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Co-expression and *In Vivo* Interaction of Serotonin$_{1A}$ and Serotonin$_{2A}$ Receptors in Pyramidal Neurons of Prefrontal Cortex

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The prefrontal cortex plays a key role in the control of higher brain functions and is involved in the pathophysiology and treatment of schizophrenia. Here we report that ~60% of the neurons in rat and mouse prefrontal cortex express 5-HT$_{1A}$ and/or 5-HT$_{2A}$ receptor mRNAs, which are highly co-localized (~80%). The electrical stimulation of the dorsal and median raphe nuclei elicited 5-HT$_{1A}$-mediated inhibitions and 5-HT$_{2A}$-mediated excitations in identified pyramidal neurons recorded extracellularly in rat medial prefrontal cortex (mPFC). Opposite responses in the same pyramidal neuron could be evoked by stimulating the raphe nuclei at different coordinates, suggesting a precise connectivity between 5-HT neuronal subgroups and 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors in pyramidal neurons. Microdialysis experiments showed that the increase in local 5-HT release evoked by the activation of 5-HT$_{2A}$ receptors in mPFC by DOI (5-HT$_{2A/2C}$ receptor agonist) was reversed by co-perfusion of 5-HT$_{1A}$ agonists. This inhibitory effect was antagonized by WAY-100635 and the prior inactivation of 5-HT$_{1A}$ receptors in rats and was absent in mice lacking 5-HT$_{1A}$ receptors. These observations help to clarify the interactions between the mPFC and the raphe nuclei, two key areas in psychiatric illnesses and improve our understanding of the action of atypical antipsychotics, acting through these 5-HT receptors.

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

Introduction

The prefrontal cortex is involved in higher brain functions such as working memory or behavioral inhibition (Fuster, 2001; Miller and Cohen, 2001). Its medial aspect in rodents (medial prefrontal cortex, mPFC) controls, via the excitatory axons of pyramidal neurons, the activity of many subcortical areas, including the midbrain aminergic nuclei [ventral tegmental area (VTA), raphe nuclei and locus coeruleus] which, in turn, project back to the prefrontal cortex (Groenewegen and Uylings, 2000). Prefrontal function and metabolism is altered in patients with severe psychiatric disorders (Weinberger *et al.*, 1994; Andreasen *et al.*, 1997; Drevets, 2001) and cognitive deficits in schizophrenia patients are mediated by derangements in brain circuits involving the prefrontal cortex (Bertolino *et al.*, 2000; Elvevag and Goldberg, 2000). Consistent with the pivotal role of dopamine in prefrontal function (Glowinski *et al.*, 1984; Williams and Goldman-Rakic, 1995; Goldman-Rakic, 1996; Robbins, 2000; O'Donnell, 2003), neuroimaging studies have revealed abnormalities in the ascending dopamine pathways in schizophrenia (Laruelle *et al.*, 1996; Abi-Dargham *et al.*, 2002). Also, a reduced density of dopamine axons in deep cortical layers has been reported in post-mortem samples from schizophrenic patients (Akil *et al.*, 2000).

Less is known, however, on the role of serotonin (5-hydroxytryptamine, 5-HT) in prefrontal function, despite the fact that this area is innervated by 5-HT axons and is highly enriched in various receptors, notably the 5-HT$_{1A}$ and 5-HT$_{2A}$ subtypes (Azmitia and Segal, 1978; Pazos and Palacios, 1985; Pazos *et al.*, 1985; Blue *et al.*, 1988; Pompeiano *et al.*, 1992, 1994; Hall *et al.*, 2000; Talvik-Lotfi *et al.*, 2000; Martinez *et al.*, 2001; Arango *et al.*, 2002). Clues about a role for serotonergic transmission – and in particular, of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors – in the normal and pathological function of the prefrontal cortex are numerous, such as (i) some hallucinogens (e.g. LSD, DOI) are 5-HT$_{2A}$ agonists and atypical antipsychotics are 5-HT$_{2A}$ antagonists (Kroeze and Roth, 1998; Meltzer, 1999), (ii) prefrontal 5-HT$_{2A}$ receptors are involved in working memory (Williams *et al.*, 2002), (iii) 5-HT$_{1A}$ receptors are involved in anxiety (Heisler *et al.*, 1998; Parks *et al.*, 1998) and learning (Harder and Ridley, 2000) and (iv) some 5-HT receptors are abnormal in the frontal lobes of psychiatric patients (Arango *et al.*, 1997; Sargent *et al.*, 2000; Gurevich *et al.*, 2002). Furthermore, 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors mediate the changes in prefrontal dopamine release induced by atypical antipsychotics (Ichikawa *et al.*, 2001).

Both receptors are expressed by pyramidal neurons (Kia *et al.*, 1996; Willins *et al.*, 1997; Jakab and Goldman-Rakic, 1998, 2000; De Felipe *et al.*, 2001; Martín-Ruiz *et al.*, 2001). 5-HT$_{2A}$ receptors are also present in GABA interneurons (Willins *et al.*, 1997; Jakab and Goldman-Rakic, 2000) and putative catecholaminergic axons in mPFC (Miner *et al.*, 2003). 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors mediate, respectively, the hyperpolarizing and depolarizing actions of 5-HT on prefrontal neurons *in vitro*, although both actions have been reported for 5-HT$_{2A}$ receptors (Araneda and Andrade, 1991; Tanaka and North, 1993; Aghajanian and Marek, 1997, 1999; Zhou and Hablitz, 1999). Given the potential relevance of both receptors in the function of the prefrontal cortex, we studied their possible co-expression in prefrontal neurons and the effects of their *in vivo* activation at cellular and circuit level in the brain of rats and mice. Additional interest to study the localization and function of such receptors in prefrontal cortex stems from the observations that (i) mPFC pyramidal neurons project to the ventral tegmental area (Thierry *et al.*, 1983), including those sensitive to the 5-HT$_{2A/2C}$ agonist 1-[2,5-dimethoxy-4-iodophenyl-2-aminopropane] (DOI; Puig *et al.*, 2003) and (ii) the firing and terminal release of dopamine cells in the VTA is controlled by 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors (Lejeune and Millan, 1998; Ichikawa *et al.*, 2001). Overall, a better knowledge of the localization and function of both receptors in prefrontal cortex may improve our understanding of the pathophysiology and treatment of schizophrenia.

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Materials and Methods

Drugs

5-HT oxalate, 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT), alpha-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionate [(*S*)-AMPA], buspirone, cirazoline, DOI, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), mianserin, pargyline, pertussis toxin, ritanserin and *N*-[2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl]-*N*-(2-pyridyl) cyclohexane carboxamide·3HCl (WAY-100635) were from Sigma/RBI (Natick, MA). Alnespirone (S-20499) was from Institut de Recherches Internationales Servier. *R*-(−)-2-{4-[(chroman-2-ylmethyl)-amino]-butyl}-1,1-dioxo-benzo[d]isothiazolone·HCl (BAY × 3702)and ipsapirone were from Bayer AG. Citalopram·HBr was from Lundbeck A/S and *R*-(+)-alpha-(2,3-dimethoxyphenyl)-1-[4-fluorophenylethyl]-4-piperidinemethanol (M100907; Lilly code LY 368675) and fluoxetine were from Eli Lilly & Co.

For the assessment of local effects in microdialysis experiments, drugs were dissolved in the perfusion fluid and applied by reverse dialysis at the stated concentrations. Concentrated solutions (1 mM; pH adjusted to 6.5–7 with $NaHCO_3$ when necessary) were stored at −80°C and working solutions were prepared daily by dilution in artificial cerebrospinal fluid (CSF). Concentrations are expressed as free bases. The concentrations of 5-HT_{1A} receptor agonists were chosen according to their relative affinity and intrinsic efficacy for 5-HT_{1A} receptors (BAY × 3702 > 8-OH-DPAT > buspirone ≈ ipsapirone ≈ alnespirone). Table 1 shows the drugs used and their primary pharmacological activity. The concentrations of DOI and AMPA were chosen from previous data (Martín-Ruiz *et al.*, 2001). Control rats and mice were perfused for the entire experiment with artificial CSF. The bars in the figures show the period of drug application (corrected for the void volume of the system). In electrophysiological experiments, drugs were administered i.v. and the effects were assessed in one neuron per animal. After this procedure was completed, animals were killed by an overdose of anesthetic.

Animals

Male albino Wistar rats weighing 250–320 g and C57BL/6 mice, 10–12 weeks old at the time of experiments, were used (Iffa Credo, Lyon, France). 5-HT_{1A} receptor knockout KO(−/−) mice (referred onwards as KO) had the same genetic background than their wild-type (WT) counterparts (C57BL/6) and were engendered as previously generated at Princenton University (Parks *et al.*, 1998). From this initial source a stable colony was grown in the animal facility of the University of Barcelona School of Medicine. 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 Institutional Animal Care and Use Committee.

Stereotaxic coordinates were taken from bregma and duramater according to the atlas of Paxinos and Watson (1998) for rat and Franklin and Paxinos (1997) for mouse. Additionally, we used the brain maps (Swanson, 1998) for nomenclature of the cortical areas. The location of probes and stimulation electrodes was verified histologically.

Table 1
Drugs used

Drug	Activity
8-OH-DPAT	5-HT_{1A} agonist
BAY × 3702	5-HT_{1A} agonist
Alnespirone	5-HT_{1A} agonist
Buspirone	5-HT_{1A} agonist
Ipsapirone	5-HT_{1A} agonist
WAY-100635	5-HT_{1A} antagonist
DOI	5-$HT_{2A/2C}$ agonist
M100907	5-HT_{2A} antagonist
Ritanserine	5-$HT_{2A/2C}$ antagonist
Mianserine	5-HT_{2A} and α_2-adrenoceptor antagonist
Ketanserine	5-$HT_{2A/2C}$ antagonist
Cirazoline	α_1-Adrenoceptor agonist
Prazosin	α_1-Adrenoceptor antagonist
Fluoxetine	selective serotonin reuptake inhibitor
EEDQ	Alkylating agent of G-protein coupled receptors

Tissue Preparation

Frozen whole brains from rats and from 5-HT_{1A} receptor KO and WT mice were used. Tissue sections, 14 µm thick, were cut using a microtome-cryostat (HM500-OM Microm, Walldorf, Germany), thaw-mounted onto 3-aminopropyltriethoxysilane (APTS; Sigma, St Louis; MO)-coated slides, and kept at −20°C until use.

Synthesis of Hybridization Probes

Different oligonucleotides were used to detect 5-HT_{1A} and 5-HT_{2A} receptor mRNA in rat brain (Pompeiano *et al.*, 1992, 1994). Four oligonucleotides complementary to different regions of the mRNA coding for the rat 5-HT_{1A} receptor were used simultaneously, corresponding to the 5′ untranslated region (bases 82–122), amino terminus (bases 123–171), third cytoplasmic loop (bases 885–933) and carboxy terminus (bases 1341–1389) (Albert *et al.*, 1990). Three oligonucleotides complementary to the mRNA coding for 5-HT_{2A} receptor, corresponding to the amino terminus (bases 669–716), third cytoplasmic loop (bases 1882–1520) and carboxy terminus (bases 1913–1960) (Pritchett *et al.*, 1988) were used. 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.

Each 5-HT_{2A} receptor oligodeoxyribonucleotide (2 pmol) was individually labeled at its 3′ end with [^{33}P]α-dATP (>3000 Ci/mmol; Amersham Pharmacia Biotech, Little Chalfont, UK) using terminal deoxynucleotidyltransferase (TdT; Roche Diagnostics GmbH, Mannheim, Germany). Labeled probes were purified through QIAquick Nucleotide Removal columns (QIAGEN GmbH, Hilden, Germany). The four 5-HT_{1A} receptor mRNA probes (100 pmol of each) (Pompeiano *et al.*, 1992) were labeled by 3′-end tailing with TdT and digoxigenin-11-dUTP (Roche Diagnostics GmbH). Digoxigenin (Dig)-labeled oligonucleotides were purified as mentioned above.

In Situ *Hybridization Histochemistry Procedure*

The protocol for double-label *in situ* hybridization histochemistry was based on previously described procedures (Tomiyama *et al.*, 1997; Landry *et al.*, 2000) and has been reported elsewhere (Serrats *et al.*, 2003). For hybridization, the radioactively labeled and Dig-labeled probes were pooled at final individual concentrations of ~1.5 nM. Dig-labeled 5-HT_{1A} hybridized oligonucleotides were visualized with alkaline-phosphate-conjugated anti-digoxigenin-F(ab) fragment antibody color reaction. Sections were then dipped into Ilford K5 nuclear track emulsion (Ilford, Mobberly, UK), exposed in the dark at 4°C for 6 weeks and then developed.

Tissue sections were examined 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. Micrography was performed using a digital camera (DXM1200 3.0, Nikon) and analysis software (soft Imaging system GmbH, Münster, Germany). 5-HT_{1A} mRNA positive neurons were identified as cellular profiles showing dark staining (alkaline phosphatase reaction product) clearly distinguishable from background. 5-HT_{2A} mRNA positive neurons were identified as aggregations of grouped silver grains that were at least three times greater than background. Figures were prepared for publication using Adobe Photoshop software (Adobe Software, Mountain View, CA).

