Reboxetine: Functional Inhibition of Monoamine Transporters and Nicotinic Acetylcholine Receptors

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ABSTRACT

The present study determined whether repeated administration of the antidepressant and selective norepinephrine (NE) uptake inhibitor reboxetine resulted in an adaptive modification of the function of the NE transporters (NETs), serotonin (5-HT) transporters, or dopamine (DA) transporters. Because antidepressants may be effective tobacco smoking cessation agents and because antidepressants have recently been shown to interact with nicotinic acetylcholine receptors (nAChRs), the interaction of reboxetine with nAChRs was also evaluated. Repeated administration of reboxetine (10 mg/kg i.p., twice daily for 14 days) did not alter the potency or selectivity of reboxetine inhibition of \(^{[3]}\)HNE, \(^{[3]}\)HD\(A\), or \(^{[3]}\)H5-HT uptake into striatal or hippocampal synaptosomes (IC\(_{50}\) values = 8.5 nM, 89 \(\mu\)M, and 6.9 \(\mu\)M, respectively). In a separate series of experiments, reboxetine did not inhibit (\(K_i > 1 \mu\)M) \(^{[3]}\)Hmethyllycaconitine, \(^{[3]}\)Hcytisine, or \(^{[3]}\)Hepibatidine binding to rat whole brain membranes. However, at concentrations that did not exhibit intrinsic activity, reboxetine potently inhibited (IC\(_{50}\) value = 7.29 nM) nicotine-evoked \(^{[3]}\)HNE overflow from superfused hippocampal slices via a noncompetitive mechanism. In the latter experiments, the involvement of NET was eliminated by inclusion of desipramine (10 \(\mu\)M) in the superfusion buffer. Reboxetine also inhibited (IC\(_{50}\) value = 650 nM) nicotine-evoked \(^{86}\)Rb\(^+\) efflux at reboxetine concentrations that did not exhibit intrinsic activity in this assay. Thus, in addition to inhibition of NET function, reboxetine inhibits nAChR function, suggesting that it may have potential as a smoking cessation agent.

The efficacy of reboxetine, a well tolerated antidepressant in clinical use in Europe (Berzewski et al., 1997; Montgomery, 1997), has been linked to inhibition of neurotransmitter uptake, specifically at the norepinephrine (NE) transporter (NET). Reboxetine is a highly selective inhibitor of NET, relative to the serotonin transporter (SERT) and the dopamine transporter (DAT; Montgomery, 1997; Wong et al., 2000). Like other antidepressants, reboxetine displays a characteristic delay in onset of action (Versiani et al., 1999), due to prerequisite neural adaptations (Harkin et al., 2000; Invernizzi et al., 2001). Adaptive changes in transporter function seem to be dependent on the specific antidepressant administered and transporter affected. For example, repeated administration of the SERT inhibitors citalopram and fluoxetine did not produce adaptive modifications of SERT function (Gobbi et al., 1997). In contrast, repeated administration of the DAT inhibitor bupropion, but not nomifensine, resulted in up-regulation of DAT (Tella et al., 1997). Thus, one purpose of the present study was to determine whether repeated reboxetine produced adaptive modifications in NET, SERT, or DAT function.

Many studies have correlated mood disorders with tobacco smoking (Glassman et al., 1990). Individuals who are depressed are more likely to be smokers, dependent on nicotine, experience difficulty quitting, and have greater withdrawal symptoms (Covey, 1999). Depression may also be a symptom of nicotine withdrawal, and depression associated with cessation occurs more frequently among smokers with a history of depression (Covey, 1999). Recent clinical studies report that bupropion, an antidepressant that inhibits DAT and NET function, is a useful smoking cessation therapy (Hurt et al., 1997; Jorenby et al., 1999; Shiffman et al., 2000).

The intrinsic rewarding properties of nicotine, the major alkaloid in tobacco believed to be responsible for maintenance of the smoking habit, result from activation of neuronal DA pathways (Corrigall et al., 1992). Nicotinic acetylcholine receptors (nAChRs) are located on cell bodies and terminals of the hippocampal NE pathway (Sershen et al., 1997) and the nigrostriatal DA pathway (Wonnacott, 1997). Nicotine has been shown to evoke \(^{[3]}\)HNE overflow from rat hippocampal slices (Sershen et al., 1997) and \(^{[3]}\)HD\(A\) over-
flow from rat striatal slices (Teng et al., 1997). The diversity of mRNA for nAChR subunits isolated in NE- and DA-containing neurons (Wada et al., 1989; Dineley-Miller and Patrick, 1992; Charpentier et al., 1998) supports the speculation that several nAChR subtypes may be responsible for nicotine-evoked NE and DA release. Furthermore, evidence has been obtained suggesting that nicotine-evoked NE release from hippocampus is the result of the stimulation of presynaptic αβ4* nAChRs (Clarke and Reuben, 1996; Ser- shen et al., 1997; Reuben et al., 2000), whereas at least in part, nicotine-evoked striatal DA release is via presynaptic αβ2* nAChRs (Schulz and Zigmond, 1989; Grady et al., 1992; Cartier et al., 1996; Kaiser et al., 1998). Convincing evidence suggests that nicotine-evoked [86Rb] efflux from thalamic synaptosomes is the result of stimulation of αβ2* nAChRs. The rank order of potency of nAChR agonists to inhibit carbamylcholine-evoked [86Rb] efflux from thalamic synaptosomes is highly correlated with that for inhibition of [3H]nicotine binding to thalamic membranes (Marks et al., 1995).

