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Association of changes in norepinephrine and serotonin transporter expression with the long-term behavioral effects of antidepressant drugs

Zaorui Zhao
West Virginia University

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James M. O'Donnell, Ph.D., Chair
Patrick S. Callery, Ph.D.
Han-Ting Zhang, M.D., Ph.D.
Adrienne K. Salm, Ph.D.
Jason D. Huber, Ph.D.

Department of Basic Pharmaceutical Sciences

Morgantown, West Virginia

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Abstract

Association of changes in norepinephrine and serotonin transporter expression with the long-term behavioral effects of antidepressant drugs

Zaorui Zhao

The relationship between the ability of repeated antidepressant treatment to cause down-regulation of the norepinephrine and serotonin transporters (NET, SERT) and produce antidepressant-like effects on behavior was determined. Treatment of rats with 15 mg/kg/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 two days following discontinuation of desipramine treatment when plasma and brain levels of desipramine and its major metabolite desmethyl-desipramine were not detectable. Reduced NET expression resulted in decreased norepinephrine (NE) uptake, measured *in vitro*, and increased noradrenergic neurotransmission, measured *in vivo* using microdialysis. Fourteen-day treatment of rats with 20 mg/kg/day protriptyline or 7.5 mg/kg/day sertraline reduced NET and SERT expression, measured by ^{3}H-nisoxetine/^{3}H-citalopram binding and SDS-PAGE/immunoblotting, in cerebral cortex and hippocampus and reduced the time of immobility in the forced-swim test. Six-week, but not 2-week, treatment with 20 mg/kg/day reboxetine caused down-regulation of NET and an antidepressant-like effect in the forced-swim test. Antidepressant-induced NET and SERT down-regulation was not due to the reduction of gene transcription, as determined using quantitative, real-time RT-PCR.

Since studies using cell cultures have revealed a role of protein kinase C (PKC) in NET regulation, experiments were carried out to assess the importance of this mechanism in brain tissues and determine its role in the mediation of antidepressant-like effects on behavior. It was found that the PKC activators β-PMA and bryostatin-1 reduced NE uptake in cerebrocortical slices; this was due to decreased V_{max} and unchanged K_m values. Further, bilaterally intracerebroventricular (ICV) administration of β-PMA produced a significant antidepressant-like effect on forced-swim behavior, which was reversed by co-administration of 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), a PKC inhibitor.

Overall, antidepressant-induced changes in NET/SERT expression paralleled those in the antidepressant-like effects on behavior. The antidepressant-like effects of desipramine and sertraline were blocked by inhibition of catecholamine synthesis with alpha-methyl-p-tyrosine (AMPT) and inhibition of serotonin (5-HT) synthesis with para-chlorophenylalanine (PCPA), respectively, suggesting that increased noradrenergic or serotonergic neurotransmission is an important mechanism underlying antidepressant activity. In addition, the results, which are consistent with clinical data in terms of time-course respone, suggest an important role of NET and SERT regulation in the long-term

behavioral effects of antidepressant drugs and that enhanced noradrenergic or serotonergic neurotransmission is necessary, but not sufficient, for its antidepressant actions. Understanding the mechanisms underlying NET and SERT regulation *in vivo* may suggest novel pharmacological targets for treating depression. Future studies may focus on identifying the role of PKC signaling in NET regulatio, given thaht this signaling pathway appears to be an important mediator contributing to the long-term behavioral effects of antidepressant treatment.

Dedicated to

My Parents

Jifa Zhao and Yiping Fan

My Wife

Yang Bai

AND

My beloved newborn baby boy

Devin Zhao

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5-HT	serotonin
AMPT	alpha-methyl-para-tyrosine
CRH	corticotropin releasing hormone
ECT	electroconvulsive therapy
HPA	hypothalamic-pituitary-adrenal
ICV	intracelebroventracular
IP	intraperitoneal
LC	locus celeulous
MAOI	monoamine oxidase inhibitor
NE	norepinephrine
NET	norepinephrine transporter
NK1R	neurokinin-1 receptor
NRIs	noradrenergic reuptake inhibitors
PCPA	para-chlorophenyalanine
PDE4	phosphodiesterase 4
PKA	protein kinase A
PKC	protein kinase C
RT-PCR	real-time polymerase chain reaction
SERT	sertonin transporter
SNRIs	serotonin-norepinephrine reuptake inhibitors
SSRIs	selective serotonin reuptake inhibitors

CHAPTER 1

INTRODUCTION

LITERATURE REVIEW

1.1 Depression and its treatment

The mood disorders, primarily major depressive-anxiety and bipolar disorder, are among the most prevalent forms of mental illness and are associated with dramatic reductions in work capacity, overall health, and longevity (Thase and Denko, 2008; Nestler et al., 2002). Severe depression affects 2% - 5% of the U.S. population during a single year; 20% of the population suffers from milder depression. Mood disorders are life threatening due to the high risk of suicide (Blazer, 2000; Akiskal, 2000).

Diagnosis of depression is based on symptomatic criteria in the Diagnostic and Statistical Manual (DSM-IV, 2000) and its severity can be quantified using the Hamilton depression rating scale (HAM-D) (Hamilton, 1960; Hamilton, 1967). These symptomatic criteria consist of depressed mood, irritability, low self-esteem, feelings of hopelessness, worthlessness, and guilt, decreased ability to concentrate and think, decreased or increased appetite, weight loss or weight gain, insomnia or hypersomnia, low energy, fatigue and increased agitation.

Roughly 40%-50% of the risk of depression is genetic (Sanders et al., 1999; Fava and Kendler, 2000), which makes depression a highly heritable disorder. However, the difficulty in finding specific genes implicated in depression is likely due to the fact that it is a complex psychiatric disease with many genes involved (Burmeister, 1999). Thus, any single gene might produce a relatively small effect and therefore its role would be difficult to detect experimentally. In addition, nongenetic factors are also the causes of depression. The nongenetic etiology of depression consists of stress, emotional trauma, viral infections, and random processes during brain development (Akiskal, 2000; Fava

and Kendler, 2000). Episodes of depression usually occur in the context of stress, but stress is not sufficient to produce depression; i.e., not all people developing depression do so after a serious stress experience. It appears that depression is caused by interactions between genetic predisposition and environmental factors.

The finding that depletion of brain monoamines by reserpine, which blocks vesicular monoamine storages, induces despression-like symptoms, led to speculation that NE is deficient in depression. However, only a small number of individuals given reserpine actually develop depression. Further, depletion of monoamines by AMPT in normal subjects does not induce significant changes in mood (Salomon et al., 1997). Acute treatment with antidepressants that block the NET does not produce remission of symptoms of depression, even though the extraceullar NE level is elevated. Taken together, these findings argue against a simple role for NE in the pathology of depression and indicate that monoamine deficiency itself is insufficient to cause symptoms of depression (Miller et al., 1996b). However, rapid depletion of NE by AMPT results in depression relapse in the patients who have successfully responded to desipramine, an antidepressant that blocks the NET. 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).

Most of the NE in the brain arises from the cell bodies in the locus ceruleus (LC). The projections of these neurons are diffuse and overlap, nerve terminals in a wide range of brain regions including prefrontal and cingulate cortex, hippocampus, striatum,

amygdala, and thalamus (Drevets, 2001; Liotti and Mayberg, 2001). These brain regions play an important role in the mediation of cognition, depression, and emotional memory. The function of these brain regions suggests the aspects of depression to which they may contribute. Neocortex and hippocampus might mediate the cognitive aspects of depression, e.g. impairment of memory, feeling of worthless, guilt and suicidality. The striatum and amygdala are believed to relate to emotional memory, anxiety, and reduced motivation, symptoms observed in depressed patients. The role of the hypothalamus is speculated to involve sleep, activity, appetite, energy and a loss of interest in sex in depression (Nestler et al., 2002).

The role of 5-HT in the mediation of depression parallels, in many ways, that of NE. Clinically, in untreated patients, it has been reported that reducing serotonergic activity with tryptophan depletion does not produce symptoms of depression in normal subjects (Miller et al., 1996b). However, inhibition of 5-HT synthesis with PCPA or a tryptophan-free amino-acid drink reverses the antidepressant effects of selective serotonin reuptake inhibitors (SSRIs) in depressed subjects (Miller et al., 1996a; Salomon et al., 1993).

The majority of brain serotonergic innervation originates in the dorsal and median raphe nuclei. The serotonergic neurons in these two nuclei project to almost every region of the brain, including cortical and subcortical areas. Neurons in the dorsal raphe project mostly to striatum and substantia nigra, while those in the median raphe project to septum and hippocampus. Serotonergic terminals densely innervate the limbic system.

The majority of depressed patients show improvement with antidepressant medications or electroconvulsive therapy (ECT). Specific antidepressant medications

were first developed in the 1950s, with the discovery of two classes of agents, the monoamine oxidase inhibitors (MAOI) and tricyclic antidepressants. MAOI, e.g., phenelzine and iproniazid, elevate neurotransmission by inhibition of monoamine oxidase, a major catabolic enzyme involved in the degradation of monoamine neurotransmitters. Tricyclic antidepressants, e.g. imipramine and desipramine, inhibit the reuptake of NE or 5-HT by blocking the transporters (NET, SERT). Progress in antidepressant development advanced notably with the SSRIs, e.g., fluoxetine, sertraline, paroxetine; these result in fewer serious side effects and have less overdose hazard (Murphy et al., 2000). Subsequently, a selective norepinephrine reuptake inhibitors (NRIs) antidepressant was developed, i.e., reboxetine. The dual norepinephrine-serotonin reuptake inhibitors (NSRIs), venlafaxine and duloxetine, were then developed. Studies in inpatients and outpatients comparing venlafaxine with SSRIs e.g, fluoxetine, sertraline, and paroxetine reveal higher response or remission rates with the dual inhibitor venlafaxine (Clerc et al., 1994; Dierick et al., 1996; Ballus et al., 2000; Poirier and Boyer, 1999; Mehtonen et al., 2000) although other studies have not demonstrated superior efficacy (Thase et al., 2006). α-2 Adrenergic receptor antagonists, e.g., idazoxan, yohimbine, and antipamizole, have been shown to exhibit some antidepressant-like activity, and increase neuronal firing and synaptic NE levels in conjunction with NRIs antidepressants in a synergistic manner (Dhir and Kulkarni, 2007; Schramm et al., 2001). 5-HT_{1A} receptor agonists, e.g., buspirone, gepirone, and ipsapirone, were found to have antidepressant-like effects in animal models (Schreiber and de Vry, 1993; Yocca, 1990). Further, 5-HT_{2A}/5-HT_{2C} antagonists, e.g., methysergide and cyproheptidine, produce antidepressant effects in depressed patients (Eison et al., 1990).

Rolipram, a phosphodiesterase 4 (PDE4) inhibitor, also appears to have antidepressant activity (O'Donnell, 1993; Fleishchhacker et al., 1992), but its clinical efficacy compared with other antidepressants has been questioned (Hebenstreit et al., 1989). Although rolipram increases cyclic AMP by inhibiting its degradation, its effects are not mimicked by forskolin, an activator of adenylyl cyclase (O'Donnell, 1993). Rolipram was believed to increase NE turnover (Kehr et al., 1985) and the firing rate of neurons in the LC (Scuvee-Moreau et al., 1987). The antidepressant activity of rolipram has led to the hypothesis positing an inbalance between adenylyl cyclase/protein kinase A (PKA) systems and phospholipase C/PKC systems in depression (Wachtel, 1983).

Substance P antagonists and corticotropin releasing hormone (CRH) antagonists are two new classes of putative antidepressants. MK869, a substance P antagonist that blocks neurokinin-1 receptors (NK1R), has been found to be as effective as paroxetine in a 6-week double-blind trial in outpatients (Kramer et al., 1998; Maubach et al., 1999), although there have been subsequent failures in clinical trials (Herpfer and Lieb, 2005). The clinical failures suggest that, in addition to NK1R, other neurokinin receptors might be involved in the modulation of stress-related behaviors and that exclusive blockade of the NK1R might not be sufficient to produce consistent anxiolytic and antidepressant effects. NK2 (saredutant) and NK3 (osanetant) antagonists have been found to produce anxiolytic- and antidepressant-like effects in gerbils (Salome et al., 2006). Of special interest might be compounds that block more than one receptor type, e.g., NK1/2R antagonists or NK1/2/3R antagonists (Herpfer and Lieb, 2005; Griebel et al., 2001; Steinberg et al., 2001). The other mechanism of antidepressant effect of substance P antagonist is not clear, but may be associated with desensitization of α-2 adrenergic

receptors or 5-HT_{1A} serotonergic autoreceptors (Fisher et al., 2007; Froger et al., 2001; Santarelli et al., 2001). CRH mediates hypothalamic-pituitary-adrenal (HPA) axis function, including autonomic and behavioral responses to acute and chronic stress (Owens and Nemeroff, 1993; Arborelius et al., 1999).

1.2 Delayed onset of antidepressant effects

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).

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 NET to an extent greater than would be predicted from plasma concentrations (Zhu et al., 2004; Mandela and Ordway, 2006).

α-2 Adrenergic receptors, located presynaptically on noradrenergic neurons, regulate neuronal firing and NE release by a negative feedback mechanism. Excessive synaptic

NE inhibits α-2 adrenergic receptors and subsequently reduces the release of NE. 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.

The 5-HT_{1A} serotonergic autoreceptor plays a role in the effects of SSRIs treatment, because this receptor regulates neuronal firing and 5-HT release (Pineyro and Blier, 1999; Celada et al., 2004). It has been found that somantodetric 5-HT_{1A} autoreceptors are densensitized after chronic fluoxetine or sertraline treatment, which might be one of the mechanisms underlying the SSRIs-induced antidepressant-like effect. The receptor desensitization occurs at the level of effector coupling (Ase et al., 2001); a decrease in presynaptic 5-HT_{1A} receptor G-protein coupling is observed after chronic fluoxetine treatment.

Repeated desipramine treatment causes other neuronal changes that may contribute to antidepressant activity, including increased expression of cAMP response element binding protein (CREB) and brain-derived neurotrophic factor (BDNF; Nibuya et al., 1996) and neurogenesis in the dentate gyrus of the hippocampus (Santarelli et al., 2003; Kodama et al., 2004; Chen at al., 2006; Sairanen et al., 2005). These findings could be involved in the delayed onset of antidepressants, since they require sufficient time to develop. In many cases of neuronal adaptation produced by antidepressant treatment, e.g., down-regulation of β-adrenergic receptors, the antidepressant-induced adaptation is homeostatic, i.e., 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). It appears that similar neuroadaptive changes occurr for the long-term

effects of SSRIs. 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 homogenate binding with ^{3}H-citalopram, 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 (i.e., clearance) in the CA3 region of hippocampus is reduced after chronic treatment with SSRIs such as paroxetine and sertraline; desipramine does not affect SERT function (Benmansour et al., 2002). The extracellular 5-HT 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).

1.3 Animal models of depression

The lack of fully validated animal models retards depression research to some degree; the symptoms of depression (e.g. depressed mood, feeling of worthlessness, guilty and suicidality) are not easily produced in laboratory animals. Also, the genetic causes of depression cannot be replicated in animals since the depression vulnerability genes are still unknown. The dilemma is that animal depression models are needed to explore the mechanism of depression, but such models can only be developed after mechanisms underlying depression are better understood. Animal behaviors that model particular aspects of depression, e.g. impairment of cognition, abnormal psychomotor activity, responses to stresses and pleasurable stimuli, appetite, and sleep can be

evaluated, as can behavior that exhibits sensitivity to antidepressant treatment (Table 1.1).

Table 1.1 Examples of animal models used in depression research

Model	Features
Forced-swim test	Antidepressants decrease immobility time in a cylinder of water; lack of movement thought to represent a state of despair (Porsolt et al., 1977).
Tail-suspension test	Antidepressants increase the struggling time of mice when suspended by the tail; lack of movement thought to represent a state of despair; Used in mice only.
Differential-reinforcement-low-rate 72-s operant schehdule	Antidepressants reduce the response rate and increase the reinforcement rate (O'Donnell, 2005)
Chronic mild stress	The animals subjected to random sequence of mild stresses, i.e. cold, restrain, cages tilting, disruption of light-dark cycle, develop a series of abnormal behavioral and physiological responses. These abnormalities are corrected by chronic antidepressant. (Willner, 1997)
Learned helplessness	The animals are exposed to inescapable foot shock and then develop escape deficit. This deficit is reversed by chronic antidepressants (Chen et al., 2001; Shirayama et al., 2002; Vollmayr and Henn, 2001).
Olfactory bulbectomy	Chemical or surgical lesions of olfactory cause hyperactivity and other abnormal behaviors, which are reversed by chronic antidepressants (Kelly et al., 1997)
Open space swim test	Antidepressants increase the swimming distance (Sun and Alkon, 2005).

1.4 Molecular structure and function of NET and SERT

The duration and the intensity of noradrenergic and serotonergic neurotransmitter action are controlled by plasma membrane-bound transporters. After release into the synapse, neurotransmitters activate pre- or postsynaptic receptors. The termination of synaptic transmission is mostly completed by reuptake back into neurons or uptake into glial cells by neurotransmitter transporters (Figure 1.1).

The NET and SERT belong to a family of Na^+/Cl^- dependent symporters that also include transporters for dopamine, glycine, and GABA (Amara, 1992; Masson et al., 1999). Electrochemical energy derived from the inward gradient of Na^+, provided by Na^+/K^+-ATPase, drives the intracellular accumulation of neurotransmitter via the NET or SERT. Binding of Na^+ and then Cl^- is a prerequisite for the substrate to bind to the transporter (Trendelenburgh, 1999). Following the binding of substrate, both Na^+ and Cl^- are co-transportered along with the substrate into the cytoplasm.

Both NET and SERT proteins include 12 transmembrane domains and sites for glycosylation and phosphorylation (Figure 1.2). Analysis of NET and SERT structure reveals important information regarding the residues and domains responsible for NET function, substrate binding, drug binding, phosphorylation and regulation. The NET protein consists of 617 (human NET and rat NET) or 615 (bovine NET) amino acids (Pacholczyk et al., 1991). Both amino and carboxy termini of the NET are located on the cytoplasmic side. The NET contains a large hydrophilic loop between TMD3 and TMD4, within which are located the canonical sites for N-linked glycoslation (Blakely, 1994). Inability to N-glycosylate the NET is associated with reduced protein stability, surface trafficking, and transporter activity (Melikian et al., 1994). Addition of a

carbohydrate moiety was shown to significantly increase uptake (Melikian et al., 1994; Nguyen and Amara, 1996). It is likely that glycosylated NET proteins are expressed on the membrane and non-glycosylated forms are retained in the cytoplasm, which makes the glycosylation site a poteintial domain for regulation of NET function. In addition to glycosylation sites, there are sites of potential phosphorylation by PKC within the intraceullar part of the NET. For example, the phosphorylation of threonine-258 and serine-259 located at the small intracellular loop between DM4 and DM5 is linked to PKC-mediated NET protein internalization (Jayanthi et al., 2006). Several sites of phosphorylation by other protein kinases also have been found. Threonine-30, threonine-58, and serine-502 are potential PKA phosphorylation sites. Threonine-19 and threonine-583 are potential phosphorylation sites for casein kinase II (CKII) (Jayanthi et al., 2006). The predicted topological structure of SERT is very similar with the NET. The SERT also has N-linked glycosylation sites on the large extracellular loop between TMD 3 and TMD4 as well as homologous, potential phosphorylation sites.

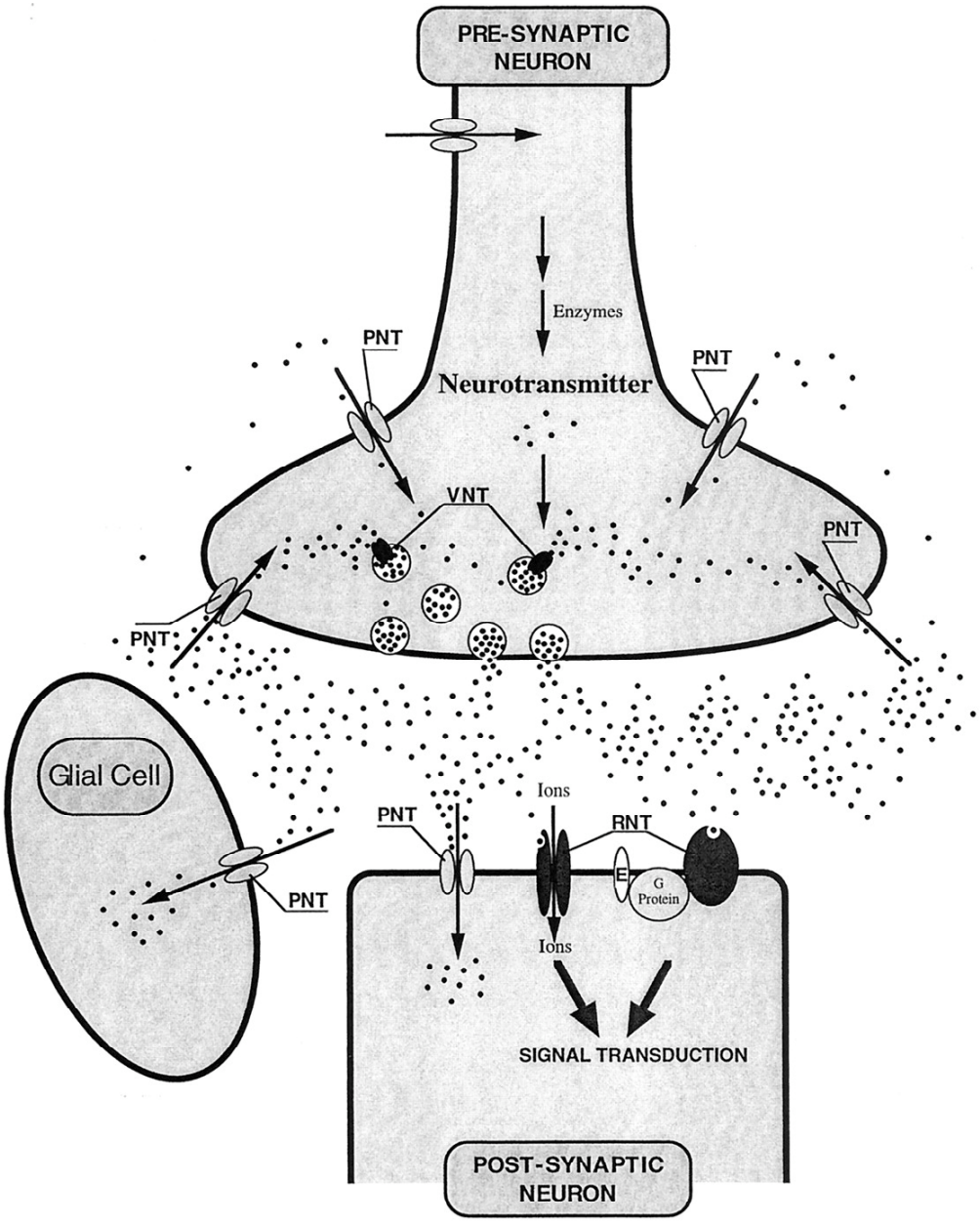


Figure 1.1 Schematic representation of neurotransmission steps at a synapse. The neurotransmitter is synthesized in the presynaptic neuron, stored in synaptic vesicles by the VNT, and released by exocytosis. The neurotransmitter then binds to receptors as an effector, and is removed from the synaptic cleft by membrane-bound neurotransmitter transporters (NET or SERT) through the reuptake process in presynaptic or postsynaptic neurons and/or glial cells (Masson et al., 1999).

