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ARTICLE

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Selective enhacement 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 α_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 α_2-adrenoceptors, thus preventing the autoreceptor-mediated negative feedback on NE activity. Our results highlight the importance of NET and α_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)

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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*. 2003; Schroeter *et al*. 2000) compared to NAc. In fact, NE axons may contribute to the removal of DA from the extracellular brain space, since NET shows a similar affinity for NE and DA (Raiteri *et al*. 1977). NET inhibitors seem to preferentially increase the extracellular DA concentration in the medial prefrontal cortex (mPFC) 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 combining the NET inhibitor reboxetine and the α_2-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 RX821002 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; $CaCl_2$, 2.52; $MgCl_2$, 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. 1*b*). 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

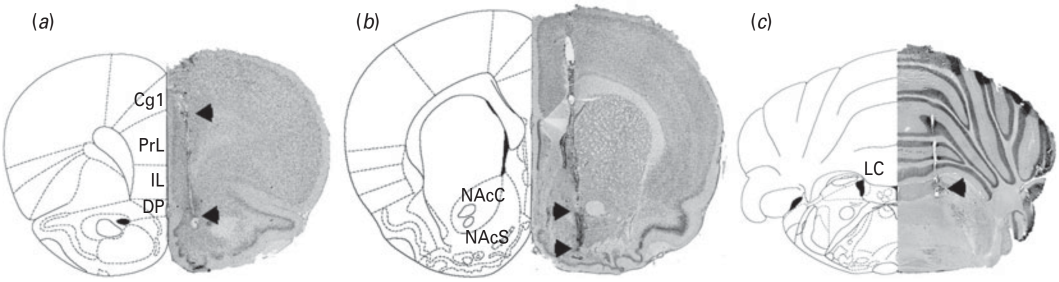

Fig. 1. Representative histological sections cut in the coronal plane at 40 μm showing the tract of a dialysis probe located within (*a*) mPFC, (*b*) NAc and (*c*) stimulating electrode in locus coeruleus (LC) of the rat. Black arrows indicate the length of microdialysis probes (4 and 1.5 mm for mPFC and NAc, respectively) and the tip of the electrode in LC. Adapted from Paxinos & Watson (1998). Cg1, cingulate area 1; PrL, prelimbic cortex; IL, infralimbic cortex; DP, dorsal peduncular cortex; NAcC, nucleus accumbens core; NAcS, nucleus accumbens shell.

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 $HClO_4$ 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-Om 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

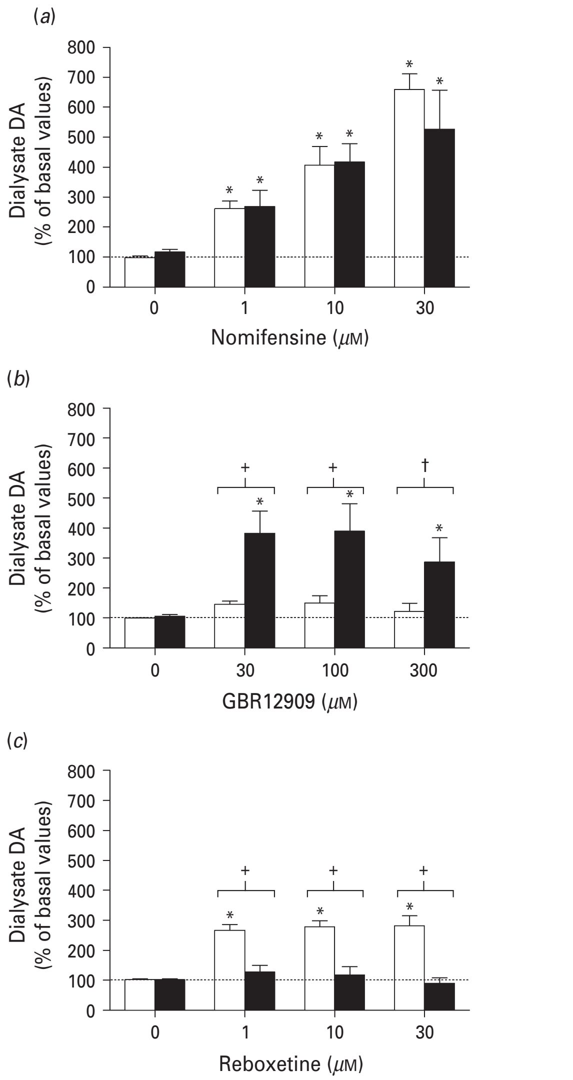

Fig. 2. Local effect of catecholamine transporter inhibitors on DA release in mPFC (□) and NAc (■) of freely moving rats. The application of artificial CSF did not alter DA levels in any area. (*a*) Nomifensine (DAT+NET inhibitor), perfused at 1, 10 and 30 μM (12 fractions each), showed a marked dose 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.

