Selective enhancement of mesocortical dopaminergic transmission by noradrenergic drugs: therapeutic opportunities in schizophrenia

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Abstract
The superior efficacy of atypical vs. classical antipsychotic drugs to treat negative symptoms and cognitive deficits in schizophrenia appears related to their ability to enhance mesocortical dopamine (DA) function. Given that noradrenergic (NE) transmission contributes to cortical DA output, we assessed the ability of NE-targeting drugs to modulate DA release in medial prefrontal cortex (mPFC) and nucleus accumbens (NAc), with the aim of selectively increasing mesocortical DA. Extracellular DA was measured using brain microdialysis in rat mPFC and NAc after local/systemic drug administration, electrical stimulation and selective brain lesions. Local GBR12909 [a selective DA transporter (DAT) inhibitor] administration increased DA output more in NAc than in mPFC whereas reboxetine [a selective NE transporter (NET) inhibitor] had an opposite regional profile. DA levels increased comparably in both regions of control rats after local nomifensine (DAT+NET inhibitor) infusion, but this effect was much lower in PFC of NE-lesioned rats (DSP-4) and in NAc of 6-OHDA-lesioned rats. Electrical stimulation of the locus coeruleus preferentially enhanced DA output in mPFC. Consistently, the administration of reboxetine + RX821002 (an \( \alpha_2 \)-adrenoceptor antagonist) dramatically enhanced DA output in mPFC (but not NAc). This effect also occurred when reboxetine + RX821002 were co-administered with haloperidol or clozapine. The preferential contribution of the NE system to PFC DA allows selective enhancement of DA transmission by simultaneously blocking NET and \( \alpha_2 \)-adrenoceptors, thus preventing the autoreceptor-mediated negative feedback on NE activity. Our results highlight the importance of NET and \( \alpha_2 \)-adrenoceptors as targets for treating negative/cognitive symptoms in schizophrenia and related psychiatric disorders.

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Introduction
Mesocortical and mesolimbic dopamine (DA) systems play a crucial role in many psychiatric disorders including schizophrenia (Carlsson, 1978). A general enhancement of brain dopaminergic neurotransmission in schizophrenia was suggested by pharmacological evidence (Creese et al. 1976; Seeman & Lee, 1975). However, current views indicate a hyperactivity of subcortical DA transmission together with a hypoactive mesocortical system (Abi-Dargham et al. 2000; Akil et al. 1999; Laruelle et al. 1996; Lewis & Lieberman, 2000; Weinberger et al. 1988).

The overall efficacy of classical (DA D_2 receptor antagonists) and atypical antipsychotics (APDs, preferential 5-HT_2A/2C vs. DA D_2 receptor antagonists) to treat positive symptoms is similar (Lieberman et al. 2005). In contrast, some APDs, and particularly clozapine, are superior to classical APDs for the treatment of negative symptoms and cognitive impairment (Kane et al. 1988; Keefe et al. 2006; Leucht et al. 2009; Meltzer & McGurk, 1999). This clinical feature has been related to the ability of atypical (but not classical)
APDs to increase DA release in the mesocortical pathway (Diaz-Mataix et al. 2005; Ichikawa et al. 2001; Kuroki et al. 1999; Rollema et al. 1997; Westerink et al. 2001). Indeed, an optimal prefrontal DA function is crucial for working memory and executive functions (Castner et al. 2000; Floresco & Magyar, 2006; Goldman-Rakic et al. 2000; Robbins & Arnsten, 2009; Vijayraghavan et al. 2007; Williams & Goldman-Rakic, 1995).

A key step determining the intensity and duration of synaptic DA signalling is the reuptake of the released transmitter into nerve terminals through high-affinity plasma membrane transporters. Previous studies indicate a lower density of DA transporter (DAT) in PFC compared to striatum (Letchworth et al. 2000; Marshall et al. 1990; Sesack et al. 1998). Conversely, the PFC contains a higher density of noradrenaline (NE) transporter (NET) (Miner et al. 2000; Schulz et al. 2000; Devoto & Flore, 2006; Kawahara et al. 2007). NET exhibits a similar affinity for NE and DA (Raiteri et al. 1990, 2006; Mazei-Robison et al. 1990), compared to NAc. In fact, NE axons may contribute to the removal of DA from the extracellular brain space, since NET shows a lower density of DA transporter (DAT) in PFC compared to striatum (Letchworth et al. 2000; Marshall et al. 1990; Sesack et al. 1998). NET inhibitors seem to preferentially increase the extracellular DA concentration in PFC compared to caudate or nucleus accumbens (NAc) (Carboni et al. 1990, 2006; Mazei et al. 2002; Pozzi et al. 1994). Furthermore, NE axons from locus coeruleus (LC) neurons may contribute to regulate extracellular DA concentration in PFC by either taking up or co-releasing DA (Devoto et al. 2001, 2005; Devoto & Flore, 2006; Kawahara et al. 2001). However, a systematic comparison of these factors in the mesocortical and mesolimbic pathways is lacking.

Here we evaluated simultaneously the contribution of noradrenergic transmission to DA reuptake and release in both mesocortical and mesolimbic pathways. As a result, we report on a marked and selective enhancement of mesocortical DA transmission by enhancement of mesocortical DA transmission by release in both mesocortical and mesolimbic pathways. As a result, we report on a marked and selective enhancement of mesocortical DA transmission by combining the NET inhibitor reboxetine and the α2-adrenoceptor antagonist RX821002.

