# Effects of Chronic Stress on Norepinephrine Clearance in the CA3 Region of the Hippocampus

Julianne Doyen

Department of Psychology

Mitte Honors Program

Texas State University – San Marcos

Approved:

_____
Heather C. Galloway
Director, Mitte Honors Program

Approved:

_____
Dr. Augustus Lumia
Department of Psychology
Supervising Professor

_____
Professor David Carpenter
Department of Psychology
Second Reader

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**Abstract**

The pathology between chronic stress, deregulation of serotonergic (5-HT) and noradrenergic (NE) neurotransmission and the onset of psychiatric disorders is poorly understood. Using *in vivo* chronoamperometry our previous findings showed 5-HT clearance is dramatically slower in chronically stressed wild-type mice, and that this reduction in clearance does not result from the loss of serotonin transporter (SERT) expression or function. NE transporters (NET) express a low affinity and high capacity for 5-HT uptake, and so can regulate 5-HT neurotransmission when high concentration of 5-HT are present in the extracellular fluid (EFC). Due to this relation, we hypothesized the reduced 5-HT clearance in stressed mice results from a downregulation in NET function and/or expression. To test this hypothesis, *in vivo* chronoamperometry was used to measure the clearance rate (μM/sec) of locally applied NE from ECF in the CA3 region of the hippocampus of anesthetized mice. Additionally, quantitative autoradiography was completed to establish if overall NET expression in the CA3 region had decreased. The forced swim test (FST, 10 minutes daily for 14 consecutive days) was administered as the stress paradigm, while unhandled mice served as controls. All experiments were conducted 24 hours after the last FST, or at matched times for non-stressed mice. Results show chronically stressed mice clear exogenously (externally) applied NE at a significantly faster rate than non-stressed controls, yet overall NET expression does not differ between the two conditions. Given the data, it is possible NET plasmallemmal expression has been upregulated in chronically stressed mice although overall protein expression has remained unchanged. Additionally, the reciprocal effects of chronic stress on 5-HT and NE clearance may be in part due to another transporter system with a low affinity for 5-HT being compromised instead of NET. In order to further elucidate the effects of chronic stress on serotonergic and noradrenergic transmission individually and in relation to their regulatory effects on each other, future studies will include administering selective norepinephrine reuptake inhibitor (SNRI) and/or a cocktail mixture (SSRI, selective serotonin reuptake inhibitor and SNRI) to index the functional tone of NETs *in vivo* in stressed and non-stressed conditions.

**Key words:** Stress, Brain, Depression, Norepinephrine, Serotonin

## 1. Introduction

An extensive body of research has found chronic stress to influence the onset and severity psychiatric disorders such as depression, anxiety and drug abuse (McEwen, 1998; Kendler et al., 1999). The relation between chronic stress and psychiatric illness was initially illuminated through clinical studies demonstrating the presence of abnormally high levels of glucocorticoids (stress hormones) in populations expressing depressive or hypertensive behaviors (Gibbons, 1964; Sachar et al., 1973; Gold et al., 1988; Checkly, 1996). Furthermore, structural imaging techniques and postmortem studies have shown individuals suffering from mood disorders (e.g. major depressive disorder (MDD), post traumatic stress disorder (PTSD), and anxiety disorders) express neural remodeling in regions essential to emotional processing and adaptive responses to stress (i.e. prefrontal cortex, amygdala, and hippocampus) (Drevets et al., 1999; Bremner et al., 2000; Drevets, 2001; Radley et al., 2004). Interestingly, animal models have found prolonged exposure to environmental stressors, or chronic administration of endogenous corticosterone (stress hormone in rodents) alone, produces reorganization in the same neuronal structures (Woolley et al., 1990; Magarinos et al., 1996; Vyas et al., 2002). Additionally, animals exhibiting neuronal remodeling or hypercortisolaemia (excess circulating glucocorticoids) express depressive and anxious behaviors (e.g. increased immobility, reduced grooming, loss of appetite, social subordination, learned helplessness etc.) considered representative of those found in human mood related disorders (Checkley, 1996; Magarinos et al., 1996; Warner-Schmidt & Duman, 2006).

