors

Introduction

The prefrontal cortex (PFC) is critically involved in many higher brain functions, including cognitive functions and behavioral control, which are altered in schizophrenic patients (Lewis and Lieberman 2000; Elvevag and Goldberg 2000; Weinberger et al. 2001). Autopsy and neuroimaging studies have revealed the existence of a reduced PFC volume, reduced layer thickness, tight packing of pyramidal neurons and reduced neuropil in the brains of schizophrenic patients (Harrison 1999; Selemon and Goldman-Rakic 1999; Lewis and Lieberman 2000). Alterations in key neurotransmitters such as glutamate, GABA, and dopamine (DA) have also been reported in PFC (Lewis and Lieberman 2000; Benes and Berretta 2001; Lewis et al. 2005). Moreover, untreated schizophrenic patients show a reduced energy metabolism in the PFC which has been related with negative symptoms (Andreasen et al. 1997; Potkin et al. 2002) yet psychotic symptoms appear associated with hyperactivity of various cortical areas, including the PFC (Catafau et al. 1994; Dierks et al. 1999; Shergill et al. 2000).

Classical antipsychotic drugs are thought to alleviate schizophrenia symptoms by dampening the overactivity of DA at D2 receptor in ventral striatum. In contrast, second generation (atypical) antipsychotics preferentially target serotonin (5-HT) receptors such as 5-HT$_{2A}$, 5-HT$_{2C}$, and 5-HT$_{1A}$ receptors (Bymaster et al. 1996; Arnt and Skarsfeldt 1998; Chou et al. 2003). These receptors are densely expressed in various subfields of the rat PFC, particularly in its medial aspect (Pompeiano et al. 1992, 1994). Recent studies have established their presence in a large proportion of PFC pyramidal and GABAergic neurons. In particular, 50-60% of the pyramidal neurons in layers II/III and V of the medial PFC (mPFC) express 5-HT$_{2A}$ and/or 5-HT$_{1A}$ receptors (Amargós-Bosch et al. 2004; Santana et al. 2004).

Anatomical and electrophysiological studies have established the presence of direct afferents from cingulated (Cg), prelimbic (PL), and infralimbic (IL) areas of the PFC to the brainstem monoaminergic nuclei, including the dorsal raphe nucleus (DR) (Aghajanian and Wang 1977; Hajós et al. 1998; Sesack et al. 1989; Peyron et al. 1998; Celada et al. 2001) and the ventral tegmental area (VTA) (Thierry et al. 1979, 1983; Tong et al. 1996, 1998; Carr and Sesack 2000). These nuclei give rise to the ascending serotonergic and dopaminergic innervation of most cortical and limbic brain regions, involved in numerous physiological functions and in severe psychiatric conditions such as major depression and schizophrenia (Carlsson 1988; Jacobs and Azmitia 1992; Weinberger et al. 1994; Williams and Goldman-Rakic 1995; Laruelle et al. 1996; Robbins 2000; Tschenkute and Schmidt 2000; Schulz 2004).

Previous studies have reported that the systemic administration of 5-HT$_{2A}$ receptor ligands such as DOI (5-HT$_{2A/2C}$ receptor agonist) or M100907 (selective 5-HT$_{2A}$ receptor antagonist) modulates the DA system (Gobert and Millan 1999; Ichikawa et al. 2001; Minabe et al. 2001; Pehek et al. 2001; Porras et al. 2002), yet the location of the receptors responsible for these effects was unknown. More recently, it was reported that the local application of DOI in mPFC increased burst firing of DA cells in the VTA and the DA output in the mesocortical DA pathway (Bortolozzi et al. 2005). Similarly, local DOI application in the mPFC enhanced the firing rate of DR 5-HT neurons and 5-HT output in rat mPFC (Martín-Ruíz et al. 2001; Amargós-Bosch et al., 2004). These observations suggested that the activity of the ascending cortical DA and 5-HT systems can be modulated by 5-HT$_{2A}$ receptor activation in pyramidal neurons projecting to midbrain.

In the present study, we provide histological support to this hypothesis by using a combination of tract-tracing techniques and subsequent in situ hybridization of 5-HT$_{2A}$ receptor mRNA in PFC sections containing retrogradely labeled neurons projecting to DR or VTA.
After surgery, rats were housed individually. Ten days after surgery, the positive-pulsed direct current for a total of 5 min (7 s on/off for 10 min).