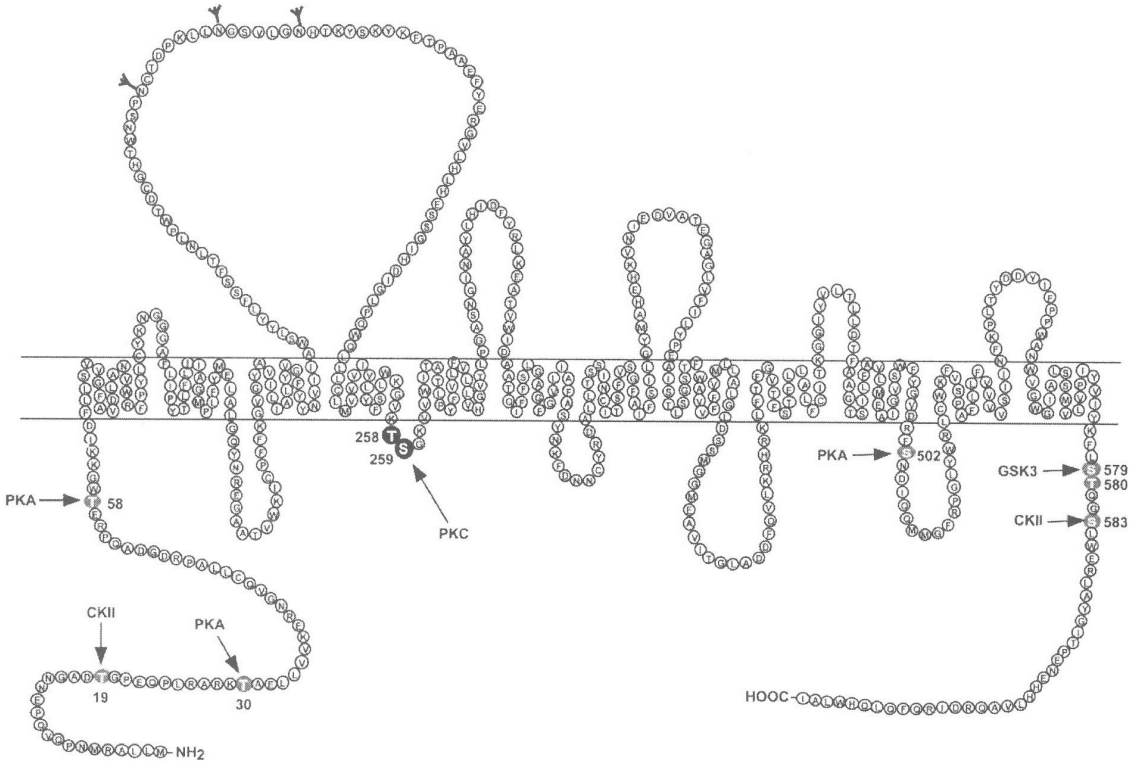


Figure 1.2 Schematic representation of the human NET. Large black circles indicate potential phosphorylation sites for PKC; large gray circles indicate potential phosphorylation sites for other kinases (Jayanthi et al., 2006).

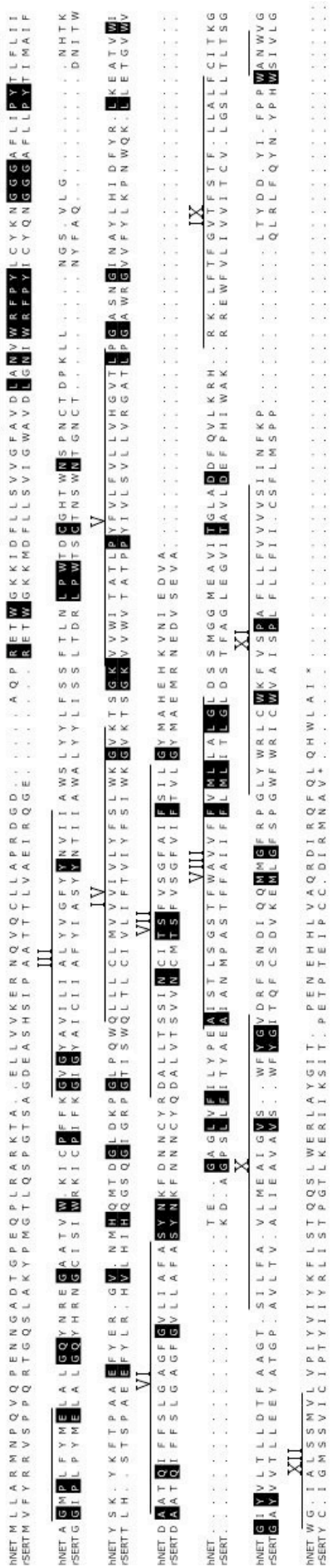
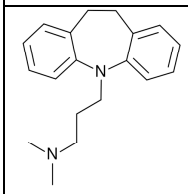
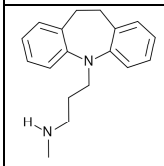
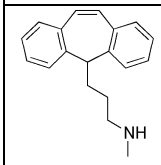
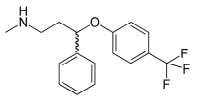
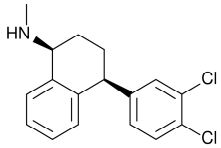
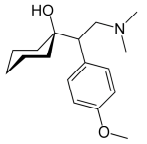
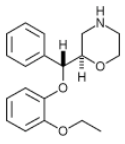


Figure 1.3 Alignment of the amino acid sequence of human NET and rat SERT. The putative α-helical membrane spanning domains (I - XII) are indicated by bars. Conserved residues are shaded (Pacholczyk et al., 1991; Blakely et al., 1991; Hoffman et al., 1991).

1.5 Noradrenergic reuptake inhibitors and selective serotonin reuptake inhibitors

NRIs and SSRIs elevate synaptic NE and 5-HT levels, respectively, by blocking the NET or SERT. The first tricyclic antidepressant, imipramine, elicited antidepressant effects by inhibiting NET and SERT with some unfavorable side effects due to antihistaminic, antiadrenergic, and anticholinergic effects (Ban, 2001). Other tricyclic antidepressants exhibit some selectivity for inhibition NE reuptake, e.g, desipramine, protriptyline, and nortriptyline, or 5-HT reuptake, e.g., clomipramine; however, the side effect profiles were still unfavorable. The introduction of fluoxetine in 1988 provided a safer treatment alternative to the tricyclic antidepressants since its side effect profile was significantly improved. However, some have questioned whether the SSRIs are as efficacious at treating depression as the tricyclic antidepressants (Song, 1993). The thought that actions on both noradrenergic and serotonergic neurotransmission may be advantageous led to the development of venlafaxine and duloxetine, which inhibit both the NET and SERT and which have a favorable side-effect profile (Shelton, 2004). The development of reboxetine, a potent, selective, and specific NE reuptake inhibitor, shifts from focusing on the 5-HT system to the NE system. In vitro, reboxetine is about 120-fold more potent at blocking NE reuptake than 5-HT reuptake (Wong et al., 2000). Extensive clinical study indicated that reboxetine is a clinically active, efficacious, and well tolerated antidepressant (Berzewdski et al., 1997).

Table 1.2 Structure of antidepressants and Ki values for inhibition of NET and SERT

Structure	Antidepressant	[^{3}H]5-HT uptake inhibition Ki (nM)	[^{3}H]NE uptake inhibition Ki (nM)
	Imipramine	20 ± 2	142 ± 8
	Desipramine	163 ± 5	3.5 ± 0.6
	Protriptyline	3.4	161
	Fluoxetine	20 ± 2	2186 ± 142
	Sertraline	3.3 ± 0.4	1716 ± 151
	Venlafaxine	102 ± 9	1644 ± 84
	*Reboxetine	1070 ± 3	8 ± 4

Data were obtained in HEK and *MDCK cells expressing hSERT and hNET (White et al., 2005; Wong et al., 2000; Sanchez and Hyttel, 1999; Zhou, 2004)

1.6 Regulation of NET

The NET is regulated by several intraceullar signaling molecules including cAMP, PKA, PKC, p38 MAPK, Ca^{2+}, CaM kinase II, protein phosphatases P1/PP2A, and ATP.

cAMP and PKA

cAMP, produced by activation of adenylyl cyclase, is a key component linking upstream G protein-coupled receptors to downstream signaling cascades, e.g., activation of PKA. Research on the regulation by cAMP and PKA of NET activity has yielded results that are not consistent across cell lines or species. For example, membrane permeable cAMP analogue 8 Br-cAMP induces a dose-dependent reduction of NET activity in bovine adrenal medullary chromaffin cells (Bunn et al., 1992). However, 8 Br-cAMP doesn't change the NET activity in SK-N-SH (Apparsundaram et al., 1998b) and SK-N-SH-SY5Y cells (Bonisch et al., 1998). Forskolin, a compound that increases intracellular cAMP by directly activating adenylyl cyclase, shows biphasic effects on NET activity in chromaffin cells. At a low concentration, forskolin increases in NET activity; while at a high concentration it inhibits NET activity (Bunn et al., 1992). Bryan-Lluka et al. (2001) found that short-term (15 min) or long-term (24 h) treatment with forskolin decreases NET activity in PC12 cells. However, in COS7 cells transiently transfected with rat NET cDNA, short-term treatment with forskolin does not affect NET activity, while long-term treatment increases the NET activity (Bryan-Lluka et al., 2001). Taken together, these data suggests that cAMP regulation is not a general mechanism in NET regulation but is cell line- and species-dependent.

PKC

The NET is the target for PKC; phosphorylation sites for this kinase have been shown for this transporter. β-PMA, a PKC activator, reduces NET activity and surface expression in HEK-293 and LLC-PK1 cells stably transfected with hNET (Apparsundaram et al., 1998a) as well as rat placental trophoblasts (Jayanthi et al., 2004). The decreased NET activity is characterized by a decrease in maximum transport velocity (V_{max}) and a slight decrease in substrate affinity (K_m). This reduction is primarily due to the internalization of surface NET protein (Jayanthi et al., 2004). Site-directed mutagenesis reveals that threonine-258 and serine-259 on the hNET are phosphorylated by PKC and that this results in transporter internalization (Jayanthi et al., 2006). NET activity also is reduced by the protein phosphatase 1/protein phosphatase 2A (PP1/2A) inhibitors okadaic acid and calyculin A; these compounds prevent dephosphorylation (Bauman et al., 2000).

P38 MAPK

A trafficking-independent mode of NET and SERT regulation that is sensitive to p38 MAPK has been identified in RBL-2H3, RN46A, and CHO cells transfected with either NET or SERT (Zhu et al., 2004; Zhu et al., 2005). NET activity measured by NE uptake assay is stimulated by anisomycin, a p38 MAPK activator. Also, SB203580, a p38 MAPK inhibitor, blocks anisomycin-induced activation of NET. In comparison with trafficking-depedent, PKC-dependent regulation in which the surface transporter density changes with unchanged catalytic activation, anisomycin treatment does not change maximal transport capacity (V_{max}) but significantly reduces the affinity (K_m), indicating a

trafficking-independent regulation mode. Moreover, a reduced displacement of [^{125}I]RTI-55, a competitive antagonist of 5-HT, from the SERT was observed, suggesting a reduced affinity of SERT for 5-HT. The observation that inhibiton of PP2A by okadaic acid and calyculin A reverses anisomycin-induced activation of the transporters indicates that PP2A is downstream of the p38 MAPK signaling pathway. At high concentrations, PP2A inhibitors reduce transporter activity, which might be due to the actions of PKC and subsequent sequestration of surface transporters.

Ca^{2+} and CaM kinase II

Extraceullar Ca^{2+} plays an important role in NET activity. NET activity in PC12 cells shows a robust increase after incubation with 0.3-10 mM $Ca2^{2+}$ (Uchida et al., 1998). Intraceullar Ca^{2+} released from endoplastic reticulum also is important in regulating NET activity. IP_3 receptors located on the membrane of the endoplasmic reticulum regulate release of Ca^{2+} stores. 2-APB, an IP_3 receptor inhibitors, reduces NET activity by inhibiting the Ca^{2+} release from endoplasmic reticulum in SH-SY5Y cells (Amano et al., 2006) and rat cerebral cortex slices (unpublished data). Ca^{2+}/calmodulin dependent kinase II (CaMKII) is able to directly phosphorylate the trasnporter and increase NET activity by translocation of NET from the cytoplasm to the membrane. Myosin light chain kinase (MLC kinase) also enhances NET activity by phosphorylation of specific amino acid residues on the transporter. CaMKII and MLC kinase inhibitors robustly inhibit Ca^{2+}-dependent increases in NET activity. External Ca^{2+} regulates transporter activity by increasing NE uptake through CaM kinase II and MLC kinase rather than decreasing NE uptake via the PKC signaling pathway, suggesting that NET activity is

regulated by both an external Ca^{2+} activation-induced pathway and an internal pathway regulating release of Ca^{2+} stores.

ATP

ATP is an important molecule in biological systems, providing the energy for a variety of cellular functions. ATP is present in the vesicles that store neurotransmitters, which raises the possibility that ATP might be a co-transmitter. Geffen and Livett (1971) found that ATP is released with NE during depolarization. Further study shows that the high-affinity uptake of NE in PC12 cells is increased in the presence of Ca^{2+} and 0.1μM ATP or ATP-gamma-S, an analog of ATP (Hardwick et al., 1989). This ATP-induced increase of NE uptake is reduced by App(NH)p, a competitive antagonist of ATP (Hendley et al., 1988). NE uptake is inhibited when the Ca^{2+} and Mg^{2+} are both absent from the incubation medium, suggesting that divalent cations are required for ATP to have its effect on NE uptake.

Regulation of the NET by neurotransmitters

The following neurotransmitters are found to be involved in the regulation of NET activity: acetylcholine, insulin, GABA, angiotensin, nerve growth factor, natriuretic peptides, and nitric oxide. Some are to be discussed below.

Acetylcholine

The muscarinic acetylcholine receptor agonist methacholine significantly reduces NET maximum transport velocity with no change in K_m value in SK-N-SH cells

(Apparsundaram et al., 1999). Methacholine treatment decreases NET binding sites measured by ^{3}H-nisoxetine in intact cells but not in total membrane factions. These studies provide evidence in support of G-protein coupled receptor-mediated regulation of the NET.

Insulin

Insulin is a hormone involved in regulating glucose homeostasis. The effect of insulin on NET activity has been studied in SK-N-SH and PC12 cells. Acute insulin treatments increases NE uptake in a time- and dose-dependent manner with enhancement of NE transport capacity (V_{max}) without an alteration in the K_m for NE (Apparsundaram et al., 2001). Acute insulin treatment decreases NE uptake in PC12 cells (Figlweicz et al., 1993a) and this decrease may be due to the effect of insulin on the transporter gene transcription, as insulin is found to decrease NET mRNA expression in LC of the rat (Figlewicz et al., 1993b). The insulin receptor is a tyrosine kinase that phosphorylates substrate proteins, including the insulin receptor substrate (IRS) family. Once phosphorylated, the IRS binds to and activates its downstream proteins including phosphatidylinositol 3-OH kinase (PI3K). Tyrosine kinase and PI3K inhibitors produce time- and concentration-dependent reduction of NE uptake, indicating that constitutive tyrosine kinase and PI3K activity is necessary for NET activity (Apparsundaram et al., 2001). Effects of tyrosine kinase and PI3K inhibitors on basal NET uptake might contribute to a loss of cell surface NET protein expression (Apparsundaram et al., 2001).

Nitric oxide

Nitric oxide is a signaling molecule in the cardiovascular system and central nervous system. After exposure to the NO donor S-nitroso-N-acetylpenicillamine (SNAP), NET activity is reduced through a cyclic GMP-independent manner in CHO cells (Kaye et al., 2000), indicating a potential regulatory role for nitric oxide in modulating NET activity. However, in SK-N-SH cells, SNAP treatment shows an elevation of uptake activity (Apparsundaram et al., 1998a). More research is needed to explore the molecular mechanism of nitric oxide.

CHAPTER TWO

Norepinephrine transporter regulation mediates long-term behavioral effects of antidepressant desipramine

This chapter is identical to a manuscript, which has been published in *Neuropsychopharmacology*, 2008 Apr 16. [Epub ahead of print]

2.1 Abstract

The relationship between the ability of repeated desipramine treatment to cause down-regulation of the norepinephrine transporter (NET) and produce antidepressant-like effects on behavior was determined. Treatment of rats with 15 mg/kg/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 two days following discontinuation of desipramine treatment when plasma and brain levels of desipramine and its major metabolite desmethyl-desipramine 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 alpha-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.

2.2 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 manifest 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 5-HT 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 alpha-methyl-p-tyrosine (AMPT) increases depressive symptoms in patients with seasonal affective disorder (Lam et al., 2001). Similarly, reduction of 5-HT 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 down-regulation 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 down-regulation of the NET (Weinshenker et al., 2002). In many cases of neuronal adaptation, e.g., down-regulation of β-adrenergic receptors, the antidepressant-induced adaptation is homeostatic, i.e., 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 down-regulates the NET in the cerebral cortex and hippocampus, reducing NE uptake, which results in increased noradrenergic neurotransmission and antidepressant-like effects on behavior.

2.3 Materials and Methods

Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 300-350g, were housed 2 per cage in a temperature-controlled room (22 - 23° C) with a 12-h on/12-h off light cycle (lights on at 6:00 AM). Food and water were freely provided. Blind observations were used throughout all behavioral testing, which was carried out from 1:00 PM – 5:00 PM 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/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 removed, and cerebral cortex and hippocampus 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 (i.e., 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), in order to determine the time of recovery. Finally, in some tests, rats were administrated 150 or 300 mg/kg AMPT (Sigma-Aldrich, St Louis, MO) 4 hours 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_3VO_4, 1 mM EDTA, 1 mM EGTA, 320 mM sucrose; pH 7.4) with a glass homogenizer and centrifuged at 1,000 × g for 20 min at 4° C. The supernatant was centrifuged at 20,000 × g for 30 min to obtain a crude synaptosomal fraction (Lin et al., 1998; Wyszynski et al., 1998; Dunah and Standaert, 2001). The

pellets were re-suspended 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 2X 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, Rockford, IL). The labeled protein bands were detected by chemiluminescence imaging (Bio-Rad, Hercules, CA) 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 (Brinkman Instruments, Westbury,

NY) and then centrifuged at 36,000 × g for 10 min at 4° C. The supernatant was discarded, the pellet re-suspended in ice-cold binding buffer, centrifuged at 36,000 × g for 10 min at 4° C, and the pellet re-suspended in binding buffer to obtain a protein concentration of 1 mg/ml as assayed using the bicinchoninic acid method (Smith, 1985; Pierce, Rockford, IL). Triplicate tubes containing 100 µl incubation buffer (total binding) or 100 µl desipramine (final concentration 50 µM; non-specific binding), 100 µl tissue sample, and 50 µl ^{3}H-nisoxetine (final concentration 5 nM; 72 Ci/mmol, PerkinElmer, Boston, MA) were incubated in a shaking ice bath for 4 h. Non-specific binding (i.e., in the presence of 50 µM desipramine) was approximately 10% of total binding. Bound ^{3}H-nisoxetine was captured by rapid filtration through glass fiber filters (GF/B Brandel, Gaithersburg, MD) that were pre-soaked in 5% polyethyleneimine, under vacuum with a cell harvester (Brandel, Gaithersburg, MD). Filters were washed twice with ice-cold binding buffer and radioactivity 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/HEPES (KRH) buffer (125 mM NaCl, 5.2 mM KCl, 1.2 mM $CaCl_2$, 1.4 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM glucose, 20 mM HEPES, 0.2 mg/ml ascorbic acid and 100 µM 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, Westbury, NY). 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, Boston, MA) 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 30,000 × g for 2 min. ^{3}H-NE in the supernatant was quantified by liquid scintillation counting. Nonspecific uptake was determined in the presence of 20 μM desipramine. Specific uptake was determined by subtracting nonspecific uptake from total uptake.

Measurement of extracellular NE in the medial prefrontal cortex (mPFC) 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 mPFC, was implanted at coordinates AP + 2.6 mm from Bregma, ML $\pm$1.4 mm from midline, DV -1.7 mm from dura. The guide cannula was anchored to the skull and an obdurator was inserted. The wound was sutured, topical antibiotic applied, and each rat returned to its home cage. On the testing day, the obdurator 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 μl/min. After a 2 h equilibration period, four 30 min samples (30 μl) were collected into tubes containing 2.5 μl of stabilizing solution (0.2 μM EDTA, 0.2 μM ascorbic acid, 0.2 M perchloric acid). NE in the microdialysis samples was measured by high-

performance liquid chromatography (HPLC) 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 desmethyl-desipramine

The measurement of desipramine and its primary metabolite desmethyl-desipramine 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 μl of plasma (either sample or blank standard) were added 100 μl of internal standard solution (imipramine 200 ng/ml) followed by 100 μl of ammonium hydroxide. The plasma was then extracted with 1 ml l-butanol (20%) in hexane (pH 11) for 15 min on a reciprocating shaker. After separation of the phases by centrifugation at 2,500 x g 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 (70:30 0.025 M KH_2PO_4:acetonitrile).

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 l-butanol (20%) n-hexane to both samples and blank brain standards. The mixture was shaken for 15 min and centrifuged at 2,500 x g 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 mm x 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 desmethyl-desipramine 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 N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) and 1 mM ethylenediamine tetra-acetic acid (EDTA) buffer (pH 7.75) for 15 sec using a Polytron homogenizer. The homogenate was centrifuged at 40,000 x g for 20 min. The supernatant was removed and the wash procedure was repeated 4 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 β-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 glass fiber 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 (ANOVA) 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 less than 0.05.

2.4 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 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 2.1A) and hippocampus ($F_{(3,16)}$=4.34, $p < 0.05$; Fig. 2.1A). Down-regulation of the NET also was demonstrated using SDS-PAGE/immunoblotting of preparations of cerebral cortex ($F_{(3,16)}$=4.75, $p < 0.05$; Fig. 2.1B) and hippocampus ($F_{(3,15)}$=13.10, $p < 0.001$; Fig. 2.1B). Importantly, this NET down-regulation was observed two days after discontinuation of chronic desipramine treatment, a time when plasma and brain concentrations of desipramine and desmethyl-desipramine were undetectable (i.e., below the 25 ng detection limit of the assay). By comparison, one hour after acute treatment with 10 mg/kg desipramine, which causes similar effects on forced-swim behavior, plasma concentrations (mean ± S.D.) were 0.5 ± 0.3 (desipramine) and 0.09 ± 0.02 (desmethyl-desipramine) µg/ml and brain concentrations were 3.4 ± 0.1 (desipramine) and 0.09 ± 0.03 (desmethyl-desipramine) µg/g protein. In parallel with the down-regulation 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, $p < 0.001$; Fig. 2.1C). This was evident two days after discontinuation of chronic desipramine treatment and paralleled the reduction of NET expression (Fig. 2.1B). 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; two days post-desipramine treatment: 40.7 ± 3.4 fmol/mg protein; n=5/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/day desipramine for 14 days and subjected to the forced-swim test 2, 5, or 8 days after the end of desipramine treatment (i.e., removal of the osmotic pumps). Rats then were 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 two days after the discontinuation of chronic desipramine treatment in the cerebral cortex ($F_{(3,19)}=8.59$, $p < 0.001$; Fig. 2.2A) and hippocampus ($F_{(3,19)}=3.22$, $p < 0.05$; Fig. 2.2A). The NET binding sites were partially recovered five days, and fully recovered eight days, post-treatment. Consistently, decreased expression of NET also was observed by SDS-PAGE/immunoblotting two days after the discontinuation of chronic desipramine treatment in the cerebral cortex ($F_{(3,12)}=6.98$, $p < 0.01$; Fig. 2.2B) and hippocampus ($F_{(3,12)} = 4.26$, $p < 0.05$; Fig. 2.2B). The down-regulation of the NET

protein expression was partially reversed five days, and completely recovered eight days, post-treatment. The time-course of NET expression and activity were paralleled by behavioral effects in the forced-swim test. Rats tested two days following the end of chronic desipramine treatment displayed a reduced duration of immobility in the forced-swim test ($F_{(3,18)}$=27.15, $p < 0.001$; Fig. 2.2C). This antidepressant-like effect was partially reversed five days and completely disappeared eight days post-treatment.

