Expression of Serotonin$_{1A}$ and Serotonin$_{2A}$ Receptors in Pyramidal and GABAergic Neurons of the Rat Prefrontal Cortex

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Serotonergic 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors are abundantly expressed in prefrontal cortex (PFC) and are targets of atypical antipsychotic drugs. They mediate, respectively, inhibitory and excitatory actions of 5-HT. The transcripts for both receptors are largely (~80%) colocalized in rat and mouse PFC, yet their quantitative distribution in pyramidal and GABAergic interneurons is unknown. We used double *in situ* hybridization histochemistry to estimate the proportion of pyramidal and GABAergic neurons expressing these receptor transcripts in rat PFC. The number of GABAergic interneurons (expressing GAD mRNA) was a 22% of glutamatergic neurons (expressing vGluT1 mRNA, considered as putative pyramidal neurons). 5-HT$_{2A}$ receptor mRNA was present in a large percentage of pyramidal neurons (from 55% in prelimbic cortex to 88% in tenia tecta), except in layer VI, where it was localized only in 30% of those neurons. 5-HT$_{2A}$ receptor mRNA was present in ~25% of GAD-containing cells except in layer VI (10%). Likewise, ~60% of glutamatergic cells contained the 5-HT$_{1A}$ receptor transcript. We also found that ~25% of GAD-expressing cells contained the 5-HT$_{1A}$ receptor mRNA. These data help to clarify the role of 5-HT in prefrontal circuits and shed new light to the cellular elements involved in the action of atypical antipsychotics.

Keywords: 5-HT$_{1A}$ receptors, 5-HT$_{2A}$ receptors, GABA interneurons, medial prefrontal cortex, pyramidal neurons

Introduction

The prefrontal cortex receives a moderate to dense serotonergic innervation from the raphe nuclei (Azmitia and Segal, 1978; Steinbusch, 1981; Blue *et al.*, 1988) and contains several serotonin (5-hydroxytryptamine, 5-HT) receptor subtypes, with a particularly high density of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors (Pazos and Palacios, 1985; Pazos *et al.*, 1985; Pompeiano *et al.*, 1992, 1994). Immunohistochemical studies have revealed the presence of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors in cortical pyramidal neurons (Kia *et al.*, 1996; Willins *et al.*, 1997; Jakab and Goldman-Rakic, 1998, 2000; Cornea-Hébert *et al.*, 1999; De Felipe *et al.*, 2001; Martín-Ruiz *et al.*, 2001) and of 5-HT$_{2A}$ receptors in GABAergic interneurons (Willins *et al.*, 1997; Jakab and Goldman-Rakic, 2000). Recent immunohistochemical studies also suggest the presence of 5-HT$_{1A}$ receptors in nearly all calbindin- and parvalbumin-positive neurons (Aznar *et al.*, 2003).

5-HT$_{1A}$ and 5-HT$_{2A}$ receptors mediate, respectively, the direct hyperpolarizing and depolarizing actions of 5-HT and selective agonists on prefrontal neurons, as assessed *in vitro* (Araneda and Andrade, 1991; Aghajanian and Marek, 1997, 1999; Zhou and Hablitz, 1999) whereas the activation of 5-HT$_{2A}$ receptors in GABAergic interneurons inhibits pyramidal neurons (Ashby *et al.*, 1990; Zhou and Hablitz, 1999). The *in vivo* physiological activation of 5-HT$_{2A}$ and 5-HT$_{1A}$ receptors excites and inhibits, respectively, pyramidal neurons in the medial prefrontal cortex (Puig *et al.*, 2003; Amargós-Bosch *et al.*, 2004), an area projecting to numerous cortical and subcortical areas (Groenewegen and Uylings, 2000). Hence, 5-HT may influence the descending excitatory input into limbic and motor structures, where the prefrontal cortex projects, through the activation of pyramidal 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors.

The exact role of serotonergic transmission in prefrontal cortex is poorly known (Robbins, 2000). However, 5-HT$_{2A}$ receptors in the dorsolateral prefrontal cortex are involved in working memory (Williams *et al.*, 2002) and recent work associates allelic variants of the 5-HT$_{2A}$ receptor with memory capacity in humans (De Quervain *et al.*, 2003). Furthermore, an excessive activation of 5-HT$_{2A}$ receptors by agonists such as LSD or DOI likely underlies the hallucinogenic properties of these compounds. On the other hand, atypical antipsychotics exert their therapeutic action, at least in part, by occupying cortical 5-HT$_{2A}$ receptors and blocking 5-HT$_{2A}$-mediated responses (Kroeze and Roth, 1998; Meltzer, 1999; Nyberg *et al.*, 1999).

5-HT$_{1A}$ receptors have long been implicated in anxiety, depression and suicide (De Vry, 1995; Artigas *et al.*, 1996; Stockmeier *et al.*, 1998). Recent data suggest an association of depression and suicide with the impaired expression of a 5-HT$_{1A}$ suppressor element (NUDR), which may lead to receptor overexpression (Lemonde *et al.*, 2003). Moreover, some atypical antipsychotics are partial agonists (Newman-Tancredi *et al.*, 1996, 2001) or behave as indirect 5-HT$_{1A}$ agonists (Ichikawa *et al.*, 2001). Finally, 5-HT$_{1A}$ receptor antagonists may be useful in the treatment of age-related cognitive impairment because of their ability to reverse drug-induced cognitive deficits (Harder and Ridley, 2000; Mello e Souza *et al.*, 2001; Misane and Ögren, 2003).

Atypical antipsychotics display a preferential occupancy of 5-HT$_{2A}$ versus dopamine D2 receptors at therapeutic doses (Nordstrom *et al.*, 1995; Nyberg *et al.*, 1999). This suggests that neurons expressing 5-HT$_{2A}$ (and possibly 5-HT$_{1A}$) receptors are the primary cellular targets of these drugs, irrespectively of an additional action on D2 receptors. We therefore examined the expression of both receptor transcripts in pyramidal and GABAergic cells of the rat prefrontal cortex using double *in situ* hybridization histochemistry. The study also improves our knowledge on cortical serotonergic transmission by identifying the cell types and anatomical localization of the neurons expressing the two main 5-HT receptors present in prefrontal cortex.

