International Journal of Neuropsychopharmacology (2011), **14**, 289–302. © CINP 2010
doi:10.1017/S1461145710000349

ARTICLE

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Simultaneous projections from prefrontal cortex to dopaminergic and serotonergic nuclei

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Abstract

Derangements of the prefrontal cortex (PFC) and of brainstem monoaminergic systems occur in depression and schizophrenia. Anatomical and functional evidence supports a PFC control of the brainstem monoaminergic systems. Similarly, the PFC contains a high density of monoamine receptors for which antipsychotic drugs exhibit high affinity. This raises the possibility that pathological or drug-induced changes in PFC may subsequently alter monoaminergic activity. Recent data indicate that a substantial proportion of PFC pyramidal neurons projecting to the ventral tegmental area (VTA) or the dorsal raphe nucleus (DR) express the 5-HT_{2A} receptor mRNA, which suggests that atypical antipsychotic drugs affect serotonergic and dopaminergic function by targeting PFC 5-HT_{2A} receptors. Using electrophysiological and tract-tracing techniques we examined whether PFC pyramidal neurons projecting to DR are segregated from those projecting to the VTA. Sequential electrical stimulation of these nuclei in anaesthetized rats evoked antidromic potentials from both areas in the same pyramidal neurons of the medial PFC (60%, $n=30$). A similar percentage of dual DR+VTA projection neurons (50%) was obtained using the reciprocal collision test ($n=85$). Similarly, tracer application (Fluoro-Gold in VTA and cholera toxin B in DR, or vice versa) retrogradely labelled pyramidal neurons in PFC projecting to VTA (81 ± 18), to DR (52 ± 9) and to both nuclei (31 ± 4, $n=5$ rats). Overall, these results indicate that the PFC may simultaneously coordinate the activity of dopaminergic and serotonergic systems within a short temporal domain, supporting a concerted modulation of the ascending serotonergic and dopaminergic activity during antipsychotic drug treatment.

Received 9 December 2009; Reviewed 13 January 2010; Revised 17 February 2010; Accepted 2 March 2010; First published online 8 April 2010

Key words: Dopamine, dorsal raphe, prefrontal cortex, pyramidal neurons, ventral tegmental area.

Introduction

The prefrontal cortex (PFC) is involved in many higher brain functions, including mnemonic processes, attention and action planning, decision-taking, behavioural inhibition and control of emotional signals (Fuster, 2001; Miller & Cohen, 2001; Romo & Salinas, 2003). PFC functions largely rely on its extensive and often reciprocal connectivity with a vast array of cortical and subcortical structures (Fuster, 2001; Groenewegen & Uylings, 2000; Miller & Cohen, 2001). In particular, the medial PFC (mPFC) is reciprocally connected with the dorsal raphe nucleus (DR), which contains most ascending serotonergic (5-HT) neurons (Aghajanian & Wang, 1977; Celada *et al.* 2001; Gabbott *et al.* 2005; Hajós *et al.* 1998; Jankowski & Sesack, 2004; Peyron *et al.* 1998; Sesack *et al.* 1989). A similar reciprocal connectivity exists with the ventral tegmental area (VTA), which contains mesocortical dopaminergic (DA) neurons (Carr & Sesack, 2000; Gabbott *et al.* 2005; Geisler & Zahm, 2005; Thierry *et al.* 1979, 1983*a*; Tong *et al.* 1996*b*). Thus, afferent excitatory pathways from mPFC to these two nuclei exert a profound influence on the activity of DA and 5-HT neurons (Aghajanian & Wang, 1977; Celada *et al.* 2001; Hajós

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et al. 1998; Thierry *et al.* 1979, 1983*a*; Tong *et al.* 1996*b*), which in turn project to cortical and limbic areas, including the PFC.

The PFC–DR–VTA connectivity is a particularly interesting subject of study due to the involvement of these brain areas in thought and affective disorders. Hence, the DA and 5-HT innervation of the PFC plays important roles in cognitive processes, including working memory and cognitive flexibility (Clarke *et al.* 2004; Robbins & Roberts, 2007; Williams & Goldman-Rakic, 1995; Williams *et al.* 2002). Moreover, alterations of the PFC have been reported in severe psychiatric conditions such as depression and schizophrenia which are also associated with derangements of both monoaminergic systems (see for review Benes & Berretta, 2001; Drevets, 2001; Harrison, 1999; Lewis *et al.* 2005; Manji *et al.* 2001; Selemon & Goldman-Rakic, 1999). Furthermore, the functional interactions between the PFC, VTA, and DR may be crucial for understanding the therapeutic action of antidepressant and antipsychotic drugs, which target transporters or receptors expressed by DA, 5-HT and PFC neurons (Arnt & Skarsfeldt, 1998; Artigas *et al.* 1996; Bymaster *et al.* 1996; Cortés *et al.* 1988; Mengod *et al.* 1989; Santana *et al.* 2004, 2009; Weiner *et al.* 1991). In particular, recent findings indicate the presence of 5-HT_{2A} receptor mRNA in a large proportion of PFC neurons projecting to the DR or VTA (Vázquez-Borsetti *et al.* 2008), which suggests that atypical antipsychotic drugs may partly exert their therapeutic effect by attenuating the activity of excitatory inputs to these midbrain nuclei. Finally, high-frequency stimulation of Brodmann area 25 (Cg25) in treatment-resistant depressives induced an immediate clinical improvement, an effect that may involve the connectivity of Cg25 with midbrain nuclei (Mayberg *et al.* 2005).

However, despite VTA-projecting and DR-projecting pyramidal neurons in the PFC being localized to the same areas (mainly prelimbic and infralimbic subdivisions), it is unknown whether these are segregated or overlapping populations. Using electrophysiological and tract-tracing techniques, we examined whether the PFC input to the VTA and the DR arises from the same projection neurons, which would raise the possibility of a concurrent PFC control of the ascending DA and 5-HT pathways.

Experimental procedures

Animals

Male albino Wistar rats (Iffa Credo, France) weighing 250–300 g were used in this study. Animals were kept in a controlled environment [12-h light/dark cycle (lights on 07:00 hours) and 22 ± 2 °C room temperature] with food and water available *ad libitum*. All experimental procedures used in this study were in strict compliance with the European Communities Council directive 86/609/EEC on 'Protection of Animals Used in Experimental and Other Scientific Purposes' of 24 November 1986 (O.J. of E.C. L358, 18/12/1986).

Electrophysiological recordings

We undertook two systematic studies to evaluate the incidence of mPFC neurons simultaneously projecting to the DR and the VTA. In the first set of experiments, we used the antidromic potential technique (Fuller & Schlag, 1976) with sequential DR/VTA stimulation while recording pyramidal neurons in mPFC. In a second set of experiments, dual-projecting neurons were identified using the reciprocal collision test (Ferino *et al.* 1987; Shadow, 1982).