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 two days after the end of chronic desipramine treatment and returned to control levels by eight days post-treatment in both cerebral cortical ($F_{(4,19)}$= 3.92, $p < 0.05$; Fig. 2.2D) and hippocampal slices ($F_{(4,21)}$=26.35, $p < 0.001$; Fig. 2.2D).

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 antidepressant-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, i.e., 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 2.3A). It was found

that the basal NE concentration was increased more than two-fold two days after the discontinuation of repeated desipramine treatment ($F_{(3,50)} = 20.14$, $p < 0.001$; Fig. 2.3B). The extracellular NE concentration returned to control values by five days post-treatment. 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 two days following the end of the repeated-treatment regimen (control: 29.8 ± 1.7; two days post-desipramine: 12.8 ± 1.8 fmol/mg protein; n=5/group; $p < 0.01$); β-1 receptor density was not different from control levels five and eight days post-treatment (27.2 ± 1.9 and 30.8 ± 3.2 fmol/mg protein, respectively; n=5/group).

Effect of catecholamine depletion on the persistent effects of desipramine treatment in the forced-swim test

In order to determine whether enhanced noradrenergic neurotransmission was necessary for the persistent antidepressant effect in the forced-swim test, a NE depletion approach was utilized, similar to that used to assess the role of noradrenergic neurotransmission in the clinical actions of desipramine (Miller et al., 1996). 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 mg/kg 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, $p < 0.001$; Fig. 2.4A). 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, $p < 0.05$; Fig. 2.4A).

2.5 Discussion

The present results show a relationship between desipramine-induced down-regulation of the NET and antidepressant effects on behavior. These exhibit similar dose-response and time-of-recovery relationships. Importantly, the down-regulation 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 down-regulation of monoamine transporters is greater than pharmacological inhibition (Klimek et al., 1997) due to its noncompetitive nature versus 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; Katz et al., 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 two-day washout, when plasma and brain antidepressant drug concentrations were not detectable. The density of postsynaptic β-1 adrenergic receptors also was decreased two days after chronic administration of 15 mg/kg/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 co-administration 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 two 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 down-regulation likely contributes to the down-regulation 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 at al. 2006; Sairanen et al., 2005).

The mechanism underlying desipramine-induced down-regulation 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 PKC- (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 protein kinase C. Activation of PKC by β-PMA down-regulates 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 down-regulation and antidepressant effects on behavior.

Whether the down-regulation 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; Zhu et al., 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 (i.e., 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 down-regulation of the SERT results in antidepressant effects on behavior, even when drug is not present. Dual norepinephrine and serotonin reuptake inhibitors, 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 monoaminergic neurotransmission. By contrast, changes in monoaminergic receptor expression often are homeostatic, i.e., 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-chlorophenylalanine 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 (l-DOPS) 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 (Fig. 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 down-regulation of the NET and antidepressant-like effects on behavior. NET down-regulation, which was evident for at least two days following discontinuation of treatment, persisted even in the absence of detectable levels of desipramine or its major metabolite in plasma or brain. NET down-regulation 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 (Dreyets, 2000). Also consistent with clinical data, it was found that the antidepressant-like effect that resulted from NET down-regulation 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 down-regulation 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.

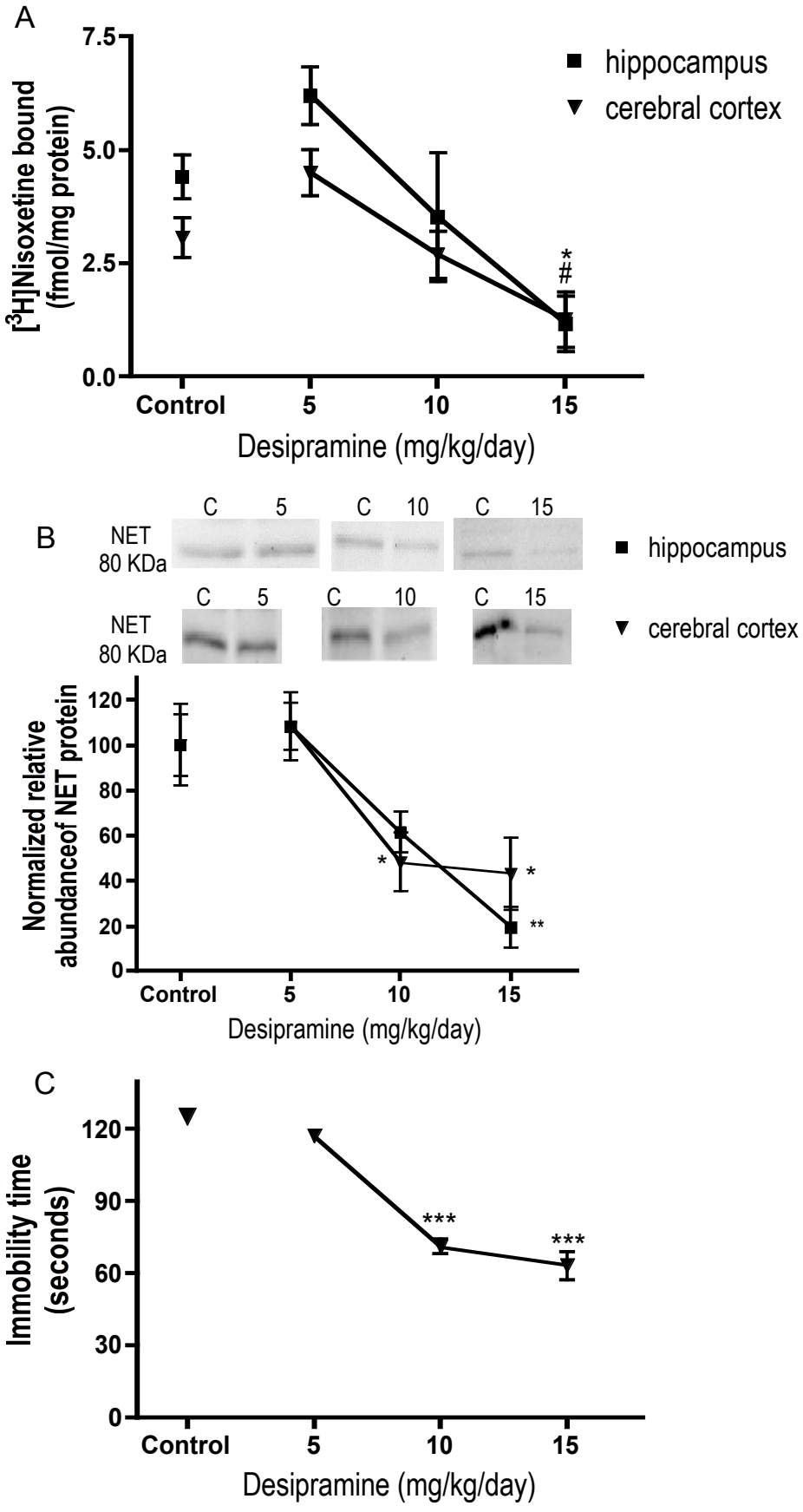
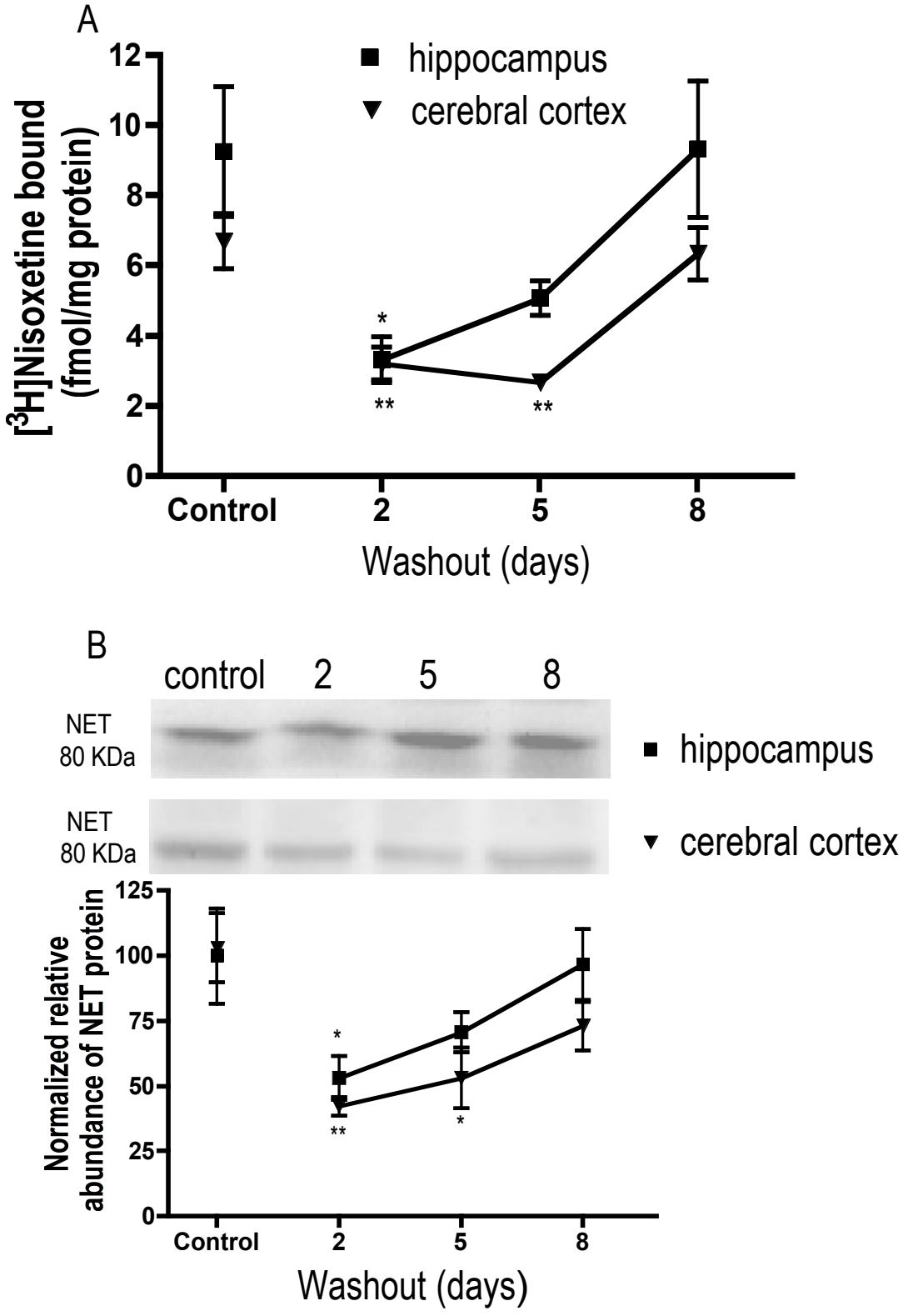
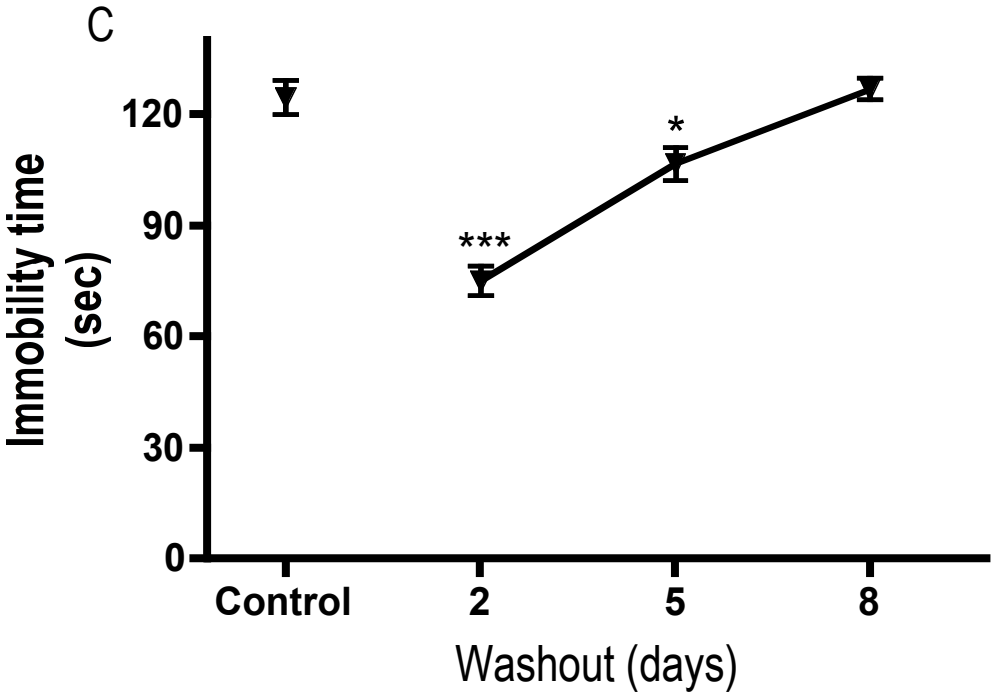

A
7.5
5.0
2.5
0.0
[3H]Nisoxetine bound (fmol/mg protein)
hippocampus
cerebral cortex
*
#
Control
5
10
15
Desipramine (mg/kg/day)
B
C 5 C 10 C 15
NET 80 KDa
C 5 C 10 C 15
NET 80 KDa
hippocampus
cerebral cortex
120
100
80
60
40
20
0
Normalized relative abundanceof NET protein
*
*
**
Control
5
10
15
Desipramine (mg/kg/day)
C
120
90
60
30
0
Immobility time (seconds)
***
***
Control
5
10
15
Desipramine (mg/kg/day)

Figure 2.1 Dose-response functions were determined for the ability of chronic desipramine treatment to reduce NET expression in the cerebral cortex and hippocampus, determined by ^{3}H-nisoxetine binding (A) and SDS-PAGE /immunoblotting with antisera against the NET (B), and to reduce immobility time in the forced-swim test (C). All measures were determined two days after the end of the chronic treatment period, when brain and plasma concentrations of desipramine and its metabolite desmethyl-desipramine were not detectable (i.e., 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.


A
hippocampus
cerebral cortex
[3H]Nisoxetine bound
(fmol/mg protein)
12
10
8
6
4
2
0
Control
2
5
8
Washout (days)
B
control
2
5
8
NET
80 KDa
NET
80 KDa
hippocampus
cerebral cortex
Normalized relative
abundance of NET protein
125
100
75
50
25
0
Control
2
5
8
Washout (days)


C
Immobility time (sec)
120
90
60
30
0
***
*
Control
2
5
8
Washout (days)

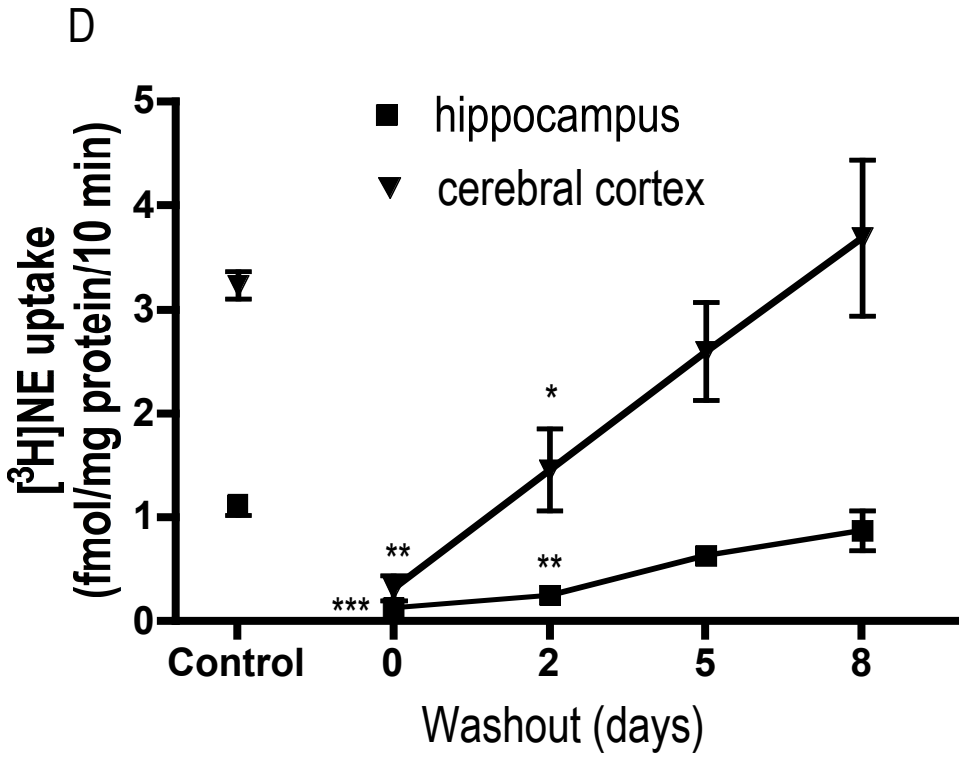
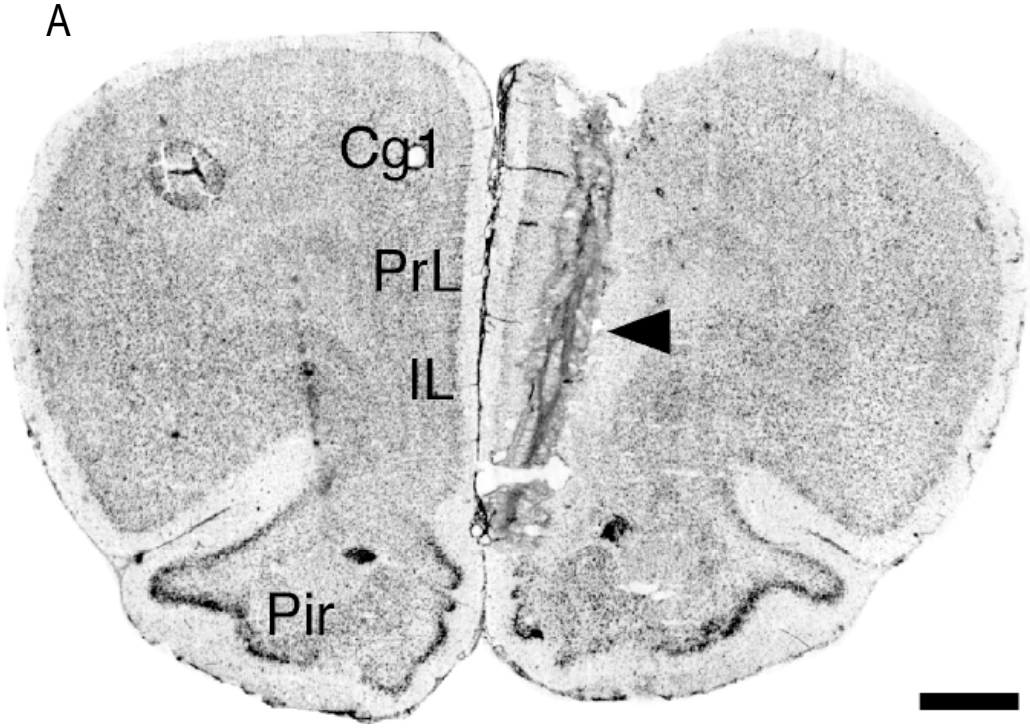

D
[3H]NE uptake (fmol/mg protein/10 min)
5
4
3
2
1
0
hippocampus
cerebral cortex
*
**
**
***
Control
0
2
5
8
Washout (days)

Figure 2.2 The recovery time-course of chronic desipramine on NET expression in the cerebral cortex and hippocampus, measured by ^{3}H-nisoxetine binding (A) and SDS-PAGE/immunoblotting with antisera against the NET (B). The persistent antidepressant-like effect in the forced-swim test was evident two days after the end of the chronic treatment period, but dissipated by eight days (C). The uptake of ^{3}H-NE into tissue slices, which reflects the activity of the NET, was reduced two days after discontinuation of desipramine treatment, and returned to the control level after five to eight days (D). Data shown are means ± SEM of 5-10 rats per group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. control.

A


Cg1
PrL
IL
Pir

B

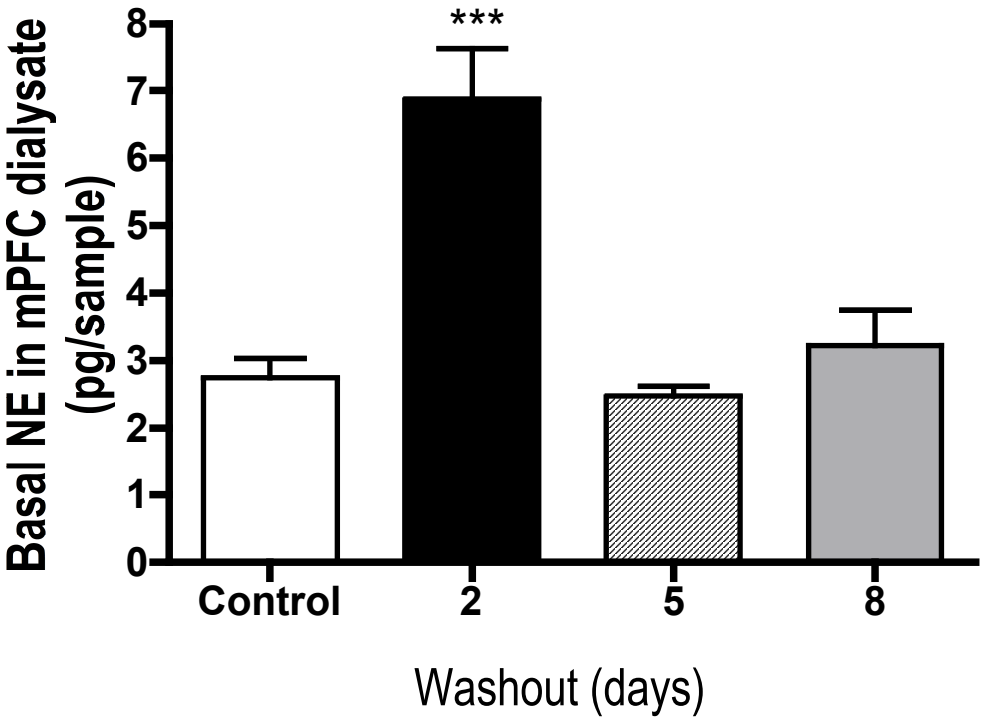
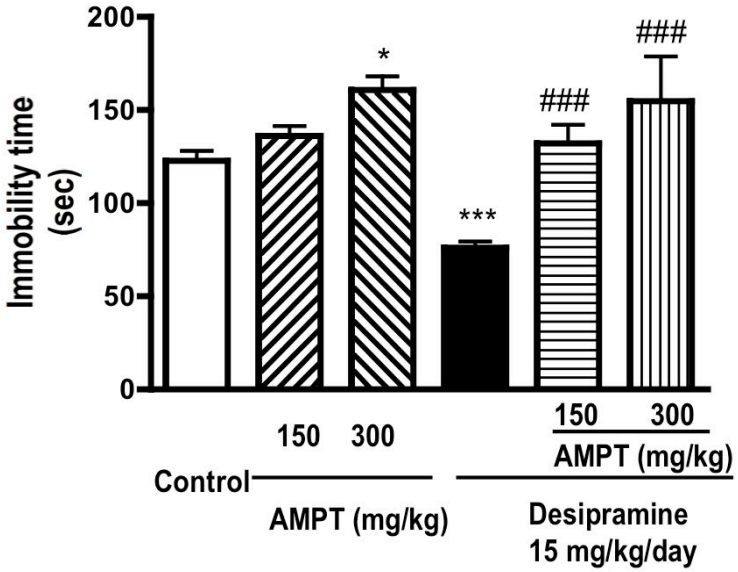

Basal NE in mPFC dialysate (pg/sample)
***
0
1
2
3
4
5
6
7
8
Control
2
5
8
Washout (days)

Figure 2.3 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 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/day desipramine treatment with 2 days washout significantly elevated the tonic baseline of NE in mPFC, measured by *in vivo* microdialysis (B). Data shown are means ± SEM of 11-12 rats per group. ***$p < 0.001$.

