ARTICULOS PUBLICADOS EN REVISTAS ELECTRÓNICAS:

TRABAJO 1:

Control of dorsal raphe serotonergic neurons by the medial prefrontal cortex: Involvement of serotonin$_{1A}$, GABA$_A$, and glutamate receptors.

Pau Celada, M. Victoria Puig, Josep M. Casanovas, Gemma Guillazo y Francesc Artigas (2001). *The Journal of Neuroscience* 21: 9917-9929

Texto completo: **http://www.jneurosci.org/cgi/content/full/21/24/9917**

TRABAJO 2:

Control of serotonergic function in medial prefrontal cortex by serotonin-$_{2A}$ receptors through a glutamate-dependent mechanism.

Raúl Martín-Ruiz*, M. Victoria Puig*, Pau Celada, David A. Shapiro, Bryan L. Roth, Guadalupe Mengod y Francesc Artigas (2001)

The Journal of Neuroscience 21: 9856-9866 (*primeros autores)

Texto completo: **http://www.jneurosci.org/cgi/content/full/21/24/9856**

TRABAJO 3:

***In vivo* modulation of the activity of pyramidal neurons in the rat medial prefrontal cortex by 5-HT_{2A} receptors: Relationship to thalamocortical afferents.**

M. Victoria Puig, Pau Celada, Llorenç Díaz-Mataix y Francesc Artigas (2003) *Cerebral Cortex* 13:870-882.

Abstract:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=pubmed&dopt=Abstract&list_uids=12853374

Texto completo (sólo accesible a suscriptores de la revista *Cerebral Cortex*):

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TRABAJO 4:

Co-expression and i*n vivo* interaction of serotonin$_{1A}$ and serotonin$_{2A}$ receptors in pyramidal neurons of prefrontal cortex

Mercè Amargós-Bosch*, Analía Bortolozzi*, M. Victoria Puig*, Jordi Serrats, Albert Adell, Pau Celada, Miklos Toth, Guadalupe Mengod y Francesc Artigas (2004). *Cerebral Cortex* 14:281-299 (*primeros autores).

Abstract:

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=pubmed&dopt=Abstract&list_uids=14754868

Texto completo (sólo accesible a suscriptores de la revista *Cerebral Cortex*):

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Modulation of the activity of pyramidal neurons in rat prefrontal cortex by raphe stimulation *in vivo*: Involvement of serotonin and GABA

M. Victoria Puig, Francesc Artigas, Pau Celada

Department of Neurochemistry, Institut d' Investigacions Biomèdiques de Barcelona (CSIC), IDIBAPS, 08036 Barcelona, Spain

Serotonin is involved in psychiatric disorders exhibiting abnormal prefrontal cortex (PFC) function (e.g., major depression, schizophrenia). We examined the effect of the stimulation of the dorsal and median raphe nuclei (DR and MnR, respectively) on the activity of PFC neurons. Electrical stimulation of DR/MnR inhibited 66% (115/173) of pyramidal neurons in the medial PFC (mPFC). The rest of the cases exhibited orthodromic excitations, either pure (13%) or preceded by short-latency inhibitions (20%). Excited neurons had a lower pre-stimulus firing rate than those inhibited. Excitations evoked by MnR stimulation had a shorter latency than those evoked by DR stimulation. WAY-100635 (5-HT_{1A} antagonist) and the selective $GABA_A$ antagonist picrotoxinin partially antagonized DR/MnR-evoked inhibitions, suggesting the involvement of 5-HT_{1A}- and $GABA_A$-mediated components. The presence of a direct DR/MnR-mPFC GABAergic component is suggested by the short latency of evoked inhibitions (9±1 ms), faster than those evoked in the secondary motor area (20±3 ms) and that of antidromic spikes evoked by DR/MnR stimulation in mPFC pyramidal neurons (15±1 ms). Stimulation of the DR/MnR with paired pulses enhanced the duration of inhibitions and turned some excitations into inhibitions. Thus, the DR/MnR control the activity of mPFC pyramidal neurons *in vivo* in a complex manner, involving 5-HT-mediated excitations and GABA- and 5-HT-mediated inhibitions.

Keywords: 5-HT_{1A} receptors, 5-HT_{2A} receptors, dorsal raphe, medial prefrontal cortex, median raphe.

Introduction

The ascending monoaminergic systems of the brainstem innervate the prefrontal cortex (PFC) in the mammalian brain and play an important role in the control of higher brain functions (Gronewegen and Uyllings, 2000; Fuster, 2001). In particular, dopamine is critically involved in working memory and cognition through a complex control of the activity of pyramidal neurons (Glowinski et al., 1984; Williams and Goldman-Rakic, 1995; Goldman-Rakic, 1996; Yang and Seamans, 1996; Robins, 2000; Tzschentke, 2001; O' Donnell, 2003; Wang et al., 2003). There is also growing evidence that the serotonergic pathways originating in the dorsal and median raphe nuclei (DR and MnR, respectively) may play an important role in prefrontal function. Thus, the PFC of the rodent, primate and human brains contains several 5-HT receptors, with a particular abundance of the 5-HT_{1A} and 5-HT_{2A} subtypes (Pompeiano et al., 1992, 1994; Morales and Bloom, 1997; Hall et al., 2000; Talvik-Lotfi et al., 2000; Martinez et al., 2001; Arango et al., 2002). 5-HT_{2A} receptors in dorsolateral PFC are involved in working memory (Williams et al., 2002) and recent work associates allelic variants of this receptor with memory capacity in humans (De Quervain et al., 2003). Hallucinogens like LSD or DOI are 5-HT_{2A} receptor agonists whereas atypical antipsychotics like clozapine are 5-HT_{2A} receptor antagonits (Kroeze and Roth, 1998; Meltzer, 1999). On the other hand, 5-HT_{1A} agonists display anxiolytic/antidepressant activity in animal models (de Vry, 1995) whereas 5-HT_{1A} receptor antagonists reverse drug-induced cognitive deficits (Harder and Ridley, 2000; Mello e Souza et al., 2001; Misane and Ogren, 2003).

5-HT and selective receptor agonists modulate the excitability of cortical neurons and their discharge rate through the activation of several receptor subtypes, namely 5-HT_{1A}, 5-HT_{1B}, 5-HT_2 and 5-HT_3 (Ashby et al., 1989; Araneda and Andrade, 1991; McCormick et al., 1993; Tanaka and North, 1993; Aghajanian and Marek, 1997; Arvanov et al., 1999; Zhou and Hablitz, 1999; Férézou et al., 2002; Puig et al., 2003). *In vitro* and *in vivo* studies suggest that 5-HT_{1A} and 5-HT_{2A} receptors are key players and exert opposite effects on the excitability and firing activity of pyramidal neurons in the medial PFC (mPFC) (Araneda and Andrade, 1991; Ashby et al., 1994; Aghajanian and Marek, 1997; Puig et al., 2003; Amargós-Bosch et al., 2004). The activation of 5-HT_{1A} receptors in PFC cortex hyperpolarizes pyramidal neurons whereas that of 5-HT_{2A} receptors results in neuronal depolarization, reduction of the afterhyperpolarization and increase of excitatory postsynaptic currents (EPSCs) and of discharge rate (Araneda and Andrade, 1991; Tanaka and

North, 1993; Aghajanian and Marek, 1997, 1999; Newberry et al., 1999; Zhou and Hablitz, 1999; Puig et al., 2003; Amargós-Bosch et al., 2004). 5-HT can also activate excitatory receptors (5-HT_{2A} and 5-HT_3) in GABA interneurons (Morales and Bloom, 1997; Jakab and Goldman-Rakic, 2000) to increase a synaptic GABA input onto pyramidal neurons (Tanaka and North, 1993; Zhou and Hablitz, 1999; Férézou et al., 2002)

However, despite the wealth of *in vitro* studies on the actions of 5-HT on cortical neurons, there is little information on the relative balance of inhibitory and excitatory responses elicited by endogenous 5-HT *in vivo*. Nearly 60 % of the neurons in the PFC of the rat and mouse express the mRNAs of 5-HT_{1A} and/or 5-HT_{2A} receptors, with a high degree of co-expression (nearly 80% in most PFC areas; Amargós-Bosch et al., 2004). The vast majority of these mRNAs co-localized with vGluT1 mRNA, suggesting a major location in pyramidal neurons (Santana et al., in press). Consistent with these data, the electrical stimulation of the DR can inhibit (via 5-HT_{1A} receptors) or excite (via 5-HT_{2A} receptors) the pyramidal neurons in the mPFC (Puig et al., 2003; Amargós-Bosch et al., 2004), although the reasons determining the nature of the response (i.e., inhibitory or excitatory) are not fully understood. Here we examined the responses elicited by the physiological stimulation of the DR and MnR in pyramidal neurons of the cingulate and prelimbic areas of the mPFC which, in turn, project to the raphe nuclei, and compared these responses with those elicited in the secondary motor area (MOs) in the vicinity of the mPFC.

Materials and Methods

Animals

A total of 74 male albino Wistar rats weighing 250-320 g at the time of experiments were used (Iffa Credo, Lyon, France). They 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 experimental procedures were approved by a local Institutional Animal Care and Use Committee. Stereotaxic coordinates were taken from bregma and duramater according to the atlas of Paxinos and Watson (1998). Additionally, we used the brain maps (CD-edition; Swanson, 1998) for nomenclature of the cortical areas.

Single unit recordings

We examined the responses elicited in pyramidal neurons of the mPFC by the electrical stimulation of the DR and/or MnR in anesthetized rats. 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. Typically, recordings were made between 10 and ~45 min after additional doses of anesthetic to avoid the effects of peak concentrations of chloral hydrate during recordings. Body temperature was maintained at 37 ºC 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 sol ution (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 either of these coordinates, within the DR (AP –7.8, L 0, DV –6.5; and AP –7.3, L –2.2 with a lateral angle of 20º, DV –6.6 mm) or the MnR (AP –7.8, L 2.0 with a lateral angle of 13º, DV –8.8 mm). These angles resulted in the tip of the electrodes at DV –6.2 and –8.6 mm, respectively in the vicinity of the midline. In most experiments, two electrodes were implanted, one in DR (either location) and another one in MnR. After each implant, the electrodes were secured to the skull with glue and dental cement. Constant current electrical stimuli were generated with a Grass stimulation unit S-48 connected to a Grass SIU 5 stimulus isolation unit. Stimulating current was typically between 0.1-2 mA, 0.2 ms square pulses at 0.9 Hz. In some experiments, we recorded the same pyramidal neuron in mPFC after the sequential stimulation of the DR/MnR with single and twin pulses while keeping current intensity (0.5-1.7 mA) and frequency (0.9 Hz). Twin pulses were delivered 7 ms apart. Twin pulse stimulation of the DR has been shown to increase the cortical 5-HT release compared with single pulse stimulation (Gartside et al., 2000).

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 Sci. Inst., Tokyo, Japan). Microelectrodes were filled with 2M NaCl. Typically, *in vitro* impedance was between 4-10 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 +3.2-3.4, L -0.5 to –1.0, DV –1.0 to -4.0 below the brain surface. We systematically confirmed that only a single pyramidal neuron was recorded by a) identification by antidromic activation from DR and/or MnR and b) collision extinction with spontaneously occurring spikes (Fuller and Schlag, 1976). Neurons without antidromic activation or without spontaneous firing activity were not considered. Additionally, recordings were made in neurons of the secondary motor area (MOs; Swanson, 1998), at AP+3.2-3.4, L –2.0-2.6, DV between 0.8 and 1.4 mm (see figure 1 for localization of the recording areas in PFC). After recording the effects of DR/MnR stimulation on pyramidal activity (see below), we administered the 5-HT_{1A} receptor antagonist WAY-100635 or the $GABA_A$ receptor antagonist picrotoxinin (both from Sigma/RBI) and further post-drug recordings were made to evaluate the actions of these drugs on DR/MnR-evoked inhibitory responses. WAY-100635 and picrotoxinin were dissolved in saline at the appropriate concentrations and injected (1 ml/kg) through the femoral vein. Finally, to determine the latency of antidromic spikes traveling along serotonergic axons projecting to mPFC we recorded serotonergic neurons in the DR during electrical stimulation of the mPFC. The methods are described in full in Celada et al., (2001).

At the end of the experiments, rats were killed by an overdose of anesthetic. The placement of the stimulating electrodes was verified histologically. Rats were transcardially perfused with saline followed by 10% formalin solution (Sigma). Brains were post-fixed, sagitally sectioned (80 µm) and stained with Neutral Red. The data from rats with electrodes implanted outside the DR or MnR were not used.

Data and statistical analysis

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). For a better precision of the onset of inhibitory responses, latencies were calculated with a bin width of 1 ms. Orthodromic excitations elicited spikes with short and variable latencies and a post-stimulus firing rate superior to the mean pre-stimulus firing rate plus two times the standard deviation during at least four bins (Hajós et al., 1998). Antidromic spikes had a fixed latency and were produced by the electrical stimulation of axons of mPFC pyramidal neurons projecting to the DR and MnR (Celada et al., 2001). Inhibitions were defined by a total cessation of spikes with respect to the pre-stimulus value for at least four successive bins (Hajós et al., 1998). The onset of the inhibition was defined as the last bin containing a spike whereas the end of the inhibition was defined as the first of four bins equal to or above the pre-stimulus value. The magnitude of the inhibition was calculated as percentage of firing vs. the pre-stimululs (200 ms) firing rate. Drug effects were calculated by comparing 2-min PSTHs at basal and post-drug periods. Data are expressed as the mean ± SEM. Statistical analysis was carried out using independent and paired Student's *t*-tests. Statistical significance has been set at the 95% confidence level (two tailed).

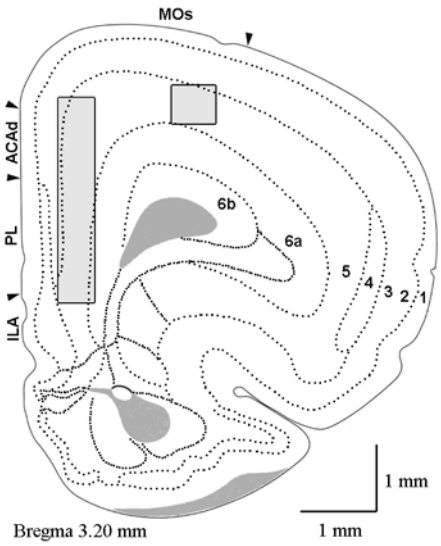

Figure 1. Schematic localization of the recording area in the dorsal anterior cingulate area (ACAd) and prelimbic area (PL) of the medial prefrontal cortex. Shown is also the localization of recordings in the secondary motor area (MOs). In both areas recordings were made mainly in layer V neurons. Section taken from *Brain Maps*, Swanson (1998).