Surgery was performed as previously described (Puig *et al.* 2003). Briefly, rats were anaesthetized (chloral hydrate, 400 mg/kg i.p.) and additional doses of chloral hydrate (80 mg/kg, 1 ml/kg) were administered i.v. Bipolar stimulating electrodes consisted of two stainless-steel enamel-coated wires (California Fine Wire, USA) with a diameter of 150 μm and *in-vitro* impedances of 10–30 kΩ. Poles were slightly separated under microscopic examination to 220–240 μm. Stimulating electrodes were stereotaxically implanted in both structures, the DR (AP -7.6 to -7.8; L -3.1 with 30° of inclination; DV -6.2 to -6.8) and VTA (AP -5.6 to -6.0; L -0.5; DV -8.2) Stereotaxic coordinates were taken from bregma and according to the atlas of Paxinos & Watson (1986). After implant, the electrodes were secured to the skull with glue and dental cement. Constant current electrical stimuli were generated with a Cibertec stimulation unit CS-20 (Cibertec, Spain) connected to two SIU 165 stimulus isolation units. The intensity of the stimulating current (0.2 ms square pulses, 0.9 Hz) was 1.16 ± 0.08 mA for DR and 1.14 ± 0.07 mA for VTA in the first set of experiments, and 0.94 ± 0.03 mA for DR and 0.98 ± 0.03 mA for VTA in the second set of experiments (reciprocal collision test). Pyramidal neurons were recorded with glass micropipettes filled with 2 M NaCl. Typically, *in-vitro* impedance was between 4 and 10 MΩ. Single unit extracellular recordings were amplified with a Neurodata IR283 (Cygnus Technology Inc., USA), post-amplified and filtered with a Cibertec amplifier (Madrid, Spain) and computed online using a DAT 1401plus interface system, Spike2 software (Cambridge Electronic Design, UK).

In the first set of experiments we systematically confirmed that only a single pyramidal neuron was recorded by identification of antidromic activation from the DR or the VTA (Fuller & Schlag, 1976). After recording antidromically evoked spikes from one of these sites (VTA or DR), the current was switched to the other site (DR or VTA, respectively). Dual-projection neurons were characterized by the presence of antidromic spikes after stimulation of both sites.

In the second set of experiments dual-projecting neurons were identified using the reciprocal collision test. We measured the reciprocal collision time, defined as the maximal interval between stimulus application at each site which produces a blockade of the second evoked antidromic spike. With this technique, it is assumed that one neuron projects to two structures when the reciprocal collision time is greater than the difference between the latencies of the two antidromic responses plus the refractory period of the second axonal branch stimulated (Ferino *et al.* 1987; Thierry *et al.* 1983*b*).

At the end of the experiments, rats were killed by an overdose of anaesthetic. The placement of the stimulating electrodes was verified histologically. The data from rats with electrodes implanted outside the DR or VTA were not used.

Tract-tracing studies

Animals were anaesthetized with sodium pentobarbital (60 mg/kg i.p.), and placed in a stereotaxic frame. Glass capillary tubes were heated, pulled and the tips broken to 30 μm outer diameter under microscopic control. The micropipettes were filled with cholera toxin B subunit (CTB, List Biological Laboratories, USA) or Fluoro-Gold™ (FG; Fluorochrome LLC, USA). A solution of CTB (2% in distilled water) was iontophoretically injected into the DR ($n=4$) or the VTA ($n=1$). The same animals subsequently received iontophoretic injections of FG (2% in 0.1 M cacodylate buffer; pH 7.3) into the VTA ($n=4$) or the DR ($n=1$), respectively. The injections were performed using a 5-μA positive-pulsed direct current for a total of 5 min (7 s on/off for 10 min). After surgery, rats were housed individually. Ten days later, the animals were anaesthetized with an i.p. overdose of sodium pentobarbital and perfused transcardially with 50 ml calcium-free Tyrode's solution (6.8 g/l NaCl, 0.4 g/l KCl, 0.32 g/l $MgCl_2 \cdot 6H_2O$, 0.10 g/l $MgSO_4 \cdot 7H_2O$, 0.17 g/l $NaH_2PO_4 \cdot H_2O$, 2.2 g/l $NaHCO_3$, 1.1 g/l glucose) containing 0.1% heparin at 37 °C, followed by 50 ml of warm (37 °C) and 300 ml of cold (4 °C) fixative (4% paraformaldehyde in 0.1 M phosphate buffer; pH 6.9). After perfusion, the skull was opened and the brain carefully removed and stored for 2 d in 10% sucrose in 0.1 M phosphate-buffered saline (PBS) (pH 7.4). The brains were then frozen by slow immersion into dry-ice chilled isopentane. Tissue sections, 14-μm thick, were cut on a microtome-cryostat (Microm HM500 OM, Germany), and mounted onto Histogrip-treated glass slides (Zymed Laboratories Inc., USA) and processed for the indirect immunofluorescence technique of Coons (1958). Thus, the sections were preincubated in 0.1 M PBS for 15 min at room temperature and then incubated for 3–4 d in a humid chamber at 4 °C with a rabbit CTB antiserum (1:1000; Sigma, USA) alone or in combination with sheep tryptophan hydroxylase (TPH) antiserum (1:100; Chemicon International, USA) or a tyrosine hydroxylase (TH) monoclonal antibody (1:1000; Diasorin, USA) in 0.1 M PBS containing 0.3% Triton X-100 and 2% BSA. Then the sections were rinsed in 0.1 M PBS and incubated for 30 min at 37 °C in the same buffer containing 0.3% Triton X-100 and appropriate combinations of the following secondary antibodies: Alexa Fluor® 546-goat anti-rabbit, Alexa Fluor 488®-goat anti-mouse, Alexa Fluor® 488-donkey anti-rabbit, AlexaFluor 546®-donkey anti-goat (all 1:500, Molecular Probes, The Netherlands) or fluorescein isothiocyanate (FITC)-donkey anti-sheep (1:50; Chemicon International). Finally, the sections were washed in PBS and coverslipped using Mowiol (Merck, Germany). The sections were observed in an epifluorescence microscope (Nikon Eclipse E1000, Japan) and a confocal microscope equipped with a spectral head (Leica SP2; Leica, Germany). In all instances, FG was detected by its native fluorescence.

