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Corresponding author: Torgny H. Svensson

Neuropsychopharmacology

Reboxetine Enhances the Olanzapine-induced Antipsychotic-like Effect, Cortical Dopamine Outflow and NMDA Receptor-Mediated Transmission.

Running title: Reboxetine enhances the efficacy of olanzapine

Monica M. Marcus PhD, Kent Jardemark PhD, Anna Malmerfelt MSc, Carl Björkholm MSc and Torgny H. Svensson MD, PhD*

Department of Physiology and Pharmacology, Section of Neuropsychopharmacology, Karolinska Institutet, S-171 77 Stockholm, Sweden

*Corresponding author:

E-mail: Torgny.Svensson@ki.se

Phone: + 46 8 524 87921

Fax: + 46 8 30 84 24

ABSTRACT

Preclinical data have shown that addition of the selective norepinephrine transporter (NET) inhibitor reboxetine increases the antipsychotic-like effect of the D_{2/3} antagonist raclopride and, in parallel, enhances cortical dopamine output. Subsequent clinical results suggested that adding reboxetine to stable treatments with various antipsychotic drugs (APDs) may improve positive, negative and depressive symptoms in schizophrenia. Here we investigated in rats the effects of adding reboxetine to the second generation APD olanzapine on: *i*) antipsychotic efficacy, using the conditioned avoidance response (CAR) test, *ii*) extrapyramidal side effect (EPS) liability, using a catalepsy test, *iii*) dopamine efflux in the medial prefrontal cortex and the nucleus accumbens, using *in vivo* microdialysis in freely moving animals, and *iv*) cortical NMDA receptor-mediated transmission, using intracellular electrophysiological recording *in vitro*. Reboxetine (6 mg/kg) enhanced the suppression of CAR induced by a suboptimal dose (1.25 mg/kg), but not an optimal (2.5 mg/kg) dose of olanzapine without any concomitant catalepsy. Addition of reboxetine to the low dose of olanzapine also markedly increased cortical dopamine outflow and facilitated prefrontal NMDA receptor-mediated transmission. Our data propose that adjunctive treatment with a NET inhibitor may enhance the therapeutic effect of low dose olanzapine in schizophrenia without increasing EPS liability and add an antidepressant action, thus in principle allowing for a dose reduction of olanzapine with a concomitant reduction of dose-related side effects, such as EPS and weight gain.

KEY WORDS: noradrenaline transporter, antipsychotic, antidepressant, dopamine, glutamate, schizophrenia

INTRODUCTION

Previous studies have shown the critical importance of optimal dopamine transmission in the prefrontal cortex for certain aspects of cognition, such as working memory, and indicate that dysfunctional dopamine D₁ receptors may contribute to cognitive impairments in schizophrenia (see Abi-Dargham, *et al* 2002; Goldman-Rakic, *et al* 2004; Abi-Dargham and Moore 2003). Moreover, glutamate, the excitatory transmitter in cortical pyramidal cells, is involved in higher mental functions, such as cognition, memory and learning, and a glutamatergic synaptic hypofunction in schizophrenia seems indicated (see Coyle, *et al* 2003), a notion recently supported by the demonstration of a reduced prefrontal expression of the *N*-methyl-D-aspartate (NMDA) receptor subunits NR1, NR2A and NR2C in schizophrenic patients (Beneyto and Meador-Woodruff 2008). In addition, several other transmitter systems are in all probability involved in the control of cognitive functions (see Briand, *et al* 2007) as well as in the pathophysiology of schizophrenia, such as the catecholaminergic systems, since elevation of central noradrenergic and secondarily dopaminergic activity, e.g. by administration of α_2 -adrenoreceptor antagonists, has been shown to improve working memory, as well as attention, learning and memory in rodents (Lapiz and Morilak 2006; Sara and Devauges 1989).

Clozapine, the prototypical atypical antipsychotic drug (APD), has been found to be superior to first (FGAs) and other second generation APDs (SGAs) in treatment-resistant schizophrenia (see Swartz, *et al* 2008; Kane, *et al* 1988; Lewis, *et al* 2006; McEvoy, *et al* 2006; Taylor and Duncan-McConnell 2000) as well as in reducing suicidality in schizoaffective disorders and schizophrenia (see Hennen and Baldessarini 2005; Meltzer, *et al* 2003). Thus, the clinical efficacy of clozapine seems superior to both FGAs and most, if not all SGAs (Davis, *et al* 2003; Leucht, *et al* 2009). Unfortunately, the use of clozapine has been associated with a risk of

Corresponding author: Torgny H. Svensson

agranulocytosis, which limits its use. Besides its relatively low affinity for the dopamine D₂ receptor, clozapine possesses high affinity for a variety of other receptors, and several serotonergic receptors, e.g. 5-HT_{2A/C}, 5-HT_{1A}, 5-HT₆, as well as α_2 -adrenoceptors have been suggested to contribute to its superior efficacy (see e.g. Meltzer and Huang 2008; Svensson 2003; Marcus, *et al* 2005). The SGA olanzapine is structurally similar to clozapine and displays higher affinity for several serotonergic receptors than for the D₂ receptor (Schotte, *et al* 1996). However, whereas clozapine possesses a high affinity for α_2 -adrenoceptors, olanzapine has a low affinity for these receptors (Shahid, *et al* 2009). Both clozapine and olanzapine elevate cortical dopamine and norepinephrine outflow to a greater extent than FGAs such as haloperidol (Devoto and Flore 2006; Li, *et al* 1998; Moghaddam and Bunney 1990; Nomikos, *et al* 1994; Westerink, *et al* 1998). In contrast to FGAs, both clozapine and olanzapine also markedly facilitate cortical NMDA receptor-mediated transmission (Jardemark, *et al* 2002; Ninan, *et al* 2003), an effect that may contribute to improve negative symptoms and cognitive dysfunction in schizophrenia (see e.g. Stone, *et al* 2007).