A


Immobility time (sec)
200
150
100
50
0
*
***
###
###
150
300
150
300
AMPT (mg/kg)
Control
AMPT (mg/kg)
Desipramine
15 mg/kg/day

B

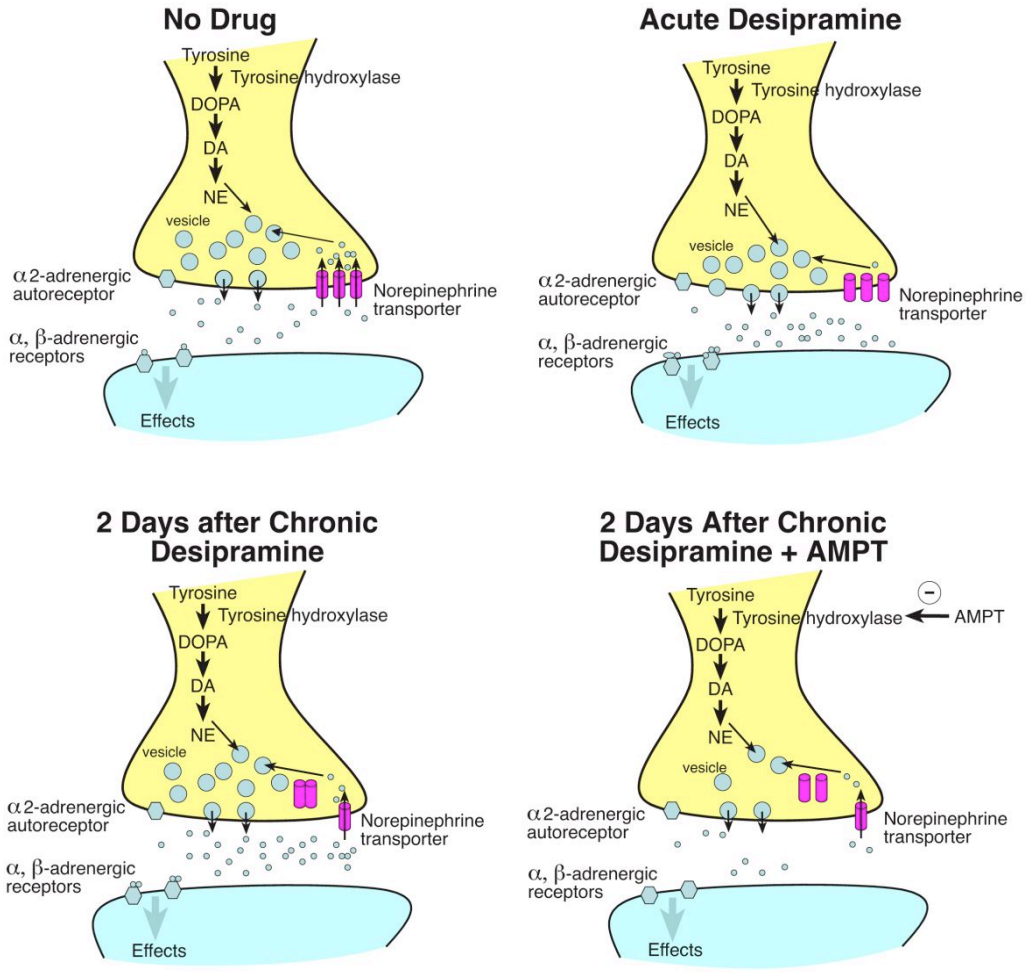

No Drug
Tyrosine
Tyrosine hydroxylase
DOPA
DA
NE
vesicle
α2-adrenergic autoreceptor
Norepinephrine transporter
α, β-adrenergic receptors
Effects
Acute Desipramine
Tyrosine
Tyrosine hydroxylase
DOPA
DA
NE
vesicle
α2-adrenergic autoreceptor
Norepinephrine transporter
α, β-adrenergic receptors
Effects
2 Days after Chronic Desipramine
Tyrosine
Tyrosine hydroxylase
DOPA
DA
NE
vesicle
α2-adrenergic autoreceptor
Norepinephrine transporter
α, β-adrenergic receptors
Effects
2 Days After Chronic Desipramine + AMPT
Tyrosine
Tyrosine hydroxylase
AMPT
DOPA
DA
NE
vesicle
α2-adrenergic autoreceptor
Norepinephrine transporter
α, β-adrenergic receptors
Effects

Figure 2.4 To examine whether enhanced noradrenergic neurotransmission was necessary for the persistent antidepressant-like effect in the forced-swim test, AMPT, an inhibitor of tyrosine hydroxylase, was administered two 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 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 down-regulated. 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 down-regulated. 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.

CHAPTER THREE

Association of changes in norepinephrine and serotonin transporter expression with the long-term behavioral effects of antidepressant drug treatment

This chapter is identical to a manuscript, which was submitted to *Neuropsychopharmacology* in July 2008.

3.1 Abstract

Previous work has shown that repeated desipramine treatment causes down-regulation of the norepinephrine transporter (NET), accompanied by persistent antidepressant-like effects on behavior. The present study examined whether this mechanism generalizes to other antidepressant drugs and also is evident for the serotonin transporter (SERT). Treatment of rats for 14 days with 20 mg/kg/day protriptyline or 7.5 mg/kg/day sertraline reduced NET and SERT expression, respectively, in cerebral cortex and hippocampus; these treatments also induced a persistent antidepressant-like effect in the forced-swim test. Increased serotonergic neurotransmission likely mediated the persistent antidepressant-like behavioral effect of sertraline, since it was blocked by inhibition of serotonin synthesis with para-chlorophenylalanine; a parallel effect was observed previously for desipramine and noradrenergic neurotransmission. Treatment with 20 mg/kg/day reboxetine for 42, but not 14, days reduced NET expression; antidepressant-like effects on behavior were observed for both treatment durations. Treatment for 14 days with 70 mg/kg/day venlafaxine, which inhibits both the NET and SERT, or 10 mg/kg/day phenelzine, a monoamine oxidase inhibitor, produced persistent antidepressant-like effects on behavior without altering NET or SERT expression. For all drugs tested, reductions of NET and SERT protein were not accompanied by reduced NET or SERT mRNA in LC or dorsal raphe nucleus, respectively. Overall, the present results suggest an important, though not universal, role for NET and SERT regulation in the long-term behavioral effects of antidepressants. Understanding the mechanisms underlying transporter regulation *in vivo* may suggest novel targets for the development of antidepressant drugs.

3.2 Introduction

Antidepressants, in general, enhance noradrenergic and/or serotonergic neurotransmission following acute administration (Millan, 2006). For many antidepressants, including the tricyclic antidepressants, serotonin reuptake inhibitors (SRIs), norepinephrine reuptake inhibitors (NRIs), and serotonin-norepinephrine reuptake inhibitors (SNRIs), this results from inhibition of the norepinephrine or serotonin transporters (NET, SERT), which clear the transmitters from the synapse (Beyer et al., 2002; Katz et al., 2004; Morilak and Frazer, 2004; White et al., 2005). Some drugs enhance monoaminergic neurotransmission by other mechanisms such as inhibition of monoamine oxidase (Laux et al., 1995; Lopez-Munoz et al., 2007) or antagonism of presynaptic α-2 adrenergic receptors (Charney et al., 1986; Dickinson, 1990; Millan et al., 2000; Artigas et al., 2001). While enhanced monoaminergic neurotransmission appears to be an effect shared by antidepressants from different chemical and pharmacological classes, its relationship to antidepressant efficacy is unclear since therapeutic effects develop gradually over time with repeated treatment (Frazer and Benmansour, 2002; Nelson et al., 2004; Katz et al., 2004).

This delayed clinical activity has raised questions regarding the relevance of the acute neurochemical effects of antidepressants, including enhancement of monoaminergic neurotransmission, to their long-term effects on behavior. It is believed that enhanced monoaminergic neurotransmission is not sufficient to produce an antidepressant effect, since this neurochemical effect is manifested early, prior to development of a clear, clinical effect. However, enhanced monoaminergic activity appears to be necessary for clinical activity. This is evident from the finding that

inhibition of synthetic enzymes for norepinephrine or serotonin results in a rapid return of symptoms in depressed patients who have been treated successfully with desipramine or fluoxetine, respectively (Charney, 1998; Miller et al., 1996a). Recent work has implicated antidepressant-induced down-regulation of NET and SERT in the brain as a mechanism that may contribute to the long-term pharmacological effects of antidepressant drugs (Hirano et al., 2005a; Thakker et al., 2005; Zhao et al., 2008). Reduced NET or SERT expression would increase monoaminergic neurotransmission, even in the absence of pharmacological inhibition of the transporters.

In a recent study, it was found that repeated treatment with desipramine, which is relatively selective for inhibition of the NET relative to the SERT, reduces the expression of the NET in cerebral cortex and hippocampus in a dose-dependent manner (Zhao et al., 2008). This was evidenced by reduced binding of ^{3}H-nisoxetine and by SDS-PAGE/immunoblotting using NET-specific antisera. The reduced expression results in reduced uptake of ^{3}H-norepinephrine into tissue slices *in vitro* and increased extracellular norepinephrine concentration in the prefrontal cortex measured *in vivo* using microdialysis. At all desipramine treatment conditions when the NET was found to be down-regulated, an antidepressant-like effect on behavior was evident in the forced-swim test. Importantly, NET down-regulation and behavioral effects were observed even when brain and plasma levels of desipramine and its major, active metabolite desmethyldesipramine were not detectable. Finally, as is observed in the clinical setting (Lam et al., 2001; Miller et al., 1996a), the persistent antidepressant-like effect of desipramine in the forced-swim test was lost when tyrosine hydroxylase was inhibited with alpha-methyl-*p*-tyrosine (AMPT; Zhao et al., 2008).

Other studies have reported that repeated treatment with antidepressant drugs reduces the expression of the NET in the brain (Benmansour et al., 2004; Wong et al., 2000). Similarly, some antidepressants, including SRIs, have been reported to reduce the expression of the SERT (Benmansour et al., 1999; Benmansour et al., 2002; Gould et al., 2003; Hirano et al., 2005a; Hirano et al., 2005b; Gould et al., 2006), resulting in enhanced serotonergic neurotransmission. Antidepressant-induced down-regulation of the NET and SERT has been observed with cell lines *in vitro* (Zhu and Ordway, 1997; Zhu et al., 1998; Horschitz et al., 2001; Zhu, 2005; Iceta et al., 2007). This suggests that the down-regulation may result from a direct effect of the drugs on the transporter and is not secondary to increased extracellular concentrations of the transmitter. Thus, NET and SERT down-regulation may be effects caused by reuptake inhibitors and not by drugs that increase monoaminergic neurotransmission by other mechanisms, such as inhibition of monoamine oxidase.

While it appears that NET and SERT down-regulation with chronic treatment is an effect shared by a number of antidepressant drugs that inhibit monoamine reuptake, it is not clear to what extent this action contributes to the long-term behavioral effects of antidepressants from different pharmacological classes; to date, this relationship has only been investigated for desipramine. The present study examined the effects of the antidepressants protriptyline, reboxetine, sertraline, venlafaxine, and phenelzine on the NET and SERT and assessed whether changes in their expression resulted in antidepressant-like effects on behavior, similar to what has been observed with desipramine (Zhao et al., 2008). Protriptyline, a tricyclic antidepressant, produces neurochemical and behavioral effects similar to those of desipramine (Crissman and

O'Donnell, 2002; O'Donnell et al., 2005). Reboxetine is an NRI that exhibits somewhat greater selectivity for the NET vs. the SERT compared to desipramine and protriptyline (Scates and Doraiswamy, 2000; Wong et al., 2000; Iversen, 2006). Sertraline is an SRI and is active in a number of antidepressant models (Benmansour et al., 1999; Benmansour et al., 2002). Venlafaxine, although classified as an SNRI, exhibits markedly greater potency for inhibition of the SERT than the NET (Iversen, 2006). Phenelzine, a monoamine oxidase inhibitor, does not interact directly with the NET or SERT, but increases monoaminergic neurotransmission and has antidepressant activity (Thase et al., 1995). Overall, the present study shows that down-regulation of the NET or SERT is produced by some antidepressants that interact with the transporters and that this effect contributes to their long-term behavioral effects; however, persistent antidepressant-like effects on behavior can, in some cases, be observed even when NET or SERT expression is not altered.

3.3 Materials and Methods

Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 300-350g, were housed 2 per cage in a temperature-controlled room (22 - 23° C) with a 12-h on/12-h off light cycle (lights on at 6:00 AM). Food and water were freely provided. Blind observations were used throughout all behavioral testing, which was carried out from 1:00 PM – 5:00 PM in a quiet room. All procedures were reviewed and approved by the Animal Care and Use Committee of West Virginia University Health Sciences Center, and are consistent with the NIH "Guidelines for the Care and Use of Laboratory Animals" (NIH publication No. 80-23, revised 1996).

Drugs administration

Rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and implanted subcutaneously with osmotic minipumps (model 2ML2 or 2ML4, Alzet Corporation, Palo Alto, CA) preloaded with either vehicle (50% saline, 40% DMSO, and 10% ethanol), protriptyline (Sigma-Aldrich, St Louis, MO), reboxetine (provided by Institut de Recherches Servier, Croissy/Seine, France), sertraline (Tocris, Ellisville, MO) or venlafaxine (Sigma-Aldrich, St Louis, MO) at concentrations that delivered 10 or 20 (protriptyline), 20 (reboxetine), 7.5 (sertraline), or 70 (venlafaxine) mg/kg/day of the free base. Minipumps were removed, under anesthesia, 14 days later. However, for some rats treated with reboxetine, minipumps were removed 21 days after implantation and new minipumps were implanted, providing 42 days of drug administration. A dose of 10 mg/kg/day (i.p.) of the irreversible monoamine oxidase inhibitor phenelzine was

administrated for 14 days. Doses of each of the drugs used were chosen based on previous reports of neuropharmacological activity (Ordway et al., 1991; Gould et al., 2006).

Rats were tested for antidepressant-like behavior in the forced-swim test and locomotor activity in the open-field test 2 days after pump removal or the last phenelzine treatment; this minimized acute drug effects. However, the behavioral tests for one group treated with 10 mg/kg/day protriptyline were performed 1 day after the removal of pumps. Following the completion of the behavioral tests, rats were sacrificed by decapitation, their brains removed, and cerebral cortex and hippocampus dissected for measurement of NET and SERT using ^{3}H-nisoxetine or ^{3}H-citalopram binding and SDS-PAGE/immunoblotting with specific antisera. Also, locus coeruleus and raphe nucleus were punched from surrounding tissue for real-time PCR measurement of NET and SERT mRNA.

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 (i.e., 1 day after cessation of antidepressant 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 chronic antidepressant 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 treated with 10 mg/kg/day protriptyline were subjected to 5-min forced-swim testing one day after the end of the antidepressant treatment regimen, with the 15-min pre-test on the last day of drug treatment. Finally, in some tests, rats were administered the tryptophan hydroxylase inhibitor *p*-chlorophenylalanine (PCPA, 300 mg/kg, i.p; Sigma-Aldrich, St Louis, MO) twice daily for three consecutive days, with the last dose given 18 h before 5-min forced-swim test. This was done to determine whether reduced serotonergic neurotransmission blocked the antidepressant-like effect observed in the forced-swim test following down regulation of the SERT; this paralleled previous experiments examining the effects of reduced noradrenergic neurotransmission on persistent antidepressant-like behavior observed following desipramine-induced down-regulation of the NET (Zhao et al., 2008).

To ensure that any changes in immobility in the forced-swim test were not secondary to increased locomotor activity, rats were observed in the open field test (Zhang et al., 2008). The floor of the open-field apparatus was divided into nine identical squares. Rats were placed individually in the center and allowed to explore for 5 min; the number of line crossings (with all four paws crossing a line) and rears were recorded. None of the treatments increased locomotor activity, so these data are not shown.

SDS-PAGE/Immunoblotting of SERT and 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_3VO_4, 1 mM EDTA, 1 mM EGTA, 320 mM sucrose; pH 7.4) with a glass homogenizer and centrifuged at 1,000 × g for 20 min at 4° C. The

supernatant was centrifuged at 20,000 × g for 30 min to obtain a crude synaptosomal fraction (Lin et al., 1998; Wyszynski et al., 1998; Dunah et al., 2001). The pellets were re-suspended 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 assay (Smith, 1985; Pierce, Rockford, IL). Equal amounts of protein from both control and treated rats were mixed with 2X 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 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, rabbit polyclonal antibody generated against a unique 22 amino acid peptide sequence mapping to the first extracellular domain of the NET (Chemicon, Temecula, CA; Zhao et al., 2008) or a peptide fragment of the C-terminus of the SERT (Santa Cruz, Santa Cruz, CA; Xie et al., 2006). After washing with TBST, membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Pierce, Rockford, IL). The labeled protein bands were detected by chemiluminescence imaging (Bio-Rad, Hercules, CA) and quantified by densitometry using an NIH image analysis program, and normalized to actin. Comparisons of treatment effects were carried out within single gels/immunoblots.

^{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). One-half of the cerebral cortex or hippocampus was 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 (Brinkman Instruments, Westbury, NY) and then centrifuged at 36,000 × g for 10 min at 4° C. The supernatant was discarded, the pellet re-suspended in ice-cold binding buffer, centrifuged at 36,000 × g for 10 min at 4° C, and the pellet re-suspended in binding buffer to obtain a protein concentration of 1 mg/ml as assayed using the bicinchoninic acid method (Smith, 1985; Pierce, Rockford, IL). Triplicate tubes containing 100 µl incubation buffer (total binding) or 100 µl desipramine (final concentration 50 µM; non-specific binding), 100 µl tissue sample, and 50 µl ^{3}H-nisoxetine (final concentration 5 nM; 72 Ci/mmol, PerkinElmer, Boston, MA) were incubated in a shaking ice bath for 4 h. Non-specific binding (i.e., in the presence of 50 µM desipramine) was approximately 10% of total binding. Bound ^{3}H-nisoxetine was captured by rapid filtration through glass fiber filters (GF/B Brandel, Gaithersburg, MD) that were pre-soaked in 5% polyethyleneimine under vacuum with a cell harvester (Brandel, Gaithersburg, MD). Filters were washed twice with ice-cold binding buffer and radioactivity determined by liquid scintillation counting.

^{3}H-citalopram binding assay

^{3}H-Citalopram binding to the SERT was performed as described previously (D'Amato et al., 1987; Gould et al., 2006). One-half of the cerebral cortex or hippocampus was 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 (Brinkman Instruments, Westbury, NY) and then centrifuged at 36,000 × g for 10 min at 4° C. The supernatant was discarded, the pellet re-suspended in ice-cold binding buffer, centrifuged at 36,000 × g for 10 min at 4° C, and the pellet re-suspended in binding buffer to obtain a protein concentration of 1 mg/ml as assayed using the bicinchoninic acid method (Smith, 1985; Pierce, Rockford, IL). Triplicate tubes containing 100 µl incubation buffer (total binding) or 100 µl fluoxetine (final concentration 50 µM; non-specific binding), 100 µl tissue sample, and 50 µl ^{3}H-citalopram (final concentration 5 nM; 79 Ci/mmol, PerkinElmer, Boston, MA) were incubated for 1 h with shaking at room temperature. Non-specific binding (i.e., in the presence of 50 µM fluoxetine) was approximately 10% of total binding. Bound ^{3}H-citalopram was captured by rapid filtration through glass fiber filters (GF/B Brandel, Gaithersburg, MD) that were pre-soaked in 5% polyethyleneimine under vacuum with a cell harvester (Brandel, Gaithersburg, MD). Filters were washed twice with ice-cold binding buffer and radioactivity determined by liquid scintillation counting.

Measurement of NET and SERT mRNA by quantitative real-time PCR

Locus coeruleus (for NET mRNA) and dorsal and median raphe nucleus (for SERT mRNA) were punched from the rat brain (Chamas et al., 1999; Chamas et al., 2004; Paxinos and Watson, 1998; Zhu et al., 2002). Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA) and was kept in RNAlater buffer (Ambion, Austin, TX) and stored at -80° C. Total RNA concentration was determined by spectrophotometry and adjusted to 0.5 µg/µl. The quality of the RNA was verified using 1% denaturing agarose gel electrophoresis. Contaminating genomic DNA was

eliminated by incubating with DNase mixture (Qiagen, Valencia, CA) at room temperature for 20 min. RNA was reverse transcribed to cDNA with high capacity cDNA archive kit (Applied Biosystems, Foster City, CA). The cDNA was subjected to real-time PCR using TaqMan universal PCR Master Mix and ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA). Specific primers and probes were purchased from pre-designed, pre-optimized TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). Quantities of NET and SERT mRNA were normalized to the 18s rRNA. Analysis was performed using ABI-7300 system SDS software.

Statistical analysis

The data were analyzed by one-way analyses of variance (ANOVA) followed by Tukey's or Bonferroni's *post hoc* tests. When simple, paired comparisons between two groups were conducted, two-tailed Student's t-tests were used. Data are presented as means ± SEM and differences are considered statistically significant when p values are less than 0.05.

3.4 Results

The neurochemical and behavioral effects of repeated antidepressant treatment were assessed 2 days after the end of the treatment period (i.e., osmotic minipump removal or discontinuation of phenelzine injections), except for some tests with protriptyline (see below). This permitted assessment of persistent effects under conditions when brain and plasma concentrations would be minimized (Zhao et al., 2008). None of the treatments increased locomotor activity, which could confound interpretation of results in the forced-swim tests; however, one day following treatment with protriptyline, locomotor activity was reduced (i.e., line crosses in the open-field test, to 65% of control; data not shown).