Cell counting and data analysis

PFC sections processed for immunohistochemical detection of CTB were analysed with the epifluorescence microscope to identify the distribution of pyramidal neurons projecting to the VTA and/or the DR. The medial and orbital cortices were then examined in more detail with the confocal microscope. Ten consecutive optical sections (1 μm each) in the vertical axis were scanned for the fluorophores present in the tissue (Alexa Fluor® 546 and FG) in adjacent frames all along the medial and the orbital prefrontal cortices. The optical sections were colourized (red for Alexa Fluor® 546 and green for FG) and merged with the confocal microscope software. The red and green images were combined to visualize double-labelled elements. Cell counting was done on the red and green images separately using the Image J 1.32j software (by Wayne Rasband, National Institute of Health, USA). The 10 optical sections were examined for each labelled

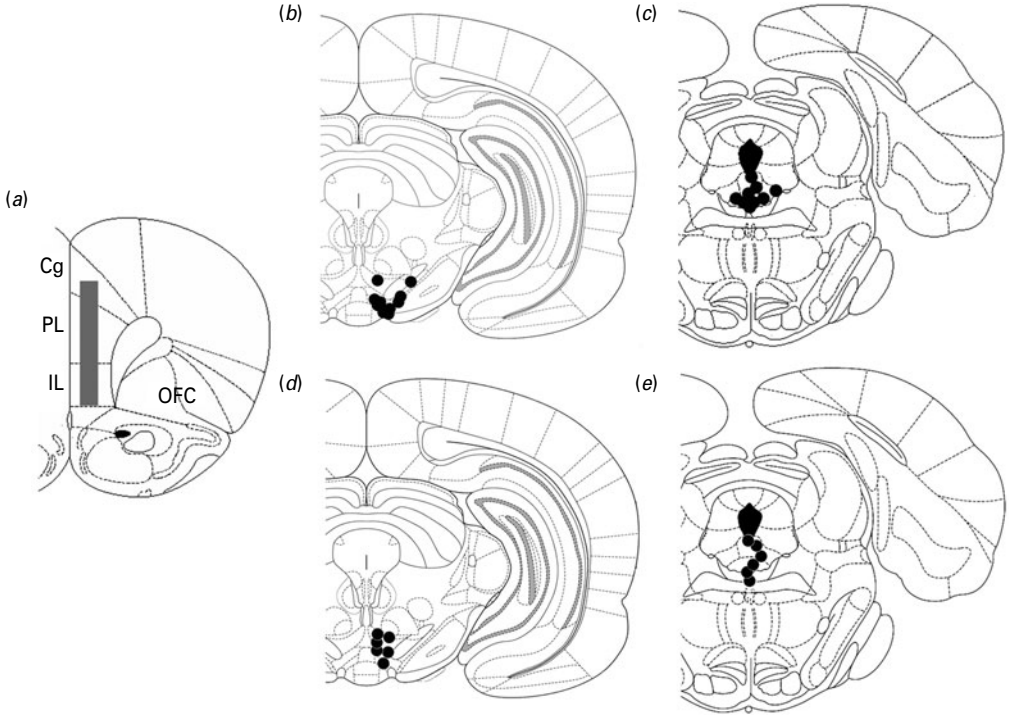

Fig. 1. (*a*) Schematic drawing of a frontal section of the rat brain at AP +3.2 mm (Paxinos & Watson, CD edition, 1997, with permission from Elsevier Science). The shaded area indicates the region where pyramidal neurons were recorded. (*b–e*) Schematic representation of the location (black dots) of the tip of bipolar electrodes used to stimulate the ventral tegmental area (*b, d*) and the dorsal raphe nucleus (*c, e*). Panels (*b*) and (*c*) correspond to expt 1 (antidromic test) and panels (*d*) and (*e*) correspond to expt 2 (reciprocal collision test) (see text). Cg, Cingulate; PL, prelimbic; IL, infralimbic; OFC, orbitofrontal cortex.

element and cell profiles were considered positive only when labelling was identified in more than six consecutive planes.

Sections through the injection sites were observed with the fluorescence microscope and photographed with a Nikon DXM1200 camera. The digital images obtained were combined using the AnalySIS Software (Soft Imaging System GmbH, Germany) to prepare Figs 4 and 6. Images were treated with Adobe Photoshop to adjust brightness, contrast and size to make the final figures.

Data are expressed as means ± S.E.M. Statistical analyses were done using Student's *t* test for independent data. Statistical significance has been set at the 95% confidence level (two-tailed).

Results

Electrophysiological experiments

Two sets of electrophysiological experiments were conducted, using the simple antidromic technique and the reciprocal collision test, respectively. Figure 1 shows the recording and stimulation sites in the two sets of experiments.

In the first set ($n = 48$ experiments), pyramidal neurons in mPFC were identified by antidromic stimulation from either the VTA or the DR. In 18 cases, the histological examination revealed that one or other electrodes were slightly misplaced. Therefore, data were taken from 30 experiments performed in 10 different rats. Data on latencies and conduction velocities correspond to the total number of experiments performed, since these variables are not affected by small differences in electrode location.

Antidromic spikes were evoked in the same pyramidal neuron by sequential stimulation of the DR and VTA in 18/30 (60%) cases. Five neurons (16.7%) were antidromically activated by DR (but not VTA) stimulation whereas seven neurons (22.3%) were activated by VTA (but not DR) stimulation. Figure 2 shows representative examples of the recording of a pyramidal neuron projecting to the VTA, the DR and both. The latencies of the antidromic spikes evoked by