Nissl staining

The cytoarchitecture of the rat and mouse prefrontal cortex was analyzed in an adjacent series of cresyl violet stained frozen sections. Nomenclature used follows that in Franklin and Paxinos (1997), Paxinos and Watson (1998) and Swanson (1998).

Receptor Autoradiography

[^{3}H]WAY-100635 (80.0 Ci/mmol) was purchased from Amersham and [^{3}H]ketanserine (80.9 Ci/mmol) from DuPont NEN (Boston, MA). The experimental incubation conditions for [^{3}H]8-OH-DPAT were as previously described (Mengod *et al.*, 1996). Nonspecific binding was defined as that remaining in the presence of 10^{-5} M 5-HT. The incubation conditions for [^{3}H]ketanserine were as described previously (Pazos *et al.*, 1987; López-Giménez *et al.*, 1997). Nonspecific binding was defined as that remaining in the presence of 10^{-5} M mianserine. After incubation and washing, tissue sections were dipped in distilled ice-cold water and dried rapidly under a cold air stream. Tissues were exposed to tritium-sensitive film (Kodak Biomax MS; Kodak, Rochester, NY) together with plastic ^{3}H-standards (^{3}H-microscales; Amersham). Exposure time was 15 days for [^{3}H]ketanserine and 30 days for [^{3}H]8-OH-DPAT. All tissue sections used for quantification of receptor sites were processed simultaneously and in the same experimental conditions. Quantitative analysis of the autoradiograms was done with a computerized image analysis system (MCID, St Catharines, Ontario, Canada).

Single Unit Recordings of Pyramidal Neurons in Rat mPFC

These experiments were aimed at examining the responses elicited in pyramidal neurons of the mPFC by the electrical stimulation of the dorsal raphe (DR) and/or median raphe (MnR) nuclei. Rats were anesthetized (chloral hydrate 400 mg/kg i.p.) and positioned in a David Kopf stereotaxic frame. Additional doses of chloral hydrate (80 mg/kg) were administered i.v. through the femoral vein. Body temperature was maintained at 37°C throughout the experiment with a heating pad. All wound margins and points of contact between the animal and the stereotaxic apparatus were infiltrated with lidocaine solution (5%). In order to minimize pulsation, the atlanto-occipital membrane was punctured to release some CSF.

Bipolar stimulating electrodes consisted of two stainless steel enamel-coated wires (California Fine Wire, Grover Beach, CA) with a diameter of 150 µm and a tip separation of ~100 µm and *in vitro* impedances of 10–30 KΩ. Stimulating electrodes were stereotaxically implanted in two different coordinates within the DR (AP –7.8, L 0, DV –6.5; and AP –7.3, L –2.2 with a lateral angle of 20°, DV –6.6 mm) and one in the MnR (AP –7.8, L 2.0 with a lateral angle of 13°, DV –8.8 mm). These angles resulted in the tip of the electrodes at DV –6.2 and –8.6 mm, respectively. After each implant, each electrode was secured to the skull with glue and dental cement. Constant current electrical stimuli were generated with a Grass S-48 stimulation unit connected to a Grass SIU 5 stimulus isolation unit. Stimulating current was typically 0.15–2 mA, 0.2 ms square pulses at 0.9 Hz. Pyramidal neurons were recorded extracellularly with glass micropipettes pulled from 2.0 mm capillary glass (WPI, Saratosa, FL) on a Narishige PE-2 pipette puller (Narishige Science Institute, Tokyo, Japan). Microelectrodes were filled with 2 M NaCl. Typically, impedance was between 4 and 10 MΩ. Single unit extracellular recordings were amplified with a Neurodata IR283 (Cygnus Technology Inc., Delaware Water Gap, PA), postamplified and filtered with a Cibertec amplifier (Madrid, Spain) and computed on-line using a DAT 1401plus interface system Spike2 software (Cambridge Electronic Design, Cambridge, UK).

Descents in mPFC were carried out at AP +3.2 to 3.4, L –0.5 to –1.0, DV –1.3 to –4.0 below the brain surface. We systematically confirmed that only a single pyramidal neuron was recorded by (i) identification by antidromic activation from DR and/or MnR and (ii) collision extinction with spontaneously occurring spikes (Fuller and Schlag, 1976). Neurons without antidromic activation or without spontaneous firing activity were not considered.

Single Unit Recordings of 5-HT Neurons in Mouse Brain

We assessed the effects of the i.v. administration of the 5-HT$_{1A}$ agonist 8-OH-DPAT and the selective 5-HT reuptake inhibitor fluoxetine on the firing rate of DR 5-HT neurons in WT and KO mice. These were performed as previously described (Sawyer *et al.*, 1985; Celada *et al.*, 1996) once adapted to mouse brain. Mice were anesthetized with chloral hydrate (400 mg/kg i.p.) and additional doses (80 mg/kg) were administered i.v. through the femoral vein. A 2 × 2 mm recording hole was drilled over the lambda suture and the sagittal sinus was ligated, cut and reflected. Single unit extracellular recordings were carried out along the midline at approximately –4.6 mm from bregma. 5-HT neurons were usually encountered at 2–3.5 mm below the brain surface and identified according to previously described electrophysiological criteria (Wang and Aghajanian, 1977). Serotonergic neurons exhibited a 2–5 ms bi- or triphasic extracellular waveform with regular firing rate and frequencies of 0.3–3.4 Hz.

Microdialysis Procedures

Microdialysis experiments in rats were conducted as described in Martín-Ruiz *et al.* (2001). Briefly, rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and stereotaxically implanted in mPFC with concentric dialysis probes equipped with a Cuprophan membrane (4 mm long) at the following coordinates (in mm): AP +3.2, L –0.8, DV –6.0. Microdialysis experiments were performed in freely moving rats the day after surgery. After a 100 min stabilization period, four fractions were collected to obtain basal values before local (reverse dialysis) or systemic administration of drugs and then successive 20 min (30 µl) dialysate samples were collected. In most experiments, the partial 5-HT$_{2A/2C}$ receptor agonist DOI was perfused alone for 2 h (six fractions), followed by its application in combination with other drugs for another 2 h period.

For mice, the manufacture of the probes was adapted from that previously described for rats. Surgical and microdialysis procedures were identical to those described for rats except for the dose of anesthesia (sodium pentobarbital, 40 mg/kg, i.p.), the length of dialysis membrane (2 mm) and the brain coordinates (in mm) of the mPFC: AP +2.2, L –0.2, DV –3.4.

The concentration of 5-HT in dialysate samples was determined by HPLC with amperometric detection, as described (Adell and Artigas, 1998). Retention time was between 3.5 and 4 min and the limit of detection was typically 1 fmol/sample. The HPLC profile from mice and rats were similar and the 5-HT peak was free from interferences.

5-HT$_{1A}$ and 5-HT$_{2A}$ Receptor Inactivation in Rats

To assess the involvement of 5-HT$_{2A}$ and 5-HT$_{1A}$ receptors in the actions of DOI and the various 5-HT$_{1A}$ agonists used, we used two different experimental strategies. On the one hand, the function of 5-HT$_{1A}$ receptors in mPFC was cancelled by the local application of pertussis toxin, which ADP-ribosylates $G_{i/o}$ proteins associated to pre- and postsynaptic 5-HT$_{1A}$ receptors (Andrade *et al.*, 1986; Innis and Aghajanian, 1987). Pentobarbital anesthetized rats were positioned in the stereotaxic frame and pertussis toxin (1 µg in 2 µl of aCSF) was infused through a 25 gauge stainless steel cannula (Small Parts Inc., Miami, FL) at two different DV coordinates (–3.0 and –5.0 mm) using the same AP and L coordinates than for probe implants. (AP +3.2, L –0.8). At each application point, the solution containing pertussis toxin was delivered over the course of 2 min using a 10 µl Hamilton syringe attached to a microinfusion pump (Bioanalytical Systems Inc., West Lafayette, IN) and the cannula was left in position for ~10 min to prevent the solution from surging back. Control rats were subjected to the same procedure but no pertussis toxin was applied. Microdialysis experiments were performed 2–3 days after pertussis toxin. In the rats examined 2 days later, a dialysis probe was implanted 1 h after the application of pertussis toxin, to avoid a second surgical procedure. However, in rats examined 3 days later, probes were implanted the day before microdialysis experiments (i.e. 2 days after pertussis toxin administration) to avoid an excessive gliosis resulting from probe implant 3 days later.

A second strategy to examine the involvement of 5-HT$_{2A}$ and 5-HT$_{1A}$ receptors in the actions of DOI and BAY × 3702, respectively, was the use of EEDQ. Since this agent alkylates several G-coupled aminergic receptors, we used the following approach to selectively protect 5-HT$_{2A}$ or 5-HT$_{1A}$ receptors from the effect of EEDQ. Rats were implanted with microdialysis probes in the mPFC, as above. At 3–4 h

after implants, the probes were perfused with the 5-$HT_{2A/2C}$ antagonist ritanserin (300 μM) for 3 h at 1.5 μl/min. One hour after beginning of the perfusion, EEDQ was dissolved in ethanol:water (1:1) and administered to the rats (6 mg/kg, i.p.). It was hypothesized that the occupation of 5-HT_{2A} receptors by ritanserin in the area sampled by the microdialysis probe would prevent the inactivation of the receptor by EEDQ. This strategy has been previously used *in vitro* (Gozlan *et al.*, 1994). Similarly, to protect 5-HT_{1A} receptors, the same procedure was applied, but the selective 5-HT_{1A} antagonist WAY-100635 was perfused before EEDQ administration instead of ritanserin. Microdialysis experiments were performed on the following day.

Data and Statistical Analysis

The statistical analysis of histological data was performed using one-way analysis of variance (ANOVA) followed by post-hoc testing to determine regional differences in the expression of 5-HT_{1A} and 5-HT_{2A} receptor. Only cellular profiles showing great abundance of both transcripts were considered to co-express both receptors. Cells with a dense labeling of 5-HT_{1A} receptor mRNA and occasional silver grains or vice versa (e.g., as shown in Fig. 1*I*) were not considered to co-express both receptors.

The responses in prefrontal pyramidal neurons evoked by DR and MnR stimulation were characterized by measuring the magnitude and duration of inhibitory and excitatory responses from peristimulus-time histograms (PSTH; 4 ms width). Orthodromic excitations elicited spikes with short and variable latencies with a success rate >10% (Celada *et al.*, 2001). These have been previously characterized in Puig *et al.* (2003). Inhibitions were defined by either a total cessation of spikes or a 75% decrease in the number of spikes with respect to the prestimulus value for at least four successive bins (Hajós *et al.*, 1998). These were pharmacologically characterized by reversal with the selective 5-HT_{1A} antagonist WAY-100635. The offset of the inhibition was defined as the first of four bins equal to or above the prestimulus value. Changes in firing rate of 5-HT neurons were assessed using paired Student's *t*-test or repeated-measures ANOVA. These were quantified by averaging the values after i.v. drug injection (omitting the first minute). ED_{50} values were calculated with the GraphPad Prism program (San Diego, CA).

Microdialysis results are expressed as fmol/fraction (uncorrected for recovery) and shown in figures as percentages of basal values (individual means of four pre-drug fractions). Statistical analysis was carried out using one- or two-way ANOVA for repeated measures of the 5-HT values followed by Duncan test. Typically, experiments consisted in the perfusion of DOI or S-AMPA by reverse dialysis in mPFC for 2 h followed by another 2 h period in which 5-HT_{1A} agonists were co-perfused. Drug effects were assessed using 5-HT values in four pre-drug fractions plus the six post-drug (2 h) fractions. When appropriate, average 5-HT values were calculated and compared using student's *t*-test or ANOVA. Data are expressed as the mean ± SEM. Statistical significance has been set at the 95% confidence level (two tailed).

Results

Co-expression of 5-HT_{1A} and 5-HT_{2A} Receptors

Rat Prefrontal Cortex

We used double *in situ* hybridization to examine the presence of 5-HT_{1A} and 5-HT_{2A} receptor mRNAs in rat prefrontal cortex. Figure 1 (*A*,*B*) shows the presence of a large number of cells containing both transcripts in superficial, middle and deep layers. At this magnification, a marked overlap in the distribution of both receptors was observed in various cortical areas, such as secondary motor area (MOs), dorsal anterior cingulate area (ACAd), prelimbic area (PL) and infralimbic area (ILA), as well as in the piriform cortex (PIR) or taenia tecta (TT). This was also evident at higher magnification in the cingulate, prelimbic and infralimbic areas of the medial prefrontal cortex (mPFC), which contain numerous cells expressing 5-HT_{1A} and/or 5-HT_{2A} receptor mRNAs (Fig. 1*D*,*E*,*G*). However, internal layers (VI) contain fewer cells expressing 5-HT_{2A} than 5-HT_{1A} receptors (compare left sides of panels Fig. 1*D* and *E*). As evidenced from Nissl-stained sections (Fig. 1*C*,*F*) the proportion of cells expressing 5-HT_{1A} and/or 5-HT_{2A} receptors was high in all prefrontal areas. Figure 1*G* and *H* show the presence of abundant cells containing both receptor transcripts in the infralimbic (Fig. 1*G*) and piriform cortices (Fig. 1*H*). Figure 1*I* and *J* show at a higher magnification, the cells in infralimbic cortex (Fig. 1*I*) and taenia tecta (Fig. 1*J*) expressing both receptor transcripts as well as occasional cells in which 5-HT_{1A} and 5-HT_{2A} receptors were not co-localized.