Results

Responses in mPFC pyramidal neurons elicited by DR/MnR stimulation

We performed 173 experiments in which we examined the effect of the stimulation of the DR or MnR on pyramidal neurons of the cingulate and prelimbic areas of the PFC. In 115/173 cases (66%) pure inhibitory responses were recorded, while in 23/173 cases (13%), pure orthodromic excitations were observed. The rest of responses (35/173, 20%) were biphasic, with an orthodromic excitation preceded by an

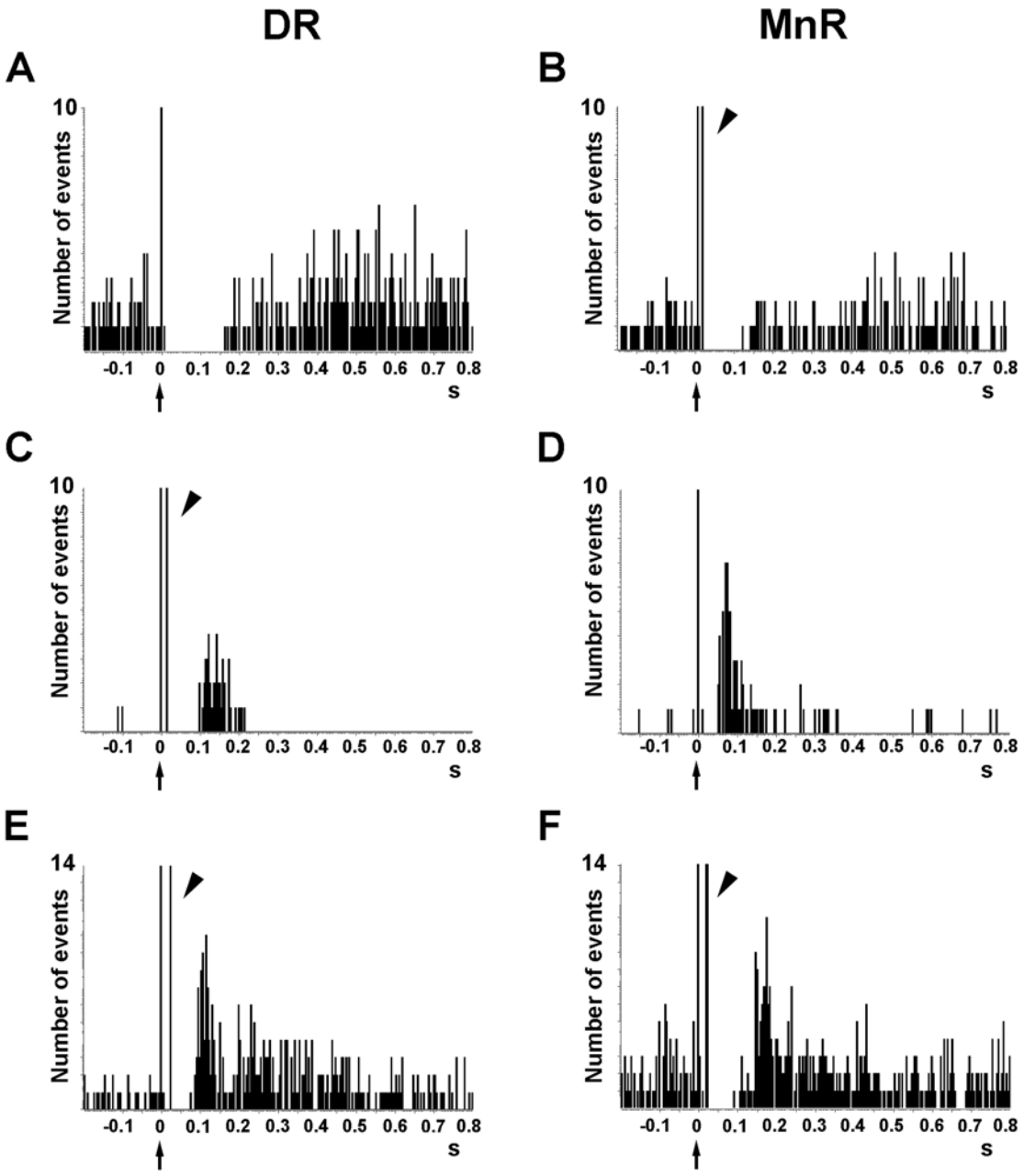

Figure 2. Representative examples of the responses evoked in pyramidal neurons of the anterior cingulate and prelimbic areas of the mPFC by the electrical stimulation of the DR and MnR (see Methods). A and B show short latency long duration inhibitory responses. C and D show pure excitatory responses, which are often seen in pyramidal neurons with a low firing rate. These orthodromic excitations have a similar duration after DR or MnR stimulation but the latency is significantly lower after MnR stimulation, as in the example seen in the figure (see also Table 1). E and F show orthodromic excitations preceded by short latency inhibitions (biphasic responses). Note the presence of antidromic spikes in the units recorded (B, C, E, F). The units in A, D were antidromically activated at currents higher than those used to evoke a pyramidal response. Each PSTH consists of 110 triggers (2 min). Bin size is 4 ms. The arrow in the abcissa denotes the stimulus artifact (time = 0). The arrowheads indicate antidromic spikes.

Table 1. Characteristics of the pure inhibitory and excitatory responses evoked in mPFC pyramidal neurons by raphe stimulation

mPFC responses	DR stimulation	MnR stimulation
Inhibition	***n = 70***	***n = 45***
Latency (ms)	9.9 ± 0.8	8.6 ± 1
Duration (ms)	166 ± 10	153 ± 16
Pre-stimulus firing (spikes/s)	2.18 ± 0.28	2.53 ± 0.49
Post-stimulus Firing (spikes/s)	0.36 ± 0.08	0.35 ± 0.07
% firing during inhibition [a]	12 ± 1	14 ± 2
Orthodromic excitation	***n =13***	***n = 10***
Latency (ms)	75 ± 11	45 ± 5*
Duration (ms)	120 ± 27	76 ± 14
Pre-stimulus Firing (spikes/s)	0.58 ± 0.34	1.23 ± 0.50
Post-stimulus Firing (spikes/s)	4.66 ± 1.40	7.12 ± 1.95
Succes rate (%)[a]	48 ± 15	47 ± 12

**p < 0.05 vs. DR*

For consistence with previous reports (Puig et al., 2003; Amargós-Bosch et al., 2004), we give the intensity of the response as percent of pre-stimulus firing for inhibitions and as success rate for excitations (i.e., percent concordance with each stimulus delivered).

inhibition of short latency and duration. Figure 2 shows representative examples of the three different types of responses obtained after stimulation of the DR and MnR.

Inhibitions had a latency of 9 ± 1 ms and a duration of 161 ± 9 ms (n = 115). Firing rate during the inhibition was reduced on average to 13% of pre-stimulus firing rate. Table 1 shows the characteristics of these inhibitions, classified according to the stimulation site (DR or MnR). There were no significant differences in the characteristics of the inhibitions between those elicited by DR stimulation (n = 70) or MnR stimulation (n = 45). In 26 cases we examined the response of the same pyramidal neuron to the stimulation of the DR and MnR in rats implanted with two stimulating electrodes. When a response was triggered in a pyramidal neuron by DR or MnR stimulation, it was recorded for 2-4 min and then the stimulation current was switched to the stimulation electrode implanted in the other nucleus and the response of the unit was recorded for a similar period of time. The statistical analysis (paired *t*-test) of this subpopulation of neurons showed no significant differences between the effects of the stimulation of both raphe nuclei.

Some of these neurons (n = 15) were antidromically activated from both the DR and the MnR at the currents used, showing that some pyramidal neurons can simultaneously control the activity of both serotonergic nuclei. In this subgroup, the latency of the antidromic spikes was 16 ± 1 ms and 14 ± 1 ms from the DR and MnR, respectively (non-significant difference).

Pyramidal neurons excited by DR/MnR stimulation (n = 23) were located at the same DV coordinates than those inhibited, i.e., near 2.5 mm below brain surface. However, the units inhibited by the DR/MnR stimulation had a higher pre-stimulus firing rate than those excited (2.3 vs. 0.9 spikes/s; n = 115 and 23, respectively; $p < 0.0006$; Table 1). The duration, pre- and post-stimulus firing rates and success rate of the orthodromic excitations did not differ between stimulation site (DR vs. MnR). However, unlike the inhibitions, the latency of the excitations was significantly lower when stimulating the MnR (45 ± 5 vs. 75 ± 11 ms, n = 10 and 13, respectively; $p < 0.05$, Student's *t*-test) (Table 1).

A subgroup of pyramidal neurons exhibited biphasic responses to DR/MnR stimulation. In 35 experiments, the orthodromic excitations were preceded by short latency inhibitions. Table 2 shows the characteristics of these responses. There were no significant differences between the responses elicited by DR or MnR stimulation, with the exception of the duration of the excitations, which was slightly but significantly greater when the DR was stimulated (96 ± 6 ms for the DR vs. 71 ± 7 ms for the MnR; n = 22 and 13, respectively; $p < 0.02$; Student's *t*-test). The success rate was also similar for the DR- or MnR-induced excitations (58 ± 8% vs. 55 ± 8%, respectively).

Table 2. Characteristics of the biphasic responses evoked in mPFC pyramidal neurons by raphe stimulation

Biphasic responses	DR stimulation	MnR stimulation
	n = 22	***n = 13***
Pre-stimulus firing (spikes/s)	1.34 ± 0.23	2.37 ± 0.57
Inhibition		
Latency (ms)	10.4 ± 1.1	9.1 ± 1.3
Duration (ms)	79 ± 6	67 ± 10
Post-stimulus firing (spikes/s)	0.17 ± 0.05	0.33 ± 0.1
% firing during inhibition	10 ± 2	11 ± 2
Orthodromic excitation		
Latency (ms)	90 ± 7	88 ± 11
Duration (ms)	95 ± 6	71 ± 7*
Post-stimulus firing (spikes/s)	6.14 ± 0.83	8.05 ± 1.15
Success rate (%)	58 ± 8	55 ± 8

*p < 0.02 vs. DR

Table 3. Comparison of pure and biphasic responses in mPFC neurons

	Pure inhibitions	Biphasic inhibitions	Pure excitations	Biphasic excitations
DR				
n	70	22	13	22
Latency (ms)	9.9 ± 0.8	10.4 ± 1.1	75 ± 11	90 ± 7
Duration (ms)	166 ± 10	79 ± 6**	120 ± 27	95 ± 6
% response[a]	12 ± 1	10.4 ± 2	48 ± 15	58 ± 8
			(1928 ± 477)	(612 ± 69**)
MnR				
n	45	13	10	13
Latency (ms)	8.6 ± 1	9.1 ± 1.3	45 ± 5	88 ± 11*
Duration (ms)	153 ± 16	67 ± 10**	76 ± 14	74 ± 9
% response[a]	14 ± 1.7	11 ± 2.2	47 ± 12	55 ± 8
			(1055 ± 282)	(432 ± 59*)

**p < 0.01 vs. pure responses
*p < 0.03 vs. pure responses

[a]For consistence with previous reports (Puig et al., 2003; Amargós-Bosch et al., 2004), we give the intensity of the response as percent of pre-stimulus firing for inhibitions and as success rate for excitations (i.e., percent concordance with each stimulus delivered). The values in parentheses are the intensity of excitations calculated as that of inhibitions (i.e., percent of pre-stimulus firing during the time of the excitation).

A comparison of the characteristics between the pure and biphasic inhibitory responses revealed a similar latency but significantly lower duration of the biphasic responses (79 ± 6 vs. 166 ± 10 ms for the DR stimulation; 67 ± 10 vs. 153 ± 16 ms for the MnR stimulation; $p < 0.01$ in both cases). Biphasic excitations had a similar duration but slightly longer latency (90 ± 7 vs. 75 ± 11 ms for the DR, n.s.; 88 ± 11 vs. 45 ± 5 ms for the MnR, $p < 0.004$), i.e., as if the preceding inhibition had delayed the excitation in neurons showing a biphasic response (Table 3).

As seen in Table 1, the characteristics of the inhibitory and excitatory responses did not differ between stimulation sites, with the exception of the latency of the orthodromic excitations, lower for MnR stimulation. When the type of response elicited (pure excitations vs. inhibitions) were compared depending on the stimulation site, a Chi-square analysis revealed no overall differences between the stimulation of the DR at AP -7.8 mm and that at the MnR (also at an AP

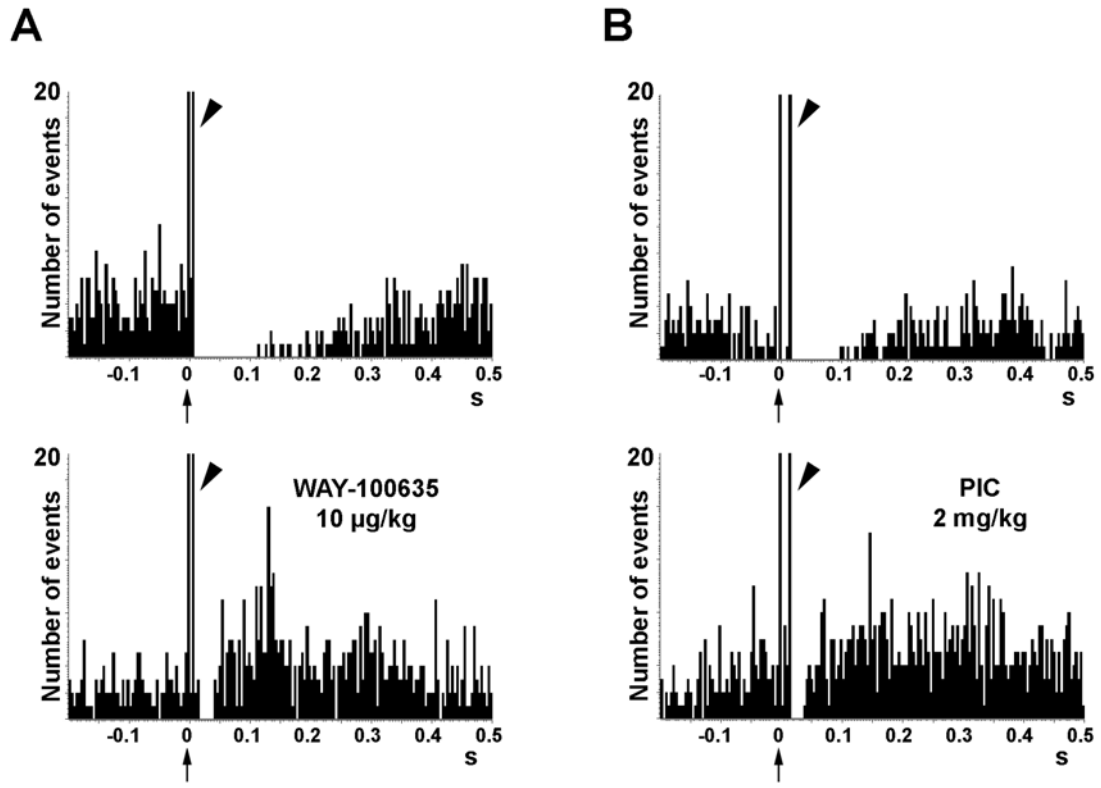

Figure 3. Antagonism of the DR/MnR-evoked inhibitory responses in mPFC by the 5-HT_{1A} receptor antagonist WAY-100635 (WAY) and the $GABA_A$ receptor antagonist picrotoxinin (PIC). Upper panels correspond to PSTHs obtained in basal conditions and lower panels, to those after drug treatment. The response in A was obtained by DR stimulation at 1 mA and shows an antidromic spike at 10 ms. This inhibitory response had a latency of 9 ms. The firing rate, expressed as percentage of pre-stimulus value, was 19% in basal conditions and 215% after WAY-100635 administration. The inhibitory response of this unit was particularly sensitive to WAY-100635 administration. On average, WAY-100635 administration did not reverse the inhibitory response between 0 and 69 ms (n = 8; Amargós-Bosch et al., 2004). The response in B was obtained by DR stimulation at 2 mA and shows an antidromic spike at 14 ms. This inhibitory response had a latency of 13 ms. The firing rates were 27% and 153% of the pre-stimulus value in basal conditions and after picrotoxinin, respectively. Note the overall increase in firing rate after picrotoxinin administration. Each PSTH consists of 110 triggers. Bin size is 4 ms.

coordinate of -7.8 mm). The corresponding ratios between inhibitions and pure orthodromic excitations were 70/13 for the DR and 45/10 for the MnR ($p = 0.86$). However, the stimulations performed in the DR at a more rostral coordinate (AP -7.3 mm) yielded a significantly greater proportion of excitations than those at AP -7.8, both in DR and MnR (4/8 vs. 70/13, $p < 0.001$; 4/8 vs. 45/10, $p < 0.002$).

Pharmacological characterization of DR/MnR-elicited responses in mPFC

The pyramidal excitations induced by the electrical stimulation of the DR/MnR were reversed by the treatment with the selective 5-HT_{2A} receptor antagonist M100907 (Puig et al., 2003; Amargós-Bosch et al., 2004). Likewise, inhibitions were partly blocked by WAY-100635 administration (10-60 µg/kg i.v.) (Amargós-Bosch et al., 2004) (Fig. 3A). An early component of the inhibitions (up to 69 ± 32 ms; Amargós-Bosch et al., 2004) could not be blocked by WAY-100635. The failure to block this earlier component cannot be ascribed to an insufficient dose since higher doses of WAY-100635 (e.g., 100-200 µg/kg i.v.) were even less effective, perhaps as a result of some partial agonist activity of this agent at these doses (Martin et al., 1999). We therefore reasoned that the WAY-100635-insensitive, low latency inhibitory responses might be due to an increased GABAergic input onto pyramidal neurons, resulting from various sources, such as the activation of 5-HT receptors in local interneurons or the activation of direct GABAergic inputs from the DR (see Discussion). Likewise, since pyramidal neurons in mPFC project to the raphe nuclei, a GABAergic component might also result from stimulus-evoked antidromic spikes in collaterals of pyramidal axons impinging on local GABAergic interneurons. We preliminarily examined the

presence of these possible GABA inputs by various means.

The presence of a GABAergic component is supported by the antagonism of the DR/MnR-induced inhibitions by the $GABA_A$ receptor antagonist picrotoxinin (1-2.5 mgkg. i.v; 2.0 mg/kg average; n = 4) (Fig. 3B). Picrotoxinin significantly reduced the duration of the inhibition elicited by DR/MnR stimulation from 114 ± 10 ms to 32 ± 4 ms ($p < 0.004$). Higher picrotoxinin doses could not be used due to its reversal of the anesthesia, which prevented to examine the effects of a full $GABA_A$ receptor blockade on these responses.