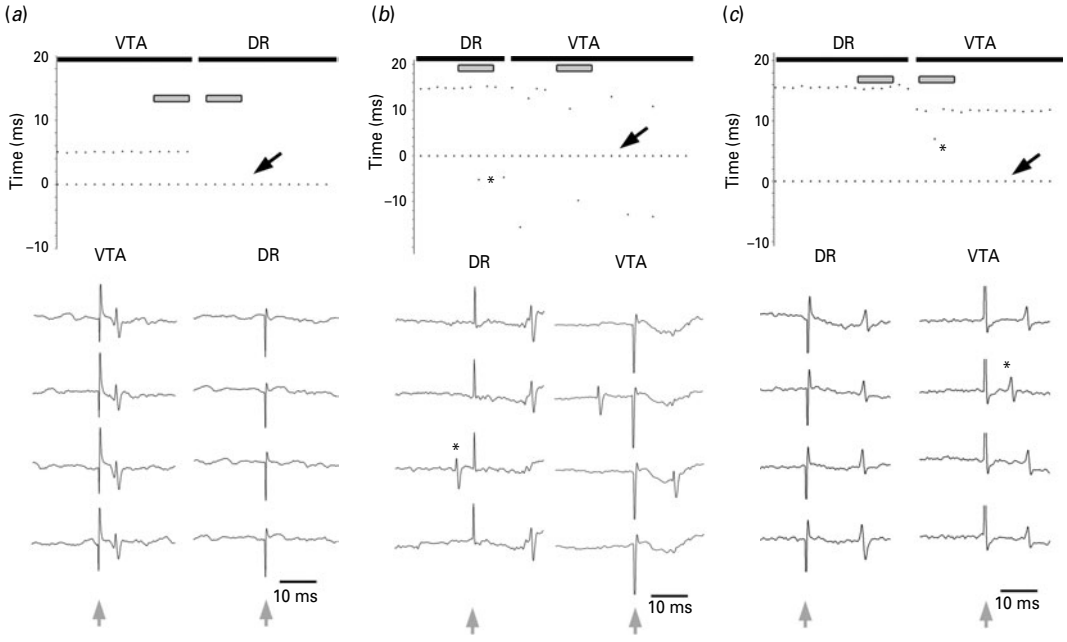

Fig. 2. Identification of pyramidal neurons in the medial prefrontal cortex (mPFC) by antidromic activation from the dorsal raphe nucleus (DR) and ventral tegmental area (VTA). Upper panels are raster plots. In these, ordinates are in ms and represent the time at which an antidromic potential (each dot) is recorded in a PFC pyramidal neuron when stimulating the DR or VTA (stimulation times are shown by black bars). Dots at $t = 0$ correspond to the stimulus artifact (shown with a black arrow). The neurons in panels (*a–c*) project to the VTA, DR and VTA + DR, respectively, as shown by the existence of antidromic potentials (dots) at a fixed latency from the stimulus when the respective nuclei are stimulated. Lower panels: actual spike traces corresponding to the time periods indicated in raster plots, immediately before and after switching the stimulating current from the DR to the VTA electrode. Asterisks denote antidromic spikes missing due to collisions with ongoing spontaneous action potentials during stimulation of the DR or VTA. Grey arrows indicate the stimulus artifact. (*a*) Example of a mPFC pyramidal neuron showing antidromic potentials after stimulation from the VTA (latency 4.4 ms) but not from DR. (*b*) Example of a mPFC pyramidal neuron showing antidromic potentials after stimulation from the DR (latency 15 ms) but not from the VTA. (*c*) Example of a mPFC pyramidal neuron showing antidromic potentials after stimulation from the DR (latency 15.8 ms) and from the VTA (latency 11.6 ms).

VTA stimulation were significantly lower than those evoked by DR stimulation (Table 1).

In a second set of experiments, using the reciprocal collision test, a total of 85 neurons were antidromically activated from either the DR or VTA. Of these, 20 were only activated from DR, 23 only from VTA and 42 from both nuclei (23%, 27%, 50%, respectively) (Fig. 3). Table 1 shows the latencies of antidromic spikes obtained with the reciprocal collision test.

Tract-tracing studies

In order to examine for the presence of PFC pyramidal neurons with simultaneous projections to the DR and the VTA, we conducted a series of tract-tracing experiments in which CTB and FG were microiontophoretically applied in the DR and VTA, respectively ($n = 4$ rats). We also examined one animal where the two tracers were switched (CTB in the VTA and FG in the DR) to examine whether this provided comparable results.

Figure 4 shows fluorescence microscope images depicting the localization of the tracers in the DR (CTB) and the VTA (FG) as well as the schematic representation of the localization of pyramidal neurons in PFC projecting to one or both nuclei ($n = 4$). In these rats, the tracers were confined within the respective nuclei, as shown by the co-localization of the tracer and the markers of 5-HT neurons of the DR (TPH) and DA neurons of the VTA (TH).

CTB application in the DR labelled a large number of neurons in layer V of the prelimbic and infralimbic subdivisions of the mPFC (Fig. 4) as well as a more moderate number of neurons in the anterior cingulate as well as in lateral and ventral orbitofrontal cortex. Labelled neurons were found in both hemispheres.

Table 1. Latencies of antidromic spikes evoked in medial prefrontal cortex (mPFC) pyramidal neurons by stimulation of midbrain monoaminergic nuclei

Stimulation site	Latency (ms)
Expt 1 (antidromic activation)	
DR ($n=31$)	13.7 ± 0.8
VTA ($n=35$)	7.9 ± 0.7*
Expt 2 (reciprocal collision test)	
All neurons	
DR ($n=61$)	12.6 ± 0.5
VTA ($n=65$)	7.6 ± 0.7*
Dual-projection neurons	
DR ($n=42$)	12.1 ± 0.6
VTA ($n=42$)	7.5 ± 0.7*

DR; Dorsal raphe nucleus; VTA, ventral tegmental area. Data (mean $\pm$ S.E.M.) are latencies of antidromic spikes reaching layer V mPFC neurons by stimulation of bipolar electrodes located in the DR and VTA (see Fig. 1). The intensity of the stimulating current (0.2 ms square pulses, 0.9 Hz) was 1.16 ± 0.08 mA for DR and 1.14 ± 0.07 mA for VTA in the first set of experiments, and 0.94 ± 0.03 mA for DR and 0.98 ± 0.03 mA for VTA in the second set of experiments (reciprocal collision test).
* $p < 0.0002$ *vs.* DR, Student's t test.

A few neurons were labelled in the agranular insular cortex, adjacent to those in the orbitofrontal cortex.

FG application into the VTA also labelled a large number of neurons in the prelimbic and infralimbic mPFC (Fig. 4). With the exception of the orbitofrontal cortex, which contained only a few FG-labelled neurons, the localization of VTA-projecting neurons was very similar to that of neurons projecting to the DR, in the cingulate, prelimbic and infralimbic subdivisions. In contrast to injections in the DR, FG application in the VTA mostly labelled neurons in the ipsilateral hemisphere, and, only a few VTA-projecting neurons were found in the contralateral side. Figure 5 shows the actual localization of mPFC pyramidal neurons projecting to DR (Fig. 5*a*), VTA (Fig. 5*b*) or both (Fig. 5*b*).

Figure 6 shows fluorescence microscope images depicting the localization of the tracers in the DR and VTA of the rat injected with the inverse tracer combination (e.g. FG in the DR and CTB in VTA). This resulted in a distribution of labelled neurons similar to that described above. A large number of FG-labelled neurons (DR-projecting) were found in the medial and orbitofrontal cortex of both hemispheres. Similarly, CTB application in the VTA labelled a large number of neurons in the ipsilateral mPFC and no neurons in the orbitofrontal cortex. Very few CTB-labelled cells were detected in the contralateral mPFC. Table 2 shows the absolute number of DR-projecting and VTA-projecting neurons in the ipsilateral PFC of the five rats examined. On average, the percentage of dual-projection neurons was $63 \pm 6\%$ for those projecting to DR and $42 \pm 6\%$ for those projecting to VTA.

Discussion

The present study presents electrophysiological and histological evidence supporting the existence of a substantial population of mPFC pyramidal neurons that simultaneously project to the VTA and DR. The proportion of dual-projection neurons can be estimated to be 50–60% according to the electrophysiological experiments. This was confirmed by the results from histological experiments, which also revealed the existence of a considerable population of mPFC neurons projecting to the DR and VTA. Thus, the present results indicate that the ascending 5-HT and DA pathways are under a concurrent control of their activity by the same pyramidal neurons in the mPFC.