The antidepressant drug reboxetine, a selective norepinephrine reuptake inhibitor (NRI), has been claimed to improve drive and motivation, as well as social and cognitive functioning in depressed patients (Ferguson, *et al* 2003; Kasper 1999; Montgomery 1997; Schatzberg 2000). A cognitive-enhancing effect of the selective NRI atomoxetine has also been observed in healthy volunteers (Chamberlain, *et al* 2006). In rodents, addition of reboxetine to the D_{2/3} antagonist raclopride has been shown to significantly enhance the antipsychotic-like effect of the FGA raclopride without increasing its extrapyramidal side effect (EPS) liability, as well as to markedly increase cortical dopamine output (Linner, *et al* 2002). Moreover, reboxetine has recently been found to enhance the effect of raclopride also on cortical NMDA receptor-mediated transmission

Corresponding author: Torgny H. Svensson

(Jardemark *et al*, unpublished observation). Thus, preclinical results propose that concomitant norepinephrine transporter (NET) inhibition may provide means to augment the efficacy of FGAs in schizophrenia

Here we have examined in rats whether concomitant NET inhibition by means of reboxetine might enhance the efficacy of olanzapine, and potentially mimic some of the preclinical effects of clozapine. The antipsychotic effect was assessed by using the conditioned avoidance response (CAR) paradigm, a preclinical test with high predictive validity for clinical antipsychotic effect (Arnt 1982; Wadenberg and Hicks 1999). EPS liability was examined by using a catalepsy test. Dopamine outflow in the medial prefrontal cortex (mPFC) and the nucleus accumbens (NAC) was measured by *in vivo* microdialysis in freely moving animals. Finally, the effects on cortical NMDA receptor-mediated transmission were examined using intracellular electrophysiological recording in pyramidal cells *in vitro*.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (~250 g upon arrival) were used for behavioral and microdialysis experiments, whereas male Sprague-Dawley rats (~70 g upon arrival; B&K Universal, Sollentuna, Sweden) were used for *in vitro* electrophysiological experiments. The animals were housed under standard laboratory conditions with a temperature of 21°C and relative humidity of 55-65%, with food (R34, Ewos, Södertälje, Sweden) and water *ad libitum*. For the behavioral tests, the animals were kept on a reversed 12/12 h light/dark cycle (lights off at 6:00 AM), whereas for the other experiments, animals were maintained on a 12/12 h light/dark cycle (lights on at 6:00 AM). The animals were acclimatized for at least 5 days prior to experiments. Experiments were approved by, and conducted in accordance with, the local Animal Ethics Committee, Stockholm North and the Karolinska Institutet, Sweden.

Conditioned Avoidance Response Behavior

The CAR methodology used has previously been described in detail (Linner et al 2002; see Wadenberg and Hicks 1999). In brief, rats were trained and tested in conventional shuttle boxes (530×250×225 mm), divided into two compartments of equal size by a partition with an opening (Salmi, *et al* 1994). Upon presentation of the 80 dB white noise (Lafayette Instruments, Lafayette, IN, USA) conditioned stimulus (CS), the rats had 10 s to move from one compartment of the shuttle box into the other. If the rat remained in the same compartment for more than 10 s, an intermittent electric shock (intershock interval 2.5 s, shock duration 0.5 s) of ~0.4 mA, i.e. the unconditioned stimulus (UCS), was presented to the grid floor until an escape was performed. If

Corresponding author: Torgny H. Svensson

the rat did not respond within 50 s, the trial was terminated, i.e. escape failure. The position of the rat was automatically transferred to the computer and avoidance (response to CS within 10 s), escape (response to CS + UCS), escape failure (failure to respond within 50 s) and intertrial crosses were recorded. The animals were trained for five days, each session consisted of ~20 trials randomly distributed over 15 min. Only animals reliably performing >85% avoidance were included in the study. Experiments were preceded by a pre-test and experiment sessions, lasting 10 min, were conducted at 20, 90 and 240 min after last injection. Experimental days were separated by at least two non-experimental days. The animals were tested in a counterbalanced change-over design serving as their own controls (Li, 1964).

Catalepsy Measurements

Catalepsy measurements were performed in a dimly lit room at 30, 90 and 120 min after drug administration (see e.g. Linner, *et al* 2002). The rat was placed on an inclined grid (60°) and allowed 30 s of adaptation before observations started. These were performed for a maximum of 2.5 min and the time until the rat initiated a movement by one of its paws was recorded. Catalepsy was scored from 0 to 5, according to the time (square root transformation) the rat remained immobile (min): 0 = 0.00-0.08; 1 = 0.09-0.35; 2 = 0.36-0.80; 3 = 0.81-1.42; 4 = 1.43-2.24; 5 \geq 2.25 (Ahlenius and Hillegaard, 1986). The different treatments were blinded to the observer.

***In Vivo* Microdialysis**

Our procedures for microdialysis have previously been described (Frånberg *et al*, 2008). In brief, rats were anesthetized with a cocktail of Hypnorm® (0.315 mg/ml fentanyl citrate and