Materials and Methods

Tissue Preparation

Male albino Wistar rats weighing 250–320 g were used (Iffa Credo, Lyon, France). Animals were kept in a controlled environment (12 h light-dark cycle and 22 ± 2°C room temperature) with food and water provided *ad libitum*. Animal care followed the European Union regulations (O.J. of E.C. L358/1 18/12/1986) and was approved by the local Institutional Animal Care and Use Committee. The rats were killed by decapitation and the brains rapidly removed, frozen on dry ice and stored at –20°C. Tissue sections, 14 µm thick, were cut using a microtome-cryostat (HM500 OM; Microm, Walldorf, Germany), thaw-mounted onto APTS (3-aminopropyltriethoxysilane; Sigma,St Louis, MO) coated slides and kept at –20°C until use.

Hybridization Probes

The oligodeoxyribonucleotide probes used were as follows. For 5-HT_{1A} receptor mRNA four oligonucleotides were simultaneously used, complementary to bases 82–122, 123–171, 885–933 and 1341–1389 (Albert *et al.*, 1990). For the mRNA coding for 5-HT_{2A} receptor the three oligonucleotides used were complementary to bases 669–716, 1882–1520 and 1913–1960 (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.

Glutamatergic cells were identified by the presence of the vesicular glutamate transporter vGluT1 mRNA with two oligonucleotides complementary to bases 127–172 and 1756–1800 (GenBank accession No. U07609). GABAergic cells were identified by the presence of the enzyme synthesizing GABA, glutamic acid decarboxylase (GAD), that in adult brain exists as two major isoforms, GAD65 and GAD67. Two oligonucleotides for each isoform mRNA were made: bp 159–213 and 514–558 (GenBank accession No. NM_012563) and bp 191–235 and 1600–1653 (GenBank accession No. NM_017007). They were synthesized and HPLC purified by Isogen Bioscience BV (Maarsden, The Netherlands).

Each 5-HT_{1A} and 5-HT_{2A} receptor oligonucleotide was individually labeled (2 pmol) at its 3′-end with [^{33}P]-dATP (>2500 Ci/mmol; DuPont-NEN, Boston, MA) using terminal deoxynucleotidyltransferase (Roche Diagnostics GmbH, Mannheim, Germany), purified by centrifugation using QIAquick Nucleotide Removal Kit (Qiagen GmbH, Hilden, Germany). GAD and vGluT oligonucleotides (100 pmol) were non-radioactively labeled with the same enzyme and Dig-11-dUTP (Boehringer Mannheim) according to a previously described procedure (Schmitz *et al.*, 1991).

In Situ Hybridization Histochemistry Procedure

The protocols for single- and double-label *in situ* hybridization were based on previously described procedures (Tomiyama *et al.*, 1997; Landry *et al.*, 2000) and have been already published (Serrats *et al.*, 2003a). Frozen tissue sections were first brought to room temperature, fixed for 20 min at 4°C in 4% paraformaldehyde in phosphate-buffered saline (1× PBS: 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 136 mM NaCl, 2.6 mM KCl), washed for 5 min in 3× PBS at room temperature, twice for 5 min each in 1× PBS and incubated for 2 min at 21°C in a solution of predigested pronase (Calbiochem, San Diego, CA) at a final concentration of 24 U/ml in 50 mM Tris–HCl pH 7.5, 5 mM EDTA. The enzymatic activity was stopped by immersion for 30 s in 2 mg/ml glycine in 1× PBS. Tissues were finally rinsed in 1× PBS and dehydrated through a graded series of ethanol. For hybridization, the radioactively-labeled and the non-radioactively 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 tRNA and 500 µg/ml salmon sperm DNA. The final concentrations of radioactive and Dig-labeled probes in the hybridization buffer were in the same range (~1.5 nM). Tissue sections were covered with hybridization solution containing the labeled probe(s), overlaid with Nescofilm coverslips (Bando Chemical Ind., Kobe, Japan) and incubated overnight at 42°C in humid boxes. Sections were then washed four times (15 min each) in 1× SSC at 60°C and once in 1× SSC at room temperature for 30 min.

Development of Radioactive and Non-radioactive Hybridization Signal

Hybridized sections were treated as described by Landry *et al.* (2000). Briefly, after washing, the slides were immersed for 30 min in a buffer containing 0.1 M Tris–HCl pH 7.5, 1 M NaCl, 2 mM $MgCl_2$ and 0.5% bovine serum albumin (Sigma) and incubated overnight at 4°C in the same solution with alkaline-phosphate-conjugated anti-digoxigenin-F(ab) fragments (1:5000; Boehringer Mannheim). Afterwards, they were washed three times (10 min each) in the same buffer (without antibody) and twice in an alkaline buffer containing 0.1 M Tris–HCl pH 9.5, 0.1 M NaCl and 5 mM $MgCl_2$. Alkaline phosphatase activity was developed by incubating the sections with 3.3 mg nitroblue tetrazolium and 1.65 mg bromochloroindolyl phosphate (Gibco BRL, Gaithersburg, MD) diluted in 10 ml of alkaline buffer. The enzymatic reaction was blocked by extensive rinsing in the alkaline buffer containing 1 mM EDTA. The sections were then briefly dipped in 70 and 100% ethanol, air-dried and dipped into Ilford K5 nuclear emulsion (Ilford, Mobberly, Cheshire, UK) diluted 1:1 with distilled water. They were exposed in the dark at 4°C for 6 weeks and finally developed in Kodak D19 (Kodak, Rochester, NY) for 5 min and fixed in Ilford Hypam fixer (Ilford).

Specificity of the Probes

The specificity of the hybridization signals has been previously established and published (Pompeiano *et al.*, 1992, 1994; Serrats *et al.*, 2003a). These controls included the following procedures. (i) The thermal stability of the hybrids obtained was checked for every probe. (ii) 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. (iii) Since we synthesized more than one probe for each mRNA analyzed, the hybridization signal obtained with each oligonucleotide for the same mRNA was identical at both regional and cellular levels when used independently. (iv) To assure the specificity of the non-radioactive hybridization signal, we compared the results obtained with the same probe radioactively labeled.

Analysis of the Results

Tissue sections were examined in bright- and dark-field in a Wild 420 macroscope (Leica, Heerbrugg, Germany) and in a Nikon Eclipse E1000 microscope (Nikon, Tokyo, Japan) equipped with bright- and dark-field condensers for transmitted light and with epi-illumination. Micrography was performed using a digital camera (DXM1200 3.0; Nikon) and analySIS Software (Soft Imaging System GmbH, Germany). Bright-field images were captured with transmitted light. Dark-field images were captured with Darklite illuminator (Micro Video Instruments, Avon, MA). The figures were prepared for publication using Adobe Photoshop software (Adobe Software, Mountain View, CA).