Responses evoked by DR/MnR stimulation in the secondary motor area (MOs)

We examined the effects of the stimulation of the DR and MnR on neurons of MOs, which, in common with the cingulate and prelimbic areas (Santana et al., in press). Recordings were of the PFC, contains a large abundance of 5-HT_{1A} and 5-HT_{2A} receptors in pyramidal neurons made at DV -0.8 to -1.4 mm, corresponding mainly to layer V. A total of 65 recordings were made, of which only one was a pure orthodromic excitation and five were biphasic responses. The excitations in these biphasic responses had a greater latency than the 5-HT_{2A}-mediated excitations observed in mPFC (306 ± 33 ms; range 232-424 (n = 5) vs. 75 ± 11 ms for DR- and 45 ± 5 ms for MnR-evoked excitations). The rest were inhibitions, whose characteristics are shown in Table 4.

Figure 4 shows representative PSTHs of neurons in mPFC and MOs inhibited by the stimulation of DR and MnR. Only two antidromic responses were observed.

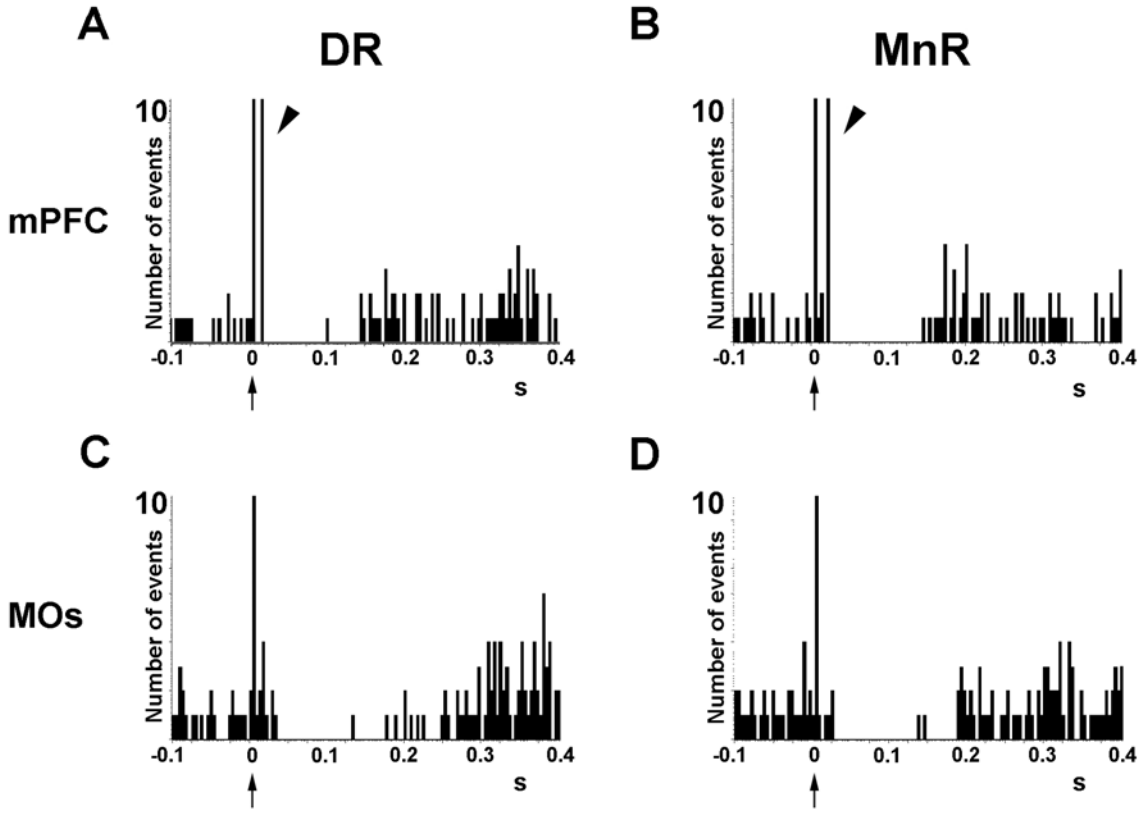

Figure 4. Comparison of the inhibitory responses evoked in mPFC (A, B) and MOs (C, D) by the electrical stimulation of the DR and MnR. Characteristics are as follows: A) Stimulation at 2 mA; latency 4 ms; duration 140 ms; 5% of pre-stimulus firing, B) Stimulation at 1 mA; latency 11 ms; duration 148 ms; 0% of pre-stimulus firing, C) Stimulation at 1 mA; latency 31 ms; duration 232 ms; 19% of pre-stimulus firing, D) Stimulation at 1 mA; latency 20 ms; duration 156 ms; 3% of pre-stimulus firing. Note the antidromic spikes (arrowheads; latency 13 and 18 ms in A and B, respectively) in the units recorded in mPFC (but not MOs), consequence of the reciprocal connectivity of the mPFC and the raphe nuclei (only two cells out of a total of 65 in MOs were found to be antidromically activated from the raphe nuclei; see Fig. 5C). For a better visualization of the inhibitions, PSTHs have been drawn between –100 and +400 ms. Each PSTH consists of 110 triggers. Bin size is 4 ms.

Table 4. Comparison of the characteristics of inhibitory responses in mPFC and secondary motor area (MOs)

	DR stimulation		MnR stimulation	
	mPFC	***MOs***	***mPFC***	***MOs***
	(n = 70)	*(n = 30)*	*(n = 45)*	*(n = 29)*
Latency (ms)	9.9 ± 0.8*	20.8 ± 2.9	8.6 ± 1*	20.4 ± 3.3
Duration (ms)	166 ± 10	184 ± 13	153 ± 16	161 ± 15
Pre-stimulus firing (spikes/s)	2.18 ± 0.28	2.52 ± 0.41	2.53 ± 0.49	2.06 ± 0.22
Post-stimulus Firing (spikes/s)	0.36 ± 0.08	0.46 ± 0.11	0.35 ± 0.07	0.26 ± 0.05
% firing during inhibition	12 ± 1	13 ± 2	14 ± 2	10 ± 2

*p < 0.003 vs. MOs

Onset of inhibitory responses in mPFC and MOs

When comparing the latencies of inhibitory responses we observed a marked difference between both areas: 21 ± 3 in MOs vs. 9 ± 1 ms in mPFC, n = 59 and 115, respectively; p < 0.0001) (Fig. 4). This difference was also statistically significant when considering the inhibitions evoked by the DR or MnR independently (Table 4). The rest of characteristics (duration, percent of basal firing, etc.) were comparable in both recording areas.

The latency of inhibitions in mPFC was significantly lower than that of a) antidromic spikes evoked in DR 5-HT neurons by mPFC stimulation (24 ± 1 ms;), b) the antidromic spikes evoked in mPFC pyramidal neurons by DR/MnR stimulation (15 ± 1 ms) and c) the inhibitions evoked by DR/MnR stimulation in the MOs (21 ± 3 ms) (Table 5). Moreover, the latencies of inhibitions in the two identified projection neurons found in MOs were 48 and 20 ms, above the latencies of the respective antidromic spikes, 7 and 9 ms, respectively (Fig. 5).

We conducted 9 additional experiments in which a higher number of triggers was used per each PSTH (342 ± 66 on average, corresponding to 6.3 ± 1.2 min per each PSTH). This procedure would drastically reduce the possibility that the lower latency in mPFC were due to a random lack of spikes around the stimulus artifact and antidromic spikes. The latency of antidromic spikes was 15 ± 1 ms (n = 9) and that of the inhibition was 13 ± 1 ms (n = 9) (p < 0.0006, paired Student's *t*-test). Figure 5 shows two representative examples.

Effects of WAY-100635 on inhibitory responses in mPFC and MOs

A second difference between the inhibitory responses in mPFC and MOs was the sensitivity to 5-HT_{1A} receptor blockade with WAY-100635. In the mPFC, an earlier component (up to ~70 ms) remained insensitive to WAY-100635 (Amargós-Bosch et al., 2004). However, in MOs, inhibitions were more sensitive to WAY-100635. Of the six units examined, three inhibitions were partially reversed with 20-30 µg/kg WAY-100635 (from 163 ± 17 to 111 ± 23 ms) whereas the other three were fully blocked with 40-80 mg/kg WAY-100635 (from 205 ± 54 ms to 0 ms) (Fig. 6).

Single vs. twin pulse stimulation

Previous data (Amargós-Bosch et al., 2004) indicate that pyramidal neurons in the mPFC can respond with excitations or inhibitions depending on the stimulation site in the raphe nuclei. This suggests that, despite 5-HT_{1A} and 5-HT_{2A} receptors are largely co-expressed in pyramidal neurons, certain neuronal subgroups

Table 5. Comparison of the latencies of antidromic and orthodromic responses in the mPFC-DR/MnR circuit

	Latency (ms)	n
Antidromic responses		
DR 5-HT neurons (from mPFC)	24 ± 1	25
Pyramidal neurons (from DR/MnR)	15 ± 1	95
DR/MnR-evoked inhibitions		
mPFC	9 ± 1*	115
MOs	21 ± 3	59

*p < 0.002 vs. the rest of values

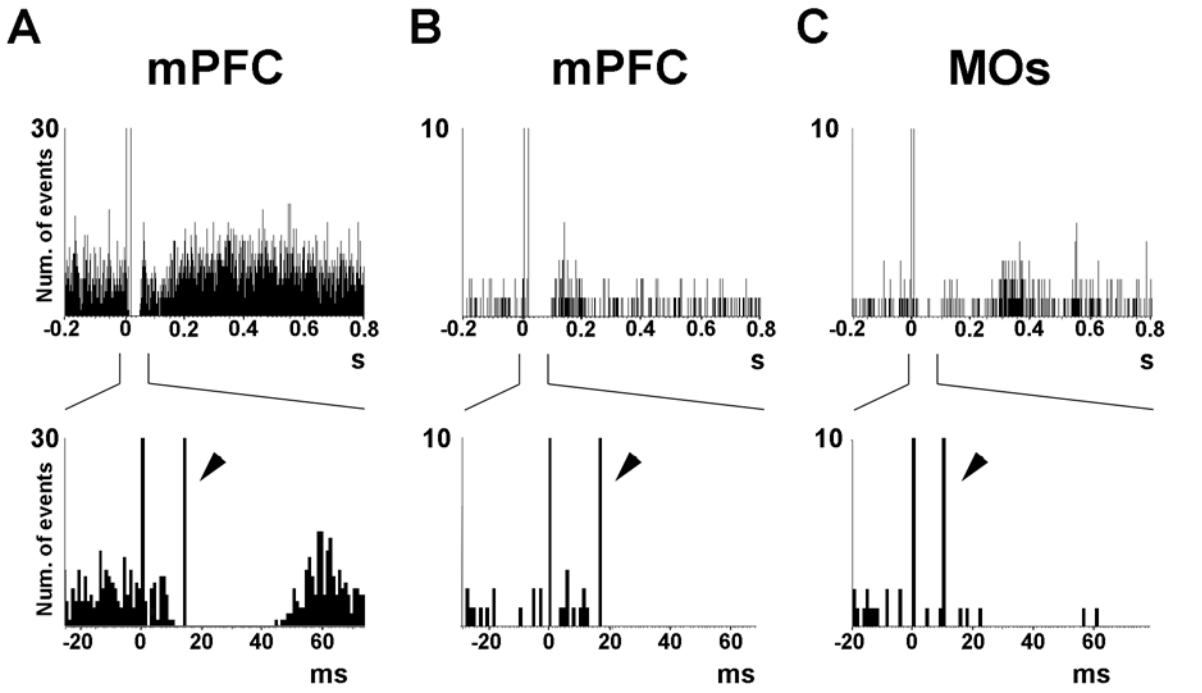

Figure 5. PSTHs of a midbrain raphe-evoked inhibitions in mPFC (A, B) and MOs (C) (lower panels show an enlargement of the time period around the stimulus). A) The stimulation of the DR at 1 mA evoked a short latency, short duration inhibition in mPFC (32 ms; 8% of pre-stimulus firing rate). The latency of the inhibition was 11 ms whereas the antidromic spike (arrowhead) had a latency of 15 ms. PSTH made of 718 triggers (~13 min recording). B) Short latency inhibition evoked in mPFC by DR stimulation at 1 mA (duration 78 ms, 3% of pre-stimulus firing rate). The latency of the inhibition was 12 ms and that of the antidromic spike was 15 ms. PSTH made of 617 triggers (~11 min). C) Inhibition evoked in an identified projection neuron in MOs by MnR stimulation at 1 mA (duration 257 ms, 38% of pre-stimulus firing rate). Despite this neuron projected to midbrain, as denoted by the antidromic spike (latency 9 ms), the latency of the inhibition was 19 ms. PSTH made of 102 triggers (~2 min). Note the absence of spikes before the antidromic spike in the inhibitions recorded in pyramidal neurons of the mPFC, which precludes that the onset of these inhibitory responses is triggered by antidromic invasion of pyramidal collaterals and further activation of local GABA interneurons. In contrast, the two projection neurons found in MOs (out of 65) had a latency greater than the antidromic spike, as in the example shown in C. .Bin size is 1 ms.

within the raphe complex may project to 5-HT_{1A} or 5-HT_{2A} receptor-rich areas in pyramidal neurons. In support of this view, here we observed that a more rostral location of the stimulating electrode within the DR resulted in a higher proportion of excitations in mPFC. However, since the affinity of 5-HT for 5-HT_{2A} receptors is lower than for 5-HT_{1A} receptors (Peroutka and Snyder, 1979; Hoyer et al., 1985) the type of response could also be determined by the concentration of 5-HT reached in mPFC after raphe stimulation. It should be noted that the mean currents of inhibitory and excitatory responses were 1.20 ± 0.05 mA (n = 115) and 1.20 ± 0.13 mA (n = 23), respectively. Therefore, we performed a series of experiments in which the same pyramidal neurons in mPFC were recorded after stimulation of the DR/MnR with single and twin pulses while keeping stimulation frequency (0.9 Hz) and intensity. A total of 32 experiments were performed. Sixteen stimulations were performed in the DR (-7.8 mm) which yielded 7 inhibitions, 3 pure excitations and 6 biphasic responses after single pulse stimulation. Twin pulse stimulation markedly enhanced the duration of the inhibitions (Table 6) and converted two pure excitations and 2 biphasic responses into inhibitions (Figure 7). The rest of responses were unaltered. Of the sixteen single pulse stimulations performed in the MnR, 12 resulted in inhibitions and 4 in biphasic responses. When the same neurons were recorded after twin pulse stimulation, the inhibitory responses were enhanced and 2 of the biphasic responses were converted into inhibitions (Table 6, Figure 7).

We examined the effect of WAY-100635 administration on the inhibitions evoked by twin pulse stimulation (n = 5). The pre-stimulus firing was 1.6 ± 0.4 spikes/s, the latency of the inhibitory response was 11 ± 1 ms and the stimulation, the duration of the inhibition duration was 180 ± 76 ms.

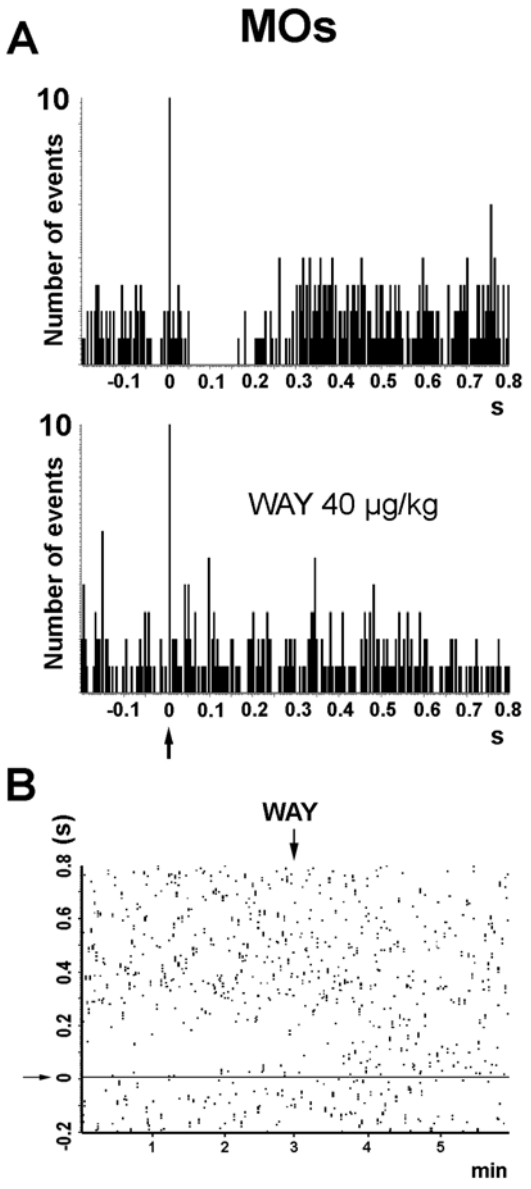

Figure 6. Effect of 5-HT_{1A} receptor blockade on a MnR-evoked inhibition in MOs (1 mA). A) PSTHs in basal conditions (upper panel) and after WAY-100635 administration (40 µg/kg i.v.) (lower panel). Inhibition latency: 46 ms, duration: 160 ms, 13% of pre-stimulus firing during inhibition. B) A raster display shows the inhibition and the rapid effect of WAY-100635 administration (arrow). The horizontal line in B corresponds to the vertical line in A and denote the stimulus artifact. Note the full blockade of the MnR-evoked inhibition by WAY-100635. Each PSTH corresponds to 110 triggers. Bin size is 4 ms.

