Regulation of the Human Norepinephrine Transporter by Cocaine and Amphetamine

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ABSTRACT

Certain antidepressant and psychostimulant drugs block the uptake of norepinephrine from the synaptic cleft by inhibiting norepinephrine transporter (NET) function. The effects of chronic occupation of the NET by these drugs on NET expression are poorly understood. We previously described down-regulation of the NET in cultured cells after continuous exposure to the tricyclic antidepressant desipramine. Here, the effects of structurally unrelated NET ligands, cocaine and amphetamine, on levels of NET and on NET function in HEK-293 cells transfected with human NET cDNA were investigated. All drug exposures were followed by incubation in drug-free media before harvesting and assays. Exposure of intact cells to cocaine for 3 days did not significantly affect the B_{max} or K_{D} of [3H]nisoxetine binding to NET in membrane homogenates, and did not alter levels of NET immunoreactivity or NET mRNA. In contrast, incubation of cells with amphetamine significantly reduced [3H]nisoxetine binding to NET and levels of NET immunoreactivity in a time-dependent manner, although levels of NET mRNA appeared to be unaffected. Exposures to cocaine or amphetamine resulted in significant reductions of [3H]norepinephrine uptake, although the magnitude of the reduction produced by amphetamine was much greater than cocaine. The effects of structurally unrelated NET ligands, cocaine and amphetamine, on levels of NET mRNA and on NET protein levels were also reduced by exposure of cells to high concentrations of norepinephrine, although norepinephrine exposures were accompanied by changes indicative of cellular toxicity. Cocaine and amphetamine have distinctly different effects on NET expression after continuous exposure. The ability of only certain drugs to down-regulate the NET may provide clues to the unique therapeutic effects of antidepressants that are NET ligands.

The norepinephrine transporter (NET) is responsible for the neuronal reuptake of norepinephrine (NE) and is located presynaptically on noradrenergic nerve terminals. Reuptake of NE by the NET contributes to the termination of noradrenergic transmission (Barker and Blakely, 1995). Treatment of rats with drugs that alter noradrenergic transmission results in up- or down-regulation of the NET (Lee et al., 1983). Based on these findings, it has been proposed that a change in the number or function of the NET on the noradrenergic neuron may in turn cause changes in the amount or longevity of NE in and around the synaptic cleft, and thereby may be a mechanism by which noradrenergic transmission is biologically regulated (Lee et al., 1983). Moreover, abnormal regulation or expression of the NET could contribute to the development of psychiatric illnesses, e.g., major depression, because alterations in the concentration of NE in the central nervous system have been suggested to play an important role in the pathophysiology of these illnesses. In fact, abnormal levels of the human NET have been observed in major depression (Klimek et al., 1997). The molecular mechanisms responsible for regulating the expression and function of the NET remain poorly understood.

The NET is a site of action of many antidepressants and chronic administration of rats with NET inhibitor antidepressants appears to regulate the expression of the NET (Bauer and Tejani-Butt, 1992). Local infusion of NET inhibitors dramatically increases the extracellular concentration of NE in the brain (L’Heureux et al., 1986; Gustafson et al., 1991). Therefore, NET regulation induced by NET inhibitor exposure could be secondary to occupation of the NET by the ligand, i.e., as a result of elevated levels of synaptic NE and subsequent activation of one or more of the synaptic receptors for NE. However, continuous exposure of intact, NET-expressing PC12 cells (a clonal cell line of rat pheochromocytoma cells) or HEK-293 cells transfected with human NET cDNA (293-hNET), to the NET inhibitors desipramine or nisoxetine down-regulates the NET (Zhu and Ordway, 1997; Zhu et al., 1998). Because these cells lack synaptic contacts, NET down-regulation may occur as a direct result of occupa-

ABBREVIATIONS: NET, norepinephrine transporter; NE, norepinephrine; 293-hNET, HEK-293 cells transfected with human NET; SSC, standard saline citrate; KRH, Krebs-Ringer HEPES.

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tion of the transporter by these antidepressants. The ability of desipramine to down-regulate the NET in cells transfected with the NET cDNA, as well as in cells expressing the native NET gene, suggests that the regulation of the NET by inhibitors is a fundamental property of the NET (Zhu et al., 1998).