Corresponding author: Torgny H. Svensson

10 mg/ml fluanisone; Janssen-Cilag Ltd, Saunderton, UK) and Dormicum® (5 mg/ml midazolam; Roche AB, Stockholm, Sweden) diluted in distilled water [1:1:2; 5 ml/kg, intraperitoneal (i.p.)] mounted in a stereotaxic frame, and implanted with dialysis probes in the mPFC or NAC [anteroposterior (mm): +3.2, +1.6; mediolateral: -0.6, -1.4; dorsoventral: -5.2, -8.2], respectively, relative to bregma and dural surface (Paxinos and Watson, 1998). Dialysis occurred through a semi-permeable membrane (Filtral AN69, Hospal Industrie, Meyzieu, France) with an active surface length of 4 mm (mPFC) or 2 mm (NAC). Dialysis experiments were conducted ~48 h after surgery in awake freely moving rats. The dialysis probe was perfused with a physiological perfusion solution (in mM: 147 NaCl, 3.0 KCl, 1.3 CaCl₂, 1.0 MgCl₂, 1.0 NaHPO₄, pH 7.4) at a rate of 2.5 µl/min, set by a microinfusion pump (Harvard Apparatus, Holliston, MA, USA). Dialysate samples were collected over 30 (mPFC) or 15 min (NAC). Online quantification of dopamine was accomplished by high performance liquid chromatography (HPLC) coupled to electrochemical detection (ESA Bioscience, Chelsford, MA, USA), with a detection limit of ~0.2 fmol/min. The injector (Valco Instruments, Houston, TX, USA) were directed by a computerized system, Totalcrom WS version 6.3 (Perkin Elmer, Wellesley, MA, USA). Separation of dopamine and metabolites was achieved by reversed phase liquid chromatography. The mobile phase consisted of 55 mM sodium acetate buffer (pH 4.0), 12% methanol and 0.55 mM octanesulfonic acid and delivered by an HPLC pump (Model 2150, Pharmacia LKB, Sweden) on a C-18 column (Nucleocil 150/75×4.6 mm, 5 µm), flow rate 0.8 ml/min. After separation, the analyte was passed through a guard cell with an applied oxidizing potential of 50 mV to reduce baseline. Samples were quantified by sequential oxidation and reduction in a high sensitive analytical cell (model 5011; ESA Bioscience) that was controlled by a potentiostat (Coulchem II model 5200; ESA Bioscience) with applied potentials of 400 mV and -200 mV for detection of metabolites and dopamine, respectively. Injection of drug was

Corresponding author: Torgny H. Svensson

performed after a stable outflow (<10% variation) of dopamine and metabolites. Baseline was calculated as the average of the last two (mPFC) or four (NAC) pre-injection values. The placement of the probe was later verified under microscope in slices stained with neutral red.

***In vitro* Electrophysiological Experiments**

Our procedures for electrophysiological experiments have previously been described (Konradsson, *et al* 2006). Briefly, the rats were decapitated while under halothane anesthesia (AstraZeneca AB, Södertälje, Sweden). The brains were quickly removed and cooled in ice-cold Ringer's solution (in mM: 126 NaCl, 2.5 KCl, 2.4 CaCl₂, 1.3 MgCl₂, 1.2 NaH₂PO₄, 10 D-glucose, 18 NaHCO₃, pH 7.4) aerated by 95% O₂ : 5% CO₂. The brains were cut coronally into 450 µm slices, using a Vibroslice (Campden model MA 752, World Precision Instruments, FL, USA), and kept submerged in aerated Ringer's solution at room temperature for >1 h to allow for recovery. A slice containing mPFC (approximately anterioposterior +3.2 mm from bregma) was transferred to a recording chamber (32°C), and held submerged between two nylon nets. The chamber was continuously perfused with aerated Ringer's solution, flow rate 1.5-2.5 ml/min. Electrodes were pulled from borosilicate glass capillaries (i.d. 0.58 mm; Clark Electromedical Instruments, Pangbourne, UK) by using a horizontal electrode puller (Model P-87, Sutter Instruments, San Rafael, CA, USA). Recording electrodes were filled with 2 M KAc (55-120 MΩ), and used for recording with an Axoclamp 2A amplifier (Molecular Devices, CA, USA). Penetrations of cells by sharp electrodes were performed blindly. The electrophysiological criteria for distinguishing presumed pyramidal from non-pyramidal neurons have been described previously (Arvanov, *et al* 1997; Connors and Gutnick 1990). It is very rare to impale fast-spiking interneurons with a relatively low resistance microelectrode (McCormick, *et al* 1985),

Corresponding author: Torgny H. Svensson

which might account for not recording fast-spiking non-pyramidal cells. The presumed pyramidal cells of the mPFC have relatively long spike duration (1–3 ms at half-maximum spike amplitude) and show a pronounced spike-frequency adaptation in response to constant-current depolarization pulses. Single electrode voltage-clamp (holding potential -60 mV) was performed in the discontinuous mode with a sampling rate of 5-6.2 kHz. The voltage-clamp recordings were acquired using digital/analogue sampling and acquisition software (Clampex version 9.2, Molecular Devices, CA, USA). During the voltage-clamp recordings of NMDA (10-15 μ M)-evoked currents, tetrodotoxin (0.5 μ M, to block the action potentials), glycine (1 μ M, to enhance the NMDA-induced responses), and bicuculline (5 μ M, to block the GABA_A responses) were included in the Ringer's solution. All drugs used were diluted in Ringer's solution and administered via bath perfusion. The amplitude of the NMDA-induced current after drug or drug combinations was divided by the amplitude of the control NMDA-induced current for calculation of drug effect on prefrontal NMDA transmission.

Statistical Analysis

The data from the behavioral experiments are not normally distributed and accordingly non-parametric tests were used. Thus, for the CAR data, i.e. avoidance behavior and intertrial crosses, we used the Friedman two-way analysis of variance (ANOVA) followed by Wilcoxon matched-pairs signed-ranks test, and for the catalepsy data the Kruskal-Wallis one-way ANOVA followed by Mann-Whitney U-test was employed. Statistical evaluation of microdialysis data over time was performed by means of a two-way (treatment \times time) ANOVA for repeated measures. To analyze difference between different treatments, we also measured the overall effects (AUC= area under curve), i.e. interval 60-240 min for mPFC and 45-240 min for NAC. The overall effects were statistically evaluated by one-way ANOVA, followed by planned

Corresponding author: Torgny H. Svensson

comparisons test. One-way ANOVA was also used to detect differences between baseline values. Statistical evaluation of the electrophysiology experiments were performed by paired t-test, and, for multiple group comparisons, one-way ANOVA followed by the post hoc Tukey HSD test. In all statistical measures $p < 0.05$ was considered significant. The statistical evaluations were performed by using Statistica version 8.0 (StatSoft Inc., USA).

Drugs

For *in vivo* experiments olanzapine was dissolved in a minimal amount of acetic acid with a 5.5% glucose solution added to volume and reboxetine was dissolved in saline (0.9% NaCl) and administered by i.p. injections. Bicuculline methiodide, glycine and NMDA were purchased from Sigma-Aldrich, St. Louis, USA and tetrodotoxin from Tocris, Bristol, UK. For the electrophysiological experiments, stock solutions of olanzapine and reboxetine (dissolved in dimethyl sulfoxide) were prepared.