We used total cell counts obtained from adjacent Nissl-stained sections to calculate the proportion of cells in prefrontal cortex expressing 5-HT_{1A} and/or 5-HT_{2A} receptor mRNAs. These values ranged from 53 to 65% (Table 2). The co-expression of both receptors was very high (80–90%) in all cortical areas examined, except in layer VI (38%; Table 2).

Mouse Prefrontal Cortex

The mouse mPFC showed the presence of abundant cells in which both 5-HT_{1A} and 5-HT_{2A} receptor transcripts were present, with a marked overlap in their distributions (Fig. 2*A*,*B*). The proportion of cells expressing one or the other receptor (or both) varied between 52% in layer VI to 71% in cingulate area (Table 3). The proportion of cells containing both receptor mRNAs was 76% in the cingulate area, 72% in the infralimbic area, 48% in layer VI and 82% in the piriform area (Table 3). The percentage of co-expression in deep layers was significantly lower than that in the other areas examined. Figure 2 shows at a higher magnification cells co-expressing both receptors in infralimbic (*C*) and cingulate (*D*) areas as well as a scarce number of cells expressing only 5-HT_{1A} or 5-HT_{2A} receptors.

We performed autoradiographic studies in wild-type (WT) and 5-HT_{1A} receptor knockout [KO(−/−)] mice to examine whether the genetic deletion of 5-HT_{1A} receptors had altered 5-HT_{2A} receptors in prefrontal cortex. The density of 5-HT_{2A} receptor binding sites in the cingulate area (see Materials and Methods) was 476 ± 19 fmol/mg protein in WT mice and 510 ± 13 fmol/mg protein in 5-HT_{1A} receptor KO(−/−) mice (n.s.).

Opposite In Vivo *Action of 5-HT_{1A} and 5-HT_{2A} Receptors on Pyramidal Cells*

Pyramidal neurons in the mPFC, systematically identified by antidromic stimulation from the DR and MnR were recorded extracellularly. The electrical stimulation of the raphe nuclei could excite and inhibit pyramidal neurons. Here we describe only the characteristics of the responses which were pharmacologically characterized by the use of selective antagonists. 5-HT_{2A}-mediated excitations (n = 10) were reversed by M100907 (0.2–0.6 mg/kg i.v.; Fig. 3*A*,*B*). Excitations had a mean latency of 71 ± 8 ms and a mean duration of 101 ± 8 ms. The success rate dropped after M100907 administration from 47 ± 8% to 11 ± 3% (P < 0.001). In all cases, orthodromic and antidromic spikes were simultaneously recorded, showing the existence of a strong reciprocal raphe–mPFC interaction. Two additional neurons (out of 12) were excited by DR stimulation but the response was not reversed by M100907 (up to 0.6 mg/kg).

Likewise, the electrical stimulation of the DR and MnR inhibited mPFC pyramidal neurons. The 5-HT_{1A} antagonist WAY-

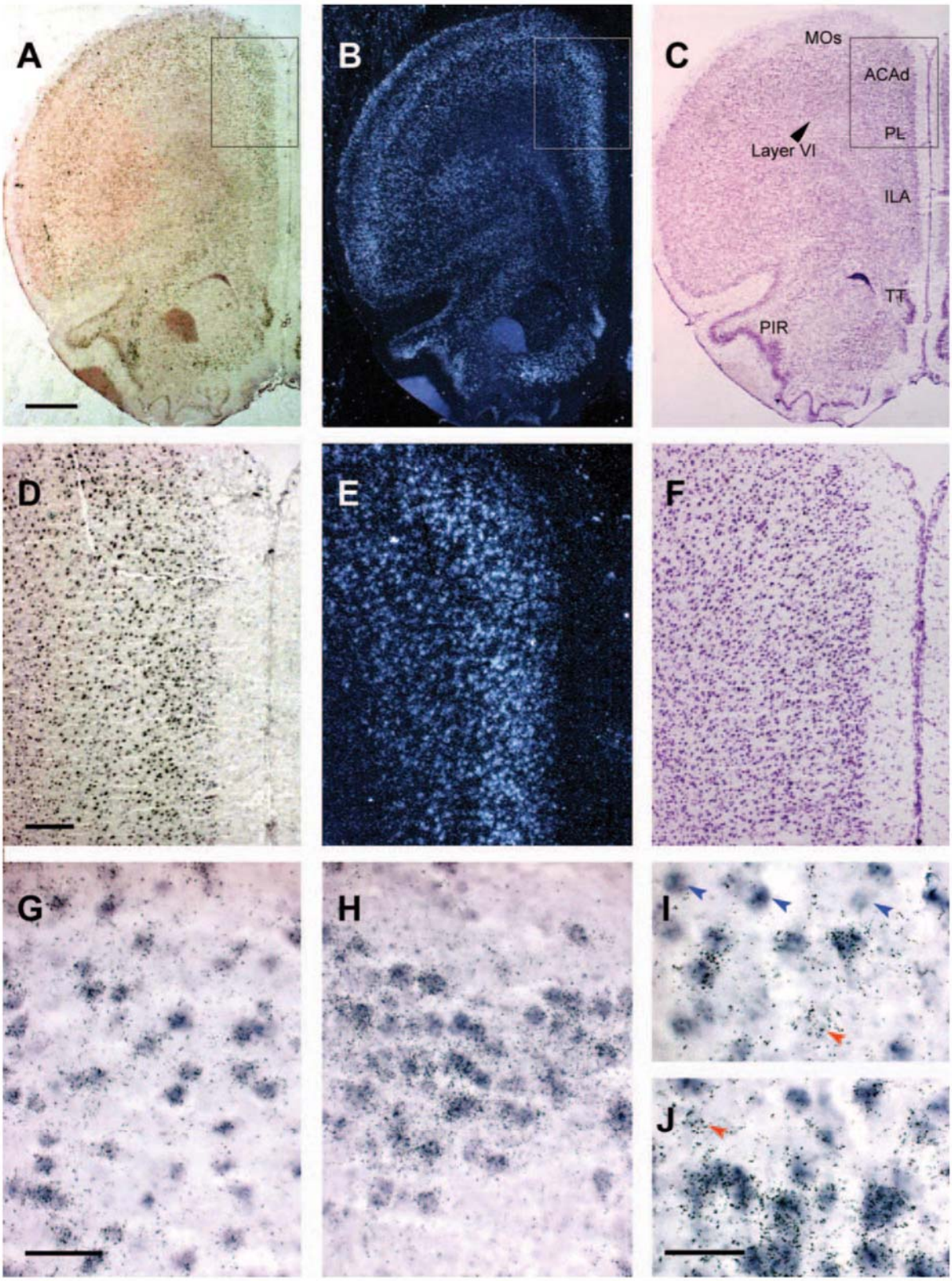

Figure 1. Localization of 5-HT_{1A} and 5-HT_{2A} receptor mRNAs in the rat prefrontal cortex using double *in situ* hybridization histochemistry. (*A–C*) Coronal sections of rat prefrontal cortex showing a large number of cells expressing (*A*) 5-HT_{1A} receptors (Dig-labeled oligonucleotides) or (*B*) 5-HT_{2A} receptors (dark field; ^{33}P-labeled oligonucleotides); (*C*) an adjacent Nissl-stained section. Note the abundant presence of cells expressing both receptors in upper and middle cortical layers, as well as in piriform cortex (PIR) and taenia tecta (TT). Cells in deep layers (VI) express preferentially 5-HT_{1A} receptor mRNA. (*D–F*) show enlargements of the marked area in panels (*A–C*). (*D*, *E*) show the presence of a large number of cells containing 5-HT_{1A} and 5-HT_{2A} receptor transcripts in cingulate (ACAd) and prelimbic (PL) cortex. (*G–J*) show individual cells expressing both receptor transcripts. Occasional cell profiles containing only 5-HT_{1A} (blue arrowheads) or 5-HT_{2A} receptor mRNAs (red arrowheads) were seen in the infralimbic cortex (*G*, *I*), piriform cortex (*H*) and taenia tecta (*J*). Bar size is 1 mm in (*A–C*), 250 μm in (*D–F*), 50 μm in (*G*, *H*) and 30 μm in (*I*, *J*).

Table 2
Co-expression of 5-HT_{1A} and 5-HT_{2A} receptor mRNA in rat prefrontal cortex

	MOs	ACAd	ILA	Layer V	PIR	TT
5-HT1A and 5-HT2A mRNAs	83.2 ± 0.9%	81.8 ± 1.7%	79.3 ± 1.6%	38.1 ± 1.5%**	90.0 ± 0.8%*	87.7 ± 1.0%*
	51	*50*	*42*	*21*	*56*	*57*
5-HT1A mRNA	11.5 ± 1.0%	14.6 ± 1.9%	16.7 ± 1.3%	59.4 ± 1.8%**	7.4 ± 1.0%*	9.5 ± 0.8%*
	7	*9*	*9*	*33*	*5*	*6*
5-HT2A mRNA	4.8 ± 0.6%	4.0 ± 0.5%	3.8 ± 0.2%	3.4 ± 1.1%	2.7 ± 0.6%	2.8 ± 0.7%
	3	*2*	*2*	*1*	*1*	*2*
Σ% cells expressing 5-HT_{1A} and/or 5-HT_{2A} receptor mRNAs	*61*	*61*	*53*	*55*	*62*	*65*

Cortical areas designated according to Paxinos and Watson (1998) and Swanson (1998). MOs, secondary motor area; ACAd, dorsal anterior cingulate area; ILA, infralimbic area (dorsal part); PIR, piriform cortex; TT, taenia tecta. Layer VI denotes deep areas of the sensorimotor cortex at prefrontal level, as shown in Fig. 1*C* (Swanson, 1998).
Data are means ± SEM of five rats (each individual measure is the mean of four consecutive sections) and represent the percentage of the counted cells expressing both, or each mRNA receptor subtype individually. Values in italics are the percentage of the cells in each prefrontal area expressing each receptor (or both), calculated as the ratio between the number of cells expressing either (or both) receptors(s) and the total number of cells in the same field counted in adjacent sections (Nissl staining). The latter value was 1026 ± 56 cells/field (field was 1 mm^2).
*$P < 0.05$ versus cingulate and infralimbic areas; **$P < 0.05$ versus all other areas (post-hoc test after ANOVA).

Table 3
Co-expression of 5-HT_{1A} and 5-HT_{2A} receptor mRNA in mouse prefrontal cortex

	Cingulate	Infralimbic	Layer VI	Piriform
5-HT1A and 5-HT2A mRNAs	75.6 ± 1.8	72.5 ± 2.5	47.6 ± 3.7**	82.1 ± 0.9
	54	*41*	*25*	*46*
5-HT1A mRNA	15.4 ± 2.0	18.5 ± 2.1	44.2 ± 3.5**	10.9 ± 1.6
	11	*10*	*23*	*6*
5-HT2A mRNA	8.3 ± 0.8	8.1 ± 1.8	7.1 ± 1.1	6.3 ± 0.7
	6	*5*	*4*	*4*
Σ cells expressing 5-HT_{1A} and/or 5-HT_{2A} receptor mRNAs	*71*	*56*	*52*	*56*

Cortical areas designated according to Franklin and Paxinos (1997).
Data are means ± SEM of five mice (each individual measure is the mean of two consecutive sections) and represent the percentage of the counted cells expressing both, or each mRNA receptor subtype individually. Values in italics: see legend to Table 2 (number of cells per field = 1540 ± 130). **$P < 0.05$ versus all other areas.

100635 (10–60 μg/kg i.v) reversed these inhibitions in 8/10 cases examined (Fig. 3*C*,*D*). WAY-100635 did not reverse a short latency/duration inhibition (see Discussion for an explanation of the putative mechanisms involved). We therefore omitted this earlier component in the calculations. 5-HT_{1A}-mediated inhibitions had a latency of 69 ± 32 ms and a duration of 181 ± 84 ms. Within this time period, raphe stimulation inhibited mPFC pyramidal neurons by 87 ± 15%. The administration of WAY-100635 reversed this inhibition to −30 ± 80%, i.e. 5-HT_{1A} receptor blockade unveiled a latent excitation, which was noticeable in four out of the eight units whose inhibition was blocked by WAY-100635, as in the example shown in Figure 4*F*. This suggests that some of the recorded units were concurrently excited and inhibited by raphe stimulation, although the overall effect was inhibitory.

The high co-expression of 5-HT_{1A} and 5-HT_{2A} receptors in prefrontal neurons seemed paradoxical in view of their opposite cellular responses to 5-HT. We hypothesized that 5-HT might excite or inhibit a given pyramidal neuron depending on (i) the amount of 5-HT released, or (ii) the existence of specific connections between the stimulated 5-HT neurons and pyramidal compartments enriched in one or other receptor (see Discussion). We tested the first possibility by recording pyramidal neurons after single or twin pulse stimulation of the DR/MnR. The results are given in a separate report (M.V. Puig *et al.*, in preparation). In general, twin pulse stimulation, which increases 5-HT release (Gartside *et al.*, 2000) also enhanced the magnitude of the inhibitory effect.

