

Control of serotonergic neurons in rat brain by dopaminergic receptors outside the dorsal raphe nucleus

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Abstract

We studied the control of dorsal raphe (DR) serotonergic neurons by dopaminergic transmission in rat brain using microdialysis and single unit extracellular recordings. Apomorphine (0.5–3.0 mg/kg s.c.) and quinpirole (0.5 mg/kg s.c.) increased serotonin (5-HT) output in the DR and (only apomorphine) in striatum. These effects were antagonized by 0.3 mg/kg s.c. SCH 23390 (in DR and striatum) and 1 mg/kg s.c. raclopride (in DR). 5-HT_{1A} receptor blockade potentiated the 5-HT increase produced by apomorphine in the DR. Apomorphine (50–400 µg/kg i.v.) increased the firing rate of most 5-HT neurons, an effect prevented by SCH 23390 and raclopride. Quinpirole (40–160 µg/kg i.v.) also enhanced the firing rate of 5-HT neurons. When applied in the DR, neither drug increased the 5-HT output in the DR or striatum.

Likewise, micropressure injection of quinpirole (0.2–8 pmol) failed to increase the firing rate of 5-HT neurons. *In situ* hybridization showed that the dopamine (DA) D₂ receptor transcript was almost absent in the DR and abundant in the substantia nigra (SN) and the periaqueductal grey matter (PAG). Using dual probe microdialysis, the application of tetrodotoxin or apomorphine in SN significantly increased the DR 5-HT output. Thus, the discrepancy between local and systemic effects of dopaminergic agonists and the absence of DA D₂ receptor transcript in 5-HT neurons suggest that DA D₂ receptors outside the DR control serotonergic activity.

Keywords: DA receptors, extracellular recordings, 5-hydroxytryptamine release, 5-HT_{1A} receptors, *in situ* hybridization, microdialysis.

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The serotonergic (5-hydroxytryptamine, 5-HT) and dopaminergic systems interact in many ways in mammalian brain. The serotonergic transmission facilitates the release of striatal dopamine (DA), although the receptor subtype(s) involved remain to be fully clarified (Blandina *et al.* 1988; Chen *et al.* 1991; Benloucif *et al.* 1993; Galloway *et al.* 1993; Bonhomme *et al.* 1995; de Deurwaerdère *et al.* 1995; 1997, 1998). There is also anatomical and functional evidence of an interaction between the serotonergic dorsal raphe nucleus (DR) and the dopaminergic mesencephalic nuclei (Azmitia and Segal 1978; Van der Kooy and Hattori 1980; Stern *et al.* 1981; Hökfelt *et al.* 1984; Kalén *et al.* 1988; Peyron *et al.* 1995). The pars reticulata of substantia nigra (SN), containing the dendrites of nigrostriatal dopaminergic neurons, receives a very dense serotonergic input from the DR (Fallon and Loughlin 1995) which modulates the activity of striatonigral GABAergic afferents (Stanford and Lacey 1996). On the other hand, the electrical stimulation of the SN suppressed the firing activity of DR serotonergic neurons (Stern *et al.* 1981). At postsynaptic

level, the striatum is a major area for the convergence of ascending dopaminergic and serotonergic inputs from the midbrain, together with glutamatergic inputs from the cerebral cortex and thalamic nuclei (Azmitia and Segal 1978; Björklund and Lindvall 1984; Graybiel 1990).

Serotonergic agents modify the activity of dopaminergic cell units (Ugedo *et al.* 1989; Nedergaard *et al.* 1991; Arborelius *et al.* 1993, 1994; Lejeune and Millan 1998) and

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Abbreviations used: DA, dopamine; DR, dorsal raphe nucleus; 5-HT, 5-hydroxytryptamine or serotonin; 8-OH-DPAT, (+/-)-8-hydroxy-2-(di-*N*-propylamino)tetralin; PAG, periaqueductal grey matter; SCH 23390, (*R*)(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine-HCl; SN, substantia nigra; WAY 100635, *N*-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-*N*-(2-pyridinyl)cyclohexane carboxamide-3HCl.

the microinfusion of 5-HT in the ventral tegmental area increased DA release in *N. accumbens* (Guan and McBride 1989). Conversely, the stimulation of dopaminergic receptors by apomorphine increased the 5-HT output in the DR and forebrain (Ferré *et al.* 1994; Matsumoto *et al.* 1996; Mendlin *et al.* 1998) and the local application of L-DOPA in the SN enhanced the local 5-HT output (Thorré *et al.* 1998). These observations suggest that dopaminergic and serotonergic systems modulate each other to act in a co-operative manner in the forebrain. However, the localization of the DA receptors involved in the apomorphine-induced increase in 5-HT output is not known. Mendlin *et al.* (1998, 1999) blocked the striatal increase in 5-HT output induced by behavioural activation and by the systemic apomorphine administration with local application of the DA D₂ antagonist raclopride. However, the local application of the DA D₂-like agonist quinpirole in a wide range of concentrations did not modify the striatal 5-HT output (Ferré *et al.* 1994; Abellán *et al.* 2000b).

To further study the dopaminergic control of 5-HT neurons of the DR, we have conducted the present *in vivo* study using single unit extracellular recordings of 5-HT neurons in the DR and intracerebral microdialysis in the DR and striatum. This has been complemented by an *in situ* hybridization analysis to examine the presence of cells expressing DA D₂ receptors in the DR and neighbouring areas. The results support the proposal that DA D₂-like receptors outside the DR increase the activity of DR 5-HT neurons. These are possibly located (among other areas) in the SN and the periaqueductal grey matter (PAG).

Materials and methods

Animals

Male albino rats weighing 280–320 g (Iffa Credo, Lyon, France) and 200–300 g (University of the Basque Country) were used in microdialysis and electrophysiological experiments, respectively. Animals were kept in a controlled environment (12-h light-dark cycle and 22 ± 2°C). Food and water were provided *ad libitum*. Animal care followed the European Union regulations (OJ of EC L358/1 18/12/1986).

Drugs and reagents

5-HT oxalate, 8-OH-DPAT (+/-)-8-hydroxy-2-(di-*N*-propylamino)-tetralin, apomorphine-HCl, quinpirole-HCl, SCH 23390 [*R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine-HCl] tetrodotoxin (TTX), veratridine and WAY 100635 *N*-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-*N*-(2-pyridinyl)-cyclohexanecarboxamide-3HCl were from RBI (Natick, MA, USA). Citalopram-HBr and raclopride were kindly provided by Lundbeck A/S (Copenhagen-Valby, Denmark) and Astra (Södertälje, Sweden), respectively. Other materials and reagents were from local commercial sources. Drugs were dissolved in saline and injected s.c. (microdialysis experiments) or i.v. (single unit recordings). Doses are expressed as free bases. Control rats

received vehicle. For the assessment of local effects in microdialysis experiments, the drugs were dissolved in the perfusion fluid and applied by reverse dialysis. Concentrated solutions (1 mM; pH adjusted to 6.5–7 with NaHCO₃ when necessary) were stored frozen (–80°C) and working solutions were prepared daily by dilution.

Surgery and microdialysis procedures

An updated description of the microdialysis procedures used can be found in Adell and Artigas (1998). Briefly, anaesthetized rats (sodium pentobarbital, 60 mg/kg i.p.) were stereotaxically implanted with concentric microdialysis probes equipped with a Cuprophan membrane in the DR (AP –7.8, L –3.1, DV –7.5, with an angle of 30°; probe tip 1.5 mm), dorsal striatum (AP +0.2, L –3.0, DV –8.0; probe tip 4.0 mm) or the SN (AP –5.3, L +2.4, DV –8.4; probe tip 1.5 mm) (coordinates in mm from bregma and duramater; Paxinos and Watson 1986). At 20–24 h after surgery, probes were perfused at 0.25 µL/min with artificial cerebrospinal fluid (125 mM NaCl, 2.5 mM KCl, 1.26 mM CaCl₂ and 1.18 mM MgCl₂). After a 1-h stabilization period, four fractions were collected to obtain basal values before local or systemic administration of drugs. Successive 20-min (5 µL) dialysate samples were collected. Unless otherwise indicated, the perfusion fluid was supplemented with 1 µM of the selective 5-HT reuptake inhibitor citalopram. This enhanced the detectability of 5-HT in dialysate samples and enabled to measure drug effects on 5-HT release, because in absence of an uptake blocker the 5-HT collected in brain dialysates is representative of the equilibrium between release and reuptake (Jackson and Abercrombie 1992; Romero *et al.* 1998). To avoid the enhancement of 5-HT release in the DR induced by handling and injection procedures (Adell *et al.* 1997), the rats were gently manipulated before performing the microdialysis experiments. To examine the role of dopaminergic receptors in the DR (or SN) on the 5-HT output in striatum (or DR), groups of rats were implanted with two probes, as described above. Drugs (apomorphine, quinpirole or TTX) were dissolved in the dialysis fluid, perfused through the probe located in the DR or SN and their effects on local and distal 5-HT output were examined. Control rats were perfused for the whole experiment with artificial CSF but sham changes of the perfusion syringes were equally done.