**Fluorescence Microscopy**

Chemical fixatives were applied for fluorescence microscopy (Nikon Eclipse E1000, Nikon, Tokyo, Japan). Cell counting was performed on a Zeiss Axioskop microscope (Carl Zeiss, Jena, Germany) with a 100x Plan Neofluar objective, and cells were counted manually. All experiments were performed in accordance with the guidelines for the Care and Use of Laboratory Animals of the Spanish Council for Scientific Research (CEC) and the European Communities Council Directive on the Protection of Animals Used for Scientific Purposes (86/609/EEC). Animal experiments were conducted in compliance with the guidelines for the Care and Use of Laboratory Animals of the Spanish Council for Scientific Research (CEC) and the European Communities Council Directive on the Protection of Animals Used for Scientific Purposes (86/609/EEC). Animal work was carried out in the animal facilities of the School of Medicine of the University of Barcelona and all protocols used were approved by the Ethical Committee for Animal Research and the "Departament de Medi Ambient i Habitatge" of the Catalan Government (Generalitat de Catalunya).

Animals were deeply anesthetized with sodium pentobarbital (60 mg/kg ip), and placed in a stereotaxic frame (David Kopf Instruments). Glass capillary tubes were heated and pulled with a Narishige PE-2 pipette puller (Narishige Sci. Inst. Tokyo, Japan). Tips were broken to 30 μm diameter under microscopic control. The micropipettes were then filled with a solution of cholecystokinin B subunit (CTB, 2% in distilled water, List Biological Laboratories, Campbell, CA). CTB was iontophoretically injected into the DR (AP: +7.6 to +7.8; L: -3.1 with a vertical 30° angle; DV: -6.2 to -6.8; n = 3) or the VTA (AP: -5.0 to -6.0; L: -0.5; DV: -8.2; n = 3). Stereotaxic coordinates were taken from the atlas of Paxinos and Watson (1986). The iontophoretic injections were performed using a 5 μA positive-pulsed direct current for a total of 5 min (7 s on/off for 10 min). After surgery, rats were housed individually. Ten days after surgery, the animals were anesthetized with an ip overdose of sodium pentobarbital and perfused transcardially with 50 mL of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Tissue sections, 14 μm thick, were cut on a cryostat (Microm HM500 OM, Walldorf, Germany) and thaw-mounted onto glass slides pretreated with HistoBond (Zymed Laboratories Inc., San Francisco, CA).

CTB Immunohistochemistry on Hybridized Sections

After the in situ hybridization protocol, tissue sections were kept for 15 min in Tris-HCl buffer (0.1 M pH 7.5) containing 1 M NaCl, 2 mM MgCl2, 0.1% BSA, and 2% DMSO. They were then incubated 30 min at 37°C with rabbit anti-CTB antiserum (1:100; Sigma, St. Louis, MI). Subsequently, the sections were washed 3 times (5 min each) and then incubated 30 min with biotinylated goat anti-rabbit immunoglobulin (1:100, Vector Laboratories). After three 5-min washes, the sections were incubated for 1 hour in Vectorstain Elite ABC solution (Vector Laboratories) in a 1% H2O2 solution in Tris (pH 7.5). Then, the sections were washed 3 times and immersed in a solution of 0.5 mg/mL 3,3′-diaminobenzidine (DAB, Sigma-Aldrich) in Tris-HCl 0.05 M pH 7.5 containing 1 ml/mL H2O2 until color development (in all cases less than 10 min). The reaction was stopped by 2 rinses in buffer. CTB staining appeared as brown/orange precipitate in the soma and proximal dendrites of retrogradely labeled neurons. The sections were then briefly dipped in 100% ethanol, followed by 1 minute in 70% ethanol, 1 minute in 50% ethanol, followed by a 1 minute in 30% ethanol, followed by a 1 minute in 10% ethanol, followed by a 1 minute in 5% ethanol, and finally in 100% Mounting media (Prolong Gold Antifade Mounting Media, Invitro) were then applied to the sections, sealed with nail polish, and allowed to dry overnight in a barrier chamber.