RESULTS

Effects of Reboxetine, Olanzapine and the Combination of Reboxetine and Olanzapine on CAR Behavior

The overall effect showed a statistical significant suppression of CAR at 20 min [χ^2 (5)= 28.71, $p<0.001$, Fig 1A] and 90 min [χ^2 (5)= 27.24, $p<0.001$, Fig 1B, $n= 10$], whereas at 240 min all animals were back to baseline performance. Compared to control, i.e. saline + vehicle, olanzapine 1.25 mg/kg produced a small but significant suppression of CAR at 90 min ($p<0.01$), and olanzapine 2.5 mg/kg at both 20 and 90 min ($p<0.01$). Reboxetine 6 mg/kg had no effect on CAR behavior when given alone. However, the same dose of reboxetine enhanced the effect of a low dose of olanzapine 1.25 mg/kg (20 min; $p<0.05$), but not a high dose of olanzapine 2.5 mg/kg.

The drug-induced suppression of CAR was accompanied by a concomitant decrease in intertrial crosses. There was a statistical significant overall effect for the intertrial crosses at 20 min [χ^2 (5)= 11.95, $p<0.05$] and 90 min [χ^2 (5)= 13.25, $p<0.05$, $n= 10$, Table 1]. At 20 min there was a statistically significant decrease in intertrial crosses after saline + olanzapine 1.25 mg/kg, reboxetine + olanzapine 1.25 mg/kg and reboxetine + olanzapine 2.5 mg/kg, whereas at 90 min only reboxetine + olanzapine 2.5 mg/kg resulted in a statistically significant decrease. No escape failures were recorded at any time point for any of the treatments.

Effects of Reboxetine, Olanzapine and the Combination of Reboxetine and Olanzapine in the Catalepsy Test

Reboxetine (6 mg/kg), olanzapine (1.25 and 2.5 mg/kg) and the combination of reboxetine and olanzapine were tested in the catalepsy test [30 min: $H(5)= 10.15$, $p= 0.071$; 60 min: $H(5)= 11.35$, $p<0.05$; 120 min: $H(5)= 3.91$, $p= 0.56$; Table 2]. Compared to control, i.e. saline + vehicle, reboxetine 6 mg/kg ($p<0.001$) as well as olanzapine 2.5 mg/kg ($p<0.01$) reached statistically significant level at 60 min (Fig 2). However, the median catalepsy scores were all below 2, and in accordance with previous studies an animal was considered cataleptic only with a score greater than or equal to 2 (Wadenberg, *et al* 2001).

Effects of Reboxetine, Olanzapine and the Combination of Reboxetine and Olanzapine on Dopamine Output in the mPFC and the NAC

The mean \pm SEM basal extracellular dopamine levels in the mPFC and NAC were 0.32 ± 0.03 fmol/min ($n= 26$) and 2.62 ± 0.22 fmol/min ($n= 23$), respectively. No statistically significant differences between mean baseline concentrations of dopamine output were found between different treatments group within the same brain region studied. Control injections, i.e. saline and vehicle, had no effect on dopamine output in any of the regions investigated.

In the mPFC, the maximal dopamine increase for reboxetine 6 mg/kg was 320% (at 60 min), for olanzapine 1.25 mg/kg 102% (at 60 min), for the combination of reboxetine 6 mg/kg and olanzapine 1.25 mg/kg 773% (at 60 min) and for the control group (saline + vehicle injections) 16% (at 60 min). Statistical evaluation for dopamine output in the mPFC (Fig 3A) revealed a significant treatment ($F_{3,22}=13.89$, $p<0.001$), time ($F_{8,176}=30.47$, $p<0.001$) as well as interaction (treatment x time) effect ($F_{24,176}=11.02$, $p<0.001$). In order to assess the effects of the two drugs in combination, we analyzed the overall effect following injection of the second drug, AUC (60–240 min). The overall effect was statistically significant ($F_{3,22}=14.21$, $p<0.001$; Fig

3B). Compared to control, i.e. saline + vehicle, reboxetine as well as the combination of reboxetine and olanzapine were significantly higher ($p < 0.01$ – 0.001). The combination of reboxetine and olanzapine was statistically higher than both reboxetine ($p < 0.01$) and olanzapine ($p < 0.001$), when given alone.

In the NAC, the maximal dopamine increase for reboxetine 6 mg/kg was 30% (at 225 min), for olanzapine 1.25 mg/kg 172% (at 60 min), for the combination of reboxetine 6 mg/kg and olanzapine 1.25 mg/kg 202% (at 60 min) and for the control group (saline + vehicle injections) 18% (at 30 min). Statistical evaluation for dopamine output in the NAC (Fig 3C) revealed a significant treatment ($F_{3,19}=13.24$, $p < 0.001$), time ($F_{16,304}=5.69$, $p < 0.001$) as well as interaction (treatment x time) effect ($F_{48,304}=2.33$, $p < 0.001$). In order to assess the effects of the two drugs in combination, we analyzed the overall effect following injection of the second drug, AUC (45–240 min). The overall effect was statistically significant ($F_{3,19}=13.37$, $p < 0.001$; Fig 3D). Compared to control, i.e. saline + vehicle, olanzapine as well as the combination of reboxetine and olanzapine were significantly higher ($p < 0.01$ – 0.001). The combination of reboxetine and olanzapine was statistically higher than reboxetine ($p < 0.001$), when given alone.

Effects of Reboxetine, Olanzapine and the Combination of Reboxetine and Olanzapine on Cortical NMDA-Induced Currents

Intracellular voltage clamp was used to record NMDA-induced currents in pyramidal cells of layer V and VI in the prelimbic region of the rat mPFC. The characterization of pyramidal cells was performed according to criteria previously described (see above Arvanov, *et al* 1997; Konradsson, *et al* 2006).

