Expression of 5-HT$_{2A}$ receptors in prefrontal cortex pyramidal neurons projecting to nucleus accumbens. Potential relevance for atypical antipsychotic action

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Abstract

The prefrontal cortex (PFC) is involved in higher brain functions altered in schizophrenia. Classical antipsychotic drugs modulate information processing in cortico-limbic circuits via dopamine D2 receptor blockade in nucleus accumbens (NAc) whereas atypical antipsychotic drugs preferentially target cortical serotonin (5-HT) receptors. The brain networks involved in the therapeutic action of atypical drugs are not fully understood. Previous work indicated that medial PFC (mPFC) pyramidal neurons projecting to ventral tegmental area express 5-HT_{2A} receptors suggesting that atypical antipsychotic drugs modulate dopaminergic activity distally, via 5-HT_{2A} receptor (5-HT_{2A}-R) blockade in PFC. Since the mPFC also projects heavily to NAc, we examined whether NAc-projecting pyramidal neurons also express 5-HT_{2A}-R. Using a combination of retrograde tracing experiments and *in situ* hybridization we report that a substantial proportion of mPFC-NAc pyramidal neurons in rat brain express 5-HT_{2A}-R mRNA in a layer- and area-specific manner (up to 68% in layer V of contralateral cingulate). The functional relevance of 5-HT_{2A}-R to modulate mPFC-NAc projections was examined in dual-probe microdialysis experiments. The application of the preferential 5-HT_{2A}-R agonist DOI into mPFC enhanced glutamate release locally (+66±18 %) and in NAc (+74±12 %) indicating that cortical 5-HT_{2A}-R activation augments glutamatergic transmission in NAc. Since NAc integrates glutamatergic and dopaminergic inputs, blockade of 5-HT_{2A}-R by atypical drugs may reduce cortical excitatory inputs onto GABAergic neurons of NAc, adding to dopamine D2 receptor blockade. Together with previous observations, the present results suggest that atypical antipsychotic drugs may control the activity of the mesolimbic pathway at cell body and terminal level.

Key words: Atypical antipsychotics; Dopamine; Mesolimbic pathway; Pyramidal neurons; Schizophrenia; Serotonin receptors

Abbeviations: CTB, cholera toxin B; DA, dopamine; FG, Fluoro-Gold; mRNA, messenger RNA; mPFC, medial PFC; NAc, nucleus accumbens; PFC, prefrontal cortex; -R, receptor; VTA, ventral tegmental area

1. Introduction

Schizophrenia is a severe psychiatric illness causing a large economic burden in developed societies (Kessler et al., 2005; Knapp et al., 2004; Smith, 2011). Alterations in several brain regions/networks have been reported in schizophrenia. In particular, the prefrontal cortex (PFC) is critically involved in many higher brain functions, including cognitive/executive functions and behavioral control, which are deeply altered in schizophrenic patients (Elvevag and Goldberg, 2000; Lewis and Lieberman, 2000; Weinberger et al., 2001). Autopsy and neuroimaging studies have revealed the existence of a reduced PFC volume, reduced layer thickness, reduced neuropil and tight packing of cortical neurons in the brains of schizophrenic patients (Harrison, 1999; Lewis and Lieberman, 2000; Selemon and Goldman-Rakic, 1999). A reduced energy metabolism in PFC has been related with negative symptoms (Andreasen et al., 1997; Potkin et al., 2002) whereas psychotic symptoms appear associated with hyperactivity of various cortical areas, including the PFC (Catafau et al., 1994; Dierks et al., 1999; Shergill et al., 2000). Alterations in key neurotransmitters such as glutamate, GABA and dopamine (DA) have also been reported in PFC (Benes and Berretta, 2001; Lewis et al., 2005; Lewis and Lieberman, 2000). In particular, hyperactive subcortical (mesolimbic) and hypoactive mesocortical DA function have been reported (Abi-Dargham et al., 2002; Breier et al., 1997; Laruelle et al., 1996; Lewis and Lieberman, 2000)

Classical antipsychotic drugs are thought to ameliorate psychotic symptoms by blocking an excessive activation of DA D2 receptors (D2-R) located in ventral striatum (nucleus accumbens –NAc– and adjacent structures). This therapeutic action is associated to severe motor side effects due to D2-R blockade in dorsal striatum. Likewise, D2-R blockade induces negative symptoms in healthy individuals, possibly by dampening cortical DA transmission (Artaloytia et al., 2006). In contrast, atypical antipsychotic drugs -and particularly clozapine, the gold standard in antipsychotic treatments (Leucht et al., 2009)- produce much lesser D2-R occupancy and preferentially target serotonin (5-HT) receptors such as $5\text{-}HT_{2A}$, $5\text{-}HT_{2C}$ and $5\text{-}HT_{1A}$ receptors of which $5\text{-}HT_{2A}$-R appear to play a key role (Arnt and Skarsfeldt, 1998; Artigas, 2010; Bymaster et al., 1996; Chou et al., 2003). These 5-HT receptors (in particular $5\text{-}HT_{2A}$-R) are mainly cortical and are densely expressed in various subfields of the rat PFC, particularly in its medial aspect (Pompeiano et al., 1992, 1994). Several studies have established their presence in a large proportion of PFC pyramidal and GABAergic neurons in rodent and human brains (Amargós-Bosch et al., 2004; de Almeida and Mengod, 2007, 2008; Santana et al., 2004). The physiological release of 5-HT excites and inhibits the activity of pyramidal neurons

in mPFC via 5-HT$_{2A}$-R and 5-HT$_{1A}$-R, respectively (Amargós-Bosch et al., 2004; Puig et al., 2005). Likewise, the systemic administration of the preferential 5-HT$_{2A}$ agonist DOI also evokes an overall increase of pyramidal neuron activity in mPFC (Puig et al., 2003).

The brain networks involved in the therapeutic action of atypical drugs are not fully understood. In particular, the pathways by which dopamine D2 receptor blockade (mainly subcortical) and 5-HT$_{2A}$ receptor blockade (mainly cortical) modulate brain function to evoke clinical antipsychotic effects are unclear. Previous microdialysis studies have indicated an important role of 5-HT$_{A1}$-R and 5-HT$_{2A}$-R in the control of DA release in cortical and subcortical regions, which contributes to atypical antipsychotic action (Bortolozzi et al., 2010; Diaz-Mataix et al., 2005; Ichikawa and Meltzer, 1991; Ichikawa and Meltzer, 1995; Ichikawa and Meltzer, 2000; Ichikawa et al., 1995, 2001; Kuroki et al., 1999; Li et al., 2005; Yamamoto et al., 1994; see for review see Meltzer and Huang, 2008). However, the fact that systemic drug administration was used in most of these studies hampers the precise identification of the neuronal pathways involved.