Evidence suggests the correlation between chronic stress and mental illness results in part from the prolonged exposure to increased concentrations of glucocorticoids compromising serotonergic (5-HT) and noradrenergic (NE) neurotransmission (Kolasa et al., 1992; Hayley et al., 2005). These relations seem probable when considering antidepressant drugs increase extracellular concentrations of 5-HT and NE in addition to reducing glucocorticoid levels (Checkley, 1996). Furthermore, when taken for prolonged durations some antidepressant drugs have been found to reverse certain types of neuronal damage (e.g. dendritic atrophy) caused by prolonged exposure to high levels of stress hormones (Magarinos et al., 1999). However, the pathology between chronic stress, dysfunction of monoamine (e.g. 5-HT, NE, dopamine)

systems and the onset of affective, anxiety and addictive disorders is poorly understood and is the purpose of further investigation in the present study.

## 2. Background

### *2.1 Stress response*

Although research has linked chronic stress to the progression of mental and physical illnesses, stress on an acute level is essential to stabilizing homeostasis, promoting adaptation and ultimately ensuring survival of the organism. Commonly, the term homeostasis refers to the maintenance of physiological systems within a narrow range of set points (Seyle, 1973). McEwen agrees in that he views homeostasis as regulation of those systems necessary for life on a consistent basis (e.g. temperature regulation, glucose levels, pH etc.), but adds the term "allostasis" to refer to more dramatic physiologic measures taken to promote adaptation (while maintaining homeostasis) when facing a significant life challenge or stressor (2004; Koob & LeMoal, 2001). Stress, in its most general sense, refers to the *biochemical* alarm system activated when a significant discrepancy exists between what the organism expects to happen based on previous experience and what is actually taking place (Levine, 1991). The two neural systems acting in parallel to mediate stress response across species (human and non-human animals) includes the sympathetic nervous system (SNS) (sometimes termed sympathic-adrenomedullary axis) and the hypothalamic-pituitary adrenocortical (HPA) axis (Fuchs, 2003; de Kloet et al., 1998). The SNS is a short-term response to stress in that when activated its effects last only minutes, while HPA axis activation has more long-term effects that last from a few minutes to weeks. The type of stressor (psychological or physiological in nature), familiarity of the stressor, duration of stressor, and perceived control or predictability of the stressor all influence what regulatory actions these two neural circuits take in restoring homeostasis.

The rapid "fight or flight" response of the SNS functions to increase vigilance and prepare the bodily organs within seconds for physiological threats that require immediate action (e.g. increase heart rate, blood flow, oxygen intake etc.). With the SNS, potentially dangerous sensory stimuli bypass cognitive processing and act instead directly on the amygdala (fear response) and the paraventricular nucleus (PVN) of the hypothalamus (regulates endocrine system). Excitation of these two structures stimulates parvocellular cells in the PVN and the central nucleus of the amygdala to release corticotrophin-releasing hormone (CRH; also termed

corticotrophin-releasing factor) along efferent pathways to the locus coeruleus (LC, main structure for NE production and secretion) located in the pons region of the brainstem. CRH is an excitatory neurotransmitter that signals the LC to release NE throughout sympathetic nerve endings directly innervating major organs (e.g. heart, lungs, adrenal glands, liver etc.) in the body. This in turn excites the SNS organs to increase or inhibit their rate of action. SNS stimulation of the adrenal medulla (the internal portion of the adrenal glands) triggers chromaffin cells therein to secrete the catecholamines epinephrine (EPI, also termed adrenaline) and norepinephrine (NE, analogous to noradrenalin) into the circulatory system. The release of EPI and NE into the bloodstream enables the SNS to excite peripheral tissues it cannot itself directly stimulate (e.g. alters blood flow) or further accentuate the excitatory signal directly communicated to organs by the sensory nerve endings (e.g. EPI increases heart rate).