Recently, several antidepressants have been reported to act as nAChR antagonists in assays assessing receptor function. Specifically, desipramine, nisoxetine, and citalopram have been shown to inhibit nicotine-evoked [3H]NE overflow from rat hippocampal slices (Hennings et al., 1997, 1999) and bupropion inhibited acetylcholine-induced currents in rat αβ2, αβ2, and α7 nAChRs expressed in Xenopus oocytes (Slemmer et al., 2000). Also, bupropion and several other antidepressants, e.g., fluoxetine, sertraline, paroxetine, and nefazodone, were shown to inhibit carbachol-evoked [86Rb] efflux from human muscle-type nAChRs (αβ6) in TE671/RD cells, to inhibit carbachol-evoked [86Rb] efflux from human autonomic nAChRs (αβ4α5 ± β2) in SH-SY5Y neuroblastoma cells, and to inhibit nicotine-evoked [86Rb] efflux from chick V274T mutant α7-nAChR heterologously expressed in native nAChR-null SH-EP1 epithelial cells (Fryer and Lukas, 1999a,b). Although reboxetine has been established as a potent and selective inhibitor of NET, its interaction with nAChRs has not been investigated previously. Thus, the second purpose of the present study was to evaluate the interaction of reboxetine with native nAChRs containing α7, α4, and α3 subunits by determining reboxetine-induced inhibition of [3H]methyllycaconitine (MLA), [3H]cytisine, and [3H]epibatidine binding, respectively, using whole rat brain membranes, inhibition of nicotine-evoked 3H overflow from superfused hippocampal and striatal slices preloaded with [3H]NE or [3H]DA, respectively, and inhibition of nicotine-evoked [86Rb] efflux from thalamic synaptosomes.

Materials and Methods

Subjects. Male Sprague-Dawley rats (200–250 g) were obtained from Harlan (Indianapolis, IN) and were housed two per cage with free access to food and water. Experimental protocols were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky and at Pharmacia Corporation.

Chemicals. Reboxetine CH3SOCH (a racemic mixture of R,R- and S,S-[2-α-[2-ethoxyphenox]benzyl]-morpholine sulfate) was synthesized and provided by Pharmacia Corporation (Kalamaooz, MI). (±)-Buproprion HCl, (−)-cytisine, desipramine HCl, (±)-epibatidine dihydrochloride, fluoxetine HCl, GBR-12909 HCl, methyllycaconitine citrate, nomifensine maleate, S-(−)-nicotine ditartrate, paraglyine HCl, tetrodotoxin (TTX), HEPES, bovine serum albumin, and catechol were purchased from Sigma-Aldrich (St. Louis, MO). [3H]Cytisine HCl [hexahydro-1,5-methano-8H-pyrido-[1,2-a][1,5]diazocin-8-one, 3,5-(3H); specific activity, 35 Ci/mmol], [3H]epibatidine ([5,6-bicycloheptenyl-3H]-(+)-epibatidine; specific activity, 48 Ci/mmol), [3H]NE (norephinephrine, levorotatory-[3H]; specific activity, 14.4 Ci/mmol), [3H]DA (dihydroxyphenethylamine 3,4-ethyl-2-[N-7H]; specific activity, 25.6 Ci/mmol), [3H]5-HT [hydroxytryptamine creatine sulfate 5-[1,2-3H(N)]; specific activity, 27.5 Ci/mmol], and [86Rb]Cl (specific activity, 55.2 mCi/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). [3H]MLA (1α,1β,6β,16β)-20-ethyl-1.6,14,16-tetramethoxy-4-[[2-(3H)-3H]-methyl-2,5-dioxo-1-pyrrolidinyl]benzoylxoyethylacacetam-7,8-diol; specific activity, 25 Ci/mmol) was purchased from Tocris Cookson (Ballwin, MO). α-D-Glucose was purchased from Aldrich Chemical Co. (Milwaukee, WI). l-t-Absorbic acid was purchased from AnalaR-BHD Ltd. (Poole, Dorset, UK). Potassium phosphate monobasic was purchased from Mallinckrodt (Paris, KY), and TS-2 tissue solubilizer was purchased from Research Products International (Mount Prospect, IL). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA).

[3H]Neurotransmitter Uptake Assays. [3H]NE and [3H]5-HT uptake assays were performed using hippocampal synaptosomes, and [3H]DA uptake assays were performed using rat striatal synaptosomes. Striata or hippocampi from an individual rat were homogenized in 20 ml of ice-cold sucrose solution (0.32 M sucrose and 5 mM NaHCO3, pH 7.4) with 16 passes of a Teflon pestle homogenizer (clearance 0.003 inch). The homogenate was centrifuged at 2000g, 10 min, 4°C, and the resulting supernatant was centrifuged (20000g, 15 min, 4°C). The resulting pellet was resuspended in 24 ml of ice-cold assay buffer (125 mM NaCl, 5 mM KCl, 1.5 mM MgSO4, 1.25 mM CaCl2, 1.5 mM KH2PO4, 10 mM α-glucose, 25 mM HEPES, 0.1 mM EDTA, 0.1 mM pargyline, and 0.1 mM ascorbic acid, saturated with 95% O2/5% CO2, pH 7.4). The final protein concentration was 400 μg/ml, as determined by protein dye binding using bovine serum albumin as a standard (Bradford, 1976). Assays were performed in duplicate in a total volume of 500 μl. Aliquot portions of synapticosomal suspension (50 μl) were added to tubes containing 350 μl of buffer and 50 μl of one of nine concentrations of drug (reboxetine, desipramine, fluoxetine, or GBR-12909) followed by 50 μl of [3H]NE, [3H]5-HT, or [3H]DA (final concentration of 0.1 μM). Accumulation proceeded for 10 min at 34°C. Accumulation was terminated by addition of 3 ml of ice-cold buffer containing catechol (1 mM) and rapid filtration through Whatman GF/B glass fiber filters presoaked with catechol (1 mM). Filters were washed three times with ice-cold buffer, transferred to scintillation vials, cocktail was added, and radioactivity was determined by liquid scintillation spectroscopy (model B1600 TR scintillation counter; Packard Instrument Company, Inc., Downer's Grove, IL).