Methodological considerations

In agreement with pilot observations (Puig *et al.* 2003), the use of the antidromic activation/collision test technique enabled a systematic identification of PFC pyramidal neurons projecting simultaneously to the DR and VTA. A similar proportion was obtained using the reciprocal collision test (Ferino *et al.* 1987; Shadow, 1982; Thierry *et al.* 1983*a*), which uses more stringent experimental conditions to avoid the misidentification of previously silent projection neurons recorded extracellularly that might be activated by antidromic spikes. This technique was used to identify the existence of efferent neuronal projections with branching axons in different structures, including the PFC. In a pioneering study, Thierry *et al.* (1983*a*) reported on different patterns of dual-projection neurons in mPFC innervating several structures such as mediodorsal thalamus, VTA, substantia nigra, superior colliculus, central grey and habenula. The present study is in accord with the view that PFC pyramidal neurons have branching axons to simultaneously coordinate the activity of various subcortical structures. The latencies found herein for single and dual-projection neurons do not differ and are within the range of those previously reported for mPFC pyramidal neurons projecting to the 5-HT and DA nuclei (Celada *et al.* 2001; Peterson *et al.* 1990; Puig *et al.* 2003; Thierry *et al.* 1983*a*).

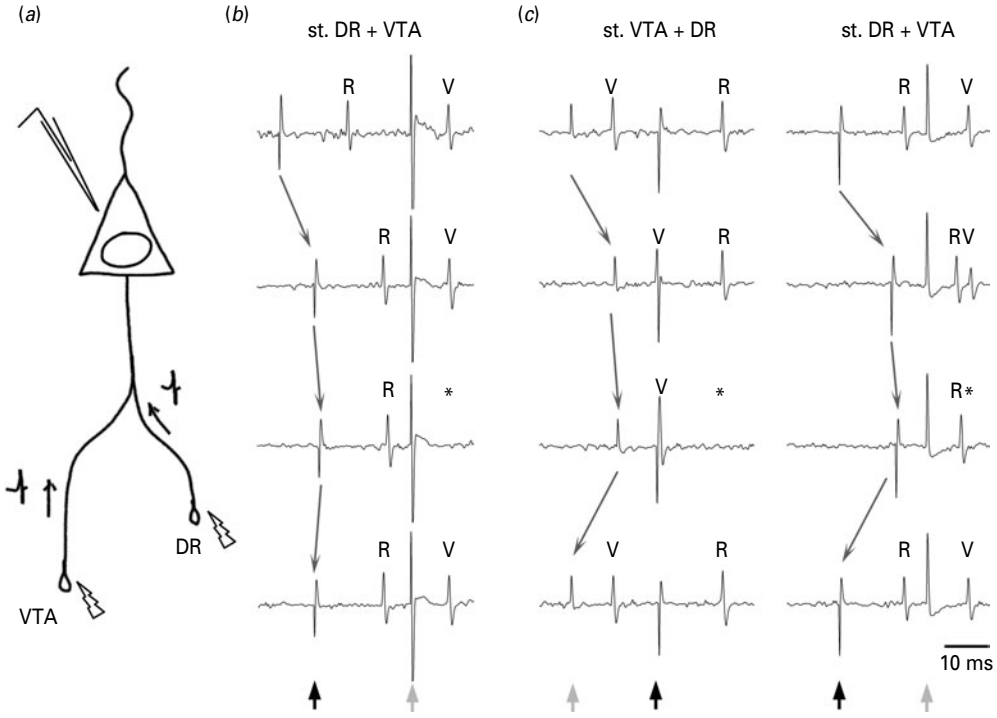

Fig. 3. Identification of pyramidal neurons in medial prefrontal cortex projecting to dorsal raphe nucleus (DR) and ventral tegmental area (VTA) by antidromic activation from these areas and reciprocal collision test. (*a*) Diagram of the stimulating and recording sites. (*b*) Example of a pyramidal neuron showing antidromic potentials after stimulation from DR (shown by R; latency 15.4 ms) and from VTA (shown by V; latency 8.2 ms). The asterisk denotes a missing antidromic spike when the distance between two consecutive stimulus was 21 ms (collision test). This neuron, when stimulated from VTA had a refractory period of 2 ms. (*c*) Left panel: example of reciprocal collision test showing a neuron antidromically activated from VTA (latency 9.1 ms) and DR (latency 14.4 ms). The refractory period when stimulated from DR was 2.2 ms. The asterisk denotes a missing antidromic spike when the distance between consecutive stimuli was 9 ms (collision test). Right panel: the reversal in the order of stimulation also induced a blockade of the second antidromic spike in the same neuron. Stimulus artifacts induced after DR or VTA stimulations are shown with a black or grey arrow, respectively.

A significantly lower latency was found for the PFC–VTA pathway than for the PFC–DR pathway, which may be due to the shorter distance between mPFC and VTA and the proximity of the latter nucleus to the median forebrain bundle (MFB), implying that fast-conduction myelinized axons exist for most of the trajectory between the mPFC and VTA.

The use of the tract-tracing techniques provided essentially the same results as electrophysiological techniques, indicating that a substantial proportion of PFC neurons project simultaneously to the DR and VTA. Pyramidal neurons projecting to the DR and/or VTA were mainly found in layer V of the infralimbic, prelimbic and cingulate cortices, in accord with previous reports in the literature using either electrophysiological or tract-tracing techniques (Aghajanian & Wang, 1977; Celada *et al.* 2001; Gabbott *et al.* 2005; Geisler & Zahm, 2005; Hajós *et al.* 1998; Jankowski & Sesack, 2004; Peyron *et al.* 1998; Sesack *et al.* 1989; Thierry *et al.* 1979, 1983*b*; Tong *et al.* 1996*b*, 1998). Similarly, more DR- than VTA-projecting neurons were found in the orbitofrontal cortex, which is in accord with previously reported data (Gabbott *et al.* 2005; Geisler & Zahm, 2005; Peyron *et al.* 1998).

The technique of microiontophoretic application allows a restricted application of the tracers (see e.g. Vázquez-Borsetti *et al.* 2008, using the same methodology as the present study). One possible limitation of the use of FG and CTB is their uptake by damaged fibres of passage close to the application site. CTB has been reported to be taken up by fibres of passage (Chen & Aston-Jones, 1995). Most studies using FG have reported no uptake (Pieribone & Aston-Jones, 1988; Schmued & Fallon, 1986; Schmued & Heimer, 1990), whereas one study found FG uptake after pressure injection (Dado *et al.* 1990). This potential

