Norepinephrine Transporter Regulation Mediates the Long-Term Behavioral Effects of the Antidepressant Desipramine

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The relationship between the ability of repeated desipramine treatment to cause downregulation of the norepinephrine transporter (NET) and produce antidepressant-like effects on behavior was determined. Treatment of rats with 15 mg/kg per day desipramine reduced NET expression, measured by ^{3}H-nisoxetine binding and SDS–PAGE/immunoblotting, in cerebral cortex and hippocampus and reduced the time of immobility in the forced-swim test. The antidepressant-like effect on forced-swim behavior was evident 2 days following discontinuation of desipramine treatment when plasma and brain levels of desipramine and its major metabolite desmethyldesipramine were not detectable. Reduced NET expression resulted in reduced norepinephrine uptake, measured *in vitro*, and increased noradrenergic neurotransmission, measured *in vivo* using microdialysis. Overall, the dose–response and time-of-recovery relationships for altered NET expression matched those for production of antidepressant-like effects on behavior. The importance of increased noradrenergic neurotransmission in the persistent antidepressant-like effect on behavior was confirmed by demonstrating that it was blocked by inhibition of catecholamine synthesis with α-methyl-*p*-tyrosine. The present results suggest an important role for NET regulation in the long-term behavioral effects of desipramine and are consistent with clinical data suggesting that enhanced noradrenergic neurotransmission is necessary, but not sufficient, for its antidepressant actions. Understanding the mechanisms underlying NET regulation *in vivo* may suggest novel targets for therapeutic intervention in the treatment of depression.
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INTRODUCTION

The therapeutic actions of antidepressant drugs develop gradually over time with repeated treatment (Frazer and Benmansour, 2002; Nelson *et al*, 2004; Wong and Licinio, 2001). This is sometimes referred to as the 'therapeutic lag' and appears to be a graded response that is somewhat symptom dependent, rather than a lack of any therapeutic effect followed by its emergence (Katz *et al*, 2004; Frazer, 2000). This delayed activity has raised questions regarding the relevance of the acute neurochemical effects of antidepressants, such as enhancement of monoaminergic neurotransmission, to their long-term effects on behavior. It has been argued that since the acute neurochemical effects are manifested early, they cannot, in and of themselves

mediate the slower developing therapeutic effects. However, while enhancement of monoaminergic activity may not be sufficient to produce antidepressant effects, it does appear to be necessary. This is evident from the finding that inhibition of synthetic enzymes for norepinephrine (NE) or serotonin results in a rapid return of symptoms in depressed patients treated successfully with desipramine or fluoxetine, respectively (Charney, 1998; Miller *et al*, 1996a). Further, inhibition of catecholamine synthesis with α-methyl-*p*-tyrosine (AMPT) increases depressive symptoms in patients with seasonal affective disorder (Lam *et al*, 2001). Similarly, reduction of serotonin synthesis with *para*-chlorophenylalanine or a tryptophan-free amino-acid drink reverses symptom remission induced by serotonin reuptake inhibitors (SRIs; Salomon *et al*, 1993).

Both pharmacokinetic and pharmacodynamic mechanisms may underlie the progressively developing effects of antidepressants. Drugs such as desipramine have relatively long half-lives and their metabolites often have even longer half-lives (Ziegler *et al*, 1978). Thus, accumulation of both parent drug and active metabolites can occur with repeated treatment (Ordway *et al*, 2005). Further, it appears that the

lipophilic nature of many antidepressants, including desipramine, contributes to their accumulation in membranes, where they have the potential to interact with the NE transporter (NET) to an extent greater than would be predicted from plasma concentrations (Zhu et al, 2004; Mandela and Ordway, 2006).

At early stages of desipramine treatment, noradrenergic neurotransmission is enhanced as a direct consequence of transporter blockade (See et al, 1992). Subsequently, dynamic, adaptive neuronal changes occur, such as downregulation of β-adrenergic receptors (Frazer and Conway, 1984; Ordway et al, 1988; Duncan et al, 1993), desensitization of presynaptic α-2 adrenergic receptors (Sacchetti et al, 2001), and downregulation of the NET (Weinshenker et al, 2002). In many cases of neuronal adaptation, eg downregulation of β-adrenergic receptors, the antidepressant-induced adaptation is homeostatic, ie in opposition to the acute drug effect. This contrasts with the progressive, unidirectional development of antidepressant effects in the clinical setting (Katz et al, 2004). Further, studies to date indicate that α-2 adrenergic autoreceptors remain largely functional after chronic desipramine treatment (Garcia et al, 2004; Lapiz et al, 2007b) and likely cannot account for time-dependent changes resulting from such treatment. However, antidepressant-induced NET regulation appears to be consistent with the clinical data. It shows a time course consistent with the delayed therapeutic effects and parallels the gradual onset of antidepressant-induced effects on depressive symptoms (Benmansour et al, 2002; Katz et al, 2004). Repeated, but not acute, treatment with desipramine reduces NET expression in brain, determined by ^{3}H-nisoxetine binding and western blotting (Benmansour et al, 2004; Zhu et al, 2002). This may be a direct effect of desipramine, rather than being secondary to increased synaptic concentrations of NE, since it also occurs in vitro with PC12 and human neuroblastoma cells (Zhu and Ordway, 1997; Zhu et al, 2005), as well as HEK-293 cells transfected with the NET (Zhu et al, 1998).

The goal of the present study was to determine the relationship between effects of repeated desipramine treatment on NET expression and function in the brain and its antidepressant-like effects on behavior. This was determined by assessing the effects of repeated desipramine treatment on NET expression and function in cerebral cortex and hippocampus, noradrenergic neurotransmission using in vivo microdialysis, and effects on behavior using the forced-swim test. The results indicate that repeated treatment with desipramine downregulates the NET in the brain, reducing NE uptake, which results in increased noradrenergic neurotransmission and antidepressant-like effects on behavior.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (Harlan, Indianapolis, IN), weighing 300–350 g, were housed two per cage in a temperature-controlled room (22–23°C) with a 12-h on–off light cycle (lights on at 0600 hours). Food and water were freely provided. Blind observations were used throughout all behavioral testing, which was carried out from 1300–1700 hours in a quiet room. All procedures were reviewed and approved by the Animal Care and Use Committees of West Virginia University Health Sciences Center and the University of Texas Health Sciences Center at San Antonio, and are consistent with the NIH 'Guidelines for the Care and Use of Laboratory Animals' (NIH Publication No. 80-23, Revised 1996).

