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Title: Noradrenergic antidepressants increase cortical dopamine: Potential use in augmentation strategies

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Abstract: Most antidepressant treatments, based on serotonin (5-HT) and/or norepinephrine (NE) transporter blockade, show limited efficacy and slow onset of action, requiring the use of augmentation strategies. Here we report on a novel antidepressant strategy to selectively increase DA function in prefrontal cortex (PFC) without the potential tolerance problems associated to DA transporter blockade. This approach is based on previous observations indicating that extracellular DA in rat medial PFC (mPFC) -but not in nucleus accumbens (NAc)- arises from noradrenergic terminals and is sensitive to noradrenergic drugs. A low dose of reboxetine (3 mg/kg i.p.; NE reuptake inhibitor) non-significantly increased extracellular DA in mPFC. Interestingly, its combined administration with 5 mg/kg s.c. mirtazapine (non-selective α_2 -adrenoceptor antagonist) increased extracellular DA in mPFC ($264 \pm 28\%$), but not in NAc. Extracellular NE (but not 5-HT) in mPFC was also enhanced by the combined treatment ($472 \pm 70\%$). Repeated (x3) reboxetine+mirtazapine administration produced a moderate additional increase in mPFC DA and markedly reduced the immobility time (-51%) in the forced-swim test. Neurochemical and behavioral effects of the reboxetine+mirtazapine combination persisted in rats pretreated with citalopram (3 mg/kg, s.c.), suggesting its potential usefulness to augment SSRI effects. In situ hybridization c-fos studies were performed to examine the brain areas involved in the above antidepressant-like effects, showing changes in c-fos expression in hippocampal and cortical areas. BDNF expression was also increased in the hippocampal formation. Overall, these results indicate a synergistic effect of the reboxetine+mirtazapine combination to increase DA and NE function in mPFC and to evoke robust antidepressant-like responses.

Dear Dr. Frenguelli,

I have uploaded a manuscript we would like to submit for publication in Neuropharmacology. The study reports on very marked antidepressant properties of a combination of two commercially available drugs, reboxetine and mirtazapine. The study is based on the recent observation by our group that noradrenergic drugs selectively increase dopamine release in prefrontal cortex. This finding likely derives from the fact that a large proportion of dopamine release in prefrontal cortex arises from noradrenergic axons, being sensitive to NET and alpha2-adrenoceptor blockade. Here we used reboxetine and mirtazapine, targeting NET and alpha2-adrenoceptors, respectively, to synergistically increase cortical dopaminergic neurotransmission, circumventing the tolerance problems derived from DAT blockade in ventral striatum. (N. Accumbens).

The results show that this drug combination evokes marked changes in preclinical variables predictive of clinical antidepressant effects, such as an increase of NA and DA release in prefrontal cortex (but not in N. accumbens) and a marked reduction in immobility in the forced swim test. Neither effect was obtained with either drug alone, indicating a synergistic interaction between them. Moreover, the drug combination increased hippocampal BDNF expression after repeated (x3) administration.

We think that these results have a potentially high translational value in the field of depression and may be of interest for the readers of the journal. Likewise, given the lack of specific treatments for negative symptoms and cognitive deficits in schizophrenia, the selective increase in PFC dopamine may be of interest in this field and may prompt the use of this drug combination in schizophrenia patients with a predominance of negative symptoms.

Finally, I should mention that the companies manufacturing both drugs are totally unaware of the present study. Drugs were chosen exclusively due to their pharmacological properties.

Below you will find four potential expert reviewers.

Looking forward to your reply

Sincerely,

Prof. Francesc Artigas

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Noradrenergic antidepressants increase cortical dopamine:

Potential use in augmentation strategies

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Running title: Noradrenergic antidepressants and dopamine

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Abstract: 249 words; introduction: 456 words; materials and methods: 922 words; results: 1123 words; discussion 1495 words.

Text (excluding abstract, acknowledgments, financial disclosures, legends and references): 4006 words

References: 84

Figures: 5

Supplemental material 1

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Noradrenergic antidepressants increase cortical dopamine: Potential use in augmentation strategies