To determine the effect of repeated reboxetine administration on [3H]neurotransmitter uptake, groups of rats were injected (i.p.) twice daily (9:00 AM and 4:00 PM) with reboxetine (10 mg/kg) or 0.9% (w/v) saline vehicle for 14 days. One day after the last injection of reboxetine or saline, inhibition of [3H]NE, [3H]5-HT, or [3H]DA uptake was assessed using tissue from one rat from each of the reboxetine treatment and control groups.

[3H]MLA, [3H]Cytisine, and [3H]Epibatidine Binding Assays. [3H]MLA was used to probe the αβ2* nACh (Davies et al., 1999) and [3H]cytisine to probe the α4* nACh (Pabreza et al., 1990). Binding of [3H]epibatidine in the presence of cytisine (30 μM) was used to probe the α3* nACh subtype (Xiao et al., 1998). Rat whole brain, minus cerebellum, was dissected quickly, weighed, and homogenized in 9 vol/g wet weight of ice-cold 0.32 M sucrose with 10 passes of a rotating Teflon pestle homogenizer (clearance 0.003 inch; setting 50, 099CK4; Glas-Col, Terre Haute, IN). The homogenate was centrifuged (1000g, 10 min, 4°C). The resulting supernatant was centrifuged (20000g, 20 min, 4°C). The pellet was resuspended to a final protein concentration of 1 to 8 mg/ml. The homogenate (5 ml)
Reboxetine Inhibition of Nicotine

To determine the mechanism of the reboxetine-induced inhibition of nicotine-evoked \(^{3}H\)NE overflow, a Schild analysis was performed in which hippocampal slices were superfused in the absence or presence of a single concentration of reboxetine (10 nM, 100 nM, or 1 \(\mu M\)), and the concentration effect for nicotine was determined. These experiments were performed as described above, except that slices from an individual rat were superfused for 30 min in the absence or presence of a single concentration of reboxetine (10 nM–1 \(\mu M\)), and subsequently, one of six concentrations of nicotine (1 nM–100 \(\mu M\)) was added to the buffer containing reboxetine. Superfusion continued for an additional 60 min. Each slice from a single animal was exposed to only one concentration of reboxetine and only one concentration of nicotine. These experiments also used a repeated measures design, such that the concentration effect for nicotine was determined in both the absence and presence of reboxetine using hippocampal tissue from a single rat, and a minimum of six rats for each reboxetine concentration. Additionally, one slice in each experiment was superfused in the absence of reboxetine and nicotine, constituting the buffer control condition. Slices and superfusate were processed as described previously.

Fractional release for each superfusate sample was calculated by dividing the tritium collected in each sample by the total tritium present in the tissue at the time of sample collection. Fractional release was expressed as a percentage of total tritium in the tissue at the time of sample collection. Basal \(^{3}H\) overflow was calculated from the average of the tritium collected in the three 5-min samples just before the addition of reboxetine. Reboxetine- and nicotine-evoked \(^{3}H\) overflow was calculated by summing the increases in collected tritium that resulted from exposure to drug and subtracting the basal overflow.

\(^{86}\)Rb \(^{+}\) Efflux Assay. The effects of reboxetine on \(^{86}\)Rb efflux were determined using previously published methods (Miller et al., 2000). Thalamus was homogenized and centrifuged (1000g, 10 min, 4°C). The supernatant fraction was centrifuged (12,000g, 20 min, 4°C) to obtain the synaptosomal fraction. Synaptosomes were incubated for 30 min in 35 \(\mu l\) of buffer (140 mM NaCl, 1.5 mM KCl, 2.0 mM CaCl\(_2\), 1.0 mM MgSO\(_4\), and 20 mM \(\alpha\)-glucose, pH 7.5) containing 4 \(\mu l\) of \(^{86}\)Rb\(^{+}\). \(^{86}\)Rb\(^{+}\) uptake was terminated by filtration of the synaptosomes onto glass fiber filters (6 mm, type A/E; Gelman Instrument Co., Ann Arbor, MI) under gentle vacuum (0.2 atm), followed by three washes with buffer (0.5 ml each). Subsequently, each filter with \(^{86}\)Rb\(^{+}\)-loaded synaptosomes (39 \(\pm\) 4.8 \(\mu g\) of protein/\(\mu l\)) was placed on a 13-mm glass fiber filter (type A/E) mounted on a polyelectrolyte platform. \(^{86}\)Rb\(^{+}\) efflux assay buffer (125 mM NaCl, 5 mM CaCl\(_2\), 1.5 mM KCl, 2.0 mM CaCl\(_2\), 1.0 mM MgSO\(_4\), and 25 mM HEPES, 20 mM \(\alpha\)-glucose, 0.1 \(\mu M\) TTX, and 1.0 g/l bovine serum albumin, pH 7.5) was superfused at a rate of 2.5 ml/min. TTX and CaCl\(_2\) were included in the assay buffer to block voltage-gated Na\(^+\) and K\(^+\) channels, respectively, and to reduce the rate of basal \(^{86}\)Rb\(^{+}\) efflux. The ability of reboxetine (1 nM–100 \(\mu M\)) to inhibit \(^{86}\)Rb\(^{+}\) efflux evoked by 1 \(\mu M\) nicotine was determined. In previous experiments, this concentration of nicotine was the lowest concentration that produced maximal \(^{86}\)Rb\(^{+}\) efflux (Miller et al., 2000). After 8 min of superfusion, basal samples (sample/18 s) were collected for 2 min. Subsequently, synaptosomes were superfused for 3 min in the absence or presence of one of six concentrations (1 nM–100 \(\mu M\)) of reboxetine. Then, nicotine (1 \(\mu M\)) was added to buffer, and superfusion continued for an additional 3 min, followed by superfusion for 3 min with buffer in the absence of either drug. Each aliquot part of thalamic synaptosomes was exposed to only one concentration of reboxetine. In each experiment, one synaptosomal aliquot part was also exposed to nicotine (1 \(\mu M\)) in the absence of reboxetine, and one synaptosomal aliquot part was superfused in the absence of either drug to determine basal \(^{86}\)Rb\(^{+}\) efflux during the course of the experiment. Samples were analyzed by liquid scintillation spectroscopy (model B1600 TR scintillation counter; Packard Instrument Company, Inc.). To determine the basal rate of \(^{86}\)Rb\(^{+}\) efflux, an exponential decay curve was used to fit the data points proceeding and