**Materials and methods**

**Animals**

Male Wistar rats (250–320 g, Iffa-Credo, France) were maintained on a 12-h light/dark cycle (lights on 07:00 hours), room temperature 22 ± 2°C, with food and water available *ad libitum*. Animal care followed European Union regulations (O. J. of E. C. L358/1 18/12/1986) and was approved by the Institutional Animal Care and Use Committee of the School of Medicine, University of Barcelona.

**Drugs and reagents**

Desipramine hydrochloride, GBR12909 dihydrochloride, haloperidol, nomifensine maleate, N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP-4), 6-hydroxydopamine hydrochloride (6-OHDA) and RX281002 hydrochloride were purchased from Sigma (Spain). Clozapine, fluoxetine hydrochloride and reboxetine mesylate were obtained from Tocris (UK). Drugs were dissolved in artificial cerebrospinal fluid [aCSF (mm): NaCl, 125; KCl, 2.5; CaCl2, 2.5; MgCl2, 1.18] and distilled water for local and systemic administration (pH adjusted to 6–7), respectively. Clozapine was dissolved in few drops of glacial acetic acid and diluted with saline. DSP-4, 6-OHDA and GBR12909 solutions were prepared prior to use. 6-OHDA was dissolved in water containing 0.1% ascorbic acid. All reagents used were of analytical grade and obtained from Merck (Germany).

**Microdialysis procedures**

Microdialysis experiments were conducted as previously described (Bortolozzi et al. 2005; Diaz-Mataix et al. 2005). Briefly, concentric dialysis probes were implanted under pentobarbital anaesthesia (60 mg/kg i.p.) at the following brain coordinates (in mm): mPFC, AP +3.2, L –0.8, DV –6.0, 4-mm membrane length; or NAc, AP +1.6, L –1.1, DV –8.0, 1.5-mm membrane length (Paxinos & Watson, 1998). The probe in NAc samples included core and shell subdivisions (Fig. 1b). Groups of rats were implanted with two probes ipsilaterally in mPFC (as above) and NAc (AP +1.6, L –3.9, DV –7.5, with a lateral 20° angle). Experiments were performed in freely moving rats ~20 h after surgery except those involving the electrical stimulation of LC (see below). Probes were perfused with aCSF pumped at 1.5 µl/min. After an initial 30-min stabilization period, four baseline samples were collected (20 min/fraction) before local or systemic drug administration. Control groups were perfused with aCSF or injected with vehicle.

To examine the effects of electrical stimulation of LC on DA release, a stimulating electrode was implanted in LC at AP –2.0 (from lambda; nose down 15° from horizontal plane), L –1.2, DV –7.2 (Mateo et al. 1998). The bipolar stimulating electrode 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. Additionally, two dialysis probes were implanted in mPFC and NAc (as above). On the following day, rats were anaesthetized with chloral hydrate (400 mg/kg i.p. followed by supplementary doses of 50–70 mg/kg, h i.p.). Ten-min
dialysate fractions were collected (flow rate 3.0 μl/min) and body temperature was maintained at 37 °C with a heating pad. LC was phasically stimulated for 20 min after four basal fractions: 0.1-ms pulses delivered at 20 Hz for 250 ms every 1 s (average frequency 5 Hz) at 0.7 mA for 20 min using a Grass stimulation unit S-48 (Devoto et al. 2005; Florin-Lechner et al. 1996).

Brain dialysate fractions were collected on microvials containing 5 μl of 10 mM HClO4 and rapidly injected into the HPLC equipment as described previously (Diaz-Mataix et al. 2005). DA was detected amperometrically (+0.7 V) (Hewlett-Packard 1049, USA) with a limit of detection of 2–3 fmol/sample. At the end of the experiments, animals were killed by an anaesthetic overdose. Brains were quickly removed and frozen on dry ice before sectioning (40 μm) with a cryostat (HM500-0m Microm, Germany). Coronal brain sections were stained with Neutral Red to verify the correct placement of probes and electrodes (Fig. 1).

Brain lesions

To examine the relative contribution of noradrenergic and dopaminergic systems to the release of DA in mPFC and NAc, we performed specific lesions: (a) treatment with the NE neurotoxin DSP-4, and (b) lesion of ventral tegmental area (VTA) DA system with 6-OHDA. To lesion NE neurons, DSP-4 (40 mg/kg i.p.) was administered 60 min after the injection of fluoxetine (10 mg/kg i.p.) and GBR12909 (20 mg/kg s.c.), to protect serotonin (5-HT) and DA neurons, respectively (Bortolozzi & Artigas, 2003; Dailly et al. 2006; Fritschy & Grzanna, 1989). Microdialysis experiments were performed 5 d after DSP-4 administration in awake rats implanted with a single probe in mPFC or NAc.

For 6-OHDA lesions, rats were pre-treated 60 min before with fluoxetine (10 mg/kg i.p.) and desipramine (25 mg/kg s.c) to protect 5-HT and NE neurons, respectively (Robinson & Whishaw, 1988; Tseng et al. 2005). Rats were unilaterally injected with 6-OHDA (total dose 8 μg/1 μl) in two locations within the VTA [AP -5.2, L -2.2 (10°), DV -7.8; AP -5.8, L -1.9 (10°), DV -7.7]. The injection rate was 1 μl/min, followed by a 3-min pause before slowly withdrawing the infusion cannula. Microdialysis experiments were performed 8 d later in rats implanted with two microdialysis probes, one in mPFC (or NAc) ipsilateral to the lesioned VTA and the other one in the contralateral mPFC (or NAc), used as a control [mPFC: AP +3.2, L ±1.5 (10°), DV -5.7; NAc: AP +1.6, L ±3.9 (20°), DV -7.5].