Cell counting was performed manually at the microscope with the help of analySIS Software. Dig-labeled cells were considered positive when a dark precipitate was clearly distinguished from background. Only cellular profiles showing great abundance of the corresponding 5-HT receptor mRNA and the cell type identifier (either GAD or vGluT1 mRNAs) were considered to be double-labeled. Cells with a dense Dig labeling and occasional silver grains (or vice versa) were not considered to co-express both transcripts. Analysis of variance (ANOVA) and *post hoc* Tukey's test were performed using GraphPad Prism software (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

The prefrontal cortex contains a large number of cells expressing the 5-HT_{1A} and 5-HT_{2A} receptor transcripts in various cortical fields, such as the secondary motor area (MOs), dorsal anterior cingulate area (ACAd), prelimbic (PrL) and infralimbic areas (ILA), as well as in the tenia tecta (TT) and

piriform cortex (PIR) (Fig. 1; see also Fig. 2 for the localization of these areas). A particularly high expression was noted in the latter two areas as well as in intermediate layers of the prelimbic and cingulate cortices. There was a marked overlap in the distribution of both receptor transcripts in most areas, with the exception of a lower expression of 5-HT_{2A} receptor

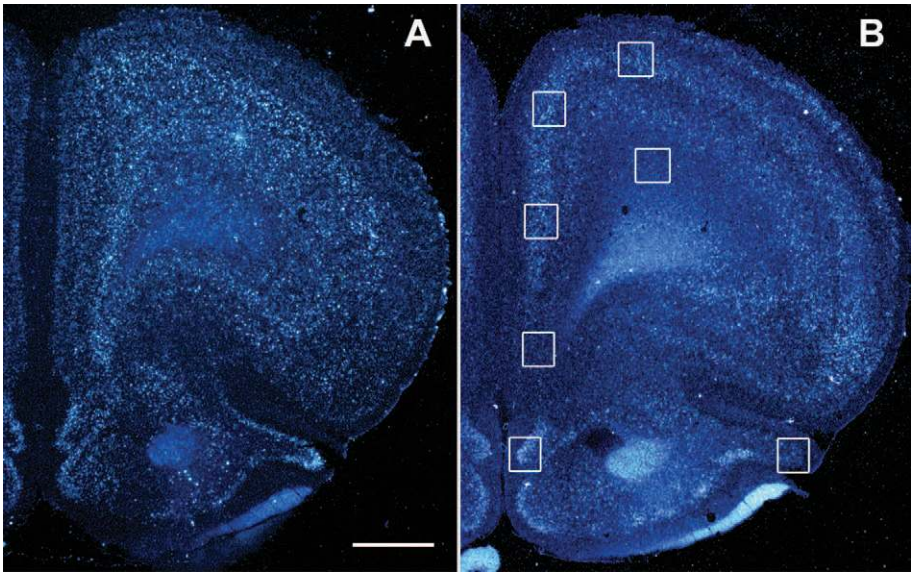

Figure 1. Dark-field photomicrographs showing the localization of (*A*) 5-HT_{1A} and (*B*) 5-HT_{2A} receptor mRNAs in the rat prefrontal cortex using *in situ* hybridization histochemistry. The sections correspond approximately to AP +3.0 mm (Paxinos and Watson, 1998). Both receptor transcripts were labeled with ^{33}P-labeled oligonucleotides. Large number of cells in superficial and middle cortical layers of the secondary motor area (MO), dorsal anterior cingulate (ACAd) and prelimbic (PrL) areas, as well as in tenia tecta (TT) and piriform cortex (PIR) expressed either receptor. Previous results revealed a very marked co-localization of both receptor mRNAs in most prefrontal areas (Amargós-Bosch *et al.*, 2004). The ventral part of the infralimbic (ILA) area and layer VI contain a lower number of cells expressing 5-HT_{2A} receptors compared with 5-HT_{1A} receptors. Open squares mark the approximate areas where cell counts were performed (see location of the corresponding areas in Fig. 2). Scale bar = 1 mm.