Upon twin pulse increased up to 407 ± 82 ms ($p < 0.01$ vs. single pulse). The administration of WAY-100635 (40-80 µg/kg i.v.) significantly reduced the duration of the inhibition evoked by twin pulse stimulation to 237 ± 67 ms ($p < 0.003$). Likewise, the magnitude of the inhibition was reduced from 93 ± 2% to 19 ± 2% ($p < 0.02$). When considering the earlier part of the inhibition (not blocked by WAY-100635; up to 237 ± 67 ms), the change was non-significant (from 93 ± 2% to 88 ± 7%). As observed with single pulse stimulations, WAY-100635 could not fully abolish the inhibitory response in mPFC evoked by twin pulse stimulation of the DR (Fig. 8)

Discussion

The present study confirms and extends previous *in vivo* observations on the reciprocal control of the PFC and the midbrain raphe nuclei, which are involved in psychiatric diseases like major depression, schizophrenia or some anxiety disorders. Indeed, the DR/MnR project to several rat cortical areas, with an enrichment in the frontal pole (Azmitia and Segal, 1978; O'Hearn and Molliver, 1984; Blue et al., 1988). On the other hand several groups have reported that the mPFC projects to the raphe nuclei (Aghajanian and Wang, 1977; Sesack et al., 1989; Takagishi and Chiba, 1991; Hajós et al., 1998; Peyron et al., 1998) and the electrical stimulation of the mPFC exerts a profound influence on DR 5-HT neurons (Hajós et al., 1998; Celada et al., 2001).

The mean firing rate of the pyramidal neurons found in this study was similar to that found in other studies recording PFC cells extracellularly (Ceci et al., 1993; Hajós et al., 2001) but lower than in some studies using intracellular recordings. Thus, Lewis and O'Donnell (2002) and Trantham et al., (2002) reported mean values of ~4 spikes/s for pyramidal neurons in the same area. The range of values in these studies (0-28 spikes/s) suggests that different pyramidal types were recorded. Indeed, cells with a regular discharge pattern are the majority (~70%) and have a firing rate <1 spike/s, lower than that of burst-firing neurons when recorded *in vitro* (Dégenètais et al., 2002). Moreover, there seems to be a relationship between firing pattern and area of projection (e.g. PFC cells projecting to the nucleus accumbens exhibit a burst-firing mode; Yang et al., 1996). Hence, it cannot be excluded that the antidromic identification from midbrain in the present study may have resulted in a selection of slowly, regular firing pyramidal cells. Additionally, methodological differences between extra- and intracellular recordings may also contribute to this difference.

The present observations agree with previous *in vitro* observations on the control of the activity of PFC neurons (see Introduction). Earlier *in vivo* observations indicated an inhibitory effect of DR

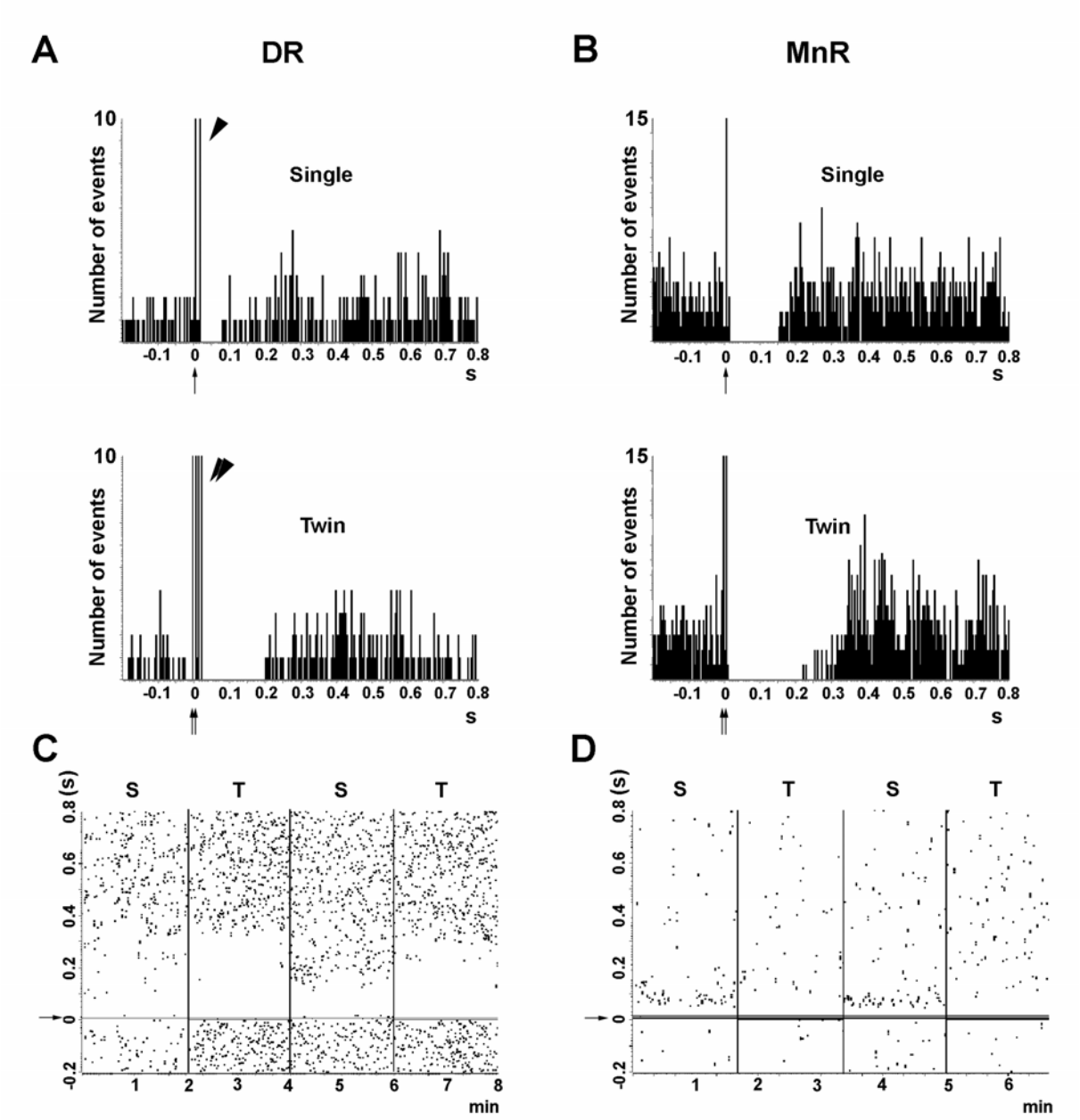

Figure 7. Enhancement of the DR- and MnR-evoked inhibitions in mPFC by twin pulse stimulation. Twin pulses were given 7 ms apart. The pyramidal neuron in A was inhibited by stimulation of the DR at 1.7 mA (latency 19 ms, duration 70 ms, 13% of pre-stimulus firing during inhibition). Twin pulse stimulation at the same intensity evoked a longer duration response (181 ms, 0% pre-stimulus firing; see lower panel in A).. Similarly, the unit in B was inhibited by MnR stimulation at 1 mA (latency 14 ms, duration 170 ms, 8% of pre-stimulus firing during inhibition). Twin pulse stimulation evoked a longer duration response (318 ms, 11% pre-stimulus firing; see lower panel in B). Each PSTH consists of 120 triggers. Bin size is 4 ms. Arrows denote the stimulus artifact and arrowheads show the presence of antidromic spikes. C and D are raster displays showing the reversibility of the responses after single (S) and twin (T) pulse stimulation. C shows the enhancement of an inhibitory response after DR stimulation (1 mA) by twin pulse stimulation. This unit was antidromically activated from MnR at 1 mA. D shows the abolishment of an excitation in a biphasic response by twin pulse stimulation in the MnR (1 mA, latency of antidromic spike = 9 ms).

Table 6. Effect of single and twin pulse stimulation on DR/MnR-evoked inhibitions in mPFC pyramidal neurons

Inhibitory responses in mPFC	DR stimulation *(n = 7)*		MnR stimulation *(n = 12)*	
	Single	***Twin***	***Single***	***Twin***
Duration (ms)	178 ± 56	378 ± 91**	153 ± 25	313 ± 36**
Pre-stimulus firing (spikes/s)	1.45 ± 0.39	1.68 ± 0.46	3.27 ± 1.22	3.03 ± 1.23
Post-stimulus Firing (spikes/s)	0.29 ± 0.12	0.1 ± 0.04**	0.49 ± 0.16	0.29 ± 0.15**
% firing during inhibition	15 ± 5	7 ± 3*	15 ± 3	7 ± 2*

*p < 0.03 vs. single pulses
**p < 0.008 vs. twin pulses

and MnR stimulation on rat prefrontal neurons (Mantz et al., 1990). More recent studies show that the electrical stimulation of the DR inhibits -via 5-HT_{1A} receptors- and excites -via 5-HT_{2A} receptors- pyramidal neurons in the rat mPFC (Puig et al., 2003; Amargós-Bosch et al., 2004). Also, a recent study reported 5-HT_{1A} receptor-mediated inhibitory responses in putative pyramidal neurons of the infralimbic area after DR/MnR stimulation (Hajós et al., 2003). These *in vivo* observations are consistent with the high density of these serotonergic receptors in rat mPFC (Pompeiano et al., 1992, 1994; López-Giménez et al., 1997). Nearly half of the neurons in PFC co-express 5-HT_{1A} and 5-HT_{2A} receptor mRNAs (Amargós-Bosch et al., 2004). To a large extent, these receptor mRNAs are present in cells also expressing vGLUT1 mRNA, which suggests a predominant pyramidal localization (Santana et al., in press). Light and electronic microscope studies have shown a preferential localization of 5-HT_{2A} receptors in apical dendrites and cell bodies of cortical pyramidal neurons (Jakab and Goldman-Rakic, 1998, 2000; Jansson et al., 2001; Martín-Ruiz et al., 2001; Miner et al., 2003; but see Cornéa-Hébert et al., 1999). However, conflicting results have been reported for 5-HT_{1A} receptors. Using different antibodies, some groups have reported a homogenous distribution in pyramidal neurons (Kia et al., 1996; Riad et al., 2000) while others have shown a preferential localization in the axon hillock of cortical and hippocampal pyramidal neurons (Azmitia et al. 1996; De Felipe et al., 2001; Czyrack et al., 2003; David E. Lewis, personal communication).

Responses evoked by DR/MnR stimulation in PFC neurons

Three types of responses were observed in mPFC: pure inhibitory, excitatory and biphasic, with a predominance of the former responses. Inhibitions appear to be mediated by two main components, a) a 5-HT_{1A} receptor-mediated, WAY-100635-sensitive inhibition, and b) a $GABA_A$ receptor-mediated, picrotoxinin-sensitive inhibition. Additionally, part of these inhibitions may be mediated by the after-hyperpolarization period (Yang et al., 1996) evoked by the antidromic spike in mPFC pyramidal neurons, which may perhaps explain the inability of WAY-1001635 and picrotoxinin to fully suppress the DR/MnR-evoked inhibitions.

On the other hand, pure excitations have been shown to be mediated by the activation of 5-HT_{2A} receptors (Puig et al., 2003; Amargós-Bosch et al., 2004). Serotonergic neurons contain vGluT3 (Gras et al, 2002; Herzog et al., 2004) and can make glutamatergic synapses *in vitro* (Johnson, 1994). Therefore, the possibility exists that DR/MnR stimulation may have evoked glutamate-mediated excitations. However, this seems unlikely in our experimental conditions since these excitations were blocked by the selective 5-HT_{2A} antagonist M100907. Also, their latency and duration was greater than that expected from a glutamatergic input, even taking into account the slower conduction velocity of serotonergic neurons (Maurice et al., 1998; Celada et al., 2001).

Intriguingly, the observed proportion of inhibitory and excitatory responses is discordant with the very large (~80%) co-localization of 5-HT_{1A} and 5-HT_{2A} receptor mRNAs in pyramidal neurons of several PFC areas, such as the dorsal anterior cingulate and prelimbic areas, where recordings have been made (Amargós-Bosch et al., 2004). This inconsistency cannot be explained by an incomplete translation of the 5-HT_{2A} receptor mRNA into the corresponding protein, since the rat mPFC contains a very high receptor density, as labeled with the selective antagonist [^{3}H]MDL 100907 (López-Giménez et

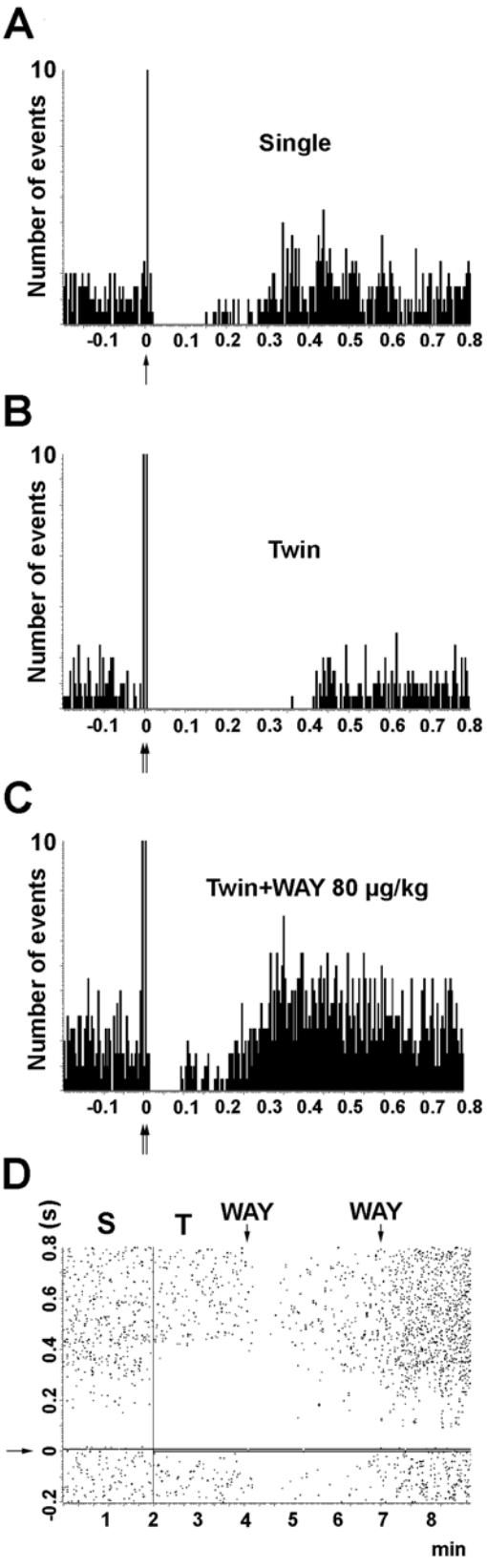

Figure 8. Enhancement of an inhibitory response in a pyramidal neuron of the mPFC by twin pulse stimulation of the DR (1 mA; the neuron was antidromically activated from DR at 2 mA with a latency of 17 ms). Basal inhibition is shown in A (latency 12 ms, duration 282 ms, 18% of pre-stimulus firing during inhibition). Twin pulse stimulation enhanced the duration of the inhibition up to 412 ms (3% of pre-stimulus firing). Note the two stimulus artifacts (7 ms apart; indicated by arrows in the abcissa). The panel in C shows the partial reversal of the effect of twin pulse stimulation by the 5-HT_{1A} receptor antagonist WAY-100635 (80 µg/kg i.v.) which reduced the duration of the inhibition to 268 ms. Each PSTH is composed of 110 triggers. Bin size = 4 ms. D is a raster display of the PSTHs shown in A-C. The time of administration of WAY-100635 (WAY) is shown by vertical arrows (40 µg/kg i.v. and 80 µg/kg i.v. cumulative doses).

al., 1997). The preferential inhibitory action of 5-HT was also observed in early microiontophoretic and stimulation studies (Ashby et al., 1994; Mantz et al., 1990; see Jacobs and Azmitia, 1992 for review) and may perhaps reflect the localization of 5-HT_{1A} receptors in the pyramidal axon hillock (see above). Such a localization, coincident with the cortical GABAergic axo-axonic synapses between chandelier cells on the pyramidal axon hillock (Somogyi et al., 1998; De Felipe et al., 2001), would assign a prominent inhibitory role to 5-HT_{1A} receptors in the control of pyramidal activity, as observed in the present study. Yet this interpretation must await the settling of the existing controversy on the cellular localization of the 5-HT_{1A} receptors.