Chronic Desipramine Administration

Rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and implanted subcutaneously with osmotic minipumps (model 2ML2, Alzet Corporation, Palo Alto, CA) preloaded with either vehicle (50% saline, 40% DMSO, and 10% ethanol) or desipramine (Sigma-Aldrich, St Louis, MO) at a concentration that delivered 5, 10, or 15 mg/kg per day of the free base (Benmansour et al, 2004). Minipumps were removed, under anesthesia, 14 days later. Rats were tested for antidepressant-like behavior in the forced-swim test 2–8 days after pump removal and discontinuation of desipramine treatment. Following the completion of the behavioral tests, rats were killed by decapitation, their brains were removed, and cerebral cortex and hippocampus were dissected for neurochemical analyses.

Forced-Swim Test

The forced-swim test was carried out as described previously (Porsolt et al, 1977; Zhang et al, 2006). Plexiglas cylinders (40 cm high and 18 cm in diameter) were filled with water (30 cm depth, 22–23°C); at this depth, rats could not touch the bottom of the cylinder with their tails or hind limbs. On day 1 (ie 1 day after cessation of desipramine treatment), the rats were pre-tested for 15 min to develop an immobility posture after initial escape-oriented movements (Cryan et al, 2002). On day 2 (2 days after cessation of desipramine treatment), rats were subjected to the 5-min forced-swim test and immobility time was recorded; immobility was defined as floating on the surface of the water with the only movement being that required to keep the head above water. Some rats were subjected to 5-min forced-swim testing 5 or 8 days after the end of the desipramine treatment regimen (15-min pre-test after 4 or 7 days, respectively), to determine the time of recovery. Finally, in some tests, rats were administrated 150 or 300 mg/kg AMPT (Sigma-Aldrich) prior to the 5-min forced-swim test, to examine the effects of catecholamine depletion on chronic desipramine-induced changes in immobility.

SDS–PAGE/Immunoblotting of NET Protein in the Rat Brain

Cerebral cortex and hippocampus were homogenized in TEVP buffer (10 mM Tris base, 5 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM ethylenediamine tetra-acetic acid (EDTA), 1 mM EGTA, 320 mM sucrose; pH 7.4) with a glass homogenizer and centrifuged at 1000g for 20 min at 4°C. The supernatant was centrifuged at 20 000g for 30 min to obtain a crude synaptosomal fraction (Lin et al, 1998; Wyszynski et al, 1998; Dunah and Standaert, 2001). The pellets were

resuspended and solubilized with RIPA buffer (50 mM Tris, 150 mM NaCl, 2% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 0.1% Triton X-100; pH 8.0) containing protease inhibitors (Roche, Indianapolis, IN). Protein content of membrane lysate was measured using the bicinchoninic acid protein assay (Smith et al, 1985; Pierce, Rockford, IL). Equal amounts of protein from both control and treated rats were mixed with $2 \times$ Laemmli sample buffer (Bio-Rad, Hercules, CA) and boiled at 100°C for 3 min. Samples were loaded onto 8% polyacrylamide gels for electrophoresis and, after separation, transferred to the nitrocellulose membranes. The membranes were blocked in TBST buffer containing 5% nonfat milk for 2 h at room temperature, washed with TBST buffer, and incubated overnight at 4°C with a specific polyclonal antibody generated against a unique 22 amino-acid peptide sequence mapping to the first extracellular domain of the NET (Chemicon, Temecula, CA). After washing with TBST, membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Pierce). The labeled protein bands were detected by chemiluminescence imaging (Bio-Rad) and quantified by densitometry. Comparisons of treatment effects were carried out within single gels.

^{3}H-Nisoxetine Binding Assay

^{3}H-Nisoxetine binding to the NET was carried out as described previously (Tejani-Butt et al, 1990; Bryan-Lluka et al, 2001). Briefly, half of the entire cerebral cortex and hippocampus were homogenized in 5 ml of ice-cold binding buffer (50 mM Tris base, 120 mM NaCl, 5 mM KCl; pH 7.4) using a Polytron (Brinkmann Instruments, Westbury, NY) and then centrifuged at $36\,000g$ for 10 min at 4°C. The supernatant was discarded, the pellet resuspended in ice-cold binding buffer, centrifuged at $36\,000g$ for 10 min at 4°C, and the pellet resuspended in binding buffer to obtain a protein concentration of 1 mg/ml as assayed using the bicinchoninic acid method (Smith et al, 1985; Pierce). Triplicate tubes containing $100\,\mu$l incubation buffer (total binding) or $100\,\mu$l desipramine (final concentration $50\,\mu$M; nonspecific binding), $100\,\mu$l tissue sample, and $50\,\mu$l ^{3}H-nisoxetine (final concentration 5 nM; 72 Ci/mmol; PerkinElmer, Boston, MA) were incubated in a shaking ice bath for 4 h. Nonspecific binding (ie in the presence of $50\,\mu$M desipramine) was approximately 10% of total binding. Bound ^{3}H-nisoxetine was captured by rapid filtration through fiberglass filters (GF/B Brandel, Gaithersburg, MD) that were pre-soaked in 5% polyethyleneimine, under vacuum with a cell harvester (Brandel). Filters were washed twice with ice-cold binding buffer and radioactivity was determined by liquid scintillation counting.

^{3}H-NE Uptake Assay

Uptake assays were carried out as described previously (Apparsundaram et al, 1998a, b; Vizi et al, 2004). Fresh rat cerebral cortex and hippocampus were placed in ice-cold Krebs-Ringer/N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) (KRH) buffer (125 mM NaCl, 5.2 mM KCl, 1.2 mM CaCl$_2$, 1.4 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 5 mM glucose, 20 mM HEPES, 0.2 mg/ml ascorbic acid, and

$100\,\mu$M pargyline; pH 7.4) gassed with 95% O$_2$/5% CO$_2$. The tissues were cut into 0.4 mm slices using a McIlwain tissue chopper (Brinkmann Instruments). Slices were separated by gentle shaking and washed with oxygenated KRH buffer. Uptake assays were performed by incubating the tissue slices with 30 nM ^{3}H-NE (10.9 Ci/mmol, PerkinElmer) for 10 min at 37°C. Assays were terminated by rapid washing of slices with 0.5 ml of ice-cold KRH buffer three times. Buffer was aspirated and slices were then sonicated in 10% trichloroacetic acid and centrifuged at $30\,000g$ for 2 min. ^{3}H-NE in the supernatant was quantified by liquid scintillation counting. Nonspecific uptake was determined in the presence of $20\,\mu$M desipramine. Specific uptake was determined by subtracting nonspecific uptake from total uptake.