At the end of the experiments, rats were killed by an overdose of sodium pentobarbital. The placement of the dialysis probes was examined by perfusion of Fast Green dye and visual inspection of the probe track after cutting the brain at the appropriate levels. The data of animals with the probes outside the structures of interest were not included in the calculations. The concentration of 5-HT in dialysate samples was determined by HPLC method, as described (Adell and Artigas 1998). The composition of HPLC eluant was as follows: 0.15 M NaH₂PO₄, 1.3 mM octyl sodium sulphate, 0.2 mM EDTA (pH 2.8 adjusted with phosphoric acid) plus 27% methanol. 5-HT was separated on a 3-µm ODS 2 column (7.5 cm × 0.46 cm; Beckman, San Ramon, CA, USA) and detected amperometrically with a Hewlett Packard 1049 detector (oxidation potential +0.6 V). Retention time was 3.5–4 min. The absolute detection limit for 5-HT was 0.5–1.0 fmol/sample. Dialysate 5-HT values were calculated by reference to standard curves run daily. In some experiments, the samples of rats treated locally with apomorphine in the DR were analyzed with a slight modification of the above HPLC method. Briefly, run time was increased to 45 min per

sample to avoid the interferences produced by apomorphine and other minor peaks in the HPLC trace.

Extracellular recordings of DR 5-HT neurons

The rats were anaesthetized with chloral hydrate (400 mg/kg, i.p.). A tracheal cannula was inserted and a jugular vein catheter was placed for additional injections of anaesthetic and other drugs. The rats were placed in a David Kopf stereotaxic frame (DKI, Tujunga, CA, USA) and rectal temperature was maintained at 37°C by means of an electric heating pad. After exposing the skull, a 4-mm burr

hole was drilled in the midline over the DR. The sagittal sinus was ligated, cut and reflected. Single unit extracellular recordings from DR neurons were made as previously described (Wang and Aghajanian 1982). The recording electrode was an Omegadot single-barrel glass micropipette (List Biological Labs, Campbell, CA, USA) filled with a 2% solution of Pontamine sky blue in 0.5% sodium acetate. The impedance of the electrode (tip diameter 1–2 µm), measured in 0.9% NaCl at 135 Hz, was 2–6 MΩ. The electrode was placed in the midline 1 mm anterior to lambda and 4.5–6.0 mm ventral to the exposed tissue, according to the atlas

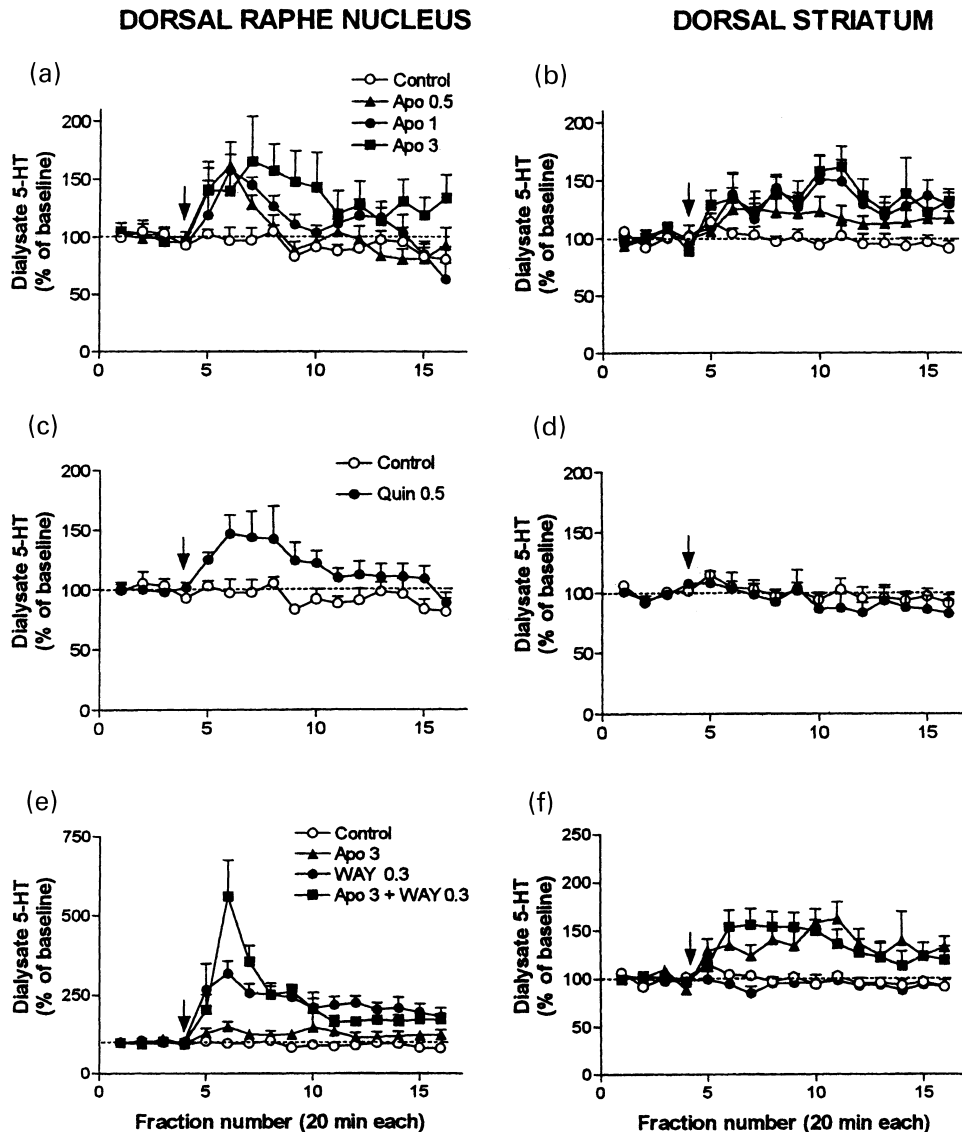


Fig. 1 Effect of the administration of apomorphine and quinpirole on the 5-HT output in the dorsal raphe nucleus (a, c and e) and dorsal striatum (b, d and f). The subcutaneous administration of apomorphine (Apo; 0.5, 1 and 3 mg/kg s.c.; injections marked by arrows) increased the 5-HT output in the dorsal raphe nucleus (a) and dorsal striatum (b). The administration of the selective DA D2-like agonist quinpirole (Quin, 0.5 mg/kg) enhanced the 5-HT output in the dorsal raphe nucleus (c) but not in dorsal striatum (d). The

increase in 5-HT output produced by 3 mg/kg apomorphine in the dorsal raphe nucleus was potentiated by the administration of 0.3 mg/kg s.c. WAY 100635 (WAY). The administration of WAY 100635 alone increased significantly the 5-HT output in the dorsal raphe nucleus but not in dorsal striatum (note the different scale in e and f). Data are mean \pm SE of 5–8 rats/group. Control rats (open circles, $n = 5$ in dorsal raphe nucleus and 6 in striatum) were injected with saline. See text for statistical analysis.

of Paxinos and Watson (1986). The signal from the recording electrode was passed through a high-input impedance amplifier, delivered to an audiometer and continuously monitored on an oscilloscope. Single neuronal spikes were discriminated in a custom-made unit, counted in an electronic rate counter, sent to a computer generating interspike time interval histograms and recorded on a pen chart recorder as spikes per 10 s. In experiments assessing the effects of i.v. administration of drugs, only one cell per rat was recorded. DR serotonergic cells were identified by their long duration action potentials and a regular, slow (0.5–3 spikes/s) discharge rate. We administered the selective 5-HT_{1A} receptor agonist 8-OH-DPAT at the end of the experiments to additionally confirm the serotonergic nature of the recorded cells. A 5- μ A cathodal current was passed through the recording electrode in order to leave a blue spot at the recording site. The brains were fixed by intracardial perfusion with 4% formalin and frozen sections (50- μ m thick) were stained with neutral red and examined microscopically for histological verification of the recording site.