The NET is also a target of psychostimulants and drugs of abuse, such as cocaine and amphetamine (Azzaro et al., 1974). Like antidepressant drugs, cocaine and amphetamine block the transport of NE, thereby elevating extracellular concentrations of NE and potentiating the activation of postsynaptic receptors (Amara and Sonders, 1998), although amphetamine is also a substrate for the NET (Bönisch, 1984). However, despite the fact that cocaine and amphetamine are potent inhibitors of NE uptake acutely (Engberg and Svensson, 1979; Ritz et al., 1990), their long-term effects on the NET have not been fully characterized. In this study, we examined the effect of continuous exposure of 293-hNET cells to cocaine and amphetamine on NET protein levels and NET function. In addition, the effect of NE, the natural substrate of NET, on NET protein has been investigated. This investigation aimed to extend previous observations by answering the following questions. 1) Do other inhibitors of NE transport down-regulate the NET? 2) Does exposure to NET substrates have effects on NET expression and function opposite to the effects of inhibitors?

Materials and Methods

Cell Culture and Drug Exposure. The 293-hNET cells (courtesy of Randy Blakely, Vanderbilt University, Nashville, TN) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), l-glutamine (2 mM), and geneticin (G418, 250 μg/ml) at 37°C in 95% humidified air with 5% CO₂. Culture medium and supplements were obtained from Life Technologies (Grand Island, NY). Drug exposures for binding assays and Western blots were started the 3rd day after each subculture, when cells had reached confluency. Media containing cocaine, amphetamine, NE, or no drug additions, respectively, were directly added to flasks and changed daily. In all experiments, exposures were followed by a 4-h incubation in fresh drug-free medium (to facilitate removal of NET ligands). After this postincubation, cells were harvested and collected by centrifugation at 1000g for 10 min. After resuspending in fresh drug-free media, cell pellets were stored at −80°C until use. Microscopic examination of cells for possible toxic effects of cocaine, amphetamine, and NE was routinely conducted as described in Zhu and Ordway (1997). The number of viable 293-hNET cells per milliliter was counted for all groups after cell harvesting. Cell viability was determined by Trypan blue exclusion.

Binding Assay. The binding of [3H]nisoxetine to 293-hNET cell membranes was assayed as described in Zhu and Ordway (1997). Briefly, frozen cells were washed twice with ice-cold PBS, homogenized in ice-cold buffer (50 mM Tris, 120 mM NaCl, 5 mM KCl, pH 7.4) using a Polytron (setting 6, 20 s), and centrifuged. After three washes following centrifugations at 40,000g for 30 min, the final pellet was resuspended in ice-cold incubation buffer (50 mM Tris, 300 mM NaCl, 5 mM KCl, pH 7.4). The reaction mixture included the membrane preparation (about 30 μg of protein) and [3H]nisoxetine (DuPont-New England Nuclear, Boston, MA) at 2.5 nM for single point assays and at concentrations ranging from 0.1 to 10 nM in saturation assays. Nonspecific binding was defined with mazindol (1 μM). The mixture was incubated at 0°C for 4 h and the reaction was stopped by the addition of 5 mM ice-cold incubation buffer, followed by rapid filtration through glass fiber filters (#25; Schleicher & Schuell, Keene, NH) presoaked in 0.3% polyethyleneimine. Radioactivity was counted by liquid scintillation spectrometry. The protein concentration in the final preparation was measured by a modified method of Lowry (Peterson, 1977).

Immunoblotting. Immunoblotting was performed as described by Melikian et al. (1994) with minor modifications. Cells were washed twice with ice-cold PBS and lysed for 30 min (4°C, shaking) in 800 μl of ice-cold RIPA buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, pH 7.4) supplemented with soybean trypsin inhibitor (1 mg/ml), o-phenanthroline (1 mM), leupeptin (1 μg/ml), iodoacetamide (1 mM), pepstatin A (1 μM), and phenylmethylsulfonyl fluoride (250 μM). Solubilized extracts were centrifuged (20,000g, 10 min, 4°C). SDS-polyacrylamide gel electrophoresis of supernatants was performed on a 7.5% gel that was then electroblotted (35 V, 16 h, 4°C) to Hybond-C super nitrocellulose membranes. After blocking, the membranes were incubated in turn with 0.5 μg/ml affinity-purified primary antibody N430 (Melikian et al., 1994, courtesy of Randy Blakely), in a 5% milk solution with 0.1% Tween 20, 0.1% NaN₃ (1 h, 22°C), and with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:3000 dilution in Tris-buffered saline with 5% milk solution and 0.1% Tween 20, 1 h, 22°C). After stripping, membranes were incubated with mouse anti-antibody (1:5000; Chemicon Inc., Temecula, CA) and horseradish peroxidase-conjugated goat anti-mouse antibody (1:3000). Immunoreactive bands were detected by enhanced chemiluminescence (Amersham Corporation, Arlington Heights, IL). The relative intensity (relative optical density × pixel area) of autoradiographic bands was estimated using gel analysis software and a computer-assisted image analysis system (MCID M2; Imaging Research, Inc., St. Catherines, Ontario, Canada). Measurements were made within the linear range of the film. All Western blot results were reproducible. The results were performed a minimum of two times to assure that the results were reproducible.