In Situ Hybridization Histochemistry Procedure

Sections through the PFC lesioned rats injected with CTB were hybridized with probes against the 5-HT2A receptor mRNA. We used simultaneously 3 oligonucleotide probes complementary to bases 128-170, 1380-1427, and 939-987 (GenBank accession no. NM_017254) (Pritchett et al., 1988). These probes were synthesized on a 380 Applied Biosystem DNA synthesizer (Foster City Biosystem, Foster City, CA) and purified on a 20% polyacrylamide/8 M urea preparative sequencing gel. The oligonucleotides (2 pmol each) were labeled at their 3′-end with [32P]dATP (>2500 Ci/mmol; Perkin-Elmer, Boston, MA) using terminal deoxynucleotidyltransferase (Roche Diagnostics GmbH, Mannheim, Germany), and then purified by centrifugation using QiAquick Nucleotide Removal Kit (Qiagen GmbH, Hilden, Germany). The protocols for in situ hybridization were based on previously described procedures (Tomiyama et al. 1997). Brieﬂy, frozen tissue sections were first brought to room temperature, ﬁxed for 20 min at 4°C in 4% paraformaldehyde in Dulbecco’s PBS (1x dPBS: 8 mM Na2HPO4, 1.4 mM KH2PO4, 136 mM NaCl, 2.6 mM KCl), washed for 5 min in 3x PBS at room temperature, twice for 5 min each in 1x dPBS and incubated for 2 min at 21°C in a solution of predigested probe (Zymed Laboratories Inc., San Francisco, CA) and a ﬁnal concentration of 24 U/mL in 50 mM Tris-HCl pH 7.5, 5 mM ethylenediaminetetraacetic acid. The enzymatic activity was stopped by immersion for 30 s in 2 mg/mL glycine in 1x dPBS. Tissue sections were ﬁnally rinsed in 1x dPBS and dehydrated through a graded series of ethanol. For hybridization, the labeled probes were diluted in a solution containing 50% formamide, 4× SSC (1× SSC: 150 mM NaCl, 15 mM sodium citrate), 1× Denhardt’s solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 10% dextran sulfate, 1% sarkosyl, 20 mM phosphate buffer pH 7.0, 250 μg/mL yeast RNA and 500 μg/mL salmon sperm DNA. Sections were covered with hybridization solution containing the labeled probe, overlaid with Nesconcell coverslips (Bando Chemical Ind., Kobe, Japan) and incubated overnight at 42°C in humid boxes. Sections were then washed 4 times (45 min each) in washing buffer (0.6 M NaCl, 10 mM Tris-HCl pH 7.5) at 60°C and once in the same buffer at room temperature for 30 min.

The specificity of the hybridization signal obtained with the probes used has been previously established (Pompeiano et al. 1994). These controls included the following procedures: 1) the thermal stability of the hybrids obtained was checked for every probe, 2) for a given oligonucleotide probe, the hybridization signal was completely blocked by competition of the labeled probe in the presence of 50-fold excess of the same unlabeled oligonucleotide, and 3) the distribution of the hybridization signal at the regional and cellular levels obtained with the 3 probes used independently was identical.

Analysis of the Results

Tissue sections processed for in situ hybridization followed by immunohistochemical detection of CTB were examined in a Nikon Eclipse E1000 microscope (Nikon, Tokyo, Japan). Cell counting was performed manually at the microscope with the help of “analySIS” software (Soft Imaging System GmbH, Germany) in 1-3 coronal sections of each rat corresponding to approximately 3.2 mm from the lesion, according to atlases of the rat brain (Paxinos and Watson 1986; Swanson 1999) DAB-labeled cells were considered positive when a dark precipitate was clearly distinguished from background. Only

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CTB-labeled cells showing high abundance of 5-HT2A receptor mRNA signal (densities of silver grains 3-3-fold greater than average background) were considered positive for both markers. Using this procedure, we cannot discard that the number of 5-HT2A receptor-bearing neurons has been somehow underestimated. We preferred this to a false-positive identification of 5-HT2A receptor-expressing neurons. Because the presence of high densities of autoradiographic silver grains might sometimes mask DAB-labeling, cells in the preparations were systematically examined at different focal planes to see CTB-immunoreactivity at lower planes and hybridization signal at upper planes. Micrography was performed using a digital camera (Color View 12) and the software “analySIS” on a Nikon Eclipse E1000 microscope (Nikon) or an Olympus NewCast microscope (Olympus, NL). Digital images were treated with Adobe Photoshop (Adobe Software, Mountain View, CA) to adjust only brightness, contrast and size to make the final figures.