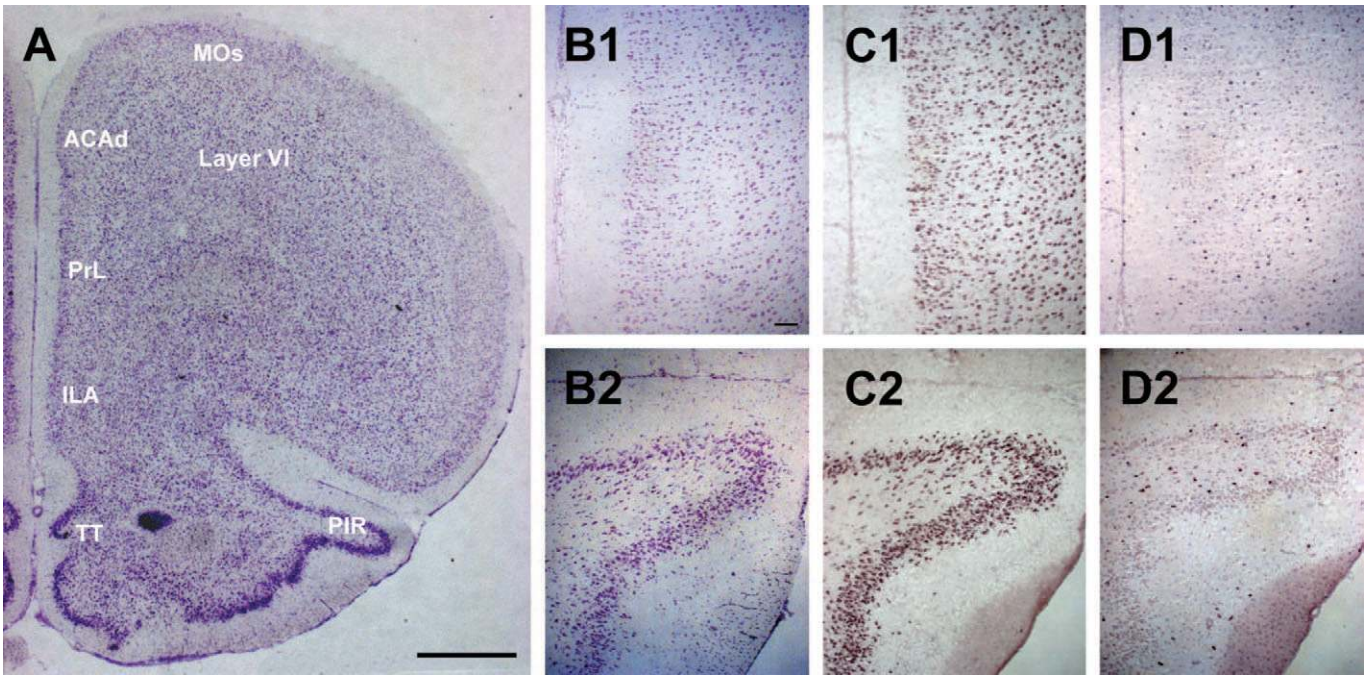

Figure 2. (*A*) Nissl-stained section of rat prefrontal cortex showing the various areas where the expression of 5-HT_{1A} and 5-HT_{2A} receptors has been studied. *B1* and *B2* show, at a higher magnification, Nissl-stained sections corresponding to the prelimbic area (see midline on the left side) and piriform cortex. *C1* and *C2* correspond to the same areas and show the presence of vGluT1-positive cells (Dig-labeled oligonucleotides). *D1* and *D2* correspond to the same areas and show the presence of GAD-positive cells (Dig-labeled oligonucleotides). Note the large abundance of pyramidal neurons, labeled with vGluT1 mRNA in intermediate and deep layers of the prelimbic area, contrasting with the total absence in layer I, near the midline. GAD mRNA-positive cells were scattered throughout the prefrontal cortex, as shown here in the prelimbic area. The observed ratio between GAD- and vGluT1-positive cells was 1:4.6. Scale bars: 1 mm (*A*); 100 μm (*B1–D2*).

mRNA in layer VI compared with that of 5-HT_{1A} receptors. In coronal sections more caudal than those shown in Figure 1, cells in layer VIb and claustrum also expressed the 5-HT_{2A} receptor mRNA (not shown). Likewise, the ventral part of the infralimbic area contained many more cells expressing 5-HT_{1A} than 5-HT_{2A} receptors.

We examined the labeling of cells in the prefrontal cortex containing the vGluT1 and GAD mRNAs (Fig. 2). High densities of pyramidal cells, as labeled by vGluT1 mRNA, were found at various cortical levels. Dense clusters of these cells were observed in the tenia tecta (not shown) and piriform cortex (Fig. 2*C2*), which also showed a greater density of label compared with that in other cortical areas, such as the prelimbic area (Fig. 2*C1*) or the anterior cingulate. In contrast, no vGluT1-expressing cells were seen in layer I (Fig. 2*C1*). GAD-expressing cells were scattered throughout the prefrontal cortex, including layer I, near the midline (Fig. 2*D1*). We estimated the proportion of vGluT1 and GAD-positive cells by reference to Nissl-stained adjacent sections. The percentage of vGluT1-labeled cells was 75 ± 5% of all Nissl-stained cells whereas the corresponding value for GAD-positive cells was 16 ± 1% (data from three rats; each individual value is the average of three adjacent sections except for the Nissl-stained section, which were duplicate sections). The calculated ratio between vGluT1- and GAD-expressing cells was 4.6.

There was a remarkable co-expression of the 5-HT_{1A} receptor mRNA with vGluT1 mRNA in all areas examined (Fig. 3). As observed in panels *A* and *B*, many vGluT1-positive cells in the dorsal anterior cingulate and in the prelimbic areas, respectively, expressed the 5-HT_{1A} receptor transcript. Figure 3*C1–C2* show enlargements of a few double-labeled cells in the dorsal anterior cingulate. We also found a much more moderate proportion of GAD mRNA-containing cells which also expressed the 5-HT_{1A} receptor mRNA. These cells were found scattered throughout the various areas of the prefrontal cortex and did not follow any particular pattern of distribution. Figure 3 shows the presence of such GABAergic cells in the prelimbic area (Fig. 3*D*) and tenia tecta (Fig. 3*E*). At a higher magnification, GAD-positive cells in the prelimbic area (Fig. 3*F1*) and orbitofrontal cortex (Fig. 3*F2*) expressing 5-HT_{1A} receptors are also shown. Figure 4 shows additional GAD-positive neurons in the prelimbic area which also express the 5-HT_{1A} receptor mRNA.

As observed for 5-HT_{1A} receptors, there was also a large expression of the 5-HT_{2A} receptor transcript in vGluT1 mRNA-positive cells in most prefrontal areas (Fig. 5), such as prelimbic area (Fig. 5*A*) or tenia tecta (Fig. 5*B*). Figure 5*C1* and *C2* show, at a higher magnification, vGluT1-positive cells expressing the 5-HT_{2A} receptor transcript, which was also present in GAD-positive cells from the prelimbic area (Fig.