A second, possibly $GABA_A$ receptor-mediated, inhibitory component was involved in the DR/MnR-evoked inhibitions of mPFC-pyramidal cells. Three different sources of GABA might account for these results. First, 5-HT has been shown to activate excitatory 5-HT_{2A} and 5-HT_3 receptors in mPFC GABAergic interneurons, thus increasing a GABAergic input onto pyramidal neurons (Ashby et al., 1989, 1990; Tanaka and North, 1993; Zhou and Hablitz, 1999; Férézou et al., 2002; Puig et al., submitted for publication). Second, due to the reciprocal anatomical connectivity of the mPFC and the raphe nuclei, the stimulation of descending pyramidal fibers projecting to the DR/MnR may result in antidromic invasion of pyramidal collaterals in mPFC and the subsequent activation of local GABA inputs onto the recorded pyramidal neurons. Third, the stimulation of the DR/MnR may stimulate non-serotonergic inhibitory afferents to the

mPFC. While not discarding the first two possibilities, the present data support the latter possibility. Hence, 20% in the DR and 40% in the MnR of cortically-projecting cells and one third of raphe-cortical axons are non-serotonergic (O'Hearn and Molliver, 1984; Kosofsky and Molliver, 1987). More recent studies have also identified these DR-containing projection cells (Li et al., 2001) and the presence of a GABAergic projection from the DR to the mPFC has been described (Jankowski and Sesack, 2002). This GABAergic projection would be analogous to that existing from the ventral tegmental area to the mPFC, as evidenced by electrophysiological and anatomical studies (Pirot et al., 1992; Carr and Sesack, 2000). Interestingly, in the former study, the stimulation of the ventral tegmental area evoked a subgroup of inhibitory responses in pyramidal neurons of the cingulate and prelimbic areas with latencies $\leq$8 ms, in analogy with those found herein.

We show here that the electrical stimulation of the DR/MnR evokes a short latency inhibitory response in pyramidal neurons that cannot be accounted for by the latency of serotonergic axons. On the other hand, this early inhibitory component seems unlikely to be due to antidromic invasion of the mPFC and further activation of local GABA neurons because the latency of antidromic spikes evoked in pyramidal cells was significantly greater than the latency of DR/MnR-evoked inhibitions (see Table 5). Interestingly, the inhibitory responses evoked in the MOs, which contains a density of cells expressing 5-HT_{1A} receptors comparable to that in mPFC (Amargós-Bosch et al., 2004) did not show this early component (latency of 21 ± 3 ms in MOs vs. 9 ± 1 ms in mPFC), a difference that cannot be explained by the short distance between both recording areas. On the other hand, the inhibitory responses in MOs were more sensitive to WAY-100635 administration. Actually, three of these inhibitions were completely reversed by 5-HT_{1A} receptor blockade, a fact never observed in mPFC. Interestingly, a recent study (Hajós et al., 2003) reported that DR/MnR- evoked inhibitions in putative pyramidal neurons of the rat infralimbic cortex had a latency >25 ms and were fully blocked by WAY-100635, as observed here in MOs, but not in the prelimbic or cingulate areas. These functional differences possibly reflect the distinct afferent and efferent projections of the prelimbic and infralimbic areas of the PFC (Groenewegen and Uylings, 2000; Vertes, 2004) and suggest that the DR/MnR-mPFC GABAergic input (Jankowski and Sesack, 2002) is restricted to the cingulate and prelimbic areas.

Overall, these observations suggest the presence of a non-serotonergic, possibly GABAergic, component evoked by the DR/MnR stimulation in the mPFC. The observed inhibitory latency is consistent with that of projection GABAergic neurons in other brain areas (Paladini et al., 1999). However, due to the complex nature of these *in vivo* experiments, we could not increase the picrotoxinin dose above 2.5 mg/kg i.v. since it reversed the effects of anaesthesia so that only a partial blockade of the inhibitory response was achieved. Further experiments are required to assess the presence of this putative GABAergic control of mPFC neurons by the raphe nuclei.

The characteristics of the DR/MnR-evoked excitations in mPFC are totally similar to those previously shown to be reversed by the 5-HT_{2A} receptor antagonist M100907. The mechanisms involved have been discussed elsewhere (Puig et al., 2003; Amargós-Bosch et al., 2004; see above). Interestingly, the latencies of the MnR-evoked excitations were lower than those observed after stimulation of the DR whereas the duration was similar. The exact reasons for this difference are unknown but may lie in anatomical differences between DR and MnR serotonergic neurons, which also exhibit a different sensitivity to neurotoxins (Kosofsky and Molliver, 1987; Mamounas and Molliver, 1988; O'Hearn et al., 1988). In the latter study, the selective lesion of fine axons by MDA (3,4-methylenedioxyamphetamine) unveiled a marked overlapping with beaded axons in the same territories of frontal cortex, in agreement with the present observation that PFC neurons are under control of DR and MnR neurons.

Biphasic responses possibly reflect the coexistence of 5-HT_{1A} and 5-HT_{2A} receptors in the same pyramidal neurons (Amargós-Bosch et al., 2004) and the temporal summation of DR/MnR-evoked inhibitory (very short latency, long duration) and excitatory responses (longer latency, shorter duration). However, as also observed for the pure orthodromic excitations, the proportion of biphasic responses is much lower than the observed 80% co-expression of the corresponding mRNAs, which again supports the predominance of inhibitory responses. Additionally, the presence of a GABAergic component cannot be ruled out.

What determines the type of response?

Hence, despite 5-HT can excite or inhibit pyramidal neurons, the reasons determining the emergence of one or other type of response are unclear. One of the limitations of the present study lies in the reciprocal connectivity of the mPFC and raphe nuclei (see above). One might

argue that a GABAergic component of the inhibitions, resulting from the antidromic invasion of pyramidal axon collaterals, may artifactually increase the proportion of inhibitory responses. While the presence of such component cannot be excluded, the inhibitions/excitations ratio in MOs (almost devoid of projections to DR; Peyron et al., 1998; this study) was even greater, which suggests that this is not a major determinant of the higher proportion of inhibitions in mPFC in our experimental conditions. A second limitation in the study is the use of anesthesia, which may alter cortical activity and hence the excitability of pyramidal neurons to incoming inputs, thereby altering the proportion of inhibitory vs. excitatory responses. We tried to minimize fluctuations in the level of anesthetic by performing the recordings within a time span after administration of additional doses of chloral hydrate.

Interestingly, the units excited had a significantly lower pre-stimulus firing rate than those inhibited, which suggests that 5-HT may physiologically increase the firing of pyramidal neurons with a low activity and depress the activity of neurons with a higher activity. Since 5-HT_{2A} and 5-HT_{1A} receptors may be present in different cellular compartments (see above), we reasoned that certain serotonergic axons, passing near 5-HT_{2A} receptor-rich apical dendrites (Jansson et al., 2001), may increase the excitability of pyramidal neurons, as observed *in vitro* (Aghajanian and Marek, 1997), rendering them more sensitive to incoming glutamatergic inputs. On the contrary, 5-HT released by axons passing near the axon hillock of pyramidal neurons would exert a dramatic inhibitory effect, switching off the propagation of action impulses (Amargós-Bosh et al., 2004). In that study, we observed that some pyramidal neurons in mPFC exhibited different responses to the stimulation of the DR or MnR at different coordinates. Here we observed that there was no difference between the mean effects (inhibition/excitation ratio) of the stimulation of DR and MnR (both at AP –7.8 mm) whereas the stimulation of the DR at a more rostral coordinate (AP -7.3 mm) yielded a significantly increased number of cells exhibiting excitations. Both observations tend to support the view that certain 5-HT neurons or neuronal subgroups may have a more excitatory effect than others on mPFC pyramidal neurons. However, the validity of this conclusion seems limited by methodological reasons, such as the pass of some DR fibers pass through the MnR and the stimulation of fibers *en passage* through or near both nuclei. Despite this limitation, we observed clear differences (e.g., latency of excitations in the present study or different responses in the same cells in Amargós-Bosch et al., 2004) which suggest the presence of a topologically defined connectivity between 5-HT and mPFC pyramidal neurons.

The possibility that a higher release of 5-HT in mPFC would result in an increased number of excitations (as suggested by the lower affinity of 5-HT for 5-HT_2 vs. 5-HT_{1A} receptors; Peroutka and Snyder, 1979; Hoyer et al., 1985) is not supported by the present study. On the contrary, twin pulse stimulation, which enhances 5-HT release, increased the magnitude of the inhibitions, as previously observed (Gartside et al., 2000) and turned some excitations into inhibitions. This was a fully reversible effect, which indicates that it is determined by the actual extracellular concentration of 5-HT released by nerve impulses. As previously observed with single pulses, twin pulse-evoked inhibitions had a very short latency and were only partly blocked by WAY-100635 administration, suggesting the presence of a non-serotonergic component of inhibitions that was also enhanced by twin-pulse stimulation.

Finally, since we antidromically identified pyramidal neurons from midbrain, it cannot be excluded that cells projecting to other brain areas (e.g., nucleus accumbens, thalamus, amygdala, etc.) may respond to DR/MnR stimulation in a different manner. Hence, despite the large co-expression of 5-HT_{1A} and 5-HT_{2A} receptor mRNAs, it could be that the influence of both receptors depends on the firing pattern of the cells recorded (e.g., slow, regular spiking cells might be more easy to inhibit than those with showing burst firing).

Conclusions

Despite the presence of excitatory 5-HT_{2A} receptors in a large percentage of PFC neurons and their co-expression with 5-HT_{1A} receptors, the present study shows that the main effect of physiologically released 5-HT in PFC is inhibitory, perhaps due to the suggested localization of 5-HT_{1A} receptors in the axon hillock of pyramidal neurons. A greater increase of 5-HT release in mPFC, as elicited by twin pulse stimulation of the DR and MnR does not increase the proportion of excitatory responses but, on the contrary, transforms them into inhibitions. Lastly, inhibitory responses evoked in mPFC involve 5-HT_{1A} receptor- and $GABA_A$-receptor-mediated components whereas in MOs only the serotonergic component appears to be present. Various sources of GABA may account for the inhibitions in mPFC, yet the very

short latency of the DR/MnR-evoked inhibitions suggests the presence of a GABAergic projection from the DR/MnR to the cingulate and prelimbic areas of mPFC.

Notes

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In vivo excitation of GABA interneurons in the medial prefrontal cortex through 5-HT$_3$ receptors

Victoria Puig*, Noemí Santana*, Pau Celada, Guadalupe Mengod and Francesc Artigas

Department of Neurochemistry, Institut d' Investigacions Biomèdiques de Barcelona (CSIC), IDIBAPS, 08036 Barcelona, Spain
*The first two authors contributed equally to this study

Serotonin (5-HT) controls pyramidal cell activity in prefrontal cortex (PFC) through various receptors, in particular, 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors. Here we report that the physiological stimulation of the raphe nuclei excites local, putatively GABAergic neurons in the prelimbic and cingulate areas of the rat PFC *in vivo*. These excitations had a latency of 36±4 ms and a duration of 69±9 ms and were blocked by the i.v. administration of the 5-HT$_3$ receptor antagonists ondansetron and tropisetron. The latency and duration were shorter than those elicited through 5-HT$_{2A}$ receptors in pyramidal neurons of the same areas. Double *in situ* hybridization histochemistry showed the presence of GABAergic neurons expressing 5-HT$_3$ receptor mRNA in PFC. These cells were more abundant in the cingulate, prelimbic and infralimbic areas, particularly in superficial layers. The percentage of GAD mRNA-positive neurons expressing 5-HT$_3$ receptor mRNA in prelimbic cortex was 40%, 18%, 6% and 8% in layers I, II-III, V and VI, respectively, a distribution complementary to that of cells expressing 5-HT$_{2A}$ receptors. Overall, these results support an important role of 5-HT in the control of the excitability of apical dendrites of pyramidal neurons in the medial PFC through the activation of 5-HT$_3$ receptors in GABAergic interneurons.

Keywords: 5-HT$_{2A}$ receptors, 5-HT$_3$ receptors, medial prefrontal cortex, GABA interneurons, pyramidal neurons

Introduction

The prefrontal cortex (PFC) plays a key role in higher brain functions (Fuster, 2001; Miller and Cohen, 2001) and controls, via the excitatory axons of pyramidal neurons, the activity of many subcortical motor and limbic areas (Groenewegen and Uylings, 2000). The activity of projection pyramidal neurons is controlled, among other areas, by the brainstem monoaminergic systems. In particular, the mesocortical dopaminergic system is involved in working memory and cognition through a complex control of the activity of pyramidal neurons (Willliams and Goldman-Rakic, 1995; Goldman-Rakic, 1996; O' Donnell, 2003; Wang et al., 2003).

There is also increasing evidence that the ascending serotonergic pathways originating in the dorsal and median raphe nuclei (DR and MnR, respectively) may play an important role in prefrontal function. Indeed, prefrontal neurons in various species express several 5-HT receptor subtypes (Pompeiano et al., 1992, 1994; Morales and Bloom, 1997; Hall et al., 2000; Talvik-Lotfi et al., 2000; Martinez et al., 2001; Arango et al., 2002; Amargós-Bosch et al., 2004), which suggests a role for 5-HT in the function of PFC. Hence, 5-HT$_{2A}$ receptors in dorsolateral PFC are involved in working memory (Williams et al., 2002) and recent work associates allelic variants of this receptor with memory capacity in humans (De Quervain et al., 2003). Hallucinogens like LSD or DOI are 5-HT$_{2A}$ receptor agonists whereas atypical antipsychotics are 5-HT$_{2A}$ receptor antagonists (Kroeze and Roth, 1998; Meltzer, 1999). On the other hand, 5-HT$_{1A}$ receptor antagonists reverse drug-induced cognitive deficits (Harder and Ridley, 2000; Mello e Souza et al., 2001; Misane and Ogren, 2003).

5-HT$_3$ receptors appear also to be involved in the cortical actions of 5-HT. Hence, 5-HT$_3$ receptor antagonists display pro-cognitive actions (Staubli and Xu,1995). These agents have been also reported to display anxiolytic and antipsychotic activity in animal models (Higgins and Kilpatrick, 1999) and to improve the therapeutic action of antipsychotics in schizophrenic patients (Sirota et al., 2000), perhaps through changes in dopamine release (Blandina et al., 1989; Chen et al., 1992; De Deuwardère et al., 1998). Likewise, the atypical antispycotic clozapine is an antagonist of 5-HT$_3$ receptors (Watling et al., 1989; Edwards et al., 1991).

Early microiontophoretic studies showed that 5-HT and 5-HT$_3$ receptor agonists suppressed pyramidal activity in rat PFC through the activation of 5-HT$_3$ receptors by a direct action (Ashby et al., 1989; 1991, 1992). However, more recent *in vitro* studies indicate that 5-HT may increase IPSCs in cortical pyramidal neurons by activation of 5-HT$_3$ receptors likely as a result of a fast synaptic excitation of local GABAergic neurons (Zhou and Hablitz, 1999; Férézou et al., 2002). The latter observations are consistent with the presence of 5-HT$_3$

receptors in GABAergic interneurons in the rat telencephalon (Morales et al., 1996; Morales and Bloom, 1997). Likewise, in macaque cortex, 5-HT_3 receptors are expressed by a subpopulation of calbindin- and calretinin-positive interneurons (Jakab and Goldman-Rakic, 2000). To gain further insight on the actions of 5-HT in PFC, we examined the localization of 5-HT_3 receptors in GABA interneurons of the rat PFC and the effects of the physiological stimulation of the DR on the activity of such neurons recorded *in vivo*.

Materials and Methods

Animals and tissue preparation

Male albino Wistar rats weighing 250-320 g were used (Iffa Credo, Lyon, France). These 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 ethics committee. Stereotaxic coordinates were taken from bregma and duramater according to the atlas of Paxinos and Watson (1998). We used the brain maps of Swanson (1998) for nomenclature of cortical areas.

Tissue preparation

Rats used in electrophysiological experiments were sacrificed by an anesthetic overdose. The location of stimulation electrodes was verified histologically (Neutral Red staining). The rats used for *in situ* hybridization histochemistry were sacrificed by decapitation, the brains rapidly removed, frozen on dry ice and stored at -20ºC. Tissue sections, 14-µm thick, were cut using a microtome-cryostat (Microm HM500 OM, Walldorf, Germany), thaw-mounted onto APTS (3-aminopropyltriethoxysilane, Sigma,St Louis, MO, USA)-coated slides and kept at –20ºC until use.