E

\[ E = \frac{1}{-\lambda} \ln(1 - \frac{O}{T}) \]

where \(E\) is the equilibrium constant, \(\lambda\) is the decay constant, \(O\) is the concentration of the drug, and \(T\) is the concentration of the substrate. The fraction of the drug associated with the substrate is then given by the ratio of the concentration of the drug to the concentration of the substrate.

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following superfusion with reboxetine and nicotine (SigmaPlot 2000; SPSS, Inc., Chicago, IL). Drug-evoked increases in \(^{86}\text{Rb}^-\) efflux were calculated as the fractional increase above baseline. Increases were summed to obtain total \(^{86}\text{Rb}^-\) efflux during the period of superfusion with reboxetine and/or nicotine, and normalized to \(^{86}\text{Rb}^-\) content in the corresponding synaptosomal sample to reduce variability within and between experiments.

**Data Analysis.** For the \(^3\text{H}\) neurotransmitter uptake assays, IC\(_{50}\) values for each individual concentration-response curve were generated using nonlinear regression and a sigmoidal curve fit (GraphPad Prism, version 3.0; GraphPad Software, San Diego, CA). For the \(^3\text{H}\) neurotransmitter uptake assays, comparisons of IC\(_{50}\) values for reboxetine with desipramine, fluoxetine, or GBR-12909 were analyzed by independent group t tests. To assess the effect of repeated reboxetine treatment on inhibition of \(^3\text{H}\) neurotransmitter uptake, two-way analysis of variance (ANOVA) was performed on the IC\(_{50}\) values with repeated drug treatment (reboxetine or saline) and inhibitor (reboxetine versus desipramine, fluoxetine, or GBR-12909) as between-group factors (SPSS version 9.0; SSPS, Inc.). Where appropriate, Tukey post hoc tests and simple main effect analyses (p < 0.05) were performed.

In competition binding studies, the inhibition constant (K\(_i\)) was calculated from the binding isotherms for \(^3\text{H}\)MLA, \(^3\text{H}\)cytisine, and \(^3\text{H}\)epibatidine binding using a nonlinear regression curve fit program according to the Cheng and Prusoff equation. For the \(^3\text{H}\) overflow assays, intrinsic activity of reboxetine and the ability of reboxetine to inhibit nicotine-evoked \(^3\text{H}\) overflow were analyzed via one-way repeated measures ANOVA with reboxetine concentration as a within-subject factor. EC\(_{50}\) values for intrinsic activity and IC\(_{50}\) values for inhibition of nicotine-evoked \(^3\text{H}\) overflow were determined via nonlinear regression, which fit the mean data to a sigmoidal concentration-response curve. The mechanism of inhibition of \(^3\text{H}\)NE overflow was determined via Schild analysis and analyzed via three-way repeated measures ANOVA with nicotine concentration and the absence or presence of reboxetine as within-subject factors, and reboxetine concentration as a between-groups factor. The intrinsic activity of reboxetine on \(^{86}\text{Rb}^-\) efflux and the ability of reboxetine to inhibit nicotine-evoked \(^{86}\text{Rb}^-\) efflux were analyzed via one-way repeated measures ANOVA with reboxetine concentration as a within-subject factor.

**Results.**

Repeated Administration of Reboxetine Does Not Alter Selectivity as a NET Inhibitor. The concentration of reboxetine that inhibited \(^3\text{H}\)NE uptake by \(50\%\) (IC\(_{50}\)) was 8.5 nM. IC\(_{50}\) values for reboxetine inhibition of \(^3\text{H}\)DA and \(^3\text{H}\)5-HT uptake were 89 and 6.9 \(\mu\)M, respectively (Fig. 1). Thus, reboxetine was 3 to 4 orders of magnitude more potent as an inhibitor of \(^3\text{H}\)NE uptake than \(^3\text{H}\)DA and \(^3\text{H}\)5-HT uptake. Significant differences were not found between reboxetine and desipramine (IC\(_{50}\) value = 17.0 nM) to inhibit \(^3\text{H}\)NE uptake \(F\_(0,12) = 0.20, p = 0.85;\) Fig. 1). GBR-12909 (IC\(_{50}\) value = 1.5 \(\mu\)M) was significantly more potent \((t\_d = 2.62, p < 0.05)\) than reboxetine in inhibiting \(^3\text{H}\)DA uptake, and fluoxetine (IC\(_{50}\) value = 0.4 \(\mu\)M) was significantly more potent \((t\_d = 15.84, p < 0.001)\) than reboxetine in inhibiting \(^3\text{H}\)5-HT uptake (Fig. 1). Thus, reboxetine is a highly potent and selective \(^3\text{H}\)NE uptake inhibitor.