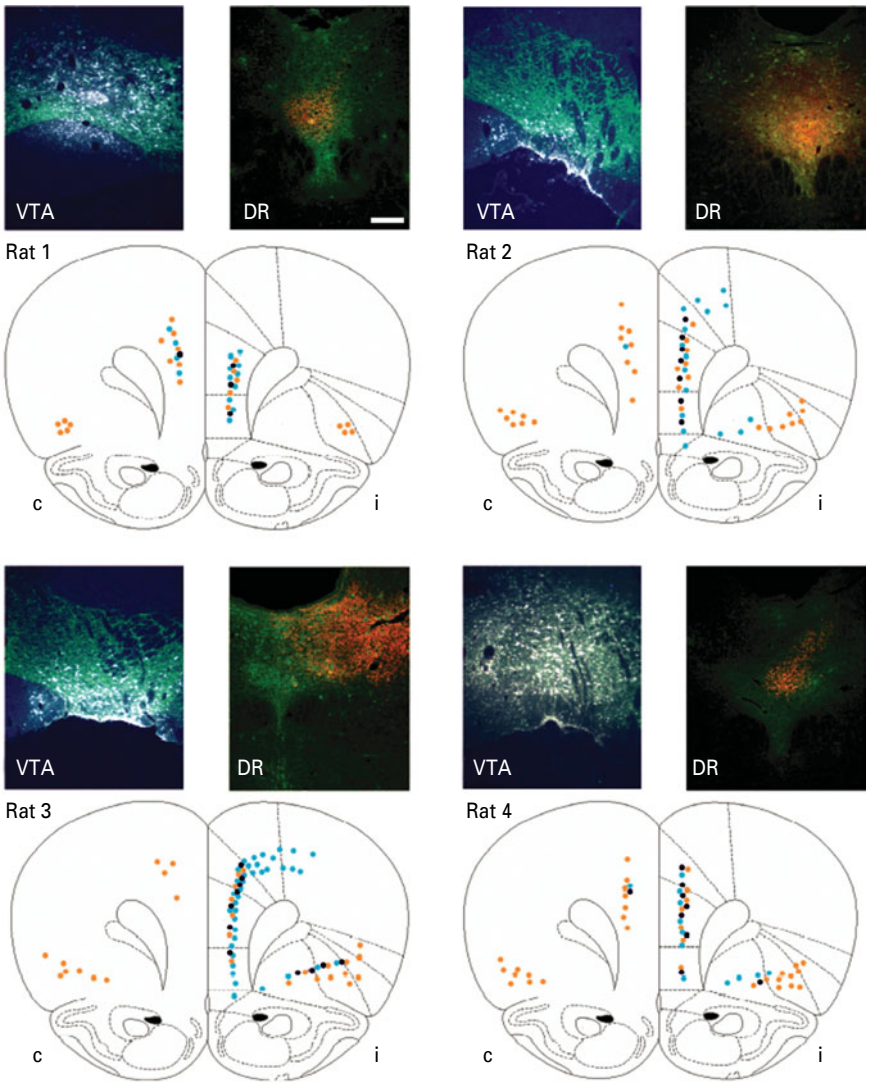

Fig. 4. Retrograde labelling of prefrontal cortex (PFC) neurons after microiontophoretic application of Fluoro-Gold (FG) in the ventral tegmental area (VTA) and cholera toxin B subunit (CTB) in the dorsal raphe nucleus (DR). For each rat (nos. 1–4), schematic representations of coronal sections at the PFC level (+3.2 mm from bregma; Paxinos & Watson, 1986) illustrate the distribution of cells retrogradely labelled with FG (light blue dots), CTB (orange dots) or both tracers (black dots). Every dot represents five labelled neurons. Note the presence of numerous neurons projecting to the VTA in the prelimbic and infralimbic cortices, extending dorsally to the cingulate and secondary motor areas and ventrally to the dorsal peduncular and orbital cortices in the hemisphere ipsilateral (i) to the injection site. Only a few VTA-projecting neurons are found in the prelimbic cortex of the contralateral (c) side. Neurons projecting to the DR are detected in both the ipsilateral and contralateral hemispheres, mainly concentrated in the prelimbic and insular agranular cortices. CTB-labelled cells are also be encountered in the orbital, infralimbic and cingulate cortical areas. The location of injection sites in each rat is illustrated in the corresponding photomicrographs obtained from sections through the brainstem which show tyrosine hydroxylase immunoreactivity (green) and FG fluorescence (white) in the VTA and tryptophan hydroxylase immunoreactivity (green) and CTB immunoreactivity (red) the DR. Images were obtained with a Colorview camera in a Nikon Eclipse fluorescence microscope and were overlapped using AnalySIS software. Bar, 200 μm.

limitation is minimized by the microiontophoretic application of the tracer, as used in the present study. This procedure resulted in a tracer distribution within the boundaries of the DR and VTA as observed by the coincidence of the tracer with TPH and TH immunoreactivity. Moreover, tracer uptake by damaged MFB

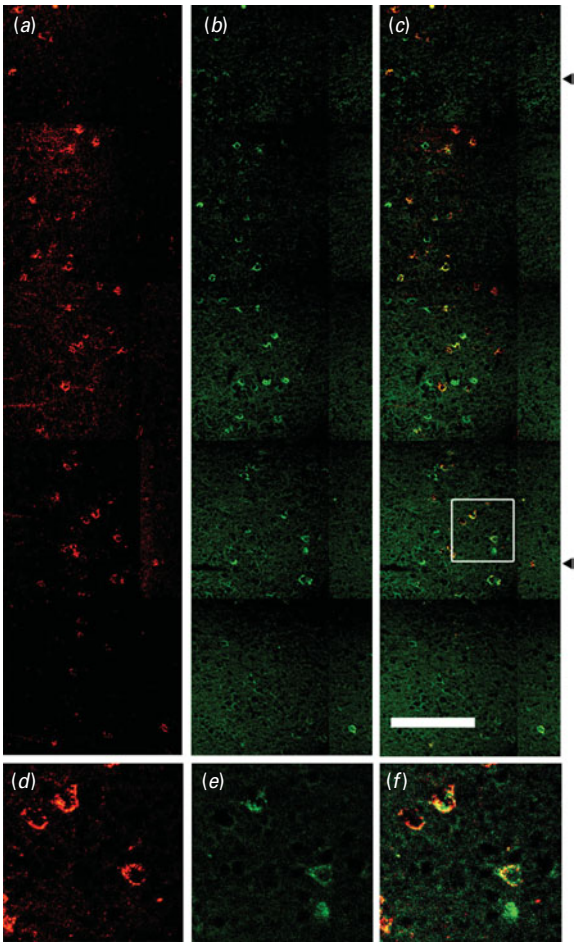

Fig. 5. Confocal photomicrographs of a prefrontal cortex (PFC) coronal section of a rat (no. 4; see Fig. 3) that received cholera toxin B subunit (CTB) in the dorsal raphe nucleus (DR) and Fluoro-Gold (FG) in the ventral tegmental area (VTA), after incubation with CTB antiserum. FG was visualized by its own florescence. The images in panels (*a–c*) are montages of 10 consecutive optical sections (1 μm each) colourized and merged with the confocal software. Panels (*d–f*) show enlargements of the boxed area in panel (*c*). (*a, d*) CTB-immunoreactive cell bodies are observed along layer V of the mPFC. (*b, e*) FG fluorescence shown in green is also present in numerous cells in layer V. (*c, f*) Superimposed images show the presence of CTB (red), FG (green) or both tracers (yellow). Black arrows on the right mark the limit between the cingulate and the prelimbic cortices (upper arrow) and the limit between the prelimbic and infralimbic cortices (lower arrow). Bar, 200 μm.