Anatomical and functional studies indicate the existence of a 5-HT$_{2A}$-R-mediated control of DA neurons in the ventral tegmental area (VTA). On the one hand, PFC pyramidal neurons project densely to the VTA (Gabbott et al., 2005; Sesack et al., 1989) making synapses onto dopaminergic and GABAergic neurons (Carr and Sesack, 2000; Sesack and Pickel, 1992). These glutamatergic inputs are important to switch discharge of DA neurons from tonic to phasic (burst) discharge (Gariano and Groves, 1988; Murase et al., 1993; Tong et al., 1996a). On the other hand, a large percentage of mPFC pyramidal neurons projecting to midbrain structures, including the VTA and the dorsal raphe nucleus, express 5-HT$_{2A}$-R (Vázquez-Borsetti et al., 2009). Consistent with these anatomical observations, the local stimulation of 5-HT$_{2A}$-R in mPFC enhanced DA burst firing and DA release in rodent brain (Bortolozzi et al., 2005; Bortolozzi et al., 2010). These observations indicate that PFC 5-HT$_{2A}$-R distally control VTA DA neuronal activity and raise the possibility that atypical antipsychotic drugs may attenuate an excessive dopaminergic activity in schizophrenia by antagonizing excitatory 5-HT$_{2A}$-R in pyramidal neurons projecting to VTA. This pharmacological effect would help atypical antipsychotic drugs to reduce dopaminergic function without the massive blockade of striatal DA D2-R produced by classical antipsychotic drugs (Kapur et al., 1999).

The ventral striatum is a brain structure with two main sources of innervation, descending excitatory inputs from prefrontal cortex using glutamate as neurotransmitter, and ascending dopaminergic inputs from the VTA (mesolimbic pathway). Hence, the mPFC projects heavily to

NAc (Gabbott et al., 2005) where descending glutamatergic terminals synapse on spiny neurons also receiving dopaminergic inputs (Sesack and Pickel, 1992). These observations suggest that 5-HT$_{2A}$-R in mPFC pyramidal neurons might also be involved in the control of NAc neurons, and therefore in the modulation of signals along basal ganglia circuits. We tested this hypothesis using a combination of tract-tracing techniques and *in situ* hybridization to examine whether mPFC pyramidal neurons projecting to NAc express 5-HT$_{2A}$-R. The functional relevance of 5-HT$_{2A}$-R in the activity of descending mPFC-NAc afferents has been assessed using dual-probe microdialysis.

2. Materials and Methods

2.1. Surgery and tissue preparation.

A total of 5 male albino Wistar rats (Iffa Credo,Lyon, France) weighting 250–275 g were used. Animals were kept in a controlled environment (12 h light-dark cycle and 22 ± 2 °C room temperature) with food and water provided *ad libitum*. Animal care followed the European Union regulations (O.J. of E.C. L358/118/12/1986) and protocols used were approved by the Ethical Committee for Animal Research of the University of Barcelona and the ''Departament de Medi Ambient i Habitatge'' from the Catalan Government (Generalitat de Catalunya).

Animals were deeply anesthetized with sodium pentobarbital (60 mg/kg ip), and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Glass capillary tubes were heated and pulled with a Narishige PE-2 pipette puller (Narishige Sci. Inst., Tokyo, Japan). Tips were broken to 30 μm diameter under microscopic control. Such micropipettes were filled with a solution of cholera-toxin B subunit (CTB, 2% in distilled water; List Biological Laboratories, Campbell, CA, USA) or Fluoro-Gold (FG, 2% in cacodylate buffer, pH 3.8; Fluorochrome LLC, Denver, CO, USA). Retrotracers were injected into the nucleus accumbens (NAc) at stereotaxic coordinates 1.7 mm AP, -1.6 mm L, -6.8 mm DV from bregma, according to the rat brain atlas of Paxinos and Watson (1998). The injections were done by microiontophoresis using a Midgard Precision current source device (Stoelting, Wood Dale, IL, USA) using a 5 μA positive-pulsed direct current for a total of 5 min (7 s on/off for 10 min). After surgery animals were housed individually to prevent any risk of injury from other animals. Fifteen days after surgery rats were deeply anesthetized and perfused transcardially with 120 mL of calcium-free Tyrode's solution (6.8 g/L NaCl; 0.4 g/L KCl; 0.32 g/L MgCl$_2$·6H$_2$O; 0.10 g/L MgSO$_4$·7H$_2$O; 0.17 g/L NaH$_2$PO$_4$·H$_2$O; 2.2 g/L NaHCO$_3$; 1.1 g/L glucose) containing 0.1 % heparin at room temperature, followed by 60 mL of fixative (4% paraformaldehyde in 0.1 M

phosphate buffer, pH 6.9) at room temperature and 300 mL of cold (4 °C) fixative. The brains were quickly removed, immersed in fixative for 90 min, kept in 10 % sucrose in 0.1 M phosphate buffer for 2 days at 4 °C, and finally frozen in isopentane at -30 °C.

Fourteen μm thick slices were cut using a microtome-cryostat (Microm HM560, Walldorf, Germany) and thaw-mounted onto microscope glass-slides pretreated with HistoGrip (Invitrogen, Frederick, MD, USA) and kept at -20 °C until used. The correct placement of tracer injection in the NAc was verified by direct observation of FG fluorescence at the microscope or by immunohistochemical labeling of CTB (see procedure described below). Figure 1 illustrates the precise location of microinjections performed in the different animals used.

2.2. Histochemistry

Tissue sections were hybridized with oligodeoxyribonucleotide probes complementary to 5-HT$_{2A}$ receptor mRNA. We used 3 oligonucleotide probes complementary to bases 669-716, 1923-1970, and 1482-1529 (GenBank accession no. X13971.1) (Pritchett et al., 1988). The specificity of the hybridization signal obtained with these probes has been previously established (Pompeiano et al., 1994) using the following control procedures: 1) the thermal stability of the hybrids obtained was checked for every probe, 2) for every oligonucleotide probe, the hybridization signal was completely blocked by competition of the labeled probe in the presence of 50-fold excess of the same unlabeled oligonucleotide, and 3) the 3 probes used independently demonstrated identical distributions of hybridization signal at regional and cellular levels. All oligonucleotides were synthesized and HPLC purified by Isogen Bioscience BV (Maarsden, The Netherlands). Each probe (2 pmol) was individually labeled at the 3'-end with [^{33}P]α-dATP (>2500 Ci/mmol; Perkin-Elmer, Boston, MA, USA) using terminal deoxynucleotidyltransferase (Roche Diagnostics GmbH, Mannheim, Germany) and then purified by centrifugation using QIAquick Nucleotide Removal Kit (Qiagen GmbH, Hilden, Germany).

The protocol for *in situ* hybridization combined with immunohistochemistry was based on previously described procedures (Tomiyama et al., 1997) and has been described in detail (Vázquez-Borsetti et al., 2009). Briefly, frozen tissue sections were first brought to room temperature, washed in 3x Dulbecco's PBS (dPBS; 1x dPBS: 8 mM Na$_2$HPO4, 1.4 mM KH$_2$PO$_4$, 136 mM NaCl, 2.6 mM KCl) and twice in 1x dPBS, incubated 2 min at 20 ºC in a solution of predigested Pronase (Calbiochem, San Diego, CA, USA) at a final concentration of 24 U/mL in 50 mM Tris-HCl pH 7.5, 5 mM ethylenediaminetetraacetic acid (EDTA). The enzymatic activity