Electrophysiological recordings

We assessed the effects of the electrical stimulation of the DR at physiological rates on the activity of non-pyramidal neurons in the dorsal anterior cingulate and prelimbic areas of the rat PFC. Descents were carried out at AP +3.2 to +3.4, DV –1.1 to -3.6 below the brain surface. For the recording of 5-HT_3-expressing GABAergic neurons, the lateral coordinate was adjusted between -0.2 and –0.5 mm in order to target cells in the border between layers I and II-III, which show the greater abundance of cells expressing this receptor, as observed in *in situ* hybridization experiments (see below). To this end, the sinus was retracted to allow recording near the midline. As in previous studies, pyramidal neurons were identified by antidromic activation from projection areas of the medial prefrontal cortex (mPFC), such as the DR (at two different coordinates) or the mediodorsal thalamus (AP –2.8, L –0.5, DV –5.3), up to 2 mA, and collision extinction with spontaneously occurring spikes (Fuller and Schlag, 1976). Non-projecting units which were spontaneously active with a slow firing rate were considered candidates for the examination of the *in vivo* effects of 5-HT through 5-HT_3 receptors (see below). To this end, the DR (tip coordinates: AP –7.8, L –0, DV –6.5) was stimulated at 0.5-1.7 mA, 0.2 ms square pulses, 0.9 Hz. Peristimulus time histograms (PSTH) were constructed in baseline conditions and after the administration of the 5-HT_3 receptor antagonists ondansetron (gift from VITA-INVEST, Sant Joan Despí, Spain) and tropisetron (Sigma).

Single-unit extracellular recordings were performed as follows. Rats were anesthetized (chloral hydrate 400 mg/kg i.p.) and positioned in an stereotaxic apparatus (David Kopf). Additional doses of chloral hydrate (80 mg/kg) were administered i.v. through the femoral vein. Body temperature was maintained at 37 ºC 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. Putative GABAergic 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 Sci. Inst., Tokyo, Japan). Microelectrodes were filled with 2M NaCl. Typically, impedance was between 4-10 MΩ. Bipolar stimulating electrodes consisted of two stainless steel enamel-coated wire (California Fine Wire, Grover Beach, CA) with a diameter of 150 µm and a tip of separation of ~ 100 µm and in vitro impedance of 10-30 KΩ. Constant current electrical stimuli were generated with a Grass stimulation unit S-48 connected to a Grass SIU 5 stimulus isolation unit. 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, U.K).

Oligonucleotide probes

The oligodeoxyribonucleotide probes used were complementary to the following bases: 669-716, 1482-1520, and 1913-1960 of the rat 5-HT_{2A}

receptor mRNA (Pritchett et al., 1988); 728-772 and 1001-1045 of the rat 5-HT_{3A} receptor subunit mRNA (GenBank Acc. No U59672); 159-213 and 514-558 of the GAD65 mRNA (GenBank Acc. No NM_012563); 191-235 and 1600-1653 of the GAD67 mRNA (GenBank Acc. No NM_017007); 127-172 and 1756-1800 of the vGluT1 mRNA (GenBank Acc. No U07609). The probes for 5-HT_{2A} receptor and GAD67 were synthesized on a 380 Applied Biosystem DNA synthesizer (Foster City Biosystem, Foster City, CA, USA) and purified on a 20% polyacrylamide / 8 *M* urea preparative sequencing gel. The rest of the probes were synthesized and HPLC purified by Isogen Bioscience BV (Maarsden, The Netherlands).

Oligonucleotides were individually labeled at their 3'-end either with [^{33}P]-dATP (>2500 Ci/mmol; DuPont-NEN, Boston, MA, USA) or with Dig-11-dUTP (Boehringer Mannheim) using terminal deoxynucleotidyltransferase (Roche Diagnostics GmbH, Mannheim, Germany), purified by centrifugation using QIAquick Nucleotide Removal Kit (QIAGEN GmbH, Hilden, Germany).

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., 2003). Frozen tissue sections were first brought to room temperature, fixed for 20 min at 4ºC in 4% paraformaldehyde in phosphate-buffered saline (1x PBS: 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 136 mM NaCl, 2.6 mM KCl), washed for 5 min in 3x PBS at room temperature twice for 5 min each in 1x PBS, and incubated for 2 min at 21ºC in a solution of predigested pronase (Calbiochem, San Diego, CA, USA) 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 sec in 2 mg/ml glycine in 1x PBS. Tissues were finally rinsed in 1x 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, 4x SSC (1x SSC: 150 mM NaCl, 15 mM sodium citrate), 1x 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 (approximately 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 42ºC in humid boxes. Sections were then washed four times (45 min each) in a buffer containing 0.6 M NaCl and 10 mM Tris-HCl (pH 7.5) at 60ºC.

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 4ºC in the same solution with alkaline-phosphatase-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, USA) 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, Chesire, UK) diluted 1:1 with distilled water. They were exposed in the dark at 4°C for 4 weeks, and finally developed in Kodak D19 (Kodak, Rochester, NY, USA) 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 2003). These controls included: 1) the thermal stability of the hybrids obtained was checked for every probe, 2) for a given oligonucleotide probe, the hybridization signal was completely blocked by competition of the labeled probe in the presence of 50-fold excess of the same unlabeled oligonucleotide, 3) 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 regional and cellular levels when used independently, and 4) 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

The responses in putative GABAergic neurons evoked by DR stimulation were characterized by measuring the delay, magnitude and duration of excitatory responses from peristimulus-time histograms (PSTH) (4-ms bin width). Orthodromic excitations elicited spikes with short and variable latencies with a success rate greater than 10% (Celada et al., 2001). Success rate in PSTHs were corrected by the pre-stimulus firing. Drug changes were assessed with paired Student's *t*-test.

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 also captured with Darklite illuminator (Micro Video Instruments, Avon, MA, USA). The figures were prepared for publication using Adobe Photoshop software (Adobe Software, Mountain View, CA, USA).

Cell counting was performed manually at the microscope with the help of analySIS Software. Only cellular profiles showing great abundance of both transcripts were considered to co-express both mRNAs. Cells with a dense labeling of GAD mRNAs and occasional silver grains were not considered to co-express both receptors. $p < 0.05$ was considered statistically significant.

Results

5-HT$_3$-mediated excitations of local neurons in mPFC

The present experiments were initiated in parallel to the study of the effect of DR/MnR stimulation on pyramidal neurons of mPFC mediated by 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors (Puig et al., 2003; Amargós-Bosch et al., 2004; Puig et al., submitted). Pyramidal neurons were recorded at a lateral coordinate typically between -0.5 and -1.0 mm. During these experiments, encompassing ~230 neurons, we occasionally found cells that 1) were excited by DR/MnR stimulation but were not antidromically activated from the midbrain and thalamus, and 2) exhibited excitations with a latency and duration shorter than those typically elicited through the activation of 5-HT$_{2A}$ receptors. Because of the presence of 5-HT$_3$ receptors in GABA interneurons in rat telencephalon (Morales and Bloom, 1997), we hypothesized that these excitations might be due to the activation of 5-HT$_3$ receptors. Five units were recorded at this location, whose excitations were reversed by 5-HT$_3$ receptor antagonists. Based on these initial observations, we carried out *in situ* hybridization experiments to determine the location of cells expressing the 5-HT$_3$ receptor mRNA. Once these results were available (see below), additional descents were systematically performed at a more central coordinate, between -0.2 and -0.5 mm, aiming at cells expressing 5-HT$_3$ receptors in superficial layers (I-III). In all cases, only slow spiking neurons, not antidromically activated from the DR or the mediodorsal thalamus were considered to be potential candidates to examine the effects of DR stimulation upon 5-HT$_3$ receptors. A total of 14 excitations were considered to be potentially attributable to 5-HT$_3$ receptor activation and blockade was successfully attempted in 11 cases with the 5-HT$_3$ receptor antagonists ondansetron and tropisetron. Since other 5-HT receptors might potentially contribute to these excitations, here we report only the data of those cells whose excitations were reversed by these antagonists.

The electrical stimulation of the DR at a physiological rate (0.9 Hz, 0.2 ms square pulses) resulted in orthodromic excitations of slow spiking putative GABAergic neurons. Fast-spiking neurons (>10 spikes/s) were not excited by DR stimulation (data not shown). The characteristics of the recorded neurons, as well as the latency and duration of the excitations are given in Fig. 1 and Table 1. Unlike fast-spiking cells, these neurons exhibited a slow firing rate (<3 spikes/s), as recorded extracellularly, with a mean firing rate of 1.7 ± 0.3 spikes/s (n = 11; one neuron per rat). The latency and duration of these excitations were significantly lower than those elicited by DR stimulation, using the same parameters, in pyramidal neurons recorded in layers III-V of cingulate and prelimbic areas (Table 1, Fig. 2). The latter excitations were mediated by 5-HT$_{2A}$ receptor activation, since

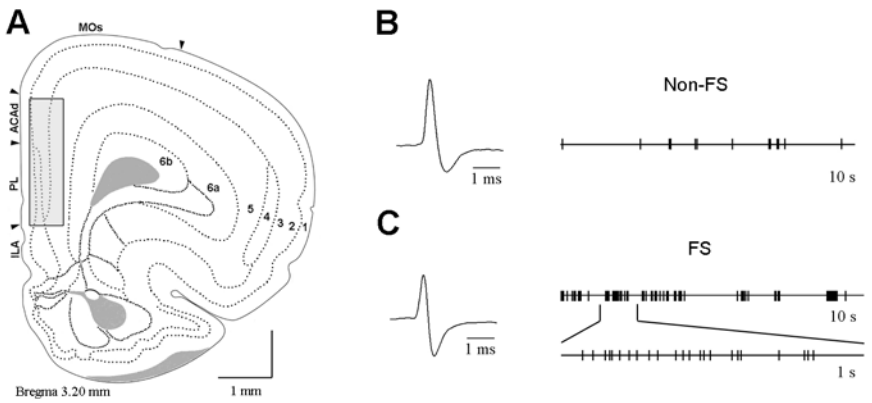

Figure 1. (A) Section drawing taken from Swanson (1998) showing the localization of the units recorded (shaded rectangle in the cingulate and prelimbic areas of PFC). These units were not antidromically activated from DR or mediodorsal thalamus and showed orthodromic activation from the DR that was blocked by the 5-HT_3 receptor antagonists. (B, C) Extracellular recordings of putative GABAergic neurons in mPFC. B) Representative waveform (average of 10 sweeps) and firing pattern of a non-fast spiking (non-FS) neuron whose orthodromic activation from the DR was blocked by 5-HT_3 receptor antagonist administration. C) Representative waveform (average of 10 sweeps) and firing pattern of a spontaneously fast-spiking (FS) neuron. Firing rates of these two units were 1.3 and 10.6 spikes/s, respectively.

Table 1. Characteristics of 5-HT_3 and 5-HT_{2A} receptor-mediated excitations induced by stimulation of the DR

	5-HT_3	5-HT_{2A}
Firing rate (spikes/s)	1.7 ± 0.3	1.1 ± 0.8
Latency (ms)	36 ± 4*	71 ± 8
Duration (ms)	69 ± 9*	101 ± 8
Success rate (%)	68 ± 11*	38 ± 8
Localization (DV, mm)	2.1 ± 0.3	2.2 ± 0.1
Number	11	10

*p < 0.05, paired Student's *t* test
Data from 5-HT_{2A} receptor-mediated excitations taken from Amargós-Bosch et al. (in press).

they were blocked by the i.v. administration of the 5-HT_{2A} receptor antagonist M100907 (Puig et al., 2003; Amargós-Bosch et al., 2004). Moreover, the success rate was significantly greater for the 5-HT_3 receptor- than for 5-HT_{2A} receptor-mediated excitations at the same current (68 ± 11% vs. 38 ± 8, p < 0.04).

We used the 5-HT_3 receptor antagonists ondansetron and tropisetron to examine whether the DR-induced excitations were mediated by 5-HT_3 receptors. Considering all cases (n = 11), 5-HT_3 receptor blockade significantly blocked these excitations, reducing the success rate from 68 ± 11% to 26 ± 8% on average (p < 0.006). Ondansetron was used to reverse the excitations in 7 cases (from 73 ± 16% to 29 ± 11%; p < 0.001). Typically, excitations were blocked by 0.5-2 mg/kg i.v. except in one unit which required 3 mg/kg. Tropisetron was used in 4 units (from 59 ± 12% to 20 ± 9% success rate; p < 0.003). Three of them were blocked at 0.5-1 mg/kg i.v. whereas one was blocked at 3 mg/kg i.v. Figure 3 shows the reversal of the excitations in two different units by ondansetron and tropisetron, respectively.

5-HT_3 receptor-mediated responses have been shown to desensitize after exposure to 5-HT and 5-HT_3 receptor agonists *in vitro* (Zhou and Hablitz, 1999). Here we examined whether physiological amounts of 5-HT released by raphe stimulation could elicit a similar desensitizing response *in vivo*. To this end, we calculated the success rate for 1-min intervals at different times after the onset of the stimulation. The corresponding values were 114 ± 14, 108 ± 16 and 101 ± 17 %, for the 2^{nd}, 3^{rd} and 4^{th} minutes after beginning the raphe simulation, taking 100 % as the success rate during the first minute.

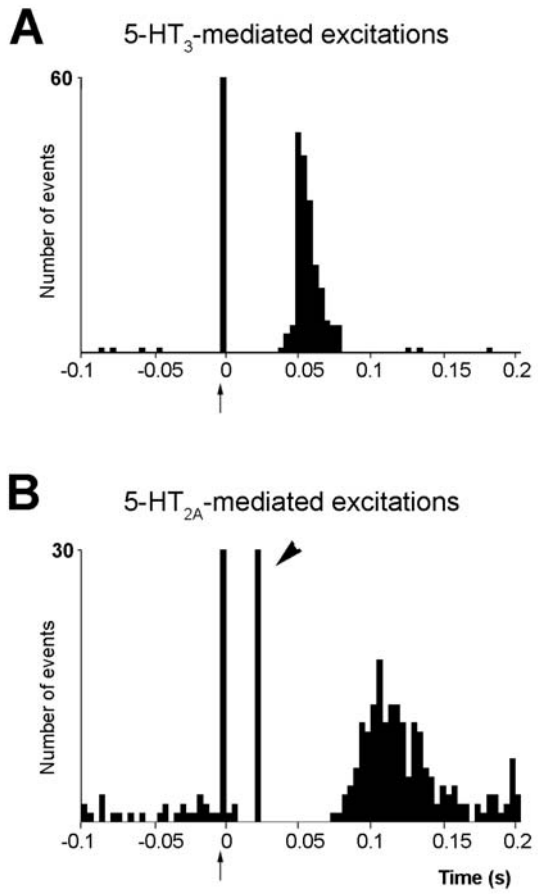

Figure 2. Peristimulus time histograms showing the orthodromic excitations elicited by the electrical stimulation of the DR on (A) a putatively GABAergic, 5-HT_3 receptor-containing neuron and (B) on a pyramidal neuron in the prelimbic PFC identified by antidromic stimulation. Both responses were selectively blocked by the administration of the respective antagonists ondansetron (A) and M100907 (B) (not shown). Note that, as many pyramidal neurons in mPFC, this unit had antidromic (arrowhead) and orthodromic responses to DR stimulation as a result of the reciprocal connectivity and functional interaction between the DR and mPFC (Puig et al., 2003). The latency and duration of the 5-HT_3-mediated responses in putative GABAergic neurons was significantly lower than those evoked by 5-HT_{2A} receptor activation in pyramidal neurons. The concordances of the units shown are 85% (A) and 43% (B). Each peristimulus consists of 200 triggers; bin size is 4 ms. The arrow at zero abcissa marks the stimulus artifact.

Expression of 5-HT_3 receptors in GABA interneurons

The presence of cells expressing the 5-HT_3 receptor transcript in various areas of the rat PFC is illustrated in figure 4. These cells were present in all cortical layers, although they had a preferential localization in superficial layers. In particular, they were more abundant in the cingulate, prelimbic and infralimbic areas as well as in primary and secondary motor areas (Figs. 4B-4C). A smaller number of cells were also present in piriform cortex and adjacent olfactory areas. Some cells were also present in layer VI of medial and motor cortices whereas layers III-V of these areas as well as the tenia tecta were almost without or with a much smaller population of neurons expressing 5-HT_3 receptors. Most cells positive for the 5-HT_3 receptor had a high level of expression, as judged from the large density of silver grains, corresponding to the ^{33}P-labeled oligonucleotides used to hybridize with the mRNA (Figs. 4D, 5). This was more marked than that of 5-HT_{2A} receptors in GABAergic neurons in the same prefrontal areas observed using the same methodology (Santana et al., in press). This difference may indicate a higher density of 5-HT_3 receptors per cell although methodological reasons may also account (e.g., a higher hybridization of the oligonucleotides complementary to the 5-HT_3 receptor mRNA).