RNA Isolation. The acid guanidinium-phenol-chloroform method was used to isolate RNA from cultured cells (Chomczynski and Sacchi, 1987). Cells were washed with 1× PBS and lysed directly in the culture flasks (on ice) by the addition of a denaturing solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). Sodium acetate (2 M, pH 4), water-saturated phenol, and chloroform-isoamyl alcohol mixture (49:1) were added to the lysate, followed by shaking vigorously for 10 s and cooled on ice for 15 min. After centrifugation (10,000g, 20 min, 4°C), the aqueous phase was mixed with 1 ml of isopropanol, and placed at −20°C for at least 1 h to precipitate RNA. After recentrifugation the resulting RNA pellet was dissolved in solution D and precipitated with isopropanol at −20°C for 1 h. The RNA pellet was resuspended in 75% ethidium, sedimented, and vacuum dried (10 min). The final RNA preparation was dissolved in 50 μl of diethylpyrocarbonate-treated water and electrophoresed in a formaldehydeagarose (1%) mini-gel (Farrell, 1993).

Northern Blot Analysis. The formaldehyde gel was blotted onto nylono-66 filters. Hybridization was performed overnight at 42°C in 1 ml of a formamide prehybridization/hybridization solution (5× SSC, 5× Denhardt’s solution, 50% w/v formamide, 1% w/v SDS, and 100 μg/ml denatured herring sperm DNA) per 10 cm² of membrane. A human NET cDNA probe was obtained by HindIII/XbaI digestion of pcDNA1-hNET (Eshleman et al., 1997), resulting in a 0.86-kb fragment beginning 1.06 kb after the start codon and extending 65 base pairs after the translational stop codon. Human NET and human β-actin cDNA probes (Clontech, Palo Alto, CA) were prepared by random primer synthesis with [α-32P]dCTP (Promega, Madison, WI). Blots were washed in an equal volume of 2× SSC/0.1% SDS to a final stringency of 0.1× SSC/0.1% SDS at 68°C, rinsed with 2× SSC at 22°C. Blots were exposed to film (Fuji Photo Film Co., Ltd., Tokyo) for 1 h. A single band (1.9 kb), corresponding to the labeled human NET mRNA, was observed in all experiments. All Northern blots were performed a minimum of two times to assure that the results were reproducible.
Exposure to cocaine (binding to the NET were also not significantly affected by exposure periods. The measurement of homogenates. Each column represents data from four to five separate placements) buffer. Protein concentrations of lysed cell preparations were measured by the method of Lowry et al. (1951). Statistics. \( B_{\text{max}} \) and \( K_D \) values were computed using nonlinear regression analysis (Prism 1.0; GraphPad Software, Inc., San Diego, CA). Data were subjected to a single-factor ANOVA (SuperANOVA program; Abacus Concepts, Inc., Berkeley, CA) and are presented as means ± S.E. In the presence of significant \( F \) values, individual comparisons between means were made using the Student-Newman-Keuls test.

Results

Effect of Cocaine on NET Expression. To examine the effect of cocaine on the NET, intact 293-hNET cells were exposed to medium containing different concentrations of cocaine for 3 days. After a 4-h wash in drug-free medium, crude homogenates of cells were prepared and \(^{[3]H}\)nisoxetine binding was performed. \( B_{\text{max}} \) values of \(^{[3]H}\)nisoxetine binding were not significantly affected by cocaine exposures (\( F_{2,25} = 1.98, P = .18 \), Fig. 1), although there was a tendency for reductions at concentrations in the \( B_{\text{max}} \) of \(^{[3]H}\)nisoxetine binding to the NET were not affected by cocaine \( B_{\text{max}} \) values of \(^{[3]H}\)nisoxetine binding to the NET were not significantly affected by exposure to cocaine (\( F_{2,25} = 0.85, P = .57 \)).

To further confirm \(^{[3]H}\)nisoxetine binding results, Western blots were performed using an antibody against the human NET. Solubilized proteins, from the same cell exposures used for radioligand binding experiments, were loaded onto SDS-polyacrylamide gels, and were electrophoresed and probed with N430 antibody (Melikian et al., 1994). Similar to \(^{[3]H}\)nisoxetine binding results, there were no changes in the signal intensity of the 80-kDa bands (representing human NET immunoreactivity, Melikian et al., 1994) at any exposure concentration of cocaine (Fig. 2).