was stopped by immersion for 30 s in 2 mg/mL glycine in 1x dPBS. Tissues were finally rinsed in 1x dPBS and dehydrated through a graded series of ethanols. For hybridization, the labeled probes were diluted in a solution containing 50 % formamide, 4x SSC (1x SSC: 150 mM NaCl, 15 mM sodium citrate), 1x Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 10% dextran sulfate, 1% sarkosyl, 20 mM phosphate buffer pH 7.0, 250 µg/mL yeast tRNA and 500 µg/mL salmon sperm DNA. Tissue sections were covered with hybridization solution containing the labeled probes, overlaid with Nescofilm coverslips (Bando Chemical Ind., Kobe, Japan) and incubated overnight at 42 ºC in humid boxes. Sections were then washed 4 times (45 min each) in washing buffer (0.6 M NaCl, 10 mM Tris-HCl pH 7.5) at 60 ºC and once in the same buffer at room temperature for 15 min. After the *in situ* hybridization protocol, tissue sections were kept for 15 min in buffer A (0.1 M Tris-HCl, pH 7.5, containing 1 M NaCl, 2 mM $MgCl_2.6H_2O$ and 2% albumin bovine serum (BSA: Sigma-Aldrich, Germany)). Then sections were incubated overnight in a humid chamber at 4 ºC with rabbit antiserum anti-CTB (1:1000; Sigma, St. Louis, MI, USA) or a rabbit serum anti-FG (1:1000 Chemicon, Billerica, MA, USA) in buffer A. Afterward the sections were washed three times in buffer B (0.1 M Tris-HCl, pH 7.5, containing 1 M NaCl, 2 mM $MgCl_2.6H_2O$), incubated for 30 min at 37ºC with biotinylated goat anti-rabbit IgG (1:100, Vector Laboratories, Burlingame, CA, USA), washed three times 5-min in buffer B and incubated in Vectastain Elite ABC solution (Vector Laboratories) for 30 min at 37ºC. Finally, the sections were washed three times 5 min and immersed in a solution of 0.5 mg/mL 3,3-diaminobenzidine (DAB, Sigma) in Tris-HCl 0.05 M, pH 7.5, containing 1 µL/mL H_2O_2 until color development (usually 10 min). After three 5-min washes the sections were briefly dipped in 70 and 100% ethanol, air-dried and dipped into Ilford K5 nuclear emulsion (Ilford, Mobberly, Cheshire, UK) diluted 1:1 with distilled water. They were exposed in the dark at 4 ºC for 4 weeks and were finally developed in Kodak D19 (Kodak, Rochester, NY) for 5 min, fixed in Ilford Hypam fixer (Ilford) for 5 min, washed, and cover-slipped using Mowiol (Merck, Darmstad, Germany).

2.3. Microdialysis

Microdialysis experiments were conducted as previously described (López-Gil et al., 2007). Briefly, rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and concentric dialysis probes with a Cuprophan membrane were stereotaxically implanted in mPFC (3.2 mm AP, –0.6 mm L, –6.0 mm DV from bregma, membrane length 4 mm) and contralateral NAc (1.7 mm AP, +1.2 mm L, –6.8 mm DV from bregma, membrane length 2 mm). Coordinates were taken according to the rat brain atlas from Paxinos and Watson (1998). Microdialysis

experiments were performed in freely moving rats the day after surgery. After a 180 min stabilization period, six 20-min fractions were collected to obtain basal values before local administration of DOI in mPFC by reverse dialysis to stimulate 5-HT$_{2A}$-R, as previously reported (Amargós-Bosch et al., 2004; Martín-Ruiz et al., 2001). The concentration of DOI was selected from previous studies showing maximal effects on 5-HT and DA release in mPFC when DOI was applied by reverse dialysis at nominal concentrations of 100-300 µM (Martín-Ruiz et al., 2001; Bortolozzi et al., 2003; Bortolozzi et al., 2005; Bortolozzi et al.,2010). Control rats received the perfusion fluid (artificial CSF) for the whole experiment.

Dialysate samples of 30 μl were divided into two fractions for the determination of DA (20 μl) and glutamate (10 μl). At the end of the experiments, animals were killed by an overdose of sodium pentobarbital. Brains were quickly removed, frozen in dry ice and 30 μm coronal sections were taken with a cryostat (HM560 Microm, Germany) in order to confirm the correct position of the dialysis probes. DA concentration in dialysate samples was determined by HPLC with electrochemical detection (Hewlett Packard 1049, Palo Alto, CA, USA, +0.6 or +0.7 V) as recently described (Masana et al., 2011). Glutamate concentration was determined using a HPLC system which consisted of a Waters 717plus autosampler, a Waters 600 quaternary gradient pump, and a Nucleosil 5-mM ODS column (10 cm x 0.4 cm; Teknokroma, Spain), as previously described (López-Gil et al., 2007)

2.4. Data Analysis

Tissue sections processed for *in situ* hybridization followed by immunohistochemical detection of retrograde tracers were analyzed with the aid of a stereological microscope Olympus BX-51 (Olympus, Tokyo, Japan) equipped with a digital camera (DP71, Olympus) and stereological VIS software (Visiopharm Integrator System, Hoersholm, Denmark) to evaluate the proportion of retrogradely labeled neurons that expressed 5-HT$_{2A}$ receptor mRNA. Cell counting was performed in 2-5 coronal sections of each rat, corresponding to approximately +3.0 mm AP from bregma, according to the rat brain atlas from Paxinos and Watson (1998). The limits of the different mPFC areas and layers were established for each tissue section and outlined on the computer screen. Then, DAB-labeled cells in each region were counted. Afterward the presence of hybridization signal was evaluated on retrogradely labeled cells. The preparations were examined at different depths to ascertain the presence or absence of tracer immunoreactivity under accumulations of autoradiographic grains corresponding to *in situ* hybridization signal and in order to avoid eventual false positive or negative counts. Only cells

showing densities of silver grains 3-fold higher than average background were considered positive for 5-HT$_{2A}$ receptor mRNA.

Micrographs were taken using a digital Olympus DP71 camera in a BX-51 microscope (Olympus) and a digital Olympus DP72 camera in a Nikon Eclipse E1000 microscope (Nikon, Tokyo, Japan). Figures were prepared using Photoshop software (Adobe Software, Mountain View, CA). Micrographs were only modified for brightness, contrast and size.

In microdialysis experiments, individual basal values were averaged from pretreatment values and expressed in percentage. Statistical analysis was assessed by two-way ANOVA with treatment and time as factors. Post-hoc Newman–Keuls tests were used to compare the effects of DOI with the corresponding control group. Results are given as mean ± SEM of 7-9 rats per group. The level of statistical significance was set at 95%.

3. Results

The histological data reported here correspond to 5 rats in which retrotracers (FG or CTB) were microiontophoretically applied into the core/shell subdivisions of the NAc (Fig. 1). This resulted in the labeling of mPFC pyramidal neurons in layer V of both hemispheres and layer II and outer layer III ipsilateral to the injection site, as illustrated in Figure 2. Few isolated neurons were seldom identified in contralateral layer II. Along the mPFC, CTB- or FG-immunoreactive cells were located mainly in the prelimbic (PL) and infralimbic (IL) areas and in lower density in the anterior cingulate cortex (Cg). The distribution of cells retrogradely labeled in the mPFC when either FG or CTB were used as tracers was highly comparable (see Supplementary figure 1).

In sections processed for *in situ* hybridization, the presence of 5-HT$_{2A}$-R mRNA was established by dense clusters of silver grains, clearly distinguishable from background (Fig. 3A). Abundant cell profiles containing 5-HT$_{2A}$-R mRNA hybridization signal were found in the Cg, PL and IL subfields of the mPFC. Grain densities were highest in layers II-III and lower in layers V-VI, whereas no 5-HT$_{2A}$ receptor mRNA signal was detected in layer I.