The concentration-response curve for the effects of olanzapine as well as other atypical APDs, on the NMDA-induced currents recorded has been shown to display an inverted U-shaped curve and the maximal effect of olanzapine were observed at 5nM (see Ninan, *et al* 2003). Here we examined a potential enhancement by reboxetine of a sub-maximal concentration of olanzapine, i.e. 3 nM. First, reboxetine in various concentrations (5, 20, 200 and 1000 nM) was tested alone, and was generally found to produce very small effects on the NMDA-induced currents regardless of concentrations (data not shown). Plasma concentration in patients treated with reboxetine has been shown to be 660 ± 346 nM (mean \pm SD; Öhman *et al*, 2001). Reboxetine displays an extensive (>97%) binding to plasma proteins (Fleishaker 2000) and, since the amount of drug present in cerebrospinal fluid (CSF) tends to be equivalent at steady-state to the non-protein-bound drug concentration in plasma, 20 nM was chosen as a clinically relevant concentration.

There was a small but significant facilitation of the NMDA-induced currents by 20 nM reboxetine [117.7 ± 4.2 (mean \pm SEM), n=7, t(6)=4.18, p<0.01, Fig 4], whereas 3 nM olanzapine did not reach statistical significance [118.2 ± 7.3 , n=5, t(4)=2.49, p=0.07]. The facilitating effect on NMDA-induced currents by the combination of reboxetine 20 nM and olanzapine 3 nM was highly significant [170.0 ± 6.0 , n=5, t(4)=11.62, p<0.001]. Group comparison [F(treatment: 2,14)=26.3, p<0.001] revealed statistical significant differences between reboxetine 20 nM and the combination of reboxetine 20 nM and olanzapine 3 nM (p<0.001) and between olanzapine 3 nM and the combination of reboxetine 20 nM and olanzapine 3 nM (p<0.001).

DISCUSSION

Our results demonstrate that the selective NET inhibitor reboxetine may significantly enhance the antipsychotic-like effect of a low, but not a high, dose of olanzapine, without inducing catalepsy. In principle, these data suggest the possibility of a dose reduction of olanzapine with maintained antipsychotic effect. The olanzapine-induced dopamine output in the mPFC, but not in the NAC, was significantly increased by reboxetine. Finally, reboxetine was also shown to potentiate the effect of a low concentration of olanzapine on prefrontal glutamatergic NMDA receptor-mediated transmission. The preferential enhancement of cortical dopamine and NMDA receptor-mediated transmission propose that addition of reboxetine to a sub-maximal dose of olanzapine may not only enhance the antipsychotic effect, but also ameliorate negative and depressive symptoms and cognitive impairment in schizophrenia (c.f. Introduction).

Reboxetine has previously been found to enhance the antipsychotic-like effect and cortical dopamine output of a low dose of the selective $D_{2/3}$ antagonist raclopride, which by itself did not produce a sufficient antipsychotic effect (Linner, *et al* 2002). Here, we found that the same dose of reboxetine also enhanced the antipsychotic-like effect and cortical dopamine output induced by a low dose of olanzapine, which also when given alone was far from producing a sufficient antipsychotic-like effect in the CAR model. The low doses of both raclopride and olanzapine used in these experiments produce approximately 60-65% occupancy of the D_2 receptor (Marcus, *et al* 2005; Wadenberg, *et al* 2001). The doses needed for sufficient antipsychotic effect in the CAR model of both raclopride and olanzapine, when given alone, require approximately 75% D_2 receptor occupancy (Wadenberg, *et al* 2000, 2001). In

Corresponding author: Torgny H. Svensson

schizophrenic patients treated with clozapine, the striatal dopamine receptor occupancy is rather low, i.e. approximately 45% (Kessler, *et al* 2006; Nordström, *et al* 1995), indicating that also other receptors than D₂ receptors contribute to its efficacy (see Svensson 2003). In schizophrenic patients treated with FGAs the D₂ receptor occupancy is approximately 70-80% (Farde, *et al* 1988; Nordström, *et al* 1995), and a similar level of D₂ receptor occupancy is also seen in patients treated with olanzapine (Tauscher, *et al* 1999; Zipursky, *et al* 2005). At high D₂ receptor occupancy levels the risk of adverse side-effects such as EPS and prolactin elevation are increased (Kapur, *et al* 2000). In addition, a high degree of D₂ blockade *per se* may generate detrimental effects on both cognition and mood in healthy volunteers (Saeedi, *et al* 2006) as well as in schizophrenic patients (Carpenter Jr 1996). Therefore, a lower D₂ receptor occupancy, which may be achieved by reducing the dose of olanzapine, may in principle even contribute to ameliorate cognitive and negative symptoms. The present data thus indicate that this goal may be attainable by adjunctive reboxetine treatment.

In similarity with reboxetine, the α_2 -adrenoceptor antagonist idazoxan has been shown to enhance the antipsychotic-like effects of raclopride and olanzapine, as well as haloperidol and risperidone, without inducing catalepsy. In addition, the prefrontal dopamine output induced by these APDs was markedly enhanced by idazoxan (Hertel, *et al* 1999; Marcus, *et al* 2009; Wadenberg, *et al* 2007). Furthermore, to a similar magnitude as that of clozapine, idazoxan enhanced the effects of both raclopride and risperidone on prefrontal NMDA-receptor mediated transmission, an effect that has been shown to be dopamine-dependent and executed via the dopamine D₁ receptor (Chen and Yang 2002; Marcus, *et al* 2005, 2009; Ninan and Wang 2003). The increased prefrontal availability of catecholamines induced by adjunctive treatment with

idazoxan is probably due to a co-release of dopamine and norepinephrine that is controlled by α_2 -adrenoceptors located on norepinephrine nerve terminals (Devoto, *et al* 2001).