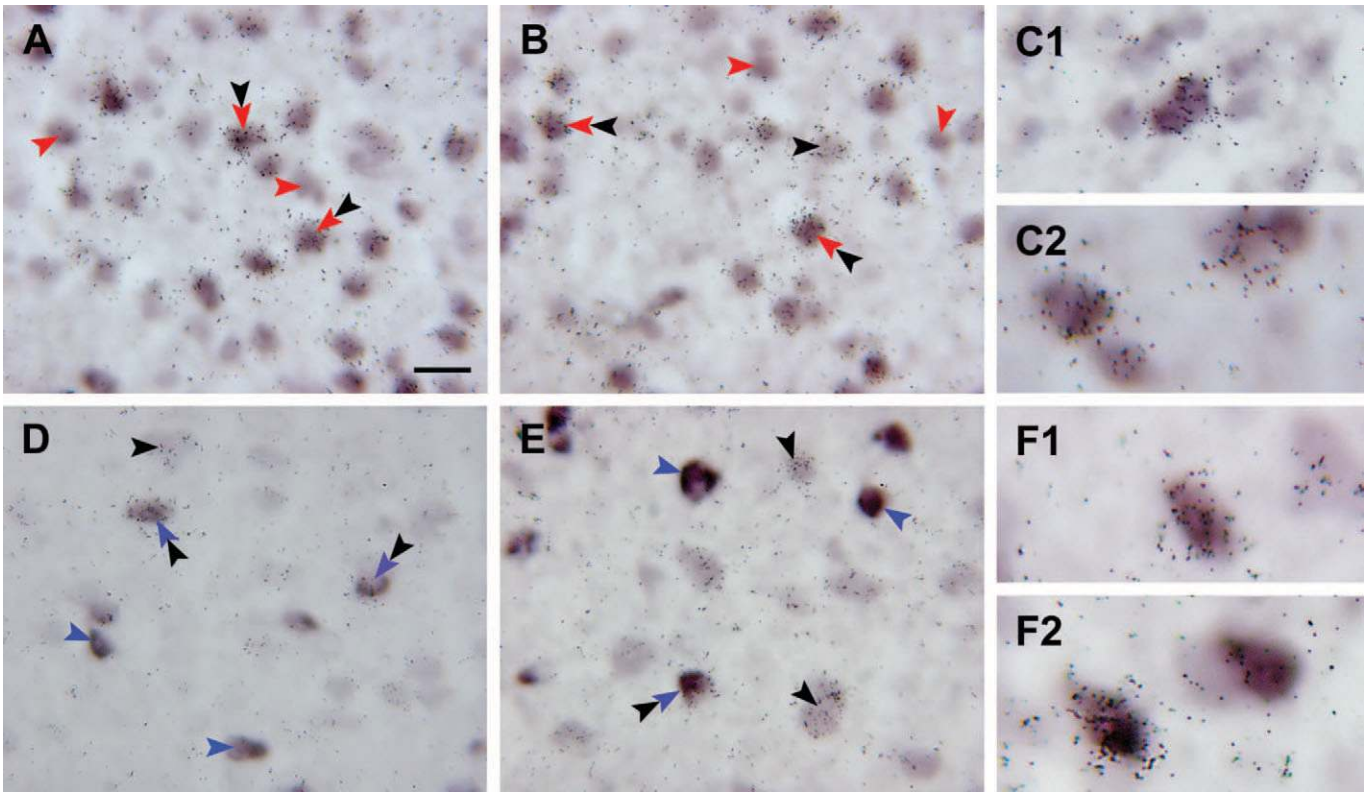

Figure 3. Upper row (*A–C*): low and high magnification photomicrographs showing the presence of 5-HT_{1A} receptor mRNA (^{33}P-labeled oligonucleotides) in pyramidal cells, identified by the presence of vGluT1 mRNA (Dig-labeled olligonucleotides). *A* and *B* show respectively, the presence of abundant cells expressing both transcripts in deep layers of the cingulate area and prelimbic area, respectively. Red arrowheads mark cells positive for vGluT1 mRNA, black arrowheads mark cells positive for 5-HT_{1A} receptor mRNA. Double labeled cells are marked by both arrowheads. For the sake of simplicity, only a few cells of each type are marked. A majority of glutamatergic cells expressed the 5-HT_{1A} receptor mRNA, as denoted by the double labeling. Note also the presence of non-glutamatergic cells expressing the 5-HT_{1A} receptor mRNA (black arrowheads). *C1* and *C2* show individual cells expressing both transcripts in the dorsal anterior cingulate. Lower row (*D–F*): the 5-HT_{1A} receptor mRNA was also found in GABAergic cells throughout the prefrontal cortex. *D* and *E* show a few double labeled cells in the prelimbic area and piriform cortex, respectively. Blue arrowheads mark cells positive for GAD mRNA and black arrowheads mark cells positive for 5-HT_{1A} receptor mRNA. Some fouble labeled cells are marked by both arrowheads. *F* shows, at a higher magnification, individual GABAergic cells expressing the 5-HT_{1A} receptor in the prelimbic area (*F1*) and orbitofrontal cortex (*F2*). Scale bar = 20 μm (*A*, *B*, *D*, *E*); 10 μm (*C*, *F*).

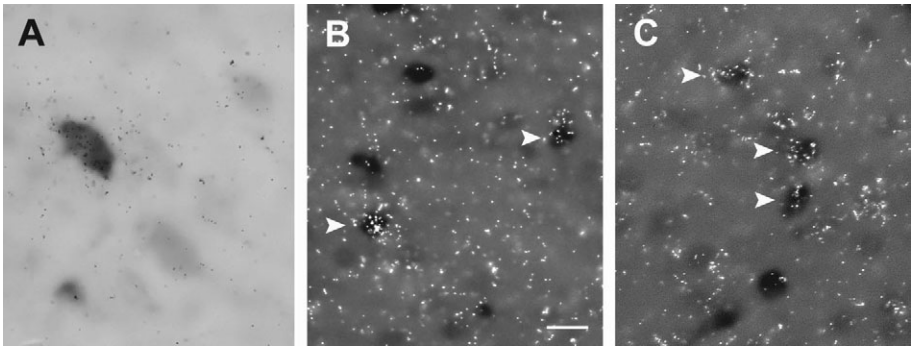

Figure 4. Bright (*A*) and dark-field (*B*, *C*) photomicrographs showing GABAergic neurons expressing the 5-HT_{1A} receptor mRNA in the prelimbic area of prefrontal cortex. White arrowheads in *B* and *C* mark GAD-positive cells (Dig-labeled nucleotides, seen as dark large spots) expressing the 5-HT_{1A} receptor mRNA (^{33}P-labeled oligonucleotides, seen as white dots over the cells). Scale bar = 13 μm (*A*); 20 μm (*B*, *C*).