The combination of tracer immunostaining and *in situ* hybridization allowed the simultaneous detection of NAc-projecting cell bodies and 5-HT$_{2A}$-R mRNA expression in the mPFC (Fig. 3B). Single-labeled cell profiles were found that contained only 5-HT$_{2A}$ receptor mRNA hybridization signal (Fig. 3C), only tracer immunoreactivity (Fig. 3D) or both (Fig. 3E). Figure 3F-N illustrates the presence of the three types of labeling in the different layers (V contralateral as well as II

and V ipsilateral to the injection side, from left to right) of the Cg, PL and IL subdivisions of the mPFC (upper, middle and lower panels, respectively).

The numbers of retrogradely labeled neurons and double-labeled neurons found along the mPFC were determined with the aid of a stereological microscope and are reported in Table 1. The percentage of tracer-positive cells that expressed 5-HT$_{2A}$ receptors showed little deviation in the 5 rats used in the study and was heterogeneous across the subfields of the mPFC, with a dorso-ventral gradient. Hence, the Cg area, both in the ipsi- and contralateral hemispheres, showed the highest percentage of double-labeled cells, followed by PL and IL areas. Likewise, the percentage of double-labeled cells was greater in layer V (particularly of the contralateral hemisphere) than in layers II/III. This difference was particularly noticeable in the Cg subfield. Overall, the percentage of double-labeled cells varied between 68 ± 3 % (Cg, contralateral layer V) and 32 ± 4 % (IL, ipsilateral layer V). However, given the greater number of NAc-projecting cells in PL, the higher percentage of double-labeled cells in Cg had little impact in the overall percentage in mPFC, which was very similar to that in PL (Table 1). Two-way repeated-measures ANOVA of the percentage of double-labeled cells revealed a significant effect of the mPFC area ($F_{2,24}$= 81.5, p<0.00001) but not of the layer or layer x area interaction.

The functional relevance of the 5-HT$_{2A}$-R expression in mPFC cells projecting to NAc was assessed using dual-probe microdialysis. The local administration of DOI (300 µM) in the mPFC by reverse dialysis (see Methods) significantly increased extracellular glutamate up to 166 ± 18 % and 174 ± 12 % of baseline in the mPFC and NAc, respectively (p<0.05 in both areas; n=8 and 7, respectively). This was accompanied by an increase of extracellular DA to 226 ± 21 % in mPFC (p<0.05, n=7). In contrast, extracellular DA experienced a small, non-significant decrease to 84 ± 4 % of baseline in NAc (n=9) (Fig. 4).

4. Discussion

The present study shows that 1) a relatively high percentage (40-42%) of pyramidal neurons in the various mPFC subfields that project to NAc express 5-HT$_{2A}$-R, 2) these neurons are located in the ipsilateral (layers II/outer III and V) and contralateral (layer V) hemispheres, and 3) 5-HT$_{2A}$-R in mPFC modulate excitatory transmission in NAc via this descending pathway. These results add to previous observations by our group (Vázquez-Borsetti et al., 2009) showing that mPFC pyramidal neurons projecting to VTA also express 5-HT$_{2A}$-R. Overall, the results of both studies are relevant for the mode of action of atypical antipsychotic drugs and suggest that these agents may exert their therapeutic action by regulating PFC excitatory inputs onto the

mesolimbic VTA-NAc pathway. Yet, the different cellular connectivity in mPFC-VTA and mPFC-NAc pathways (Carr and Sesack, 2000; Sesack and Pickel, 1992) implies a differential control of mPFC inputs on mesolimbic DA and GABA neurons (see below).

4.1. Methodological Aspects

CTB- and FG-immunoreactive cells were clearly visible in tissue sections previously processed for in situ hybridization, showing a distribution in the various areas of mPFC in close agreement with previous studies (Brog et al., 1993; Gabbott et al., 2005; Pinto and Sesack, 2000; Sesack and Pickel, 1992). Hence, NAc-projecting neurons were found in layer V of both hemispheres and layer II/III of the hemisphere ipsilateral to the application site, mainly in the PL and IL areas and in lower numbers in the Cg area. The distribution of NAc-projecting cells in mPFC was similar with the two tracers used (CTB and FG) yet the absolute number of identified cells was lower when using CTB (Table 1). This may reflect a lower uptake of CTB by descending mPFC-NAc fibers or immunoreactivity differences between the two tracers in the present experimental conditions. Hence, as indicated in a previous study (Vázquez-Borsetti et al., 2009) the number of CTB-immunoreactive neurons in mPFC is lower when CTB immunohistochemistry is carried out in sections previously subjected to in situ hybridization. However, these differences do not appear to be relevant for the conclusions of the study, since the percentage of double-labeled cells was totally comparable among rats injected with FG or CTB.

The distribution of 5-HT_{2A}-R mRNA found herein was identical to that previously reported (Amargós-Bosch et al., 2004; Pompeiano et al., 1994; Santana et al., 2004) with an expression of the transcript in layers II-III of the Cg, PL and IL subfields of the mPFC. Double in situ hybridization studies reported that this receptor mRNA is present in 50-60% of the pyramidal neurons and 20-40% of GABAergic neurons in these PFC layers (Santana et al., 2004). The analogy between the present and reported distribution indicates that the in situ hybridization procedure reliably labeled 5-HT_{2A}-R mRNA-expressing cells when the hybridization procedure was followed by immunostaining of CTB or FG in the same tissue sections. Some previous studies have reported the use of riboprobes in combination with tract tracers (Barroso-Chinea et al., 2008; Hur and Zaborszky, 2005; Pérez-Manso et al., 2006; Yokota et al., 2007). Together with a previous study (Vázquez-Borsetti et al., 2009), the present results show that oligonucleotides can be also reliably combined with tracer immunohistochemistry to identify proteins expressed in relatively low levels, such as monoamine receptors.

4.2. Relevance of 5-HT$_{2A}$-R activation for excitatory neurotransmission in NAc

In vivo microdialysis experiments indicate that the stimulation of 5-HT$_{2A}$-R in mPFC by the local application of the preferential 5-HT$_{2A}$-R agonist DOI increased extracellular glutamate concentrations locally and in NAc. This observation suggests that of 5-HT$_{2A}$-R activation in mPFC increases excitatory inputs onto medium spiny GABAergic neurons of the NAc, thus activating direct and indirect inhibitory inputs onto output nuclei of the basal ganglia and inducing downstream changes in basal ganglia function (Schmidt and Kretschmer, 1997; Gerfen, 2000).

5-HT$_{2A}$-R activation results in neuronal depolarization, reduction of the afterhyperpolarization and increase of excitatory postsynaptic currents and pyramidal discharge in PFC (Aghajanian and Marek, 1997, 1999; Amargós-Bosch et al., 2004; Araneda and Andrade, 1991; Puig et al., 2005; Tanaka and North, 1993; Villalobos et al., 2005). 5-HT can also activate 5-HT$_{2A}$ receptors in GABA interneurons to increase a synaptic GABA input onto pyramidal neurons (Tanaka and North, 1993; Zhou and Hablitz, 1999). Hallucinogens such as DOI, DOB or LSD also modify PFC activity through the activation of 5-HT$_{2A}$ receptors, an effect sensitive to antipsychotic drugs when examined (Aghajanian and Marek, 1997, 1999; Celada et al., 2008; González-Maeso et al., 2007; Lambe and Aghajanian, 2007; Puig et al., 2003; Villalobos et al., 2005).