Pressure microinjection into the DR

For the local administration of quinpirole, a thick-walled pipette with a calibrated inner diameter (Blaubrand; Intramark, Houston, TX, USA) was glued to a recording electrode, according to Akaoka *et al.* (1992). The calibrated pipettes were filled with Dulbecco's buffered saline solution containing (in mM): NaCl 136.9, KCl 2.7, NaH₂PO₄ 8.1, KH₂PO₄ 1.5, MgCl₂ 0.5 and CaCl₂ 0.9 (pH 7.4), with or without quinpirole. Drug ejection was performed by applying pressure pulses (100–200 ms) with a solenoid-controlled pneumatic device (PicospritzerTM II, General Valve Corporation; Fairfield, NJ, USA). The volume injected was measured by monitoring the movement of the fluid in the calibrated pipette. In each rat we checked the sensitivity of the last recorded neuron to the i.v. administration of 8-OH-DPAT.

In situ hybridization histochemistry

Oligonucleotide probes complementary to the mRNA coding for the rat dopamine D₂ receptor (bases 1212–1245, Bunzow *et al.* 1988; Mengod *et al.* 1989), the 5-HT transporter (bases 612–659, Blakely *et al.* 1991) and glutamic acid decarboxylase (GAD) (bases 1537–1590, Kobayashi *et al.* 1987) were used. Oligonucleotides were labelled at the 3' end with terminal deoxynucleotidyltransferase (Boehringer Mannheim, Mannheim, Germany) and [³²P] α -dATP (3000 Ci/mmol; DuPont-NEN, Boston, MA, USA) to specific activities of 2–3 \times 10⁴ Ci/mmol. The labelled probes were purified with Qiaquick columns (Qiagen, Valencia, CA, USA).

Tissue treatment before hybridization was essentially as described (Vilaró *et al.* 1992). For hybridization, labelled probes were diluted to a final concentration of 2 \times 10⁷ cpm/mL in a solution containing 50% formamide, 4 \times SSC (1 \times SSC: 150 mM NaCl, 15 mM sodium citrate), 1 \times Denhardt's solution (0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 10% dextran sulphate, 1% sarkosyl, 20 mM phosphate buffer pH 7.0, 250 mg/mL yeast tRNA, 500 mg/mL salmon sperm DNA. Tissue sections (14 μ m thick) were covered with 80 mL of hybridization solution, overlaid with Nescofilm coverslips, and incubated overnight in humid boxes at 42°C. Sections were washed four times (45 min each) in 600 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 mM EDTA at 60°C, dehydrated in graded ethanols and dried. Hybridized sections were dipped in liquid emulsion (LM1,

Amersham) and exposed for 4 weeks for the 5-HT transporter mRNA, 5 weeks for the GAD mRNA and 7 weeks for the dopamine D₂ receptor mRNA.

Data treatment and statistics

The concentration of 5-HT in dialysates (mean \pm SE) is expressed as fmol/fraction and represented in figures as percentages of baseline (average of 3–4 predrug fractions). The statistical analysis was performed using one- or two-way analysis of variance (ANOVA) for repeated measures of raw data (fmol/fraction) followed by Tukey's test where appropriate. We analysed the effect of the independent factor (treatment group), the repeated factor (time) and the interaction between them. The latter assessed whether the change in 5-HT over time differs between treatment groups (e.g. treated versus controls). Thus, a significant *p*-value of the treatment or the time-treatment interaction indicates differences in the effects of two or more treatments on 5-HT output. Averaged values of

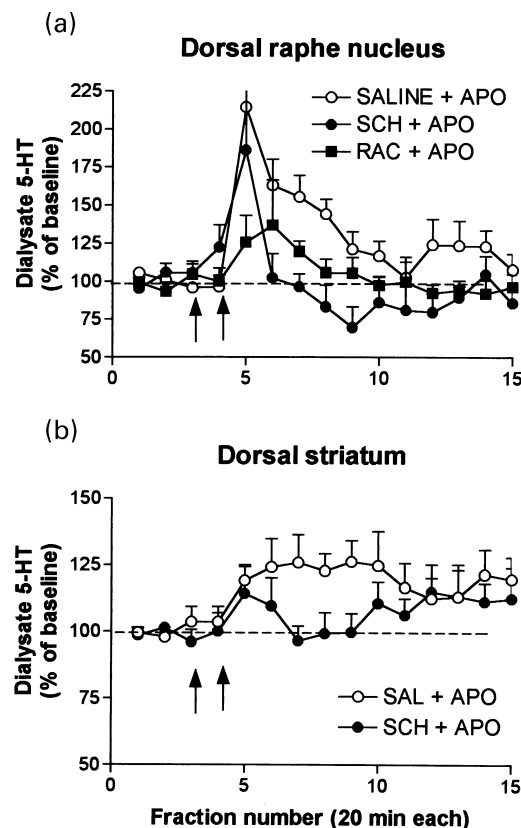


Fig. 2 The administration of the DA D₁ receptor antagonist SCH 23390 (0.3 mg/kg s.c., first arrow) antagonized the increase in 5-HT output produced by the administration of apomorphine (0.5 mg/kg s.c., second arrow) in the dorsal raphe nucleus (a) and striatum (b). Likewise, the DA D₂ receptor antagonist raclopride (1 mg/kg s.c., first arrow) antagonized, though to a lesser extent than SCH 23390, the effect of apomorphine in the dorsal raphe nucleus. Note that the Y-axis scales are different in (a) and (b) due to the increase in 5-HT output elicited by the repeated injection procedure in two experimental groups in the dorsal raphe nucleus. Data are mean \pm SEM of 4–7 rats/group. See text for statistical analysis.

post-treatment periods (100 min; fractions 6–10) have been used to examine the effects of DA receptor antagonists on the increase in 5-HT output produced by apomorphine. In these calculations, the data from fraction 5 (first post-treatment fraction) were omitted from the calculations due to the increase in 5-HT (Adell *et al.* 1997) produced by the two-injection procedure in the DR. In electrophysiological recordings, the individual firing rates during baseline and drug periods were averaged and computed using 1-min periods and compared using paired Student's *t*-tests. Statistical significance has been set at the 95% confidence level (two-tailed).

Results

Baseline 5-HT output

Baseline dialysate 5-HT concentrations in presence of 1 μM citalopram were 35.3 ± 1.9 , 18.3 ± 1.0 and 27.2 ± 4.1 fmol/fraction in the DR, dorsal striatum and SN ($n = 104$, 79 and 11, respectively). In absence of citalopram, the corresponding values in DR and striatum were 6.5 ± 1.0 and 2.7 ± 0.5 fmol/fraction ($n = 13$ and 11, respectively). The dialysate 5-HT values in the DR were significantly

greater than in the striatum in presence of citalopram ($p < 0.00001$, Tukey's test post ANOVA).