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Most antidepressant treatments, based on serotonin (5-HT) and/or norepinephrine (NE) transporter blockade, show limited efficacy and slow onset of action, requiring the use of augmentation strategies. Here we report on a novel antidepressant strategy to selectively increase DA function in prefrontal cortex (PFC) without the potential tolerance problems associated to DA transporter blockade. This approach is based on previous observations indicating that extracellular DA in rat medial PFC (mPFC) –but not in nucleus accumbens (NAc)– arises from noradrenergic terminals and is sensitive to noradrenergic drugs. A low dose of reboxetine (3 mg/kg i.p.; NE reuptake inhibitor) non-significantly increased extracellular DA in mPFC. Interestingly, its combined administration with 5 mg/kg s.c. mirtazapine (non-selective α_2 -adrenoceptor antagonist) increased extracellular DA in mPFC ($264 \pm 28\%$), but not in NAc. Extracellular NE (but not 5-HT) in mPFC was also enhanced by the combined treatment ($472 \pm 70\%$). Repeated (x3) reboxetine+mirtazapine administration produced a moderate additional increase in mPFC DA and markedly reduced the immobility time (-51%) in the forced-swim test. Neurochemical and behavioral effects of the reboxetine+mirtazapine combination persisted in rats pretreated with citalopram (3 mg/kg, s.c.), suggesting its potential usefulness to augment SSRI effects. *In situ* hybridization *c-fos* studies were performed to examine the brain areas involved in the above antidepressant-like effects, showing changes in *c-fos* expression in hippocampal and cortical areas. BDNF expression was also increased in the hippocampal formation. Overall, these results indicate a synergistic effect of the

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1. Introduction

Major depression is a severe psychiatric syndrome with high prevalence and socio-economic impact (Andlin-Sobocki et al., 2005; Kessler et al., 2005; Smith, 2011). Current antidepressant treatments show moderate response and remission rates (~50% and ~30%, respectively; Trivedi et al., 2006) making necessary augmentation strategies or more aggressive interventions for treatment-resistant patients (Carney and Geddes, 2003; Mayberg et al., 2005; Puigdemont et al., 2011).

The brainstem serotonergic (5-HT) and noradrenergic (NE) systems are involved in the pathophysiology and treatment of major depression (Nestler et al., 2002). The limited efficacy and slow onset of action of the selective serotonin reuptake inhibitors (SSRI) and the dual serotonin and norepinephrine reuptake inhibitors (SNRI) is partly attributable to the activation of presynaptic autoreceptors (5-HT_{1A/1B} receptors in 5-HT neurons and α_2 -adrenoceptors in NE neurons) by the increased 5-HT/NE produced by reuptake blockade (Artigas et al., 1996; Mateo et al., 1998). Repeated antidepressant treatment desensitizes autoreceptors and normalizes monoamine release (Bel and Artigas, 1993; Blier and de Montigny, 1994; Mateo et al., 2001). Hence, the combined administration of monoamine reuptake inhibitors and autoreceptor antagonists synergistically increases extracellular 5-HT/NE concentrations, thus mimicking chronic antidepressant effects (Artigas et al., 1996; Invernizzi and Garattini, 2004; Mateo et al., 1998). This strategy has been applied to augment SSRI effects, using the non-selective 5-HT_{1A}/ β -adrenoreceptor blocker pindolol (Artigas et al., 1994; Perez et al., 1997; see metaanalyses in Ballesteros and Callado, 2004;

Portella et al., 2011; Whale et al., 2010). However, pindolol is not useful in treatment-resistant patients (Pérez et al., 1999) making necessary alternative augmentation strategies.

The dopaminergic system plays a key role in many physiological (e.g., cognition, reward) and pathological processes (schizophrenia, drug addition, etc.). Thus, abnormal dopamine (DA) function likely underlies certain depressive symptoms, such as cognitive dysfunction, lack of motivation and anhedonia (Goldman-Rakic, 1999; Goldman-Rakic et al., 2004). Indeed, an increased DA function may be beneficial for antidepressant action (D'Aquila et al., 2000; Lavergne and Jay, 2010). However, DA transporter (DAT) blockade may not be a convenient strategy due to the profound plastic changes in synaptic function (Jones et al., 1998) and the possibility of inducing tolerance or abuse (Kuhar et al., 1991; Sora et al., 2001; Woolverton and Johnson, 1992).

Here we explored the possibility to selectively enhance prefrontal cortex (PFC) DA function in order to evoke antidepressant-like effects using a noradrenergic drug combination. This strategy is based on the observation that a substantial fraction of extracellular DA in PFC derives from noradrenergic axons (Devoto and Flore, 2006; Devoto et al., 2001, 2005; Kawahara et al., 2001; Masana et al., 2011) being also sensitive to noradrenergic drugs (Masana et al., 2011). Thus, we examined the antidepressant properties of the combination of two marketed antidepressant drugs, reboxetine (NET inhibitor) and the non-selective α_2 -adrenoceptor antagonist mirtazapine.

2. Materials and Methods

2.1. Animals

Male Wistar rats (250–320 g, Iffa-Credo, Lyon, France) were maintained at 12h light/dark cycle, 22 ± 2 °C room temperature, and 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 (Spain).