In a separate series of experiments, rats were administered reboxetine (10 mg/kg) or saline twice daily for 14 days, and \(^3\text{H}\) neurotransmitter uptake was subsequently determined. Repeated reboxetine injection did not alter reboxetine- or desipramine-induced inhibition of \(^3\text{H}\)NE uptake \(F\_(1,12) = 0.543, p = 0.48;\) Table 1, reboxetine- or GBR-12909-induced inhibition of \(^3\text{H}\)DA uptake \(F\_(1,16) = 0.041, p = 0.84;\) Table 1, and reboxetine- or fluoxetine-induced inhibition of \(^3\text{H}\)5-HT uptake \(F\_(1,12) = 0.439, p = 0.52;\) Table 1. Thus, repeated reboxetine injection did not alter the potency or selectivity of reboxetine to inhibit \(^3\text{H}\)NE uptake.

**Reboxetine Does Not Inhibit \(^3\text{H}\)MLA, \(^3\text{H}\)Cytisine, or \(^3\text{H}\)Epibatidine Binding to Rat Whole Brain Membrane Preparations.** To validate the nAChR binding assays, concentration-dependent inhibition of binding was determined using several standard competitors, MLA, nicotine, cytisine, and epibatidine (Table 2). Neither reboxetine nor bupropion significantly inhibited binding of these three nAChR radioligands (Table 2).

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![Fig. 1](image-url) Reboxetine inhibits \(^3\text{H}\)NE and \(^3\text{H}\)5-HT uptake into rat hippocampal synaptosomes and \(^3\text{H}\)DA uptake into rat striatal synaptosomes. Data are expressed as the mean (±S.E.M.) represented in picomoles per minute per milligram of specific \(^3\text{H}\)NE, \(^3\text{H}\)5-HT, or \(^3\text{H}\)DA uptake (n = 4–6 rats).
TABLE 1
Repeated reboxetine injection does not alter inhibition of [3H]NE, [3H]DA, or [3H]5-HT uptake by reboxetine or other selective transport inhibitors.

<table>
<thead>
<tr>
<th>Uptake Inhibitor</th>
<th>IC50 (CI)</th>
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<tbody>
<tr>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>Saline-treated</td>
<td>Reboxetine 5.8 nM (1.7–20.0 nM)</td>
</tr>
<tr>
<td>Reboxetine-treated</td>
<td>Desipramine 18.0 nM (10.0–31.0 nM)</td>
</tr>
<tr>
<td></td>
<td>Reboxetine 2.0 nM (0.5–4.5 nM)</td>
</tr>
<tr>
<td></td>
<td>Desipramine 2.8 nM (1.4–5.7 nM)</td>
</tr>
<tr>
<td>DA</td>
<td></td>
</tr>
<tr>
<td>Saline-treated</td>
<td>Reboxetine 100 μM (0.64–160 μM)</td>
</tr>
<tr>
<td>Reboxetine-treated</td>
<td>GBR 12909 5.5 μM (2.4–5.3 μM)</td>
</tr>
<tr>
<td></td>
<td>Reboxetine 103 μM (82–130 μM)</td>
</tr>
<tr>
<td></td>
<td>GBR 12909 3.8 μM (3.5–4.2 μM)</td>
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<tr>
<td>5-HT</td>
<td></td>
</tr>
<tr>
<td>Saline-treated</td>
<td>Reboxetine 4.9 μM (3.3–7.1 μM)</td>
</tr>
<tr>
<td></td>
<td>Fluoxetine 0.21 μM (0.11–0.40 μM)</td>
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<tr>
<td>Reboxetine-treated</td>
<td>Reboxetine 5.5 μM (2.9–11.0 μM)</td>
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<tr>
<td></td>
<td>Fluoxetine 0.22 μM (0.12–0.38 μM)</td>
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</table>

*p < 0.05, different from reboxetine within the same treatment group (n = 5–8 rats/group).

Reboxetine Noncompetitively Inhibits Nicotine-Evoked [3H]Overflow from Superfused [3H]NE-Preloaded Hippocampal Slices. In a concentration-dependent manner, reboxetine increased [3H] overflow from superfused rat hippocampal slices preloaded with [3H]NE (Table 3). A significant main effect of reboxetine concentration was found [F(6,30) = 10.67, p < 0.001], and post hoc tests revealed greater [3H] overflow after superfusion with 100 μM reboxetine compared with control (0 M) or compared with lower concentrations (1 nM–10 μM) of reboxetine. Furthermore, [3H] overflow from slices superfused with 1 nM to 10 μM reboxetine was not different from control (Table 3), indicating that these concentrations of reboxetine produced no intrinsic activity.

In a concentration-dependent manner, reboxetine inhibited nicotine (10 μM)-evoked [3H] overflow from hippocampal slices preloaded with [3H]NE (Fig. 2). An IC50 value of 7.3 nM was obtained. Because 100 μM reboxetine intrinsically evoked [3H] overflow, data from slices superfused in the presence of this concentration of reboxetine were not included in the analysis of the inhibition of the effect of nicotine. A significant main effect of reboxetine concentration was found [F(5,25) = 5.19, p < 0.01], and post hoc tests revealed that 0.1 to 10 μM reboxetine significantly inhibited nicotine-evoked [3H] overflow. Evaluation of the time course (Fig. 2, inset) revealed that reboxetine (10 nM–1 μM) did not increase fractional [3H]NE release compared with fractional release from control slices, which were not exposed to reboxetine. Addition of nicotine to the buffer resulted in an immediate increase in fractional release, which returned to basal levels by the end of the superfusion period. The time course also illustrates the concentration-dependent inhibition produced by reboxetine, such that 1 μM reboxetine completely inhibited the stimulation produced by this concentration of nicotine.