Enhancement of the 5-HT release by systemic apomorphine and quinpirole

The administration of the non-selective DA receptor agonist apomorphine (0.5, 1 and 3 mg/kg s.c.) resulted in a stereotyped behaviour (mainly sniffing, licking and grooming) and enhanced the 5-HT output in the DR ($F_{15,270} = 6.81$, $p < 0.0001$ time effect; $F_{45,270} = 1.69$, $p < 0.006$ time-dose interaction; Fig. 1a) and dorsal striatum ($F_{3,26} = 3.52$, $p < 0.03$ dose effect; $F_{15,390} = 4.12$, $p < 0.0001$ time effect; Fig. 1b). Where the lower dose was used, the maximal elevation of the striatal 5-HT output occurred soon after administration (40 min). Higher doses (1 and 3 mg/kg s.c.) elicited a more persistent elevation, with the maximal increase occurring 2 h after administration (Fig. 1b). In the DR, the differences in 5-HT output at the three doses used were more conspicuous than in striatum and maximal elevations occurred at 40–60 min post-administration. Beyond this time point, the 5-HT output declined rapidly at 0.5 mg/kg but remained elevated until

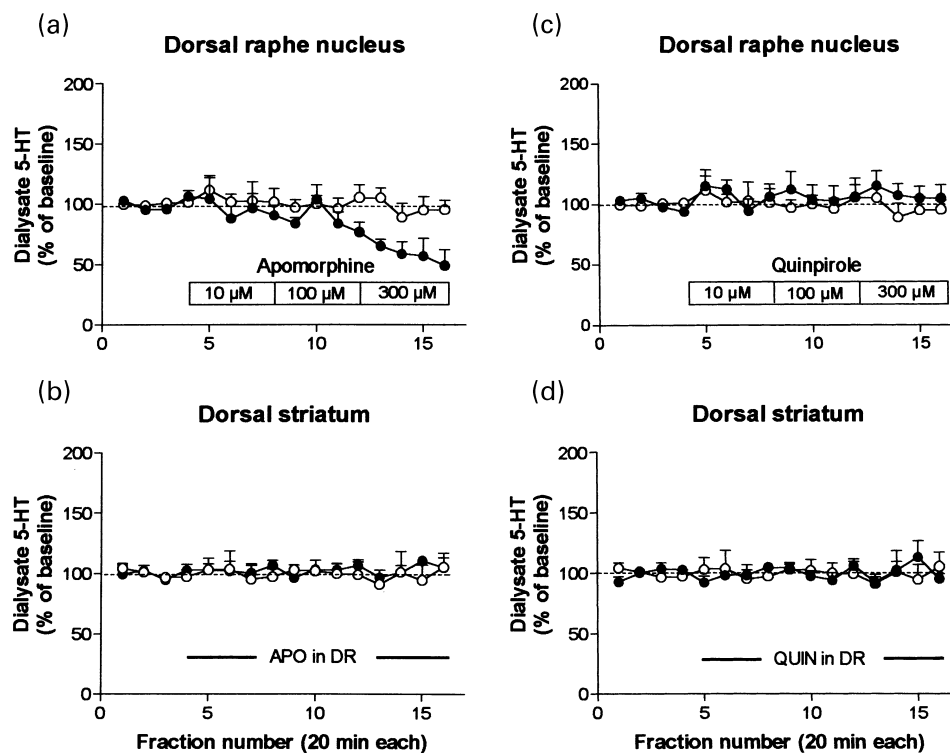


Fig. 3 (a and b) The perfusion of apomorphine (10, 100 and 300 μM) in the dorsal raphe nucleus of rats bearing two dialysis probes did not alter the 5-HT output in the dorsal striatum and reduced it in the dorsal raphe nucleus at 300 μM (filled circles, $n = 5$). Control rats (open circles, $n = 7$ in dorsal raphe nucleus, $n = 5$ in striatum) were perfused with artificial CSF throughout the experiment. (c, d) The perfusion of quinpirole (QUIN; 10, 100 and

300 μM) in the DR of rats bearing two dialysis probes did not alter the 5-HT output in the DR or dorsal striatum (filled circles, $n = 10$ in DR; $n = 6$ in striatum). Control rats (open circles, $n = 7$ in DR, $n = 5$ in striatum) were perfused with artificial CSF throughout the experiment. The lower number of rats in striatum is due to sample lost during the HPLC analysis.

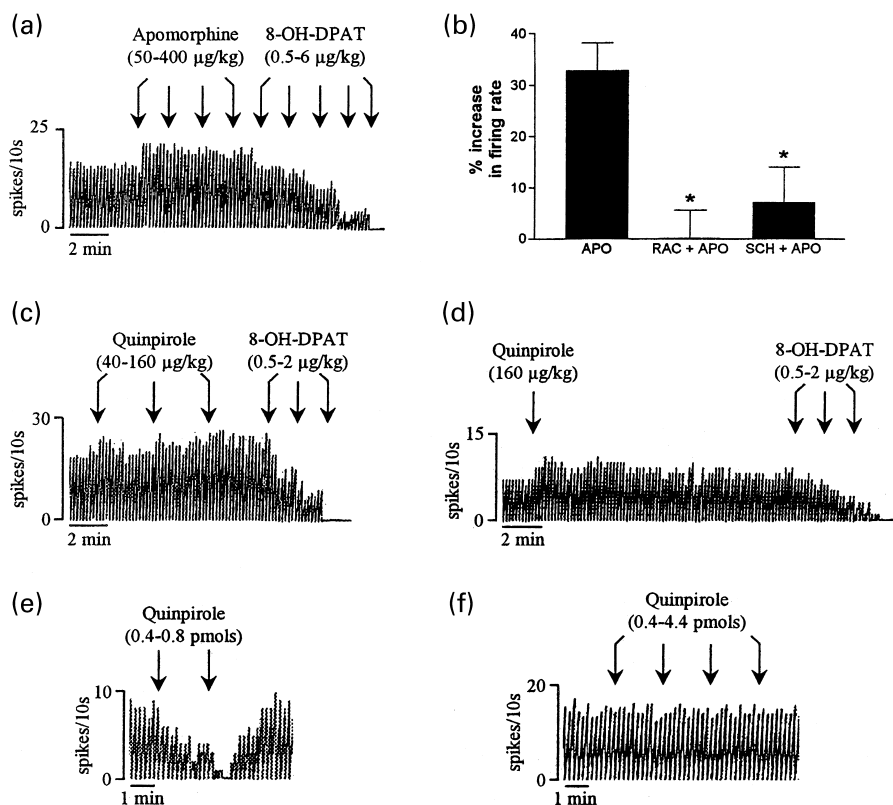


Fig. 4 (a) Integrated firing rate histograms showing the effect of cumulative doses of apomorphine (50–400 μg/kg i.v.) on the activity of one serotonergic neuron in the dorsal raphe nucleus. The serotonergic nature of the units recorded (one per rat) was further tested by cumulative injections of the selective 5-HT_{1A} agonist 8-OH-DPAT, which markedly suppressed the firing activity. (b) The previous treatment with raclopride (125 μg/kg i.v. injected 4 min before apomorphine) or SCH 23390 (400 μg/kg i.v. injected 4 min before apomorphine) significantly prevented the increase in serotonergic firing rate elicited by apomorphine. The increase in firing rate elicited by apomorphine corresponds to the cells excited by this drug (c and d). Representative integrated firing rate histograms showing the

effect of the i.v. injection of quinpirole on the activity of identified 5-HT neurons of the dorsal raphe nucleus. (c) and (d) show the effect of cumulative doses (40–160 μg/kg i.v.) and of a single 160 μg/kg i.v. dose on the activity of two different units, respectively. 8-OH-DPAT (0.5–2 μg/kg i.v.) fully suppressed the firing activity. (e) and (f) Integrated firing rate histograms showing the effect of the micropressure injection of quinpirole (arrows) on the electric activity of 5-HT neurons of the dorsal raphe nucleus. Shown are representative examples of quinpirole-sensitive (e; cumulative dose: 0.8 pmol) and a quinpirole-insensitive (f; cumulative dose: 4.4 pmol) 5-HT neurons.

the end of the experiment (4 h postadministration) in the rats treated with 3 mg/kg apomorphine.

To examine whether the presence of citalopram interfered the effects of apomorphine, a group of rats was treated with this agent but in this case the dialysis probes were perfused with an artificial CSF devoid of citalopram. The administration of 0.5 mg/kg apomorphine ($n = 6$) elevated significantly the striatal 5-HT output with respect to controls (maximal increase $192 \pm 20\%$ of controls; $n = 5$) ($F_{15,135} = 2.02$, $p < 0.02$ time effect; $F_{15,135} = 2.97$, $p < 0.0004$ time–dose interaction). The 5-HT output in the DR was also increased by apomorphine (1 and 3 mg/kg) in absence of citalopram, to a maximum of $150 \pm 16\%$ of baseline ($F_{15,165} = 2.07$, $p < 0.015$ time effect).