Unlike the SNS, the HPA axis is activated by the collaborative input from limbic (i.e. amygdala and hippocampus) cortical structures (i.e. prefrontal cortex). This cognitive input further stimulates the parvocellular neurons in the PNV to release CRH to the medial eminence of the hypothalamus where it is then transported along the hypophyseal portal veins to the anterior portion of the pituitary gland. The presence of CRH triggers the anterior pituitary to secrete adrenocorticotrophic hormone (ACTH) into the circulatory system, which transports the hormone to the adrenal cortex (outer portion of the adrenal glands). Upon reaching the adrenal cortex, ACTH stimulates the release of corticosteroids (i.e. glucocorticoids and mineralocorticoids) into the circulatory system to coordinate the organism's metabolic and behavioral responses to stress. Of particular importance to this process is the glucocorticoid steroid corticosterone (in rodents, cortisol in humans), which influences arousal, metabolism, anti-inflammation, and immune function by directly altering protein synthesis and cell excitability (De Kloet et al., 2005).

### *2.2. Chronic stress and allostatic load*

Corticosterone (CORT) ability to alter cell physiology, tissue morphology and influence behavior is not restricted to the periphery. Since it is a highly lipophilic molecule, it is capable of crossing the cell membrane and binding directly to intracellular receptors that regulate DNA transcription. Likewise, CORT can cross the blood-brain barrier and activate corticosteroid receptors (i.e. mineralocorticoid receptors and glucocorticoid receptors) modulating the activity

of neuronal pathways involved in emotional processing, circadian rhythms and the regulation of stress response. The densest populations of mineralocorticoid receptors (MR) or glucocorticoid receptors (GR) are present in the hypothalamus, anterior pituitary, amygdala, and hippocampus (De Kloet et al., 1998; De Kloet et al., 2005). MR has a ten fold higher affinity (attraction) for CORT than GR, which results in an absolute occupation of MR prior to GR binding (Reul et al., 1985; Fuchs et al., 2003). Due to this complimentary relation, MR are believed to maintain circadian rhythmicity at basal concentrations of CORT and initiate the onset of stress responses once fully occupied, while GR are thought to be the primary mechanisms signaling stress response inhibition since they are only active when CORT concentrations are elevated (e.g. during circadian peaks or periods of stress) (De Kloet et al., 1987; Reul et al., 1987).

The densest co-expression of MR and GR across species exists in the hippocampus (Patel et al., 2000; De Kloet et al., 2005; Herman, 1993), which is a primary inhibitory structure in the negative feedback regulation of the HPA axis (Lopez et al., 1998). Additionally, this limbic structure plays a vital role in emotional processing and in key aspects of learning and memory. When increasing CORT levels occupy hippocampal GR, the hippocampus stimulates the PVN to inhibit parvocellular neurosecretory neurons therein from further secreting CRH (Herman et al., 1992). As previously mentioned CRH initiates and perpetuates SNS and HPA axis activity during an organism's response to threatening or stressful stimuli. By inhibiting the release of CRH from the PVN, the hippocampus is able to indirectly prevent further secretion of ATCH from the anterior pituitary into the circulatory system. Without continued ACTH stimulation, the adrenal cortex stops releasing CORT thus reducing circulatory concentrations of the steroid. This reduction in CORT levels slows or halts the steroid's influences on cellular activity, which eventually brings the organism back to homeostatic conditions. In an acute stress response, CORT levels peak approximately 15 – 30 minutes post activation of the HPA axis and then slowly decline over 60 – 90 minutes due to the neuro-regulatory effects of the negative feedback loop (McEwen, 2004; De Kloet et al., 2005). However, in chronic stress conditions the HPA axis becomes over-stimulated causing cellular mechanisms regulating the stress response to become unresponsive and/or morphologically altered.

Animal studies have found prolonged exposure to stress paradigms (e.g. psychosocial stress, forced swim test etc.) downregulates (decreases) GR expression and/or function throughout the hippocampal formation (Johren et al., 1994; Meyer et al., 2001), which has been