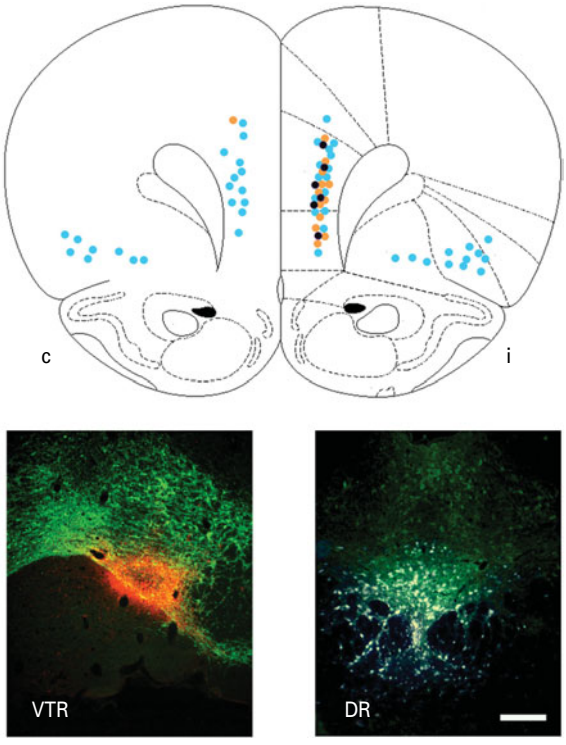

Fig. 6. Retrograde labelling of prefrontal cortex neurons after microiontophoretic application of cholera toxin B subunit (CTB) in the ventral tegmental area (VTA) and Fluoro-Gold (FG) in the dorsal raphe nucleus (DR). Schematic representation of a coronal section at the prefrontal cortex level (+3.2 mm from bregma; Paxinos & Watson, 1986) illustrating the distribution of cells retrogradely labelled with FG (blue dots), CTB (orange dots) or both tracers (violet dots) in rat no. 5. Every dot represents five labelled neurons. The distribution of VTA- and DR-projecting cells is comparable to that described in Fig. 3. The location of injection sites is illustrated in photomicrographs obtained from sections through the brainstem of the same rat which show tyrosine hydroxylase immunoreactivity (green) and CTB immunoreactivity (red) in the VTA and tryptophan hydroxylase immunoreactivity (green) and FG fluorescence (white) in the DR. Images were obtained with a Colorview camera in a Nikon Eclipse fluorescence microscope and were overlapped using AnalySIS software. Bar, 200 μm.

fibres after application in the VTA would have resulted in a more widespread distribution of labelled neurons in the PFC, particularly in the primary and secondary motor areas, that contain the largest proportion of corticospinal fibres of all PFC subdivisions (Gabbott *et al.* 2005). Only one rat (rat no. 3; see Fig. 3) contained some labelled neurons in the secondary motor area labelled by the tracer application in the VTA. Yet in this case, the number of labelled neurons was higher in the mPFC (cingulate, prelimbic, infralimbic) than in the motor PFC.

Moreover, the ratio between the number of VTA-projecting neurons and DR-projecting neurons in

Table 2. Labelling of medial prefrontal cortex neurons by tracer application (CTB or FG) in the DR and VTA

	VTA	DR	Both
Rat no. 1	49	22	15 (31%, 68%)
Rat no. 2	147	47	38 (26%, 81%)
Rat no. 3	88	75	39 (44%, 52%)
Rat no. 4	57	50	33 (58%, 66%)
Rat no. 5 (FG in VTA; CTB in DR)	65	68	32 (49%, 47%)
Average	81 ± 18	52 ± 9	31 ± 4 (42%, 63%)

CTB, Cholera toxin B subunit; FG, Fluoro-Gold; DR, dorsal raphe nucleus; VTA, ventral tegmental area.
Data are numbers of neurons labelled by CTB application in the VTA and FG application in the DR (rat nos. 1–4) or vice versa (rat no. 5) in individual sections of the PFC (approx. bregma +3.2; Paxinos & Watson, 1986).
Values in parentheses are the percentages of double-labelled neurons projecting to VTA and DR, respectively.

the mPFC was 1.5 on average (Table 2), which is in close agreement with that reported using single applications of wheat germ agglutinin conjugated with horseradish peroxidase in each site (VTA/DR ratios of 3.28, 1.21 and 1.02 for the infralimbic, prelimbic and cingulate cortices, respectively; see table 3 in Gabbott *et al.* 2005). Based on this evidence, it is most likely that the present methods specifically labelled pyramidal neurons in mPFC projecting to the DR and VTA.

Notwithstanding the reliability of the two techniques used in the present study, a quantitative difference was noted. The percentage of dual-projection neurons obtained with the antidromic activation technique was 50–60% which was very similar (63%) to that obtained by tract-tracing techniques when results were expressed by reference to the total of DR-projecting neurons. A lower proportion (42%) was obtained when expressing the data by reference to VTA-projecting neurons, which are more abundant in mPFC. One possible reason for this difference is the smaller size of the VTA compared to the DR, which extends ~2 mm along the rostrocaudal axis. Here it is possible that only a small proportion of the actual number of PFC neurons projecting to the DR had been labelled by tracer application in this nucleus compared with those labelled by tracer application in the VTA. This would result in an apparently greater number of VTA-projecting neurons in the mPFC, as observed, and therefore result in a comparatively lower percentage of dual-projection neurons when expressed by reference to VTA. Moreover, it may be that electrical stimulation activates myelinized axons in the VTA that would not take up the tracers.

Functional and therapeutic implications

Several previous studies have examined the collateralization of PFC axons innervating subcortical structures (Cassell *et al.* 1989; Ferino *et al.* 1987; Gabbott *et al.* 2005; Pinto & Sesack, 2000; Thierry *et al.* 1983*a*) with varying results. Some studies have reported little collateralization of cortico-accumbens neurons (Pinto & Sesack, 2000), whereas others have found a significant branching of PFC axons innervating the contralateral PFC and several subcortical structures (Cassell *et al.* 1989; Ferino *et al.* 1987; Thierry *et al.* 1983*a*).

The results of the present study closely match those of the original study by Thierry *et al.* (1983*a*), who reported a very high proportion of branched axons of neurons projecting to the VTA (ventromedial tegmentum, as designated in the 1980s) and substantia nigra compacta. Overall, this indicates that a subpopulation of PFC neurons projecting to the brainstem show extensive collateralization, which would allow for the synchronization of the activity of several midbrain nuclei following PFC activation.