2.2. Drugs and reagents

All HPLC reagents used were of analytical grade and obtained from Merck (Darmstadt, Germany). DA·hydrochloride, NE·bitartrate and 5-HT·oxalate were from Sigma/RBI (Madrid, Spain). Reboxetine·mesylate and mirtazapine were from TOCRIS (Avonmouth, UK). Citalopram·hydrobromide was generously donated by H. Lundbeck A/S (Copenhagen-Valby, Denmark). Drugs were dissolved in saline (pH adjusted to 6-7) in a final volume of 1-2 ml/kg for systemic administration. Doses used were: reboxetine 3 mg/kg i.p., mirtazapine 5 mg/kg s.c. and citalopram 3 mg/kg s.c. (expressed as free bases). In experiments involving the repeated administration of reboxetine, mirtazapine and citalopram, drugs were dissolved together and given i.p. to avoid an excessive number of injections and volume of vehicle.

2.3. *In vivo* microdialysis experiments

Microdialysis experiments were conducted as previously described (Masana et al., 2011). Briefly, concentric dialysis probes were implanted under pentobarbital anesthesia (60 mg/kg, i.p.) at the following brain coordinates (in mm, from bregma and duramater; Paxinos and Watson, 1998): mPFC, AP +3.2, L -0.8, DV -6.0, 4 -m membrane length; and NAc, AP +1.6, L -1.1, DV -8.0, 1.5-mm membrane length. Microdialysis experiments were performed 20-24 h after surgery in freely-moving rats, except in experiments involving repeated treatment (see section 3.2 in Results). Probes were connected to the perfusion pump delivering artificial cerebrospinal fluid at 1.5 μ l/min (see Masana et al., 2011 for details). Experiments detailed in section 3.1 involved dual implants (mPFC and NAc), to examine drug effects in both regions of the same animals.

Monoamine concentrations in dialysate samples were determined by HPLC with electrochemical detection (Hewlett Packard 1049, Palo Alto, CA, USA, +0.6 or +0.7 V) as described (Bortolozzi and Artigas, 2003; Masana et al., 2011).

Absolute detection limits were 1–3 fmol monoamine/sample. At the end of experiments, animals were killed by a pentobarbital overdose. Brains were quickly removed, frozen in dry ice and sectioned (40 μ m) with a cryostat (HM500-Om Microm, Walldorf, Germany). Coronal brain sections were stained with neutral red to verify the correct placement of probes. Only data from rats with probes correctly implanted in mPFC and NAc were used.

2.4. Behavioral experiments

2.4.1. Locomotor activity

Motor activity was measured using an Open-Field (OF) apparatus (35 x 35 x 40 cm) with black plastic walls dimly illuminated (50-60 lx). Locomotor activity was recorded during 1 h by a videocamera connected to a computer (Videotrack, View Point, Lyon, France) and automatically measured by the video-tracking software, as described (Scorza et al., 2010).

2.4.2. Forced swimming test (FST)

We used the modified rat FST (Cryan et al., 2002a, 2005). Rats were handled daily 1 week before test. In the pre-test session, rats were placed in a clear methacrylate cylinder (20 cm wide x 46 cm high) filled with water ($24\pm 1^{\circ}\text{C}$; 30 cm depth) for 15 min. Rats were returned to their home cages after being dried off, first with paper towel followed by 15 min into a cage with a heating pad. The test was conducted 24 h later in the same cylinder for 5 min and videotaped from above. Climbing, swimming and immobility behavior were scored every 5 s as previously defined (Detke et al., 1995). Time spent before the first immobility score (latency) was also measured (Espejo and Minano, 1999; Pliakas et al., 2001) and time-course representation was also used for graphic representation of individual activity patterns (Chandramohan et al., 2008). Drugs were injected 23.5, 5 and 1 h before test.

2.5. *In situ* hybridization experiments

The effects of reboxetine+mirtazapine administration were examined after acute (1 h before sacrifice) and repeated administration (23.5, 5 and 1 h before sacrifice, as in FST experiments). Controls received saline with the same time schedule. Rats were killed by decapitation with a guillotine. The brains were

rapidly removed, frozen on dry ice, and stored at -20°C. Brain tissue sections, 14 µm thick, were cut using a microtome-cryostat, thaw-mounted onto APTS (3-aminopropyltriethoxysilane, Sigma, St Louis, MO, USA)-coated slides and kept at -20°C until use. *c-fos* oligonucleotide probe was complementary to bases 131–178 (GenBank ID: NM 022197). BDNF oligonucleotide probe was complementary to bases 1188-1237 (GenBank ID: NM 007540). Labeling of the probes ([³³P]-dATP (>2500 Ci/mmol; DuPont-NEN, Boston, MA), tissue sectioning and *in situ* hybridization procedures were carried out as described (Kargieman et al., 2007; Santana et al., 2011). Hybridized sections were exposed to Biomax MR film (Kodak, Sigma-Aldrich, Madrid, Spain) for 7 (*c-fos*) or 2 days (BDNF) with intensifying screens. Relative optical densities (R.O.D.) were measured with the AIS computerized image analysis system (AIS, Imaging Research Inc.), also used to acquire pseudocolor images. Images were processed with Photoshop (Adobe Systems, Mountain View) and shown in the figures as acquired. Individual values of optical densities were calculated as the mean of 2-4 sections per rat (N=4 rats per treatment group).