DA values (repeated measures) followed by Newman–Keuls *post-hoc* test. Basal DA dialysate levels and tissue monoamine contents were compared using Student's *t* test. Data are expressed as means ± S.E.M. The significance level was set at $p<0.05$.

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. 2*a*). 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 ($F_{3,32}=27.13$, $p<0.00001$) 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. 2*b*). 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 ($F_{1,32}=27.92$, $p<0.00001$) 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. 2*c*). 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 ($F_{1,32}=60.07$, $p<0.0001$) and concentration ×

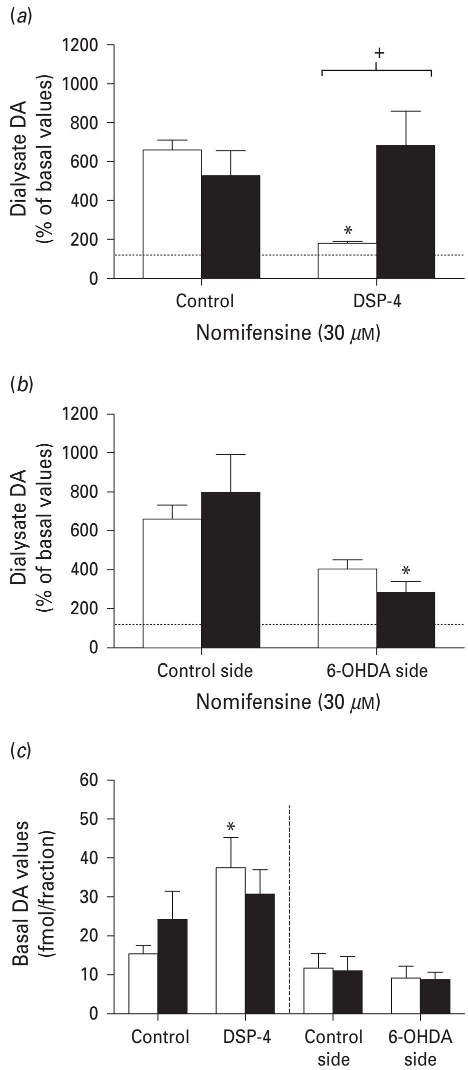

Fig. 3. Local effect of nomifensine 30 μM in mPFC (□) and NAc (■) of rats underlying specific lesions. (*a*) Noradrenergic lesion with DSP-4 (40 mg/kg i.p., 5 d before experiments) changed DA response to nomifensine in mPFC but not NAc, compared to control groups. (*b*) VTA was unilaterally lesioned 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 ($F_{3,32} = 7.19$, $p < 0.001$). *Post-hoc* test (Newman–Keuls) revealed significant differences between NAc and mPFC at all concentrations.

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. 3*c*).

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. 3*a*). 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 ± 2272 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. 3*b*). 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