The efficacy of DSP-4 and 6-OHDA to lesion NE and DA systems, respectively, was assessed by analysis of NE and DA in brain tissue by HPLC-ED (Adell et al. 1989; Bortolozzi & Artigas, 2003). The effect of lesions was examined in brain areas containing a substantial innervation of NE (PFC, for DSP-4 lesion) and DA (NAc, for 6-OHDA lesion). At the end of the microdialysis experiments, rats were killed and their brains were quickly removed and placed over a cold plate. In these animals, probe location was assessed by visual inspection with a low power magnification microscope. Brains were sectioned at 1-mm-wide coronal sections and PFC (for DSP-4 lesion) and NAc (for 6-OHDA lesion) were carefully dissected out. Only rats with more than 90% depletions in NE (DSP-4) or DA (6-OHDA) were included.

Statistical analysis

Microdialysis results are expressed as fmol/30-μl fraction and shown as percentages of baseline. Area under the curve (AUC) of selected time-periods was also used. Statistical analysis was performed using one- or two-way ANOVA of AUC or
inhibitor) was perfused at increasing concentrations (30, 100 and 300 μM) and produced a significant increase of DA levels in both areas in a concentration-dependent manner (Fig. 2b). The maximal DA increase (AUC$_{6-16}$) was 660 ± 52% in mPFC and 527 ± 130% in NAc at 30 μM (n = 5 each). Two-way ANOVA of AUCs revealed a significant effect of concentration (F$_{1,27} = 27.13$, p < 0.0001) and non-significant effects of region and concentration × region interaction. Post-hoc test (Newman–Keuls) revealed significant differences among all tested concentrations.

Unlike nomifensine, significant regional differences were found for the selective DAT (GBR12909) and NET (reboxetine) inhibitors after local administration. Local GBR12909 infusion markedly increased DA output in NAc and evoked a minor increase in mPFC (Fig. 2b). Mean DA elevations at 30, 100 and 300 μM, expressed as percentage of baseline, were respectively: (a) mPFC (n = 6): 146 ± 11, 150 ± 24 and 122 ± 28 and (b) NAc (n = 4): 382 ± 75, 390 ± 91 and 287 ± 80. Two-way ANOVA of AUCs showed a significant effect of the drug concentration (F$_{3,32} = 6.39$, p < 0.01), region (F$_{1,27} = 27.92$, p < 0.0001) and concentration × region interaction (F$_{3,32} = 3.19$, p < 0.05). Post-hoc test (Newman–Keuls) revealed significant differences between NAc and mPFC at concentrations of 30 and 100 μM and a marginal difference (p = 0.052) at 300 μM.

Local reboxetine application elevated extracellular DA in mPFC but not in NAc (Fig. 2c). Reboxetine effects at 1, 10 and 30 μM, expressed as percentage of baseline, were respectively: (a) mPFC (n = 6): 267 ± 19, 278 ± 21, and 282 ± 33 and (b) NAc (n = 4): 129 ± 21, 117 ± 28, and 90 ± 19. Two-way ANOVA showed a significant effect of concentration (F$_{3,32} = 8.50$, p < 0.001), region (F$_{1,27} = 60.07$, p < 0.0001) and concentration × region interaction.

Results

Local effect of DAT and NET inhibitors on DA output in mPFC and NAc

Perfusion of aCSF did not significantly alter DA output in mPFC and NAc of awake rats. The mean baseline concentrations of DA dialysate samples from mPFC and NAc (single probe experiments) were 19 ± 1 fmol/fraction (n = 33) and 19 ± 3 fmol/fraction (n = 27), respectively.

Local infusion of the DAT + NET inhibitor nomifensine (1, 10 and 30 μM) increased extracellular DA in both areas in a concentration-dependent manner (Fig. 2a). The maximal DA increase (AUC$_{6-16}$) was 660 ± 52% in mPFC and 527 ± 130% in NAc at 30 μM (n = 5 each). Two-way ANOVA of AUCs revealed a significant effect of concentration (F$_{3,32} = 27.13$, p < 0.0001) and non-significant effects of region and concentration × region interaction. Post-hoc test (Newman–Keuls) revealed significant differences among all tested concentrations.

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Fig. 2. Local effect of catecholamine transporter inhibitors on DA release in mPFC (□) and NAc (■) of freely moving rats. The application of artificial CSF did not alter DA levels in any area. (a) Nomifensine (DAT + NET inhibitor), perfused at 1, 10 and 30 μM (12 fractions each), showed a marked dose effect without regional differences at any concentration. (b) GBR12909 (a selective DAT inhibitor) was perfused at increasing concentrations (30, 100 and 300 μM, five fractions each) and produced a significant increase of DA levels in NAc, but not in mPFC. (c) Reboxetine (a selective NET inhibitor) was perfused at increasing concentrations (1, 10 and 30 μM, four fractions each) and increased DA output in mPFC, but not in NAc. Bars show the AUC calculated by averaging DA percentage of baseline values in fractions 6–16 for nomifensine, and three DA values for each concentration for GBR12909 and reboxetine. Data are expressed as mean ± S.E.M., n = 4–7 rats/group, except for 1 μM nomifensine in NAc (n = 3). * p < 0.05 vs. respective control (0 μM). + p < 0.01 for mPFC vs. NAc and † p = 0.05. See text for detailed statistical analysis.
Noradrenergic contribution to mesocortical DA

Effects of selective brain lesions on the modulation of DA output in mPFC and NAc induced by nomifensine

We further explored the effect of nomifensine in the mPFC and NAc of rats underlying specific lesions with (a) the NE neurotoxin DSP-4 and (b) unilateral lesion of VTA DA system with 6-OHDA, respectively.