2.6. Statistical analysis

Microdialysis results are expressed as fmol/30 µl fraction (uncorrected for recovery) or as a percentage of basal values. Area under the curve (AUC) of selected time periods was also used. Statistical analysis of neurochemical, behavioral and histological data was carried out using Student's *t*-test, one- or two-way ANOVA for repeated or independent measures, as appropriate,

followed by post-hoc Newman-Keuls test. Data are expressed as means $\pm$ SEM. Significance level was set at $p<0.05$.

3. Results

3.1. Effects of acute reboxetine+mirtazapine treatment on extracellular monoamine concentrations in mPFC and NAc

Baseline concentrations (fmol/fraction) of monoamines in dialysate samples from mPFC and NAc (dual probe experiments) were respectively: a) DA: 13 ± 2 (n=34) and 8 ± 1 (n=32); b) NE: 3.5 ± 0.2 (n=9) and 1.9 ± 0.4 (n=10); and c) 5-HT: 4.0 ± 1.0 (n=11) and 1.9 ± 0.3 (n=10).

The administration of vehicle, 3 mg/kg i.p. reboxetine, or 5 mg/kg s.c. mirtazapine alone did not significantly alter the DA output in mPFC (Fig. 1A,1B). However, the combined administration of reboxetine+mirtazapine evoked a marked and significant increase of DA in mPFC (264 ± 28 % of baseline; $F_{3,22}=9.47$, $p<0.001$), significantly different from the rest of groups (post-hoc Newman-Keuls test) (Fig. 1A). In contrast, neither drug (alone or in combination) significantly altered the DA output in NAc (Fig. 1B).

SSRI are used as first choice antidepressant treatments. Thus, we examined whether citalopram pre-treatment modified the effect of the reboxetine+mirtazapine combination, to potentially augment clinical SSRI effects. Citalopram pre-treatment (3 mg/kg s.c.) evoked a moderate, non-significant, increase of DA output in mPFC and did not alter the ability of reboxetine+mirtazapine to increase it (Fig. 1C). Two-way ANOVA of mPFC DA

revealed a significant effect of reboxetine+mirtazapine treatment ($F_{1,19}=25.36$, $p<0.0001$) but not citalopram pre-treatment or interaction effect. Neither treatment altered the DA output in NAc (Fig. 1D).

Figs. 1E, 1F and S1 show the change of the extracellular monoamine concentrations in mPFC and NAc, respectively, produced by the acute reboxetine+mirtazapine combination (AUC of 5 fractions, Fig. S1). The combined treatment evoked a very marked increase in DA and NE, but not 5-HT concentrations in mPFC (Fig. 1E). One-way ANOVA of mPFC data revealed a significant effect of the treatment for DA ($F_{1,13}=24.72$, $p<0.001$) and NE ($F_{1,7}=40.93$, $p<0.001$) output, but not for 5-HT output. In NAc, only NE was significantly increased by the reboxetine+mirtazapine combination ($F_{1,8}=15.85$, $p<0.01$) (Fig. 1F).

3.2. Effects of the repeated reboxetine+mirtazapine combination on extracellular monoamine concentrations in mPFC

Given the preferential effects of the reboxetine+mirtazapine combination on mPFC DA output, subsequent microdialysis experiments were carried out only in this region. Repeated administration of reboxetine+mirtazapine -but not of vehicle- significantly increased extracellular DA and NE, but not 5-HT, in mPFC (Fig. 2). Reboxetine+mirtazapine increased extracellular DA from 9 ± 2 to 23 ± 5 , 36 ± 3 and 35 ± 2 fmol/fraction (baseline, first, second and third administrations, respectively) (Fig. 2A). Repeated vehicle administration did not modify DA output in mPFC (10 ± 1 , 12 ± 2 , 12 ± 2 and 18 ± 4 fmol/fraction baseline, first, second and third administrations, respectively) (Fig. 2A). Two-way ANOVA

showed a significant effect of the treatment ($F_{1,6}=17.24$, $p<0.01$), number of injection ($F_{3,18}=24.76$, $p<0.0001$) and interaction between both factors ($F_{3,18}=12.46$, $p<0.001$).

The reboxetine+mirtazapine combination increased NE output from 5 ± 2 fmol/fraction to 28 ± 3 , 33 ± 3 and 30 ± 6 fmol/fraction (baseline, first, second and third administrations, respectively) (Fig. 2B). Repeated vehicle administration did not modify NE output in mPFC (4 ± 1 , 4 ± 1 , 3 ± 1 and 4 ± 1 fmol/fraction; baseline, first, second and third administrations, respectively). Two-way ANOVA showed a significant effect of the treatment ($F_{1,6}=34.34$, $p<0.01$), number of injections ($F_{3,18}=21.55$, $p<0.0001$) and interaction between both factors ($F_{3,18}=22.91$, $p<0.0001$).