The administration of the DA D₂-like receptor agonist quinpirole (0.5 mg/kg s.c.) also increased significantly the

5-HT output in the DR ($F_{15,105} = 3.15$, $p < 0.0003$; Fig. 1c). However, it failed to induce a similar increase in striatum (Fig. 1d). Given this difference between the effects of apomorphine and quinpirole in striatum, we examined whether DA D₁ receptors could be involved in the 5-HT-enhancing action of apomorphine. Groups of rats were administered with saline or the selective DA D₁ receptor antagonist SCH 23390 (0.3 mg/kg s.c.) 20 min before apomorphine. SCH 23390 significantly attenuated the increase in DR 5-HT output produced by 0.5 mg/kg s.c. apomorphine. The average post-treatment values for the saline + apomorphine and SCH 23390 + apomorphine groups were, respectively, $141 \pm 11\%$ ($n = 5$) and $87 \pm 13\%$ ($n = 4$) ($p < 0.02$, Student's *t*-test). Pretreatment with raclopride (1 mg/kg s.c., 20 min before apomorphine) also attenuated the apomorphine-induced

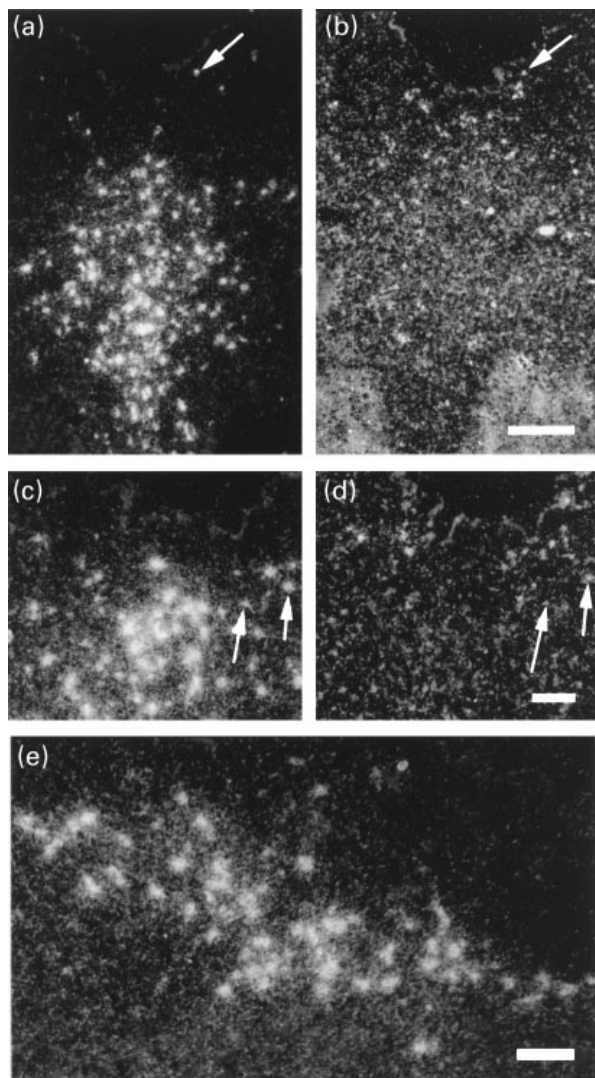


Fig. 5 Cellular localization of 5-HT transporter mRNA (a and c) and dopamine D_2 receptor (b and d) mRNA in the rat dorsal raphe nucleus (DR). Pictures are dark-field photomicrographs of emulsion-dipped consecutive sections. Note that the hybridization intensity with the dopamine D_2 receptor labelled probe as well as the number of D_2 receptor mRNA positive cells (b and d) is much lower than the one observed for the 5-HT transporter probe (a and c) in the DR, indicating a lower abundance of the DA D_2 receptor mRNA in this brain area. This is not the case in the substantia nigra pars compacta (e), where dopaminergic cells are highly enriched with the mRNA coding for the DA D_2 receptor. Arrows point to some of the few cells apparently expressing both transcripts in the DR (sections A-C and B-D are adjacent). (Bars = a, b and e 250 μm ; c, d 200 μm .)

increase of the 5-HT output in DR (Fig. 2), although the antagonism exerted by this dose was less marked than that of SCH 23390 and was only marginally significant (141 ± 11 versus $113 \pm 3\%$, $n = 5$ and 4, respectively; $p = 0.06$, Student's *t*-test). In the dorsal striatum, pretreatment

with SCH 23390 (0.3 mg/kg s.c.) 20 min before apomorphine 0.5 mg/kg also attenuated its effects on the 5-HT output (saline + apomorphine, $125 \pm 7\%$ ($n = 7$) versus SCH 23390 + apomorphine $101 \pm 7\%$ ($n = 7$), $p < 0.04$, Student's *t*-test) (Fig. 2). The pretreatment with SCH 23390 and raclopride abolished the apomorphine-induced stereotypies.

Interaction with 5-HT_{1A} autoreceptors

The effect of drugs that increase the synaptic availability of 5-HT in the DR is limited by a negative feedback mechanism involving raphe 5-HT_{1A} autoreceptors (Artigas *et al.* 1996). We therefore examined whether 5-HT_{1A} receptors restrained the apomorphine-induced increase in 5-HT output. The administration of the selective 5-HT_{1A} receptor antagonist WAY 100635 (0.3 mg/kg s.c.) markedly enhanced the 5-HT output in the DR with respect to control rats injected with saline ($F_{1,9} = 4.79$, $p < 0.06$ dose effect; $F_{15,135} = 5.03$, $p < 0.00001$ time effect; $F_{15,135} = 5.75$, $p < 0.00001$ time-dose interaction; Fig. 1e). The 5-HT output rose to 300% of baseline and declined slowly thereafter. In contrast, WAY 100635 (0.3 mg/kg s.c.) did not significantly alter the 5-HT output in striatum (Fig. 1f). The administration of WAY 100635 0.3 mg/kg s.c. in combination with apomorphine 3 mg/kg s.c. increased the 5-HT output significantly more than apomorphine alone ($F_{15,135} = 8.12$, $p < 0.00001$ time effect; $F_{15,135} = 5.99$, $p < 0.00001$ time-treatment interaction; Fig. 1e). This effect was not merely additive, since the combined treatment elevated the 5-HT output to $\approx 550\%$ of baseline. In dorsal striatum, the treatment with WAY 100635 0.3 mg/kg s.c. and apomorphine 3 mg/kg s.c. increased the 5-HT output comparably to apomorphine alone (Fig. 1f).

Effects of the local application of apomorphine and quinpirole in the DR

In rats implanted with two probes, the perfusion of apomorphine in the DR (10, 100 and 300 μM , four fractions each) reduced the 5-HT output at the higher concentration used ($F_{15,150} = 2.6$, $p < 0.002$ time effect; $F_{15,150} = 1.58$, $p = 0.083$ time-treatment interaction Fig. 3a). Apomorphine infusion in the DR did not significantly alter the 5-HT output in dorsal striatum (Fig. 3b). The perfusion of quinpirole (10, 100 and 300 μM , four fractions each; $n = 10$) in the DR did not affect significantly the 5-HT output in either region (Figs 3c and d).