Effects of protriptyline

The relationship between the effect of chronic protriptyline treatment on NET expression and its effect on behavior in the forced-swim test was examined. Treatment of rats with 20 mg/kg/day protriptyline for 14 days, with a 2-day washout period, reduced NET expression, as indicated by a reduction of the binding of ^{3}H-nisoxetine to the NET in preparations of cerebral cortex ($F_{(4,21)}= 5.75$, $p < 0.01$; Fig. 3.1A) and hippocampus ($F_{(4,21)}= 7.43$, $p < 0.001$; Fig. 3.1A). Down-regulation of the NET also was demonstrated using SDS-PAGE/immunoblotting of preparations of cerebral cortex ($F_{(2,12)}= 12.19$, $p < 0.001$; Fig. 3.1B) and hippocampus ($F_{(2,12)}= 13.81$, $p < 0.001$; Fig. 3.1B). Down-regulation of NET expression was not observed 2 days following the end of treatment with 10 mg/kg/day protriptyline for 14 days, but was seen 1 day following this treatment (Fig. 3.1A).

A persistent antidepressant-like effect in the forced-swim test was evident 2 days after discontinuation of 14-day treatment with 20 mg/kg/day protriptyline or 1 day after discontinuation of 14-day treatment with 10 mg/kg/day protriptyline ($F_{(4,25)}$= 10.00, $p < 0.001$; Fig. 3.1C); this paralleled the protriptyline-induced down-regulation of the NET. No antidepressant-like effect on behavior was observed 2 days after chronic protriptyline treatment at a dose of 10 mg/kg/day (Fig. 3.1C), consistent with the lack of NET down-regulation at this treatment condition.

In order to determine whether the down-regulation of the NET resulted from a change in gene transcription, NET mRNA expression in the locus coeruleus was measured by quantitative RT-PCR. The normalized, relative NET mRNA expression was not changed 2 days following the end of chronic treatment with 20 mg/kg/day protriptyline or 1 day following the end of chronic treatment with 10mg/kg/day protriptyline ($F_{(2,27)}$= 2.14, $p > 0.05$; Table 3.1). Similarly, repeated treatment with 15 mg/kg/day desipramine, which reduces NET protein expression (Zhao et al., 2008), also did not reduce NET mRNA in locus coeruleus ($p > 0.05$; Table 3.1).

Effects of reboxetine

The relationship between the effect of chronic reboxetine treatment on NET expression and its effect on behavior in the forced-swim test was examined. Treatment of rats with 20 mg/kg/day reboxetine for 6 weeks, with a 2-day washout, reduced NET expression, as indicated by a reduction of the specific binding of ^{3}H-nisoxetine to the NET in preparations of cerebral cortex ($F_{(2,12)}$ =23.00; $p < 0.0001$; Fig. 3.2A) and hippocampus ($F_{(2,12)}$ =3.78; $p < 0.05$; Fig. 3.2A); however, treatment for two weeks did

not alter NET expression significantly. By contrast, SERT expression (i.e., ^{3}H-citalopram binding) was not altered by reboxetine treatment, either in preparations of cerebral cortex ($F_{(2,14)}$ =1.67; $p > 0.05$; Fig. 3.2B) or hippocampus ($F_{(2,16)}$ =0.68; $p > 0.05$; Fig. 3.2B).

Down-regulation of the NET by six-week chronic reboxetine treatment also was demonstrated using SDS-PAGE/immunoblotting of preparations of cerebral cortex ($F_{(2,13)}$ =5.99; $p < 0.05$; Fig. 3.2C) and hippocampus ($F_{(2,16)}$ =2.94; $p < 0.05$; Fig. 3.2C). By contrast, this treatment did not alter SERT expression, measured by immunoblotting, in either cerebral cortex or hippocampus (Fig. 3.2D). Interestingly, repeated treatment with 15 mg/kg desipramine for 14 days reduced SERT expression in cerebral cortex and hippocampus as evidenced using ^{3}H-citalopram binding (40 – 50% reduction; data not shown).

In parallel with the down-regulation of the NET, chronic treatment with reboxetine for 6 weeks also produced a reduction in the duration of immobility in the forced-swim test, indicative of an antidepressant-like effect ($F_{(2,15)}$ =34.01; $p < 0.001$; Fig. 3.2E). Although the 2-week reboxetine treatment did not reduce NET expression in cerebral cortex or hippocampus, an antidepressant-like effect still was observed in the forced-swim test following this treatment (Fig. 3.2E).

In order to determine whether the down-regulation of NET resulted from a change in NET gene transcription, NET mRNA expression in the locus coeruleus and SERT mRNA expression in the dorsal and medial raphe nucleus were measured using quantitative RT-PCR. The normalized, relative NET mRNA expression in the locus coeruleus was elevated, about 3-fold, 2 days after discontinuation of chronic reboxetine treatment ($p <$

0.001; Table 3.1). The normalized, relative SERT mRNA expression in the dorsal and medial raphe nucleus was increased about 2.5-fold ($p < 0.01$; Table 3.1).

Effects of sertraline

Given the apparent relationship between the down-regulation of the NET by chronic treatment with NRIs and persistent antidepressant-like effects in the forced-swim test, it was of interest to also examine the relationship between changes in SRI-induced SERT expression and behavior. Rats were treated chronically with 7.5 mg/kg/day sertraline for 14 days and subjected to the forced-swim test 2 days after the end of sertraline treatment (i.e. removal of the osmotic pumps), and SERT expression was determined in several brain regions using ^{3}H-citalopram binding and Western blotting. Further, to determine whether enhanced serotonergic neurotransmission was necessary for the persistent antidepressant effect in the forced-swim test, a serotonin depletion approach was utilized, similar to that used to assess the role of serotonergic neurotransmission in the clinical actions of sertraline (Miller et al., 1996b). Rats were treated with PCPA, an inhibitor of tryptophan hydroxylase, the rate-limiting enzyme for serotonin biosynthesis. PCPA (300 mg/kg, i.p.) was administrated twice daily for three consecutive days with the last dose given 18 h before behavioral test (O'Leary et al., 2007) to rats that had been treated chronically with vehicle or sertraline.

A significant reduction of SERT binding sites, measured using ^{3}H-citalopram, was evident 2 days after the discontinuation of chronic sertraline treatment in the cerebral cortex ($F_{(3,18)}= 8.17$, $p < 0.01$; Fig. 3.3A) and hippocampus ($F_{(3,18)}= 10.06$, $p < 0.001$; Fig. 3.3A). By contrast, NET expression (i.e., ^{3}H-nisoxetine binding) was not altered by

sertraline treatment, either in preparations of cerebral cortex ($p > 0.05$; Fig. 3.3B) or hippocampus ($p > 0.05$; Fig. 3.3B).

The down-regulation of the SERT also was demonstrated using SDS-PAGE/immunoblotting of preparations of cerebral cortex ($F_{(3,16)}= 6.94$, $p < 0.01$; Fig. 3.3C) and hippocampus ($F_{(3,16)}= 14.37$, $p < 0.001$; Fig. 3.3C). By contrast, this treatment did not alter NET expression, measured by immunoblotting, in either cerebral cortex or hippocampus (Fig. 3.3D).

In parallel with the down-regulation of the SERT, chronic sertraline treatment also produced an antidepressant-like effect in the forced-swim test, demonstrated by a reduction of immobility time ($F_{(3,19)}= 19.57$, $p < 0.001$; Fig. 3.3E). Three-day, twice daily treatment with 300 mg/kg/day PCPA, to reduce serotonin neurotransmission, reversed the behavioral actions of chronic sertraline treatment, demonstrated by the increased immobility time and reduced latency to immobility (Fig. 3.3E); PCPA, when administered alone, did not affect behavior in the forced-swim test (Fig. 3.3E), nor did it alter SERT expression (Fig. 3.3C).

There was a significant effect of sertraline administration on normalized, relative SERT mRNA expression in the dorsal and median raphe nucleus ($F_{(3,18)}=28.5$, $p < 0.001$; Table 3.1). *Post hoc* tests revealed that while chronic sertraline treatment did not change the SERT mRNA expression, a significant decrease in SERT mRNA expression was found in the groups treated with PCPA alone or in combination with chronic sertraline (Table 3.1).

Effects of venlafaxine

Given that venlafaxine inhibits both the NET and SERT, i.e. it is an SNRI, it was of interest to explore the relationship between venlafaxine-induced changes in NET and SERT expression and behavior. Rats were treated chronically with 70 mg/kg/day venlafaxine for 14 days and subjected to the forced-swim test 2 days after the end of venlafaxine treatment. NET and SERT binding sites were determined in several brain regions using ^{3}H-nisoxetine and ^{3}H-citalopram binding. NET and SERT protein expression was determined by Western blotting. Treatment of 70 mg/kg/day venlafaxine did not alter NET (Fig. 3.4A) or SERT binding sites (Fig. 3.4B) in cerebral cortex and hippocampus. Consistently, NET and SERT protein expression in cerebral cortex and hippocampus was not changed after chronic venlafaxine treatment (Fig. 3.4C, 3.4D). However, in spite of the lack of any change in NET or SERT expression, an antidepressant-like effect in the forced-swim test was observed two days after the end of the chronic venlafaxine treatment period ($p < 0.001$; Fig. 3.4E).

Effects of phenelzine

To determine whether enhanced monoaminergic neurotransmission, independent of NET or SERT inhibition, was sufficient to alter expression of the transporters, the effects of the monoamine oxidase inhibitor phenelzine were determined. Rats were treated with 10 mg/kg/day phenelzine (i.p.) for 14 days and subjected to the forced-swim test 2 days after the end of the treatment period. NET and SERT expression was then assessed in cerebral cortex and hippocampus.

Phenelzine treatment did not alter NET or SERT expression in cerebral cortex or hippocampus, measured by ^{3}H-nisoxetine and ^{3}H-citalopram binding (Fig. 3.5A, 3.5B) and SDS-PAGE/immunoblotting (Fig. 3.5C, 3.5D). However, a significant decrease in immobility time in the forced-swim test was observed following chronic phenelzine treatment ($p < 0.001$; Fig. 3.5E).

3.5 Discussion

In a previous study, the relationship between desipramine-induced down-regulation of the NET and antidepressant effects on behavior was observed (Zhao et al., 2008). The down-regulation of the NET is associated with antidepressant effects on behavior, even when there is no detectable drug present to inhibit norepinephrine reuptake. Desipramine-induced down-regulation of the NET and the development of persistent antidepressant-like effects on behavior develop gradually with repeated treatment, consistent with the development of the clinical response to antidepressants (Frazer et al., 2002; Katz et al., 1996, 2004; Gelenberg and Chesen, 2000). This suggests that neural adaptation resulting in enhanced monoaminergic neurotransmission may be a key mechanism mediating antidepressant actions. Consistent with this, it was found that inhibition of catecholamine synthesis with AMPT reverses the persistent antidepressant-like effect in the forced-swim test (Zhao et al., 2008); similar effects have been observed in depressed patients treated with desipramine (Charney, 1998; Miller et al., 1996a). The results of the present study demonstrated that NET down-regulation appears to be important for the actions of protriptyline and reboxetine, two other antidepressants that interact with the NET. Further, SERT down-regulation contributes to the long-term behavioral effects of the SRI sertraline.

NET expression, measured by ^{3}H-nisoxetine binding and SDS-PAGE/immunoblotting, was reduced after 14 days of treatment with protriptyline or 42 days of treatment of reboxetine, similar to the effect observed after repeated treatment with desipramine (Zhao et al., 2008). β-1 adrenergic receptor density also is reduced after chronic desipramine, protriptyline, or reboxetine treatment (Ordway et al., 1991; Gould et

al., 2003). This suggests that NET down-regulation results in increased noradrenergic neurotransmission; this has been verified experimentally for desipramine and reboxetine treatment using *in vivo* microdialysis (Page and Lucki, 2002; Lapiz et al., 2007; Zhao et al., 2008).

NET down-regulation in response to NRI treatment, in addition to being observed *in vivo* (Zhu et al., 2002; Weinshenker et al., 2002; Zhao et al., 2008), also has been shown *in vitro* in cell lines expressing the NET. NET expression in SK-N-BE(2)M17 cells is decreased following 3 – 14 days of exposure to desipramine (Zhu et al., 1998). Chronic desipramine-induced NET down-regulation also was observed 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). Because these cells lack synaptic innervation, these findings suggest that desipramine-induced NET regulation is, at least in part, a direct effect of occupation of the NET, and not secondary to an increase in the extracellular concentration of NE. Consistent with this interpretation, it was found that chronic treatment with the monoamine oxidase inhibitor phenelzine, which increases noradrenergic neurotransmission, does not down-regulate the NET. However, it did produce antidepressant-like effects in the forced-swim test.

In the present study, it was found that repeated treatment with the SRI sertraline reduced SERT expression, measured using ^{3}H-citalopram binding and SDS-PAGE/immunoblotting. Previously, it has been found that SERT density in the CA3 region of the hippocampus, assessed by quantitative autoradiography with ^{3}H-cyanoimipramine, is decreased by 80-90% by chronic sertraline or paroxetine treatment (Benmansour, et al., 1999). After 15 days of sertraline treatment, SERT binding sites are

reduced by 80%, accompanied by a marked reduction of serotonin clearance (Benmansour et al., 2002). SERT density in the dentate gyrus, lateral nucleus of the amygdala, and dorsal raphe, measured by ^{3}H-cyanoimipramine binding, is significantly reduced after chronic paroxetine treatment (Gould et al., 2003). 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). SERT expression in mouse brain regions is reduced after chronic paroxetine treatment; this effect persists for 120 h after 21 days of treatment (Hirano et al., 2005b). In humans, it has been reported that treatment with 40 mg/day citalopram for 8 days reduces SERT binding sites in the diencephalon and brainstem (Kugaya et al., 2003). To date, it had not been determined whether persistent down-regulation of the SERT resulted in the antidepressant effects on behavior. In the present study, it was found that two days following discontinuation of sertraline treatment, when SERT expression was reduced in cerebral cortex and hippocampus, a persistent antidepressant-like effect in the forced-swim test was observed.

In HEK cells expressing the SERT, its binding sites are reduced after a 3-day incubation with 500 nM citalopram; this is accompanied by decreased maximal transporter activity (V_{max}) and an unchanged K_m value (Horschitz et al., 2001). *In vitro*, down-regulation of SERT is associated with protein kinase C (PKC) activation-induced phosphorylation of specific amino acid residues, which triggers translocation of SERT from membrane to cytoplasm (Jayanthi et al., 2004; Sung et al., 2005; Sung et al., 2007; Amano et al., 2006). Qian et al. (1997) and Lau et al. (2008) also have shown that stimulation of PKC causes internalization of cell-surface SERT protein.

While treatment with the NRI reboxetine reduced NET expression without altering the SERT, consistent with its reported selectivity (Wong et al., 2000; Millan et al., 2001; Versiani et al., 1999), desipramine treatment reduced expression of both the NET and the SERT. This may result from a lack of selectivity for desipramine for the NET at the dose tested (Iversen, 2006) or may be due to the effects of desipramine's primary, active metabolite desmethyldesipramine, which reaches concentrations similar to the parent compound (Kozisek et al., 2007). In preparations of rat brain, desmethyldesipramine exhibits 12-fold greater affinity for the SERT compared with the NET; desmethyldesipramine's affinity for the SERT is similar to desipramine's for the NET (Deupree et al., 2007). Thus, immediately after desipramine administration, when the desipramine concentration is much higher than that of desmethyldesipramine, the pharmacological effect of desipramine might be associated primarily with the NET, while later as the concentration of the longer-lived metabolite desmethyldesipramine increases, both SERT and NET may be involved.

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 monoaminergic neurotransmission. By contrast, changes in monoaminergic receptor expression often are homeostatic, i.e., in opposition to the effect of acute antidepressant treatment (Ordway et al., 1991). Preclinically, AMPT partially prevents the acute desipramine response, while a combination of AMPT and reserpine

completely prevents the behavioral effects of acute desipramine (O'Leary et al., 2007); this suggests that disruption of both catecholamine synthesis and vesicular storage and release are required to completely block desipramine's effects. PCPA, a tryptophan hydroxylase inhibitor (Koe and Weissman, 1966), completely reverses the antidepressant-like effect produced by acute treatment with the SRIs fluoxetine and citalopram (Page et al., 1999; O'Leary et al., 2007). 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 the return of symptoms of depression in depressed patients treated chronically with NRIs such as desipramine, but not SRIs (Miller et al., 1996a; Miller et al., 1996b; Bremner et al., 2003). By contrast, inhibition of serotonin synthesis with PCPA or a tryptophan-free amino acid drink reverses symptom remission caused by SRIs, but not NRIs (Miller et al., 1996b; Salomon et al., 1993).

Consistent with these clinical data, it has been found in an earlier study that inhibition of catecholamine synthesis with AMPT reverses the persistent antidepressant-like effect of desipramine in the forced-swim test (Zhao et al., 2008); changes in both noradrenergic and dopaminergic neurotransmission may contribute to this effect of AMPT (O'Donnell and Seiden, 1984). A role for noradrenergic neurotransmission is suggested by the finding that NE-deficient mice fail to respond to the behavioral effects of the NRI desipramine. Restoration of noradrenergic function by l-threo-3,4-dihydroxyphenylserine (l-DOPS) reinstates the behavioral effects of desipramine in these

mice (Cryan et al., 2004). In the present study, it was found that inhibition of serotonin synthesis with PCPA reversed the persistent antidepressant-like effect of sertraline in the forced-swim test. 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.

Whether the down-regulation of the NET or SERT is mediated by altered 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; Zhu et al., 2002). However, *in vivo*, it is found that NET mRNA expression in the locus coeruleus is elevated after chronic desipramine treatment (Szot et al., 1993). Further, it is found that the SERT mRNA expression in the raphe nucleus is unchanged following chronic treatment with SRIs (Benmansour et al., 1999; Abumaria et al., 2007). Lopez et al. (1994) found that the SERT mRNA level is increased after chronic antidepressant treatment, while Lesch et al. (1993) reported a decreased SERT mRNA level. *In vitro*, the expression of NET mRNA in human enterocyte-like cell line Caco-2 (Iceta et al., 2007) and SERT-expressing HEK293 cells (Lau et al., 2008) is unaltered after chronic SRIs treatment. In the present study, it was found that NET mRNA expression in the rat locus coeruleus was unchanged after chronic desipramine or protriptyline treatment, while it was elevated 3-fold after the 6-week reboxetine treatment regimen. Given that the change in NET protein expression produced by these drugs is reduced, i.e., in the opposite direction, it is unlikely that chronic antidepressant-induced down-regulation of the NET is regulated at the

transcriptional level. In fact, exposure to reboxetine for six weeks might trigger a compensatory increase in transcription. Changes in SERT mRNA also do not appear to account for sertraline-induced changes in SERT protein expression. However, while sertraline treatment alone did not alter SERT mRNA, PCPA alone or in combination with sertraline reduced it. Interestingly, SERT mRNA expression in the raphe nucleus was increased after the six-week reboxetine treatment; the mechanism mediating this effect and its functional significance are unclear.

It seems that down-regulation of NET or SERT is not the only mechanism mediating persistent antidepressant-like behavioral effects. Treatment of rats with 20 mg/kg/day reboxetine for two weeks did not reduce the NET expression but did produce an antidepressant-like effect in the forced-swim test 2 days after the end of the treatment period. Further, 2-week treatment with 70 mg/kg/day venlafaxine treatment failed to decrease the NET or SERT expression but still produced a persistent antidepressant-like effect in the forced-swim test. It also was reported that NET and SERT binding sites, measured using quantitative autoradiography with ^{3}H-citalopram, are not reduced in the CA3 region of hippocampus after treatment for 21 days with 15 or 70 mg/kg/day venlafaxine (Gould et al., 2006). Similarly, chronic treatment with amitriptyline, a tricyclic antidepressant that inhibits both the NET and SERT, also fails to reduce SERT and NET binding sites (Owens et al., 1997). The inability of venlafaxine to down-regulate the NET or SERT following chronic treatment is not due to a lack of efficacy, since treatment with 70 mg/kg/day causes a down-regulation of β-1 adrenergic receptors in the same rats in which no down-regulation of the transporters is observed (Gould et al., 2006). Also, chronic venlafaxine produces a dose-dependent attenuation of the

hypothermic response of rats to acute treatment with 8-OH-DPAT, a serotonin-1A receptor agonist, similar to the effects produced by sertraline and citalopram (Hensler et al., 2003; Rossi et al., 2005). The reason why chronic reboxetine and venlafaxine failed to down-regulate NET or SERT but still showed antidepressant-like behavioral effects is not known. It is possible that differential stimulation of heteroreceptors (Chiang and Aston-Jones, 1993; Mongeau et al., 1993), distinct interactions with P450 isoenzymes (Cadieux, 1999; Tanaka, 1999), different binding affinities for the transporters (Lenox and Frazer, 2002), and details regarding the nature and location of binding to the NET or SERT (Larsen et al., 2004; Schmid et al., 2001) may account for the observed differences among the effects of the antidepressants tested.

Overall, the present results demonstrate a relationship between antidepressant-induced changes in NET and SERT expression and the development of persistent antidepressant-like effects on behavior. Also, consistent with clinical data, it was found that the antidepressant-like effect that resulted from SERT down-regulation depended on the enhanced serotonergic neurotransmission, since it was lost when serotonin synthesis was inhibited. A similar dependence of desipramine-induced NET down-regulation on enhanced noradrenergic neurotransmission was demonstrated previously (Zhao et al., 2008). The present data provide support for the idea that the down-regulation of NET and SERT may contribute to the long-term therapeutic effects of antidepressant drugs. The mechanism of NET and SERT regulation appears to be due more to altered internalization and degradation than altered transcription. Understanding the molecular mechanisms mediating NET and SERT regulation *in vivo* might suggest novel targets for therapeutic intervention in the treatment of depression.

Table 3.1 Effects of chronic antidepressant treatment on NET mRNA expression in the locus coeruleus and SERT mRNA expression in the raphe nucleus

Antidepressants (mg/kg/day)	Normalized NET mRNA level in locus ceruleus	Normalized SERT mRNA level in raphe nuclei
Protriptyline		
Control	1.41 ± 0.26	N.D.
20	1.80 ± 0.33	N.D.
10	1.73 ± 0.17	N.D.
Desipramine		
Control	1.59 ± 0.54	N.D.
15	2.42 ± 0.40	N.D.
Reboxetine		
Control	1.08 ± 0.14	1.04 ± 0.09
20	3.14 ± 0.69***	2.61 ± 0.73**
Sertraline		
Control	N.D.	1.24 ± 0.34
PCPA	N.D.	0.19 ± 0.04***
7.5	N.D.	1.47 ± 0.33
PCPA+7.5	N.D.	0.42 ± 0.22***

Rats were treated chronically with antidepressants via osmotic pumps. NET and SERT mRNA were determined by the real-time PCR 2 days after discontinuation of chronic treatment (1 day post-treatment for 10 mg/kg protriptyline). Values shown are ratios relative to 18S rRNA (mean ± SEM; n = 5/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N.D., not determined.