Effects of Amphetamine on the NET Expression. Amphetamine inhibits the uptake of NE by the NET, but differs from cocaine in that it is also a substrate of the NET. 293-hNET cells were exposed to amphetamine to examine its effect on the NET. Exposures of cells to amphetamine for 3 days, followed by 4-h washouts resulted in significant reductions in the \( B_{\text{max}} \) of \(^{[3]H}\)nisoxetine binding to NETs (\( F_{3,28} = 5.81, P < .001 \), Fig. 3) at concentrations greater than 1 \( \mu M \). \( K_D \) values of \(^{[3]H}\)nisoxetine binding to the NET were unaffected by amphetamine exposures (\( F_{3,28} = 2.35, P > .05 \)). Exposure concentrations of 1, 10, and 100 \( \mu M \) amphetamine reduced the \( B_{\text{max}} \) of \(^{[3]H}\)nisoxetine binding to the NET to nearly the same extent (reductions of 39, 40, and 38%, respectively). Western blotting revealed a similar pattern of concentration-independent reductions in NET protein, as indicated by obvious decreases of signal intensities of the 80-

Fig. 1. Effect of exposure of intact 293-hNET cells to cocaine for 3 days on \( B_{\text{max}} \) and \( K_D \) values of \(^{[3]H}\)nisoxetine binding to NET in membrane homogenates. Each column represents data from four to five separate experiments. There was no significant difference in both \( B_{\text{max}} \) and \( K_D \) values between any of the groups.

Fig. 2. A, top, ECL photograph of immunoblotted NET in 293-hNET cells exposed to different concentrations of cocaine for 3 days. Each well was loaded with 25 \( \mu g \) of total protein and blots were probed with the N430 antibody. C, control group. Bottom, the same membrane was also probed with anti-actin antibody (after stripping) as an internal control. The positions of standard molecular mass markers in kilodaltons are indicated on the left of the blot. B, relative densities of 80- and 56-kDa bands of the film shown in A measured as optical density × pixel area.
kDa band in groups exposed to 1, 10, and 100 μM amphetamine. Western blotting also revealed a modest reduction of NET immunoreactivity in the cells exposed to 0.1 μM amphetamine (Fig. 4).

The time course of the effect of amphetamine exposure on NET expression was carried out by exposure of 293-hNET cells to 1 μM amphetamine. Amphetamine produced a time-dependent reduction in the $B_{max}$ of $[^3H]niosoxetine$ binding to NETs ($F_{2,8} = 16.11, P < .001$). A significant reduction in the $B_{max}$ of binding to NET was observed on the 2nd day after exposure of cells to amphetamine, and $B_{max}$ values continued to decline for the 3rd day of exposure (Fig. 5). The reductions of the $B_{max}$ of $[^3H]niosoxetine$ binding after 2- and 3-day exposures to amphetamine was 31 and 46%, respectively. $K_D$ values of $[^3H]niosoxetine$ binding to NET were not significantly changed after treatment with amphetamine for any time period (Fig. 5).

**Effect of Cocaine and Amphetamine on the NET mRNA in 293-hNET Cells.** Northern blotting analysis of mRNA extracted from 293-hNET cells exposed to cocaine and amphetamine was performed by hybridization of filters with a human NET cDNA probe. The same filters were then hybridized to a β-actin cDNA probe (Clontech), after stripping, to verify RNA loads. Autoradiograms of these blots (Fig. 6) showed that human NET mRNA levels were not obviously affected by exposure of cells to cocaine and amphetamine at the concentrations tested.

**Effects of NE on Expression of NET.** The effect of exposure to the natural substrate of NET, NE, on the expression of NET in 293-hNET cells was examined. Exposure of cells to NE for 3 days caused a concentration-dependent reduction in the $B_{max}$ of $[^3H]niosoxetine$ ($F_{4,10} = 7.77, P < .01$), with exposure concentrations of 100 and 1000 μM NE producing significant reductions of 64 and 67%, respectively (Fig. 7). In parallel experiments, 3-day exposures to NE at 1 and 10 μM had no effect on the human NET immunoreactivity (80-kDa band), whereas exposures to 100 and 1000 μM NE reduced the intensity of this band (Fig. 8). Furthermore, it is interesting to note that the signal intensity of 55-kDa bands was dramatically increased, an effect not observed for any other drug exposures in the present study or previous studies (Zhu et al., 1998). It is possible that this 55-kDa band and a previously reported 54 kDa, a biosynthetic precursor of the 80-kDa form of human NET (Melikian et al., 1994, 1996), are the same protein. However, it is unknown whether the increased intensity of 55-kDa species represents the enhanced precursor that has failed to mature owing to the presence of higher concentrations of NE. It is also possible that this increased 55-kDa species resulted from the degra-
dation of the 80 kDa NET protein triggered by higher concentrations of NE, a situation that would suggest that this species may represent one kind of degradation form of hNET rather than a precursor. More study is needed to address these possibilities. Examination of the time course of the effect of NE (100 μM) exposure on NET revealed a significant reduction (40%) in the $B_{\text{max}}$ of [3H]nisoxetine after a single day of exposure. A maximum reduction occurred after 2 days of NE exposure ($F_{3,8} = 8.41, P < .01$). $K_D$ values were not significantly affected by any of the NE exposures (Figs. 7 and 9).