In a previous report, the infusion of apomorphine (10–100 μM) induced a transient increase of the 5-HT output in the DR (Ferré and Artigas 1993). To examine the reasons of this discrepancy, we conducted two additional experiments in which apomorphine (100 μM) was infused in the DR using two slightly different positions of the probes, i.e. aimed at the centre of the DR and in a slightly more lateral coordinate, to sample the lateral wings of the DR, as described in Ferré *et al.* (1994). Apomorphine infusion did

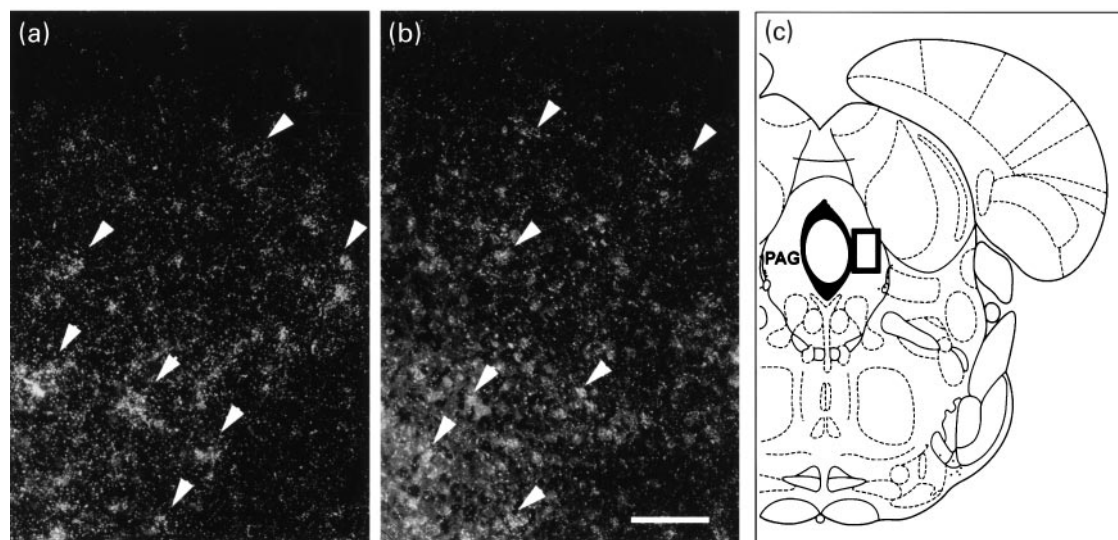


Fig. 6 Cellular localization of GAD mRNA (a) and dopamine D2 receptor mRNA (b) in the periaqueductal grey matter (PAG). Pictures are dark-field photomicrographs of emulsion-dipped sections. Box in (c) (modified from Paxinos and Watson atlas; CD-ROM edition) illustrates the anatomical level. Observe the relative

abundance of GAD positive cells (arrowheads) with a high hybridization labelling in PAG (a) and the existence of D2 receptor positive cells (arrowheads) in this same area (b) with lower levels of expression and abundance. Note that unlike in the previous figure, sections shown in (a) and (b) were not consecutive. (Bar = 100 μ M.)

not significantly increase the 5-HT output in either location ($n = 4$ rats/group; data not shown).

Single unit recordings: effects of the administration of dopaminergic agents on serotonergic cell firing

The administration of saline did not affect the firing rate of DR 5-HT neurons. The non-selective DA receptor agonist apomorphine (50–400 μ g/kg i.v.) increased the firing rate in six out of the nine neurons tested. There was little difference between the effects of low and high doses of apomorphine (Fig. 4a). Paired Student's *t*-test revealed a significant difference between the baseline firing rate and that after the administration of 100 μ g/kg ($p < 0.008$, $n = 5$) or 200 μ g/kg ($p < 0.001$, $n = 6$). Irrespective of the dose at which maximal effect was attained, this was significantly different from baseline ($p < 0.002$, $n = 6$). Two other neurons were inhibited and one remained unaffected by the i.v. administration of apomorphine. When considering all 5-HT neurons, the effect was marginally significant ($p = 0.10$). The further treatment with the selective 5-HT_{1A} receptor agonist 8-OH-DPAT (0.5–6 μ g/kg) suppressed the firing activity in all units tested. The DA D₂ receptor antagonist raclopride (125 μ g/kg i.v.) slightly (–19%) reduced the firing rate of DR 5-HT neurons, although this change was not significant and, injected 4 min before, prevented the increase in firing rate induced by cumulative doses of apomorphine (100–600 μ g/kg i.v.; $n = 4$) (Fig. 4b). Likewise, the DA D₁ receptor antagonist SCH 23390 (400 μ g/kg i.v.) non-significantly reduced serotonergic firing rate (–20%) and, injected 4 min before,

it also antagonized the increase in cell firing elicited by the injection of apomorphine (100–400 μ g/kg i.v.; $n = 4$; Fig. 4b).

The i.v. administration of the DA D₂-like agonist quinpirole also enhanced the firing rate of 5-HT neurons of the DR, although this effect was somewhat less marked than that of apomorphine (Figs 4c and d). At 80 μ g/kg, quinpirole significantly increased the firing rate of 5-HT cells compared to baseline ($p < 0.025$, $n = 5$) whereas at a higher dose (160 μ g/kg) the effect was marginally significant ($p = 0.095$, $n = 6$).

Micropressure injection of Dulbecco's solution (used as vehicle) did not alter the firing rate of 5-HT neurons. We used veratridine as a positive control to depolarize 5-HT neurons. The injection of veratridine (0.4–3.2 pmol) increased the firing rate of all 5-HT neurons tested ($n = 5$; maximal effect 1277% at 0.8 pmol in one neuron; data not shown). The administration of quinpirole by micropressure injection affected 5-HT neurons in a bimodal manner. In 16 cells of seven different rats, quinpirole (0.2–8 pmol) markedly inhibited the serotonergic firing rate (Fig. 4e). The mean inhibition was 53% (maximal 85% at 8 pmol injected; average of four rats). However, in five neurons of the same rats, the application of quinpirole was without effect up to 6 pmol (Fig. 4f). Neither neuron was excited by the application of quinpirole. Both quinpirole-sensitive and -insensitive 5-HT neurons were found randomly in descents along the midline. At the end of the experiments in each rat, we administered 8-OH-DPAT to confirm the serotonergic nature of the last neuron recorded.

In situ hybridization studies

Rat brain coronal sections at different anterior–posterior levels of the DR were treated as described (see Methods) and hybridized with probes complementary to the mRNA encoding the DA D₂ receptor. 5-HT cells in adjacent sections were labelled with a ³⁵S-oligonucleotide probe directed against the 5-HT transporter. Figure 5 shows *in situ* hybridization autoradiograms of the DR at two different magnifications (Figs 5a–b, c–d). A large number of 5-HT neurons were labelled by the oligonucleotide probe directed against the 5-HT transporter (Figs 5a and c). However, very few cells showed the presence of the DA D₂ transcript (Figs 5b and d). The substantia nigra pars compacta showed abundant dopaminergic neurons that were labelled by the same oligonucleotide probe (Fig. 5e). In the DR, very few neurons (marked by arrows) appeared to be labelled by both oligonucleotide probes in adjacent sections (Figs 5a–d).

In the PAG adjacent to the DR there was an abundance of cells containing the transcript for the DA D₂ receptor (Fig. 6a). This area was also very rich in GABAergic

neurons, as labelled by the transcript for GAD, the GABA-synthesizing enzyme (Fig. 6b) (unlike in the previous figure, these sections are not adjacent).

Dual probe experiments in SN and DR

The mismatch between the local and systemic effects of apomorphine and quinpirole suggested that the dopaminergic receptors responsible for the systemic effect of these drugs were not located in the DR. Given the presence of a large density of DA D₂ receptors in the SN and the existence of anatomical and functional relationships between the SN and the DR (see Introduction) we performed dual probe experiments to examine whether dopaminergic receptors in the SN could be involved in the effects of systemic apomorphine administration. Control rats perfused with artificial CSF for the whole experiments did not show any change in the 5-HT output in either region. The infusion of TTX (10 μM) in the SN markedly reduced the 5-HT release in this structure compared to controls ($F_{9,90} = 8.24$ $p < 0.00001$ time effect; $F_{9,90} = 8.56$ $p < 0.00001$ time–treatment interaction) and evoked a parallel increase in the DR ($F_{9,63} = 3.14$, $p < 0.005$, time–treatment interaction) (Figs 7a and b). Likewise, the application of apomorphine (10–100 μM) in the SN significantly enhanced the 5-HT in the DR compared with controls ($F_{9,72} = 2.88$, $p < 0.005$, time–treatment interaction; Fig. 8). In this case, the 5-HT concentration in samples of SN during the application of apomorphine could not be determined due to the presence of interfering peaks in the HPLC trace.