Rats pretreated with DSP-4 showed a 90% depletion of tissue NE level (85 ± 8 vs. 894 ± 92 pmol/g of wet tissue in PFC, p < 0.00001) without a significant change in tissue DA in this area. DSP-4-lesioned rats had higher basal values (fmol/fraction) of dialysate DA in mPFC (37 ± 8, n = 6) and NAc (31 ± 6, n = 6), compared to their respective controls in mPFC (15 ± 2, n = 5) and NAc (24 ± 7, n = 5), yet only the DA increase in mPFC reached statistical significance (p < 0.05) (Fig. 3c).

Local infusion of nomifensine (30 μM) by reverse dialysis increased mPFC DA output in control rats to 660 ± 52% of baseline, but to a much lower extent (180 ± 11% of baseline) in mPFC of DSP-4-pretreated rats (Fig. 3a). However, DSP-4 pretreatment did not alter the ability of nomifensine to increase extracellular DA in NAc (DA increase of 528 ± 130% and 682 ± 176% in control and DSP-4-pretreated rats, respectively). Two-way ANOVA revealed a significant effect of lesion × region interaction (F_{1,18} = 7.48, p < 0.05).

Post hoc t test revealed that the effect of nomifensine in mPFC of DSP-4-lesioned rats was significantly different to the other groups.

The unilateral dopaminergic VTA lesion by 6-OHDA was assessed in the same rats comparing lesion side (ipsilateral to 6-OHDA application) with control side (contralateral to 6-OHDA application). Tissue DA and NE levels were significantly decreased to 93% and 71%, respectively in NAc (492 ± 174 vs. 7226 ± 2227 pmol/g of DA in wet tissue and 298 ± 53 vs. 1019 ± 1173 pmol/g of NE in wet tissue, p < 0.01). Basal DA values (fmol/fraction) in the control and lesioned sides were: (a) control mPFC: 12 ± 4, (b) lesioned mPFC: 9 ± 3, (c) control NAc: 11 ± 4 and, (d) lesioned NAc: 9 ± 2 (n = 5–6). Non-significant differences of DA output were found between control and lesioned sides.

Simultaneous local nomifensine (30 μM) infusion by reverse dialysis in both control and lesioned mPFC increased DA output to 660 ± 72% (control mPFC) and 405 ± 47% (lesioned mPFC) of baseline (Fig. 3b). On the other hand, nomifensine (30 μM) perfusion enhanced DA output to 798 ± 194% of baseline in control NAc and to 284 ± 69% in lesioned NAc. Two-way ANOVA revealed a significant effect of lesion (F_{1,19} = 11.03, p < 0.01) and non-significant effects of region and lesion × region interaction. Post hoc Newman–Keuls

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**Fig. 3.** Local effect of nomifensine 30 μM in mPFC (■) and NAc (▲) of rats underlyingspecific lesions. 
(a) Noradrenergic lesion with DSP-4 (40 mg/kg i.p., 5 d before experiments) changed DA response to nomifensine in mPFC but not NAc, compared to control groups. (b) VTA was unilaterally lesioned with 6-OHDA (8 μg/1 μl, 8 d before experiments) and the contralateral mPFC and NAc were used as control. 6-OHDA lesion produced a significant change to nomifensine in NAc-lesioned (6-OHDA ipsilateral side) compared to control side. Bars show AUC values, averaged DA values in fractions 6–16, expressed as percentage of baseline. (c) Basal DA values (fmol/fraction) in the mPFC and NAc of control and lesioned rats (DSP-4 and 6-OHDA). Data are expressed as mean ± s.e.m., n = 5–6 per group. *p < 0.05 vs. respective control groups and +p < 0.01 for mPFC vs. NAc. See text for statistical analysis.

region interaction (F_{1,18} = 7.19, p < 0.001). Post hoc test (Newman–Keuls) revealed significant differences between NAc and mPFC at all concentrations.
test indicated that only DA values in lesioned NAc were statistically different from control NAc.

**Electrical stimulation of LC differentially increases DA output in mPFC and NAc**

We then assessed the effect of electrical stimulation of LC on DA output in mPFC and NAc of the same rats. Mean DA basal values (fmol/10-min fraction) were 15 ± 3 (n = 18) and 17 ± 3 (n = 17) in mPFC and NAc, respectively. Burst LC stimulation (n = 5) significantly elevated extracellular DA in mPFC and NAc compared to animals with misplaced electrodes, including pericoeruleus area (n = 9–10) and sham control rats (n = 3, no current was passed through the LC electrode) (Fig. 4). The DA output in mPFC showed a sharp rise during the stimulation period which declined rapidly. Maximal DA increase was 244 ± 44% of baseline. Two-way ANOVA revealed significant effects of the treatment (F<sub>2,15</sub> = 6.17, p < 0.05), time (F<sub>8,120</sub> = 8.94, p < 0.0001) and treatment × time interaction (F<sub>16,120</sub> = 3.78, p < 0.0001). Burst LC stimulation induced a modest and slow increase of DA release in NAc (153 ± 20% of baseline). Two-way ANOVA indicated a significant effect of time (F<sub>8,120</sub> = 2.13, p < 0.05) and treatment × time interaction (F<sub>16,120</sub> = 1.89, p < 0.05) and non-significant effects of stimulation and time. Post-hoc Newman–Keuls test indicated that DA output in mPFC during LC stimulation was significantly greater than in control rats.