**Effect of Cocaine, Amphetamine, and NE on Uptake of [3H]NE in 293-hNET Cells.** To verify that the alterations in [3H]nisoxetine binding to NET and NET protein levels reflect changes in the capacity of 293-hNET cells to transport NE, the uptake of [3H]NE was measured in the intact 293-hNET cells exposed to different concentrations of cocaine, amphetamine, or NE for 3 days. As for all other experiments, exposures were followed by a 4-h washout in drug-free media. As a control comparison, the uptake of [3H]alanine was measured in parallel (same passage of cells, exposure to the same compounds on the same experiment day). In contrast to binding experiments and Western blotting, exposure of 293-hNET cells to 1, 10, and 100 μM cocaine significantly reduced the uptake of [3H]NE in a concentration-independent manner ($F_{7,40} = 54.6, P < .01$, Table 1). Uptake of [3H]NE was significantly inhibited by exposure to 10 and 100 μM amphet-

**Fig. 6.** Top, autoradiograph obtained by Northern blot analysis illustrating the lack of effect of cocaine and amphetamine on the human NET mRNA in 293-hNET cells. Isolated RNA (20 μg each) from cells exposed to 0 (control, C), 10, and 100 μM cocaine and 1, 10, and 100 μM amphetamine for 3 days were loaded onto the gel and hybridized with 32P-labeled human NET cDNA. Bottom, the same filter was again hybridized with 32P-labeled β-actin cDNA after stripping.

**Fig. 7.** Effect of exposure of intact 293-hNET cells to NE on $B_{\text{max}}$ and $K_D$ values of [3H]nisoxetine binding to NET in membrane homogenates. Each column represents data from four separate experiments. Asterisks indicate significant differences from the control group ($P < .01$). There were no significant differences in $K_D$ values between any of the groups.

**Fig. 8.** A, top, ECL photograph of immunoblotted NET in 293-hNET cells exposed to different concentrations of NE for 3 days. Each well was loaded with 25 μg of total protein and blots were probed with the N430 antibody. C, control group. Bottom, the same membrane was also probed with anti-actin antibody (after stripping) as an internal control. The positions of standard molecular mass markers in kilodaltons are indicated on the left of the blot. B, relative densities of 80- and 56-kDa bands of the film shown in A measured as optical density × pixel area.

**Fig. 9.** Time course of the effect of NE exposure (100 μM) on the specific binding of [3H]nisoxetine (2.5 nM) to NET. Each column represents data obtained from three separate experiments. Asterisks indicate significant differences from the control group (*$P < .05$, **$P < .01$).
The effect of cocaine and amphetamine exposures (3 days) on the uptake of \(l-[^3H]\)norepinephrine (50 nM) and \(l-[^3H]\)alanine (50 nM) in 293-hNET cells

<table>
<thead>
<tr>
<th>Drug Exposure ((\mu M)) (n = 4)</th>
<th>Control</th>
<th>Cocaine</th>
<th>Amphetamine</th>
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<tbody>
<tr>
<td>(l-[^3H])Norepinephrine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.28 ± 0.07(^a)</td>
<td>1.06 ± 0.03(^b)</td>
<td>1.04 ± 0.02(^b)</td>
<td>0.80 ± 0.04(^b)</td>
</tr>
<tr>
<td>(l-[^3H])Alanine</td>
<td>0.56 ± 0.02</td>
<td>0.55 ± 0.03</td>
<td>0.56 ± 0.01</td>
</tr>
</tbody>
</table>

\(^a\) Uptake of \(l-[^3H]\)norepinephrine and \(l-[^3H]\)alanine is expressed in pmol/mg of protein/min.

\(^b\) \(P < .01\), compared with control group.