associated with hippocampal signaling to the PVN becoming suppressed (Fuchs et al., 2003). When hippocampal tone is reduced, the PVN is disinhibited enabling the structure to continue releasing CRH for longer durations of time. Since CRH stimulates the release of ACTH and CORT, prolonged CRH secretion correlates with elevated levels of circulating CORT and a delayed return to basal conditions (Herman et al., 1992). In addition to changing GR regulatory effects on the HPA axis, chronic exposure to elevated CORT is neurotoxic to hippocampal cells and causes the structure to atrophy (decrease in structural volume). Within the hippocampus, atrophy is most significant in the CA3 region where there is evidence of dendritic atrophy and in the dentate gyrus where the proliferation of new neurons (termed neurogenesis) is reduced and even cell death results (Sapolsky and Plotsky, 1990; McEwen, 2004). Evidence also shows HPA axis deregulation leads to amygdala hyperactivity and an increase in the structure's volume due to dendritic sprouting (McEwen, 2004). Excitation of the amygdala acts as a positive feedback on the PVN meaning it stimulates the secretion of CRH and triggers SNS and HPA axis activity. As the inhibitory feedback of the hippocampus on the HPA axis is reduced as a result of chronic stress and the excitatory feedback of the amygdala on the HPA axis is increased, the organism progressively moves farther away from having a system capable of restoring itself to homeostatic conditions (termed allotatic load) (McEwen, 2004).

*2.3. Chronic stress and deregulation of monoamine neurotransmission*

In many affective and anxiety disorders, chronically elevated CORT and morphological changes in limbic structures (i.e. hippocampus, amygdala) are accompanied by the biochemical deregulation of serotonergic (5-HT) and noradrenergic (NE) neurotransmission (Ressler & Nemeroff, 2000). Both 5-HT and NE systems originate in the pons-medulla region of the brainstem (raphe nucleus (RN) for 5-HT and locus coeruleus (LC) for NE) and have commonality in the subcortical (limbic) and cortical structures their ascending pathways innervate (e.g. amygdala, prefrontal cortex, hippocampus, hypothalamus etc.) (Levitt et al., 1984). The functional roles of 5-HT and NE in mood regulation, learning and memory formation, inhibition, mental illness and stress response has largely come to light through the development of antidepressant drugs and the conduction of lesion studies. Research suggests a regulatory relation exists between the two systems in which LC-NE activity increases vigilance during stress response, and that RN-5HT activity inhibits the excitatory response of the LC-NE

and its associated structures (Ressler & Nemeroff, 2000; Mongeau et al., 1997). For instance, studies have shown direct stimulation of the amygdala or the LC will elicit an excitatory response in the other structure (Feldman & Weidenfeld, 1998). Evidence suggests this circular relation is regulated in part by 5-HT ascending pathways densely innervating the amygdala to inhibit the excitatory loop from over activating stress responses (Tork, 1990; Feldman & Weidenfeld, 1998). Furthermore, studies have found decreased 5-HT is significantly correlated with increased levels of LC activity, aggression, impulsivity and suicide (Mann, 1999; Stanley et al., 2000; Ressler & Nemeroff, 2000). Considering how many neuronal structures 5-HT and NE neurotransmission and SNS and HPA axis systems have in common, the possibility of discourse in any one of these pathways triggering deregulation in the others seems probable. A number of studies have found 5-HT signaling is altered by glucocorticoids, which makes deregulation in 5-HT neurotransmission a likely trigger or propagator for dysfunction in the other systems involved in stress responses (e.g. SNS, HPA axis, NE neurotransmission etc.). However, the specific mechanism by which CORT alters 5-HT signaling is not known at this time.

*2.4. Serotonergic neurotransmission*

Evidence showing serotonin transporter (SERT or 5-HTT) density is reduced as a result of high anxiety in individuals diagnosed with major depressive disorder (MDD) and generalized anxiety disorder (Iny et al., 1994; Arango et al., 2002) suggests the mechanism is a primary candidate for CORT activity and 5-HT signaling deregulation. The SERT is a membrane bound protein that terminates 5-HT neurotransmission by clearing 5-HT from the extracellular fluid (ECF) (Blakely et al., 1991). In addition, it is a major target for antidepressants drugs (SSRI, selective serotonin reuptake inhibitors) and drugs of abuse (e.g. methamphetamines and cocaine), which work by blocking the transporter from clearing 5-HT from the ECF thus increasing extracellular concentrations of the neurotransmitter and prolonging the 5-HT signal. Furthermore, a polymorphism of the SERT gene (SLC6A4 in the 5' promoter region) transcribes for a 50 – 60% reduction in SERT expression for carriers of the *s* allele (*s/l* or *s/s*) compared to those who are homozygous for the *l* allele (*l*/*l*) (Lesch et al., 1996). A number of clinical studies have associated the reduction in SERT expression with increased resistance to antidepressant drugs (Lee et al. 2004; Rausch, 2005), increased anxiety (Hariri et al., 2006; Wurtma, 2005), and substance abuse (Feinn and Kranzler, 2005). In regards to stress and psychiatric illness, Caspi et

al. (2003) found carriers of the *s* allele expressed a significantly higher rate of depressive and suicidal episodes as a result of stressful life events compared to those who are homozygous for the *l* allele.