Finally, two-way ANOVA of DA output of the stimulated groups revealed significant effects of time (F<sub>8,12</sub> = 11.67, p < 0.00001) and time × region interaction (F<sub>8,12</sub> = 2.39, p < 0.05). Post-hoc Newman–Keuls test indicated that the DA output in the mPFC during LC stimulation was significantly greater than in NAc.

**Selective enhancement of cortical DA output by noradrenergic drugs in APD pre-treated rats**

Overall, the above results suggest that (1) the extracellular DA concentration is distinctly regulated in mPFC and NAc, and (2) NE terminals markedly contribute to the control of mPFC DA (but not of NAc DA) either by co-releasing DA and/or taking up DA via NET.

We next studied the feasibility of selectively increasing mesocortical DA transmission through NE-acting drugs. We conducted two sets of experiments in rats implanted with two microdialysis probes (mPFC and NAc). Baseline DA concentrations in mPFC and NAc (double-probe experiments) were 9 ± 1 fmol/fraction (n = 69) and 9 ± 2 fmol/fraction (n = 62), respectively.

In the first set of experiments, the selective NET inhibitor reboxetine (30 μM) was locally applied by reverse dialysis in mPFC and NAc followed by the systemic administration of the selective α<sub>2</sub>-adrenoceptor antagonist RX821002 (1 mg/kg s.c.) 2 h later, to dis-inhibit the autoreceptor-mediated negative feedback on NE neuron activity. Figure 5 shows the increase in...
extracellular DA (AUCs) obtained in mPFC and NAc during local reboxetine application plus its combination with RX821002. Two-way ANOVA revealed a significant effect of treatment ($F_{2,18}=79.18$, $p<0.0001$) and region ($F_{1,18}=50.40$, $p<0.0001$) as well as a significant treatment $\times$ region interaction ($F_{2,18}=20.74$, $p<0.0001$).

In the second set of experiments, both drugs were given systemically (reboxetine, 3 mg/kg i.p.; RX821002, 1 mg/kg s.c.). The effect of the reboxetine + RX821002 combination was examined alone and in rats pretreated with APD drugs: haloperidol (classical, 0.1 mg/kg s.c.) and clozapine (APD, 3 mg/kg s.c.). The whole set of data are shown in Fig. 6 and were analysed using two-way ANOVA with treatment and time as main factors (main effects are shown in Table 1). The AUC data of relevant fractions (12–16) for each combination treatment were also calculated and analysed by two-way ANOVA with treatment and region as main factors (Fig. 7).

**Vehicle + reboxetine + RX821002**

Vehicle injections did not alter DA output in mPFC or NAc. The administration of either reboxetine or RX821002 alone moderately enhanced DA output, yet their combined administration dramatically increased DA levels in mPFC ($869\pm 139$% of baseline, $n=5$) but not in NAc ($188\pm 33$%, $n=4$) (Fig. 6a,b; see Table 1 for detailed statistical analysis). *Post-hoc* $t$ tests (Newman–Keuls) revealed a significant difference of the reboxetine + RX821002 treatment vs. the rest of experimental groups in mPFC. No significant differences were found for DA values in NAc.

Two-way ANOVA of AUC data (Fig. 7a) revealed a significant effect of treatment ($F_{3,30}=20.6$, $p<0.00001$), region ($F_{1,30}=21.0$, $p<0.0001$) and region $\times$ treatment interaction ($F_{3,30}=14.9$, $p<0.00001$).

**Clozapine + reboxetine + RX821002**

The systemic administration of clozapine elevated DA output to $241\pm 27$% of baseline in mPFC ($n=4$, data are AUC$_{4–16}$). In NAc, systemic clozapine administration did not significantly alter DA output ($99\pm 13$%, $n=4$).

The combination of clozapine + reboxetine + RX821002 markedly increased DA output to $781\pm 123$% of baseline in mPFC ($n=6$). This effect was significantly greater than that of clozapine alone and that of clozapine + reboxetine (Newman–Keuls test post-ANOVA) (Fig. 6b, Table 1). In NAc, the administration of reboxetine + RX821002 increased the effect of clozapine to $335\pm 127$% of baseline ($n=5$) (Fig. 6d, Table 1). No significant differences between treatments were found in NAc.

When comparing AUC data within regions (Fig. 6b), two-way ANOVA revealed a significant effect of treatment ($F_{3,30}=11.9$, $p<0.0001$), region ($F_{1,30}=10.5$, $p<0.005$) and a marginal significance of region $\times$ treatment interaction ($F_{3,30}=2.5$, $p=0.078$).

**Haloperidol + reboxetine + RX821002**

The systemic administration of haloperidol, given alone, did not alter the DA output in mPFC ($116\pm 12$%, $n=9$, AUC$_{4–16}$), while in NAc it significantly increased DA levels to $162\pm 20$% of baseline ($n=7$).

The combination of haloperidol + reboxetine + RX821002 induced a large elevation of DA levels in mPFC to $1375\pm 275$% of baseline ($n=6$). This effect was significantly greater than that of haloperidol alone or that of the combined administration of reboxetine + RX821002 (post-hoc Newman–Keuls; Fig. 6c, Table 1). In NAc, the co-administration of haloperidol + reboxetine + RX821002 increased DA levels to $238\pm 43$% of baseline ($n=5$) (Fig. 6f, Table 1). No significant differences between treatments were found for DA values in this region.