Previous experiments show that the local administration of DOI in mPFC increased neuronal activity in brain areas such as the dorsal raphe (5-HT neurons) or the VTA (DA neurons) (Bortolozzi et al., 2005; Martín-Ruiz et al., 2001) that receive direct excitatory inputs from mPFC (Aghajanian and Wang, 1977; Celada et al., 2001; Gabbott et al., 2005; Geisler and Zahm, 2005; Hajos et al., 1998; Jankowski and Sesack, 2004; Peyron et al., 1998; Sesack et al., 1989; Thierry et al., 1983; Thierry et al., 1979; Tong et al., 1996b; Tong et al., 1998). These mPFC glutamatergic inputs are important for phasic activity of these monoaminergic neurons (Celada et al., 2001; Levine and Jacobs, 1992; Tong et al., 1996b). In agreement with these observations, the local application of DOI in mPFC increased extracellular glutamate in NAc, most likely as a result of the activation of 5-HT$_{2A}$-R on NAc-projecting mPFC pyramidal neurons. Excitatory inputs from mPFC to NAc are thought to play a role in reward by activating medium spiny GABAergic neurons (Britt et al., 2012). Likewise, in agreement with the DOI-mediated activation of mPFC pyramidal neurons projecting to midbrain (Bortolozzi et al., 2005; Bortolozzi et al., 2010; Martín-Ruiz et al., 2001), DOI application in mPFC increased DA release in mPFC. Interestingly, DA release in NAc remained unchanged. Overall, these observations are in agreement with the existence of a reciprocal connectivity between mPFC pyramidal neurons

and VTA DA neurons of the mesocortical (but not mesolimbic) pathway (Carr and Sesack, 2000) (Fig. 5).

4.3. Functional implications

The present results show that $5\text{-HT}_{2A}\text{-R}$ are involved in the communication between two important brain areas in schizophrenia, the PFC and the NAc, both integrated in basal ganglia circuits, e.g. PFC → basal ganglia (NAc, ventral pallidum, subthalamic nucleus, substantia nigra reticulata) → mediodorsal/centromedial thalamus → PFC. On the one hand, excitatory afferents from PFC control the activity of many cortical and subcortical structures reciprocally connected with the PFC -with the exception of the basal ganglia, feeding back to the cortex via the thalamus- (Groenewegen and Uylings, 2000). The PFC exerts a top-down control of brain activity through these excitatory afferents (Fuster, 2001; Miller and Cohen, 2001) and PFC alterations have been reported in schizophrenia that may well underlie negative symptoms and cognitive deficits in schizophrenia (see Introduction). Thus, $5\text{-HT}_{2A}\text{-R}$ blockade in PFC by atypical antipsychotic drugs may distally control the activity cortical and subcortical brain areas directly innervated by the PFC, as indicated above.

On the other hand, basal ganglia play a major role in brain function, including motor, cognitive and affective domains (Alexander and Crutcher, 1990; Breakefield et al., 2008; DeLong and Wichmann, 2007; Middleton and Strick, 2000). Anatomical and functional abnormalities of these brain structures have been reported in psychiatric disorders, including schizophrenia (Aouizerate et al., 2004; Hwang et al., 2006; Manoach et al., 2000; Mittal et al., 2010; Ring and Serra-Mestres, 2002). Indeed, the mesolimbic dopaminergic hyperactivity responsible for psychotic symptoms (Breier et al., 1997; Laruelle et al., 1996) likely results in alterations of the information processing along basal ganglia circuits.

A limitation of the present study is that we do not demonstrate that atypical antipsychotic drugs specifically block $5\text{-HT}_{2A}\text{-R}$ in pyramidal neurons projecting to NAc. However, this effect may be assumed in view that 1) atypical antipsychotic drugs block the effects of DOI on 5-HT and DA release in mPFC, driven by mPFC-midbrain pathways (Bortolozzi et al., 2003, 2010), and 2) PFC pyramidal neurons in different layers (i.e., projecting to cortical and subcortical targets) equally express $5\text{-HT}_{2A}\text{-R}$ (Pompeiano et al., 1994; Satana et al., 2004).

4.4 Conclusion

The present data may help to understand the complex effects of atypical antipsychotic drugs on dopaminergic and glutamatergic neurotransmission in basal ganglia circuits. However, since

atypical drugs target several monoamine receptors not examined in the present study, our results provide an incomplete view on this matter. As an example, the increased DA release in NAc produced by atypical antipsychotic drugs can be canceled by co-administration with the 5-HT_{1A}-R receptor agonist 8-OH-DPAT (Ichikawa and Meltzer, 2000), which supports the involvement of 5-HT_{1A}-R in these effects. These functional observations suggest a more complex circuitry than that reported (Carr and Sesack, 2000) and are unlikely to involve the PFC-NAc connectivity examined in the present study. Moreover, most atypical antipsychotic drugs show high affinity for 5-HT_{2C} receptors and some act as inverse agonists at this receptor, an aspect that deserves further studies.

Despite these limitations, 5-HT_{2A}-R blockade by atypical antipsychotic drugs may contribute to normalize an abnormal information processing in the basal ganglia in schizophrenia. Hence, that blockade of 5-HT_{2A} receptors by atypical drugs may reduce cortical excitatory inputs onto GABAergic neurons of nucleus accumbens, thus complementing dopamine D2-R blockade in this structure. Both pharmacological actions may add to each other to modulate information processing along basal ganglia circuits. Together with previous observations, the present results support the view that atypical antipsychotic drugs may control the activity of the mesolimbic pathway at cell body and terminal level. This may account for the superior efficacy of clozapine and some other second-generation drugs over classical neuroleptics, only blocking DA D2-R (Leucht et al., 2009).

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Table 1: 5-HT$_{2A}$ receptor expression in mPFC neurons projecting to NAc.

	Rat #	Cg Tr+	Cg Tr+2A+	Cg %	PL Tr+	PL Tr+2A+	PL %	IL Tr+	IL Tr+2A+	IL %	PFC Tr+	PFC Tr+2A+	PFC %
contralateral layer V	1	16	11	**68.8**	143	90	**62.9**	56	18	**32.1**	215	119	**55.3**
	2	33	20	**60.6**	167	74	**44.3**	48	15	**31.3**	248	109	**44.0**
	3	21	15	**71.4**	203	101	**49.8**	66	26	**39.4**	290	142	**49.0**
	4	9	7	**77.8**	107	48	**44.9**	35	11	**31.4**	151	66	**43.7**
	5	3	2	**66.7**	34	15	**44.1**	12	4	**33.3**	49	21	**42.9**
				67.7±3.4			**49.2±3.6**			**33.5±1.5**			**47.0±2.4**
ipsilateral layer II/III	1	23	18	**78.3**	118	50	**42.4**	74	28	**37.8**	215	96	**44.7**
	2	26	14	**53.8**	109	40	**36.7**	45	15	**33.3**	180	69	**38.3**
	3	15	7	**46.7**	134	60	**44.8**	57	24	**42.1**	206	91	**44.2**
	4	10	5	**50.0**	73	24	**32.9**	34	11	**32.4**	117	40	**34.2**
	5	3	2	**66.7**	40	16	**40.0**	15	5	**33.3**	58	23	**39.7**
				59.1±5.9			**39.3±2.1**			**35.8±1.8**			**40.2±1.9**
ipsilateral layer V	1	25	16	**64.0**	179	96	**53.6**	61	28	**45.9**	265	140	**52.8**
	2	37	23	**62.2**	189	72	**38.1**	71	19	**26.8**	297	114	**38.4**
	3	23	13	**56.5**	165	70	**42.4**	81	25	**30.9**	269	108	**40.1**
	4	19	12	**63.2**	171	70	**40.9**	68	17	**25.0**	258	99	**38.4**
	5	4	3	**75.0**	44	18	**40.9**	20	6	**30.0**	68	27	**39.7**
				64.2±3.0			**43.2±2.7**			**31.7±3.7**			**41.9±2.8**