The effect of norepinephrine exposure (3 days) on the uptake of \(l-[^3H]\)norepinephrine (50 nM) and \(l-[^3H]\)alanine (50 nM) in 293-hNET cells

<table>
<thead>
<tr>
<th>Drug Exposure ((\mu M)) (n = 4)</th>
<th>Control</th>
<th>Norepinephrine Exposure ((\mu M)) (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(l-[^3H])Norepinephrine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.34 ± 0.01(^a)</td>
<td>1.33 ± 0.05</td>
<td>1.34 ± 0.02</td>
</tr>
<tr>
<td>(l-[^3H])Alanine</td>
<td>0.56 ± 0.03</td>
<td>0.58 ± 0.02</td>
</tr>
</tbody>
</table>

\(^a\) Uptake of \(l-[^3H]\)norepinephrine and \(l-[^3H]\)alanine is expressed in pmol/mg of protein/min.

\(^b\) \(P < .01\), compared with control group.

P > .05, respectively, Tables 1 and 2), implying that the effects of these ligands are specific for the NET.

The effects of exposures to cocaine (100 \(\mu M\)), amphetamine (1 \(\mu M\)), and NE (100 \(\mu M\)) on the kinetics of NE uptake were also examined (Fig. 10). Nonlinear regression analysis demonstrated that the concentration for half-maximum saturation of uptake (\(K_m\)) of \([^3H]\)NE in control 293-hNET cells was 540 ± 60 nM, similar to that (457 nM) reported by Pacholszyk et al. (1991). Exposures to cocaine, amphetamine, or NE caused small, but not statistically significant, changes in the \(K_m\) of \([^3H]\)NE (cocaine, 830 ± 130 nM; amphetamine, 980 ± 220 nM; NE, 800 ± 160 nM). Maximum uptake velocities (\(V_{\text{max}}\)) after amphetamine and NE exposures were significantly reduced by 33 and 57% (\(P < .001\)), respectively, compared with that of control (Fig. 10; \(V_{\text{max}}\) values for control, 528 ± 9; amphetamine, 353 ± 12; NE, 229 ± 29 in units of pmol/mg of protein/min). Cocaine exposure produced a nonsignificant, 9% reduction in the \(V_{\text{max}}\) (478 ± 5 pmol/mg of protein/min). These kinetic data are consistent with binding and Western blot studies, indicating that exposure to amphetamine and NE reduces the number of functional NET. It is noteworthy that the small increases in \(K_m\) values observed after drug exposures are sufficient to produce substantial (~33%) reductions in uptake when measured at low substrate concentrations (computed from \(V/\text{max} = L/(L + K_m)\)), such as was performed in experiments used to evaluate NE and alanine uptake after drug exposures (Tables 1 and 2). Hence, significant reductions of uptake observed at 50 nM \([^3H]\)NE likely result from reductions in \(V_{\text{max}}\) as well as from increases in the \(K_m\). A change in \(K_m\) is a likely explanation for significant reductions of 50 nM \([^3H]\)NE after cocaine exposure measured as described above, and suggest that some residual cocaine may be retained in the media, despite attempts to wash it away. Cocaine exposure did not alter the \(K_D\) of \([^3H]\)nisoxetine (Fig. 1), as determined using binding assays that use repeated homogenizations, centrifugations, and washings.

**Microscopic Examination of 293-hNET Cells after Treatments.** Possible toxic effects of drugs were assessed by microscopic examination of 293-hNET cell morphology and by the counting of viable cells after exposures. No gross morphological changes in drug-treated 293-hNET cells were observed. With the exception of NE exposures, at no time point (or drug concentration) were there differences between drug-exposed 293-hNET cells and cells cultured in drug-free media in terms of the number of 293-hNET cells per milliliter. However, a possible toxic effect of NE on the cell line was observed. Generally, after 293-hNET cells reach confluence, cells continue to grow and become highly condensed, clustering above the monolayer, until harvesting. As with exposures to other drugs in this study, incubation of cells with different concentrations of NE was begun when cells reached confluence. Cells exposed to NE at concentrations of 100 and 1000 \(\mu M\) stopped growing, identified by reduced consumption of medium and reduced cell counts at harvesting. For example, cell numbers in the control group, and groups exposed for 3 days to 100 \(\mu M\) NE, or 1000 \(\mu M\) NE were 4.77 (± 0.64) \(\times 10^7\), 2.88 (± 0.34) \(\times 10^7\) and 2.63 (± 0.24) \(\times 10^7\)/flask, respectively. However, no change in the cell morphology (microscopic evaluation) or obvious cell death (as determined by the lack of cells floating in the medium) in the confluent cells was observed after the NE exposures.
Discussion