5-HT output in mPFC (Fig. 2C) was unaltered by repeated reboxetine+mirtazapine administration (2.2 ± 0.2 , 2.1 ± 0.2 , 3.7 ± 1.7 and 2.3 ± 0.5 fmol/fraction; baseline, first, second and third administrations, respectively) or repeated vehicle administration (1.7 ± 0.2 , 1.6 ± 0.2 , 1.8 ± 0.3 and 1.9 ± 0.5 fmol/fraction; as above).

3.3. Antidepressant-like effect of reboxetine+mirtazapine in the forced swimming test (FST)

Fig. 3a shows the effects of the various treatments used on FST variables (total immobility score, swimming and climbing behaviors). At the dose used, reboxetine and mirtazapine alone did not significantly reduce FST variables. However, its combined administration evoked a synergistic 51% reduction in

immobility ($F_{3,30}=3.31$, $p<0.05$) with a non-significant increase in climbing or swimming scores.

The administration of 3 mg/kg i.p. citalopram did not reduce immobility. However, the subsequent treatment of the reboxetine+mirtazapine combination induced a marked decrease in immobility (46% of citalopram-treated rats, $p<0.01$; Student's *t*-test), and an increase in climbing (125% of citalopram-treated rats, $p<0.05$; Student's *t*-test)

We further examined the latency to immobility (Fig. 3B). This variable has been used as a measure of the time spent by rodents fighting against an aversive situation (Castagne et al., 2009; Espejo and Minano, 1999; Pliakas et al., 2001). The reboxetine+mirtazapine combination –but not either drug alone– induced a very marked increase of this variable (from 81 to 153 seconds; $F_{3,30}=7.62$, $p<0.001$). As observed with the reduced immobility time, the increased latency persisted in citalopram-pretreated rats (from 53 to 134 seconds; $p<0.05$, Student's *t*-test)

Fig. 3c shows the individual behavior of all rats used in FST experiments (Chandramohan et al., 2008). The behavior of each rat is depicted by a horizontal line during the 5-min observation period. Each 5-s color-coded pixel represents the predominant behavior of the rat (black = climbing; grey = swimming; white = immobility). Rats treated with reboxetine+mirtazapine, alone or in combination with citalopram, exhibited the most active behaviors (greater density of black/grey pixels).

The effects of the reboxetine+mirtazapine combination in the FST are not due to an increase in motor activity, since the combination treatment did not alter the

distance moved in the open-field nor the number of rearings, yet it moderately decreased grooming behavior (data not shown).

3.4. Effects of acute and repeated reboxetine + mirtazepine treatment on *c-fos* mRNA expression

c-fos expression was used to assess the brain areas involved in the antidepressant-like effects of the reboxetine+mirtazapine combination. Relative optical densities (R.O.D.) were measured in coronal brain sections (see section 2.5 in Methods) and results are expressed as percentages of vehicle-treated rats. Fig. 4A shows representative sections at various AP levels of vehicle- and reboxetine+mirtazapine-treated rats. Fig. 4B shows the effect of the single and repeated administration of reboxetine+mirtazapine on *c-fos* expression in representative brain areas. Single administration significantly increased *c-fos* expression in the entorhinal cortex and reduced it in mPFC and hypothalamus as shown by two-way ANOVA of R.O.D. values: effect of region (repeated measure; $F_{8,48}=11.73$, $p<0.001$) and region by treatment interaction ($F_{8,48}=11.73$, $p<0.001$; Fig. 4B, upper panel). Repeated reboxetine+mirtazapine administration significantly elevated *c-fos* expression in the entorhinal cortex and the dentate gyrus, and normalized *c-fos* expression in the mPFC and hypothalamus: effect of treatment ($F_{1,5}=7.34$, $p<0.05$), region (repeated measure, $F_{8,40}=2.94$, $p<0.05$) and region by treatment interaction ($F_{8,40}=2.94$, $p<0.05$) (Fig. 4B, lower panel).

3.4.1. Effects on BDNF and *c-fos* expression in the hippocampal formation

We carried out an additional study to examine the effect of the repeated reboxetine+mirtazapine combination on BDNF and *c-fos* expression in various hippocampal subfields at a more rostral AP coordinate (-2.8 mm; Fig. 5A). Two-way ANOVA indicated a significant increase of BDNF mRNA in CA1 and CA2 areas: region effect ($F_{3,18}=6.48$, $p<0.01$) and region by treatment interaction ($F_{3,18}=6.48$, $p<0.01$). *c-fos* expression was also increased in the dentate gyrus (two-way ANOVA, region effect ($F_{3,18}=7.41$, $p<0.01$) and region by treatment interaction effect ($F_{3,18}=4.96$, $p<0.05$)).