Values are average numbers (mean of 2-5 sections per rat) of retrotracer-positive neurons (**Tr+**) counted in the different areas of the mPFC and the entire mPFC as determined by FG- or CTB-immunohistochemistry. Retrogradely labeled neurons that are also positive for 5-HT$_{2A}$ receptor mRNA (**Tr+2A+**) were identified by the presence of the 2 markers (see Methods). Only neurons with unambiguous labeling for both markers were considered positive, as in the examples shown in Figure 3E. Individual and average (mean ± SEM) ratios of Tr+2A+/Tr+ are provided as percentages (**%**).
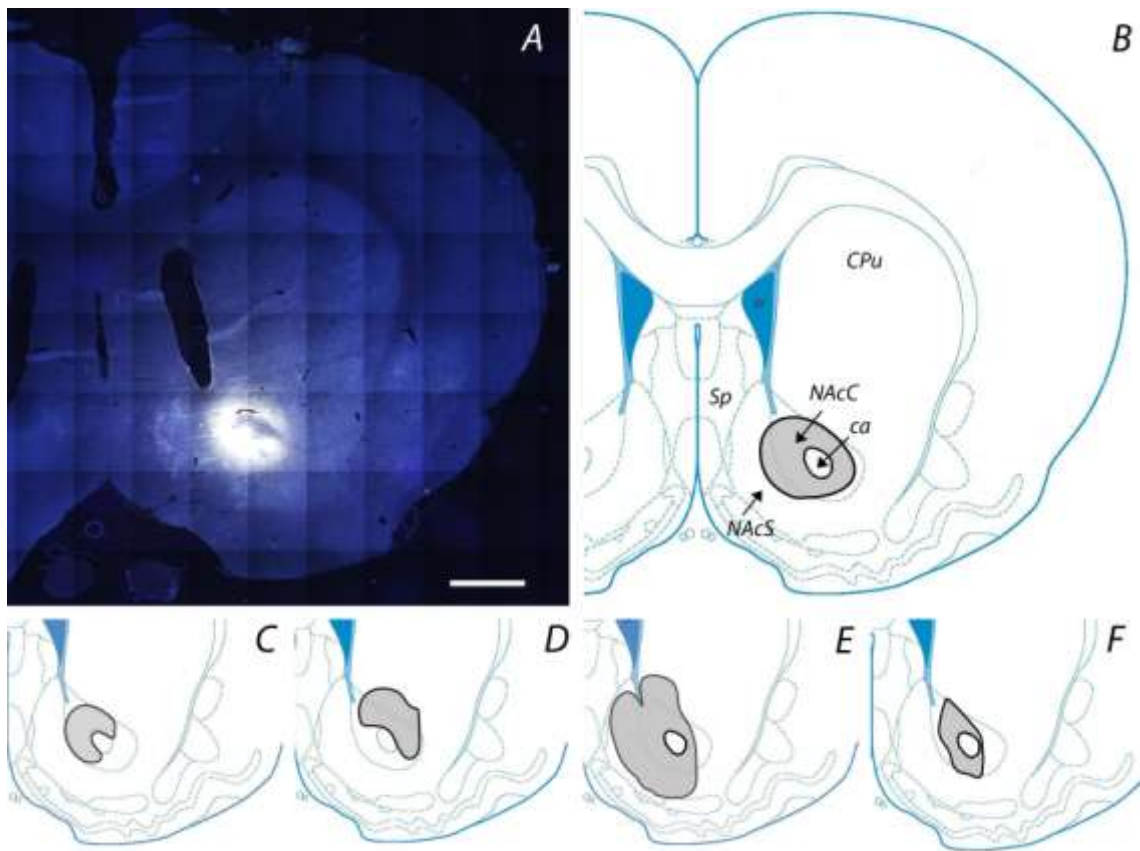
Figure 1. Localization of retrotracer injection sites in the nucleus accumbens. (*A*) Micrograph of the injection site where FG is visualized by its own fluorescence. Bar: 1 mm. (*B-F*) Schematic representations of the nucleus accumbens area in a coronal section of the rat brain at the level of the image shown in panel *A* (AP +1,68 mm from bregma), where shaded areas illustrate the precise location and extent of tracer injections in the