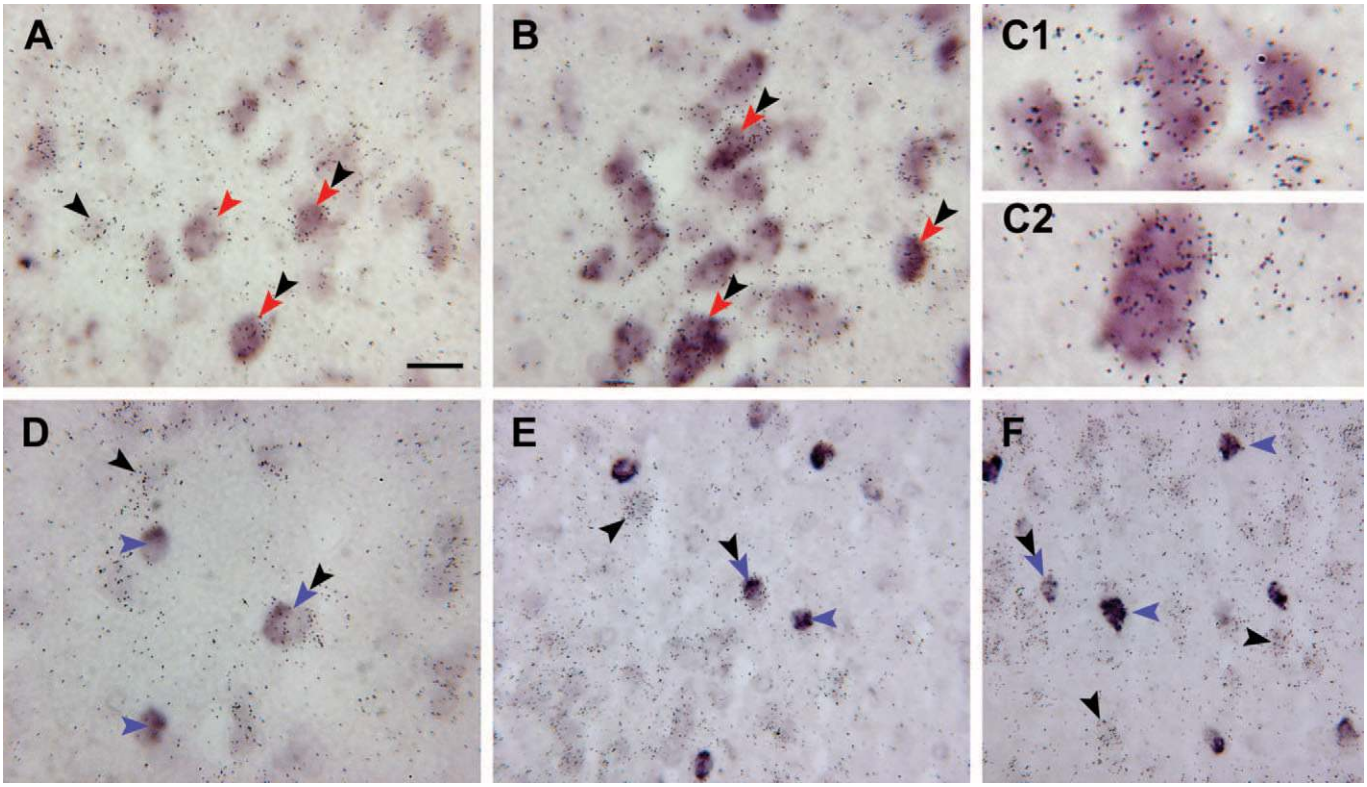

Figure 5. Upper row (*A–C*): low and high level magnification photomicrographs showing the presence of 5-HT_{2A} receptor mRNA (^{33}P-labeled oligonucleotides) in pyramidal cells, identified by the presence of vGluT1 mRNA (Dig-labeled olligonucleotides). *A* and *B* show, respectively, the presence of abundant cells expressing both transcripts in the prelimbic area and tenia tecta. Red arrowheads mark some cells positive for vGluT1 mRNA, black arrowheads mark cells positive for 5-HT_{2A} receptor mRNA. Double labeled cells are marked by both arrowheads. A large number of glutamatergic cells expressed the 5-HT_{2A} receptor mRNA, as denoted by the double labeling. Note also the presence of non-glutamatergic cells expressing the 5-HT_{2A} receptor mRNA (black arrowhead). *C1* and *C2* show individual cells expressing both transcripts in the piriform cortex (*C1*) and prelimbic area (*C2*). Lower row (*D–F*): as opposed to pyramidal neurons, only a small percentage of GAD-containing cells (~20% on average) expressed the 5-HT_{2A} receptor transcript. Blue arrowheads mark cells positive for GAD mRNA and black arrowheads mark cells positive for 5-HT_{2A} receptor mRNA. Some double labeled cells are marked by both arrowheads. *D* shows a field in the prelimbic area containing a GABAergic neuron expressing the 5-HT_{2A} receptor mRNA. *E* and *F* show two different fields, in the piriform cortex and prelimbic area, respectively, showing abundant non-GABAergic neurons expressing the 5-HT_{2A} receptors. Occasional GABAergic cells expressing the 5-HT_{2A} receptor were observed (double arrowhead). Scale bar = 20 μm (*A*, *B*, *D*); 50 μm (*E*, *F*); 10 μm (*C*).

5*D*,*F*) or piriform cortex (Fig. 5*E*). However, as evidenced in Figure 5*D–F*, the majority of 5-HT_{2A}-expressing cells are non-GABAergic.

Table 1 shows the percentages of glutamatergic and GABAergic cells expressing 5-HT_{1A} and 5-HT_{2A} receptor transcripts in the various areas of prefrontal cortex. The approximate location of the fields where cell counts were performed are shown in Figure 1. Forty to sixty per cent of vGluT1-positive cells expressed 5-HT_{1A} receptors in the various prefrontal areas examined, with a maximum in tenia tecta (63%). The corresponding values for the 5-HT_{2A} receptor mRNA were similar on average, although the maximal value reached 81% in tenia tecta. This proportion dropped dramatically in the more ventral part of the infralimbic area (compare Fig. 1*A*,*B*), where only 12% of glutamatergic cells expressed the 5-HT_{2A} receptor mRNA versus 40% expressing the 5-HT_{1A} receptor mRNA. Like-

Table 1
Expression of 5-HT_{1A} and 5-HT_{2A} receptor transcripts in pyramidal (vGluT1 mRNA-positive) and GABAergic (GAD mRNA-positive) cells in rat prefrontal cortex

	vGluT1 mRNA		GAD mRNA	
	5-HT_{1A} mRNA	5-HT_{2A} mRNA	5-HT_{1A} mRNA	5-HT_{2A} mRNA
MOs	54 ± 4	60 ± 2	28 ± 6	28 ± 10
ACAd	54 ± 3	66 ± 5	22 ± 4	32 ± 2
PrL	61 ± 2	51 ± 3	20 ± 1	34 ± 1
ILA[a]	40 ± 4*	12 ± 1**	22 ± 4	22 ± 3
TT	63 ± 6	81 ± 3***	24 ± 1	24 ± 2
PIR	60 ± 2	50 ± 3	21 ± 6	24 ± 2
Layer VIa	54 ± 3	26 ± 3^{+}	23 ± 4	11 ± 3^{++}

Data are means of three rats (each individual measure is the mean of four consecutive sections) and represent the percentage of the counted cells expressing the mRNAs of each 5-HT receptor in pyramidal (vGluT1 mRNA-positive) and GABAergic (GAD mRNA-positive) cellular profiles. The average numbers of vGluT1 mRNA-expressing cells per field were: 30 ± 1 (MO), 32 ± 1 (ACAd), 44 ± 2 (PrL), 44 ± 2 (ILA), 56 ± 1 (TT), 85 ± 4 (PIR), 44 ± 2 (Layer VI). The respective figures for GAD mRNA-expressing cells were: 12 ± 1, 15 ± 1, 16 ± 1, 18 ± 1, 12 ± 1, 10 ± 1 and 13 ± 1 cells per field (due to the lower abundance of GABAergic cells, these were counted at a lower magnification).