4. Discussion

We recently reported that the combination of the NET inhibitor reboxetine and the selective α_2 -adrenoceptor antagonist RX821002 (not available for human use) evoked a marked and selective increase of extracellular DA in PFC (Masana et al., 2011). Here we confirm and extend these previous observations using the non-selective α_2 -adrenoceptor antagonist mirtazapine, a marketed antidepressant drug. The neurochemical, behavioral and gene expression effects evoked by this antidepressant drug combination suggest its usefulness to treat PFC- and catecholamine-dependent depressive symptoms (e.g., cognitive impairment, lack of motivation, psychomotor retardation, etc.) Likewise, an enhanced DA function in PFC may be a useful way to treat cognitive deficits and negative symptoms in schizophrenic patients, which lack specific pharmacological treatments.

The present neurochemical data (Figs. 1, 2) agree with previous observations indicating that noradrenergic drugs can enhance DA transmission in PFC

(Carboni et al., 1990; Devoto et al. 2001, 2005; Devoto and Flore 2006; Masana et al., 2011; Mazei et al., 2002; Pozzi et al., 1994). In particular, a recent report from our group (Masana et al., 2011) showed that DA output in mPFC (but not in NAc), was enhanced by reboxetine (selective NE reuptake inhibitor) and nomifensine (dual NE/DA reuptake inhibitor), but not by the selective DA reuptake inhibitor GBR12909. Moreover, electric stimulation of the *locus coeruleus* enhanced extracellular DA in mPFC and not in NAc. In agreement with a major contribution of noradrenergic axons, extracellular DA in mPFC was sensitive to α_2 -adrenoceptor blockade with RX821002, as observed with the PFC NE output (Invernizzi and Garattini, 2004; Mateo et al., 1998; Ortega et al., 2010; Sacchetti et al., 1999).

After more than two decades of antidepressant drug development based on single or dual targets (e.g., SSRI, SNRI), current trends are focused on multi-target drugs that *i)* reduce the efficacy of negative feed-back mechanisms operating in monoaminergic neurons, and/or *ii)* increase the function of all monoamine systems (e.g., triple reuptake inhibitors). The first approach is based on drug combinations targeting several monoaminergic elements that overcome SSRI/SNRI limitations (Artigas et al., 1994, 1996; Blier et al., 2010; Carpenter et al., 2002; Chen and Skolnick, 2007; Lopez-Munoz et al., 2006; Millan, 2009; Papakostas et al., 2006; Trivedi et al., 2006). On the other hand, triple reuptake inhibitors increase extracellular concentrations of 5-HT, NE and DA, without the severe side effects of MAO inhibitors. These drugs appear to be superior in terms of shorter onset of action than conventional antidepressants (Chen and Skolnick, 2007; Millan, 2009). However, the blockade of DA transporter may involve tolerance or abuse problems (Haddad, 1999).

The present study shows the feasibility of selectively increasing cortical (but not mesolimbic) catecholaminergic transmission using marketed drugs. Hence, as proposed (Masana et al., 2011), NET blockade would increase the extracellular NE concentration, resulting in an enhanced activation of terminal (and possibly somatodendritic) α_2 -adrenoceptors, which would attenuate noradrenergic cell firing and NE release. Blockade of α_2 -adrenoceptors would counteract autoreceptor-mediated negative feedback, leading an enhanced NE release. DA, stored in the same synaptic vesicles as NE, would therefore be subjected to the same processes (Masana et al., 2011).

In contrast to the marked increase of extracellular DA and NE concentrations in the mPFC, the reboxetine+mirtazapine combination did not alter extracellular DA and only increased moderately extracellular NE in NAc. This effect may be due to the different regional NE innervation in rat brain and to the greater contribution of DA axons to extracellular DA in NAc. Hence, the *locus coeruleus* is the main NE source for noradrenergic PFC innervation whereas it has a low contribution in NAc, where the nucleus of the solitary tract is the main NE source (Berridge et al., 1997; Delfs et al., 1998; Foote et al., 1983; Seguela et al., 1990). On the other hand, the large density of DA fibers in NAc –compared to PFC– minimizes the noradrenergic contribution to extracellular DA in the NAc.

The marked increase in extracellular DA and NE in PFC produced by reboxetine+mirtazapine was accompanied by a substantial reduction of the immobility score in the FST. This effect most likely resulted from the synergistic interaction between both drugs. Although various neurotransmitters and receptors have been implicated in antidepressant response in the FST (Cryan et al., 2002b; Page et al., 1999, 2003; Reneric and Lucki, 1998), the parallel

changes in PFC DA/NE release and FST variables induced by the reboxetine+mirtazapine combination support the view that an increased cortical DA –and likely NE- function underlies the behavioral action of this drug combination. The neurochemical and behavioral effects of reboxetine+mirtazapine were unaffected by citalopram pretreatment, suggesting that this drug combination can be efficiently used as an augmentation strategy in patients treated with –but not responding to- SSRI.