different rats used in this study, as assessed by FG fluorescence (*B-D*) or CTB immunostaining (*E,F*). Drawings were taken from Paxinos and Watson (1997) with permission from Academic Press. Abbreviations: ca: anterior commissure; CPu: caudate-putamen; NAcC: nucleus accumbens, core; NAcS: nucleus accumbens, shell; Sp: septum.
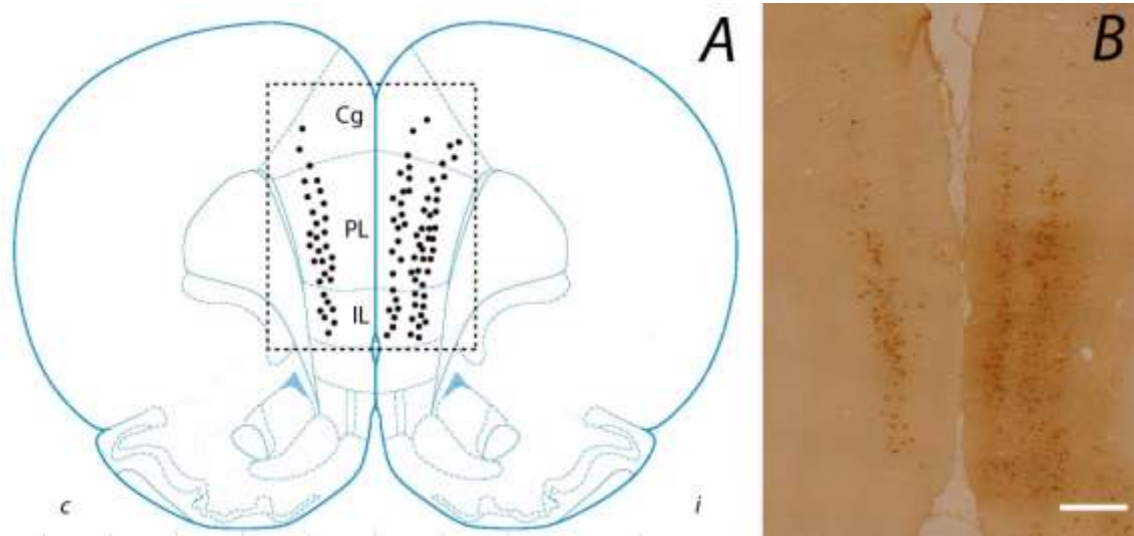
Figure 2. Retrograde labeling in mPFC after tracer application in NAc. (*A*) Schematic drawing of a coronal section of mPFC illustrating the average numbers and distribution of cells that project to NAc. Every dot represents 6 cells. The box indicates the borders of image in panel *B*. (*B*) Low-power magnification micrograph through the mPFC showing the presence of CTB-immunoreactive cell bodies in contralateral (c) layer V and ipsilateral (i) layers II/III and V in relation to the tracer injection hemisphere. Cg: cingulate cortex; IL: infralimbic area; PL: prelimbic area. Bar: 200 μm.
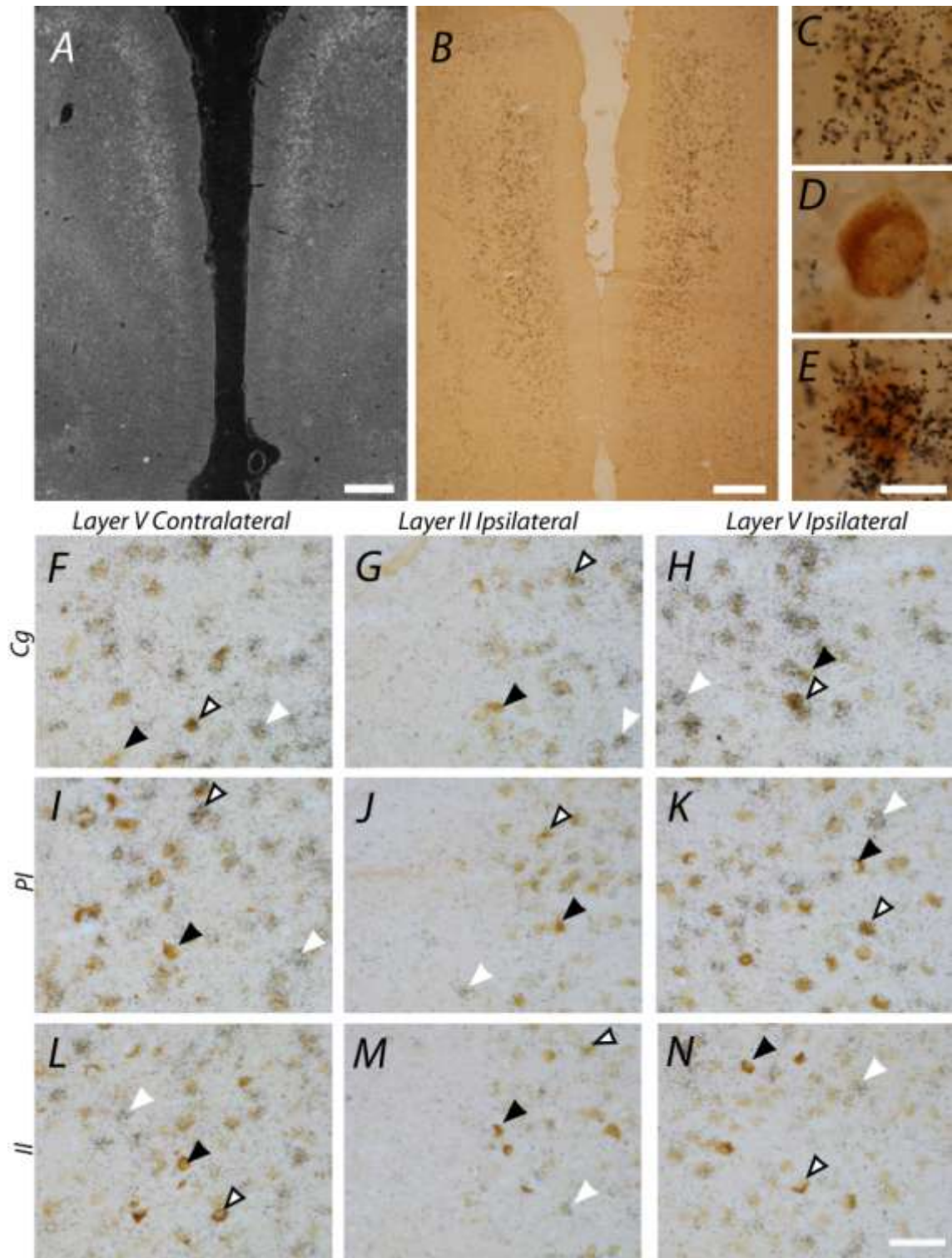
Layer V Contralateral   Layer II Ipsilateral   Layer V Ipsilateral