Measurement of Extracellular NE in the Medial Prefrontal Cortex by In Vivo Microdialysis

At the same time the osmotic pumps were implanted, rats were placed in a stereotaxic frame with the incisor bar set at -3.3 mm. A microdialysis guide cannula (CMA/12; CMA Microdialysis, North Chelmsford, MA), aimed at the medial prefrontal cortex (mPFC), was implanted at coordinates AP $+2.6$ mm from bregma, ML ±1.4 mm from midline, DV -1.7 mm from dura. The guide cannula was anchored to the skull and an obturator was inserted. The wound was sutured, topical antibiotic was applied, and each rat was returned to its home cage. On the testing day, the obturator was removed and a microdialysis probe, with molecular weight cutoff of 20 kDa and 4 mm of active membrane, was inserted into the guide cannula; the probe extended 4 mm beyond the tip of the guide, centering the active membrane within the mPFC. The probe was perfused with artificial cerebrospinal fluid (147 mM NaCl, 2.5 mM KCl, 1.3 mM CaCl$_2$, 0.9 mM MgCl$_2$; pH 7.4) at a flow rate of $1.0\,\mu$l/min. After a 2 h equilibration period, four 30 min samples ($30\,\mu$l) were collected into tubes containing $2.5\,\mu$l of stabilizing solution ($0.2\,\mu$M EDTA, $0.2\,\mu$M ascorbic acid, 0.2 M perchloric acid). NE in the microdialysis samples was measured by high-performance liquid chromatography with coulometric detection (Coulochem2, ESA Inc., East Chelmsford, MA). The amount of NE in each sample was quantified against a calibration curve run daily, ranging from 0 to 25 pg, with a detection limit of 0.5 pg.

Measurement of Plasma and Brain Desipramine and Desmethyldesipramine

The measurement of desipramine and its primary metabolite desmethyldesipramine was carried out using a modification of a method described previously (Argenti and D'Mello, 1994). Plasma and brain were collected for analysis and stored at -70°C until assay. To $500\,\mu$l of plasma (either sample or blank standard) was added $100\,\mu$l of internal standard solution (imipramine 200 ng/ml) followed by $100\,\mu$l of ammonium hydroxide. The plasma was then extracted with 1 ml 1-butanol (20%) in hexane (pH 11) for 15 min on a reciprocating shaker. After separation of the phases by centrifugation at $2500g$ at 25°C for 3 min, the organic layer was transferred to fresh glass culture tubes and evaporated to dryness with nitrogen gas at 34°C. Each

sample was reconstituted with 100 µl mobile phase (0.025 M KH$_2$PO$_4$: acetonitrile, 70 : 30).

Half of the brain was homogenized in 8 ml carbonate buffer (0.1 M NaCO$_3$, pH 12). Aliquots (2 ml) of homogenates were transferred to glass tubes and 2 µg internal standard (imipramine) was added along with 3 ml 1-butanol (20%) n-hexane to both samples and blank brain standards. The mixture was shaken for 15 min and centrifuged at 2500g at 25°C for 3 min. The organic phase was transferred to a glass tube and the extraction was repeated again. The organic phase was evaporated by nitrogen gas and reconstituted to a 100 µl volume with mobile phase.

Chromatography was conducted using a reversed-phase column (Waters Symmetry Shield RP C$_{18}$ 4.6 × 150 mm, Milford, MA) and a 50 µl injector loop. The column temperature was ambient and the flow rate was set at 1.2 ml/min. The fluorescence of the eluent was monitored at an excitation wavelength of 260 nm with an emission wavelength of 360 nm. For desipramine and desmethyldesipramine determinations, recovery during extraction exceeded 90% and the lower limit of quantification was 25 ng.

Measurement of β-Adrenergic Receptor Density

β-Adrenergic receptor density was determined as described previously (O'Donnell, 1990). The right cerebral cortex was homogenized in 2 mM HEPES and 1 mM EDTA buffer (pH 7.75) for 15 s using a Polytron homogenizer. The homogenate was centrifuged at 40 000g for 20 min. The supernatant was removed and the wash procedure was repeated four times. The final pellet was suspended in SHM buffer (10 mM HEPES, 150 mM NaCl, 10 mM MgCl$_2$; pH 7.75). Saturation experiments were carried out to determine the density of the β-1 and -2 adrenergic receptors. Membrane suspensions were incubated in triplicate at 37°C for 30 min with ^{125}I-pindolol (50–1000 pM) alone, ^{125}I-pindolol and 100 nM CGP20712A (to block binding of IPIN to β-1 adrenergic receptors), or ^{125}I-pindolol plus 100 µM isoproterenol (to define nonspecific binding). The reaction was stopped by the addition of 6 ml of ice-cold SHM, and the samples were filtered over a vacuum through Schleicher and Schuell No. 25 fiberglass filters. The filters were washed twice with 3 ml of ice-cold SHM buffer and counted for radioactivity using a gamma counter at an efficiency of 75%.

Statistical Analysis

The data were analyzed by one-way analyses of variance followed by Tukey's or Bonferroni's post hoc tests. Data are presented as means ± SEM and differences are considered statistically significant when p-values are < 0.05.