Two-way ANOVA of AUC data (Fig. 7c) revealed a significant effect of treatment ($F_{3,30}=18.2$, $p<0.00001$), region ($F_{1,30}=19.5$, $p<0.0001$) and region $\times$ treatment interaction ($F_{3,30}=14.2$, $p<0.00001$).
Discussion

The results of the present study confirm and extend previous observations on a differential regulation of DA release in mPFC and NAc. We systematically compared the effect of agents modulating extracellular DA in both pathways. The data support a significant contribution of noradrenergic neurotransmission to DA release in mPFC compared to NAc. The different mechanisms involved in the control of the active (extracellular) DA fraction in both areas offer new therapeutic opportunities to treat non-psychotic symptoms in schizophrenia, associated with a reduced cortical dopaminergic function. Hence, we demonstrate that noradrenergic drugs dramatically and selectively enhance mesocortical DA, using a strategy previously shown to potentiate the effects of reuptake blockers on serotonergic (Artigas et al. 1996) and noradrenergic systems (Mateo et al. 1998).

Relative contribution of NE neurotransmission to DA output in mPFC and NAc

The effects of DAT and/or NET inhibitors agree with previous observations indicating that NET inhibitors increase extracellular NE and DA in PFC, but not in

![Graphs showing the effect of combination treatment with NE-targeting drugs on DA release in rats.](https://example.com/figure6.png)

Fig. 6. Effect of the combination treatment with NE-targeting drugs [reboxetine (Reb), 3 mg/kg i.p.; and RX821002 (RX), 1 mg/kg s.c.] on DA release in rats pretreated with (a, b) vehicle (Veh), (c, d) clozapine (Clz 3 mg/kg s.c.) and (e, f) haloperidol (Hal 0.1 mg/kg s.c.). Control groups received vehicle injections ($n=4–6$; except for Veh/Veh + Veh, where $n=3$). Left (a, c, e) and right (b, d, f) panels correspond to the effects in mPFC and NAc, respectively. Data are expressed as mean ± s.e.m. Statistical analyses shown in Table 2.
Table 1. Statistical analyses of the combination treatment of NE-targeting drugs, reboxetine and RX821002, on dopamine release in vehicle, clozapine and haloperidol pre-treated rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>mPFC</th>
<th>NAc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Effect</td>
<td>F</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veh + Veh</td>
<td>93 ± 19 (6)</td>
<td>T&lt;sub&gt;3.15&lt;/sub&gt; = 19.0</td>
</tr>
<tr>
<td>Reb + Veh</td>
<td>195 ± 38 (5)</td>
<td>T&lt;sub&gt;15.248&lt;/sub&gt; = 33.6</td>
</tr>
<tr>
<td>Veh + RX</td>
<td>234 ± 42 (4)</td>
<td>T x t&lt;sub&gt;15.248&lt;/sub&gt; = 19.0</td>
</tr>
<tr>
<td>Reb + RX</td>
<td>869 ± 139* (6)</td>
<td></td>
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<tr>
<td>Clozapine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veh + Veh</td>
<td>197 ± 30 (4)</td>
<td>T&lt;sub&gt;3.15&lt;/sub&gt; = 5.0</td>
</tr>
<tr>
<td>Reb + Veh</td>
<td>180 ± 46 (4)</td>
<td>T&lt;sub&gt;15.248&lt;/sub&gt; = 24.2</td>
</tr>
<tr>
<td>Veh + RX</td>
<td>348 ± 67 (5)</td>
<td>T x t&lt;sub&gt;15.248&lt;/sub&gt; = 10.3</td>
</tr>
<tr>
<td>Reb + RX</td>
<td>781 ± 123* (4)</td>
<td></td>
</tr>
<tr>
<td>Haloperidol</td>
<td></td>
<td></td>
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<tr>
<td>Veh + Veh</td>
<td>120 ± 16 (6)</td>
<td>T&lt;sub&gt;3.15&lt;/sub&gt; = 18.8</td>
</tr>
<tr>
<td>Reb + Veh</td>
<td>323 ± 40 (5)</td>
<td>T&lt;sub&gt;15.248&lt;/sub&gt; = 29.2</td>
</tr>
<tr>
<td>Veh + RX</td>
<td>197 ± 18 (6)</td>
<td>T x t&lt;sub&gt;15.248&lt;/sub&gt; = 16.0</td>
</tr>
<tr>
<td>Reb + RX</td>
<td>1375 ± 275* (9)</td>
<td></td>
</tr>
</tbody>
</table>

Reb, Reboxetine; RX, RX821002; Veh, vehicle; n.s., not significant.

Data (effect) are given as percentage of baseline in each experimental group (AUC<sub>12–16</sub>). Number of animals in each group is given in parentheses.

* Significantly different to all other treatments.
+ Significantly different to all treatments except for Veh + RX.

Data have been analysed using two-way ANOVA with treatment (I) and time (t) as main factors.

Noradrenergic contribution to mesocortical DA

NAc or dorsal striatum (Bymaster et al. 2002; Carboni et al. 1990, 2006; Di Chiara et al. 1992; Mazei et al. 2002; Pozzi et al. 1994; Tanda et al. 1994). The local application of nomifensine, with similar affinity for DAT and NET (PDSP database: http://pdsp.med.unc.edu/pdsp.php; Bymaster et al. 2002), increased comparably dialysate DA in mPFC and NAc. However, GBR12909 preferentially increased DA in NAc, suggesting a poor contribution of DAT-containing fibres to extracellular DA in mPFC, a view consistent with the low density of DAT in PFC (Sesack et al. 1998) compared to NAc or dorsal striatum (Letchworth et al. 2000; Marshall et al. 1990). In contrast, mPFC contains a higher density of NET (Miner et al. 2003; Schroeter et al. 2000) than the NAc. Hence, the NAc core contains scarce NE fibres (Seguela et al. 1990) and those in NAc shell mainly arise from the nucleus tractus solitarius (Berridge et al. 1997; Delfs et al. 1998). This, together with the similar affinity of NE and DA for NET (Gu et al. 1994; Raiteri et al. 1977) may account for the effect of reboxetine, which increased DA output only in mPFC. Despite NE axons being infrequently apposed to DA axons in PFC (Miner et al. 2003), newly released DA may diffuse trans-synaptically to reach NET sites, as observed for DA itself (Sesack et al. 1998) (see Fig. 8a).