Results

The data reported here correspond to 6 rats in which CTB was microiontophoretically applied into the VTA or the DR (n = 3 for each site). In all cases, the applied CTB was confined within the respective nucleus, as shown by the colocalization of the tracer and the markers for dopaminergic neurons of the VTA (TH) and serotonergic neurons of the DR (TPH). Figure 1 shows representative fluorescence microscope images illustrating the localization of CTB in the VTA (1A–C) and the DR (1D–F) of 2 of the rats used in the study. Schematic drawings in Figure 2 illustrate the location of the different microinjections performed in the VTA and DR.

The application of CTB in the DR or the VTA resulted in the labeling of pyramidal neurons in layer V and superficial aspect of layer VI in the mPFC, including the Cg, PL, and IL subdivisions. CTB-labeled neurons were also found in the orbitofrontal cortex (OFC). These cells were located mainly in the ventral OFC of VTA-injected rats whereas they extended more laterally, reaching the agranular insular cortex in rats injected with CTB in the DR. Tracer application in the DR resulted in the labeling of PFC neurons in both hemispheres, whereas the application in the VTA labeled almost exclusively neurons in the ipsilateral hemisphere. Figure 3 shows the schematic location of CTB-retrogradely labeled neurons in the PFC of 2 rats (V2 and D2 in Tables 1 and 2, respectively) injected with the tracer in the VTA (V2, Fig. 3A) and in the DR (D2, Fig. 3B), respectively. Each dot corresponds to 3 CTB-labeled pyramidal neurons.

Figure 4 shows 5-HT2A receptor mRNA labeling in VTA-projecting neurons in the mPFC. The presence of the 5-HT2A receptor mRNA was established by dense clusters of silver grains, clearly distinguishable from background. As previously observed (Amargós-Bosch et al. 2004; Santana et al. 2004), a large proportion of cells in the Cg, PL, and IL subdivisions of the PFC were found that contained the 5-HT2A receptor transcript, mainly in layers II/III and V. No 5-HT2A receptor-positive cells were found in layer I and a lower abundance of 5-HT2A receptor-positive cells was observed in layer VI compared with layers III–V. In occasions, the density of silver grains made difficult the detection of CTB-immunoreactivity. In these cases, and because CTB-immunoreactivity and silver grains are localized at different depths of the tissue preparation, a careful inspection of individual cells was performed by examining different planes of the preparation, which contain the CTB signal (lower plane) and the emulsion for in situ hybridization (upper plane). Table 1 shows the number of CTB-positive cells and the percentage of double-labeled cells in the mPFC and ventral OFC of the 3 rats injected with CTB in the VTA. The percentage of CTB-positive cells that expressed 5-HT2A receptors in the mPFC was rather high and homogenous (43–60%; 54 ± 6% on average). Two of the rats also exhibited a moderate-high (45–65%) percentage of CTB-positive neurons expressing the 5-HT2A receptor in the ventral OFC. No unambiguous CTB-positive cells could be detected in the third animal. It is likely that these individual differences depend on the size and precise site of injection of CTB within the VTA. Representative examples of single-labeled (CTB- or 5-HT2A receptor mRNA positive) and double-labeled (CTB- and 5-HT2A receptor mRNA positive) cells are shown at a high magnification in Figure 4C–E.

A large proportion of CTB-positive pyramidal neurons in the mPFC and OFC of both hemispheres as labeled by tracer application in the DR also contained 5-HT2A receptor transcripts (Figs 5 and 6 Table 2). The percentage of CTB-positive cells that expressed 5-HT2A receptors was also high and showed little variability in the 3 rats examined. Representative examples of single-labeled (CTB- or 5-HT2A receptor mRNA positive) and double-labeled (CTB- and 5-HT2A receptor mRNA positive)
positive) cells are shown at a high magnification in Figures 5C--E and 6C--E.