The vast majority of 5-HT_3 receptor-expressing cells also contained the transcript for GAD (Dig-labeled), as observed in double *in situ* experiments (Fig. 5). The estimated proportion of GAD + 5-HT_3 receptor-expressing cells vs. the total of GABAergic (GAD-expressing) cells in layers I-III was 16.1 ± 0.3%, 23.9 ± 3.5% and 19.1 ± 3.6% in the cingulate, prelimbic and infralimbic areas, respectively (means of 3 rats; each value is the average of 3 sections; the average number of GAD-positive cells per field was between 29 and 33). However, the localization of these cells was not homogeneous in the various fields examined and sometimes appeared as clusters (several cells per field, as shown in Figs. 4C and 5C, for instance). Unlike GAD-positive cells, the number of those double-labeled cells fell rapidly at a greater lateral coordinate. Table 2 shows the number of cells expressing GAD mRNA, 5-HT_3 receptor mRNA and double-labeled cells in the various cortical layers of the prelimbic area. ANOVA showed a significant effect of the layer on the number of cells expressing GAD ($F_{3,8}$ = 88.3, $p < 0.000005$), 5-HT_3 receptors ($F_{3,8}$ = 14.2, $p < 0.0002$) and the percentage of double-labeled cells ($F_{3,8}$ = 127.7, $p < 0.000001$). Post-hoc Tukey *t*-test revealed significant differences between layers. Thus, the number of double-labeled cells was significantly greater in layers II-III than in the rest of layers. However, due to the lower number of GABAergic neurons in layer I compared to other layers, the percentage of double labeled cells was greater in this layer (40% in layer I vs. 18% in layers II-III and 6-8% in deeper layers; Table 2).

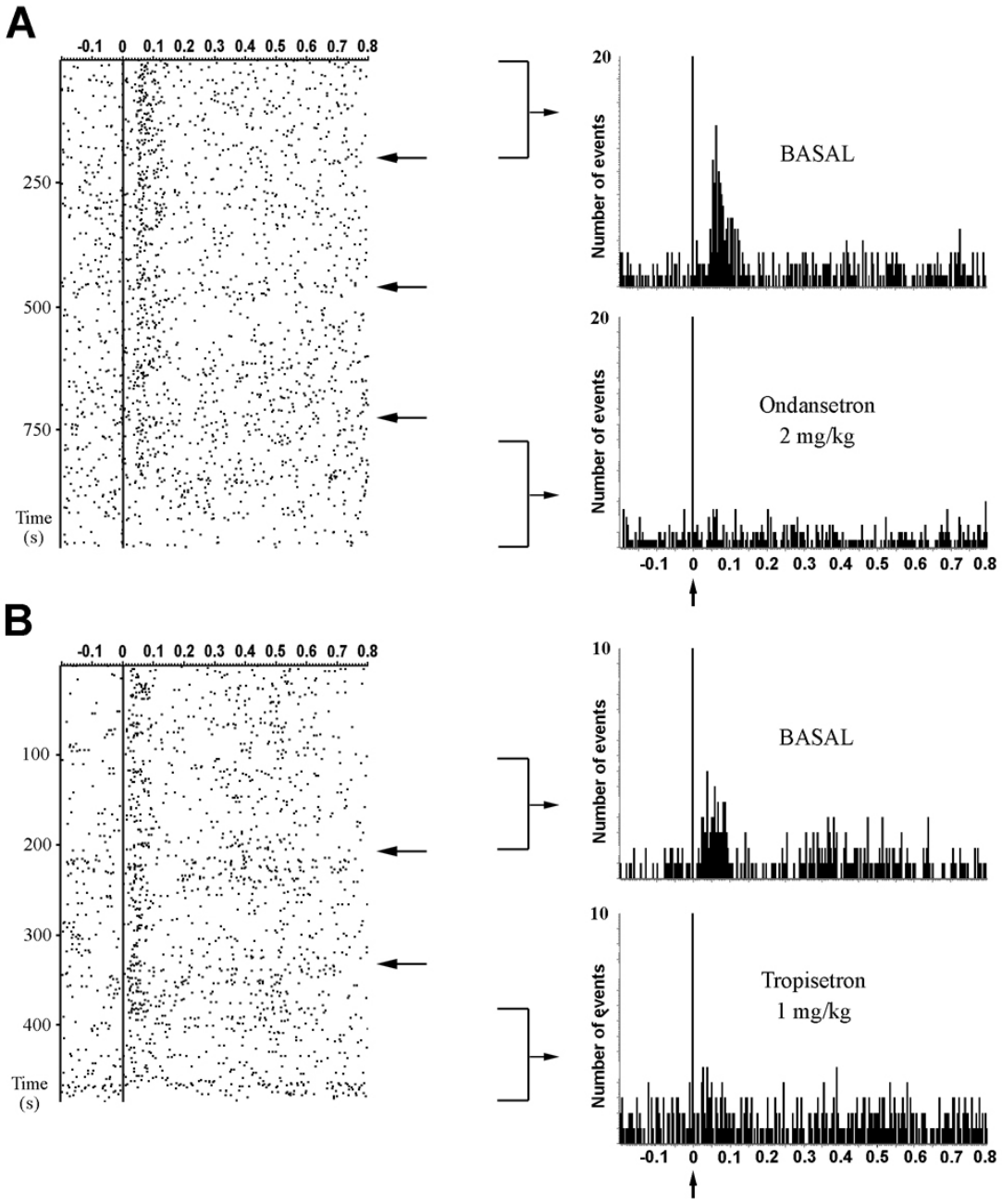

Figure 3. Blockade of the 5-HT-induced excitations in putative GABAergic neurons by the 5-HT_3 antagonists ondansetron and tropisetron. (A) Raster display and PSTHs of a neuron in basal conditions and after the administration of ondansetron (0.5-2 mg/kg i.v. cumulative doses; injection time shown by arrows in the raster display). Note the complete suppression of the DR-induced orthodromic excitation by ondansetron. Each PSTH corresponds to 200 triggers; bin size 4 ms. (B) Raster display and PSTHs of a neuron in basal conditions and after the administration of tropisetron (0.5-1 mg/kg i.v. cumulative doses; injection times shown by arrows in the raster display). Note the marked suppression of the DR-induced orthodromic excitation induced by tropisetron. Each PSTH corresponds to 100 triggers; bin size 4 ms. The brackets denote the times in the raster displays at which PSTH have been constructed. X-axis units are in seconds.

We observed a minority of cells expressing the 5-HT_3 receptor mRNA which apparently were not positive for GAD mRNA (Table 2). This suggests that the 5-HT_3 receptor transcript may also be present in a few non-GABAergic neurons. A pilot double *in situ* hybridization experiment showed the presence of some 5-HT_3 receptor-positive cells which also contained the vGluT1 mRNA, yet these results require further confirmation. Likewise, it cannot

Table 2. Expression of 5-HT_3 receptors in prelimbic cortex

	Layer I	Layers II-III	Layer V	Layer VI
GAD mRNA	6.6 ± 0.4*	22.1 ± 1.2	26.7 ± 1.5	23.9 ± 0.1
GAD + 5-HT_3 receptor mRNAs	2.6 ± 0.2†	4.1 ± 0.5*	1.5 ± 0.3	2.0 ± 0.1
5-HT_3 receptor mRNA alone	0.2 ± 0.1	0.4 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
% double labeled cells	40 ± 2*	18 ± 2^{+}	6 ± 1	8.2 ± 0.4

Data are number of cells expressing the corresponding mRNAs in the various cortical layers in the prelimbic area (mean ± SEM of 3 rats). The values for each rat were calculated by averaging the number of cells in three consecutive fields per section (3 sections per rat) as observed at x40 magnification in a Nikon Eclipse E1000 microscope.
*$p < 0.05$ vs. the rest of layers; $^{+}p < 0.05$ vs. deeper layers; $^{\dagger}p < 0.05$ vs. layers II-V (Tukey *t*-test)
Layer and area nomenclature according to Swanson (1998).

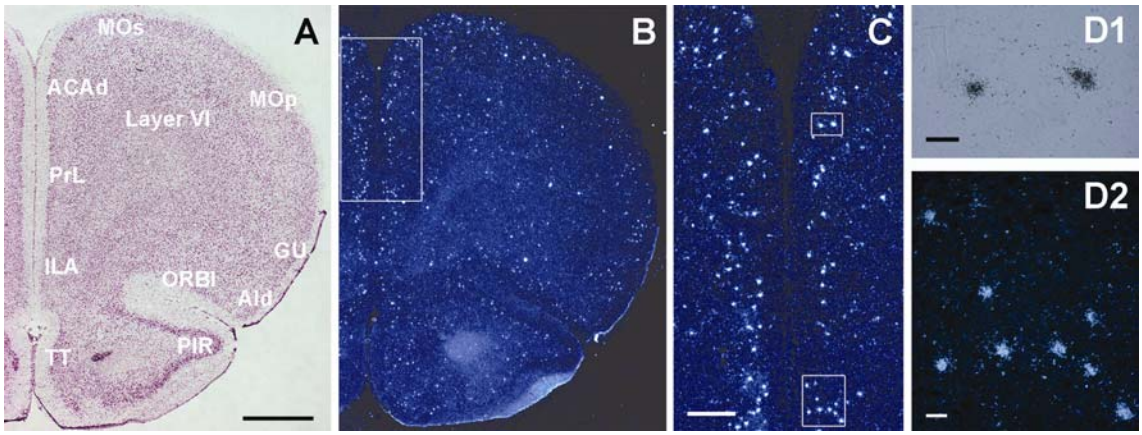

Figure 4. Visualization of 5-HT_3 receptor mRNA in the rat prefrontal cortex. A) Nissl stained section consecutive to B used as anatomical reference for the areas where 5-HT_3 receptors are expressed. B) Macroscopic dark-field image from an emulsion-dipped coronal section showing the localization of cells containing 5-HT_3 receptor mRNA. Note the preferential expression in superficial cortical layers in all cortical areas. Frame in B limits the approximate area shown at higher magnification in panel C. High magnification bright field (D1) and dark field (D2) photomicrographs of the areas marked in C, showing individual cells expressing 5-HT_3 receptors. Note the abundance of silver grains. ACAd, anterior cingulate (dorsal); AId, agranular insular (dorsal); GU, gustatory area; ILA, infralimbic area; MOp, primary motor area; MOs, secondary motor area; Layer VI; ORBl, orbital area (lateral); PIR, piriform area; PrL, prelimbic area; TT, taenia tecta. Bar size is 1 mm in A, B and 250 µm in C and 30 µm in D1 and D2.

be excluded that those are GABAergic cells with a faint labeling (e.g., poor penetration of the oligonucleotides or the antibody against digoxygenin).

5-HT_{2A} and 5-HT_3 receptors mediate direct excitatory responses of 5-HT on the cells expressing these receptors (see Introduction). A previous immunohistochemical study suggested the localization of these two 5-HT receptors in different subpopulations of GABAergic interneurons in monkey neocortex (Jakab and Goldman-Rakic, 2000). We therefore examined the localization of cells expressing these receptors in the mPFC. As observed in the prelimbic area of PFC (Fig. 6), 5-HT_3 receptor-expressing cells were located near the midline, in layers I-III. Shown in the same figure are the cells positive for vGluT1 (Fig. 6A) and GAD (Fig. 6B) mRNAs in layers I-VI of this cortical area. GAD-positive cells were present in all layers, including layer I where, as expected, pyramidal cells (vGluT1-expressing) were absent. On the other hand, cells expressing 5-HT_{2A} receptors were located mainly in layers III-V, an area where the 5-HT_3 receptor mRNA was much less abundant (Fig. 6C-6D). The 5-HT_{2A} receptor transcript is expressed by ~60% of pyramidal (vGluT1-positive) cells and by ~25% of GABAergic cells (GAD -positive) (Santana et al., in press). However, the

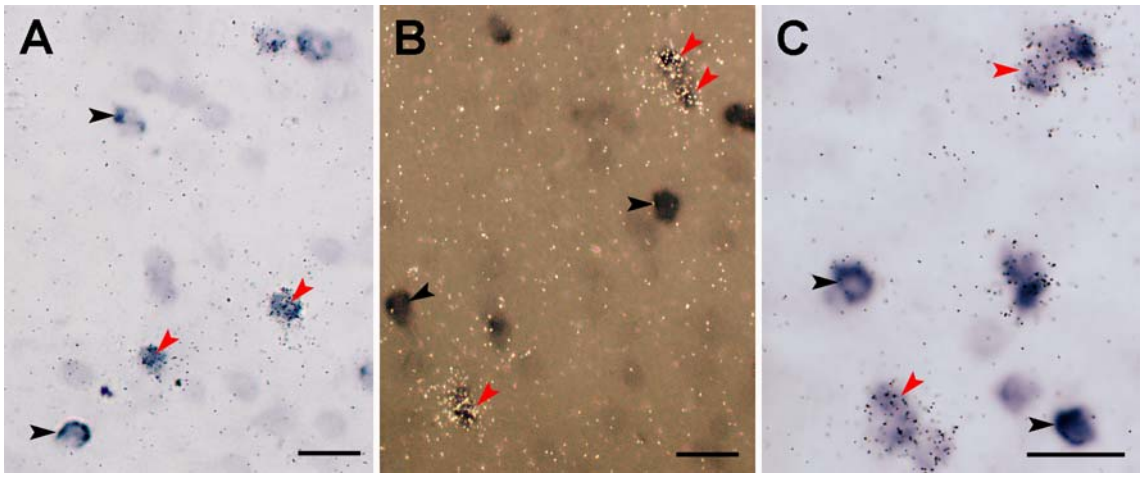

Figure 5. High magnification photomicrographs showing the detection in layers II-III of prelimbic and cingulate areas of 5-HT_3 receptor mRNA using ^{33}P-labeled oligonucleotides (silver grains) in GABAergic cells, visualized by hybridization with Dig-labeled oligonucleotides complementary to GAD mRNA (dark precipitate). A and C are bright-field photomicrographs showing the presence of several double-labeled cells (red arrowheads) as well as GABAergic cells not expressing the 5-HT_3 receptor (black arrowheads) in the infralimbic (A) and prelimbic (C) areas. B is a dark-field photomicrograph showing three double-labeled cells with a very dense labeling of the 5-HT_3 receptor transcript (silver grains are seen as yellowish dots) in the dorsal anterior cingulate area. Bar size is 20 µm.

proportion between 5-HT_{2A} receptors in GAD- and vGluT1-positive cells is similar in all areas of the PFC and therefore, the total population of cells positive for the 5-HT_{2A} receptor mRNA is representative of that in GABAergic neurons. The conspicuous absence of cells containing the 5-HT_{2A} receptor mRNA in layer I indicates that the GABAergic neurons close to the midline express 5-HT_3 but not 5-HT_{2A} receptors.

Discussion

The present study shows that 1) endogenous 5-HT excites putative GABAergic interneurons in the medial PFC through the activation of 5-HT_3 receptors, and 2) GAD- and 5-HT_3 receptor-expressing cells are mainly located in superficial layers of the PFC, a location different –but complementary- to that of GABAergic neurons expressing 5-HT_{2A} receptors. These results add to previous studies in rat PFC indicating that 5-HT modulates the activity of cortical microcircuits in various ways, either directly through the activation of excitatory and inhibitory receptors in pyramidal neurons or indirectly, through the activation of excitatory receptors in selected populations of GABAergic interneurons.

Methodological considerations

Previous studies examining the cellular phenotypes expressing 5-HT_3 receptors used GABA immunoreactivity to label GABAergic interneurons (Morales et al., 1996; Morales and Bloom, 1997). Here we identified GABAergic neurons by the presence of GAD67 or GAD65 mRNAs. 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 (Esclapez et al., 1993; 1994; Erlander et al., 1991; Feldblum et al., 1993). On the other hand, the cloning and characterization of glutamate vesicular transporters, vGluT1, vGluT2 and vGluT3, in rat brain (Takamori et al., 2000, 2001; Gras et al., 2002) has enabled to histologically identify a glutamatergic neuronal phenotype (Fremeau et al., 2001, Gras et al., 2002; Takamori et al., 2001; Oliveira et al., 2003). In particular, most rat cortical cells express very high levels of vGluT1 mRNA (Gras et al., 2002, Ziegler et al., 2002), which supports the use of vGluT1 to identify cortical glutamatergic pyramidal neurons.