NRIs have, in depressed patients, been suggested to improve drive and motivation as well as cognitive impairment due to increased availability of cortical catecholamines (see Introduction). The increased availability of dopamine in the prefrontal cortex seen after administration of a selective NRI may largely be explained by the fact that dopamine has a high affinity for the NET, which thus contributes to clearance of dopamine from the extracellular space in the cortex (Carboni, *et al* 1990; Pozzi, *et al* 1994). Thus, following administration of a NET inhibitor, both norepinephrine and dopamine extracellular levels increase. The increased availability of dopamine in the prefrontal cortex may not only contribute to improve the effect on cognitive symptoms, but can probably also, in the presence of a D_2 antagonist, contribute to enhance the antipsychotic effect *per se*. Previous clinical studies show that low doses of L-dopa used as adjunct treatment to conventional APDs may enhance their therapeutic effect in schizophrenic patients (see Jaskiw and Popli 2004), and experimental data reveal that adding low doses of L-dopa to raclopride not only caused a preferentially increased prefrontal cortical dopamine output, but also potentiated the antipsychotic-like effect of the $D_{2/3}$ antagonist (Eltayb, *et al* 2005). Therefore, addition of drugs that are able to increase the availability of cortical monoamines, e.g. reboxetine and idazoxan, to APDs may both enhance the antipsychotic effect as well as improve negative or depressive symptoms and certain aspects of cognitive impairment.

Clinical studies using adjunctive treatment with NET inhibitors to APDs have show inconsistent results. Whereas reboxetine when added to a stable treatment with APDs, including both FGAs and SGAs, it was found to improve both positive and negative as well as depressive symptoms in schizophrenic patients (Raedler, *et al* 2004), whereas reboxetine as add-on treatment

to haloperidol did not cause any significant improvement (Schutz and Berk 2001). In addition, adjunctive treatment with the NET inhibitor atomoxetine to schizophrenic patients treated with SGAs was found to activate certain brain areas related to working memory (Friedman, *et al* 2008). Interestingly, a recent study found no improvement of processing speed and accuracy by co-administration of reboxetine with olanzapine in schizophrenic patients (Poyurovsky, *et al* 2009), but the authors concluded that a more comprehensive test battery, including tasks such as working memory, verbal memory and social cognition, might have been more useful. Needless to say, these clinical results are obtained from small pilot studies in patients maintained on effective doses of various APDs. However, the present study suggests that the potential benefits of adjunctive NET inhibition would be obtained at reduced dosage of olanzapine rather than at standard dose levels. Significantly, quetiapine, with a structure similar to clozapine and olanzapine, has an active metabolite, nor-quetiapine, which is a potent NET inhibitor that has been identified and visualized in the primate brain (Nyberg, *et al* 2007). Thus, the recently demonstrated antidepressant effect of quetiapine (Bandelow, *et al* 2009; Bauer, *et al* 2009; Cutler, *et al* 2009) as well as its utility in bipolar depression may be related to concomitant NET inhibition in combination with a low D₂ occupancy (Kessler, *et al* 2006). Finally, clinical data indicate that adding a NET inhibitor such as reboxetine to olanzapine in the treatment of schizophrenia attenuates the olanzapine-induced weight gain (Poyurovsky, *et al* 2003, 2007).

Consequently, further studies on NET inhibition as add-on treatment to APDs in the treatment of schizophrenia seem warranted. Specifically, the present data propose that the addition of a NET inhibitor to olanzapine treatment might allow for a dose reduction, yet with maintained and even broadened therapeutic effects and a concomitant reduction of dose-related side effects of olanzapine, such as EPS and weight gain. To our knowledge no clinical study

Corresponding author: Torgny H. Svensson

employing this approach has as yet been published.

Corresponding author: Torgny H. Svensson

DISCLOSURE/ CONFLICT OF INTEREST

The authors declare that there is no conflict of interest. Monica M. Marcus, Anna Malmerfelt and Torgny H. Svensson are employees at the Karolinska Institutet, Carl Björkholm is a PhD student at the Karolinska Institutet. Kent Jardemark is employee at the Karolinska Institutet and Pronexus Analytical AB.

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Reboxetine is widely used in over 60 countries, however the FDA has declined the approval of reboxetine in the US.

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Corresponding author: Torgny H. Svensson

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TABLES

Table 1

Effects of reboxetine (6 mg/kg i.p.) and olanzapine (1.25, 2.5 mg/kg i.p.) on intertrial crosses in rats.

Drugs (mg/kg)	pretest	20 min	90 min	240 min
saline + vehicle	16.0 ± 4.5	13.0 ± 4.0	7.0 ± 4.5	12.5 ± 7.0
reboxetine (6) + vehicle	17.5 ± 9.0	7.5 ± 5.5	6.0 ± 4.0	8.0 ± 5.0
saline + olanzapine (1.25)	19.5 ± 8.5	7.0 ± 3.5*	6.0 ± 2.0	7.5 ± 4.0
reboxetine (6) + olanzapine (1.25)	17.0 ± 7.5	5.0 ± 5.5*	6.5 ± 6.5	8.0 ± 4.0
saline + olanzapine (2.5)	16.0 ± 2.5	5.0 ± 5.5	2.0 ± 2.0	6.0 ± 3.5
reboxetine (6) + olanzapine (2.5)	12.5 ± 6.0	3.0 ± 5.5*	2.5 ± 2.0**#	3.5 ± 3.5

The results are shown as medians ± semi-interquartile range. Animals (n=10) are serving as their own control in a change-over design (Li, 1964). * p<0.05, ** p<0.05 vs. saline + vehicle; # p<0.05 vs. reboxetine + vehicle.

Table 2

Effects of reboxetine (6 mg/kg i.p.) and olanzapine (1.25, 2.5 mg/kg i.p.) on catalepsy in rats.

Drugs (mg/kg)		Score at 30 min	Score at 60 min	Score at 120 min
saline	+ vehicle	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.25
reboxetine (6)	+ vehicle	0.5 ± 0.5	1.0 ± 0.0	0.5 ± 1.0
saline	+ olanzapine (1.25)	0.0 ± 0.0	0.0 ± 0.25	1.0 ± 0.5
reboxetine (6)	+ olanzapine (1.25)	0.0 ± 0.0	0.5 ± 0.75	1.0 ± 1.0
saline	+ olanzapine (2.5)	0.0 ± 0.0	1.0 ± 0.5	0.5 ± 0.75
reboxetine (6)	+ olanzapine (2.5)	0.0 ± 0.0	0.5 ± 0.75	1.0 ± 0.5

The results are shown as medians ± semi-interquartile range based on observations of eight animals per treatment group.