To determine the mechanism of reboxetine-induced inhibition of the effect of nicotine, the concentration response for nicotine-evoked (10 nM–1 μM) [3H] overflow from [3H]NE-preloaded hippocampal slices was determined in the absence and presence of 10 nM, 100 nM, or 1 μM reboxetine (Fig. 3). Data from this Schild analysis were analyzed via repeated measures ANOVA, which revealed a significant three-way interaction of nicotine concentration, the absence or presence of reboxetine, and reboxetine concentration [F(12,84) = 4.73, p < 0.001]. Simple main effect analyses and post hoc tests revealed that nicotine produced a concentration-dependent increase in [3H] overflow [F(6,102) = 65.51, p < 0.001]. The low concentration of reboxetine (10 nM) significantly inhibited the 10 μM nicotine-evoked [3H] overflow [F(6,30) = 2.61, p < 0.05]; however, this concentration of reboxetine did not inhibit [3H] overflow evoked by 100 μM nicotine. Reboxetine at 100 nM and 1 μM inhibited [3H] overflow evoked by 100 nM to 100 μM nicotine [F(6,30) = 4.57, p < 0.01 and F(6,30) = 11.49, p < 0.001, respectively]. Moreover, the inhibition produced by the latter concentrations of reboxetine was not surmounted by increasing concentrations of nicotine. These results indicate that reboxetine noncompetitively inhibited nicotine-evoked [3H]NE overflow from superfused hippocampal slices.

Reboxetine Evokes [3H] Overflow from Rat Striatal Slices Preloaded with [3H]DA; However, Reboxetine Does Not Inhibit Nicotine-Evoked [3H] Overflow. Two series of experiments were performed to determine the effect of reboxetine across 6 orders of magnitude concentration. The effect of reboxetine to increase [3H] overflow from [3H]DA-preloaded striatal slices is shown in Table 4. Significant main effects of reboxetine concentration were found in the two series of experiments [F(5,25) = 3.77, p < 0.05 and F(6,30) = 119.86, p < 0.001, respectively]. Post hoc tests revealed a concentration-dependent increase in [3H] overflow after superfusion with 3 to 100 μM reboxetine compared with control; 1 nM to 1 μM reboxetine did not increase [3H] overflow compared with control. Concentrations of reboxetine that did not produce intrinsic activity, also did not inhibit nicotine-evoked [3H] overflow (Table 4; F(1,11) = 3.90, p = 0.11 and F(1,11) = 1.84, p = 0.49). Thus, reboxetine did not inhibit nicotine-evoked [3H] overflow from superfused striatal slices preloaded with [3H]DA.

Reboxetine Inhibits Nicotine-Evoked 86Rb+ Efflux from Rat Thalamic Synaptosomes. Only the highest concentration (100 μM) of reboxetine significantly [F(6,18) = 13.95, p < 0.001] evoked 86Rb+ efflux above basal levels (Table 5). Thus, this high concentration was not included in the analysis of reboxetine-induced inhibition of nicotine-evoked 86Rb+ efflux (Fig. 4). Reboxetine significantly inhibited nicotine-evoked 86Rb+ efflux [F(6,18) = 6.77, p < 0.01] with an IC50 value of 650 nM. Post hoc tests revealed that 1 and 10 μM reboxetine inhibited nicotine-evoked 86Rb+ efflux, compared with control.

Discussion

The present study demonstrates that reboxetine potently and selectively inhibits [3H]NE uptake into rat hippocampal synaptosomes. [3H]NE uptake was inhibited 3 to 4 orders of magnitude more potently than either [3H]DA or [3H]5-HT uptake. The present results demonstrating that reboxetine is a highly selective inhibitor of NET are consistent with previous findings (Montgomery, 1997; Wong et al., 2000). The current results, demonstrating that repeated injection of reboxetine does not alter its potency or selectivity to inhibit [3H]NE uptake, suggest that reboxetine does not produce adaptive modifications of NET, SERT, or DAT function under...
Table 2

Reboxetine does not inhibit binding of [3H]MLA, [3H]cytisine, or [3H]epibatidine to rat whole brain membranes. Data are presented as the mean (±S.E.M.) of total 3H overflow during the 60-min period of exposure to nicotine in the absence or presence of reboxetine (10 µM). Data from the 60-min period of reboxetine and nicotine superfusion are presented in Fig. 2.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>[3H]MLA</th>
<th>[3H]Cytisine</th>
<th>[3H]Epibatidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLA</td>
<td>0.64 ± 0.08 nM</td>
<td>567 ± 50 nM</td>
<td>2903 ± 244 nM</td>
</tr>
<tr>
<td>Nicotine</td>
<td>980 ± 125 nM</td>
<td>5.3 ± 1.0 nM</td>
<td>44 ± 26 nM</td>
</tr>
<tr>
<td>Cytisine</td>
<td>1370 ± 88 nM</td>
<td>1.1 ± 0.55 nM</td>
<td>1.1 ± 0.08 nM</td>
</tr>
<tr>
<td>Epibatidine</td>
<td>11 ± 0.7 nM</td>
<td>0.08 ± 0.01 nM</td>
<td></td>
</tr>
<tr>
<td>Reboxetine</td>
<td>4 ± 4%</td>
<td>0%</td>
<td>25%</td>
</tr>
<tr>
<td>Bupropion</td>
<td>3 ± 3%</td>
<td>8 ± 4%</td>
<td>10 ± 3%</td>
</tr>
</tbody>
</table>

a Data are Kᵢ values (mean ± S.E.M.).
b Data are the mean (±S.E.M.) of the percentage of inhibition of [3H]MLA binding at 100 µM reboxetine or bupropion, and the percentage of inhibition of [3H]cytisine and [3H]epibatidine binding at 1 µM reboxetine or bupropion.

Table 3

Reboxetine (100 µM) evokes 3H overflow from rat hippocampal slices preloaded with [3H]NE. Superfusion buffer contained pargyline (10 µM) and desipramine (10 µM) from the start of the experiment. Data represent the 30-min period of superfusion in the absence and presence of reboxetine before exposure of the slices to nicotine (10 µM). Data from the 60-min period of reboxetine and nicotine superfusion are presented in Fig. 2.