Interestingly, the marked reduction of immobility produced by reboxetine+mirtazapine and its combination with citalopram was obtained with doses of both drugs that are ineffective when given alone, in agreement with data in the literature (Connor et al., 1999; Mague et al., 2003; Reneric et al., 2002). This further supports the above interpretation of the mechanism of action, likely resulting from a synergistic interaction between NET blockade and α_2 -adrenoceptor blockade. Furthermore, the present results agree with previous studies showing that the combination of desipramine (NET inhibitor) + idazoxan (α_2 -adrenoceptor antagonist) + fluoxetine (SERT inhibitor) produced a greater reduction of the immobility time, as well an increase of swimming and climbing behaviors (Reneric et al., 2001).

C-fos expression is widely used to label activated cells after physiological or pharmacological stimuli (Sagar et al., 1988). We used the expression of this early gene to examine the rat brain areas involved in the effects of the reboxetine+mirtazapine combination. Moderate changes in *c-fos* mRNA were observed. Notably, an increase in the entorhinal cortex which was accompanied by a simultaneous decrease in mPFC and hypothalamus after single reboxetine+mirtazapine administration and by an increase in the dentate gyrus

after repeated administration. However, *c-fos* expression remained unaltered in other brain areas such as nucleus accumbens or septum, which are affected by other antidepressant drugs (fluoxetine, imipramine, reboxetine) (Beck 1995; Torres et al., 1998; Svenningsson et al., 2007; Slattery et al., 2005; Miyata et al., 2005). The unchanged *c-fos* expression in the present study cannot be attributed to methodological reasons, since similar *in situ* hybridization procedures were able to detect *c-fos* changes in various brain regions after the administration of drugs that increase neuronal discharge (Kargieman et al., 2007; Lladó-Pelfort et al., 2011; Santana et al., 2011). More likely, regional differences in the effect of antidepressant drugs can be due to the different mechanism of action (i.e., SSRI vs. noradrenergic antidepressants) and, more specifically to the drug combination used herein, which simultaneously elevated PFC DA and NE function. Indeed, Slattery et al., (2005) reported an increased *c-fos* expression in the shell of NAc after single mirtazapine administration (2 mg/kg). Likewise, Miyata et al., (2005) reported an increase in *c-fos* immunoreactivity in the same region after single reboxetine administration (5 and 10 mg/kg). However, this is the first study examining the effects of the combination of both drugs.

The unaltered *c-fos* expression in mPFC after repeated reboxetine+mirtazapine administration suggests that the increase in mPFC catecholamine function is not sufficient to trigger the expression of this immediate early gene, which requires marked changes of neuronal discharge (Sagar *et al.*, 1988). In agreement, fluoxetine -which moderately increased the activity of PFC neurons (max. increase of 53%; Ceci et al., 1993)- did not alter *c-fos* expression in this area (Salchner and Singewald, 2002).

Interestingly, single and repeated administration of reboxetine+mirtazapine evoked a similar increase of *c-fos expression* in the entorhinal cortex, an effect possibly related to the antidepressant-like effects in the FST. Indeed, the entorhinal cortex is the main input and output structure for the hippocampal formation (van Groen et al., 2003), where antidepressant drugs increase the expression of neuroplasticity-associated genes, such as BDNF (Nibuya et al., 1995). Hence, the activation of the entorhinal cortex may be a previous step for the well-known antidepressant-induced change in hippocampal activity, as recently reported using high frequency stimulation of the entorhinal cortex (Stone et al., 2011). Indeed, here we found that the repeated (x3) administration of low doses of the reboxetine+mirtazapine combination evoked changes in hippocampal *c-fos* and BDNF, as observed after chronic SSRI treatments (Duman, 2004) or with a much higher reboxetine dose (20 mg/kg·day; Russo-Neustadt et al., 2004). Our observations also agree with a recent report indicating that α_2 -adrenoceptor blockade with yohimbine enhanced the effects of imipramine on neuroplasticity genes and behavioral measures of antidepressant effect (Yanpallewar et al., 2010).

In summary, we show that the combination of drugs targeting NET (e.g., reboxetine) and α_2 -adrenoceptors (e.g., mirtazapine) can be a useful antidepressant strategy to augment the effects of SSRI, by selectively increasing catecholaminergic neurotransmission in PFC. In particular, the increased DA function in PFC evoked by this drug combination may contribute to improve cognitive dysfunction and lack of motivation in depressed patients. Likewise, this may be also useful to treat cognitive deficits and negative

symptoms in schizophrenia patients, associated to a hypoactive dopaminergic function in PFC.