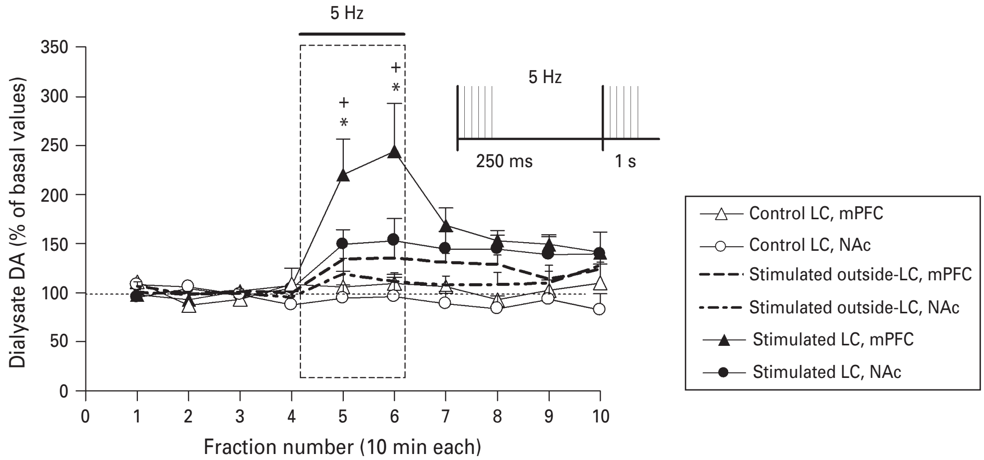

Fig. 4. Effect of electrical stimulation of LC on DA levels in mPFC and NAc of the same anaesthetized rats. LC stimulation increased DA release in mPFC to a larger extent than in NAc. Data are expressed as mean ± S.E.M., $n=3$ for control; $n=5$ for stimulated. Inset: LC was stimulated after four basal fractions in phasic mode with 0.1-ms pulses delivered at 20 Hz for 250 ms every 1 s (average frequency of 5 Hz) and 0.7 mA for 20 min. See text for statistical analysis. * $p<0.05$ *vs.* control (non-stimulated) and $^{+}$ $p<0.05$ for mPFC *vs.* NAC. Dotted lines show the effect on extracellular DA of rats with stimulating electrodes implanted outside the LC.

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 $244\pm44\%$ of baseline. Two-way ANOVA revealed significant effects of the treatment ($F_{2,15}=6.17$, $p<0.05$), time ($F_{9,135}=8.94$, $p<0.0001$) and treatment × time interaction ($F_{18,135}=3.78$, $p<0.0001$). Burst LC stimulation induced a modest and slow increase of DA release in NAc ($153\pm20\%$ of baseline). Two-way ANOVA indicated a significant effect of time ($F_{9,126}=2.13$, $p<0.05$) and treatment × time interaction ($F_{18,126}=1.89$, $p<0.05$) 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 ($F_{9,72}=11.67$, $p<0.00001$) and time × region interaction ($F_{9,72}=2.39$, $p<0.05$). *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_2-adrenoceptor antagonist RX821002 (1 mg/kg s.c.) 2 h later, to disinhibit the autoreceptor-mediated negative feedback on NE neuron activity. Figure 5 shows the increase in

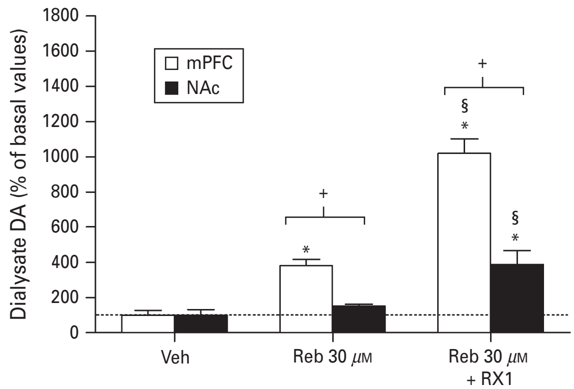

Fig. 5. Effect of the combination treatment with NE-targeting drugs [local reboxetine (Reb), 30 μM and RX821002 (RX), 1 mg/kg s.c.] on DA release in mPFC and NAc. Reboxetine was locally applied by reverse dialysis at 30 μM for the whole experiment (fractions 4–16) and RX was systemically administered 2 h after the beginning of reboxetine perfusion. Bars are AUC of four DA values for Reb and for Reb + RX. See text for statistical analysis. * $p < 0.01$ *vs.* control, § $p < 0.01$ *vs.* 30 μM Reb and + $p < 0.01$ for mPFC *vs.* NAc.

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 × 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 ± 139% of baseline, $n = 5$) but not in NAc (188 ± 33%, $n = 4$) (Fig. 6*a*, *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. 7*a*) 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 × treatment interaction ($F_{3,30} = 14.9$, $p < 0.00001$).

Clozapine + reboxetine + RX821002

The systemic administration of clozapine elevated DA output to 241 ± 27% of baseline in mPFC ($n = 4$, data are AUC_{6-16}). In NAc, systemic clozapine administration did not significantly alter DA output (99 ± 13%, $n = 4$).

The combination of clozapine + reboxetine + RX821002 markedly increased DA output to 781 ± 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. 6*b*, Table 1). In NAc, the administration of reboxetine + RX821002 increased the effect of clozapine to 335 ± 127% of baseline ($n = 5$) (Fig. 6*d*, Table 1). No significant differences between treatments were found in NAc.

When comparing AUC data within regions (Fig. 6*b*), 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 × 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 ± 12%, $n = 9$, AUC_{6-16}), while in NAc it significantly increased DA levels to 162 ± 20% of baseline ($n = 7$).