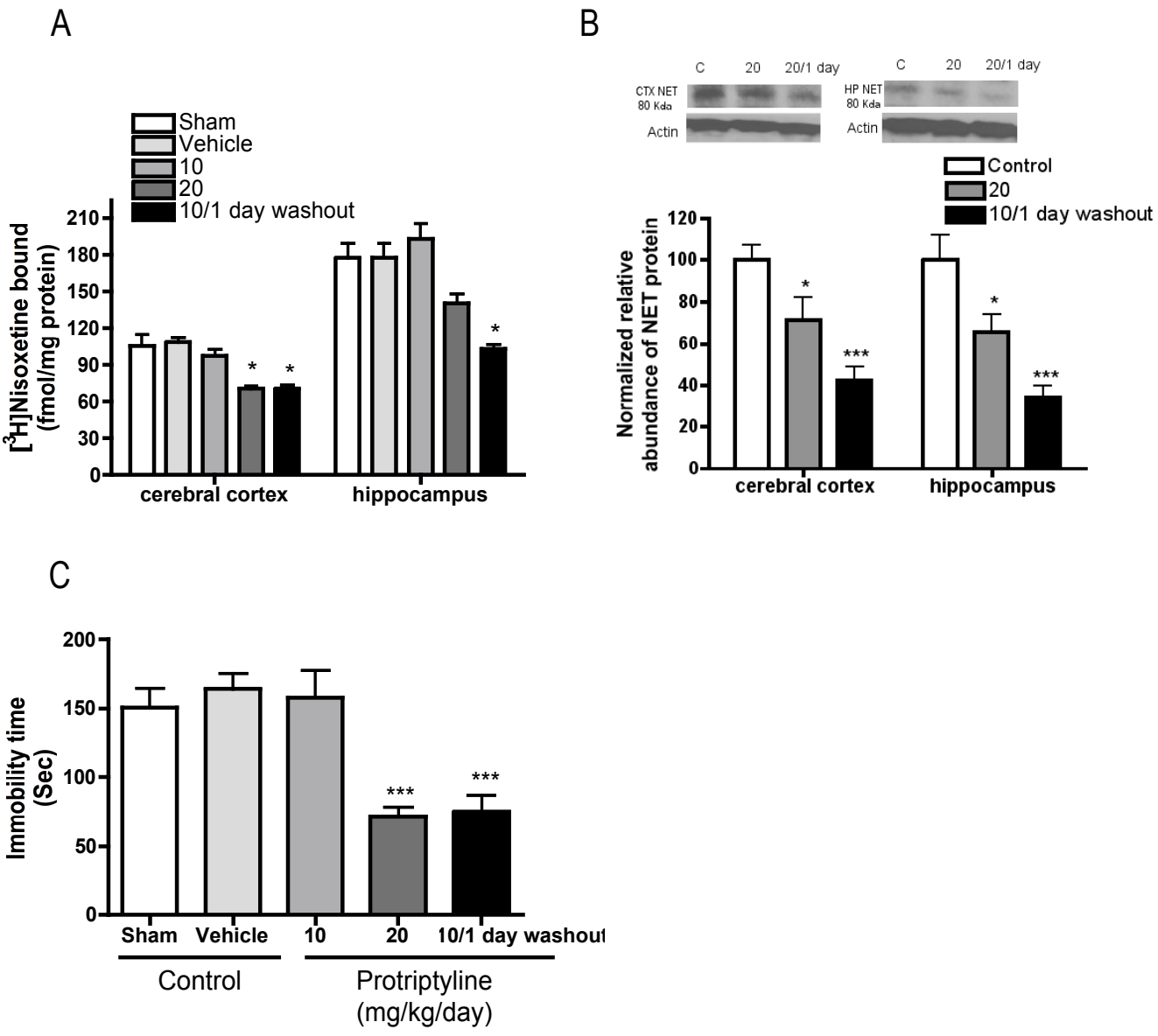


Figure 3.1 The effects of chronic protriptyline were determined for the ability to reduce NET expression in the cerebral cortex and hippocampus, determined by ^{3}H-nisoxetine binding (A) and SDS-PAGE/immunoblotting (B), and to reduce immobility time in the forced-swim test (C). All measures were determined two days after the end of the chronic treatment period except one group (10 mg/kg/day with 1 day washout). Data shown are means ± SEM of 5-8 rats per group. *$p < 0.05$; ***$p < 0.001$ vs. control.

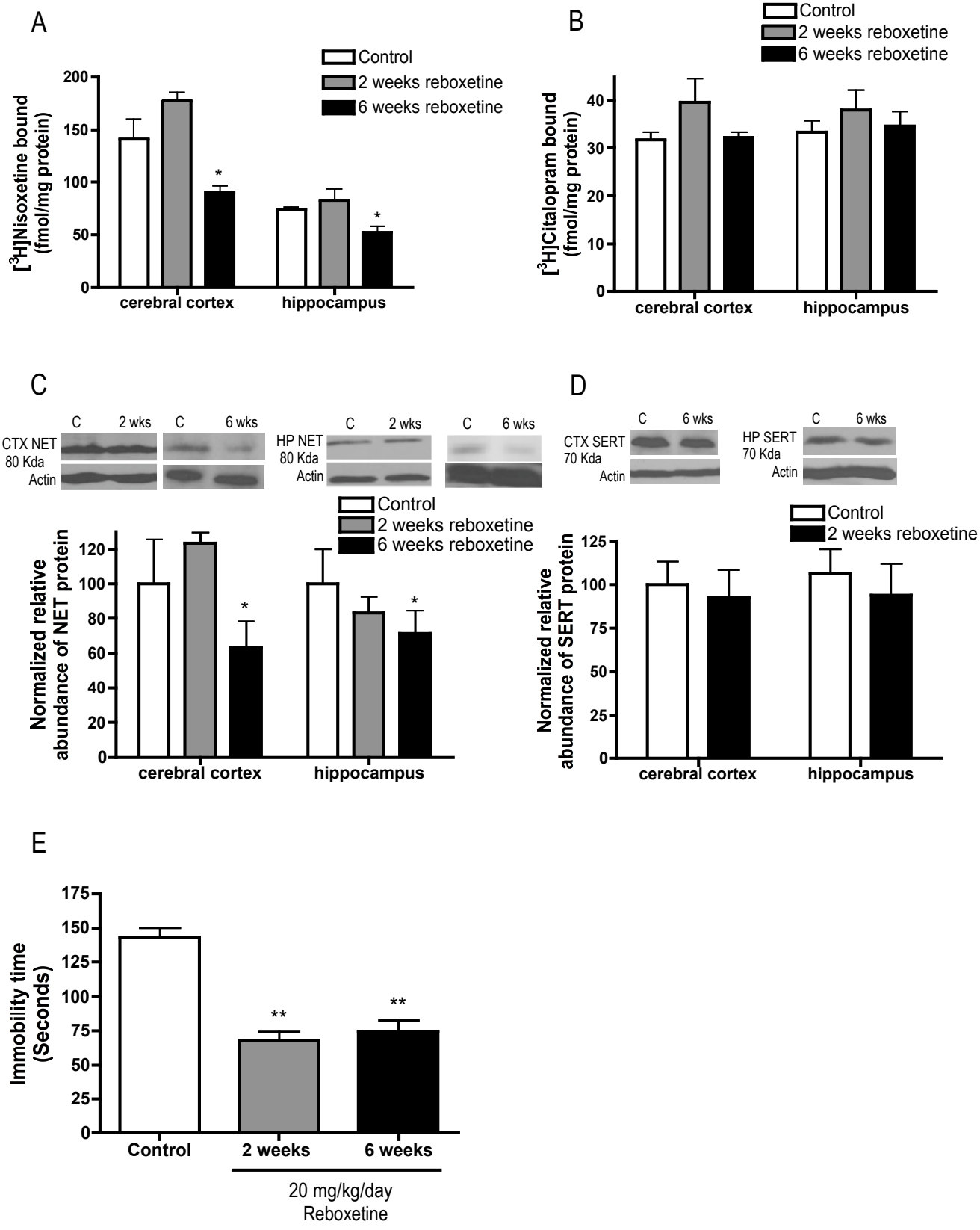

A
Control
2 weeks reboxetine
6 weeks reboxetine
[3H]Nisoxetine bound (fmol/mg protein)
200
150
100
50
0
*
*
cerebral cortex
hippocampus
B
Control
2 weeks reboxetine
6 weeks reboxetine
[3H]Citalopram bound (fmol/mg protein)
40
30
20
10
0
cerebral cortex
hippocampus
C
C
2 wks
C
6 wks
CTX NET
80 Kda
Actin
C
2 wks
C
6 wks
HP NET
80 Kda
Actin
Control
2 weeks reboxetine
6 weeks reboxetine
Normalized relative abundance of NET protein
120
100
80
60
40
20
0
*
*
cerebral cortex
hippocampus
D
C
6 wks
CTX SERT
70 Kda
Actin
C
6 wks
HP SERT
70 Kda
Actin
Control
2 weeks reboxetine
Normalized relative abundance of SERT protein
125
100
75
50
25
0
cerebral cortex
hippocampus
E
Immobility time (Seconds)
175
150
125
100
75
50
25
0
**
**
Control
2 weeks
6 weeks
20 mg/kg/day
Reboxetine

Figure 3.2 The effect of 6-week reboxetine (20 mg/kg/day) on NET and SERT expression in the cerebral cortex and hippocampus was determined using ^{3}H-nisoxetine and ^{3}H-citalopram binding, respectively. 6-week reboxetine treatment significantly reduced NET binding sites in both cerebral cortex and hippocampus, but not SERT binding sites (A, B). The NET protein expression in the cerebral cortex and hippocampus determined by SDS-PAGE/immunoblotting with antisera against the NET was significantly reduced only after 6-week treatment, but not after 2-week treatment (C). The persistent antidepressant-like effect in the forced-swim test was still evident two days after the end of the chronic treatment period (D). Data shown are means ± SEM of 5-6 rats per group. *$p < 0.05$; **$p < 0.01$ vs. control.

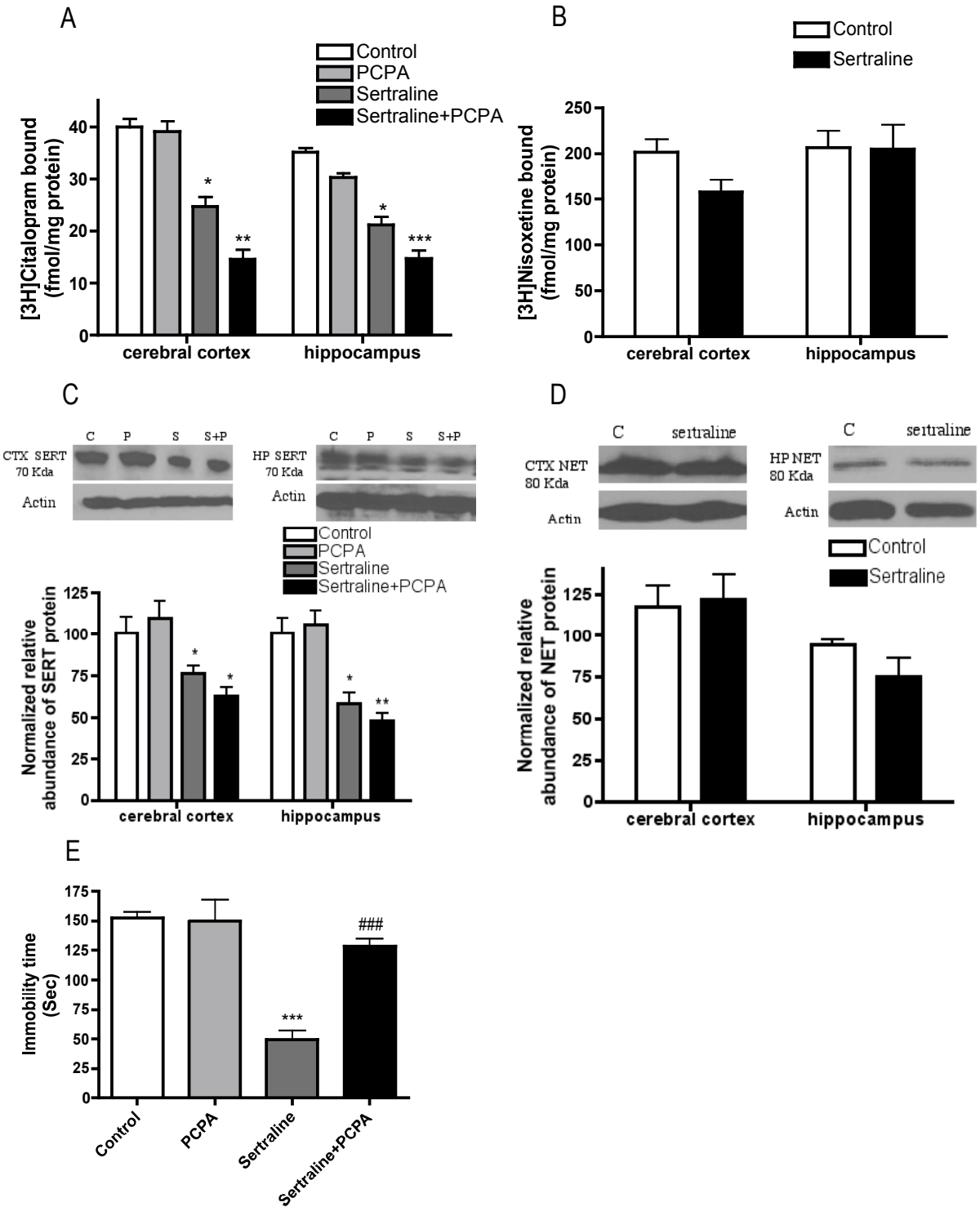

A
Control
PCPA
Sertraline
Sertraline+PCPA
[3H]Citalopram bound (fmol/mg protein)
cerebral cortex
hippocampus
B
Control
Sertraline
[3H]Nisoxetine bound (fmol/mg protein)
cerebral cortex
hippocampus
C
C P S S+P
CTX SERT 70 Kda
HP SERT 70 Kda
Actin
Control
PCPA
Sertraline
Sertraline+PCPA
Normalized relative abundance of SERT protein
cerebral cortex
hippocampus
D
C sertraline
CTX NET 80 Kda
HP NET 80 Kda
Actin
Control
Sertraline
Normalized relative abundance of NET protein
cerebral cortex
hippocampus
E
Immobility time (Sec)
###
Control
PCPA
Sertraline
Sertraline+PCPA

Figure 3.3 The SERT expression in the cerebral cortex and hippocampus measured by ^{3}H-citalopram binding (A) and SDS-PAGE/immunoblotting (C) displayed significant reduction after 2-week sertraline treatment. The NET expression in the cerebral cortex and hippocampus determined by ^{3}H-nisoxetine binding (B) and SDS-PAGE/immunoblotting (D) was not altered by chronic sertraline treatment. To examine whether enhanced serotonergic neurotransmission was necessary for the persistent antidepressant-like effect in the forced-swim test, PCPA, an inhibitor of tryptophan hydroxylase, was administered (300 mg/kg, IP) twice daily for three consecutive days with last dose given 18 h before the forced-swim test. PCPA reversed the sertraline-induced antidepressant-like effect in the forced-swim test, even at a dose where it did not affect behavior on its own (E). Data shown are means ± SEM of 5-8 rats per group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. control; $^{\#\#}p < 0.01$; $^{\#\#\#}p<0.001$ vs. sertraline alone.

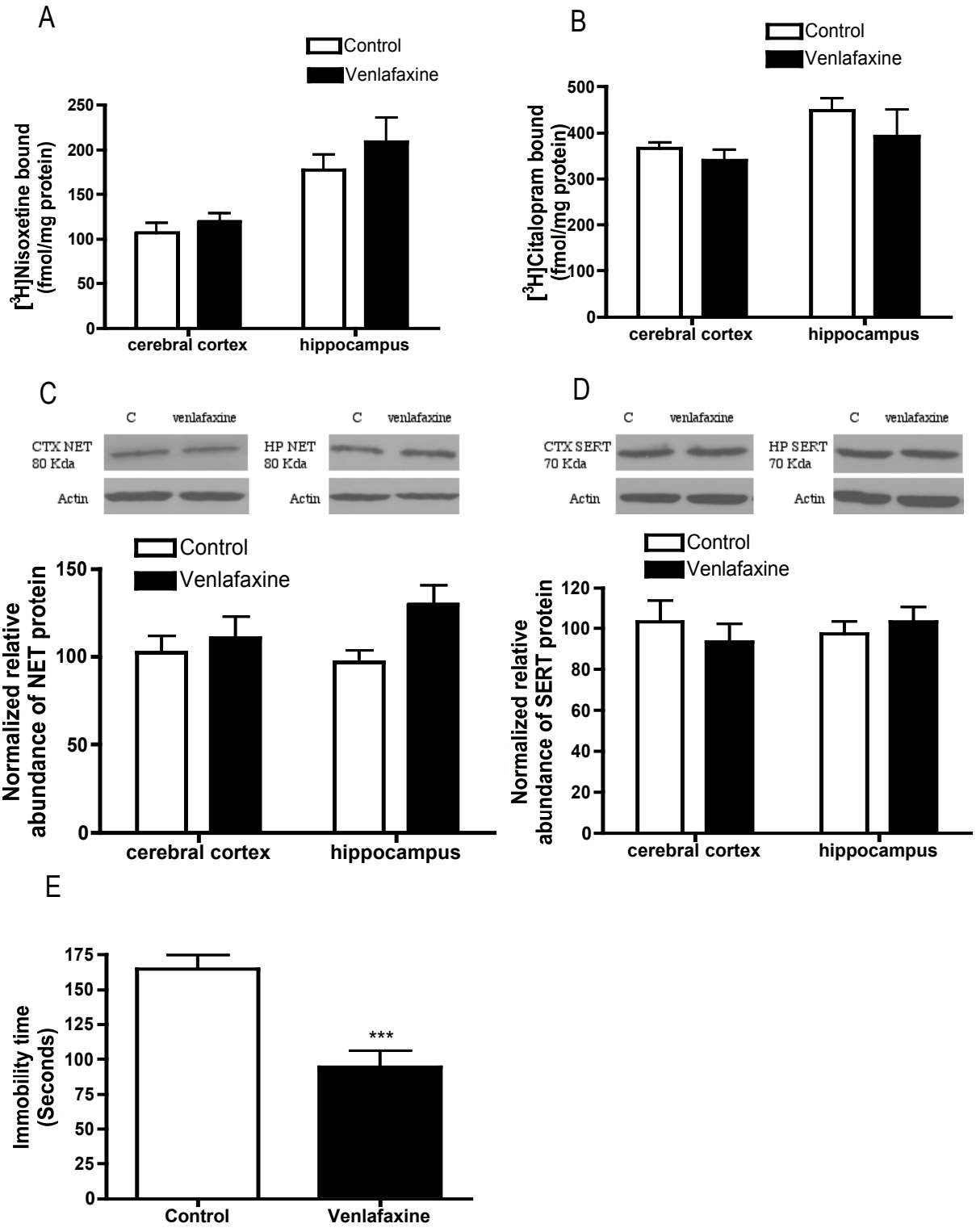

A
Control
Venlafaxine
[3H]Nisoxetine bound (fmol/mg protein)
250
200
150
100
50
0
cerebral cortex
hippocampus
B
Control
Venlafaxine
[3H]Citalopram bound (fmol/mg protein)
500
400
300
200
100
0
cerebral cortex
hippocampus
C
C
venlafaxine
CTX NET 80 Kda
HP NET 80 Kda
Actin
Actin
Control
Venlafaxine
Normalized relative abundance of NET protein
150
100
50
0
cerebral cortex
hippocampus
D
C
venlafaxine
CTX SERT 70 Kda
HP SERT 70 Kda
Actin
Actin
Control
Venlafaxine
Normalized relative abundance of SERT protein
120
100
80
60
40
20
0
cerebral cortex
hippocampus
E
Immobility time (Seconds)
175
150
125
100
75
50
25
0
***
Control
Venlafaxine

Figure 3.4 The effect of 2-week of venlafaxine (70 mg/kg/day) on NET and SERT expression in the cerebral cortex and hippocampus was determined using ^{3}H-nisoxetine and ^{3}H-citalopram binding, respectively. 2-week venlafaxine treatment did not reduce NET and SERT binding sites in both cerebral cortex and hippocampus (A, B). The NET and SERT protein expression in the cerebral cortex and hippocampus determined by SDS-PAGE/immunoblotting with antisera against the NET and SERT was not changed after chronic treatment (C, D). However, the persistent antidepressant-like effect in the forced-swim test was still evident two days after the end of the chronic treatment period (D) even though neither NET nor SERT protein expression was down-regulated. Data shown are means ± SEM of 5-6 rats per group. ***$p < 0.001$ vs. control.

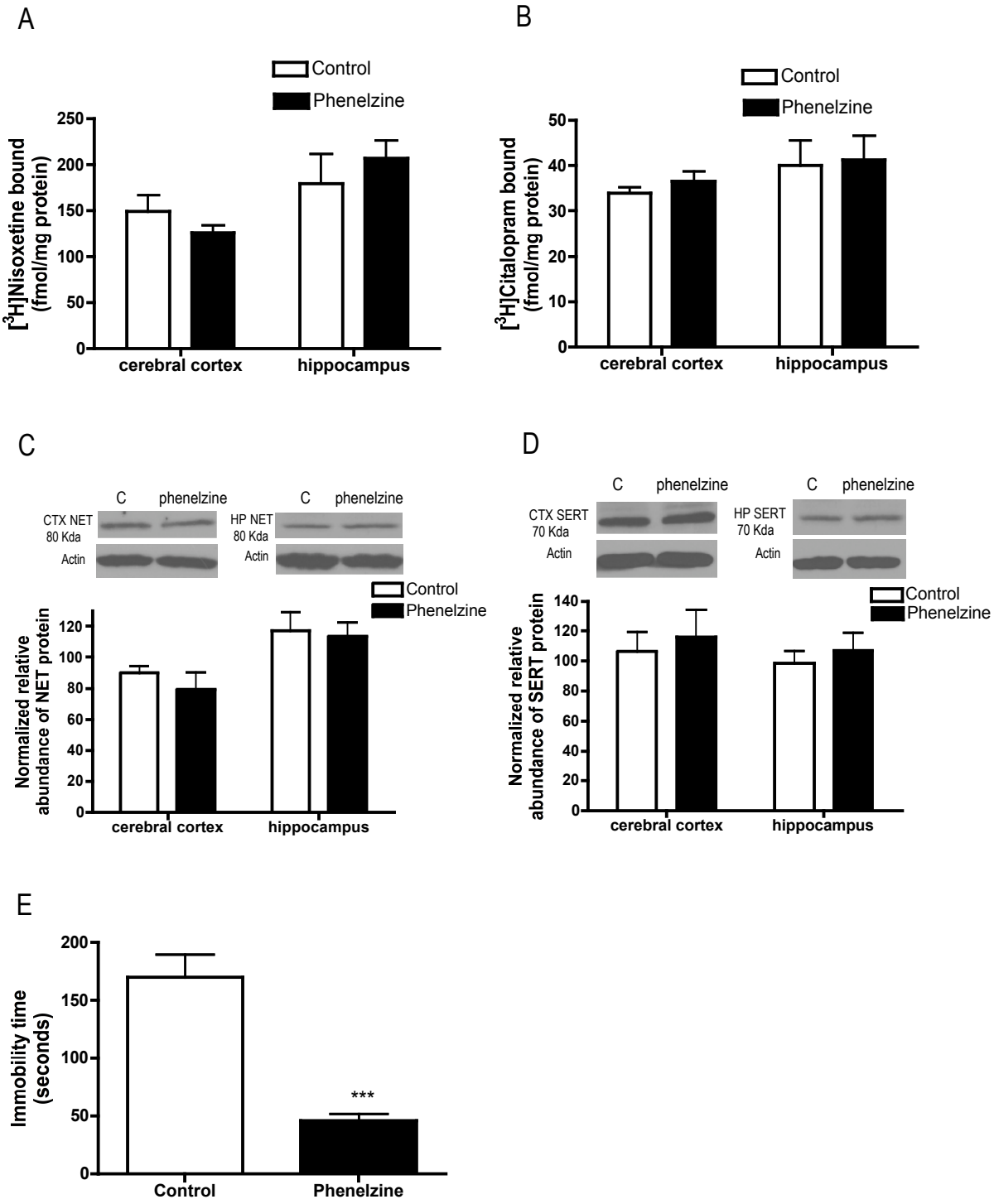

A
Control
Phenelzine
[3H]Nisoxetine bound (fmol/mg protein)
250
200
150
100
50
0
cerebral cortex
hippocampus
B
Control
Phenelzine
[3H]Citalopram bound (fmol/mg protein)
50
40
30
20
10
0
cerebral cortex
hippocampus
C
C
phenelzine
C
phenelzine
CTX NET 80 Kda
HP NET 80 Kda
Actin
Actin
Control
Phenelzine
Normalized relative abundance of NET protein
120
100
80
60
40
20
0
cerebral cortex
hippocampus
D
C
phenelzine
C
phenelzine
CTX SERT 70 Kda
HP SERT 70 Kda
Actin
Actin
Control
Phenelzine
Normalized relative abundance of SERT protein
140
120
100
80
60
40
20
0
cerebral cortex
hippocampus
E
Immobility time (seconds)
200
150
100
50
0
***
Control
Phenelzine

Figure 3.5 The NET and SERT binding sites in the cerebral cortex and hippocampus measured by 3H-nisoxetine (A) and 3H-citalopram binding (B) didn't change after 14-day phenelzine treatment. The NET and SERT protein expression in the cerebral cortex (C) and hippocampus (D) determined by SDS-PAGE/immunoblotting displayed also no change after 14-day phenelzine treatment. The persistent antidepressant-like effect was still observed in the forced-swim test 2 days after discontinuation of 14-day phenelzine treatment (E). Data shown are means ± SEM of 5-6 rats per group. ***$p < 0.001$ vs. control.