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Legends to Figures

Fig. 1. Effect of single reboxetine, mirtazapine and its combination treatment on extracellular monoamine concentration in mPFC (left) and NAc (right). Bars show the average effect of vehicle, reboxetine (Reb), mirtazapine (Mirt) and Reb+Mirt in (a) mPFC and (b) NAc. Citalopram (Cit) pretreatment did not modify the effects of Reb+Mirt on DA concentration in (c) mPFC nor (d) NAc. The Reb+Mirt combination increased extracellular NE concentration -but not that of 5-HT- in (e) mPFC and (f) NAc. Data are AUC of five 20-min fractions, expressed as percentage of baseline (see collection period in Fig. S1). N=4-8 rats/group; * $p < 0.05$ and ** $p < 0.01$ vs vehicle; $^{\zeta}p < 0.05$ vs reboxetine and mirtazapine; $^ap < 0.05$ treatment effect; n.s. non-significant.

Fig. 2. Effect of repeated reboxetine+mirtazapine combination treatment on extracellular monoamine concentration in mPFC (a) DA, (b) NE and (c) 5-HT. Bars show absolute monoamine values after administering the drug combination using the FST protocol. Data are AUC of four (baseline) and six (1st, 2nd and 3rd injection) 20-min fractions, expressed as fmol/fraction. N=4 rats/group. * $p < 0.05$ and ** $p < 0.01$ vs vehicle; $^{\#}p < 0.05$; $^{\#}p < 0.01$ vs the respective baseline and $^{\zeta\zeta}p < 0.01$ vs first injection for respective treatment.

Fig. 3. Drug effects on forced-swim test (FST) variables. Bar graphs showing the effect of treatments on (a) immobility, swimming and climbing scores and (b) latency to the first immobility count in the FST. (c) Individual raw data from FST

experiments. Each horizontal line corresponds to the behavior of a single rat. Time (abscissa) corresponds to the 5 min of FST. Each color-coded pixel represents 5 s of behavioral activity (black- climbing, grey-swimming, white-immobility). Vertical black line indicates mean latency to first immobility. N=7-10 rats/group; * $p < 0.05$ and ** $p < 0.01$ vs vehicle; # $p < 0.05$ and ## $p < 0.01$ vs citalopram; $^{\zeta\zeta}p < 0.001$ vs Reb and Mirt.

Fig. 4. Expression of *c-fos* mRNA after reboxetine+mirtazapine treatment as assessed by in situ hybridization. (a) Panels show coronal brain sections at the various AP coordinates from bregma (from left to right = 3.2, 1.6, -5.6, -7.8, -9.7 mm; Paxinos and Watson, 1988) of rats treated with vehicle or reboxetine+mirtazapine (Reb+Mirt) (single, upper panel; repeated, lower panel). Scale bar is 2 mm. (b) Bar graphs showing the change of *c-fos* mRNA relative optical density (R.O.D) in vehicle- and reboxetine+mirtazapine-treated rats (single, upper graph; repeated, lower graph). Data are percentage of the respective controls (N=4 rats/group). mPFC, medial prefrontal cortex; NAc, nucleus accumbens; Septum, lateral septum; Hypoth, dorsomedial hypothalamic nucleus, dorsal part; DG, dentate gyrus; Ent, entorhinal cortex; VTA SN, ventral tegmental area and substantia nigra; DR, dorsal raphe nucleus; LC, *locus coeruleus*. ** $p < 0.01$ and *** $p < 0.001$ vs vehicle.

Fig. 5. Representative coronal rat brain sections showing the expression of BDNF (left panels) and *c-fos* (right panels) mRNA at AP -2.8 mm (taken from bregma; Paxinos and Watson, 1988), as assessed by *in situ* hybridization. (a) Panels show the coronal brain sections of rats treated with vehicle (upper

panel) or reboxetine+mirtazapine (Reb+Mirt) (lower panel). Scale bar is 2 mm.

(b) Bars represent percentage of change of the BDNF (left) and *c-fos* (right)

mRNA relative optical density (R.O.D) compared to respective vehicle. N=4

rats/group. * $p < 0.05$ ** $p < 0.01$ and *** $p < 0.001$ vs respective vehicle.

Fig. S1. Microdialysis experiments showing the effect of reboxetine (Reb), mirtazapine (Mirt) and its combination (Reb+Mirt) on extracellular monoamines in rat mPFC. Points are mean $\pm$ SEM values of 4-8 rats/group. The box shows the fractions used to calculate AUC values in Fig. 1. *p<0.05 and **p<0.01 vs baseline.

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Highlights

- Include **3 to 5 highlights**.
- There should be a maximum of **85 characters**, including spaces, per highlight.
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Noradrenergic antidepressants selectively increase DA function in PFC but not in NAc

The combination of reboxetine and mirtazapine selectively increases DA and NE in mPFC

This evokes antidepressant-like effects (FST) and increases c-Fos and BDNF in HPC

Citalopram pretreatment does not interfere with the effects of these drugs.

Potentially useful in SSRI-resistant depression and negative symptom schizophrenia