To examine the second possibility, we conducted a series of experiments in which we recorded the same pyramidal neuron while stimulating the DR and the MnR at different sites (two in the DR, one in MnR; see Experimental Procedures). Once a pyramidal neuron was identified, stimulation began in one of these sites to evoke a response, which was recorded for 2–4 min. Then, the current was switched to an electrode implanted in another coordinate within the raphe nuclei while recording the same neuron. Due to the experimental paradigm used, we could not pharmacologically assess these responses, yet their characteristics did not differ from those blocked by 5-HT_{2A} and 5-HT_{1A} antagonists. A total of 56 experiments were done in which pyramidal responses differed – qualitatively or quantitatively – depending on the site of placement of the stimulating electrode within the raphe (three electrodes were stably implanted; see Experimental Procedures). Figure 4 (*A–E*) shows examples of several of these experiments. In 14 cases, the response changed from an excitation to an inhibition (or

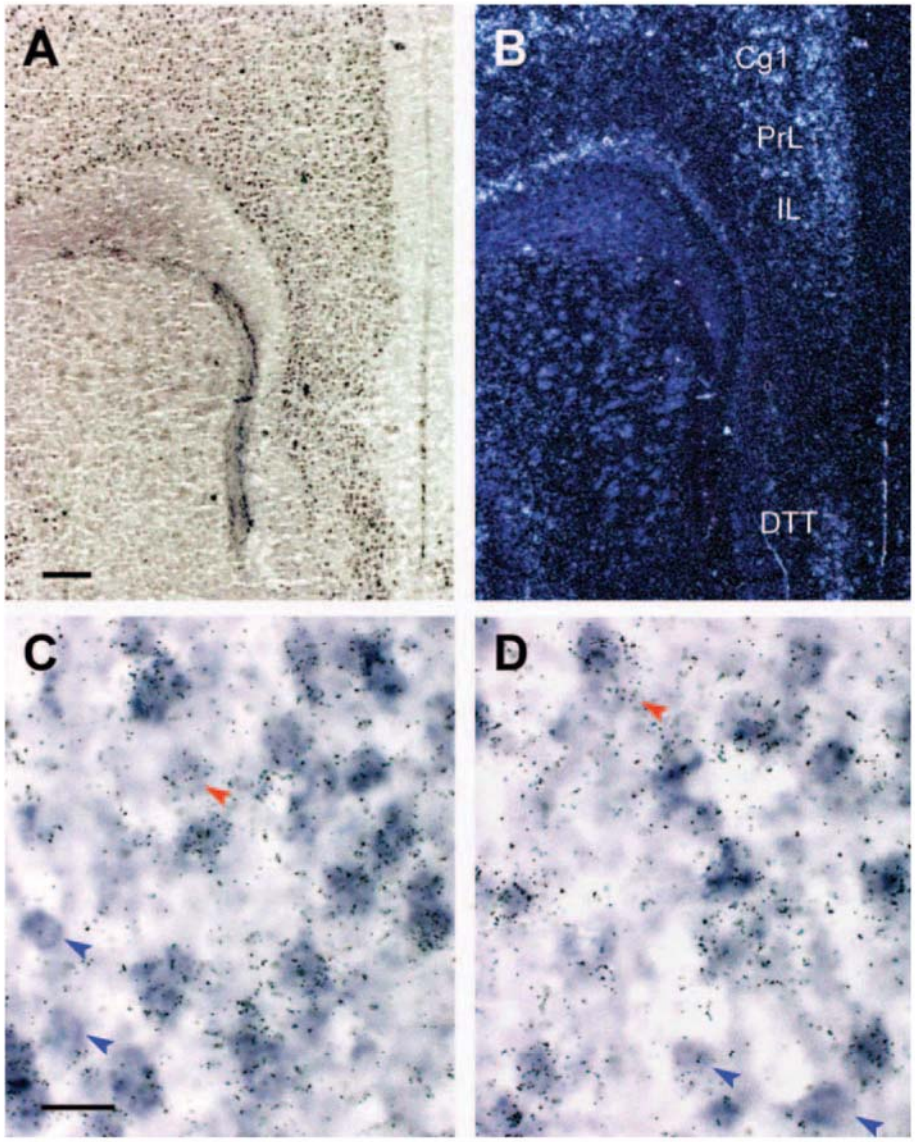

Figure 2. Low and high level magnification photomicrographs of 5-HT_{1A} and 5-HT_{2A} receptor mRNA containing cells in C57BL/6 mouse prefrontal cortex. Bright-field image of 5-HT_{1A} receptor mRNA visualized by non-radioactive *in situ* hybridization histochemistry using Dig-labeled oligonucleotides (*A*) and dark-field image of 5-HT_{2A} receptor mRNA detected using ^{33}P-labeled oligonucleotides (*B*). A large amount of cells expressing 5-HT_{1A} and 5-HT_{2A} receptor mRNA can be appreciated in the cingulate (Cg1), prelimbic (PrL) and infralimbic (IL) cortices as well as in dorsal taenia tecta (DTT). Note also the presence of a cell layer above the corpus callosum showing abundant expression of both receptors that could correspond to the cortical layer VIb. (*C*, *D*) show higher magnification images of the high degree of co-expression of both receptors in infralimbic cortex (*C*) and cingulate cortex (*D*). As in rat brain, occasional cells were seen expressing only 5-HT_{1A} (blue arrowheads) or 5-HT_{2A} receptor mRNAs (red arrowheads). Bar size is 200 μm (*A*, *B*) and 20 μm (*C*, *D*).

vice versa). In three of them, the excitation was preceded by an inhibition lasting for ~100 ms (Fig. 4*A′*). In another 14 experiments, switching the stimulation site inhibited a previously unresponsive neuron (or vice versa; Fig. 4*B′*). Also, a change from no response to excitation (or vice versa) was observed in six cases (Fig. 4*C′*). In 22 cases, the nature of the response did not change (15 inhibitions; seven excitations) but the effect size differed when switching the stimulation site (9/15 for inhibitions, 4/7 for excitations). As shown in the examples in Figure 4, excitations and inhibitions could be indistinctly evoked by DR or MnR stimulation. Lastly, to verify the reversibility of these effects, in some experiments we further switched the stimulation site back to the original one and the response was recorded for another 2 min period (n = 7). Figure 4*D–E* shows two examples of the reversibility of the responses achieved.

An additional evidence of the dual action of 5-HT on prefrontal pyramidal neurons is shown in Figure 4*F*. This cell was initially inhibited by DR stimulation at AP –7.8 mm. The administration of WAY-100635 (10 μg/kg i.v.) unveiled a latent excitation which was reversed by the selective 5-HT_{2A} antagonist M100907 (0.5 mg/kg i.v.).

In Vivo *Interaction between 5-HT_{1A} and 5-HT_{2A} Receptors at Circuit Level*

Anatomical and functional data (see Introduction) support the existence of a reciprocal connectivity and mutual control of 5-HT and prefrontal neurons (mPFC–raphe circuit). The stimula-

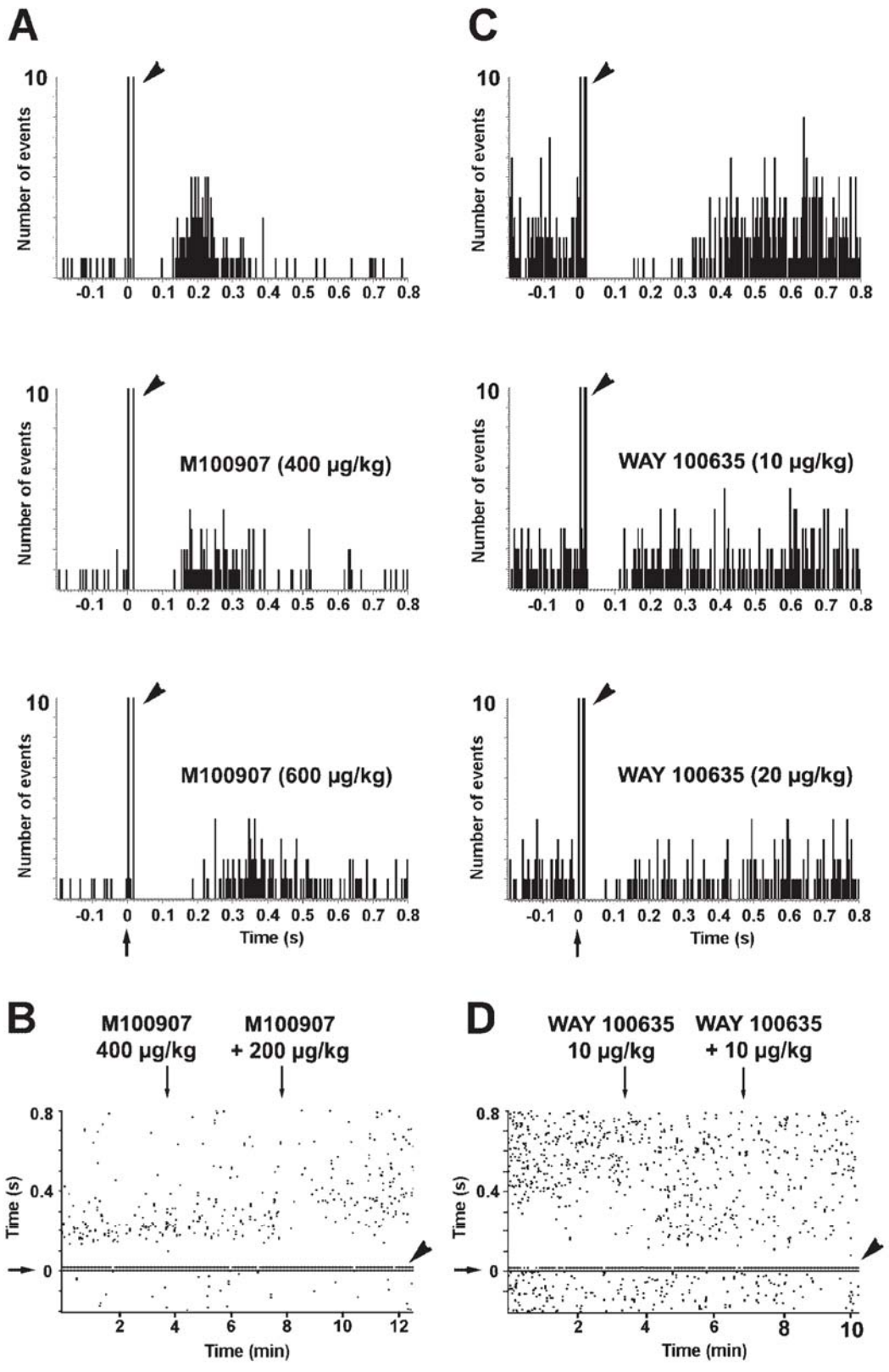

Figure 3. The electrical stimulation of the DR excited and inhibited mPFC pyramidal neurons. (*A*) Peristimulus time histograms (PSTH; 200 trials, 4 ms bin width) showing the orthodromic excitatory response in a mPFC pyramidal neuron (40% success rate; latency 96 ms, duration 152 ms) evoked by electrical stimulation of the DR (0.2 ms, 1.7 mA, 0.9 Hz). The filled arrowhead denotes the antidromic activation from the DR (latency 16 ms). Lower panels show PSTH of the same neuron after the blockade of the excitation by the 5-HT_{2A} receptor antagonist M100907 at the cumulative doses indicated (i.v.). (*B*) Raster display of the response showed in *A* (the abscissa in *A* corresponds to the ordinate in *B*). (*C*) PSTH of a pyramidal inhibition evoked by the electrical stimulation of the DR (0.2 ms, 2 mA, 0.9 Hz). The PSTH shows also antidromic activation (13 ms; filled arrowhead). The lower panels show the PSTH of the same neuron after the reversal of the inhibition by the 5-HT_{1A} antagonist WAY-100635 (10–20 µg/kg i.v.) except for a short period post-stimulus (see Discussion). (*D*) Raster display of the PSTHs shown in *C*. Collisions are seen in *B* and *D* as white gaps in the line showing the antidromic response (filled arrowhead).