Discussion

The present study examined the effects of dopaminergic agents on the activity of DR 5-HT neurons using two

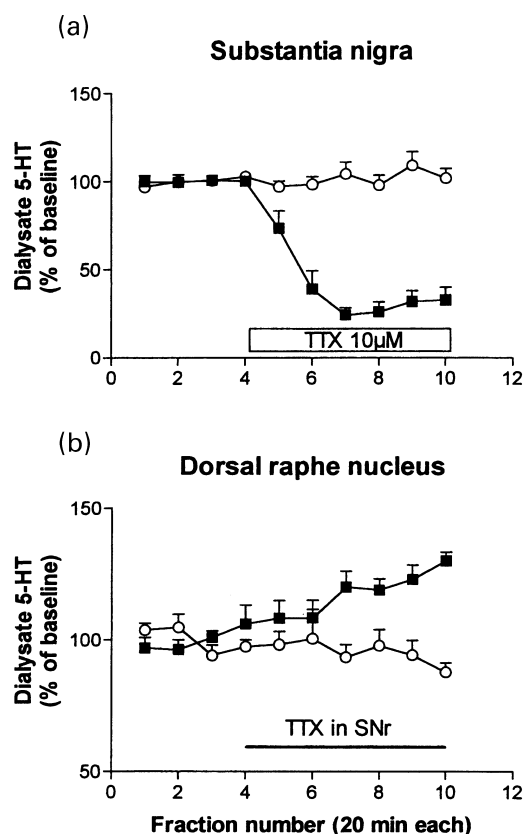


Fig. 7 Dual probe experiments in the SN and DR. The application of 10 μM TTX (●) in the SN (a) reduced markedly the 5-HT output in this area and increased it in the DR (b). Data are mean ± SE of five rats. Controls (○, $n = 4$) were perfused with artificial CSF. See Results for statistical details.

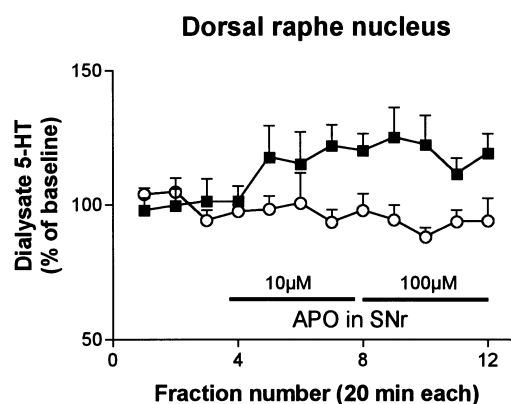


Fig. 8 In dual probe experiments, the application of 100 μM apomorphine (●) in the SN significantly raised the 5-HT output in the DR. The 5-HT content in samples from SN could not be determined due to interfering peaks in the HPLC trace (likely apomorphine impurities). Data are mean ± SE of six rats. Controls (○, $n = 4$) were perfused with artificial CSF. See Results for statistical details.

different techniques, *in vivo* microdialysis in freely moving rats and single unit recordings in the DR of anaesthetized rats. Overall, the results support a facilitatory action of DA D₁ and D₂ receptors on the activity of DR 5-HT neurons and of the 5-HT release in the DR. However, the single activation of DA D₂ receptors is not sufficient to increase the striatal 5-HT release.

In agreement with previous data (Ferré *et al.* 1994), the systemic administration of apomorphine enhanced the 5-HT output in the DR at all doses examined. This effect appears to involve DA D₁ and D₂ receptors, since it was fully antagonized by SCH 23390 and partly by raclopride. The involvement of DA D₂ receptors in the control of DR 5-HT output is also supported by the increase produced by quinpirole. The fact that SCH 23390 reduced 5-HT levels to below baseline may be indicative of a tonic control of 5-HT release in the DR by DA D₁ receptors.

Also, as previously observed at 0.5 mg/kg (Mendlin *et al.* 1998), apomorphine increased the 5-HT output in the dorsal striatum at this and higher doses. The antagonism of this effect by SCH 23390 supports the involvement of DA D₁ receptors. However, Mendlin *et al.* (1998) reported that systemic raclopride antagonized the increase in striatal 5-HT output produced by apomorphine, an observation that appears to be at variance with the unchanged striatal 5-HT output after quinpirole administration observed in the present study. A higher quinpirole dose (1 mg/kg s.c.) was also without effect in striatum and increased the 5-HT output in the DR (Martín-Ruiz and Artigas, unpublished observations). Moreover, the local application of 10 µM (Ferré *et al.* 1994) or higher (up to 1 mM) quinpirole concentrations (Abellán *et al.* 2000b) in dorsal striatum did not change the local 5-HT output. These observations raise doubts about the role of DA D₂ receptors in the control of the striatal 5-HT output. Thus, the present microdialysis experiments suggest that both DA D₁ and D₂ receptors are involved in the stimulatory effect of apomorphine on the 5-HT output in the DR but only the former appear to control the striatal 5-HT output.

In addition to increasing DR 5-HT output, apomorphine and quinpirole enhanced the firing rate of DR 5-HT neurons. Data from *in vivo* microdialysis and single unit recordings are not directly comparable due to the different routes of drug administration, the use of anaesthetized rats in single unit experiments and the distinct time scale of both techniques. However, the 5-HT release in the DR is sensitive to the propagation of nerve impulses, as it is reduced by TTX and 5-HT_{1A} agonists (Matos *et al.* 1996; Casanovas *et al.* 1997) and increased by depolarizing agents such as KCl or veratridine (Casanovas *et al.* unpublished observations). Thus, it is conceivable that the increase in DR 5-HT output produced by apomorphine results from a higher discharge rate of 5-HT neurons observed in single unit experiments. In agreement, Fornal *et al.* (1996a) observed an

increase in serotonergic firing rate after the administration of apomorphine to awake cats.

The change in 5-HT cell firing elicited by dopaminergic agents was moderate (± 20 –35%). This hindered the finding of a clear dose-effect relationship, as the effects of the lower doses employed were nearly maximal. SCH 23390 and raclopride significantly antagonized the enhancing effect of apomorphine. Thus, the facilitation of serotonergic activity by apomorphine appears to involve DA D₁ and D₂ receptors, as also observed in microdialysis experiments performed in the DR (see above). DA D₂ (but not DA D₁) receptors are present in a moderate density in the DR (Bouthenet *et al.* 1987; Palacios and Pazos 1987). This raised the possibility that they mediate the facilitatory effects of apomorphine and quinpirole on 5-HT neurons. However, the local application of these dopaminergic agonists in the DR in a wide range of concentrations did not increase the 5-HT output in this area or dorsal striatum in dual probe experiments. This is in contrast with previous observations from this laboratory showing that the application of apomorphine and quinpirole in the DR induced a moderate and transient increase in the local 5-HT output (Ferré and Artigas 1993). This discrepancy does not appear to be accountable by methodological factors such as probe type and location, perfusion fluid, analytical method, etc. Similar procedures were used in past and present studies, with the exception that the rats used herein were gently manipulated before experiments to reduce the effects of the stress/arousal associated to handling and injection on the raphe 5-HT output (Adell *et al.* 1997). In the present study, this increase was only evident in rats injected twice (e.g. antagonist experiments). It is thus possible that a distinct behavioural state of rats may play a role in this discrepancy. Interestingly, Mendlin *et al.* (1999) noted that raclopride attenuated the striatal 5-HT increase induced by behavioural activation but did not alter the basal 5-HT output in this area, as previously observed (Ferré *et al.* 1994). On the other hand, the blockade of postsynaptic 5-HT receptors attenuated the increase of the DA output in activated but not basal conditions (de Deurwaerdère and Spampinato 1999). These observations suggest a complex interaction between DA and 5-HT systems in which phasic stimuli may play an important role.