RESULTS

Effects of Repeated Desipramine Treatment on NET Expression and Behavior in the Forced-Swim Test

The relationship between the effects of chronic desipramine treatment on NET expression and its effects on behavior in the forced-swim test, a preclinical test predictive of
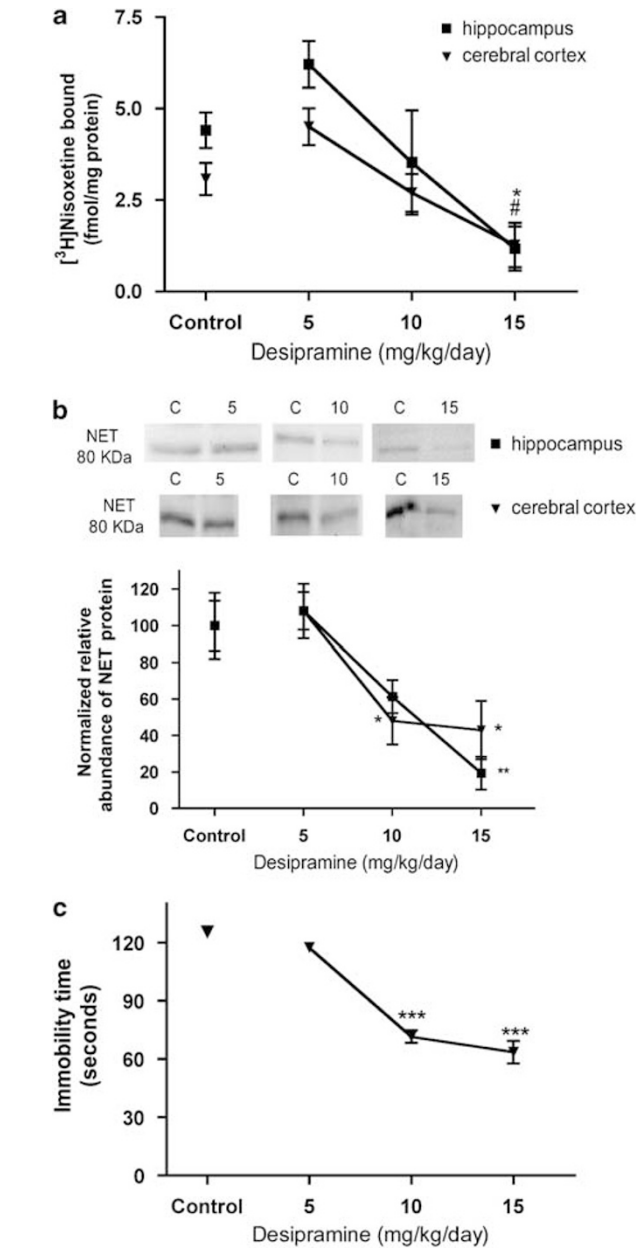
Figure 1 Dose–response functions were determined for the ability of chronic desipramine treatment to reduce norepinephrine transporter (NET) expression in the cerebral cortex and hippocampus, determined by ^{3}H-nisoxetine binding (a) and SDS–polyacrylamide gel electrophoresis (PAGE)/immunoblotting with antisera against the NET (b), and to reduce immobility time in the forced-swim test (c). All measures were determined 2 days after the end of the chronic treatment period, when brain and plasma concentrations of desipramine and its metabolite desmethyldesipramine were not detectable (ie below the 25 ng detection limit of the assay). Data shown are means ± SEM of 5–6 rats per group. *p < 0.05, **p < 0.01, ***p < 0.001 vs control.

antidepressant activity, was examined. Treatment of rats with desipramine for 14 days reduced NET expression in a dose-dependent manner, as indicated by a reduction of the specific binding of ^{3}H-nisoxetine to the NET in preparations of cerebral cortex (F$_{(3,16)}$ = 4.33, p < 0.05; Figure 1a) and hippocampus (F$_{(3,16)}$ = 4.34, p < 0.05;

Figure 1a). Downregulation of the NET also was demonstrated using SDS–PAGE/immunoblotting of preparations of cerebral cortex ($F_{(3,16)} = 4.75$, $p < 0.05$; Figure 1b) and hippocampus ($F_{(3,15)} = 13.10$, $p < 0.001$; Figure 1b). Importantly, this NET downregulation was observed 2 days after discontinuation of chronic desipramine treatment, a time when plasma and brain concentrations of desipramine and desmethyldesipramine were undetectable (ie below the 25 ng detection limit of the assay). By comparison, 1 h after acute treatment with 10 mg/kg desipramine, which causes similar effects on forced-swim behavior, plasma concentrations (mean ± SD) were $0.5 ± 0.3$ (desipramine) and $0.09 ± 0.02 \mu g/ml$ (desmethyldesipramine) and brain concentrations were $3.4 ± 0.1$ (desipramine) and $0.09 ± 0.03 \mu g/g$ (desmethyldesipramine) protein. In parallel with the downregulation of the NET, chronic desipramine also produced a dose-dependent reduction in the duration of immobility in the forced-swim test ($F_{(3,18)} = 91.23$, $p < 0.001$; Figure 1c). This was evident 2 days after discontinuation of chronic desipramine treatment and paralleled the reduction of NET expression (Figure 1b). This treatment also reduced the B_{max} for ^{125}I-pindolol binding to β-1 adrenergic receptors in cerebral cortex (control: $73.3 ± 4.6$; 2 days post-desipramine treatment: $40.7 ± 3.4$ fmol/mg protein; $n = 5$ per group; $p < 0.01$); β-2 adrenergic receptor density was unchanged.

Recovery of NET Expression and Behavioral Effects Following Discontinuation of Repeated Desipramine Treatment

Chronic treatment with desipramine reduced NET expression and produced a persistent antidepressant-like effect in the forced-swim test. Experiments were carried out to examine the relationship between changes in NET expression and behavior. Rats were treated chronically with 15 mg/kg per day desipramine for 14 days and subjected to the forced-swim test 2, 5, or 8 days after the end of desipramine treatment (ie removal of the osmotic pumps). Rats were then killed and brains regions reserved for assessment of NET expression and function; NET expression was determined using ^{3}H-nisoxetine binding and western blotting and NET activity by measurement of ^{3}H-NE uptake *in vitro*.