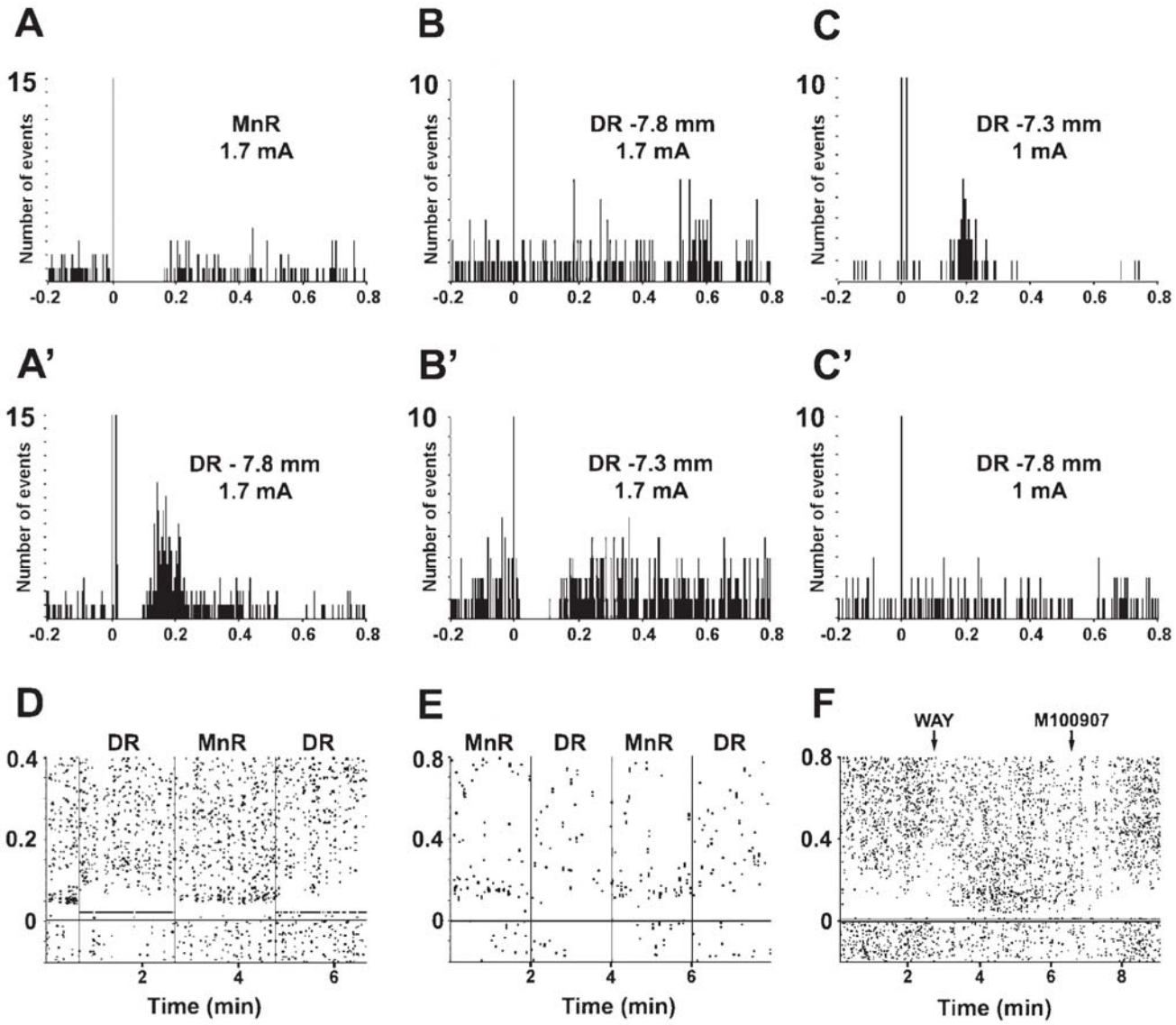

Figure 4. Representative responses of pyramidal excitations and inhibitions evoked by the sequential stimulation of the DR at different AP coordinates (bregma –7.3 and –7.8 mm) and the MnR (bregma –7.8 mm; see Experimental Procedures for DV and L coordinates). Each pair of PSTH (*A–A'* to *C–C'*) corresponds to the response elicited in three different (*A–C*) pyramidal neurons by the stimulation at two different sites. *A–A'*) MnR stimulation produces an inhibition and DR stimulation produces an inhibition followed by an excitation. (*B–B'*) Unresponsive to stimulation at DR –7.8 mm and inhibited by stimulation at DR –7.3 mm. (*C–C'*) Excited by stimulation at DR –7.3 mm and unresponsive to stimulation at DR –7.8 mm. Each PSTH consisted of approximately 100 trials. (*D, E*) Raster displays of two pyramidal neurons after the sequential stimulation at two different sites showing the reversibility of the response when switching the stimulation site (vertical lines). (*D*) An inhibition evoked by stimulation at DR –7.8 mm was followed by excitation when stimulating at MnR –7.8 mm. Switching the stimulation back to DR –7.8 mm restored the initial inhibition (current = 2 mA). Note the antidromic spikes when stimulating from the DR. (*E*) Excitation from MnR (–7.8 mm) and inhibition from DR (–7.8 mm) repeated twice. Current was 1 mA in all cases. Antidromic spikes were seen at a higher current (not shown). (*F*) Raster display of a pyramidal inhibition evoked by DR stimulation (–7.8 mm). WAY-100635 (WAY; 10 μg/kg i.v.; 1st arrow) reversed the inhibition and unveiled a latent 5-HT_{2A} receptor-mediated excitation, which was reversed by M100907 (0.5 mg/kg i.v.; 2nd arrow).

tion of excitatory (AMPA, 5-HT_{2A}) and inhibitory (5-HT_{1A}) receptors in mPFC, increases and decreases, respectively (i) pyramidal cell firing, (ii) 5-HT cell firing in the DR, and (iii) 5-HT release in mPFC (Celada *et al.*, 2001; Martín-Ruiz *et al.*, 2001; Puig *et al.*, 2003). Here we used the drug-evoked *in vivo* 5-HT release in mPFC as an overall index of the pyramidal influence on ascending midbrain 5-HT neurons.

Microdialysis Studies in Rats

A total of 123 rats were used in these studies. Baseline 5-HT values were 20.3 ± 1.0 fmol/fraction. As previously observed, local DOI application (100 μM) in rat mPFC doubled 5-HT release (n = 10, P < 0.0001; Fig. 5*A*). This effect depends on 5-HT_{2A} receptor activation (Martín-Ruiz *et al.*, 2001) and was reversed by co-perfusion of the following 5-HT_{1A} receptor agonists: 8-OH-DPAT (100 μM, n = 8), alnespirone (300 μM, n = 6), BAY × 3702 (30 μM, n = 7), buspirone (300 μM, n = 6) and ipsapirone (300 μM, n = 5; data not shown) (Fig. 5*A*). Two-way ANOVA revealed a significant effect of the group (P < 0.0025), time (P < 0.0001) and time × group interaction (P < 0.0001).

The reducing effect of BAY × 3702 was antagonized by the co-perfusion of WAY-100635 (300 μM, n = 9, P < 0.007) (Fig. 5*B*). A lower concentration of WAY-100635 (100 μM) could not reverse the effect of BAY × 3702 (n = 6, not shown). We

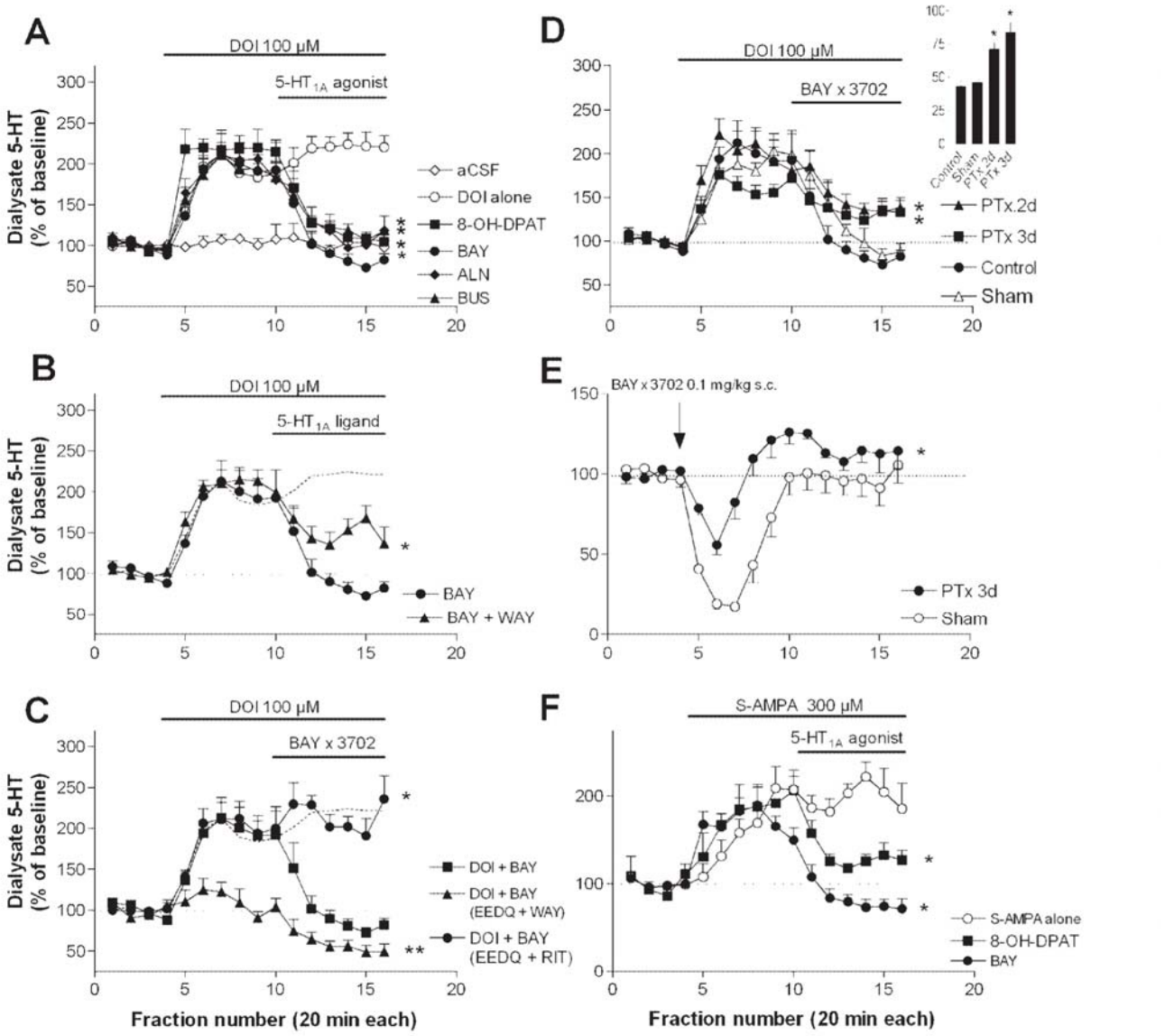

Figure 5. (*A*) The perfusion of 100 µM DOI in rat mPFC increased the 5-HT release ($n = 10$; open circles). Controls (open diamonds; $n = 5$) received aCSF. The co-perfusion of various 5-HT_{1A} agonists significantly reversed the 5-HT elevation induced by DOI: 8-OH-DPAT (100 µM, $n = 8$), BAY × 3702 (BAY; 30 µM, $n = 7$), alnespirone (ALN; 300 µM, $n = 6$), buspirone (BUS; 300 µM, $n = 6$). (*B*) The 5-HT_{1A} antagonist WAY-100635 (300 µM, $n = 9$) partly reversed the inhibitory effect of BAY × 3702 30 µM. The dotted line shows the effect of DOI alone. (*C*) The inactivation of 5-HT_{1A} receptors by EEDQ (6 mg/kg i.p., 1 day before) while protecting 5-$HT_{2A/2C}$ receptors with ritanserin 300 µM (RIT) preserved DOI's effect and cancelled that of BAY × 3702 ($n = 7$). Conversely, the protection of 5-HT_{1A} receptors with WAY-100635 300 µM during EEDQ administration (1 day before experiments) preserved the suppressing effect of BAY × 3702 on 5-HT release and markedly attenuated the increase induced by DOI ($n = 6$). (*D*) Effects of the local pretreatment with pertussis toxin (PTx) in mPFC (1 µg, 2 or 3 days before) on the inhibitory action of BAY × 3702 30 µM compared to control rats and sham-operated rats ($n = 4$). Filled triangles ($n = 5$) and squares ($n = 7$) correspond to rats receiving the toxin 2 and 3 days before, respectively. Inset: effect of BAY × 3702 expressed as pre-drug value in each group; $^*P < 0.0001$. (*E*) Effects of the systemic administration of BAY × 3702 (0.1 mg/kg s.c.) on mPFC 5-HT release in control rats and in rats pretreated with PTx 3 days before ($n = 6$ and 7, respectively) $^*P < 0.0001$ versus controls. (*F*) The local application of S-AMPA (300 µM) in mPFC increased the local 5-HT release ($n = 5$). BAY × 3702 (30 µM) suppressed the AMPA-induced 5-HT release ($n = 5$). In a small group of rats ($n = 3$), 8-OH-DPAT (100 µM) also attenuated the AMPA-induced 5-HT release. $^*P < 0.0001$ versus AMPA alone.

further examined the involvement of postsynaptic 5-HT receptors in these effects using two strategies. First, one day before microdialysis experiments, we inactivated 5-HT_{1A} receptors in rat mPFC using the chelating agent EEDQ (6 mg/kg i.p.) while protecting 5-$HT_{2A/2C}$ receptors with ritanserin (see Experimental Procedures). Under these conditions, the 5-HT-enhancing effect of DOI did not differ from that in naïve rats ($n = 7$; Fig. 5*C*) but the suppressing effect of BAY × 3702 was totally abolished ($P < 0.0001$; Fig. 5*C*). In another group of rats we protected 5-HT_{1A} receptors from EEDQ by local WAY-100635 application (300 µM) using the same temporal schedule. In these rats, DOI elicited a very modest 5-HT increment (25 ± 13%) which clearly differed from controls ($P < 0.0001$) whereas BAY × 3702 30 µM was fully effective to reduce 5-HT release (~50% of the initial baseline; Fig. 5*C*).