Several classifications of GABAergic interneurons have been made, based on their morphology, chemical neuroanatomy and electrophysiological characteristics (De Felipe, 2002; Freund 2003). Considering their firing characteristics when recorded intracellularly, GABA interneurons have been classified as fast-spiking and non-fast spiking (both regular

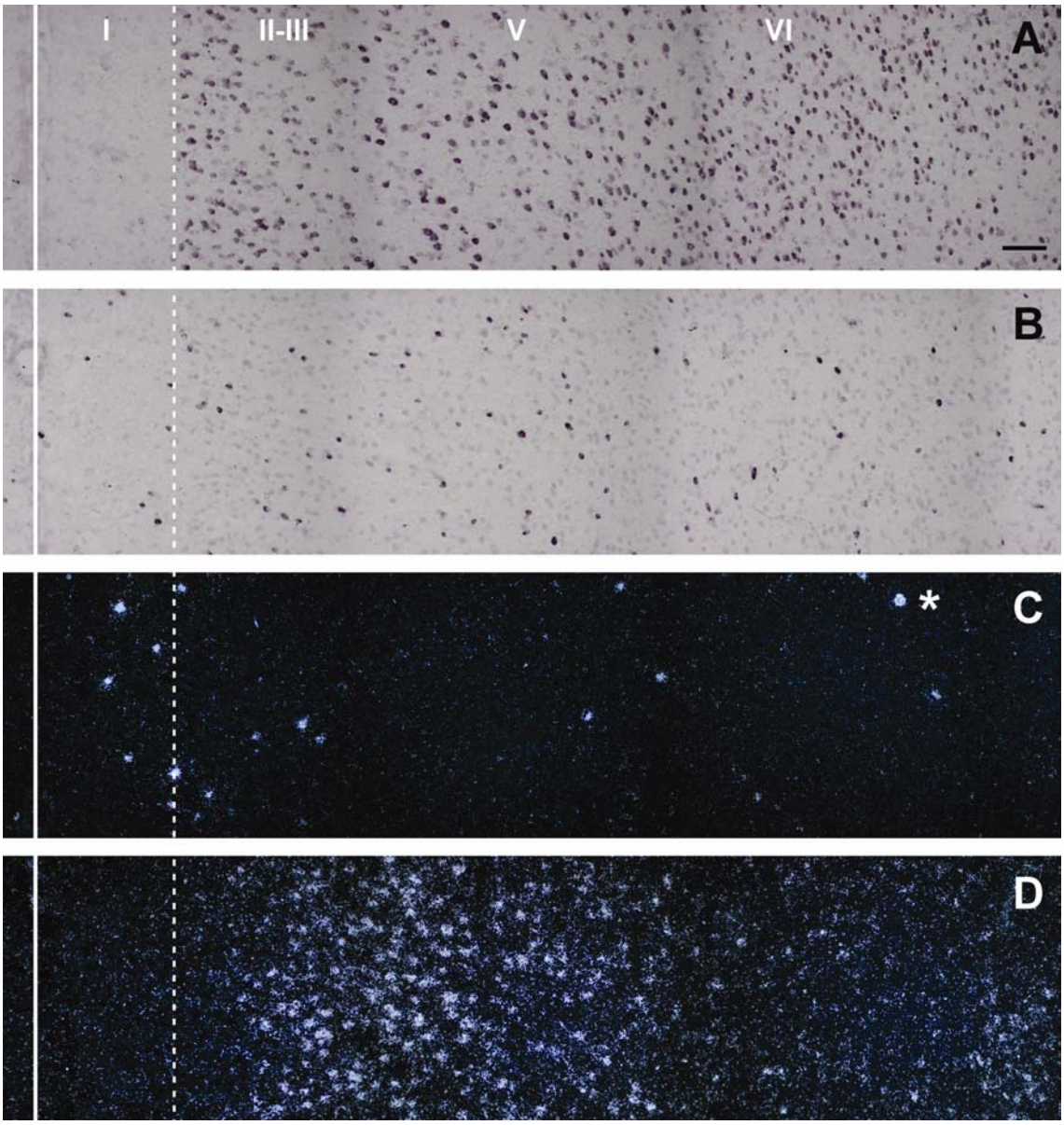

Figure 6. Composite photomicrographs showing the localization of cells expressing vGluT1 (A), GAD (B), 5-HT_3 (C) and 5-HT_{2A} (D) mRNAs through layers I-VI at the level of the prelimbic area in the rat PFC. The continuous vertical line denotes the location of the midline whereas the dotted line shows the approximate border between layer I and II. Each panel (A to D) corresponds to three consecutive microscopic fields. Pyramidal neurons (as visualized by vGluT1 mRNA) are present in layers II-VI whereas GAD mRNA-positive cells are present in all layers, including layer I. Note the different location of cells expressing 5-HT_3 (panel C) and 5-HT_{2A} receptors (panel D). 5-HT_3 receptor transcript is expressed by a limited number of cells present in layers I-III, particularly in the border between layers I and II. However, they represent a 40% of GABAergic neurons in layer I. On the other hand, cells in these locations, particularly in layer I, do not express 5-HT_{2A} receptors (note that only a small proportion of the latter receptors are expressed by GABAergic neurons; Santana et al., in press). The asterisk denotes an artifact of the emulsion, seen in the dark field. Bar is 150μm.

and irregular) cells (Cauli et al., 1997; Kawaguchi and Kondo, 2002; Férézou et al., 2002). Although extracellular recordings cannot discriminate between these cellular types, here we observed two main firing patterns of putative GABAergic interneurons, namely slow (non fast-spiking, not firing in trains, discharge rate < 3 spikes/s) and fast-spiking cells (firing in trains, discharge rate > 10 spikes/s; Constantinidis and Goldman-Rakic, 2002). Indeed, due to the inherent complexity of the *in vivo* recordings of putative GABAergic neurons, a limitation of the present study is that the recorded units were not neurochemically characterized. However, it is

unlikely that these were pyramidal neurons, in view of the following reasons. First, they were not antidromically activated from the DR or the mediodorsal thalamus, which make up two main targets of the axons of mPFC pyramidal neurons, where recordings were made (Thierry et al., 1983; Peyron et al., 1998; Groenewegen and Uylings, 2000). Second, more than half of the successful recordings were made close to the midline (0.2-0.5 mm lateral) to target the GAD- + 5-HT_3 receptor-labelled cells observed in the parallel *in situ* hybridization studies. In close agreement with the present observations, Zhou and Hablitz (1999) recorded 5-HT_3 receptor-mediated responses *in vitro* in layer I of cortical slices. Third, the DR-induced excitations were unequivocally mediated by 5-HT_3 receptor activation since they were reversed by the selective antagonists ondansetron and tropisetron. Thus, although there may be a very small proportion of 5-HT_3 receptors in non-GABAergic neurons (Morales and Bloom, 1997; this study) it is unlikely that these were recorded.

Effect of DR stimulation on putative GABAergic neurons in mPFC

Cortical microcircuits consist of principal (pyramidal) neurons and local (mainly GABAergic) interneurons that modulate pyramidal activity (Somogyi, 1998). 5-HT can modulate the activity of these microcircuits in various ways. Direct inhibitory and excitatory actions on pyramidal neurons are mediated by 5-HT_{1A} and 5-HT_{2A} receptors respectively (Araneda and Andrade, 1991; Aghajanian and Marek, 1997,1999; Zhou and Hablitz, 1999). Indirect actions are mediated by the activation of 5-HT receptors present on GABAergic interneurons and afferent terminals (heteroceptors; e.g., 5-HT_{1B}) (Ashby et al., 1990; Tanaka and North, 1993; Zhou and Hablitz, 1999). The electrical stimulation of the DR and MnR in the anesthetized rat can excite or inhibit pyramidal neurons in the cingulate and prelimbic PFC through the activation of 5-HT_{2A} and 5-HT_{1A} receptors, respectively (Puig et al., 2003; Amargós-Bosch et al., 2004). In this manner, 5-HT may influence the descending excitatory input into limbic and motor structures where the prefrontal cortex projects (Groenewegen and Uylings, 2000).

However, the role of 5-HT_3 receptors in the control of cortical neurons is less well understood. These receptors have been reported to be present in axons and in the somatodendritic region of cortical neurons (Miquel et al., 2002). The microiontophoretic application of 5-HT and selective 5-HT_3 agonists in the rat mPFC suppressed the firing of cells in layers II-III, an effect blocked by 5-HT_3 receptor antagonists (Ashby et al., 1989, 1991, 1992). Likewise, the stimulation of ascending serotonergic fibers at high frequency (15 Hz) evoked a suppression of cortical, possibly pyramidal, cells which was also blocked by 5-HT_3 antagonists (Ashby et al., 1991, 1992). Based on the inability of the microiontophoretic application of the $GABA_A$ receptor antagonist SR 95103 to block these effects, it was concluded that cortical neurons were directly inhibited through 5-HT_3 receptor activation (Ashby et al., 1989, 1991, 1992). In contrast to these reports, whole cell recordings in rat sensorimotor cortex revealed that 5-HT induces a fast synaptic excitation in a subpopulation of regular or irregular slow spiking (but not fast-spiking) VIP- and CCK-containing GABAergic interneurons in layer II (Férézou et al., 2002). These effects were mimicked by the 5-HT_3 receptor agonist m-phenylbiguanide and blocked by tropisetron, indicating the involvement of 5-HT_3 receptors, whose presence in the recorded neurons was determined by single cell RT-PCR (Férézou et al., 2002). Moreover, 5-HT and the 5-HT_3 agonist 1-(m-chlorophenyl)-biguanide increased a TTX-independent inward current in layer I interneurons (Zhou and Hablitz, 1999). This cortical effect is consistent with the ionic characteristics of the 5-HT_3 receptor (Maricq et al., 1991) and agrees with earlier data in hippocampus showing that 5-HT can excite GABA interneurons through 5-HT_3 receptors (Ropert and Guy, 1991; Kawa, 1994; McMahon and Kauer, 1997). Thus, these observations suggest that the 5-HT_3 receptor-mediated inhibitory action of 5-HT on cortical pyramidal neurons is indirect, involving an increase of local GABA inputs.

Our *in vivo* data in mPFC accord with the above *in vitro* observations (Zhou and Hablitz, 1999; Férézou et al., 2002) and indicate that 5-HT, released in the PFC by the physiological stimulation of the DR, can excite slow spiking GABAergic neurons through the activation of 5-HT_3 receptors. However, unlike to the exogenous *in vitro* application of 5-HT (Zhou and Hablitz, 1999), the response to endogenous 5-HT does not appear to desensitize, at least during the observation period used herein (4 min). The inability of the DR stimulation to evoke a similar excitation in spontaneous fast-spiking interneurons agrees with the fact that 5-HT_3 receptors are only expressed by a subpopulation of GABAergic neurons (Morales and Bloom, 1997; Férézou et al., 2002; this work). We cannot give an estimate of the proportion of cells responding to DR stimulation with 5-HT_3 receptor-mediated responses, but

indeed this is very low, consistent with the low proportion of neurons expressing 5-HT_3 receptor observed in the parallel histological study. Systematic descents in the recording area enabled to record few cells that a) were spontaneously firing, b) were not antidromically activated from the DR or the mediodorsal thalamus, and c) responded to DR stimulation with an excitation that d) was blocked by 5-HT_3 receptor antagonists. Hence, although the total number of cells reported here may appear low (n = 11), a much larger number were recorded to obtain such data. Similarly, Férézou et al. (2002) reported that only 19 out of a total of 107 attempted neurons were excited *in vitro* by 5-HT through 5-HT_3 receptors in slices of sensorimotor cortex.

The latency and duration of the 5-HT receptor-mediated excitations in putative GABAergic neurons were shorter than those observed in pyramidal neurons in the same areas of the PFC after the stimulation of the DR at the same rate (the latter are 5-HT_{2A} receptor-mediated; Puig et al., 2003; Amargós-Bosch et al., 2004). This difference may indicate a higher conduction velocity of the 5-HT fibers targeting 5-HT_3 receptors. Indeed, two main types of serotonergic axons have been reported that differ in their morphology (Kosofsky and Molliver, 1987). On the other hand, this difference could also be attributed to the ionic nature of the 5-HT_3 response which results in fast synaptic actions of 5-HT on these neurons (Maricq et al., 1991; Férézou et al., 2002). In contrast, the actions of 5-HT_{2A} receptors on neuronal excitability are mediated by metabotropic mechanisms (Aghajanian, 1995). The short latency and duration 5-HT_3 receptor-mediated activation of GABAergic inputs onto pyramidal neurons may perhaps contribute to a short-latency, 5-HT_{1A} receptor-independent inhibition observed in pyramidal neurons after the stimulation of the DR (Amargós-Bosch et al., 2004).

Localization of GABAergic neurons expressing 5-HT_3 receptors

Consistent with previous data in various telecephalic areas in rat (Morales and Bloom, 1997) and mouse brain (Hermann et al., 2002), here we found that a very large proportion of 5-HT_3 receptor is expressed by GABAergic neurons in PFC. Few non-GABAergic cells exhibited the presence of the 5-HT_3 receptor transcript. Given the larger proportion of pyramidal vs. GABAergic cells in neocortex (the latter represent a 15% of total; Beaulieu, 1993) we cannot exclude that a minority of the 5-HT_3 receptor-positive cells are pyramidal neurons.

5-HT_3 receptor-immunoreactive cells were found through all layers in frontal, temporal and parietal cortex in monkeys (Jakab and Goldman-Rakic, 2000). In contrast, these appear to be located preferentially in superficial layers in the rat, as judged from histological and functional studies (Morales and Bloom, 1997; Zhou and Hablitz, 1999; Férézou et al., 2002; this work). In particular, we show an enrichment of these cells in superficial layers of the cingulate, prelimbic and infralimbic areas of the rat PFC. This localization suggests that 5-HT_3 receptors may be the target of the dense plexus of serotonergic fibers in superficial cortical layers (Blue et al., 1988). Indeed, the expression of other cortical 5-HT receptors, such as 5-HT_{1A}, 5-HT_{2A} or 5-HT_{2C} is more marked in intermediate and deep layers (Pompeiano et al., 1992, 1994; Amargós-Bosch et al., 2004; Santana et al., in press; see also Fig. 6). Interestingly, the distribution of cells expressing 5-HT_3 and 5-HT_{2A} receptors in PFC seems complementary. The latter were expressed in glutamatergic and GABAergic neurons in layers III-V of the PFC, with a conspicuous absence in layers I-II and a low expression in layer VI (Amargós-Bosch et al., 2004; Santana et al. in press; this work). Only a small proportion of all 5-HT_{2A} receptor-expressing cells is GABAergic (Santana et al., in press), although their distribution follows the pattern of all 5-HT_{2A} receptor-containing cells. In contrast, 5-HT_3 receptor-expressing cells were found near the midline (particularly layers I-III) and -to a much lesser extent- in layer VI. 5-HT_3 receptors have been localized to calbindin- and calretinin-containing, small size GABAergic interneurons, whereas 5-HT_{2A} receptors are expressed by parvalbumin-containing large size interneurons (e.g., basket cells) (Morales and Bloom, 1997; Jakab and Goldman-Rakic, 1998, 2000). The presence of 5-HT_3 receptors in layer I GABAergic neurons, a cortical level devoid of pyramidal cell bodies (see for instance Fig. 6), suggests that 5-HT can modulate the inputs onto the apical dendrites of pyramidal neurons in PFC via 5-HT_3 receptors located in GABAergic interneurons. In this manner, 5-HT might modulate the cortico-cortical and thalamo-cortical inputs into superficial layers through an enhancement of synaptic GABAergic inputs (Krettek and Price, 1977; Linke and Schwegler, 2000; Mitchell and Cauler, 2001). On the other hand, 5-HT_{2A} receptors are involved in the feed-forward inhibition of pyramidal neurons through large, perisomatic parvalbumin-containing GABAergic neurons (Jakab and Goldman-Rakic, 2000). Thus, although the present study did not characterize the subtype(s) of GABAergic interneurons expressing 5-HT_3 and

5-HT_{2A} receptors, the distinct localization of cells expressing one or other receptor strongly supports an anatomical and functional segregation of both receptors in cortical microcircuits in the rat PFC, as observed in macaque cortex (Jakab and Goldman-Rakic, 2000). Moreover, 5-HT_3 receptor-mediated excitations are faster and last less than those induced by the activation of 5-HT_{2A} receptors, which indicates that 5-HT_{2A} and 5-HT_3 receptor-mediated responses are also temporally segregated.

In summary, the present study adds to previous *in vivo* data indicating that endogenous 5-HT, released by the physiological stimulation of the DR, is able to control the activity of neurons in the cingulate and prelimbic areas of the PFC through various cortical receptors, in particular the 5-HT_{1A}, 5-HT_{2A} and 5-HT_3 subtypes (Puig et al., 2003; Amargós-Bosch et al., 2004). The distinct temporal patterns of activation and the different cellular localizations of these receptors suggest a complex regulation of the cortical activity by 5-HT which deserves further investigation.

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