A significant reduction of NET binding sites, measured by ^{3}H-nisoxetine binding, was evident 2 days after the discontinuation of chronic desipramine treatment in the cerebral cortex ($F_{(3,19)} = 8.59$, $p < 0.001$; Figure 2a) and hippocampus ($F_{(3,19)} = 3.22$, $p < 0.05$; Figure 2a). The NET binding sites were partially recovered 5 days, and fully recovered 8 days, posttreatment. Consistently, decreased expression of NET also was observed by SDS–PAGE/immunoblotting 2 days after the discontinuation of chronic desipramine treatment in the cerebral cortex ($F_{(3,12)} = 6.98$, $p < 0.01$; Figure 2b) and hippocampus ($F_{(3,12)} = 4.26$,
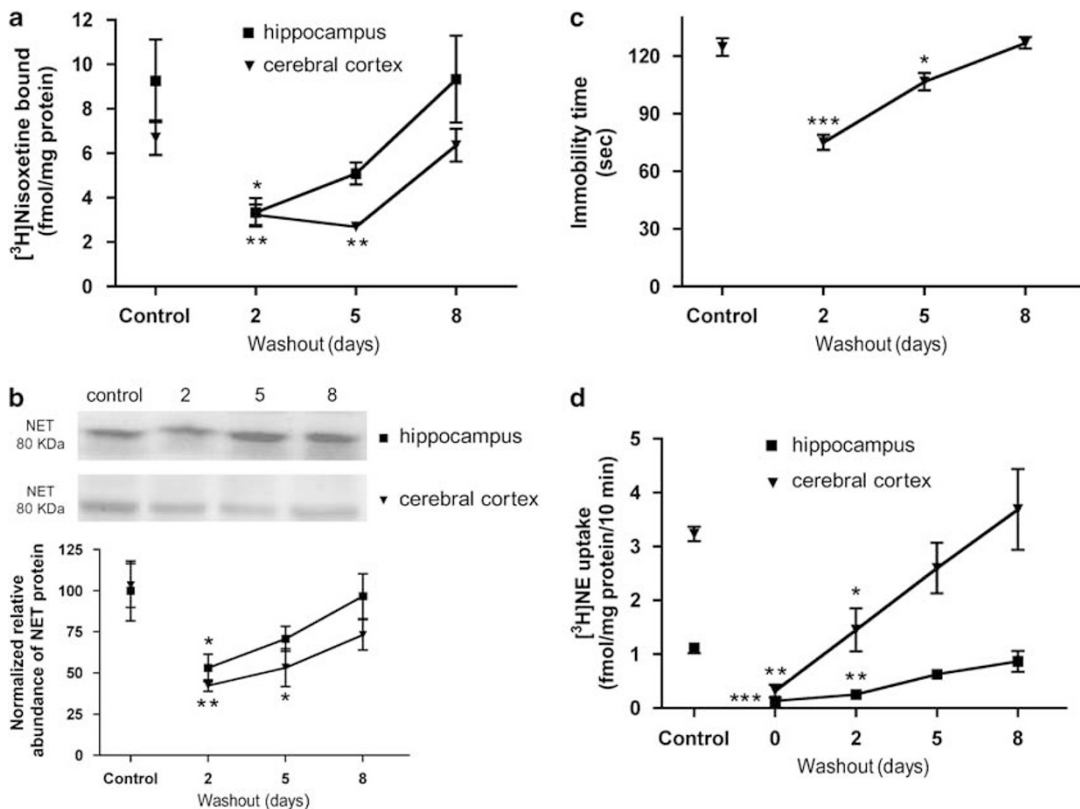
Figure 2 The recovery time course of chronic desipramine on norepinephrine transporter (NET) expression in the cerebral cortex and hippocampus, measured by ^{3}H-nisoxetine binding (a) and SDS–polyacrylamide gel electrophoresis (PAGE)/immunoblotting with antisera against the NET (b). The persistent antidepressant-like effect in the forced-swim test was evident 2 days after the end of the chronic treatment period, but dissipated by 8 days (c). The uptake of ^{3}H-NE into tissue slices, which reflects the activity of the NET, was reduced 2 days after discontinuation of desipramine treatment, and returned to the control level after 5–8 days (d). Data shown are means ± SEM of 5–10 rats per group. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ *vs* control.

$p < 0.05$; Figure 2b). The downregulation of the NET protein expression was partially reversed 5 days, and completely recovered 8 days, posttreatment. The time course of NET expression and activity was paralleled by behavioral effects in the forced-swim test. Rats tested 2 days following the end of chronic desipramine treatment displayed a reduced duration of immobility in the forced-swim test ($F_{(3,18)} = 27.15$, $p < 0.001$; Figure 2c). This antidepressant-like effect was partially reversed 5 days and completely disappeared 8 days posttreatment.

The NET activity, determined by measurement of uptake of ^{3}H-NE into cerebral cortical and hippocampal slices *in vitro*, exhibited a pattern similar to NET expression. NE uptake was significantly reduced 2 days after the end of chronic desipramine treatment and returned to control levels by 8 days posttreatment in both cerebral cortical ($F_{(4,19)} = 3.92$, $p < 0.05$; Figure 2d) and hippocampal slices ($F_{(4,21)} = 26.35$, $p < 0.001$; Figure 2d).