Furthermore, DSP-4 (Fig. 3a) almost abolished the effect of nomifensine on DA output in mPFC, but not in NAc, further supporting a preferential contribution of NE fibres to mPFC DA release. We used standard lesion procedures to extensively damage DA and NE neurons/fibres. Thus, similarly to previous studies (Bortolozzi & Artigas, 2003; Fritschy & Grzanna, 1989; Robinson & Whishaw, 1988; Tseng et al. 2005) DSP-4 and 6-OHDA depleted tissue NE and DA concentrations by ≈90%, respectively. The clear-cut mPFC–NAc difference of DSP-4 on nomifensine’s effect may reflect the aforementioned differences on NE axon densities innervating both brain structures and also their ability to release DA. Moreover, a preferential LC sensitivity to the DSP-4 lesion cannot be discounted (Dailly et al. 2006; Grzanna et al. 1989; Jonsson et al. 1981). A limitation of the present study is the lack of immunohistochemical or autoradiographic analysis of lesions, which, as in other studies (Grzanna et al. 1989;
Vos et al. (1999; Szot et al. 2010) would have permitted a detailed assessment of lesion effects on NE and DA axons.

Conversely, local nomifensine increased DA output less in NAc than in mPFC of the unilaterally 6-OHDA-lesioned rats (Fig. 3b), compared to the contralateral (unlesioned) side, respectively, suggesting that most DA output in NAc arises from VTA DA fibres. Even with the prior protection of NE fibres by desipramine prior to 6-OHDA application, a marked reduction of tissue NE was found in the ipsilateral NAc (71% for NE vs. 93% for DA), possibly due to toxin diffusion to the neighbouring median forebrain bundle. Previous studies have shown the inability of desipramine to fully protect NE axons from 6-OHDA lesions (Harden et al. 1998; King & Finlay, 1995).

Although DSP-4 and 6-OHDA markedly affected the nomifensine-induced rise in extracellular DA, baseline levels were almost unaltered, with the exception in mPFC of DSP-4 pretreated rats. This is consistent with previous literature indicating compensatory changes to maintain extracellular monoamine concentrations despite marked differences in tissue concentrations (Abercrombie & Zigmond, 1989; Robinson et al. 1994; Romero et al. 1998; Zigmond et al. 1990; see however Devoto et al. 2008).

The enhancement of DA output in mPFC (244% of baseline) after electrical stimulation of LC agrees with previous observations on a potential co-release of DA from LC NE fibres in PFC (Devoto et al. 2001, 2005; Devoto & Flore, 2006). Since rats in the present study were implanted with two probes (in mPFC and NAc), we were able to compare the effect of LC stimulation on DA output in mPFC and NAc of the same animals. LC stimulation also moderately increased (153% of baseline) the DA output in NAc although with a blunted time-course, an effect that may arise from a

1-
adrenergic stimulation of VTA DA neurons following LC stimulation (Grenhoff & Svensson, 1993). Further, a

1-
adrenoceptor blockade abolishes the behavioural effect produced by the stimulation of VTA DA neurons (Auclair et al. 2002, 2004; Darracq et al. 1998). Although an a

1-
adrenergic stimulation of mesocortical DA neurons cannot be excluded, the larger DA increase in mPFC, its temporal association with LC stimulation and the lack of effect when the stimulating electrodes were placed outside the LC supports a noradrenergic origin of the DA release in mPFC.

Thus, the present results suggest that the extracellular DA concentration in NAc mainly arise from VTA DA fibres whereas that in mPFC has a dual contribution, from the VTA and from LC NE fibres. Since DA is an intermediate metabolite in the synthesis of NE in noradrenergic neurons, the co-release of DA and NE may reflect a deficient activity of dopamine-

β-

hydroxylase in cortical noradrenergic axons, a possibility that deserves further investigation. This region-specific noradrenergic contribution to DA

![Fig. 7. Bars show the average effect of the combination treatment of reboxetine (Reb, 3 mg/kg i.p.) plus RX821002 (RX, 1 mg/kg s.c.) on DA release in mPFC (□) and NAc (■) of rats pre-treated with (a) vehicle, (b) clozapine (3 mg/kg s.c.) or (c) haloperidol (0.1 mg/kg s.c.), respectively. Data are AUC of fractions 12–16 (Fig. 5), expressed as percentage of baseline; n = 4–6, except for Veh/Veh + Veh, where n = 3. See text for statistical analysis. * p < 0.001 vs. control treatments and + p < 0.01 for mPFC vs. NAc.](http://ijnp.oxfordjournals.org/Downloaded from http://ijnp.oxfordjournals.org/ by guest on October 7, 2016)
release allows the selective modulation of mesocortical DA function.