We also inactivated the function of 5-HT_{1A} receptors with pertussis toxin (PTx) (see Experimental Procedures). Figure 5*D* shows the effect of the co-perfusion of DOI and BAY × 3702

(30 μM) in control rats (n = 7), sham-operated rats (n = 4) and in rats pretreated with PTx (1 μg) 2–3 days before (n = 5 and 7, respectively). The reducing effect of BAY × 3702, expressed as pre-drug values (1 h periods, fractions 14–16 versus 8–10) was 43 ± 4% in control rats, 46 ± 2% in sham-operated rats, 71 ± 5% and 84 ± 8% in PTx-treated rats respectively (inset in Fig. 5*D*) (P = 0.00115). PTx (3 days before) also attenuated the suppressing effect of BAY × 3702 (0.1 mg/kg, s.c.) on 5-HT release in mPFC (18% of baseline in controls, 55% in PTx-treated rats; n = 6 and 7, respectively; P < 0.0001; Fig. 5*E*), as this effect involves pre- and postsynaptic 5-HT_{1A} receptors (Casanovas *et al.*, 1999).

S-AMPA application (300 μM, n = 5) also elevated mPFC 5-HT release (P < 0.0001). The co-perfusion of 8-OH-DPAT (100 μM, n = 3) and BAY × 3702 (30 μM, n = 5) also attenuated the 5-HT-enhancing effect of S-AMPA (P < 0.0001; Fig. 5*F*).

In Vivo *Studies in WT and 5-HT_{1A} KO Mice*

We used 5-HT_{1A} KO mice (Parks *et al.*, 1998) to further verify the specificity of the responses elicited by 5-HT_{1A} agonists. Autoradiographic studies using [^{3}H]WAY-100635 showed the lack of hippocampal 5-HT_{1A} receptors in 5-HT_{1A} KO mice (Fig. 6*A*). The absence of functional 5-HT_{1A} receptors was determined using extracellular recordings of DR 5-HT neurons. Basal firing rate of DR 5-HT neurons in WT and KO mice was 1.6 ± 0.2 and 1.7 ± 0.2 spikes/s (n = 26 and 20, respectively; n.s.). 8-OH-DPAT (2–32 μg/kg i.v., cumulative doses) dose-dependently inhibited the firing rate of 5-HT neurons in WT mice (n = 10, P < 0.0001; Fig. 6*B*,*D*) with an ED_{50} value of 6.8 μg/kg but failed to significantly reduce cell firing in KO mice (n = 6; Fig. 6*C*,*D*). Similarly, the 5-HT reuptake inhibitor fluoxetine (2–32 mg/kg i.v., cumulative doses) suppressed 5-HT cell firing in WT mice (n = 4; ED_{50} = 7.6 mg/kg, P < 0.0001; Fig. 6*E*,*H*) but not in KO mice (n = 4; Fig. 6*F*–*H*). However, 5-HT neurons in KO mice were fully responsive to the α_1-adrenoceptor antagonist prazosin (up to 4.5 mg/kg i.v., ED_{50} = 2.7 mg/kg i.v., n = 5; Fig. 6*C*,*F*).

In microdialysis experiments we used 56 WT mice and 50 KO mice. Baseline 5-HT release in the mPFC was 14.1 ± 1.0 and 13.7 ± 1.0 fmol/fraction in WT and KO mice, respectively (n.s.). The perfusion of artificial CSF did not significantly alter the prefrontal 5-HT output in WT and KO mice (n = 6 and 4, respectively) although 5-HT release was less stable in KO mice (i.e. saw-tooth oscillations; Fig. 7*A*). The local application of DOI (30, 100, 300 and 500 μM) in the mPFC increased dose-dependently the 5-HT output to a similar extent in the mPFC of WT and KO mice (n = 5; Fig. 7*B*). The concentration of 100 μM was chosen for subsequent experiments. The perfusion of DOI 100 μM stably increased 5-HT release (Fig. 7*C*). In a separate report, we describe the mechanisms involved in the effect of DOI on 5-HT release in WT mice (Bortolozzi *et al.*, 2003). We used the data from some groups included in that study for statistical analysis of WT–KO differences. The perfusion of M100907 (300 μM) reversed the effect of DOI on 5-HT release in WT and KO mice (n = 5 and 7, respectively; P < 0.0001 time effect, non-significant differences between groups; Fig. 7*C*).

The perfusion of BAY × 3702 (30 μM) markedly reduced the 5-HT release in the mPFC of WT but not of KO mice (n = 5 and 10 respectively, P < 0.001; Fig. 7*D*). BAY × 3702 also reversed the 5-HT elevation induced by the local application of DOI in mPFC of WT but not KO mice (n = 8 each, P < 0.0003; Fig. 7*E*). Likewise, 8-OH-DPAT (100 μM) significantly reduced 5-HT release in WT but not KO mice (n = 6 and 4 respectively; P < 0.001; Fig. 7*D*) and reversed the DOI-induced facilitation of 5-HT release in WT but not KO mice (n = 5 and 4, respectively; data not shown).

As observed in rats (Fig. 5) the perfusion of S-AMPA in the mPFC of mice increased 5-HT release. This effect was fully counteracted by the co-perfusion of BAY × 3702 (30 μM) in WT (n = 5) but not KO mice (n = 7, P < 0.0001; Fig. 7*F*).

Discussion

The main findings of the present study are that (i) nearly 60% of the prefrontal cells contain the mRNA encoding 5-HT_{1A} and/or 5-HT_{2A} receptors, with a very high degree (80%) of co-localization, (ii) endogenous 5-HT modulates the activity of pyramidal neurons *in vivo* through these receptors, (iii) the response elicited by 5-HT (excitation or inhibition) possibly depends on the existence of precise projections from the raphe cells to areas of the pyramidal neurons rich in 5-HT_{2A} or 5-HT_{1A} receptors and (iv) drug-induced activation of 5-HT_{1A} and 5-HT_{2A} receptors in mPFC modulates the pyramidal output to the raphe nuclei – and possibly other subcortical areas – in an opposite manner.

Co-expression of 5-HT_{1A} and 5-HT_{2A} Receptors in Rodent Prefrontal Cortex

The high number of neurons expressing the 5-HT_{1A} and 5-HT_{2A} receptor mRNAs accords with the very high density of the respective proteins in rodent prefrontal cortex, particularly in its medial aspect. Previous studies revealed an overlap in the distribution of 5-HT_{1A} and 5-HT_{2A} receptors in prefrontal cortex (Pazos and Palacios, 1985; Pazos *et al.*, 1985; Pompeiano *et al.*, 1992, 1994) and suggested some coexistence in pyramidal neurons (Araneda and Andrade, 1991; Ashby *et al.*, 1994; Martín-Ruiz *et al.*, 2001). The present data indicate that the co-expression of both receptors is the general rule in the various prefrontal areas examined, except in layer VI, where 5-HT_{1A} receptor mRNA predominates. Notably, the prelimbic and infralimbic areas of the mPFC, which project to the midbrain serotonergic and dopaminergic nuclei (Hajós *et al.*, 1998; Peyron *et al.*, 1998; Carr and Sesack, 2000) contain many cells co-expressing both receptors. The vast majority of cells expressing 5-HT_{1A} or 5-HT_{2A} receptors also express vGLUT1 (N. Santana *et al.*, unpublished observations). A smaller percentage (~20%) of all GAD-expressing cells also express these receptors (N. Santana *et al.*, unpublished observations). Taking into account the percentage of GABA interneurons versus principal cells in the cortex (~15%; Beaulieu, 1993), it appears that most of 5-HT_{1A} and 5-HT_{2A} mRNAs are localized to pyramidal neurons.

We are unaware of similar data for other neurotransmitter receptors (e.g. dopaminergic) in prefrontal cortex. However, the large proportion of cells expressing 5-HT_{1A} or 5-HT_{2A} receptors suggest an important serotonergic control of prefrontal function in the rodent brain. Indeed, *in vitro* recordings have revealed that prefrontal pyramidal neurons respond to 5-HT applications with inhibitory and excitatory responses (see Introduction). Moreover, *in vivo* recordings of presumed (Hajós *et al.*, 2003) and identified pyramidal neurons in mPFC (Puig *et al.*, 2003; this work) indicates that these are very sensitive to the physiological activation of 5-HT neurons.

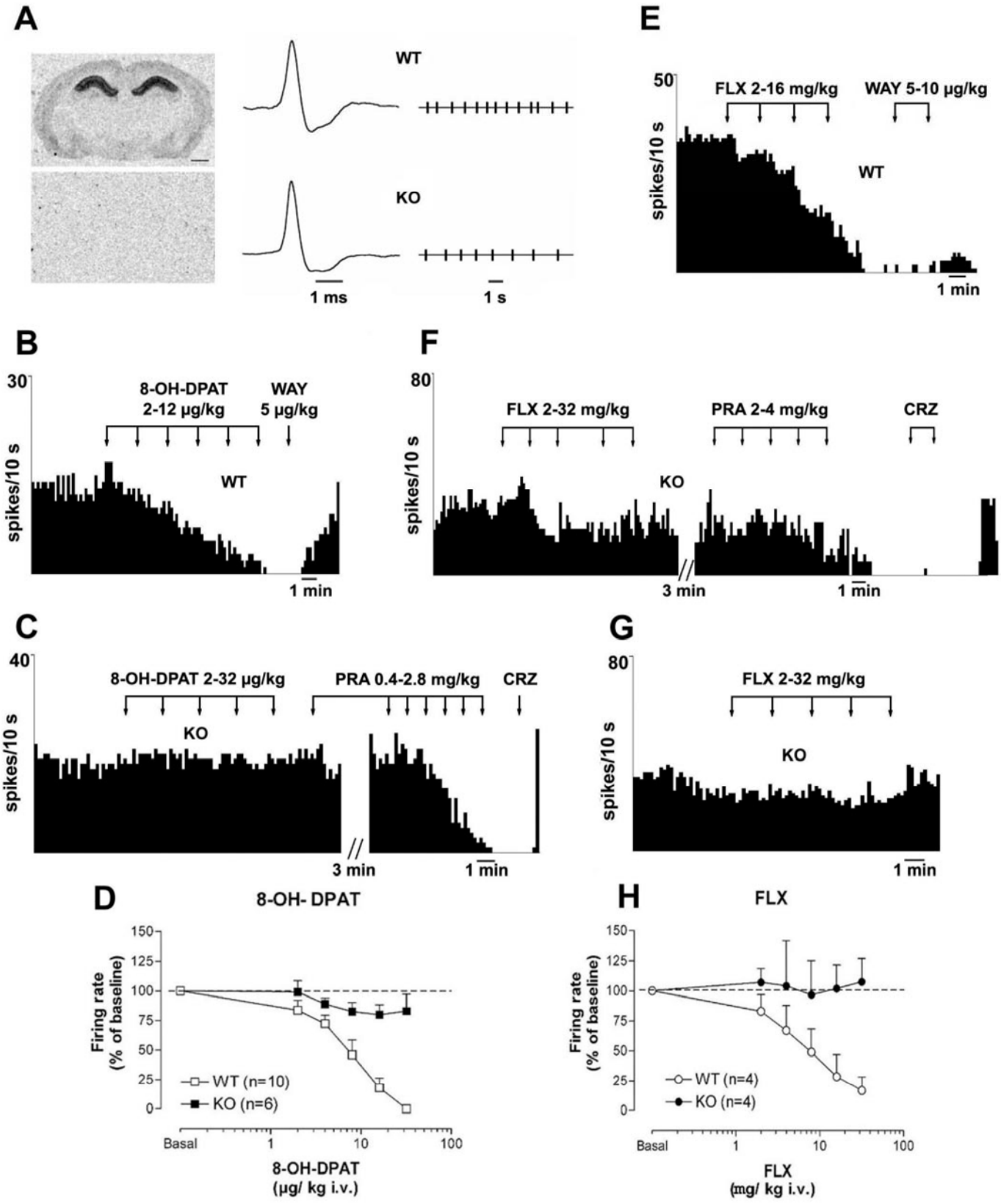

Figure 6. (*A*) Lack of hippocampal 5-HT_{1A} receptors in knockout [KO(–/–)] mice compared to wild-type (WT) mice, as labeled with [^{3}H]WAY-100635. Shown also in (*A*) are typical spikes and firing pattern recorded in DR 5-HT neurons of WT and KO mice. (*B–D*) Effects of i.v. 8-OH-DPAT administration on the firing rate of DR 5-HT neurons of WT (*B*) and KO mice (*C*). (*D*) Dose–response curve for 8-OH-DPAT in both mice groups. (*E–H*) Effects of fluoxetine on the firing of DR 5-HT neurons of WT (*E*) and KO mice (*F*, *G*). (*H*) shows the dose–response curve (WT and KO mice) for the effect of fluoxetine. 5-HT neurons in KO mice were fully responsive to the i.v. administration of the α_1-adrenoceptor antagonist prazosin (PRA). The subsequent i.v. administration of the α_1-adrenoceptor agonist cirazoline (CRZ) could reverse the prazosin-induced suppression of cell firing.