CHAPTER FOUR

Role of PKC in NET activity in cerebral cortical slices and antidepressant-like effects on behavior

4.1 Abstract

The results of studies using cell culture models have suggested a role for protein kinase C (PKC) in norepinephrine transporter (NET) regulation; experiments were carried out to begin to assess the importance of this mechanism in brain tissue, as well to determine its importance in mediation of antidepressant effects on behavior. The PKC activators β-PMA and bryostatin-1 reduced norepinephrine uptake in cortical slices *in vitro*; this was due to decreased V_{max} and unchanged K_m values. Reduced norepinephrine uptake also was observed following incubation with the PP1/2A inhibitor calyculin A and the IP_3 receptor antagonist 2-APB. It also was found that i.v. or ICV administration of β-PMA or ICV administration of bryostatin-1 produced antidepressant-like effect on forced-swim behavior. The effect of β-PMA on forced-swim behavior was reversed by co-administration of 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), a protein kinase C inhibitor. The present results suggest a role for PKC signaling in NET regulation in the rat brain and indicate that modulation of PKC activity by changes in IP_3 and PP1/2A function may contribute in the mediation of antidepressant effects on behavior. Since NET regulation appears be involved in the development of antidepressant effects on behavior, mechanisms involving PKC may offer novel targets for producing antidepressant actions.

4.2 Introduction

The NET, a target for certain antidepressants, is important in regulation of synaptic NE concentrations; the NET mediates reuptake of NE into the presynaptic terminal, which is the primary method of inactivation (Schomig et al., 1989). Chronic treatment with antidepressants that inhibit the NET, e.g. desipramine and reboxetine, down-regulates NET expression and enhances synaptic NE levels, resulting in persistent antidepressant behavioral effects (Zhao et al., 2008). The time-course of down-regulation of NET is consistent with clinical observations and provides a plausible mechanism to explain the delayed onset of action of antidepressants in patients with major depressive illness (Benmansour et al., 2002; Katz et al., 2004); it also is consistent with data that suggest that enhanced monoaminergic neurotransmission is necessary for antidepressant effectiveness (Miller et al., 1996a; Miller et al., 1996b). The molecular mechanisms underling chronic antidepressants-induced down-regulation of the NET, particularly *in vivo*, have not been fully elucidated; however, the PKC signaling pathway does appear to be involved (Jayanthi et al., 2004; Jayanthi et al., 2006).

The NET is phosphorylated by PKC, affecting its cellular localization and function (Jayanthi et al., 2006). β-PMA, a PKC activator, reduces NET activity and surface expression in HEK-293 and LLC-PK1 cells stably transfected with the human NET (Apparsundaram et al., 1998a; Apparsundaram et al., 1998b), as well as in rat placental trophoblasts (Jayanthi et al., 2004). Reduced NET activity is characterized by a decrease in the maximum transport velocity (V_{max}) with little or no change in substrate affinity (K_m). This reduction in activity is mediated primarily by the internalization of surface NET protein (Jayanthi et al., 2004). Site-directed mutagenesis reveals that threonine-258

and serine-259 residues of the human NET are PKC phosphorylation sites that control transporter internalization (Jayanthi et al., 2006). NET activity also is reduced by the protein phosphatase 1 and 2A (PP1/2A) inhibitors okadaic acid and calyculin A, which prevent de-phosphorylation of the PKC sites (Bauman et al., 2000).

The PKC activator bryostatin-1 has been suggested to be a novel antidepressant, based on preclinical data (Sun and Alkon, 2005). Antidepressant effects caused by PKC activation could be mediated by phosphorylation of a number of different proteins, given the high expression of this enzyme in the brain (Saito et al., 1988). PKC activation facilitates synaptic neurotransmission and induces rapid morphological plasticity (Pilpel and Segal, 2004), especially in the hippocampus, which has been implicated in depression and its pharmacotherapy (Drevets, 2001; Liotti and Mayberg, 2001). Some behavioral effects of PKC activation may be mediated via the regulation of NET and SERT cell surface expression, which has been demonstrated *in vitro.* If this also occurs *in vivo,* it could result in reduced monoamine transporter function and increased monoaminergic neurotransmission (Jayanthi et al., 2005).

Most studies examining PKC-induced down-regulation of NET expression and activity have been carried out *in vitro* using a variety of cell lines; it is of interest to examine this mechanism in brain and to assess possible behavioral relevance. The results of the present study addressing this issue indicate that PKC activation reduces NET activity both *in vitro* and *in vivo* and results in antidepressant-like effects in the forced-swim test.

4.3 Materials and Methods

Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 300-350g, were housed 2 per cage in a temperature-controlled room (22 - 23°C) with a 12-h on/12-h off light cycle (lights on at 6:00 AM). Food and water were freely provided. Blind observations were used for all behavioral testing, which was carried out from 1:00 PM – 5:00 PM in a quiet room. All procedures were reviewed and approved by the Animal Care and Use Committee of West Virginia University Health Sciences Center, and are consistent with the NIH "Guidelines for the Care and Use of Laboratory Animals" (NIH publication No. 80-23, revised 1996).

Intracerebroventricular (ICV) drug administration

ICV cannulation and drug administration were carried out as described previously (Zhang et al., 2003). All surgery was performed under aseptic conditions. Rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and placed in a stereotaxic apparatus (Stoelting, Wood Dale, IL). Guide cannulas (22-guage; Plastics One, Roanoke, VA) were implanted bilaterally into the lateral ventricles using the following coordinates: AP, -0.5 mm from Bregma; ML, $\pm$1.6 mm from the midline; DV, -3.9 mm from dura (Paxinos and Watson, 1986). Following a recovery period of at least one week, compounds dissolved in aCSF were injected into the lateral ventricles using a syringe pump (1 μl over 2 min).

Forced-swim test

The forced-swim test was carried out as described previously (Porsolt et al., 1977; Zhang et al., 2006; Zhao et al., 2008). 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, 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, rats were subjected to the 5-min forced-swim test 30 min after administration of drugs; immobility time was recorded, with immobility defined as floating on the surface of the water with the only movement being that required to keep the head above water.

^{3}H-NE uptake assay

Uptake assays were carried out as described previously (Apparsundaram et al., 1998; Vizi et al., 2004). Fresh rat cerebral cortex was placed in ice-cold, oxygenated (95% O_2 / 5% CO_2), Krebs-Ringer/HEPES (KRH) buffer (125 mM NaCl, 5.2 mM KCl, 1.2 mM $CaCl_2$, 1.4 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM glucose, 20 mM HEPES, 0.2 mg/ml ascorbic acid, and 100 μM pargyline; pH 7.4). Tissue was cut into 0.4 mm slices using a McIlwain tissue chopper (Brinkmann, Westbury, NY). Slices were separated by gentle shaking and washed with oxygenated KRH buffer. The slices were treated with β-PMA (SigmaAldrich, St. Louis, MO) or bryostatin-1 (SigmaAldrich, St. Louis, MO) for 30 min at 37° C. Uptake assays were performed by incubating the tissue slices with 30 nM ^{3}H-NE/NE (0.03 – 3 uM; 10.9 Ci/mmol, PerkinElmer, Boston, MA) 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 30,000 × g for 2 min. ^{3}H-NE in the supernatant was quantified by liquid scintillation counting. Nonspecific uptake was determined in the presence of 20 μM desipramine. Specific uptake was determined by subtracting nonspecific uptake from total uptake.

Statistical analysis

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 less than 0.05.

4.4 Results

Effects of β-PMA and bryostatin-1 on behavior in the forced-swim test

The PKC activator β-PMA (25 – 100 nmol/kg, i.v.) reduced the duration of immobility ($F_{(3,15)}$=6.86, $p < 0.01$; Figure 4.1A) and increased the latency to immobility ($F_{(3,15)}$=6.99, $p < 0.01$; Figure 4.1B) in the forced-swim test; this antidepressant-like effect on behavior only was observed at the 100 nmol dose. ICV administration of 20 pmol β-PMA, as well as 2 pmol bryostatin-1, another PKC activator, also reduced the time of immobility in the forced-swim test ($F_{(2,12)}$=4.58, $p < 0.01$; Figure 4.1C). Co-administration of 10 nmol H-7, a PKC inhibitor, antagonized the antidepressant-like effects of 2 pmol, but not 20 pmol, β-PMA in the forced-swim test ($F_{(5,18)}$=11.92, $p < 0.001$; Figure 4.1D); H-7 alone had no effect.

Effects of β-PMA and bryostatin-1 on NET activity

^{3}H-NE uptake into cerebral cortical slices was significantly reduced after treatment with 2 μM β-PMA *in vitro* (Figure 4.2A, 4.2B). This was due to a reduction in the V_{max} from the control value of 70.0 ± 2.6 to 45.1 ± 2.5 fmol/mg protein/10min (36% reduction; $p < 0.05$), with no change in the K_m value (control: 0.62 ± 0.04; β-PMA: 0.53 ± 0.05 μM). Similar effects were observed following treatment of cerebral cortical slices *in vitro* with 0.5 μM bryostatin-1. The V_{max} was reduced to 49.9 ± 2.2 fmol/mg protein/10min (29% reduction; $p < 0.05$), while the K_m was unchanged (0.57 ± 0.04 μM).

Effects of PP1/2A inhibitors okadaic acid and calyculin A and IP_3 receptor antagonist 2-aminoethoxydiphenyl borate (2-APB) on NET activity

Treatment of cerebral cortical slices with the PP1/2A inhibitors okadaic acid (Figure 4.4A) and calyculin A (Figure 4.4B) reduced ^{3}H-NE uptake *in vitro*. Okadaic acid produced a near-significant effect at a concentration of 10 μM ($p = 0.05$), while calyculin A was effective at concentrations at 50 μM ($p < 0.05$).

Treatment of cerebral cortical slices *in vitro* with a 50 μM concentration of the IP_3 receptor antagonist 2-APB reduced ^{3}H-NE uptake ($p < 0.01$; Figure 4.4C).

4.5 Discussion

The present findings demonstrate that PKC activators β-PMA and bryostatin-1 reduce membrane NET activity in rat cerebral cortical slices. These data are consistent with previous studies carried out in cell lines which show that both NET and SERT are regulated via a PKC-dependent pathway (Apparsundaram et al., 1998b; Jayanthi et al., 2005). Chronic treatment with desipramine or other inhibitors of NE or 5-HT appear to stimulate molecular factors in the PKC pathway resulting in its activation. Activation of PKC by β-PMA down-regulates and internalizes membrane-associated NET in cell lines *in vitro* (Jayanthi et al., 2004). The NET-PP2A-Ar complex and the NET-syntaxin 1A complex have been shown to be involved in this process (Sung et al., 2005; Sung et al., 2007).

β-PMA and bryostatin-1 also were found to produce antidepressant-like effects on behavior in the forced-swim test. Potential antidepressant-like effects of bryostatin-1 were first reported by Sun and Alkon (2005). PKC mediation of the antidepressant-like effects of β-PMA was confirmed by the finding that they were antagonized by the PKC inhibitor H7. Consistent with this interpretation, it has been reported that 4-methylcatechol, a stimulator of brain-derived neurotrophic factor (BDNF) and an indirect PKC activator, produces antidepressant-like effects on behavior (Sun and Alkon, 2008).

Previous work has shown that chronic treatment of rats with desipramine reduces the expression and activity of the NET in cerebral cortex and hippocampus, resulting in enhanced noradrenergic neurotransmission in the brain and antidepressant-like effects on behavior (Zhao et al., 2008). NET, as well as SERT, down-regulation has been demonstrated following chronic treatment with a number of other antidepressant drugs.

The down-regulation appears to be a direct effect of antidepressant interaction with the monoamine transporters and not secondary to increase synaptic concentrations of the transmitters, since this effect also is observed for cell lines *in vitro* (Oster et al., 1989; Zhu and Ordway, 1997; Zhu et al., 1998; Horschitz et al., 2001; Zhu, 2005; Iceta et al., 2007). Consistent with this, it has been found that the monoamine oxidase inhibitor phenelzine, which increases monoaminergic neurotransmission but does not interact with the NET or SERT, does not down regulate the transporters. Given the role of PKC in the control of NET and SERT trafficking and internalization, it is possible that antidepressant-like behavioral effects of PKC activators may be mediated, at least in part, via a reduction in cell-surface expression of the NET or SERT.

PKC isozymes, the main targets of phorbol ester tumor promoters, have important roles in cell-cycle regulation, cell survival, malignant transformation, apoptosis, synaptic plasticity, and neuronal injury (Griner and Kazanietz, 2007; Weinreb et al., 2004; Cejas et al., 2005). Three classes of PKCs (classical PKCs, novel PKCs, and atypical PKCs), encoded by nine PKC genes, have been identified (Dekker and Parker, 1994; Hug and Sarre, 1993; Jaken, 1996). Classical PKCs include PKCα, PKCβI, PKCβII, and PKCγ, which require calcium and diacylglycerol (DAG) for activation. Novel PKCs include PKCδ, PKCε, PKCη, and PKCθ, which require DAG. Atypical PKCs include PKCζ and PKCι, which require neither calcium nor DAG. The physiological functions of the different PKC isozymes are not known at present.

Bryostatin-1, a naturally occurring macrocyclic lacton with a chemical structure distinct from phorbol esters, potently activates PKC but induces only part of the effects resulting from phorbol ester treatment. The binding of bryostatin-1 to PKC results in

PKC activation, auto-phosphorylation, and translocation to the cell membrane. Importantly, bryostatin-1 is ineffective as a tumor promoter (Hennings et al., 1987; Ramsdell et al., 1986) and shows some favorable pharmacological profiles as an anti-cancer compound (Philip and Zonder, 1999; Davidson and Haygood, 1999; Farokhzad et al., 1998).

The involvement of PKC in the depression therapy has been implicated in several studies and malfunction of PKC signal transduction has been suggested to be involved in symptoms of depression (Akin et al., 2005). The involvement of PKC in antidepressant mechanisms also is suggested by the findings that PKC activation or phosphatase inhibition induces phosphorylation of the NET and SERT *in vitro* (Ramamoorthy et al., 1998).

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 surface NET expression in SH-SY5Y cells (Amano et al., 2006), suggesting that the IP_3 receptors/Ca^{2+} system has facilitatory effects on NET activity. Given that activation of IP_3 receptor-induced release of Ca^{2+} activates PKC and consequently reduces membrane NET activity, it seems that the regulation of the NET activity is balanced between the PLC-related signaling pathways. Another inducer of intracellular Ca^{2+} release, the ryanodine receptor, and an influx of extracellular Ca^{2+} mediated by SOC channels, does not change NET activity (Amano et al., 2006). The changes of NET activity mediated by PKC and IP_3 receptors have been only demonstrated in cell lines *in vitro*. A demonstration of the importance of this mechanism *in vivo* might suggest a novel mechanism to produce NET (or SERT) down-regulation with resulting antidepressant-like effects on behavior. The activation of

PKC signaling pathway by chronic antidepressant treatment might provide a mechanism underlying neuroadaptive changes in NET and SERT function.

Overall, the present study demonstrates a relationship between PKC activation-induced antidepressant-like effect in the forced-swim test and PKC activation-induced reduction of membrane NET activity in the rat cerebral cortical slices. This suggests the importance of future experiments to examine the means by which antidepressants alter PKC activity to affect cell surface expression of monoamine transporters and to identity molecular targets in this pathway that might provide a novel means for producing antidepressant effects on behavior.

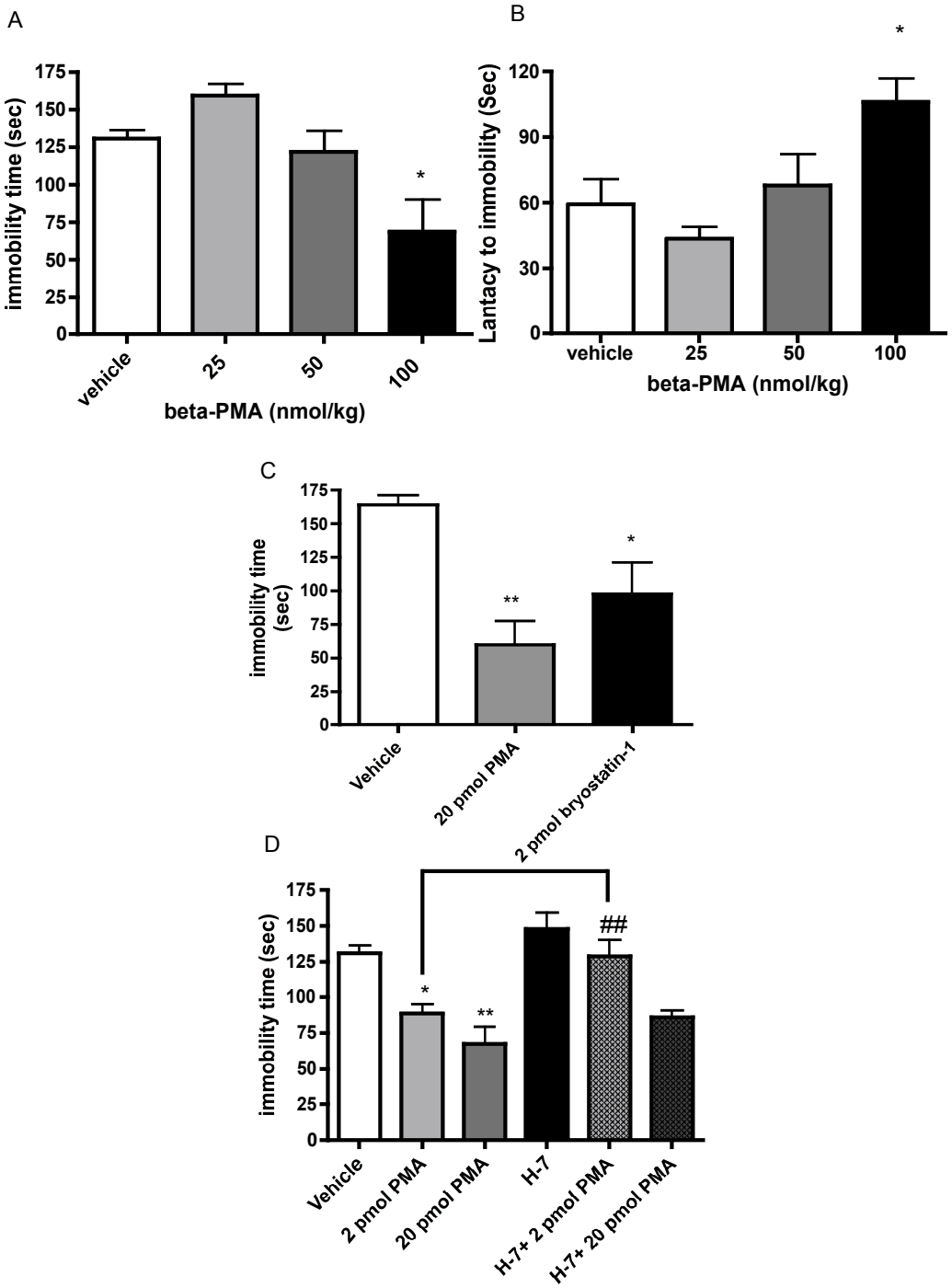
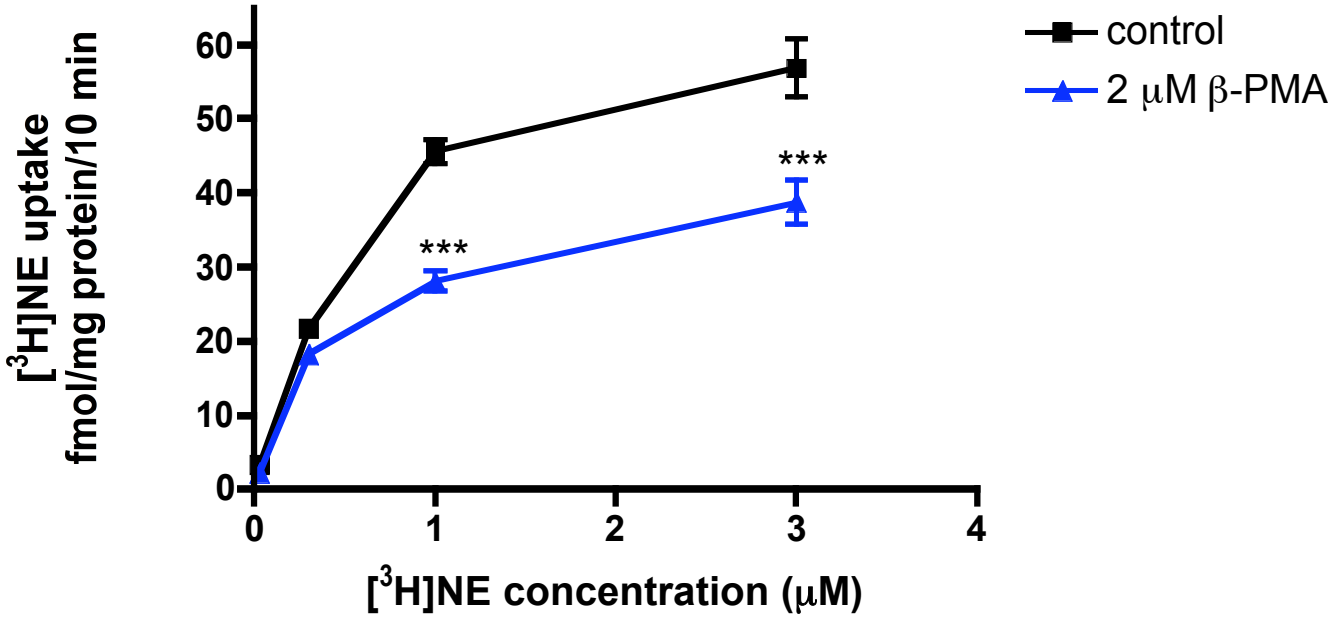

A
immobility time (sec)
175
150
125
100
75
50
25
0
*
vehicle
25
50
100
beta-PMA (nmol/kg)
B
Lantacy to immobility (Sec)
120
90
60
30
0
*
vehicle
25
50
100
beta-PMA (nmol/kg)
C
immobility time (sec)
175
150
125
100
75
50
25
0
**
*
Vehicle
20 pmol PMA
2 pmol bryostatin-1
D
immobility time (sec)
175
150
125
100
75
50
25
0
2 pmol bryostatin-1
*
**
##
Vehicle
2 pmol PMA
20 pmol PMA
H-7
H-7+ 2 pmol PMA
H-7+ 20 pmol PMA

Figure 4.1 The PKC activator β-PMA produced antidepressant-like effects on forced-swim behavior. This effect was observed 3 h after i.v. administration of β-PMA, as indicated by reduced immobility time (A) and increased latency to immobility (B). Bilateral ICV administration of 20 pmol β-PMA and 2 pmol bryostatin-1, another PKC activator, 30 min before testing reduced also immobility time in the forced-swim test (C). Bilateral ICV administration of the PKC inhibitor H-7 (20 nmol) reversed the antidepressant-like effect of 2 pmol PMA in the forced-swim test (D). Data shown are means ± SEM for 5 – 6 rats per group. *$p < 0.05$, **$p < 0.01$ vs. vehicle; $^{\#\#}p < 0.01$ vs. 2 pmol PMA alone.