<table>
<thead>
<tr>
<th>Reboxetine Concentration</th>
<th>[3H]NE Overflow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 M)</td>
<td>0.11 ± 0.042a</td>
</tr>
<tr>
<td>1 nM</td>
<td>0.27 ± 0.15</td>
</tr>
<tr>
<td>10 nM</td>
<td>0.052 ± 0.026</td>
</tr>
<tr>
<td>100 nM</td>
<td>0.239 ± 0.171</td>
</tr>
<tr>
<td>1 µM</td>
<td>0.087 ± 0.034</td>
</tr>
<tr>
<td>10 µM</td>
<td>0.17 ± 0.050</td>
</tr>
<tr>
<td>100 µM</td>
<td>1.46 ± 0.27*</td>
</tr>
</tbody>
</table>

a Data are the mean (±S.E.M.) of total 3H overflow expressed as a percentage of tissue tritium.

Fig. 3. Reboxetine noncompetitively inhibits nicotine-evoked 3H overflow from rat hippocampal slices preloaded with [3H]NE. The superfusion buffer contained pargyline (10 µM) and desipramine (10 µM) from the start of the experiment. The concentration response for nicotine (10 nM–1 µM) was determined in the absence and presence of 10 nM, 100 nM, or 1 µM reboxetine. Data were collapsed for graphical presentation. * p < 0.05, different from control (0 M reboxetine) across the corresponding nicotine concentration (n = 6 rats/group).

Fig. 2. Reboxetine inhibits nicotine (10 µM)-evoked 3H overflow from rat hippocampal slices preloaded with [3H]NE. Superfusion buffer contained pargyline (10 µM) and desipramine (10 µM) from the start of superfusion. Data are presented as the mean (±S.E.M.) of total 3H overflow during the 60-min period of superfusion with reboxetine plus nicotine. * p < 0.05, different from 0 µM reboxetine (n = 6 rats). Inset, data are presented as mean ± S.E.M. of fractional [3H]NE release during the entire collection period. Left arrow designates beginning of superfusion with reboxetine, and right arrow designates addition of nicotine to the buffer.

The current experimental conditions. Furthermore, tolerance did not develop to the reboxetine-induced inhibition of NET after repeated reboxetine administration.

The same reboxetine injection regimen (10 mg/kg/day i.p., twice daily for 14 days) used in the present 3H neurotransmitter uptake study was previously demonstrated to produce characteristic neural adaptive changes, e.g., down-regulation of β-adrenergic receptors (Harkin et al., 2000), indicating that this reboxetine injection regimen was sufficient to induce neural adaptation. Because reboxetine requires 2 to 3 weeks of treatment before its antidepressant effects are ob-
Reboxetine evokes 3H overflow from rat striatal slices preloaded with [3H]DA, but does not inhibit nicotine-evoked 3H overflow

Superfusion buffer contained pargyline (10 μM) and nomifensine (10 μM) from the start of the experiment. Slices were superfused with reboxetine for 30 min before addition of nicotine to the superfusion buffer. Superfusion with nicotine in the absence or presence of reboxetine continued for 60 min.

<table>
<thead>
<tr>
<th>Reboxetine Concentration</th>
<th>Reboxetine</th>
<th>Reboxetine + Nicotine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 M)</td>
<td>0.16 ± 0.12</td>
<td>2.03 ± 0.45</td>
</tr>
<tr>
<td>1 nM</td>
<td>0.052 ± 0.047</td>
<td>1.50 ± 0.50</td>
</tr>
<tr>
<td>10 nM</td>
<td>0.030 ± 0.016</td>
<td>1.52 ± 0.23</td>
</tr>
<tr>
<td>100 nM</td>
<td>0.020 ± 0.012</td>
<td>1.71 ± 0.26</td>
</tr>
<tr>
<td>1 μM</td>
<td>0.037 ± 0.019</td>
<td>1.25 ± 0.29</td>
</tr>
<tr>
<td>3 μM</td>
<td>0.69 ± 0.49*</td>
<td>NA</td>
</tr>
<tr>
<td>10 μM</td>
<td>1.25 ± 0.42*</td>
<td>NA</td>
</tr>
<tr>
<td>100 μM</td>
<td>36.46 ± 3.24*</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, not analyzed.

* Data are the mean (±S.E.M.) of total 3H overflow expressed as a percentage of tissue tritium.

** p < 0.05, different from control and 1 nM to 1 μM reboxetine (n = 6–12 rats).

TABLE 5
Reboxetine evokes 86Rb efflux from rat thalamic synaptosomes

Synaptosomes were superfused for a 3-min period in the absence or presence of reboxetine before addition of nicotine to buffer. Data from the period of superfusion following addition of nicotine to the buffer are presented in Fig. 4.

<table>
<thead>
<tr>
<th>Reboxetine Concentration</th>
<th>86Rb Efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 M)</td>
<td>0.04 ± 0.04*</td>
</tr>
<tr>
<td>1 nM</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>10 nM</td>
<td>0.10 ± 0.09</td>
</tr>
<tr>
<td>100 nM</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>1 μM</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>10 μM</td>
<td>0.10 ± 0.10</td>
</tr>
<tr>
<td>100 μM</td>
<td>0.78 ± 0.24*</td>
</tr>
</tbody>
</table>

* Data are presented as mean (±S.E.M.) percent 86Rb tissue content.