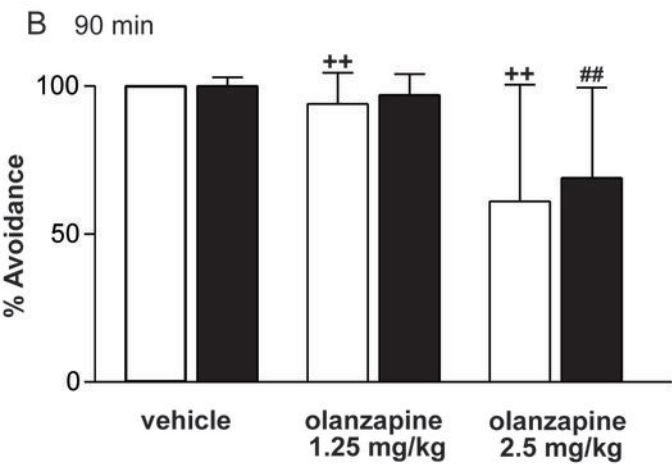
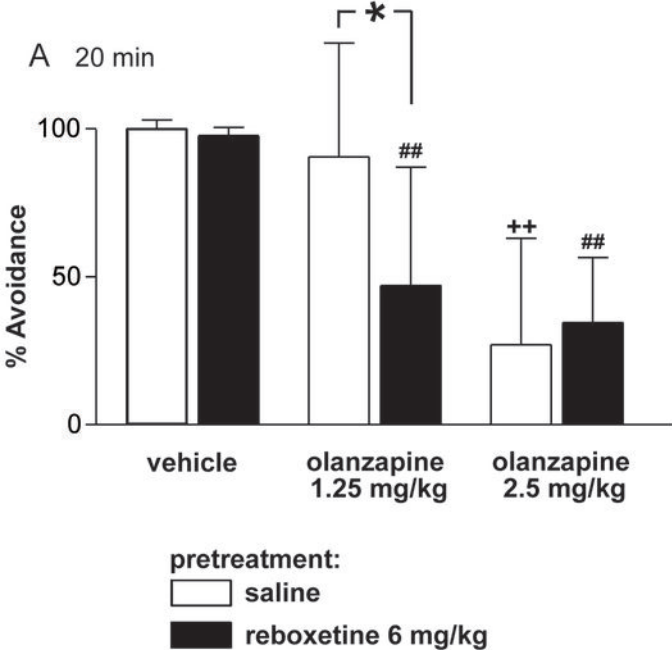
FIGURE LEGENDS

Figure 1 Reboxetine enhances the effect of a low, but not a high, dose of olanzapine on suppression of CAR. Effects on CAR behavior in rats at (A) 20 min and (B) 90 min after administration of vehicle, olanzapine 1.25 or olanzapine 2.5 mg/kg i.p. in combination with saline or reboxetine 6 mg/kg s.c. (reboxetine/saline was administered 30 min before vehicle/olanzapine). The results are presented as median (% avoidance) \pm semi-interquartile range. Animals (n=10) are serving as their own control in a change-over design (Li, 1964). ++p<0.01 vs. saline + vehicle, ##p<0.01 vs. reboxetine + vehicle, *p<0.05 saline + olanzapine vs. reboxetine + olanzapine.

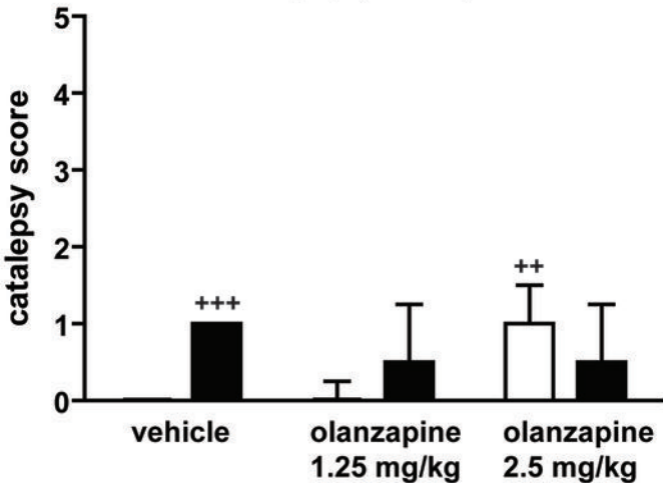
Figure 2 All treatments tested show very low propensity to induce catalepsy. Each bar represents the median catalepsy score \pm semi-interquartile range, measured at 60 min, based on observations of eight animals per treatment group. ++ p<0.01, +++ p<0.001 vs. saline + vehicle.

Figure 3 Reboxetine enhances the olanzapine-induced dopamine output in the mPFC, but not in the NAC. The effects of olanzapine 1.25 mg/kg i.p. administration on dopamine output in the mPFC (A-B) and NAC (C-D) respectively, in rats pretreated with saline or reboxetine 6 mg/kg i.p.). Fig A and C show the effects on dopamine output over time in mPFC and NAC, respectively. Arrows indicate time of reboxetine/saline and olanzapine/vehicle injections. *p<0.05, **p<0.01, ***p<0.001 compared to baseline. Fig B and D show the overall dopamine output (AUC), measured for 60-240 min (mPFC) and 45-240 min (NAC). The dotted line represents the baseline values (100%). The results are presented as mean \pm SEM. ++p<0.01, +++p<0.001 compared to control group (saline/vehicle); **p<0.01, ***p<0.001 between different treatments.

Figure 4 Reboxetine enhances the effect of a sub-maximal concentration of olanzapine on NMDA-induced currents in pyramidal cells. Bar representing the effects of reboxetine 20 nM, olanzapine 3 nM and the combination of reboxetine 20 nM and olanzapine 3 nM on NMDA induced currents in pyramidal cells of the mPFC. The results are presented as mean \pm SEM. ++p<0.05, +++p<0.001 compared to baseline, ***p<0.001 between different treatments.