The dorsal striatum is exclusively innervated by 5-HT neurons from the DR (Azmitia and Segal 1978). 5-HT_{1A} receptor antagonists augment the effects of drugs that increase extracellular 5-HT in the DR by preventing a negative feedback mediated by 5-HT_{1A} autoreceptors (Artigas *et al.* 1996). The WAY 100635-induced potentiation of the effect of apomorphine on the DR 5-HT output suggests that 5-HT_{1A} autoreceptors also limit the effect of apomorphine on the DR output. This agrees in part with the conclusion from previous experiments showing that the 5-HT_{1A} receptor antagonist WAY 100135 reversed

a moderate inhibition in striatal 5-HT output produced by apomorphine (Ferré *et al.* 1994). Although the net effect of apomorphine on striatal 5-HT output differed markedly in both studies, the fact that apomorphine + 5-HT_{1A} antagonist combinations elevated 5-HT more than apomorphine alone in one or other area supports the notion that apomorphine indirectly increases the tone on 5-HT_{1A} autoreceptors.

The increase in DR 5-HT output elicited by WAY 100635 alone is consistent with the increase in serotonergic firing rate elicited by this agent in awake cats (Fornal *et al.* 1996b) and in midbrain slices containing the DR (Corradetti *et al.* 1996) although no such effect was observed in anaesthetized rats (Gartside *et al.* 1995). WAY 100635 might antagonize a tonic self-inhibition of the activity of 5-HT neurons, this leading to an increased release in the DR. However, we cannot discard that WAY 100635 reversed a citalopram-induced inhibition of 5-HT release in the DR given the presence of the SSRI in the perfusion fluid. WAY 100635 administration markedly potentiated the increase in 5-HT output produced by a larger (50 µM) citalopram concentration that activates raphe 5-HT_{1A} autoreceptors (Romero and Artigas 1997). Yet, it is unclear whether 1 µM citalopram, which elicits a partial blockade of the 5-HT reuptake (*in vivo* EC₅₀ = 1.0 µM; Hervás *et al.* 2000) can activate sufficiently 5-HT_{1A} autoreceptors as to attenuate the local 5-HT release. Contrary to the DR, WAY 100635 did not elevate the striatal 5-HT output when given alone, as previously observed (Romero and Artigas 1997), nor did it potentiate the effect of apomorphine in striatum (Mendlin *et al.* 1998; this study) which suggests a distinct sensitivity of the 5-HT release in both areas to WAY 100635 administration.

Quinpirole excited 5-HT neurons when administered systemically but not when applied in the DR. On the contrary, micropressure injection of quinpirole inhibited more than 70% of the neurons tested. Vehicle applications were without effect and veratridine dramatically increased the firing rate of 5-HT neurons, which validates the technique used. The local effect of quinpirole would be consistent with the inhibitory responses elicited by DA D₂ receptor stimulation (Vallar and Meldolesi 1991). However, few serotonergic neurons expressed this receptor subtype in the DR, which precludes their involvement in the local effects of quinpirole. Recently, dopamine was found to depolarize 5-HT neurons *in vitro* by an action on DA D₂ receptors (Haj-Dahmane and Shen 2000) which further complicates the understanding of the DA–5-HT interaction in the DR. It is unlikely that quinpirole-sensitive neurons were dopaminergic because of their firing characteristics, response to 8-OH-DPAT (when assessed) and low number in the DR (Hökfelt *et al.* 1976; Geffard *et al.* 1987; Kalén *et al.* 1988). Moreover, all recorded neurons were found in descents along the midline, containing dopaminergic fibres

but few DA cell bodies (Geffard *et al.* 1987). It is possible that the inhibition of 5-HT neurons by quinpirole results from a non-specific effect at the local doses used. Quinpirole and 8-OH-DPAT display common behavioural actions (Ahlenius and Larsson 1997). Yet, despite the high density of 5-HT_{1A} receptors in the DR and its inhibitory role, it is unlikely that quinpirole inhibits 5-HT neurons by activation of 5-HT_{1A} autoreceptors since quinpirole displays low affinity for 5-HT_{1A} receptors (Toll *et al.* 1998) and these are present in all 5-HT DR neurons (Sotelo *et al.* 1990) whereas quinpirole only inhibited 16 of the 21 neurons tested.

The scarcity of 5-HT neurons containing the DA D₂ receptor transcript and the fact that systemic quinpirole and apomorphine increased 5-HT cell firing and release suggests that the facilitation of serotonergic transmission produced by these agents is indirect, possibly through dopaminergic receptors in areas projecting to the DR. Several such areas exist in the midbrain that exhibit a moderate or high density of DA D₂ receptors, such as the SN, the ventral tegmental area and the lateral habenula (Bouthenet *et al.* 1987; Palacios and Pazos 1987). In addition, the PAG, in close vicinity to the DR, contains dense plexuses of dopaminergic fibres (Peyron *et al.* 1995) and a large density of DA D₂ receptors (Bouthenet *et al.* 1987) as well as GABAergic afferents to the DR (Gervasoni *et al.* 2000).

The activity of DR 5-HT neurons is tonically inhibited by GABA during periods of low behavioural activity, such as the light period in rats – present experimental conditions – (Nitz and Siegel 1997; Gervasoni *et al.* 2000). The activation of inhibitory receptors such as µ-opioid or GABA_B located on these GABAergic afferents disinhibits 5-HT neurons and increases the 5-HT release in the DR (Tao and Auerbach 1994; Jolas and Aghajanian 1997; Abellán *et al.* 2000a). The presence of GAD and DA D₂ transcripts in the PAG suggests that such a mechanism might participate in the increase in cell firing and 5-HT release elicited by apomorphine and quinpirole. It remains to be established (e.g. by using double *in situ* hybridization) whether both transcripts colocalize. Also, further work is needed to examine this possibility at a functional level using techniques other than dual probe microdialysis due to the proximity of the PAG and the DR.

In addition, DA receptors in the SN could be involved in the facilitatory effects of apomorphine and quinpirole on 5-HT neurons. The electrical stimulation of the SN suppressed the firing activity of DR 5-HT neurons (Stern *et al.* 1981) and somatodendritic DA receptors in the SN were postulated to regulate the activity of the raphe-striatal pathway (Héry *et al.* 1980). The dual-probe experiments conducted in the present study support the notion that the dopaminergic receptors in the SN may be involved in the effects of apomorphine on DR 5-HT neurons. Thus, the suppression of nerve impulses in the SN by TTX increased

the 5-HT output in the DR, which supports an inhibitory role of the SN on DR activity in accordance with previous electrophysiological evidence (Stern *et al.* 1981). Since the 5-HT output in the DR is markedly reduced by the local application of TTX (Matos *et al.* 1996) it is obvious that TTX diffusion to the DR cannot account for the 5-HT increase observed. The limited spatial resolution of dialysis probes (1.5-mm tip size) does not enable to conclude which part of the SN was involved in this effect but, conceivably, TTX suppressed the nigral GABAergic output to the midbrain (Chevalier and Deniau 1990), thus disinhibiting 5-HT neurons.

Furthermore, the activation of DA receptors in the SN by apomorphine enhanced the 5-HT output in the DR. Given the greater increase in DR output produced by apomorphine (versus quinpirole) we preferred to use the former, despite its lack of selectivity for DA D₁ and D₂ receptors. Indeed, the maximal effect size in the DR was moderate (~25%). However, apomorphine was applied unilaterally, thus affecting half of the nigral neurons putatively affected by systemic administration. These results are in agreement with the observation that the application of L-DOPA by reverse dialysis in the SN of freely moving rats increased the local 5-HT output and that in striatum (Thorré *et al.* 1998) which also appears to support a facilitatory action of nigral DA on the activity of the raphe-striatal pathway. Given the non-selective actions of apomorphine and the presence of DA D₁ receptors in the SN (Levey *et al.* 1993) both receptor subtypes might be involved in apomorphine effects.

In summary, the present results provide further evidence of a complex control of the serotonergic activity by dopaminergic transmission. Both DA D₂ and D₁ receptors are involved in the control of serotonergic activity and 5-HT release in the DR, whereas only the latter appear to mediate the facilitatory effects of apomorphine in the dorsal striatum. Based on the mismatch between the effects of local and systemic applications of drugs (also supported by mRNA data) we propose that dopaminergic receptors outside the DR control the activity of serotonergic neurons. A subpopulation of these receptors appears to be located in the SN, although other brain areas cannot be discarded and in particular the PAG, rich in DA D₂ receptor and GAD transcripts.

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