Discussion

The present results indicate that a substantial proportion (>50% on average) of pyramidal neurons in the PFC projecting to the midbrain monoaminergic nuclei (DR and/or VTA) contain the 5-HT2A receptor transcript. These results provide anatomical evidence for the existence of a PFC-based, 5-HT2A receptor-mediated distal control of the activity of the ascending dopaminergic and serotonergic systems. From a psychopharmacological perspective, the present data suggest that the interaction of atypical antipsychotics with 5-HT2A receptors in the PFC may decrease the excitatory PFC input onto selected neuronal populations within the DR and the VTA and consequently modulate the activity of the ascending dopaminergic and serotonergic systems.

Methodological Considerations

5-HT2A receptors are expressed in high densities in PFC (Pazos et al. 1985; Pompeiano et al. 1994). More recent data using double in situ hybridization indicate that ~50–60% pyramidal neurons and ~20–30% GABAergic neurons in layers II–V of the mPFC contain the 5-HT2A receptor mRNA (Santana et al. 2004).

Our results are consistent with these previous observations and show the presence of a high number of 5-HT2A receptor mRNA-expressing cells in the Cg, PL, and IL subdivisions of the PFC, mainly in layers II–V and a lower abundance in layer VI, as observed previously (Pompeiano et al. 1994; Amargós-Bosch et al. 2004; Santana et al. 2004). This demonstrates that the in

Table 1

| Rat | PFC region | CTB+ | CTB+/5HT2A+ | % Double-labeled cells |
|-----|------------|------|--------------|------------------------|
| V1  | mPFC       | 24   | 10           | 43                     |
| V2  | OFC (ventral) | 17   | 10           | 59                     |
| V3  | mPFC       | 90   | 54           | 60                     |
|     | OFC (ventral) | 0    | 0            | —                      |

Note: Values are numbers of CTB-positive neurons in each structure (1–2 sections per rat), as determined by CTB-immunoreactivity (CTB+). CTB-positive neurons that are also positive for the 5-HT2A receptor transcript (CTB+/5-HT2A+), were identified by the presence of the 2 markers (see Methods). Only neurons with unambiguous labeling for both markers were considered positive, as in the examples shown in Figure 3B.

Figure 2. Schematic drawings of coronal sections through the VTA (A) and the DR (B) illustrate the precise location of CTB injections (shaded areas) in the different rats used in this study as assessed by CTB immunostaining. Drawings were taken from Swanson (1999).

Figure 3. Schematic drawings of coronal sections through the PFC illustrating the localization of CTB-retrogradely labeled cells after tracer injections in the VTA of rat V2 (A) and the DR of rat D2 (B). Every dot represents 3 labeled cell bodies. (A) CTB injections in the VTA only labeled cell bodies in the ipsilateral (i) mPFC (including Cg, PL, and IL) and medial (ventral) part of the OFC. (B) CTB injections in the DR labeled cell in the mPFC and OFC of both ipsilateral and contralateral (c) hemispheres. Drawings were taken from Paxinos and Watson (1986).
The present methodology has also been used to determine the presence of 5-HT4 receptors in PFC neurons projecting to VTA (Peñas et al., manuscript in preparation). Recent reports have used riboprobes in combination with tract tracers (Hur and Zaborszky 2005; Perez-Manso et al. 2006; Yokota et al. 2007; Barroso-Chinea et al. 2008). The present study indicates that, similarly to riboprobes, oligonucleotides can be reliably combined with tracer immunohistochemistry to identify proteins expressed at relatively low levels, such as monoamine receptors, in projection neurons.