The combination of haloperidol + reboxetine + RX821002 induced a large elevation of DA levels in mPFC to 1375 ± 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. 6*e*, Table 1). In NAc, the co-administration of haloperidol + reboxetine + RX821002 increased DA levels to 238 ± 43% of baseline ($n = 5$) (Fig. 6*f*, Table 1). No significant differences between treatments were found for DA values in this region.

Two-way ANOVA of AUC data (Fig. 7*c*) revealed a significant effect of treatment ($F_{3,39} = 18.2$, $p < 0.00001$), region ($F_{1,39} = 19.5$, $p < 0.0001$) and region × treatment interaction ($F_{3,39} = 14.2$, $p < 0.00001$).

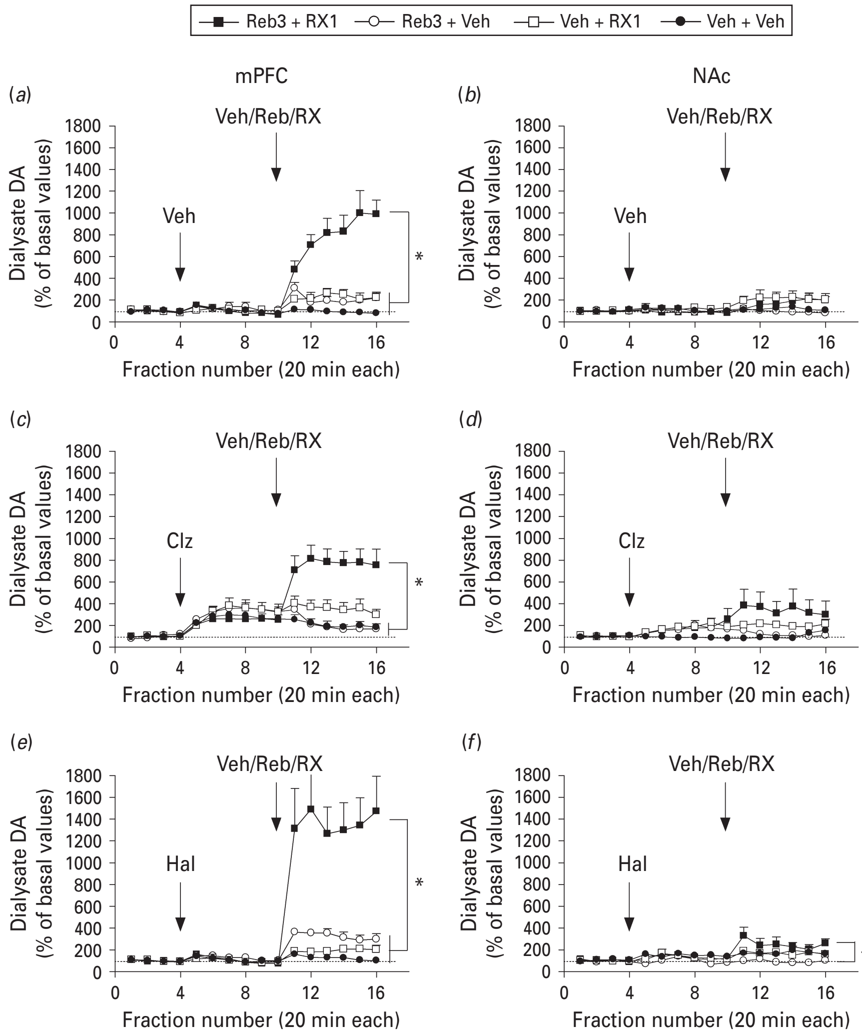

Fig. 6. Effect of the combination treatment with NE-targeting drugs [reboxetine (Reb), 3 mg/kg i.p.; and RX821002 (RX), 1 mg/kg s.c.] on DA release in rats pretreated with (*a, b*) vehicle (Veh), (*c, d*) clozapine (Clz 3 mg/kg s.c.) and (*e, f*) haloperidol (Hal 0.1 mg/kg s.c.). Control groups received 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.