Previously, we reported that exposure of NET-expressing PC12 cells and 293-hNET cells to the NET inhibitors desipramine and nisoxetine produced down-regulation of NET expression and function (Zhu and Ordway, 1997; Zhu et al., 1998). The 293-hNET cell does not have synthetic machinery for catecholamine synthesis and catecholamines were not present in incubation media during exposures to inhibitors in these studies. Hence, the regulatory effects of inhibitor exposure appear to be substrate-independent and different from the activity-dependent (or substrate-dependent) regulation of the serotonin transporter recently described by Ramamoorthy and Blakely (1999). Based on the effects of desipramine and nisoxetine on NET expression (Zhu et al., 1998), it was predicted that down-regulation is a fundamental response of NET-expressing cells to exposure to NET inhibitors. To further test this conjecture, the present study was designed to examine the effect of two structurally unrelated NET ligands on NET expression in 293-hNET cells. Cocaine, like desipramine and nisoxetine, is an inhibitor of the NET, whereas amphetamine, which inhibits transport of NE, is itself a substrate for the NET. In marked contrast to the antidepressant NET inhibitors, exposure of intact cells to cocaine (up to 100 μM) did not significantly affect NET levels, as assessed by radioligand binding and Western blotting. As was reported previously for desipramine, exposure to the NET substrate amphetamine significantly down-regulated NET density, and produced a marked reduction in [3H]NE uptake. As a comparison, the natural substrate of NET, NE, also significantly reduced the Bmax of [3H]nisoxetine binding and NET protein level, when higher concentrations were used. These results demonstrate that down-regulation of the NET is not a common effect of all NET inhibitors. Furthermore, substrates of the NET are capable of down-regulating the NET as well, although possibly by different mechanisms.

The exposure concentrations of cocaine and amphetamine that were studied (particularly those that produced effects on the NET) were in the range of plasma or brain concentrations achieved in humans or laboratory animals after administration of behaviorally active doses (Isenschmid et al., 1993; Badiani et al., 1997). Published concentrations of cocaine and amphetamine are taken from well controlled laboratory studies. It is likely that concentrations that are achieved in the street use of these drugs (e.g., during binging) are considerably higher.

The effects of cocaine exposure on the NET have received little attention, despite the fact that cocaine is a potent inhibitor of the NET. The inability of cocaine to alter radioligand binding to the NET or NET protein levels here is consistent with the study of the effects of cocaine exposure in vivo. Benmansour et al. (1992) reported that cocaine (35 mg/kg daily) administered to rats for 10 days did not affect brain [3H]nisoxetine binding. Belej et al. (1996) reported that 30 days of cocaine treatment (25 mg/kg) to rats did not affect [3H]nisoxetine binding to the NET in many brain regions, although a transient reduction of binding was observed in some specific brain regions.

The exposure of 293-hNET cells to amphetamine produced a pronounced reduction of [3H]nisoxetine binding to NET and of NET protein levels. Interestingly, decreases in [3H]nisoxetine binding to NET have been used as an indicator of toxicity to neurons by high doses of methamphetamine (Brunswick et al., 1992). Therefore, the possibility that reductions of NET observed here result from amphetamine-induced toxicity must be considered. Studies on the neurotoxic potential of amphetamine and its analogs have identified predominant effects on brain dopamine and serotonin neurons in the striatum and cerebral cortex (Ellison et al., 1978; Ricart et al., 1985; Schmidt, 1987; Melega et al., 1997), whereas noradrenergic projections seemed to be spared. This toxicity is manifested by reductions in levels of dopamine (Ricart et al., 1984) and serotonin (Ellison et al., 1978; Ricart et al., 1985), and in the maximum velocity (Vmax) of labeled dopamine and serotonin uptake in several brain regions (Ricart et al., 1980). These changes are paralleled by swollen dopamine axons and fiber degeneration within these brain regions as illustrated by histochemistry (Ellison et al., 1978; Ricart et al., 1984, 1985), and by reductions of striatal dopamine integrity indices such as tyrosine hydroxylase (Fibiger and McGeer, 1971), dopamine concentration (Melega et al., 1997), and dopamine transporter densities (Brunswick et al., 1992). In contrast, no change has been observed in levels of NE (Ricart et al., 1984, 1985). In other studies, administration of methamphetamine reduced NET (determined by [3H]mazindol binding) in the cerebral cortex (subcutaneous injection for 4 days, Zaczek et al., 1989) and in subcortical regions (subcutaneous injection every 5 h for five doses, Brunswick et al., 1992) in rats. However, in these two studies, measurements were taken only 18 h after the injection of drug (Zaczek et al., 1989) or after a single day treatment (Brunswick et al., 1992). Such findings are, therefore, more likely a result of an acute effect of the drug not related to neurotoxicity (Brunswick et al., 1992). Hence, little data are available demonstrating neurotoxicity of amphetamine on noradrenergic neurons in vivo. In the present study, we did not observe changes in cell growth rate, determined by simple cell counting at the time of harvesting, or evidence of cell death, determined by counting of viable cells, after incubation with amphetamine for 3 days. Further specificity of the effect of amphetamine on NET expression was verified by demonstrating a lack of effect of amphetamine exposure on the uptake of the amino acid alanine. Hence, the loss of NET as a result of amphetamine exposure in vitro appears to be result of a down-regulation of NET expression rather than a result of neurotoxicity.