CTB-immunoreactive cells were clearly detected in tissue sections previously treated for in situ hybridization. In the PFC, these cells were exclusively located in the medial and orbital divisions. This distribution agrees well with that reported previously using electrophysiological and tract-tracing methods for DR- and VTA-projecting neurons (Aghajanian and Wang 1977; Thierry et al. 1979; Thierry et al. 1983; Sesack et al. 1989; Peyron et al. 1998; Jankowski and Sesack 2004; Gabbott et al. 2005; Geisler and Zahm 2005). One possible limitation of the use of CTB is the uptake by damaged fibers of passage close to the application site. This might be a potential confounding factor, particularly for the identification of VTA-projecting neurons, given the proximity of the VTA to the medial forebrain bundle. CTB has been reported to be taken up by fibers of passage (Chen and Aston-Jones 1995). However, this potential limitation can be minimized by the microiontophoretic application of the tracer, as used herein. This procedure resulted in a tracer distribution within the boundaries of the DR and VTA as observed by the coincidence of the tracer with the TPH and TH immunoreactivity used to label 5-HT and DA neurons, respectively. Non-specific uptake by damaged fibers of the medial forebrain bundle during CTB application in the VTA would have resulted in labeling of pyramidal neurons in other PFC areas, particularly in the primary and secondary motor areas, that contain the largest proportion of corticospinal fibers of all PFC subdivisions (Gabbott et al. 2005). Such labeling was not observed in any of the rats, which further supports the specificity of the labeling procedure.

### Table 2

| Rat | PFC region         | CTB+ | CTB+ / 5HT2A+ | % Double-labeled cells |
|-----|--------------------|------|--------------|-----------------------|
| D1  | mPFC, ipsilateral* | 12   | 7            | 58                    |
|     | OFC, ipsilateral   | 19   | 13           | 68                    |
|     | mPFC contralateral | 19   | 13           | 68                    |
|     | OFC contralateral  | 21   | 18           | 86                    |
| D2  | mPFC, ipsilateral  | 30   | 20           | 67                    |
|     | OFC, ipsilateral   | 38   | 30           | 79                    |
|     | mPFC contralateral | 23   | 14           | 61                    |
|     | OFC contralateral  | 30   | 23           | 77                    |
| D3  | mPFC, ipsilateral  | 19   | 11           | 58                    |
|     | OFC, ipsilateral   | 42   | 26           | 61                    |
|     | mPFC contralateral | 6    | 4            | 67                    |
|     | OFC contralateral  | 27   | 22           | 81                    |

Note: Values are the numbers of CTB-positive neurons in each structure (2 sections per rat), as determined by CTB-immunoreactivity (CTB+). CTB-positive neurons that are also positive for the 5-HT2A receptor transcript (CTB+/5HT2A+), were identified by the presence of the 2 markers (see Methods). Only neurons with unambiguous labeling for both markers were considered positive, as in the examples shown in Figures 4B and 5B.

*CTB application was aimed at the midline in the DR. However, the micropipette entered the brain with an angle of 30° to avoid the sinus. Thus, we use the term ipsilateral to refer to the brain hemisphere where the micropipette first entered the midbrain.

**Figure 4.** Colocalization of 5-HT2A receptor mRNA hybridization signal and CTB-immunoreactivity in the mPFC after injection of the tracer in the VTA. (A) Clusters of autoradiographic grains corresponding to 5-HT2A receptor mRNA-containing cells are clearly visible in layers II-VI at a low magnification; the strongest signal is found in layer V neurons. The boxed area is shown in (B) at a higher magnification. (B) CTB-immunoreactivity (brown precipitate) can be seen in some cells under the hybridization signal (red-blue double arrowheads), whereas other cells display only 5-HT2A receptor mRNA signal (blue arrowheads). (C) Detail of a CTB-immunoreactive neuron which lacks the hybridization signal. (D) Detail of a double-labeled neuron containing CTB-immunoreactivity and a strong 5-HT2A receptor mRNA hybridization signal. (E) Detail of a cell profile showing 5-HT2A receptor mRNA signal without CTB-immunoreactivity. Bars: 200 µm (A), 30 µm (B), 10 µm (C-E).
Figure 5. Colocalization of 5-HT2A receptor mRNA hybridization signal and CTB-immunoreactivity in the mPFC after injection of the tracer in the DR. (A) Clusters of autoradiographic grains corresponding to 5-HT2A receptor mRNA-containing cells are clearly visible in layers II–VI at a low magnification. The boxed area is shown in (B) at a higher magnification. (B) CTB-immunoreactivity (brown precipitate) can be seen in some cells under the hybridization signal (red-blue double arrowheads) and in cells lacking hybridization signal (red arrowheads), whereas other cells display only 5-HT2A receptor mRNA signal (blue arrowheads). (C) Detail of a CTB-immunoreactive neuron which lacks the hybridization signal. (D) Detail of a double-labeled neuron containing CTB-immunoreactivity and a strong 5-HT2A receptor mRNA hybridization signal. (E) Detail of a cell profile showing 5-HT2A receptor mRNA signal without CTB-immunoreactivity. Bars: 200 μm (A), 30 μm (B), 10 μm (C–E).