Cortical areas designated according to Paxinos and Watson (1998) and Swanson (1998). MOs, secondary motor area; ACAd, dorsal anterior cingulate area; PrL, prelimbic area; ILA, infralimbic area; PIR, piriform cortex; TT, tenia tecta. Layer VIa denotes deep areas of the sensorimotor cortex at prefrontal level (Swanson, 1998). The approximate location of the counted fields is shown by small rectangles in Figure 1*B*.

[a]The data of the infralimbic area (ILA) correspond to its more ventral part, which shows a remarkable low level of 5-HT_{2A} receptor, whereas cell counts from its dorsal part are more similar to those of PrL (see Fig. 1).

$^{*}P < 0.05$ versus PrL, TT and PIR; $^{**}P < 0.05$ versus the rest of areas, except layer VIa ($P = 0.9$); $^{***}P < 0.05$ versus the rest of areas; $^{+}P < 0.05$ versus the rest of areas except ILA; $^{++}P < 0.05$ versus ACAd and PrL (Tukey test post-ANOVA).

wise, layer VI (particularly VIa) showed also a lower proportion of glutamatergic cells expressing the 5-HT_{2A} receptor transcript. One-way ANOVA showed a significant effect of the region on the density of glutamatergic cells expressing one or other receptor ($P < 0.001$), with significant differences among regions (Table 1).

Twenty to twenty-five percent of GAD-positive cells expressed the 5-HT_{1A} receptor mRNA (Table 1 and Fig. 3). There was no apparent enrichment of these double-labeled cells in any of the areas examined. 5-HT_{2A} receptors were present in a similar percentage of GABAergic cells in most areas, except in layer VI, where there was a significantly lower proportion compared with some other areas (11 versus 22–34% in the rest of regions; $P < 0.03$), as observed for the 5-HT_{2A} receptors in vGluT1-positive cells.

Discussion

The present study shows that a high proportion (>50% on average) of glutamatergic cells in the rat prefrontal cortex express 5-HT_{1A} and/or 5-HT_{2A} receptors. A smaller proportion (20–25% on average) of GABAergic cells also express 5-HT_{1A} and/or 5-HT_{2A} receptor mRNAs. The percentage of GAD-expressing cells was estimated to be a 16%, a figure very similar to the percentage of GABAergic cells in various cortical areas (15%; Beaulieu, 1993) whereas the percentage of vGluT1-positive cells was 75% of all cellular profiles in Nissl-stained sections. Taking into account the ratio between GABAergic and pyramidal neurons and the proportion of cells of each type that contain 5-HT_{1A} or 5-HT_{2A} receptor mRNAs, it follows that the actual proportion of each receptor transcript in GABAergic interneurons (compared with that in pyramidal neurons) is low, close to 10%. To our knowledge, this is the first quantitative study of the expression of these receptors at cellular level in mammalian cortex. A novel finding is the occurrence of 5-HT_{1A} receptor mRNA in cortical GABAergic interneurons in a proportion similar to that of 5-HT_{2A} receptors. Collectively, these observations provide an anatomical background to interpret the complex functional effects of 5-HT on prefrontal pyramidal cells.

Methodological Considerations

The recent cloning and further characterization of three structurally related glutamate vesicular transporters, vGluT1, vGluT2 and vGluT3, in rat brain (Takamori *et al.*, 2000, 2001; Gras *et al.*, 2002) has originated a new approach to histologically identify glutamatergic phenotype in neurons (Fremeau *et al.*, 2001; Takamori *et al.*, 2001; Gras *et al.*, 2002; Oliveira *et al.*, 2003). The distributions of vGluT1 and vGluT2 mRNAs in rat brain show a complementary pattern that agrees with the localization of glutamatergic neurons as identified by previous techniques (Ziegler *et al.*, 2002). Most of the cells in rat cerebral cortex express very high levels of vGluT1 mRNA (Gras *et al.*, 2002; Ziegler *et al.*, 2002), whereas the other two transporters are found at much lower densities. VGluT1 immunoreactivity is evenly distributed in neuropil of the cerebral neocortex, being more intense in layers I-III and V (Fujiyama *et al.*, 2001). Thus, the presence of vGluT1 can be used for the identification of most cortical glutamatergic pyramidal neurons. GABAergic neurons were identified by the presence of GAD67 or GAD65 mRNA. Immunohistochemical and *in situ* hybridization histochemistry indicate that the majority of GABA-containing neurons in the brain co-express the genes encoding the two GAD isoforms (Erlander *et al.*, 1991; Esclapez *et al.*, 1993, 1994; Feldblum *et al.*, 1993).

Expression of 5-HT_{1A} and 5-HT_{2A} Receptors in Prefrontal Cortex

The present results add to previous data showing a high degree of co-expression (80%) of 5-HT_{1A} and 5-HT_{2A} receptor mRNAs in most prefrontal areas (Amargós-Bosch *et al.*, 2004). According to the present data, a very large percentage of both mRNAs are localized in glutamatergic neurons.

The distribution of cells expressing the receptor transcripts agrees well with the regional patterns of distribution of the respective mRNA and protein, as assessed autoradiographically (Pazos *et al.*, 1985; Pompeiano *et al.*, 1992, 1994). A high density of both receptor transcripts was observed in most cortical layers except for the 5-HT_{2A} receptor mRNA in layer VI and the ventral part of the infralimbic area, expressed by a considerable lower cell number. The piriform cortex and the tenia tecta displayed a very large number of cells with a high expression of both receptor transcripts, where they also co-localize extensively (Amargós-Bosch *et al.*, 2004).

The mRNAs of both receptors are effectively translated into functional proteins, as shown by previous immunohistochemical and autoradiographic studies (see introductory section). Likewise, electrophysiological reports showed that exogenously applied 5-HT and selective agonists modulate the excitability and firing rate of cortical pyramidal neurons via these receptors (see below). Furthermore, the electrical stimulation

of the raphe nuclei at physiological rates inhibits (via 5-HT_{1A} receptors) and activates (via 5-HT_{2A} receptors) pyramidal neurons in the rat prefrontal cortex (Puig *et al.*, 2003; Amargós-Bosch *et al.*, 2004). Given the connectivity of the prefrontal cortex (Groenewegen and Uylings, 2000), this indicates that 5-HT and selective ligands of these receptors may modulate the cortical excitatory output to subcortical motor and limbic structures. Of particular interest are the many neurons of the prelimbic and infralimbic areas expressing 5-HT_{2A} and/or 5-HT_{1A} receptors, since these areas project to midbrain serotonergic and dopaminergic cells and influence their activity (Thierry *et al.*, 1983; Sesack *et al.*, 1989; Hajós *et al.*, 1998; Peyron *et al.*, 1998; Carr and Sesack, 2000; Celada *et al.*, 2001). Therefore, the present results may account for the observed effects of 5-HT_{1A} and 5-HT_{2A} agonists/antagonists on monoaminergic cell firing and transmitter release (Lejeune and Millan, 1998; Celada *et al.*, 2001; Ichikawa *et al.*, 2001; Martín-Ruiz *et al.*, 2001). In support of this view is the fact that many pyramidal neurons excited through 5-HT_{2A} receptors simultaneously project to the dorsal raphe and ventral tegmental area, as assessed by antidromic activation from both areas (Puig *et al.*, 2003).