** p < 0.05, different from control and 1 nM to 10 μM reboxetine (n = 4 rats).

Table 4 and 5

Reboxetine and nefazodone inhibited the nicotine-evoked 3H overflow. By contrast, bupropion, fluoxetine, sertraline, and antidepressants were reported to be antagonists of nAChRs (Hennings et al., 1997, 1999; Fryer and Lukas, 1999). Recently, bupropion has efficacy for smoking cessation and ameliorates some nicotine withdrawal symptoms (Hurt et al., 1997; Jorenby et al., 2000). Bupropion, fluoxetine, sertraline, paroxetine, and nefazodone inhibited the α1βγδ subtype expressed in TE671/RD cells, the α3β4α5β δ subtype expressed in SH-SY5Y neuroblastoma cells, and the chick V274T mutant α7 subtype expressed in SH-EP1 epithelial cells (Fryer and Lukas, 1999a,b). Desipramine, nisoxetine, and citalopram inhibited nicotine-evoked [3H]NE overflow from rat hippocampal slices (Hennings et al., 1997, 1999), suggesting an interaction with the α3β4α nAChR subtype. Based on these findings, it seems that different antidepressants may have different inhibitory profiles across the diversity of nAChR subtypes. Importantly, the specific nAChR subtype(s) inhibited may impart the pharmacological properties necessary for the inhibition of the reinforcing effect of nicotine. Thus, selected antidepressants may prove efficacious as smoking cessation pharmacotherapies, in part depending on their pharmacological profile as inhibitors of specific nAChR subtypes.

The present study provides an initial profile for the functional antagonism produced by reboxetine at several nAChR subtypes. Reboxetine potently inhibited (IC50 value = 7.3 nM) nicotine-evoked [3H]NE overflow from superfused hippocampal slices, suggesting antagonism at the α3β4α subtype. Furthermore, the reboxetine-induced inhibition was not surmounted by increasing concentrations of nicotine, indicative of a noncompetitive mechanism of action. It is notable that reboxetine inhibited the function of this nAChR subtype within the same concentration range that it inhibited NET function. Inclusion of desipramine in the superfusion buffer in the [3H]NE release experiments eliminated the contribution of NET function to the observed effect of reboxetine to inhibit nicotine-evoked [3H]NE release. The functional elimination of NET provided the necessary conditions to focus on the contribution of nAChR function to the observed effect of reboxetine. Thus, the present observation that reboxetine potently inhibited nicotine-evoked [3H]NE release in the presence of desipramine strongly suggests the involvement of the α3β4α nAChR. In agreement, other investigators have reported that IC50 values for inhibition of nicotine-evoked [3H]NE overflow from rat hippocampal slices are not correlated with K values for inhibition of NET function (Hennings et al., 1999). Thus, both the current studies and the work of others suggest that there is no relationship between antidepressant-induced inhibition of NET function and antidepressant-induced inhibition of the effect of nicotine to release NE from its presynaptic terminal in hippocampus.

Furthermore, the present results suggest that reboxetine selectively inhibits the α3β4α nAChR subtype because reboxetine failed to inhibit nicotine-evoked [3H]DA release from superfused rat striatal slices in the presence of nomifensine, indicating a lack of interaction with the α3β2α subtype. Ad-
ditionally, reboxetine did not inhibit binding of several nAChR radioligands, including [3H]epibatidine and [3H]MLA, indicating that reboxetine does not interact at the ligand binding site on either the α3β2* or α7* nAChR subtypes. Thus, neither functional inhibition nor inhibition of ligand binding induced by reboxetine was observed at the α3β2* nAChR site, suggesting pharmacological selectivity for reboxetine at the α7* subtype.

Regarding the α4β2* nAChR subtype, reboxetine also inhibited nicotine-evoked 86Rb efflux (IC50 value = 650 nM) from thalamic synaptosomes, a functional assay for the α4β2* nAChR. The reboxetine-induced inhibition of the α4β2* subtype was 90-fold less potent than inhibition of the α3β4* subtype. Importantly, reboxetine did not inhibit [3H]cytisine binding to rat whole brain membranes, indicating that it does not interact at the ligand binding site on the α4β2* nAChR. Taken together, these results demonstrate that reboxetine interacts with the α4β2* nAChR subtype in a noncompetitive manner, and does not directly interact with the specific site to which [3H]cytisine binds on the α4β2* protein. The present results do not rule out the involvement of NET in the reboxetine-induced inhibition of nicotine-evoked 86Rb efflux because desipramine was not included in the superfusion buffer at concentrations maximally inhibiting NET function. However, the concentration required for inhibition of α4β2* function was 100-fold greater than that which inhibits NET function, mitigating against the role of NET in this effect of reboxetine. Furthermore, the present results obtained using native receptors are consistent with previous results demonstrating that antidepressants noncompetitively inhibit α4β2 nAChRs expressed in Xenopus oocytes in the absence of NET coexpression (Slemmer et al., 2000).

Pharmacological interventions as smoking cessation agents are restricted in number. Currently, the noncompetitive nAChR antagonist mecamylamine, various forms of nicotine replacement therapy, and the nonselective DA/NE transport inhibitor and nAChR antagonist bupropion have been reported to show limited efficacy. Recent studies demonstrate that reboxetine inhibits nicotine self-administration in rats and that tolerance does not develop to the inhibitory properties of reboxetine after repeated administration (A. S. Rauhut, S. N. Mullins, L. P. Dwoskin, and M. T. Bardo, submitted for publication). The latter results suggest that reboxetine may be a good candidate for the treatment of nicotine dependence and may have utility as a smoking cessation agent. Furthermore, the present study suggests that the α3β4* nAChR may be a novel target for therapeutic intervention in smoking cessation, and that the noradrenergic system may play a more important role than previously appreciated in nicotine-induced reward. Thus, given the concordance of smoking behavior and dysphoric mood disorders, reboxetine may provide an alternative effective treatment for tobacco smoking cessation.

References


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