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 serotoninergic (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

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

	mPFC			NAc		
Treatment	Effect	*F*	*p*	Effect	*F*	*p*
Vehicle						
Veh + Veh	93 ± 19 (6)			121 ± 9 (5)		
Reb + Veh	195 ± 38 (5)	$T_{3,16} = 19.0$	< 0.0001	93 ± 12 (5)	$T_{3,14} = 3.1$	n.s.
Veh + RX	234 ± 42 (4)	$t_{15,240} = 33.6$	< 0.00001	216 ± 61 (4)	$t_{15,210} = 6.3$	< 0.00001
Reb + RX	869 ± 139* (6)	$T \times t_{45,240} = 19.0$	< 0.00001	188 ± 33 (5)	$T \times t_{45,210} = 2.3$	< 0.0001
Clozapine						
Veh + Veh	197 ± 30 (4)			110 ± 15 (4)		
Reb + Veh	180 ± 46 (4)	$T_{3,15} = 5.0$	< 0.05	108 ± 21 (4)	$T_{3,15} = 2.2$	n.s.
Veh + RX	348 ± 67 (5)	$t_{15,225} = 24.2$	< 0.00001	203 ± 23 (6)	$t_{15,225} = 4.3$	< 0.00001
Reb + RX	781 ± 123⁺ (4)	$T \times t_{45,225} = 10.3$	< 0.00001	335 ± 127 (4)	$T \times t_{45,225} = 2.2$	< 0.001
Haloperidol						
Veh + Veh	120 ± 16 (6)			174 ± 23 (5)		
Reb + Veh	323 ± 40 (5)	$T_{3,22} = 18.8$	< 0.0001	95 ± 7 (3)	$T_{3,17} = 2.7$	n.s.
Veh + RX	197 ± 18 (6)	$t_{15,330} = 29.2$	< 0.00001	167 ± 22 (6)	$t_{15,225} = 5.9$	< 0.00001
Reb + RX	1375 ± 275* (9)	$T \times t_{45,330} = 16.0$	< 0.00001	238 ± 43 (7)	$T \times t_{45,225} = 1.7$	< 0.01

Reb, Reboxetine; RX, RX821002; Veh, vehicle; n.s., not significant.
Data (effect) are given as percentage of baseline in each experimental group (AUC_{12-16}). 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 (*T*) and time (*t*) as main factors.

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. 8*a*).

Furthermore, DSP-4 (Fig. 3*a*) 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;

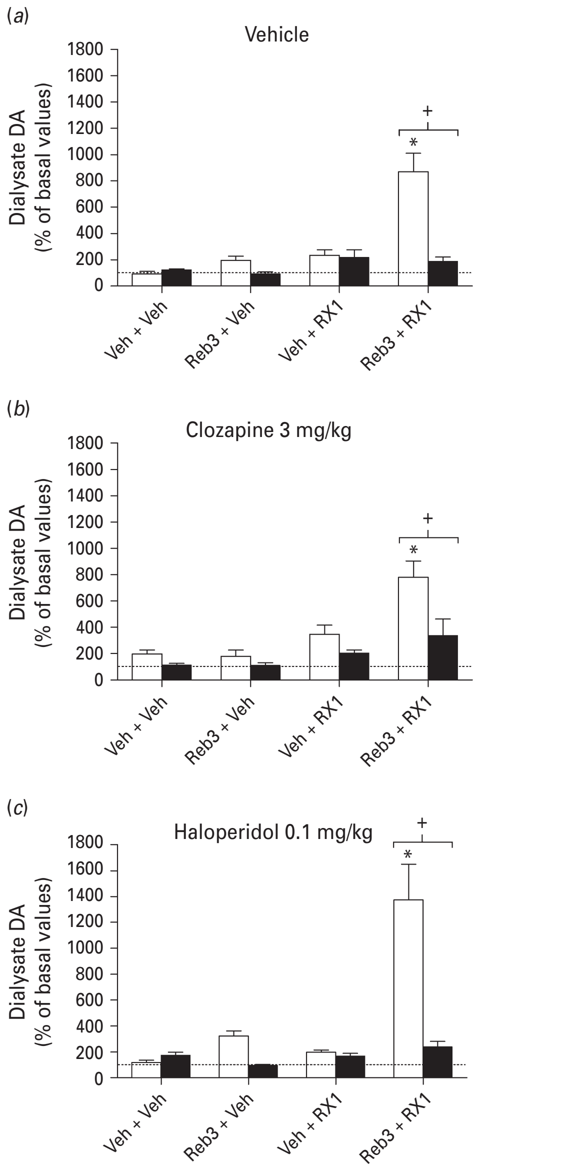

Fig. 7. Bars show the average effect of the combination treatment of reboxetine (Reb, 3 mg/kg i.p.) plus RX821002 (RX, 1 mg/kg s.c.) on DA release in mPFC (□) and NAc (■) of rats pre-treated with (*a*) vehicle, (*b*) clozapine (3 mg/kg s.c.) or (*c*) haloperidol (0.1 mg/kg s.c.), respectively. Data are 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.