A


control
2 μM β-PMA
[3H]NE uptake
fmol/mg protein/10 min
[3H]NE concentration (μM)
***
***
0
10
20
30
40
50
60
0
1
2
3
4

B

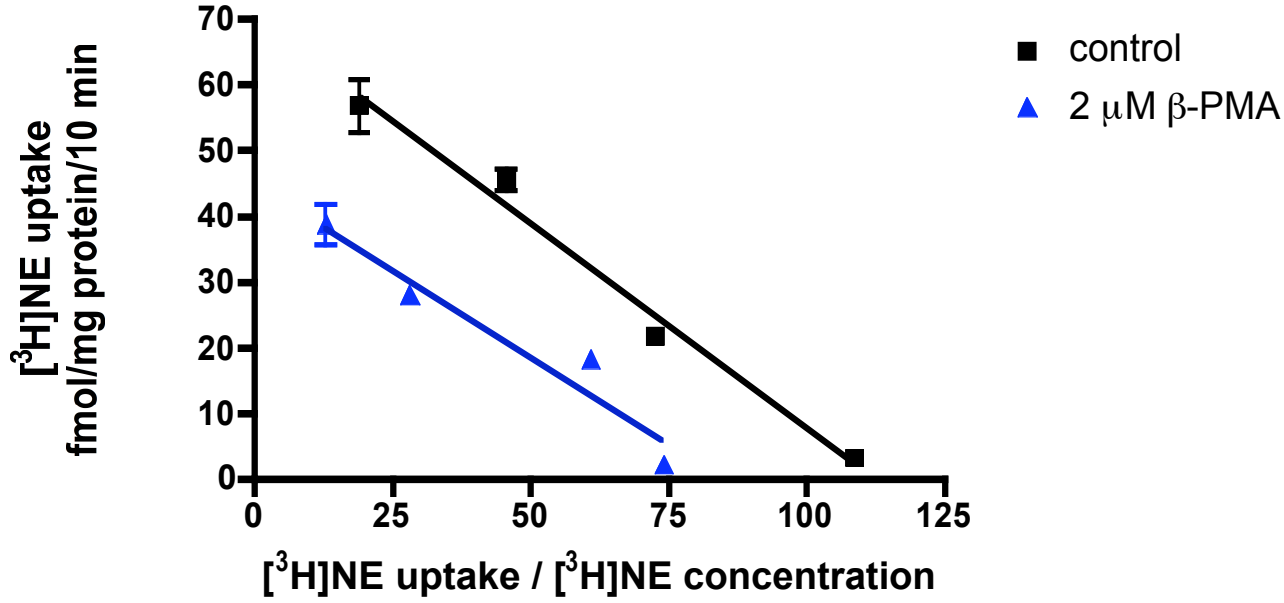
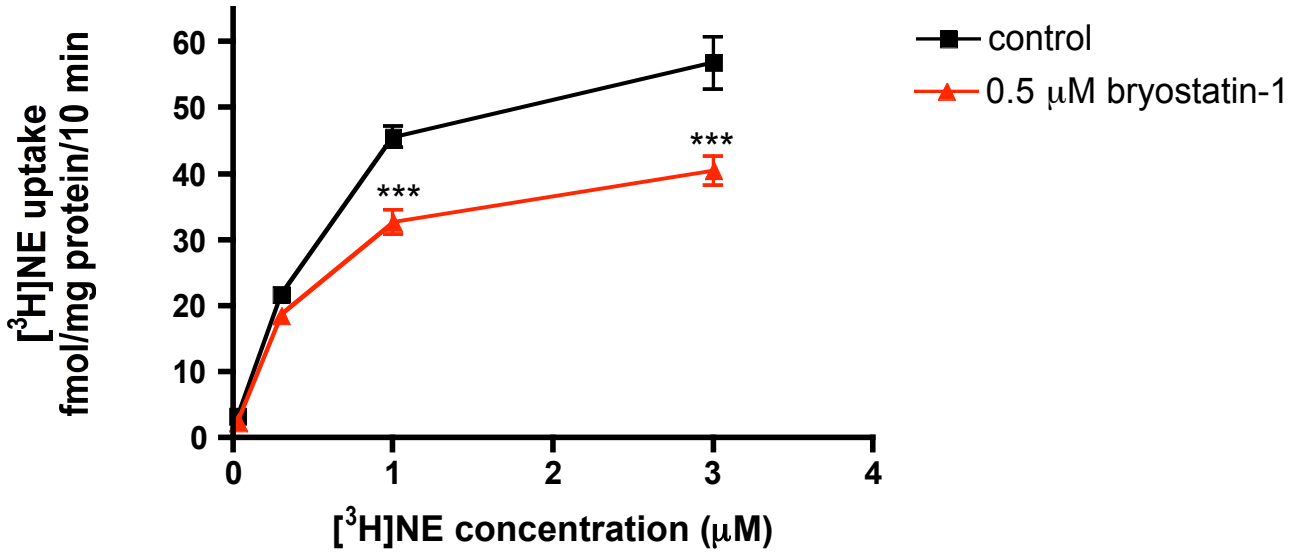

control
2 μM β-PMA
[3H]NE uptake
fmol/mg protein/10 min
[3H]NE uptake / [3H]NE concentration
0
10
20
30
40
50
60
70
0
25
50
75
100
125

Figure 4.2 The PKC activator β-PMA reduced NET activity in cerebral cortical slices *in vitro*; this was due to a reduction of the V_{max} and an unchanged K_m value. The NET activity was determined after incubation with 4 concentrations of [^{3}H]NE. Data shown are means ± SEM of 5 – 6 rats per group. *$p < 0.05$, ***$p < 0.001$ vs. control.

A


control
0.5 μM bryostatin-1
[3H]NE uptake fmol/mg protein/10 min
[3H]NE concentration (μM)
***
***
0
10
20
30
40
50
60
0
1
2
3
4

B

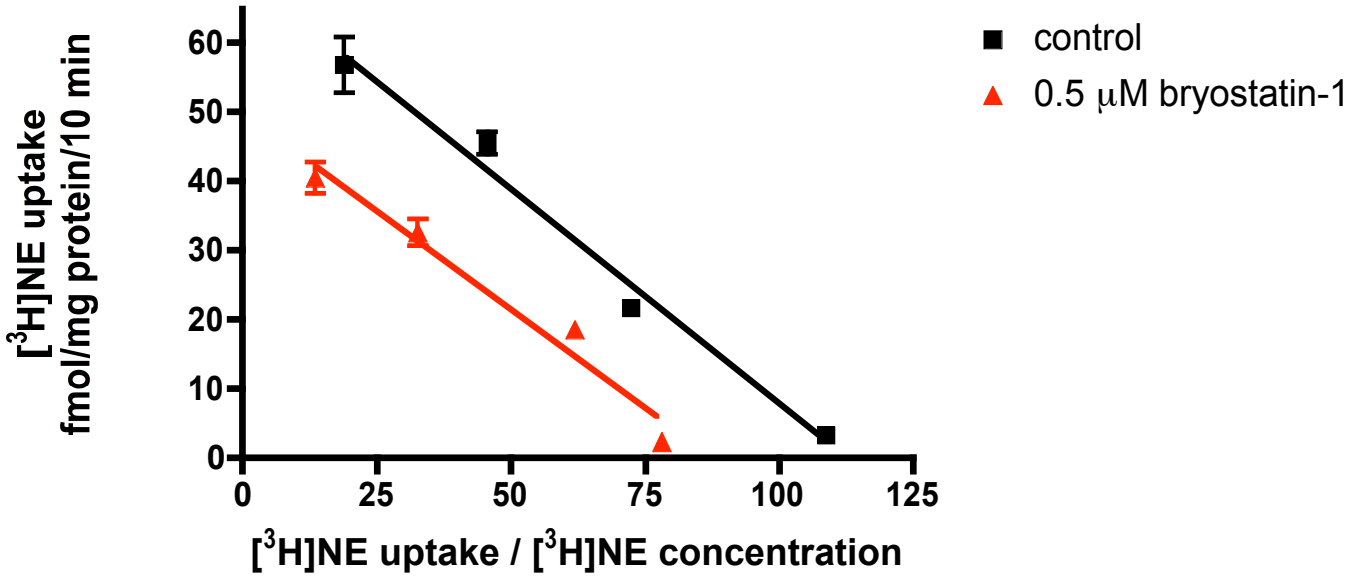
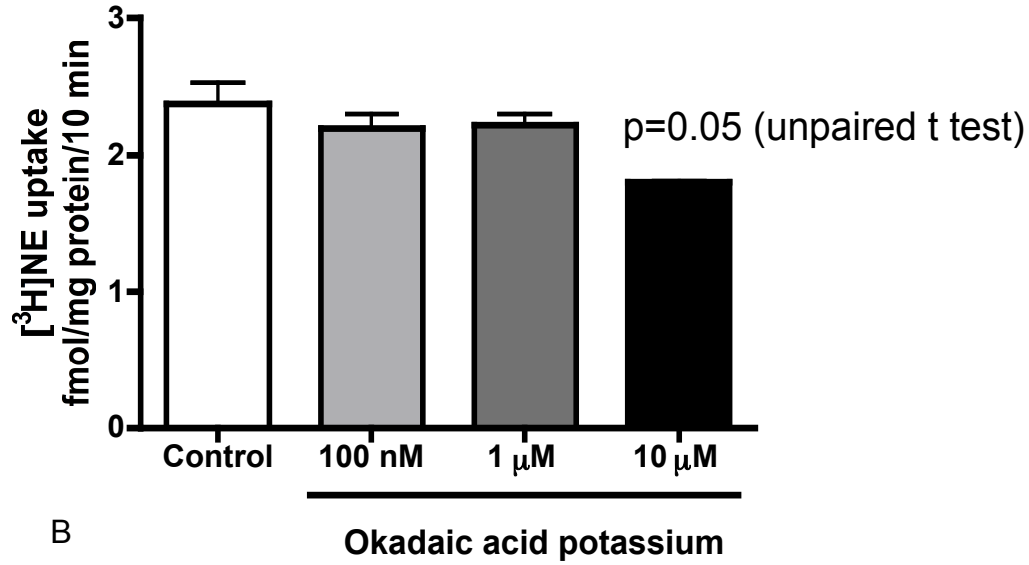
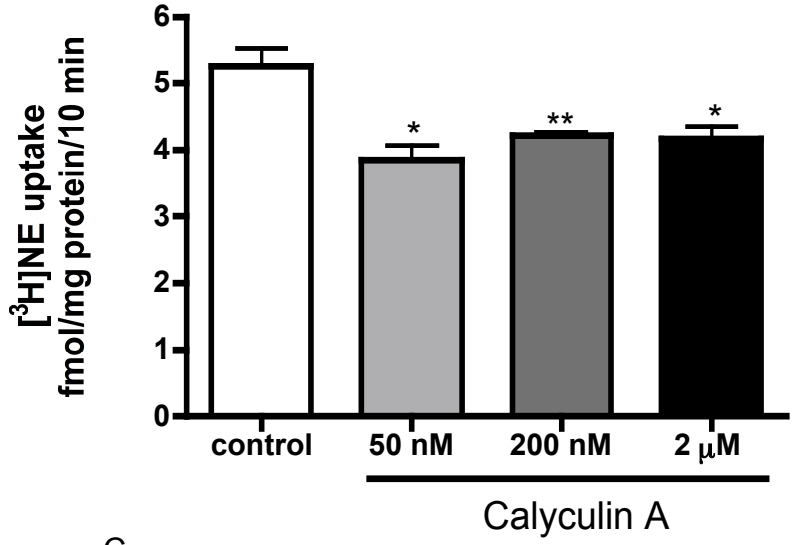

control
0.5 μM bryostatin-1
[3H]NE uptake fmol/mg protein/10 min
[3H]NE uptake / [3H]NE concentration
0
10
20
30
40
50
60
0
25
50
75
100
125

Figure 4.3 The PKC activator bryostatin-1 reduced NET activity in cerebral cortical slices; this was due to a reduction of V_{max} and an unchanged K_m value. The NET activity was determined after incubation with 4 concentrations of [^{3}H]NE/NE. Data shown are means ± SEM of 5 – 6 rats per group. *$p < 0.05$, ***$p < 0.001$ vs. control.

A


[3H]NE uptake
fmol/mg protein/10 min
3
2
1
0
Control
100 nM
1 μM
10 μM
p=0.05 (unpaired t test)

B


[3H]NE uptake
fmol/mg protein/10 min
6
5
4
3
2
1
0
*
**
*
control
50 nM
200 nM
2 μM
Calyculin A

C

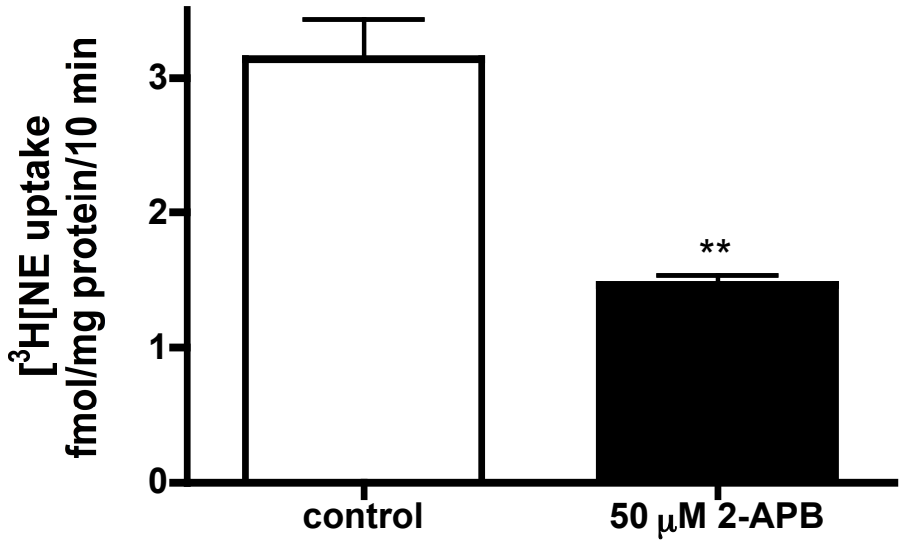

[3H]NE uptake
fmol/mg protein/10 min
3
2
1
0
**
control
50 μM 2-APB

Figure 4.4 The PP1/2A inhibitors okadaic acid and calyculin A reduced NET activity in rat cerebral cortical slices, as evidenced by a reduction in the uptake of [^{3}H]NE. The IP_3 receptor antagonist 2-APB also reduced NET activity. Data shown are means ± SEM of 5 – 6 rats per group. *$p < 0.05$, **$p < 0.01$ vs. control

Conclusions

The present study demonstrates a relatonship between antidepressant-induced down-regulation of the NET and antidepressant-like effects on behavior. This relationship also is observed for the SERT. The down-regulation of the transporters and long-term antidepressant-like behavioral effects are evidient two days following discontinuation of chronic treatment, when acute drug effects are minimized. Consitent with clinical findings, the antidepressant-like effect in the forced-swim test is reversed by inhibiton of NE or 5-HT neurotransmission, suggesting that the enhanced NE and serotonin neurontransmission is necessary, but not sufficient, to produce antidepressant-like effects on behavior. The mechanism of NET and SERT regulation by antidepressants likely is more due to altered transporter protein internalization than altered gene transcription. Further, PKC activators show some antidepressant-like effects in the forced-swim test and this is accompanied by reduced membrane NET activity in rat cerebral cortical slices. This suggests a possible PKC signaling pathway mediating NET regulation in vivo and that may be associated with antidepressant effects. Understanding molecular events that regulate NET and SERT in vivo may suggest pharmacological targets for the development of novel antidepressant drugs.

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Zaorui Zhao, Ph.D.

68 William St, Apt 1R
New Haven, CT 06511
Tel: (304) 906-8591
Email: zrzhaozrzhao@yahoo.com

Curriculum Vitae

EDUCATION

- **Ph.D.** **07/2005-08/2008**
  --- Department of Pharmaceutical and Pharmacological Sciences, West Virginia University Health Science Center, Morgantown, WV, USA

- **Ph.D Candidate** **08/2003-06/2005**

  ---Department of Pharmacology. University of Tennessee Health Science Center, Memphis, TN, USA

- **B.S.** **09/1995-06/1999**
  --- Zhejiang University, Department of Pharmacy, Hangzhou, P.R.China

RESEARCH EXPERIENCE

- **Postdoctoral research associate** **09/2008 – present**
  --- Department of Psychology, Yale University

- **Research Assistant** **07/2005 – 08/2008**
  --- Department of Pharmaceutical and Pharmacological Sciences, West Virginia University Health Science Center

Study on the involvement of norepinephrine transporter and serotonin transporter in the delayed onset of antidepressants.

- **Research Assistant** **08/2003 – 06/2005**
---Department of Pharmacology. University of Tennessee Health Science Center, Memphis, TN

- **Research Assistant** **08/1999 – 06/2003**
---Department of Pharmacology. Zhejiang Academy of Medical Sciences, Hangzhou, P.R.China

Research on the pre-clinical and clinical drug trials; New drug Pharmacokinetic and Phharmacodynamic research. Bioavailability and bioequivalence research.

- **Undergraduate internship** **02/1999 – 06/1999**
--- Department of Pharmacology, Second Military Medical University, Shanghai, P.R.China

Research on the learning and memory mechanism.

Publications

- **Zhao Z**, Baros AM, Zhang HT, Lapiz MD, Bondi CO, Morilak DA, O'Donnell JM (2008). Norepinephrine transporter regulation mediates the long-term behavioral effects of the antidepressant desipramine. *Neuropsychopharmacology* [Epub ahead of print].

- Lapiz MD, **Zhao Z**, Bondi CO, O'donnell JM, Morilak DA (2007). Blockade of autoreceptor-mediated inhibition of norepinephrine release by atipamezole is maintained after chronic reuptake inhibition. *Int J Neuropsychopharmacol*. 10(6):827-33.

- Zhou B, Zhang Q, **Zhao Z**, Li W, Li S, Jiang Y, and Yin M (2002). Effect of pregnenolone sialic glycoside on memory impairment in mice. *Academic Journal of Second Military Medical University* 23:562-563.

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- Wei Y, Zhang M, Wang K, **Zhao Z**, Liu X, Wu D, Shao Q (2003). Assessment of bioequivalence of Pidotimod Granules. Chinese Journal of New Drugs 12:461-463.

- Wei Y, Zhang M, Wang K, **Zhao Z**, Shao Q (2003). Pharmacokinetics of mifepristone after low oral dose in healthy Chinese women. The Chinese Journal of Clinical Pharmacology 19: 430-433.

Submitted manuscripts

- **Zhao Z**, Zhang H, Bootzin E, Millan M, O'Donnell JM. Association of changes in norepinephrine and serotonin transporter expression with the long-term behavioral effects of antidepressant drugs. Submitted to *Neuropsychopharmacology* on July 2008.

International Meetings

- **Zhao Z**, Zhang H, and O'Donnell JM. Role of PKC Signaling In The Regulation of The Norepinephrine Transporter In Rat Brain. *Society for Neuroscience 2007 annual meeting, San Diego, CA*

- **Zhao Z**, Zhang H, Lapiz DS, Bondi CO, Morilak DA, and O'Donnell JM. Desipramine-induced enhancement of noradrenergic neurotransmission in the medial prefrontal cortex demonstrated by in vivo microdialysis. *Society for Neuroscience 2006 annual meeting, Atlanta, GA*

- **Zhao Z**, Zhang H, Kane VB and, O'Donnell JM. Involvement of the norepinephrine transporter (NET) in the persistent behavioral effects of desipramine (DMI). *Society for Neuroscience 2005 annual meeting. Washington DC.*

- O'Donnell JM, Crissman AM, **Zhao Z**. Association of the Down-Regulation of the Norepinephrine Transporter in rat brain with the persistent behavioral effects observed following repeated treatment with desipramine. *American College of Neuropsychopharmacology 44th annual meeting 2005.*

Presentations and posters

- **Zhao Z**, Zhang H, and O'Donnell JM. (2007). Role of PKC signaling in the regulation of the norepinephrine transporter in rat brain. *West Virginia University*

Health Science Center 2007 E.J.Van Liere Convocation and research day.

- **Zhao Z**, Zhang H, and O'Donnell JM. (2006). Persistent Antidepressant Effects Produced by Desipramine-Induced Down-Regulation of the Norepinephrine Transporter in the Rat brain. *West Virginia University Health Science Center 2006 E.J.Van Liere Convocation and research day*.

Award

- 2005 - 2007 Department of Pharmaceutical and Pharmacological Sciences West Virginia University Travel award

Professional Associations

- Society for Neuroscience (SfN) since 2005
- American Society for Pharmacology and Experimental Therapeutics (ASPET) since 2008
- International Brain Research Organization, IBRO since 2005

Technical Proficiencies

- **Techniques** Animal behavioral tests, Animal surgeries including stereotaxic surgery, pump implantation, microinjection, *in vivo* microdialysis. Immunoblotting, Immunohistochemisty, fluorescence microscopy and confocal microscopy, Radioligand binding, Uptake assay, Analytical HPLC, ELISA, Real-time PCR, Reverse-transcriptase PCR, Cell culture, Plasmid transformation and transfection,

- **Software** Prism, NCBI, Pub Med, Science Direct, MS Office, Photoshop,