Figure 6. Colocalization of 5-HT2A receptor mRNA hybridization signal and CTB-immunoreactivity in the OFC after injection of the tracer in the DR. (A) Clusters of autoradiographic grains corresponding to 5-HT2A receptor mRNA-containing cells are clearly visible at a low magnification. The boxed area is shown in (B) at a higher magnification. (B) CTB-immunoreactivity (brown precipitate) can be seen in some cells under the hybridization signal (red-blue double arrowheads) and in cells lacking hybridization signal (red arrowheads), whereas other cells display only 5-HT2A receptor mRNA signal (blue arrowheads). (C) Detail of a CTB-immunoreactive neuron which lacks hybridization signal. (D) Detail of a double-labeled neuron containing CTB-immunoreactivity and 5-HT2A receptor mRNA hybridization signal. (E) Detail of a cell profile showing 5-HT2A receptor mRNA signal without CTB-immunoreactivity. Bars: 200 μm (A), 30 μm (B), 10 μm (C–E).
in addition to the DR itself, yet the percentage of double-labeled cells in the PFC was similar to the rest.

The number of CTB-immunoreactive neurons in the mPFC was lower when the CTB immunohistochemistry was conducted in sections previously subjected to in situ hybridization (data not shown). This difference may be attributed to the previous tissue exposure to the hybridizing conditions and/or the high salt concentration used in the immunohistochemical procedure. However, this limitation is unlikely to affect the conclusions of the present study, because we calculated the proportion of 5-HT2A receptor mRNA-positive neurons by reference to the total number of CTB-positive neurons visualized and not vice versa.

**Functional Implications**

A large body of data in the literature indicates that the activation of 5-HT2A receptors by 5-HT results in neuronal depolarization, reduction of the afterhyperpolarization and increase of excitatory postsynaptic currents and of the discharge rate in pyramidal neurons of the PFC (Araneda and Andrade 1991; Tanaka and North 1993; Aghajanian and Marek 1997, 1999; Amargós-Bosch et al. 2004; Puig et al. 2005; Villalobos et al. 2005). 5-HT can also activate 5-HT2A receptors in GABA interneurons to increase a synaptic GABA input onto pyramidal neurons (Tanaka and North 1993; Zhou and Hablitz 1997). Hallucinogens such as DOI DOB or LSD also modify membrane properties of pyramidal neurons and evoke marked cellular and network changes in PFC in vivo and in vitro through the activation of 5-HT2A receptors (Aghajanian and Marek 1997, 1999; Puig et al. 2003; Villalobos et al. 2005; Gonzalez-Maeso et al. 2007; Lambe and Aghajanian 2007; Celada et al. 2008).