The development of the SERT knockout mouse has been imperative to gaining a better understanding of how a reduction in SERT expression (as seen with the SERT polymorphism in humans) may predispose individuals to mental illness. Mice that are SERT wild-type (W/T, +/+) express 100% of the transporter, mice that are heterozygous (HET, +/-) for SERT express 50% of the transporter, while mice that SERT knockouts (KO, -/-) have had the gene completely removed and therefore express 0% of the transporter. To gain a better understanding of how SERT function may be altered as a result of chronic stress, we conducted preliminary studies using *in vivo* chronoamperomentry to quantify the clearance rate of 5-HT from the ECF in the CA3 region of the hippocampus of stressed and non-stressed W/T mice. Our results found 5-HT clearance was dramatically slower in stressed W/T mice compared to their non-stressed W/T counterparts (Figure 1) (Baganz et al., 2005). Furthermore, we found that there was no significant difference between the 5-HT clearance rates of stressed W/T mice when compared to non-stressed SERT knock out (KO) mice (data not shown), which would suggest a reduction in SERT expression results from prolonged exposure to CORT. However, quantitative autoradiographical analysis showed the reduction in 5-HT clearance was not associated with the loss of SERT expression between W/T stressed and non-stressed conditions (Figure 2) (Baganz et al., 2006).

Given the data presented here, it is possible another transporter system capable of clearing 5-HT from ECF in the CA3 region of the hippocampus is inhibited or down-regulated when exposed to exogenous substances (e.g. alcohol or MNDA) or increased levels of CORT. There is evidence that norepinephrine transporters (NET) and dopamine transporters (DAT) can clear 5-HT from the ECF (Shaskan and Snyder, 1970; Zhou et al., 2004). Considering the majority of studies report stress increases 5-HT release (Keeney et al., 2006; Chaouloff et al., 2006) and our preliminary studies show 5-HT clearance is reduced in W/T stressed mice, it is possible chronic exposure to CORT downregulates the expression and/or function of NET or DAT.