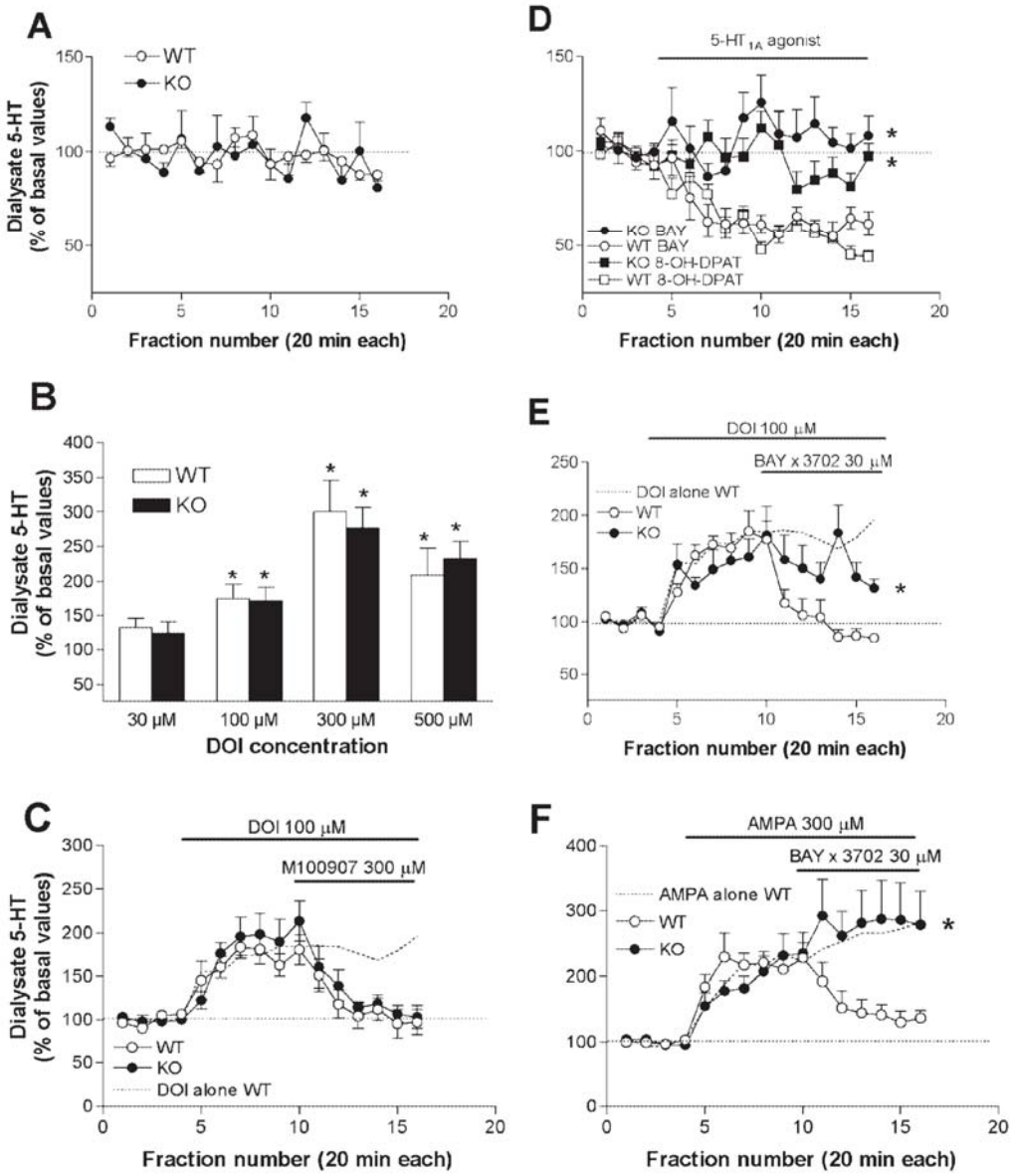

Figure 7. (*A*) The perfusion of artificial CSF did not alter the 5-HT release in mPFC of wild-type (WT) and 5-HT_{1A} receptor knockout [KO(–/–)] mice ($n = 6$ and 4, respectively). (*B*) The perfusion of the 5-$HT_{2A/2C}$ agonist DOI (30–500 µM) by reverse dialysis increased the 5-HT release in a bell-shaped manner both in WT and KO mice, with maximal effect at 300 µM ($n = 5$ each). *$P < 0.05$ versus baseline. (*C*) The co-perfusion of the 5-HT_{2A} receptor antagonist M100907 reversed the effect of DOI in WT and KO mice ($n = 5$ and 7, respectively). The dotted line shows the effect of DOI alone ($n = 11$). (*D*) The local application in mPFC of the 5-HT_{1A} agonists 8-OH-DPAT (100 µM) and BAY × 3702 (30 µM) reduced the 5-HT release in WT but not in KO mice ($n = 6$ and 4 for 8-OH-DPAT, and 5 and 10, for BAY × 3702). (*E*) The co-perfusion of BAY × 3702 30 µM reversed the 5-HT elevation induced by DOI 100 µM in the mPFC of WT but not KO mice ($n = 8$ each). (*F*) Similarly, the co-perfusion of BAY × 3702 30 µM antagonized the 5-HT elevation induced by S-AMPA 300 µM in the mPFC of WT ($n = 5$) but not in 5-HT_{1A} receptor KO mice ($n = 7$). *$P < 0.05$ versus WT.

Pyramidal Responses Elicited by Raphe Stimulation in Rats

Early microiontophoretic studies revealed predominantly inhibitory actions of 5-HT on cortical neurons (Jacobs and Azmitia, 1992). This effect may involve direct (5-HT_{1A}-mediated) and indirect (GABA-mediated) actions of 5-HT (Ashby *et al.*, 1989, 1990, 1994). *In vitro* intracellular recordings suggested that 5-HT_{1A} and 5-HT_{2A} receptors exert opposite effects on pyramidal excitability (see Introduction). Our *in vivo* observations indicate that 5-HT excites (via 5-HT_{2A} receptors) and inhibits (via 5-HT_{1A} receptors) pyramidal neurons in mPFC. All neurons recorded were activated antidromically from the DR or MnR, which supports the existence of strong reciprocal interactions between 5-HT neurons and 5-HT_{1A}/5-HT_{2A} receptor-expressing pyramidal neurons in mPFC. Moreover, in a previous study we observed that pyramidal neurons sensitive to the activation of 5-HT_{2A} receptors were also antidromically activated from the VTA (Puig *et al.*, 2003). Excitations were 5-HT_{2A} receptor-mediated since they were reversed by M100907, as recently reported (Puig *et al.*, 2003). Inhibitions lasted more than excitations and involved 5-HT_{1A} receptors, since they were reversed by WAY-100635. The earlier component of the inhibitions (not blocked by WAY-100635, even at high doses) may be due to an increase of GABA inputs mediated by the activation of 5-HT_{2A} or 5-HT_3 receptors in interneurons (Ashby *et al.*, 1989, 1990; Zhou and Hablitz,

1999). Also, since the recorded neurons were antidromically activated from the DR or MnR, it may be that antidromic stimulation enhanced prefrontal glutamate release from axon collaterals and, hence, an increase in local GABA inputs onto the recorded neurons. Moreover, Susan R. Sesack and associates have reported the presence of GABAergic projection neurons from the DR to the mPFC of the rat (Janowski and Sesack, 2002), which raises the possibility that the short latency/duration inhibitions not blocked by WAY-100635 may be due to a direct GABA synaptic input evoked by DR/MnR stimulation. Current work is examining this possibility. During the completion of the present report, Hajós *et al.* (2003) documented the existence of 5-HT_{1A} receptor-mediated inhibitions of presumed pyramidal neurons evoked by DR stimulation. However, unlike in the present report, DR-evoked inhibitions were fully blocked by WAY-100635. Differences in the location of the recorded neurons (more ventral in Hajós *et al.*, 2003) and/or strain of rats used may account for this distinct behaviour. Occasionally, WAY-100635 unveiled a prominent 5-HT_{2A}-mediated excitation (as in the example in Fig. 4*F*), suggesting that raphe stimulation simultaneously excited and inhibited the recorded neuron, although the inhibitory response prevailed.

5-HT_{2A} receptors are enriched in apical dendrites of pyramidal neurons (Willins *et al.*, 1997; Jakab and Goldman-Rakic, 1998; Cornea-Hébert *et al.*, 1999) and mediate the increase in pyramidal excitability induced by 5-HT application near the apical dendrites (Aghajanian and Marek, 1997). Yet, conflicting results have been reported for 5-HT_{1A} receptors, using two different antibodies. Azmitia *et al.* (1996) found a somatodendritic location for 5-HT_{1A} autoreceptors in raphe 5-HT neurons and a location in the axon hillock of cortical and hippocampal pyramidal neurons. This observation has been replicated by two other groups in primate and human brain (De Felipe *et al.*, 2001; David E. Lewis, personal communication). This localization is coincident with the unique cortical axo-axonic synapses such as those of GABAergic chandelier cells on the pyramidal axon hillock (Somogyi *et al.*, 1998; De Felipe *et al.*, 2001) and would assign a prominent inhibitory role to 5-HT_{1A} receptors in the control of pyramidal activity. On the other hand, a somatodendritic location of 5-HT_{1A} receptors in the DR and hippocampus has been reported using a different antibody (Kia *et al.*, 1996; Riad *et al.*, 2000). The non-synaptic nature of serotonergic transmission in mammalian cortex (Beaudet and Descarries, 1978; De Felipe and Jones, 1988) appears to be discordant with a putative presence of both receptors in the same areas of pyramidal neurons, since 5-HT released from nearby axons would indistinctly activate 5-HT_{1A} and 5-HT_{2A} receptors in the proximity of release sites. Indeed, the predominance of the inhibitory response, as observed when 5-HT is applied microiontophoretically or when enhancing cortical 5-HT release (Gartside *et al.*, 2000; Puig *et al.*, unpublished observations; see also Fig. 4*F*) suggests that 5-HT_{1A} receptors are located downstream of 5-HT_{2A} receptors in the process of spike generation. This would be consistent with a location of 5-HT_{2A} receptors on apical dendrites, as generally agreed, whereas 5-HT_{1A} receptors may be localized in cell bodies, basal dendrites and/or axon hillock. The segregation of both receptors in different compartments of the pyramidal neuron would permit that 5-HT axons terminating on (or passing near) apical dendrites would excite pyramidal neurons whereas those at lower cortical levels would result in inhibitions, as in the scheme shown in Figure 8. Additionally, it has been proposed that the activity of prefrontal pyramidal neurons is controlled by 5-HT via GABA interneurons expressing 5-HT_{2A} and 5-HT_3 receptors, located at different heights in the pyramidal tree (Jakab and Goldman-Rakic, 2000). Both proposals would be in agreement with the existence of excitatory and inhibitory inputs at different levels in cortical microcircuits regulating the output of pyramidal cells (Somogyi *et al.*, 1998).

A number of different observations appear to support the above view. First, cortical 5-HT axons differ in morphology, origin and regional brain targets (Kosofsky and Molliver, 1987; De Felipe and Jones, 1988; De Felipe *et al.*, 1991). Secondly, in rat somatosensory cortex (no such studies exist for prefrontal cortex), two dense plexuses of 5-HT axons innervate mainly layers I and Va. Between them, 5-HT axons run vertically (Blue *et al.*, 1988). More interestingly, vertical 5-HT axons run parallel to 5-HT_{2A} receptor-rich apical dendrites in rat cortex (Jansson *et al.*, 2001). Thirdly, although the synaptic relationships between 5-HT axons and 5-HT_{1A} receptors have not been studied in the rat cortex, 5-HT axons pass horizontally near 5-HT_{1A}- and $GABA_A$-receptor-rich areas in the pyramidal axon hillock in monkey and human cortex (De Felipe *et al.*, 2001). Finally, using stably implanted stimulating electrodes and switching the current between stimulation sites within the raphe, we were able to demonstrate that the response evoked in a given pyramidal neuron depends on the site of stimulation (although, as mentioned above, inhibitory responses predominate). In contrast, stimulation with twin pulses at the same stimulation current (a procedure that enhances cortical 5-HT release; Gartside *et al.*, 2000), increases the duration of the inhibitions but does not turn them into excitations as it could be expected if the response in the pyramidal cell would be solely determined by the relative affinity of 5-HT for 5-HT_{1A} and 5-HT_{2A} receptors (Puig *et al.*, in preparation). Overall, this suggests that the axons from different individual 5-HT neurons or neuronal clusters within the raphe may have specific projections to 5-HT_{1A}- or 5-HT_{2A}-receptor-rich areas of the pyramidal neuron. Indeed, further work using refined tracing techniques is required to test this hypothesis.