The present histological data, together with these functional observations suggest that activation of 5-HT2A receptors in mPFC pyramidal neurons may increase excitatory inputs onto DA and 5-HT neurons in the VTA and DR, respectively, as well as on GABAergic neurons in both nuclei. This is consistent with the existence of monosynaptic pathways from the PFC to monoaminergic and GABAergic neurons of the VTA and the DR (Aghajanian and Wang 1977; Thierry et al. 1979, 1983; Sesack et al. 1989; Tong et al. 1996, 1998; Hajós et al. 1998; Peyron et al. 1998; Celada et al. 2001; Jankowski and Sesack 2004; Gabbott et al. 2005; Geisler and Zahl 2005). It is also consistent with the presence of a high density of layer V pyramidal neurons expressing 5-HT2A receptors (Santana et al. 2004) some of which project to DR and/or VTA, as shown herein. Hence, the systemic administration of 5-HT2A receptor agonists/antagonists can distally modulate the activity of the ascending serotonergic and dopaminergic pathways originated in the DR and VTA, respectively, via PFC 5-HT2A receptors. In support of this view is also the fact that local application of the preferential 5-HT2A receptor agonist DOI in rat mPFC increased the firing rate of a subpopulation of DR 5-HT neurons, increased burst firing of DA neurons in the VTA and elevated the release of both monoamines in PFC (Martín-Ruiz et al. 2001; Bortolozzi et al. 2005). Notwithstanding the existence of this direct pathway between the PFC and the VTA involving 5-HT2A receptors, indirect pathways controlling the activity of DA neurons cannot be excluded. Thus, the electrical stimulation of the mPFC evokes monosynaptic excitatory responses as well as GABAergic and 5-HT1A receptor-mediated inhibitory responses in DR 5-HT neurons, indicating that most PFC-driven control of 5-HT neurons occurs via mono- and bisynaptic inputs (Hajós et al. 1998; Celada et al. 2001; Jankowski and Sesack 2004). However, this is not the case for DA neurons in the VTA which, in addition, to short monosynaptic excitations consistent with direct inputs from mPFC (Thierry et al. 1979), show complex biphasic responses to mPFC stimulation (Tong et al. 1996). Such biphasic responses might involve alternative pathways such as: 1) PFC to laterodorsal/pedunculopontine tegmental nuclei to VTA, and 2) PFC to nucleus accumbens to ventral pallidum to VTA. These pathways, may also be accountable for an indirect 5-HT2A receptor-mediated control of DA neurons, and both have been shown to modulate DA neuron activity via phasic and tonic inputs, respectively (Floresco et al. 2003).

The present results may have functional implications in terms of the interaction between both neurotransmitter systems (e.g., direct, PFC-mediated vs. direct DR-VTA, interactions mediated by 5-HT2A receptors in the VTA; Nocjar et al. 2002). They may also be relevant for the mechanism of action of atypical antipsychotic drugs. Most agents of this therapeutic group show greater affinity for 5-HT2A receptors than for DA D2 receptors and produce a greater occupancy of cortical 5-HT2 than of subcortical D2 receptors at therapeutic doses (Nyberg et al. 1998; Meltzer 1999). Clozapine, the prototypical representative of this group, does not reach—even at very high doses—the 70% of D2 receptor occupancy, considered to be the threshold of therapeutic efficacy for classical antipsychotics (Nordstrom et al. 1998; Kapur et al. 1999; Nyberg et al. 1999). It is unclear how 5-HT2A receptor blockade may contribute to the therapeutic action of atypical antipsychotics. Taken together, the present and previous data suggest that the blockade of 5-HT2A receptors in PFC may attenuate excitatory inputs from PFC to selected neuronal groups in the VTA, thus modulating dopaminergic activity. According to the known connectivity between mPFC and DA and GABA neurons in the VTA (Carr and Sesack 2000), this should primarily affect dopaminergic neurons projecting back to the mPFC and to limbic areas other than the nucleus accumbens as well as GABA neurons projecting to the latter nucleus. However, the recent demonstration of marked differences in the functional properties of mesocortical vs. mesolimbic DA VTA neurons (Lammel et al. 2008) and the fact that VTA GABA neurons may indirectly affect DA neuronal activity make difficult to predict the extent of the 5-HT2A receptor-mediated excitatory inputs on mesolimbic DA neurons in the VTA. Indeed, previous evidence from this laboratory indicated a widespread effect of the local (in mPFC) and systemic application of DOI in a proportion of VTA DA neurons that exceeded by and large the low abundance of mesocortical neurons (Bortolozzi et al. 2005). Also, the fact that DOI potentiates the amphetamine-induced release of DA in the nucleus accumbens (Kuroki et al. 2003) accords with the view that 5-HT2A receptor activation may also affect mesolimbic DA neurons.

In summary, the combined application of in situ hybridization and retrograde tract tracing enabled to show the presence of 5-HT2A receptor mRNA in pyramidal neurons of the medial and orbital divisions of the rat PFC that project to monoaminergic cell groups of the midbrain. This provides anatomical support for previous observations indicating a 5-HT2A receptor-mediated control of DA and 5-HT neurons and raises the possibility that atypical antipsychotic drugs may partly act by...
reducing excitatory PFC inputs onto subcortical structures, including the midbrain monoaminergic nuclei.

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