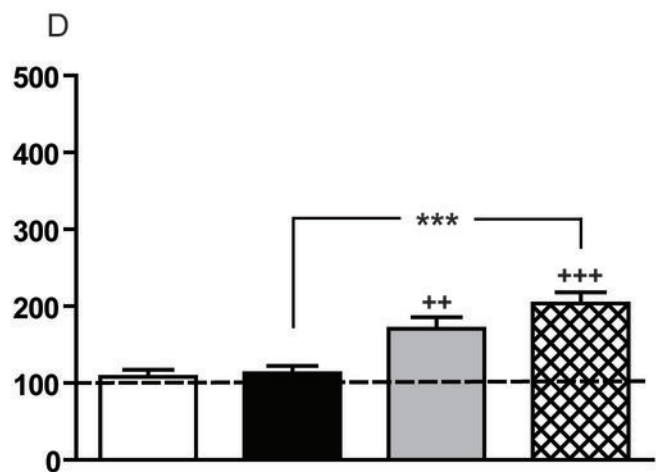
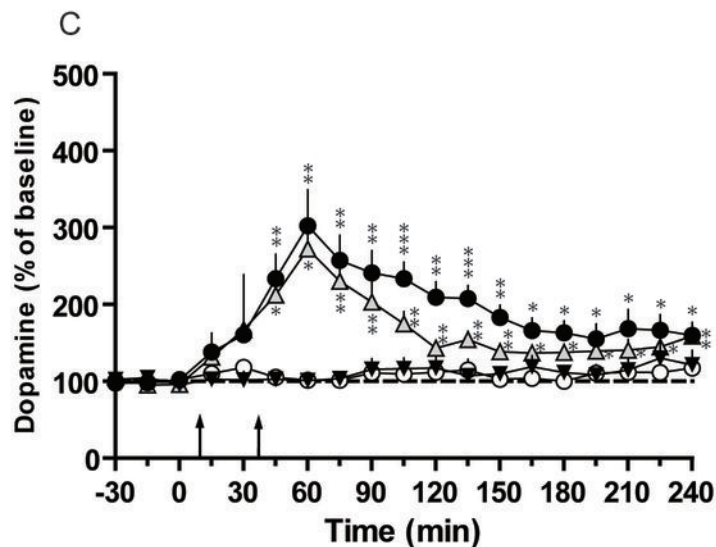
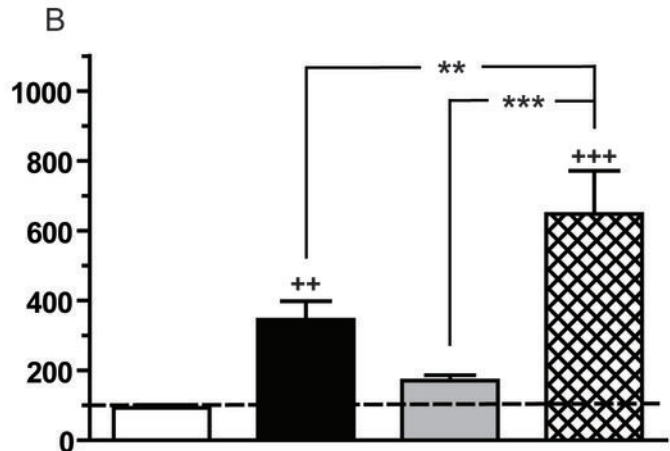
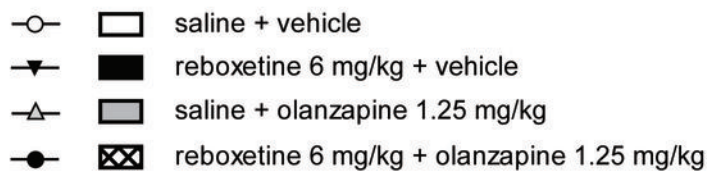
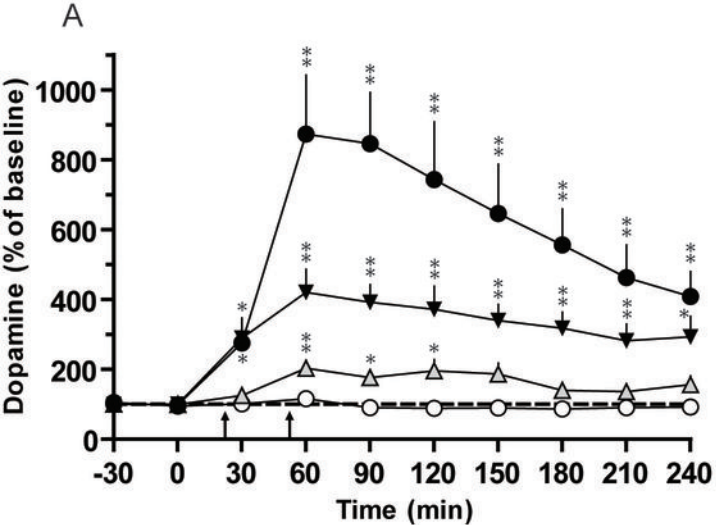
Catalepsy (60 min)



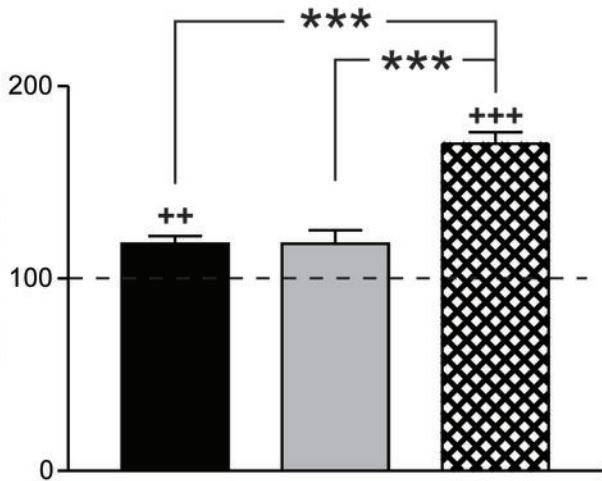
pretreatment:

□ saline

■ reboxetine 6 mg/kg



NMDA induced current
(% of control)



- reboxetine 20 nM
- olanzapine 3 nM
- reboxetine 20 nM + olanzapine 3 nM