It is presumed that changes in transcription, translation, or protein turnover may contribute to down-regulation of NET after amphetamine exposure. Expression of NET in 293-hNET cells is under the control of the powerful cytomegalovirus promoter (Galli et al., 1995). The observation of no significant changes of NET mRNA after exposure of cells to amphetamine (Fig. 6) may be accounted for by constitutively active transcription of human NET cDNA in these cells. Therefore, it is still possible that amphetamine alters NET gene expression in other cells in vitro or in neurons in vivo, which express the native NET gene. However, given the lack of changes in levels of NET mRNA despite decreases of NET protein levels (Fig. 4), enhanced degradation of NET protein is a likely mechanism for amphetamine-induced down-regulation of NET in the 293-hNET cell line. More investigation aiming to elucidate this mechanism is needed.

As a comparison to amphetamine, we investigated the possible effect of the natural substrate of NET, NE, on the
NET. Similar to amphetamine, exposure to NE produced a reduction in [3H]nisoxetine binding and NET protein levels in concentration-independent and time-dependent manners. However, Western blots revealed an increase in a 55-kDa reduction in [3H]alanine (at 1 mM NE), indicating some possible toxic effect of NE. Rosenberg (1988) reported that incubation of primary cultures of cerebral cortex with 25 and 250 μM NE for 72 h results in toxicity, produced by the products of oxidative degradation of NE. In contrast to the present study, exposure of PC12 cells to similar concentrations of NE failed to change the Bmax of [3H]nisoxetine binding and did not influence PC12 cell growth (Zhu and Ordway, 1997). A possible explanation for differences between PC12 and 293-hNET cells regarding toxic effects of NE is that 293-hNET cells have ~65 times as much NET than PC12 cells (Zhu et al., 1998). Overexpression of NET in the 293-hNET cells would be expected to result in higher intracellular concentrations of NE. Furthermore, PC12 cells express vesicular monoamine transporters to package neurotransmitters into vesicles (Liu et al., 1996). Accumulation of toxin into intracellular vesicles via vesicular monoamine transporters is a proposed cytoprotective function of these vesicles (Liu et al., 1992; Gainetdinov et al., 1998). Because 293-hNET cells are not neuronal in origin, it would be expected that they do not express vesicular monoamine transporters on intracellular vesicles. Therefore, cytosolic concentrations of NE and its metabolites would be expected to be far higher in the 293-hNET cells than in PC12 cells and, as such, may induce a toxic effect in the 293-hNET cells. In support of this contention, exposure of wild-type HEK-293 cells (lacking the transfaction with the NET gene) to the same concentrations of NE for 3 days had no toxic effects on this cell line (data not shown). Overall, these findings imply that the effects of NE on NET expression in 293-hNET cells is likely secondary to toxic effects of NE, although some regulatory effects of NE similar to amphetamine cannot be ruled out.

Cocaine and amphetamine have different regulatory actions on NET expression in 293-hNET cells. Although both drugs bind to the NET, exposure to amphetamine, but not cocaine, down-regulated the NET. These and previous data (Zhu and Ordway, 1997; Zhu et al., 1998) demonstrate that exposure to some (desipramine, nisoxetine), but not all (cocaine), inhibitors of the NET can down-regulate the NET, and that exposure to substrates (amphetamine) can also down-regulate the NET. Hence, the ability of a compound to down-regulate the NET is not necessarily related to its activity once bound to the NET. Down-regulation of the NET might contribute to the therapeutic efficacy of antidepressant drugs because down-regulation could enhance and prolong the inhibition of NET function. Hence, elucidation of the biological processes responsible for ligand-induced regulation of the NET could reveal unique pharmacological targets for inhibiting NET function.

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References


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