Extracellular NE Concentration in mPFC after Chronic Desipramine Treatment

Overall, the results described above indicate that repeated desipramine treatment reduced NET expression in cerebral cortex and hippocampus, resulting in a persistent anti-depressant-like effect on behavior that was not dependent on the presence of desipramine. Such a reduction in NET expression and function would be expected to result in enhanced noradrenergic neurotransmission in the brain since the primary method of inactivation, ie reuptake, would be impaired. To examine this, the extracellular concentration of NE was measured in the mPFC by *in vivo* microdialysis. A representative photomicrograph of a cresyl violet-stained section in which the microdialysis probe track was localized to mPFC is shown in (Figure 4a). It was found that the basal NE concentration was increased more than twofold 2 days after the discontinuation of repeated desipramine treatment ($F_{(3,50)} = 20.14$, $p < 0.001$; Figure 3b). The extracellular NE concentration returned to control values by 5 days posttreatment. This period of enhanced noradrenergic neurotransmission was consistent with that of reduced NET expression and function, as well as the persistent effects in the forced-swim test (see above). Also, consistent with persistently increased extracellular NE concentration during chronic desipramine treatment, the density of β-1 adrenergic receptors in cerebral cortex, determined by ^{125}I-pindolol (200 pM) binding, was reduced 2 days following the end of the repeated-treatment regimen (control: 29.8 ± 1.7; 2 days post-desipramine: 12.8 ± 1.8 fmol/mg protein; $n = 5$ per group; $p < 0.01$); β-1 receptor density was not different from control levels 5 and 8 days posttreatment (27.2 ± 1.9 and 30.8 ± 3.2 fmol/mg protein, respectively; $n = 5$ per group).
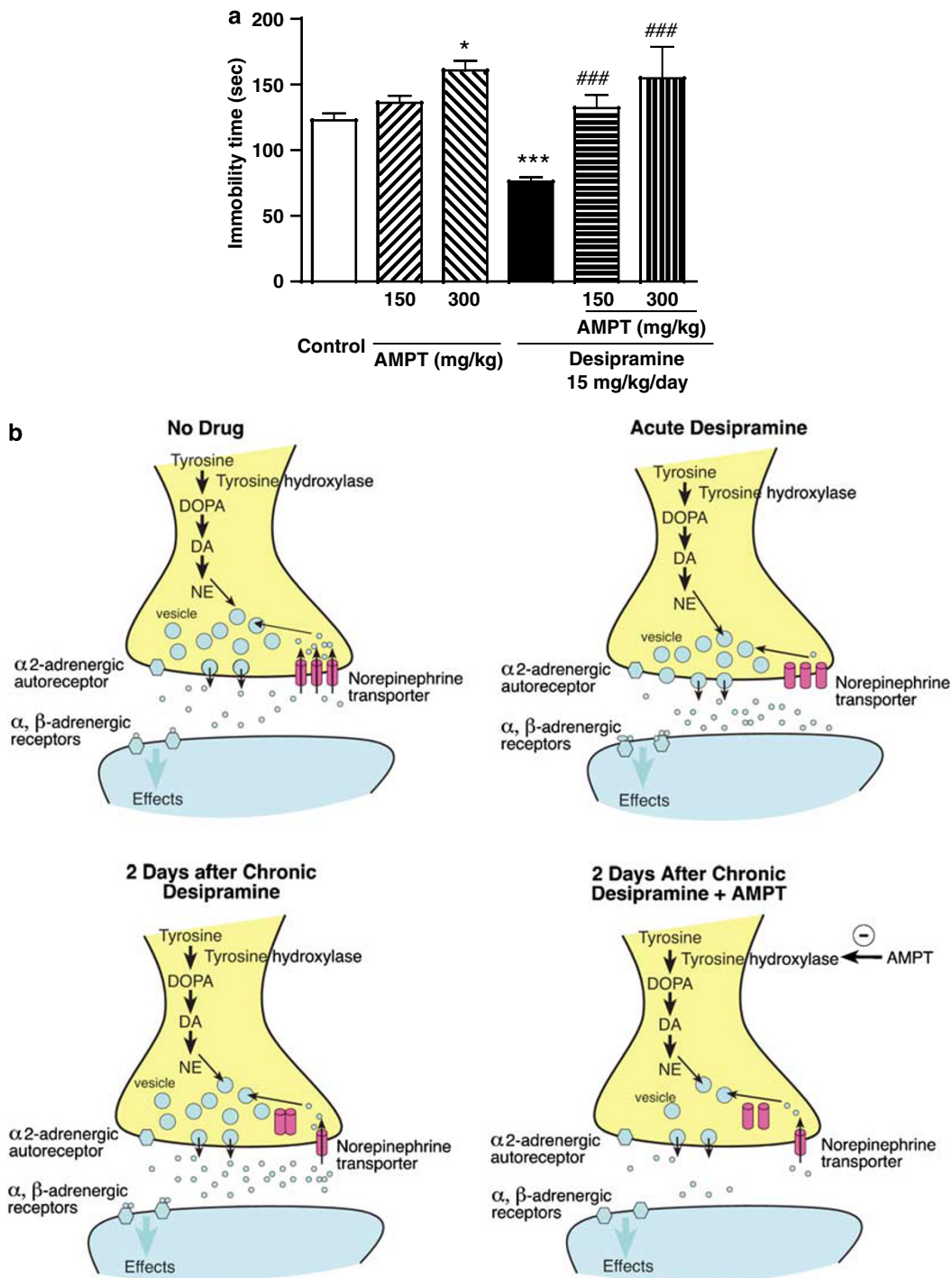
Effect of Catecholamine Depletion on the Persistent Effects of Desipramine Treatment in the Forced-Swim Test

To determine whether enhanced noradrenergic neurotransmission was necessary for the persistent antidepressant effect in the forced-swim test, an NE depletion approach was utilized, similar to that used to assess the role of
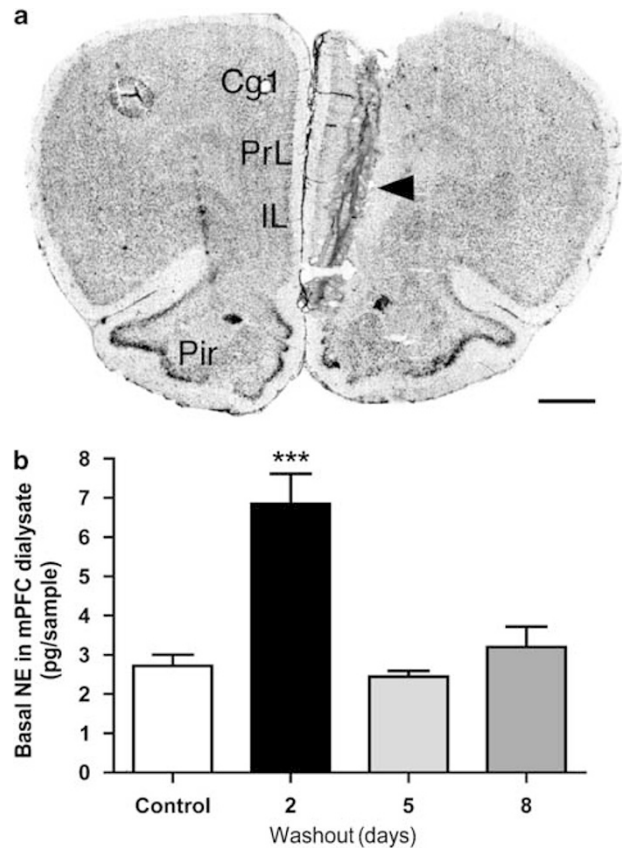
Figure 3 Representative photomicrograph of a cresyl violet-stained section, corresponding to Plate 9 in the atlas of Paxinos and Watson (1998), showing the microdialysis probe track in medial prefrontal cortex (mPFC) on the right (arrow). Brain regions are labeled on the left side for clarity. The guide cannula extended into the lower part of Cg1, and the 4 mm active probe track extended from the uppermost part of area PrL to just below area IL, thus including both major components of rat mPFC. Abbreviations: Cg1, cingulate cortex region 1; IL, infralimbic cortex; Pir, piriform cortex; PrL, prelimbic cortex (a). Chronic 15 mg/kg per day desipramine treatment with 2 days washout significantly elevated the tonic baseline of norepinephrine (NE) in mPFC, measured by *in vivo* microdialysis (b). Data shown are means ± SEM of 11–12 rats per group. ***$p < 0.001$.