Cortical microcircuits encompass pyramidal neurons and different types of GABAergic interneurons. The latter neurons are located at various levels of the pyramidal neurons and exert a local inhibitory control through GABAergic inputs onto apical dendrites, basal dendrites and cell bodies (Somogyi *et al.*, 1998). 5-HT can modulate the activity of these microcircuits in various ways. Direct inputs onto pyramidal cells involve 5-HT_{1A} and 5-HT_{2A} receptors, expressed by these neurons, whereas indirect inputs involve GABAergic neurons expressing 5-HT_{2A} and 5-HT_3 receptors (Araneda and Andrade, 1991; Tanaka and North, 1993; Aghajanian and Marek, 1997; Morales and Bloom, 1997; Willins *et al.*, 1997; Zhou and Hablitz, 1999; Jakab and Goldman-Rakic, 2000; Férézou *et al.*, 2002; Puig *et al.*, 2003; Amargós-Bosch *et al.*, 2004).

Our data indicate that ~25% of the GABAergic interneurons express the 5-HT_{2A} receptor mRNA. These cells are possibly large perisomatic interneurons (e.g. basket cells) involved in the feed-forward control of pyramidal activity, as revealed by immunohistochemical studies (Somogyi *et al.*, 1998; Jakab and Goldman-Rakic, 1998, 2000). The lower absolute number of 5-HT_{2A} receptors in interneurons compared to that in pyramidal cells (nearly 10%) would suggest that only a minority of pyramidal neurons are under this indirect control. However, marked inhibitory effects of 5-HT_{2A} receptors on pyramidal cell activity have been reported *in vitro* and *in vivo* after local or systemic application of 5-HT or 5-HT_{2A} receptor agonists (Ashby *et al.*, 1990; Zhou and Hablitz, 1999; Puig *et al.*, 2003). A possibility to circumvent this apparent contradiction may be the presence of 5-HT_{2A} receptors in networks of fast-spiking interneurons electrically connected through connexin hemichannels (Galarreta and Hestrin, 2001). Yet, this possibility has not been tested so far.

To our knowledge, the presence of 5-HT_{1A} receptor mRNA in cortical GABAergic neurons had not been previously reported. This adds an additional complexity to the ways in which 5-HT may control pyramidal activity. Recently, 5-HT_{1A} immunoreactivity was detected in most cortical parvalbumin- and calbindin-containing neurons (85–99%) and pyramidal cells (85%; Aznar *et al.*, 2003). Methodological aspects may contribute to the difference with the present results. Indeed, the specificity of some of the antibodies used in that study was unclear (e.g. 1:10 antibody against 'pyramidal/principal cells') or was not adequately tested. Indeed, serious concerns have been raised about the specificity of immunohistochemical procedures (Saper and Sawchencko, 2003). This contrasts with the rigorous controls for mRNA probes used in the present study (see Materials and Methods).

To our knowledge, a 5-HT_{1A}-mediated disinhibitory effect of 5-HT or selective agonists in cortex has not been reported previously in prefrontal cortex. The systemic administration of 8-OH-DPAT exhibited a biphasic effect on the firing rate of prefrontal cells (increase followed by decrease at high doses; Borsini *et al.*, 1995) which may be suggestive of an action on different 5-HT_{1A} receptor populations. However, the cellular elements involved remain unidentified. In contrast, there is evidence of a 5-HT_{1A} receptor-mediated modulation of excitatory postsynaptic currents (EPSCs) recorded in putative GABAergic neurons in entorhinal cortex (Schmitz *et al.*, 1998). Interestingly, raphe GABAergic cells also express 5-HT_{1A} receptor mRNA (Serrats *et al.*, 2003a,b) and 5-HT increases EPSCs in dorsal raphe 5-HT neurons *in vitro* by a TTX- and 5-HT_{1A} receptor-mediated disinhibitory mechanism (Liu *et al.*, 2000), which might be also operant in cortex.

Functional Consequences

The present data may help to clarify the anatomical substrate for the complex actions of 5-HT and ligands of 5-HT_{1A} and 5-HT_{2A} receptors in prefrontal cortex, including the atypical antipsychotic drugs. The presence of these receptors in nearly half of glutamatergic pyramidal neurons and their high degree of colocalization in the areas examined indicates that 5-HT may finely tune the activity of prefrontal neurons. Pyramidal 5-HT_{2A} receptors in the apical dendrites of these neurons can modulate excitatory glutamate inputs (Aghajanian and Marek, 1997, 1999; Martín-Ruiz *et al.*, 2001; Puig *et al.*, 2003) whereas 5-HT_{1A} receptors (perhaps located in the axon hillock; De Felipe *et al.*, 2001; Czyrak *et al.*, 2003; David E. Lewis, unpublished observations) suppress the generation of action impulses along pyramidal axons, thus reducing glutamate release in subcortical areas.

Atypical antipsychotic drugs are preferential 5-HT_{2A} receptor antagonists (Meltzer, 1999). Some also behave as direct (aripiprazole, ziprasidone) or indirect partial 5-HT_{1A} agonists (Newman-Tancredi *et al.*, 1996, 2001; Ichikawa *et al.*, 2001). The occupancy of these pyramidal receptors by atypical antipsychotics should conceivably result in a diminished excitatory input onto mesolimbic dopaminergic neurons, innervated by prefrontal afferents (Thierry *et al.*, 1983; Carr and Sesack, 2000). This effect would attenuate the presumed hyperactivity of mesolimbic dopamine neurons in schizophrenic patients (Weinberger *et al.*, 1994; Laruelle *et al.*, 1996). This attenuation would not require the high (>80%) occupancy of postsynaptic dopamine receptors produced by conventional antipsychotics, responsible for the secondary motor effects. Further anatomical studies are required to determine the areas and cellular elements targeted by pyramidal neurons expressing 5-HT_{1A} and 5-HT_{2A} receptors.

Notes

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