The mPFC also exerts a very important control of the activity of 5-HT neurons. Two independent studies revealed that more than 80% of DR 5-HT neurons responded to the electrical stimulation of the mPFC (Celada *et al.* 2001; Hajós *et al.* 1998). The responses elicited differ, possibly depending on the stimulation site. Stimulation of the infralimbic PFC resulted in a

majority of inhibitory responses in 5-HT neurons, which were not blocked by the selective 5-HT_{1A} receptor antagonist WAY-100635, while stimulation at a more dorsal site increased the proportion of excitatory responses (Hajós *et al.* 1998). However, another study, reported that mPFC stimulation evoked both 5-HT_{1A} and $GABA_A$ receptor-dependent inhibitions and AMPA and NMDA receptor-dependent excitations of 5-HT neurons (Celada *et al.* 2001). Subsequent tracing studies revealed that ventral aspects of the PFC mainly synapse on GABA cells in the DR (Jankowski & Sesack, 2004; Varga *et al.* 2001), and establish a low proportion of synaptic contacts with 5-HT cells (Jankowski & Sesack, 2004).

Thus, both electrophysiological and track-tracing studies indicate that the PFC may modulate 5-HT neuron activity via direct excitatory inputs as well as indirect inhibitory inputs involving $GABA_A$ and 5-HT_{1A} receptors. However, the electrical and chemical stimulation of the mPFC increased the activity of DR 5-HT neurons and increased DR 5-HT release (Amargos-Bosch *et al.* 2003; Celada *et al.* 2001; Martin-Ruiz *et al.* 2001) supporting the existence of overall excitatory PFC input onto DR 5-HT neurons.

Similarly, the PFC provides an important afferent excitatory pathway to the VTA, as reported by anatomical and electrophysiological studies (Carr & Sesack, 2000; Gabbott *et al.* 2005; Geisler & Zahm, 2005; Hurley *et al.* 1991; Sesack & Pickel, 1992; Thierry *et al.* 1979, 1983*b*; Tong *et al.* 1996*a*, 1998). mPFC afferents synapse onto DA and non-DA (GABA) cells with well established connectivity patterns. Thus, DA neurons projecting back to PFC and to other unidentified structures (but not to nucleus accumbens) receive monosynaptic inputs from PFC (Carr & Sesack, 2000). Similarly, VTA GABAergic neurons projecting to nucleus accumbens are innervated by direct mPFC inputs (Carr & Sesack, 2000). In this manner, mPFC afferents can regulate the activity of the mesocortical DA pathway and the mesolimbic GABAergic pathway, in addition to DA and GABA cells projecting to other structures by monosynaptic excitatory inputs (Carr & Sesack, 2000). *In-vitro* recordings in the VTA indicate that these excitatory inputs are important to elicit long-term changes in the activity of DA and GABA cells (Bonci & Malenka, 1999). *In-vivo* recordings indicate that PFC inputs are critical to evoke burst firing in DA cells (Gariano & Groves, 1988; Overton & Clark, 1997) yet this may involve both direct and indirect pathways (i.e. via laterodorsal/pedunculopontine tegmentum) (Grace *et al.* 2007).

The present data may have several important pathophysiological and pharmacological implications. The ascending DA and 5-HT systems have been implicated in emotional control, in close relationship with other structures (PFC, cingulate cortex, hippocampus, amygdala, etc.) making up the extended limbic system (Morgane *et al.* 2005). Dopamine neurons increase their activity to signal rewards or reward-associated stimuli (Schultz, 2004, 2007) and increases in DR activity have also been reported in response to future rewards (Tanaka *et al.* 2004). On the other hand, both systems are involved in cognitive processes. Among other functions, dopamine and serotonin play a role in the maintenance of persistent neuronal activity in dorsolateral PFC during working-memory tasks (Williams & Goldman-Rakic, 1995; Williams *et al.* 2002).

Based on the present results, we suggest that relevant behavioural or emotional stimuli reaching the PFC may evoke concerted changes of the activity of the ascending DA and 5-HT neurons. This simultaneous control would occur within a very short temporal domain (typically <20 ms) and would concurrently set the DA and 5-HT tone in various subcortical and cortical areas (including the PFC). These phasic PFC inputs would add to the tonic regulation of 5-HT and DA activity which is strongly dependent on inputs from locus coeruleus (via α_1-adrenoceptor activation; VanderMaelen & Aghajanian, 1983) and hippocampus (via nucleus accumbens–ventral pallidum inputs) (Grace *et al.* 2007), respectively.

Similarly, the present observations may have some implications for the pathophysiology and treatment of severe psychiatric disorders such as depression and schizophrenia. Persistent changes in the metabolic activity of the PFC in these disorders have been reported (Andreasen *et al.* 1997; Catafau *et al.* 1994; Ressler & Mayberg, 2007; Shergill *et al.* 2000). These alterations may translate into a malfunction of 5-HT and DA systems perhaps underlying depressive and psychotic symptoms. Moreover, drugs acting on receptors/transporters highly expressed in the PFC such as antidepressant or atypical antipsychotic drugs may indirectly modulate the 5-HT and DA activity via actions on PFC targets (Bortolozzi *et al.* 2003; Diaz-Mataix *et al.* 2005). In particular, atypical antipsychotic drugs have high *in-vitro* affinity for 5-HT_{2A} receptors (Arnt & Skarsfeldt, 1998; Bymaster *et al.* 1996) and produce a large occupancy of cortical 5-HT_2 receptors at therapeutic dosage (Kapur *et al.* 1999). The present results, together with our previous observations on the presence of 5-HT_{2A} receptor mRNA in $\sim$55–60% of PFC neurons projecting to the DR or VTA, suggest that

atypical antipsychotic drugs may modulate the activity of DA and 5-HT neurons in a synchronous and coordinated way, an effect possibly involved in their therapeutic activity in schizophrenia and as enhancers of the antidepressant activity of SSRIs (Zhang *et al.* 2000). Moreover, atypical antipsychotic drugs also display moderate-high affinity for other monoaminergic receptors expressed in mPFC (Pieribone *et al.* 1994; Pompeiano *et al.* 1992, 1994; Santana *et al.* 2004, 2009). Although further studies are required to examine whether these receptors are also expressed in pyramidal neurons projecting to VTA/DR, these observations support the view that atypical antipsychotic drugs may partly exert their therapeutic action by distally regulating – among others – the activity of ascending monoaminergic neurons.

Acknowledgements

This work was supported by grants SAF 2007–62378, FIS PI060264 and SENY Fundació. P.V-B. was the recipient of a FPU predoctoral fellowship from the Spanish Ministry of Education. P.C. is supported by the Researcher Stabilization Programme of the Health Department of the Generalitat de Catalunya. We thank Dr J. L. Lanciego for helpful suggestions in tracer experiments during the course of this work.

Statement of Interest

None.

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