noradrenergic neurotransmission in the clinical actions of desipramine (Miller *et al*, 1996a, b). Two days after the end of chronic desipramine treatment, when the NET expression was reduced and noradrenergic neurotransmission increased, rats were treated with the AMPT, an inhibitor of tyrosine hydroxylase, the rate-limiting enzyme for NE biosynthesis. AMPT (150 or 300 mg/kg, i.p.) was administrated 4 h before the forced-swim test to rats that had been treated chronically with vehicle or desipramine. AMPT treatment resulted in a dose-dependent loss of the persistent, antidepressant-like effect of chronic desipramine in the forced-swim test ($F_{(5,35)} = 17.64$, $p < 0.001$; Figure 4a). An acute dose of 150 mg/kg AMPT was effective in reversing the actions of chronic desipramine treatment; this dose of AMPT did not affect behavior in the forced-swim when administered alone. Administration of 300 mg/kg AMPT also reversed the persistent behavioral effect of desipramine, but when administered alone it produced a depressant-like effect on forced-swim behavior ($F_{(5,35)} = 17.64$, $p < 0.05$; Figure 4a).

Figure 4 To examine whether enhanced noradrenergic neurotransmission was necessary for the persistent antidepressant-like effect in the forced-swim test, α-methyl-*p*-tyrosine (AMPT), an inhibitor of tyrosine hydroxylase, was administered 2 days after the end of the chronic desipramine treatment regimen. AMPT reversed the antidepressant-like effect in the forced-swim test, even at a dose where it did not affect behavior on its own (a). The changes in noradrenergic neurotransmission after acute and chronic desipramine treatment are demonstrated in this diagram (b). The membrane NE transporter (NET) is blocked by acute desipramine treatment, resulting in an increased synaptic NE concentration. Two days after completion of chronic desipramine treatment, when the plasma and brain concentrations of desipramine and its major active metabolite are not detectable, the membrane NET is downregulated. Under this condition, the increase of synaptic NE and antidepressant effect on behavior result not only from the direct blocking of NET by the desipramine, but also from reduced NET expression and function. Inhibition of NE synthesis with AMPT reverses the increase in neurotransmission, resulting in a loss of antidepressant efficacy, even though the NET is downregulated. Data shown are means ± SEM of 5–7 rats per group. *$p < 0.05$, ***$p < 0.001$ *vs* control; ###$p < 0.001$ *vs* desipramine alone.

DISCUSSION

The present results show a relationship between desipramine-induced downregulation of the NET and antidepressant effects on behavior. These exhibit similar dose–response and time-of-recovery relationships. Importantly, the downregulation of the NET results in antidepressant effects on behavior, even when there is no drug present to inhibit NE transport. Consistent with this, NET knockout mice show a similar antidepressant-like effect in forced-swim and tail-suspension tests, due to reduced clearance rates of NE (Xu et al, 2000). It has been suggested that the functional consequence of downregulation of monoamine transporters is greater than pharmacological inhibition (Klimek et al, 1997) due to its noncompetitive nature vs the competitive nature of pharmacological inhibition. The results of the microdialysis experiment indicate that reduced NET expression does result in increased extracellular concentrations of NE, indicative of enhanced noradrenergic neurotransmission, similar to what is observed when the NET is inhibited acutely by desipramine (Lapiz et al, 2007a).

Clinical actions of antidepressants develop gradually over weeks of treatment (Frazer and Benmansour, 2002; Katz et al, 1996, 2004), leading to the suggestion that neuroadaptive processes underlie the long-term behavioral effects. In the present study, it was found that the expression and activity of NET were significantly reduced by repeated desipramine treatment after a 2-day washout, when plasma and brain antidepressant drug concentrations were not detectable. The density of postsynaptic β-1 adrenergic receptors also decreased 2 days after chronic administration of 15 mg/kg per day desipramine for 14 days, consistent with previous findings (Ordway et al, 1988). Desipramine-induced reduction in immobility is associated with decreased β-1 adrenergic receptors; both effects are blocked by coadministration of β-1 adrenergic receptors antagonists (Kitada et al, 1986; Mancinelli et al, 1991). Both β-1 adrenergic receptor and NET expression were significantly reduced 2 days after chronic desipramine treatment, and recovered after 8 days of withdrawal, suggesting that regulation of both NET and β-1 adrenergic receptors is involved in adaptive mechanisms for noradrenergic neurons. The increased synaptic NE concentration that results from NET downregulation likely contributes to the downregulation of β-1 adrenergic receptors (Ordway et al, 1988). Repeated desipramine treatment causes other neuronal changes that may contribute to antidepressant activity, including increased ^{3}H-GTP binding to G_s protein coupled to β-1 adrenergic receptors (Yamamoto et al, 1990), increased CREB mRNA expression (Nibuya et al, 1996), induction of BDNF expression (Nibuya et al, 1996), and increased neurogenesis in the dentate gyrus and hippocampus (Santarelli et al, 2003; Kodama et al, 2004; Chen et al, 2006; Sairanen et al, 2005).

The mechanism underlying desipramine-induced downregulation of the NET has not been fully determined. In addition to being observed in vivo (Zhu et al, 2002; Weinshenker et al, 2002), it also has been shown in vitro in cell lines expressing the NET. Using western blotting, NET expression in SK-N-BE(2)M17 cells was shown to be decreased by 3–14 days of exposure to desipramine (Zhu et al, 1998).

This suggests that desipramine-induced NET regulation is, at least in part, a direct effect and not secondary to an increase in the extracellular concentration of NE. In vitro, both the NET and serotonin transporter (SERT) have been shown to be regulated via a protein kinase C (PKC)-dependent pathway (Apparsundaram et al, 1998a, b; Jayanthi et al, 2005). Desipramine and other NE reuptake inhibitors may stimulate some specific factors in the PKC pathway and trigger a signal transduction cascade activating PKC. Activation of PKC by β-PMA downregulates and internalizes membrane-associated NET (Jayanthi et al, 2004). The NET-PP2A-Ar complex and the NET-syntaxin 1A complex have been shown to be involved in the PKC-mediated process (Sung et al, 2005; Sung and Blakely, 2007). It has been reported that inhibition of IP$_3$ receptors by 2-aminoethoxydiphenyl borate and xestospongin C reduces the V_{max} of NET, as well as NET expression (Amano et al, 2006). Given that inhibition of IP$_3$ receptors simultaneously results in PKC inhibition, it appears that the regulation of the NET is under the control of calcium/PKC-mediated mechanisms. However, this has not been demonstrated in vivo. Such a demonstration might suggest novel means to produce NET downregulation and antidepressant effects on behavior.