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. 3*b*), 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 α_1-adrenergic stimulation of VTA DA neurons following LC stimulation (Grenhoff & Svensson, 1993). Further, α_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 α_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

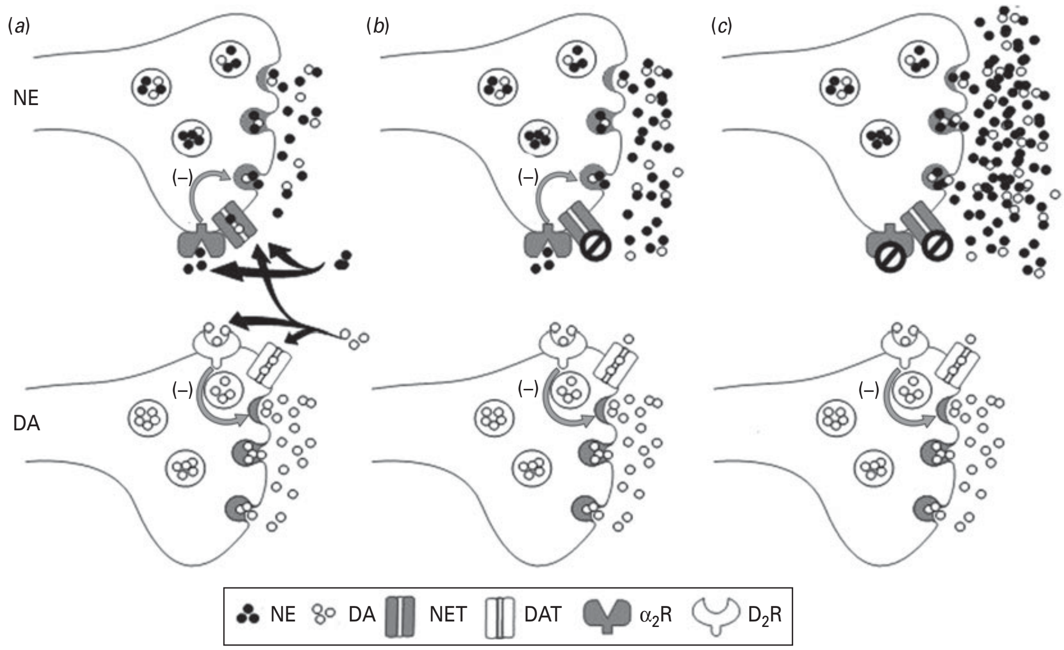

Fig. 8. Schematic representation of the contribution of NET inhibition (reboxetine) and α_2-adrenoreceptor antagonism (RX821002) in NE terminals on the control of extracellular DA levels in mPFC. (*a*) In a physiological situation, DA can be co-released with NE by noradrenergic terminals and/or taken 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 α_2-adrenoceptors. Activation of somatodendritic α_2-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 α_2-adrenoceptor-mediated negative feedback on noradrenergic release of catecholamines, and markedly potentiates the increase in extracellular DA evoked by reboxetine.

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 D_2 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 D_2 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-HT_{1A} and α_2-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).

α_2-adrenergic antagonists and NE reuptake inhibitors, acting mainly on NE neurons, increase moderately but selectively prefrontal DA output (Devoto *et al*. 2004; Gresch *et al*. 1995; Hertel *et al*. 1999*a*, *b*; Linner *et al*. 2001; Millan *et al*. 2000; Swanson *et al*. 2006; Valentini *et al*. 2004; Wadenberg *et al*. 2007). However, our results show for the first time that α_2-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 α_2-adrenoreceptor) and APDs (clozapine – with antagonist properties at α_2-adrenoreceptors). 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 ED_{50} values for occupation of their primary receptor targets ($5\text{-}HT_{2A/2C}$ and D_2, 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 $5\text{-}HT_{1A}$ receptor activation (Bortolozzi *et al*. 2010; Diaz-Mataix *et al*. 2005) although α_2-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 α_2-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 D_2 receptors by haloperidol, which would remove the D_2-mediated negative feedback on DA release following the large increase produced by reboxetine + RX821002. The lower occupancy of DA D_2 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 α_2-adrenoceptor antagonists. The latter effect occurs when these dugs 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 α_2-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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