Opposite Effects of 5-HT_{1A} and 5-HT_{2A} Receptor Activation on the mPFC–Raphe Circuit

Pyramidal neurons in prellimibc and infralimbic mPFC project to raphe 5-HT neurons, as shown by anatomical studies (e.g. Peyron *et al.*, 1998) and by the generalized antidromic responses observed during DR/MnR stimulation (Puig *et al.*, 2003; this study). We therefore used this reciprocal connectivity (mPFC–raphe circuit) to examine the interactions between prefrontal 5-HT_{1A} and 5-HT_{2A} receptors, using the *in vivo* 5-HT release in mPFC as an indicator of the prefrontal influence onto ascending 5-HT neurons. The application of DOI in mPFC enhanced – and that of 5-HT_{1A} agonists reduced – the local 5-HT release in rat and mouse brain (Casanovas *et al.*, 1999; Celada *et al.*, 2001; Martín-Ruiz *et al.*, 2001; Bortolozzi *et al.*, 2003; Puig *et al.*, 2003). The effect of DOI was interpreted as resulting from the activation of descending pyramidal inputs onto 5-HT neurons because (i) systemic DOI administration elicited an overall 2.4-fold increase in the firing of pyramidal cells in the anterior cingulate and prelimbic cortices (an area sampled by the probe in microdialysis studies) which was reversed in most cases by M100907 (Puig *et al.*,

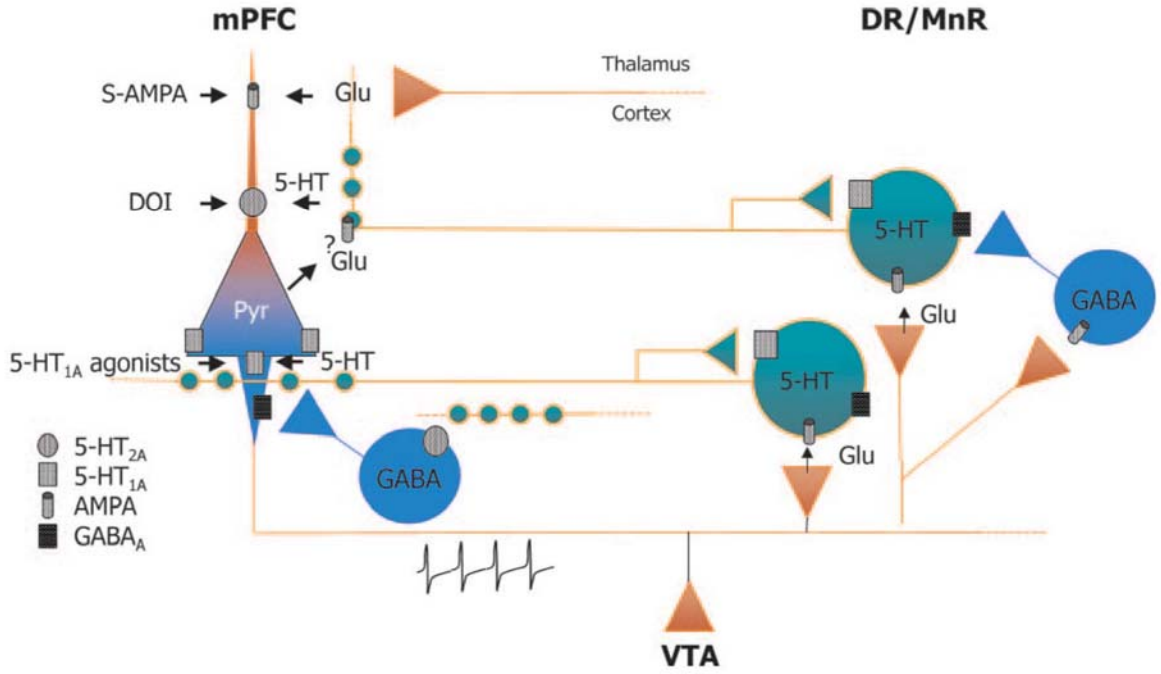

Figure 8. Schematic representation of the relationships between the mPFC and the DR involving 5-HT_{1A} and 5-HT_{2A} receptors. Endogenous 5-HT modulates pyramidal cell activity via 5-HT_{1A} and 5-HT_{2A} receptors. The latter are particularly enriched in apical dendrites of pyramidal neurons where they can facilitate AMPA inputs. A smaller population of 5-HT_{2A} receptors are expressed by large GABA interneurons. Pyramidal 5-HT_{1A} receptors may be localized in the axon hillock, together with $GABA_A$ receptors (Azmitia *et al.*, 1996; De Felipe *et al.*, 2001; David E. Lewis, personal communication) or in the somatodendritic compartment (Riad *et al.*, 2000). We suggest that the stimulation of the DR and MnR can excite or inhibit pyramidal neurons, depending on a precise topology between certain 5-HT neurons or neuronal clusters in the DR/MnR and 5-HT_{1A}- or 5-HT_{2A}-receptor-rich compartments, in agreement with anatomical studies showing an association between 5-HT axons and such receptor-rich areas (De Felipe *et al.*, 2001; Jansson *et al.*, 2001). Since the mPFC contains many neurons projecting to the DR, the pharmacological activation of 5-HT_{1A} and 5-HT_{2A} receptors modulates in an opposite way the terminal 5-HT release through changes in their activity. Although pyramidal neurons also project to midbrain GABA interneurons (particularly those in the infralimbic cortex; Varga *et al.*, 2001) and can inhibit 5-HT neurons (Celada *et al.*, 2001; Varga *et al.*, 2001), the overall influence of mPFC on 5-HT release appears to be excitatory, as judged from the effects of the pharmacological stimulation of prefrontal 5-HT_{1A} and 5-HT_{2A} receptors (Casanovas *et al.*, 1999; Celada *et al.*, 2001; Martín-Ruiz *et al.*, 2001; Bortolozzi *et al.*, 2003; this study). In addition to a distal effect (via DR) on ascending 5-HT neurons (Martín-Ruiz *et al.*, 2001), DOI could also increase local glutamatergic transmission by its excitatory action on pyramidal neurons (Puig *et al.*, 2003) whose collaterals might activate AMPA receptors putatively located on 5-HT terminals. 5-HT_{2A} receptors in pyramidal neurons can also control the activity of dopamine neurons in the ventral tegmental area (VTA) through descending excitatory axons (Carr and Sesack, 2000) since pyramidal neurons in mPFC sensitive to the activation of 5-HT_{2A} receptors also project to the VTA (Puig *et al.*, 2003).

2003) and (ii) local application of DOI in mPFC increased the firing rate of some 5-HT neurons in the DR (Martín-Ruiz *et al.*, 2001). The effect of DOI does not appear to involve thalamocortical afferents since both the DOI-induced pyramidal cell firing and mPFC 5-HT release are insensitive to extensive thalamic lesions (Puig *et al.*, 2003). Moreover, the increase in mPFC 5-HT release induced by thalamic disinhibition (to increase prefrontal AMPA inputs) was abolished by the activation of μ-opioid receptors by DAMGO yet the effect of DOI was insensitive (Puig *et al.*, 2003). Overall, this indicates that the effect of DOI is not mediated by an increase of glutamate release from thalamic afferents. However, we cannot disregard the possibility that DOI, by virtue of its overall enhancement of pyramidal cell firing, increases the excitatory input onto putative AMPA receptor on 5-HT terminals (see Fig. 8).

The stimulatory effect of local DOI application on prefrontal 5-HT release and its effects on pyramidal and 5-HT cell firing appear discordant with the reported inhibitory influence of the electrical stimulation of the mPFC on DR 5-HT neurons, which appears to involve both GABA interneurons and 5-HT_{1A} autoreceptors (Celada *et al.*, 2001; Varga *et al.*, 2001). One possibility to reconcile both sets of data is that pyramidal neurons in more ventral regions of the mPFC – i.e. infralimbic cortex, as examined in Varga *et al.* (2001) – preferentially innervated GABA interneurons within the DR. Indeed, small regional differences in the stimulation site appear to have opposite influence on the activity of *locus coeruleus* noradrenergic neurons (Sara and Hervé-Melville, 1995; Jodo *et al.*, 1998). Likewise, since only a proportion of mPFC neurons express 5-HT_{2A} receptors, it might be also possible that chemical and electrical stimulation of the mPFC differ in their overall effect on ascending 5-HT neurons.

Despite some of the above mechanisms remain to be fully clarified, microdialysis studies have shown that DOI enhances 5-HT release in mPFC whereas 8-OH-DPAT and BAY × 3702 reduce 5-HT release in the same area of the rat brain (Casanovas *et al.*, 1999; Celada *et al.*, 2001; Martín-Ruiz *et al.*, 2001; Puig *et al.*, 2003). We therefore conducted 5-HT_{2A}–5-HT_{1A} receptor interaction studies in rats to obtain a neurochemical correlate of the above electrophysiological data. Additionally, since these mechanisms involved in the DOI- and AMPA-evoked prefrontal 5-HT release appear to be substantially similar in rat and mouse brain (Bortolozzi *et al.*, 2003), we repeated these studies in WT and 5-HT_{1A} knockout mice to fully verify the specificity of the pharmacological responses elicited by 5-HT_{1A} agonists. The working hypothesis was that 5-HT_{1A} receptor activation would attenuate or cancel the increase in mPFC 5-HT release evoked by agents that increase the activity of pyramidal neurons, such as DOI and S-AMPA (Puig *et al.*, 2003).

The data obtained support this view. The activation of prefrontal 5-HT_{1A} receptors by selective agonists reversed the DOI- and S-AMPA-induced elevation in 5-HT release in rat and mouse mPFC. Conceivably, this effect was mediated by the 5-HT_{1A} agonist-induced hyperpolarization of pyramidal cells (Ashby *et al.*, 1990; Araneda and Andrade, 1991) projecting to raphe 5-HT neurons. This would oppose to the DOI- and S-AMPA-induced increase in pyramidal activity and, hence, the impulse flow along axons projecting to the DR/MnR and the terminal 5-HT release (Fig. 8). The action of DOI is mediated by postsynaptic 5-HT_{2A} receptors (Puig *et al.*, 2003). Likewise, a postsynaptic action of S-AMPA is also supported by the cancellation of its *in vitro* effects by 8-OH-DPAT in pyramidal neurons (Cai *et al.*, 2002). However, an additional presynaptic action of S-AMPA on 5-HT axons cannot be fully excluded (Whitton *et al.*, 1994), since AMPA receptors can be targeted to axon terminals at least *in vitro* (Schenk *et al.*, 2003). In any case, the 5-HT_{1A}-mediated reversal of the action of DOI and S-AMPA must necessarily involve postsynaptic 5-HT_{1A} receptors since these are not present on axon terminals (Azmitia *et al.*, 1996; Riad *et al.*, 2000).

The inhibitory action of 5-HT_{1A} agonists appeared to be exclusively mediated by 5-HT_{1A} receptors both in rats and mice since (i) it was common to five different agonists, (ii) it was prevented by the prior inactivation of 5-HT_{1A} receptors and by WAY-100635 application and (iii) it was totally absent in 5-HT_{1A} KO mice. Interestingly, the 5-HT reduction was more marked for BAY × 3702, even at the lower dose used (30 versus 100 μM of 8-OH-DPAT and 300 μM of the rest of agents) which supports a high intrinsic efficacy at postsynaptic 5-HT_{1A} receptors in cortex. The data obtained in 5-HT_{1A} knockout mice fully confirmed that BAY × 3702 and 8-OH-DPAT act exclusively at 5-HT_{1A} receptors to reduce 5-HT release in the experimental conditions used and validates the use of the such high nominal concentrations *in vivo*, necessary to elicit a substantial activation of receptors in the brain compartment when drugs are applied by reverse dialysis. Finally, the two strategies used in rats to inactivate the prefrontal 5-HT_{1A} receptors proved to be useful in attenuating or cancelling the 5-HT_{1A}-mediated response. Pertussis toxin has been used *in vivo* to inactivate pre- and postsynaptic 5-HT_{1A} receptors (Andrade *et al.*, 1986; Innis *et al.*, 1987). EEDQ has also been used to inactivate 5-HT_{1A} and other G-protein-coupled receptors (Gozlan *et al.*, 1994). The experimental approach used herein (selective local protection of 5-HT_{2A} receptors by ritanserin) permitted the preservation of the local effect of DOI while the response of BAY × 3702 was fully abolished. Conversely, the local protection of 5-HT_{1A} receptors by WAY-100635 during EEDQ administration preserved the inhibitory effect of BAY × 3702, further supporting that this action was mediated by 5-HT_{1A} receptors in the rat.

Functional Significance and Therapeutic Implications

The present data help to clarify the circuitry linking two important brain areas in psychiatric illnesses — prefrontal cortex and raphe nuclei — and suggest that 5-HT_{1A} and 5-HT_{2A} receptors play a prominent role in prefrontal function because of their presence in a high proportion of pyramidal neurons and the opposite modulation of their activity. On account of previous and present observations, we suggest that different 5-HT neurons or neuronal clusters in the DR/MnR specifically project to pyramidal compartments rich in 5-HT_{1A} and 5-HT_{2A} receptors. This precise connectivity would allow for a fine tuning of prefrontal pyramidal activity by 5-HT neurons, giving also a physiological meaning to the co-expression of both receptors.

From a therapeutic perspective, atypical antipsychotic drugs are preferential 5-HT2A receptor antagonists (Meltzer, 1999) and behave as direct (aripiprazole, ziprasidone) or indirect 5-HT_{1A} agonists (Ichikawa *et al.*, 2001). This suggests that they may act predominantly on cortical neurons expressing both receptors, reducing the excitatory output to subcortical structures putatively involved in psychotic positive symptoms (e.g. nucleus accumbens and related structures). Likewise, mPFC pyramidal neurons project to the ventral tegmental area (Carr and Sesack, 2000), including those sensitive to 5-HT_{2A} receptor stimulation (Puig *et al.*, 2003). Therefore, it is conceivable that 5-HT_{2A} receptor blockade exerted by atypical antipsychotic drugs may contribute to their therapeutic efficacy by reducing the activity of prefrontal inputs onto mesolimbic dopaminergic neurons without the extensive blockade of postsynaptic dopamine receptors exerted by classical antipsychotics, responsible for the extrapyramdial side effects.

Notes

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