Selective enhancement of DA release in mPFC: association with APD treatments

Many studies implicate prefrontal catecholamine function in cognition (Arnsten & Li, 2005; Goldman-Rakic et al., 2000; Robbins & Roberts, 2007; Sara, 2009). The present and previous observations show striking similarities between the factors governing DA and NE release in mPFC. Current views indicate a hypoactive mesocortical DA pathway in schizophrenia that may underlie negative and/or cognitive symptoms and APDs increase mPFC DA output (see Introduction). This has been considered a useful pharmacological feature accounting for the clinical superiority of some APDs in non-psychotic symptoms (Kane et al. 1988; Keefe et al. 2006; Leucht et al. 2009; Meltzer & McGurk, 1999). Actually, blockade of DA D₂ receptors is effective in treating positive symptoms, possibly related to a subcortical DA hyperactivity (Laruelle et al. 1996), but not negative/cognitive symptoms. Conversely, DA D₂ receptor blockade induces negative symptoms in healthy individuals (Artaloytia et al. 2006).

Previous reports indicate that reuptake blockade in serotonergic and noradrenergic neurons induces a very marked increase of the respective neurotransmitter in the vicinity of cell bodies in the raphe nuclei (Adell & Artigas, 1991; Bel & Artigas, 1992) and LC (Grandoso et al. 2004; Mateo et al. 1998). The excess neurotransmitter in this area activates their respective autoreceptors (5-HT₁A and α₂-adrenoceptors), which leads to a reduced neuronal activity and terminal monoamine release. Autoreceptor blockade enables the recovery of cell firing and terminal release, thus permitting the full pharmacological effect of reuptake blockade (Artigas et al. 1996; Grandoso et al. 2004; Invernizzi & Garattini, 2004; Mateo et al. 1998; Romero & Artigas, 1997). Given the marked involvement of noradrenergic fibres in the uptake/co-release of DA in mPFC, we used this strategy to selectively enhance cortical DA function (Fig. 8).

α₂-adrenergic antagonists and NE reuptake inhibitors, acting mainly on NE neurons, increase moderately

Fig. 8. Schematic representation of the contribution of NET inhibition (reboxetine) and α₂-adrenoceptor antagonism (RX821002) in NE terminals on the control of extracellular DA levels in mPFC. (a) In a physiological situation, DA can be co-released with NE by noradrenergic terminals and/or taken up by NET, given the similar affinity of the membrane transporters for both monoamines. (b) The moderate increase in extracellular DA levels in mPFC evoked by reboxetine probably results from two opposing factors, (i) an elevation resulting from NET blockade itself, and (ii) a reduction resulting from the activation of α₂-adrenoceptors. Activation of somatodendritic α₂-adrenoceptors in the LC (not shown in the figure) also contributes to attenuate catecholamine release through a reduction of the firing rate of noradrenergic neurons after systemic reboxetine administration. (c) The co-treatment with RX821002 removes the α₂-adrenoceptor-mediated negative feedback on noradrenergic release of catecholamines, and markedly potentiates the increase in extracellular DA evoked by reboxetine.
Our results show for the first time that 
Valentini et al. 2004; Gresch et al. 2004; Wadenberg et al. 2007). However, our results show for the first time that α₂-adrenoceptor blockade markedly potentiates the effect of NET inhibitors on DA output in mPFC (as also observed for cortical NE output; Swanson et al. 2006) but not in NAc (Figs 5–8).

The increase in cortical DA output also occurred when reboxetine + RX-821002 were administered in combination with classical (haloperidol – lacking appreciable affinity for α₂-adrenoceptor) and APDs (clozapine – with antagonist properties at α₂-adrenoceptors). These drug combinations did not elevate DA output in NAc or produce even a small effect compared to the marked elevation seen in mPFC. The doses used (3 mg/kg for clozapine, 0.1 mg/kg for haloperidol) are close to their ED₅₀ values for occupation of their primary receptor targets (5-HT₁A/₁B and D₂, respectively) (Schotte et al. 1993). Further, the dose of clozapine is in the lower range of those shown to enhance cortical DA release (Diaz-Mataix et al. 2005; Ichikawa et al. 2001; Rollema et al. 1997).

Clozapine increased DA output in mPFC, an effect mediated by cortical 5-HT₁A receptor activation (Bortolozzi et al. 2010; Diaz-Mataix et al. 2005) although α₂-adrenoceptor blockade has also been suggested (Ashby & Wang, 1996; Svensson, 2003). However, since the elevation of DA output produced by reboxetine + RX821002 was much larger than that of reboxetine + clozapine, it seems reasonable to assume that clozapine antagonizes α₂-adrenoceptors much less than RX821002.

Consistent with previous reports, haloperidol produced a moderate DA increase in NAc but not in mPFC (Kuroki et al. 1999; Li et al. 1998). However, the effect of haloperidol + reboxetine + RX821002 was more marked than that of the latter two drugs, possibly due to blockade of DA D₂ receptors by haloperidol, which would remove the D₂-mediated negative feedback on DA release following the large increase produced by reboxetine + RX821002. The lower occupancy of DA D₂ receptors produced by clozapine probably accounts for the lower enhancement of mPFC DA output when combined with reboxetine + RX821002.

**Therapeutic implications**

Overall, the above results indicate that (1) extracellular DA concentration is distinctly regulated in mPFC and NAc, (2) NE terminals largely contribute to the control of DA output in mPFC (but not NAc) by co-releasing DA and taking up DA via NET, and (3) a marked and selective enhancement of DA function in mPFC is feasible through the combined administration of NET blockers and α₂-adrenoceptor antagonists. The latter effect occurs when these drugs are administered alone or in combination with haloperidol or clozapine. This opens the way to perform clinical trials in which reboxetine or other NET blockers, used as antidepressants, can be combined with α₂-adrenoceptor antagonists in order to test their clinical efficacy on negative symptoms and/or cognitive dysfunction in schizophrenia and other psychiatric disorders.

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**Statement of Interest**

None.

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None.


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