Whether the downregulation of NET is mediated by altered NET gene transcription has been a point of investigation. Previous studies using HEK-293 and SK-N-BE(2)M17 cells have shown that desipramine-induced reduction of NET expression primarily is due to translocation or increased degradation of the NET, but have not ruled out altered transcription (Zhu et al, 1998, 2002). However, in vivo, it was found that NET mRNA expression in the locus ceruleus is elevated after chronic desipramine treatment (Szot et al, 1993). Further, it was found that the mRNA expression of another monoamine transporter, the SERT, in the raphe nucleus is unchanged following chronic treatment with the SRI paroxetine (Benmansour et al, 1999).

While the present study focused on NET expression and the effects of desipramine, it is likely that similar mechanisms are involved in the long-term effects of SRIs. Repeated treatment with drugs from this pharmacological class reduces SERT expression and increases serotonergic neurotransmission. Using quantitative autoradiography, a marked reduction of ^{3}H-cyano-imipramine binding to the SERT is observed after chronic paroxetine or sertraline treatment. Similarly, using ^{3}H-citalopram homogenate binding, SERT binding sites in the rat prefrontal cortex are reduced after chronic paroxetine or sertraline treatment (Gould et al, 2006). Accordingly, the SERT reuptake activity (ie clearance) in the CA3 region of hippocampus is reduced after chronic treatment with SRIs such as paroxetine and sertraline; desipramine does not affect SERT function (Benmansour et al, 2002). The extracellular serotonin concentration, measured by in vivo microdialysis, is elevated in the hippocampus and caudate nucleus in the awake monkey after 3-day fluoxetine treatment (Smith et al, 2000). However, to date, it has not been reported whether enhanced, persistent downregulation of the SERT results in antidepressant effects on behavior, even when the drug is not present. Dual NE and SRIs, which may produce persistent effects on both noradrenergic and serotonergic neurotransmission, may possess greater efficacy and a more rapid onset of action (Millan, 2006).

Among the adaptive changes that may underlie the long-term effects of antidepressant treatment, NET and SERT regulation fit with the clinical data to a considerable degree. From a functional perspective, the direction of the long-term change in transporter expression following antidepressant treatment is the same as that seen with acute treatment with an antidepressant. In both cases, there is a reduction in transporter function and enhanced mono-aminergic neurotransmission. By contrast, changes in monoaminergic receptor expression often are homeostatic, ie in opposition to the effect of acute antidepressant treatment (Ordway *et al*, 1991). Clinically, in untreated patients, it has been reported that reducing catecholamine activity with AMPT or serotonergic activity with tryptophan depletion has no obvious effects on symptoms of depression, indicating that monoamine deficiency itself is insufficient to cause symptoms of depression (Miller *et al*, 1996b). However, catecholamine depletion by AMPT results in depression relapse in the patients treated chronically with NE reuptake inhibitors such as desipramine, but not SRIs (Miller *et al*, 1996a; Bremner *et al*, 2003). By contrast, inhibition of serotonin synthesis with *para*-chlorophenyla-lanine or a tryptophan-free amino-acid drink reverses symptom remission caused by SRIs, but not NE reuptake inhibitors (Miller *et al*, 1996a; Salomon *et al*, 1993).

It was found, consistent with these clinical findings, that inhibition of catecholamine synthesis with AMPT reversed the persistent antidepressant-like effect of desipramine in the forced-swim test. Changes in both noradrenergic and dopaminergic neurotransmission may contribute to this effect of AMPT (O'Donnell and Seiden, 1984). A specific role for noradrenergic neurotransmission is suggested by the finding that NE-deficient mice fail to respond to the behavioral effects of the NE reuptake inhibitor desipramine. Restoration of NE by L-threo-3,4-dihydroxyphenylserine reinstates the behavioral effects of desipramine in these mice (Cryan *et al*, 2004). Overall, the preclinical and clinical data suggest that enhanced monoaminergic neurotransmission is necessary, but not sufficient, for producing antidepressant effects, at least for those drugs that act via inhibition of reuptake. A schema describing pharmacological mechanisms thought to underlie the effects of desipramine is shown (Figure 4b). Following acute treatment, desipramine inhibits the NET, reducing NE uptake, which results in an increased extracellular NE concentration and an antidepressant-like behavioral response. Two days following chronic desipramine treatment, when the plasma and brain levels of desipramine and its major active metabolite are not detectable, NET expression and function are reduced, resulting in enhanced noradrenergic neurotransmission and an antidepressant response. However, treatment with AMPT at this time reduces noradrenergic neurotransmission, resulting in a loss of the antidepressant-like response, consistent with clinical findings (Miller *et al*, 1996a).

Overall, the present results demonstrate a relationship between desipramine-induced downregulation of the NET and antidepressant-like effects on behavior. NET downregulation, which was evident for at least 2 days following discontinuation of treatment, persisted even in the absence of detectable levels of desipramine or its major metabolite in plasma or brain. NET downregulation resulted in reduced NE uptake, measured *in vitro*, and enhanced noradrenergic neurotransmission, measured in the mPFC *in vivo*. Neuroimaging studies have consistently implicated the mPFC in depression, and in the response to antidepressant treatment (Drevets, 2000). Also consistent with clinical data, it was found that the antidepressant-like effect that resulted from NET downregulation depended on the enhanced noradrenergic neurotransmission since it was lost when NE synthesis was inhibited. The present data provide support for the idea that NET downregulation may contribute to the long-term therapeutic effects of some antidepressant drugs. Understanding the mechanism of NET regulation *in vivo*, which *in vitro* data suggest is due more to altered internalization and degradation than altered transcription, may suggest novel targets for therapeutic intervention in the treatment of depression.

DISCLOSURE/CONFLICT OF INTEREST

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Z Zhao, A Baros, M Lapiz, C Bondi, and D Morilak declare no financial interests that could be perceived as constituting a potential conflict of interest.

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