Figure 3

Figure 3. Labeling of 5-HT$_{2A}$ receptor mRNA and tracer-immunoreactivity in the mPFC after injection of the tracer in the NAc. (*A*) Dark-field low-power magnification image of the mPFC showing the presence of 5-HT$_{2A}$ receptor mRNA signal (bright spots). (*B*) Low-power magnification image of the mPFC showing the presence of FG-immunoreactivity (brown precipitate) and the autoradiographic signal (black spots) corresponding to 5-HT$_{2A}$ receptor mRNA-containing cells. At this magnification the presence of hybridization signal hampers the visualization of tracer-immunoreactivity. (*C-E*) High-power micrographs of individual cell profiles show autoradiographic silver grains corresponding to 5-HT$_{2A}$ receptor mRNA labeling (*C*), FG-immunoreactivity (*D*) and double labeling of tracer and mRNA (*E*). (*F-N*) Micrographs illustrate details of contralateral layer V (*F, I, L*), ipsilateral layer II (*G, J, M*) and ipsilateral layer V (*H, K, N*) of the cingulate (*F-H*), prelimbic (*I-K*) and infralimbic areas (*L-N*) of the mPFC. Black arrowheads point to retrogradely labeled cells, white arrowheads to cell profiles containing 5-HT$_{2A}$ receptor mRNA hybridization signal, and black-and-white arrowheads to double-labeled cells. (Bars: *A, B*: 400 μm; *C-E*, 10 μm; *F-N*, 50 μm).
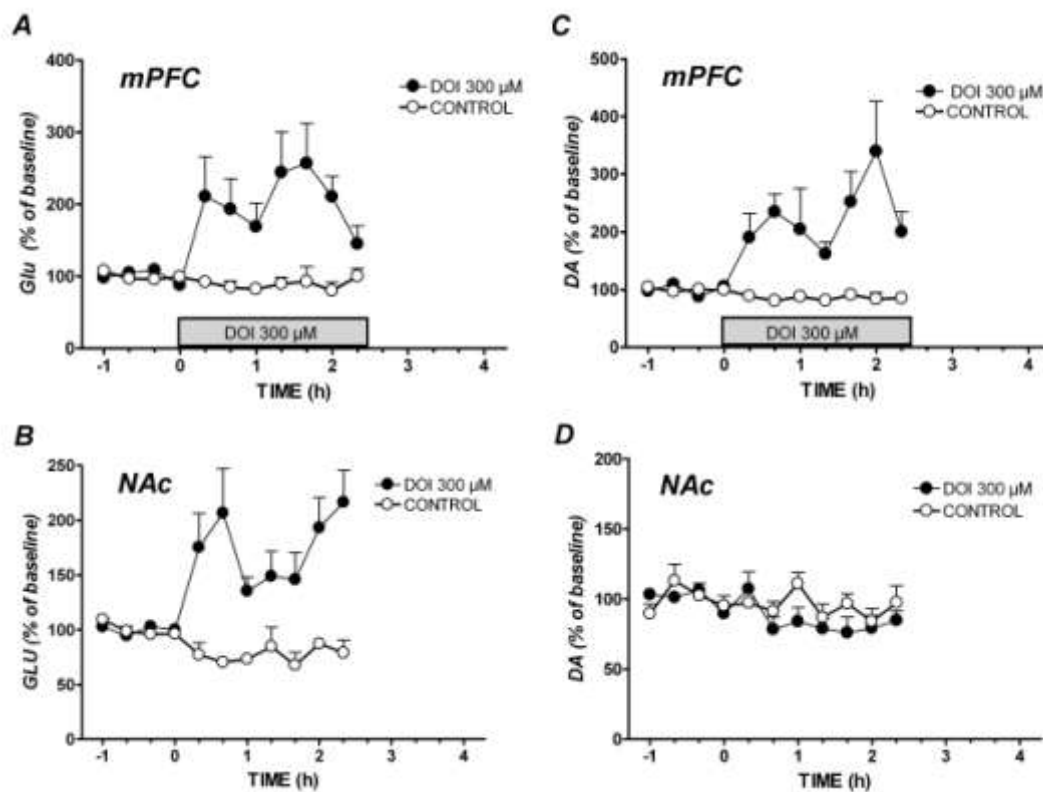
Figure 4: Effect of intra-mPFC administration of DOI on extracellular concentration of glutamate (*A-B*) and dopamine (*C-D*) in mPFC and NAc. DOI increases the concentration of GLU in both mPFC (*A*) and NAc (*B*) and the concentration of DA in mPFC (*C*) but not in NAc (*D*). Data (mean ± SEM, n=7-9 rats per group) are expressed as percentage changes of four basal predrug values. Control rats were perfused with artificial CSF for the whole perfusion period.
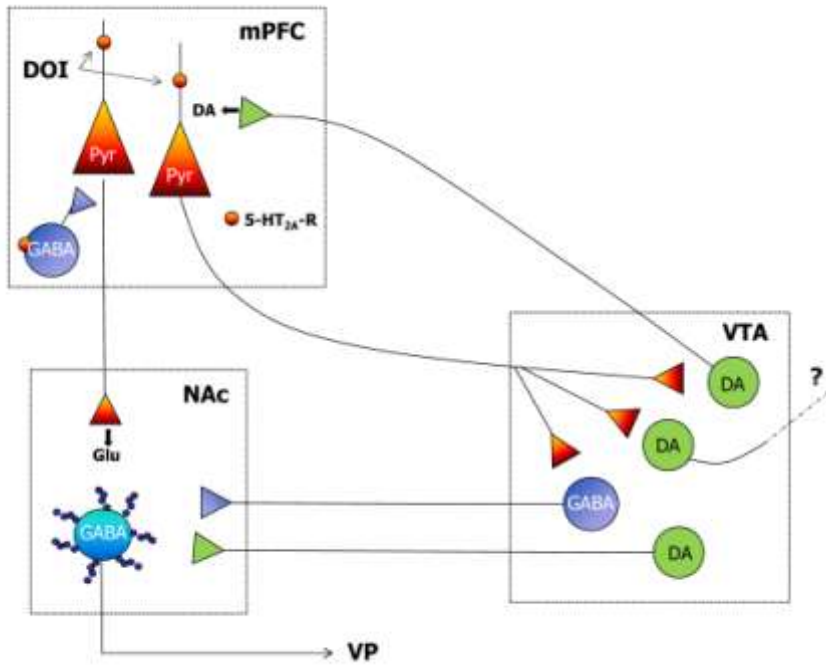
Figure 5: Schematic drawing of the connectivity between mPFC, VTA and NAc involving 5-HT$_{2A}$ receptors. According to Carr and Sesack (2000), PFC projections to the VTA synapse onto mesocortical DA neurons that project back to the PFC or GABA neurons of the mesolimbic pathway that project to the NAc, although the majority of PFC terminals within the VTA seem to target DA and GABA neurons that project to unknown target sites. We have shown that 5-HT$_{2A}$ receptors are expressed by mPFC neurons projecting to the VTA (Vázquez-Borsetti et al., 2009) as well as by those that send their axons to the NAc (present data). The present microdialysis data are in agreement with these anatomical observations.

Supplementary data
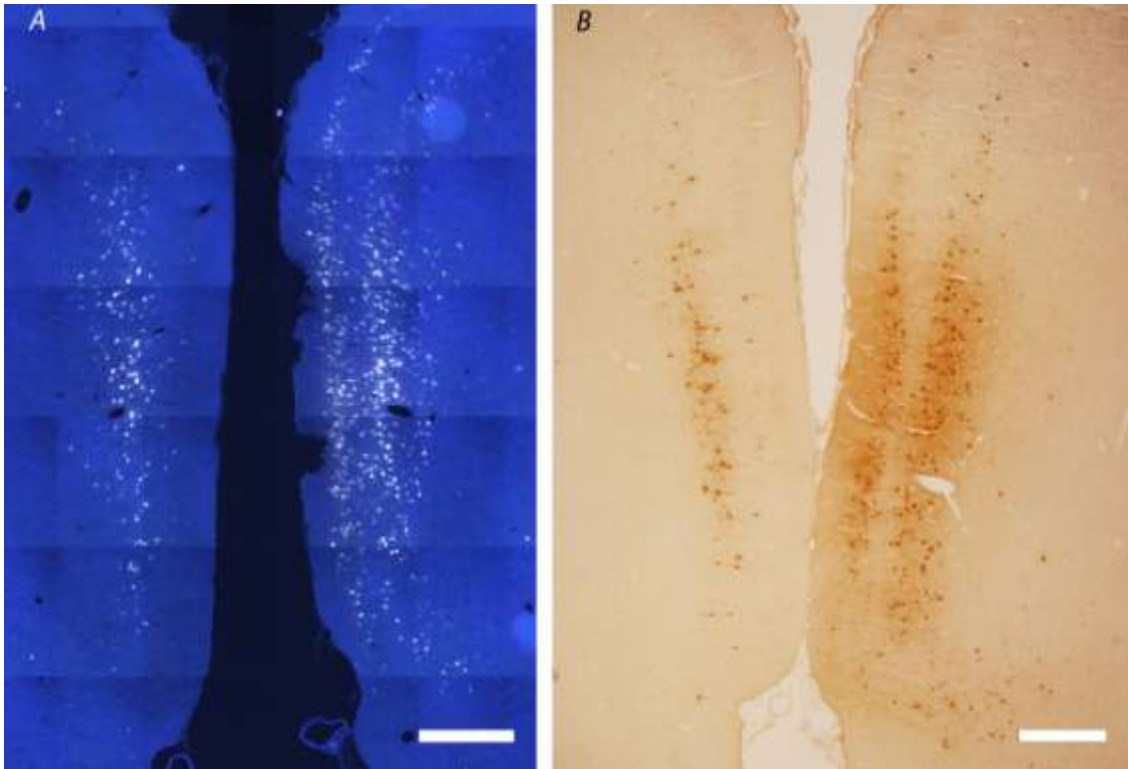
Supplemental figure 1: Comparison of retrograde labeling in the mPFC after FG (*A*) and CTB (*B*) application into the NAc. Photomicrographs show FG fluorescence (*A*) and CTB immunostaining (*B*). The distributions of retrogradely labeled cells in the different layers and areas of the mPFC obtained with the two tracers were highly omparable. (Bars: 400 μm).