**Figure 1**

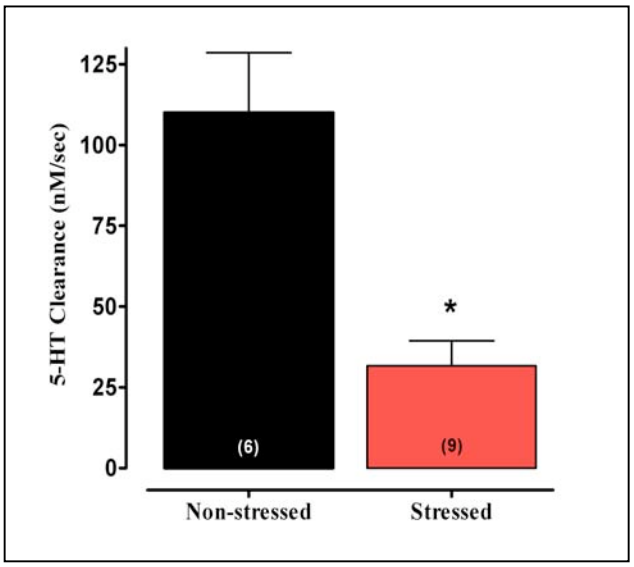


**Figure 2**

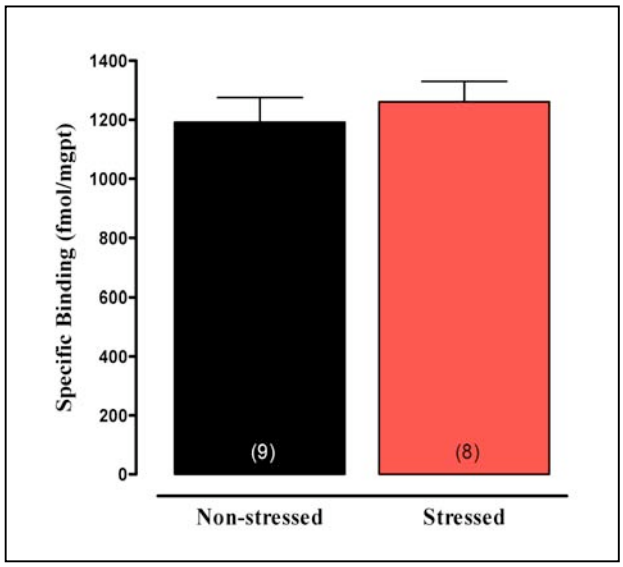


**Figure 1:** Stressed W/T mice clear 5-HT from the Eat a significantly slower rate then non-stressed mice when 5-HT was applied locally to the CA3 region of the hippocampus (*p<0.05 compared to non-stressed control condition). **Figure 2:** Specific SERT binding in the CA3 region of stressed and non-stressed mice was labeled using [3H]cyano-imipramine (1 nM), while nonspecific binding was determined in the presence of fluvoxamine (1 μM).

*2.5. Norepinephrine neurotransmission*

Similar to SERT and 5-HT, the NET regulates NE transmission by clearing the neurotransmitter form the ECF and is a primary target site for a number of antidepressant drugs (SNRI, selective norepinephrine reuptake inhibitor) (Blakely and Bauman, 2000; Frazer, 2000). Also in common with 5-HT, dysfunction of NE neurotransmission has been implicated as a contributing factor to the pathogenesis of affective and addictive disorders (Southwick et al., 1999; Ressler & Nemeroff, 2000; Charney & Manji, 2004). Furthermore, evidence shows NET expression is reduced in the brains of humans diagnosed with MDD (Klimek et al., 1997), and that chronic stress reduces NET binding in structures key to NE neurotransmission and stress response (i.e. hypothalamus, locus coeruleus, amygdala) (Zafar, 1997).

In addition to clearing NE from the ECF, the NET exhibits a low affinity but high capacity for 5-HT clearance *in vitro* (Shaskan and Snyder, 1970). Furthermore, our previous studies have show the NET is capable of clearing NE and 5-HT from the ECF in the CA3 region of the hippocampus using *in vivo* chronoamperometry (Daws et al., 1998). Using binding assays, our lab has shown the CA3 region expresses a NET to SERT density ratio of 1:4 in non-stressed W/T mice (Montanez et al., 2003). Although there is evidence that DAT is also a low affinity

high capacity transporter for 5-HT, it is not likely this mechanism is contributing to 5-HT in the CA3 region based on previous studies showing there is relatively no DAT in this region of the hippocampus (Javitch et al., 1985).

## 3. Hypothesis

Given the evidence presented, we hypothesize the reduced 5-HT clearance rate found in stressed mice results from a downregulation of NET function and/or expression. If a downregulation in NET function results, stressed mice will express significantly slower NE clearance rates compared to their non-stressed counterparts. If a reduction in NET density results, stressed mice will express significantly less NET binding compared to non-stressed mice. The hippocampus was chosen for this study because of its involvement in the limbic "mood-regulating" system, and it being an area where antidepressant drugs demonstrate both acute and regulatory effects (Blier et al., 1990). Furthermore, the hippocampus inhibits hypothalamic activity when glucocorticoid levels reach threshold concentrations, and structurally the CA3 region displays severe atrophy in many psychiatric disorders (Sapolsky and Plotsky, 1990). A modified version of the FST as originally described by Porsolt et al. (1977) was used as the stress paradigm in this study due to its common use as a behavioral test for screening antidepressant compounds (Cryan et al., 2005).

## 4. Methods and Materials

### *4.1. Apparatus*

A Plexiglas cylinder served as the swim tank in the FST stress paradigm. Electrochemical recording assemblies consisted of a single carbon fiber electrode (CFE) attached to a multi-barrel micropipette. Electrodes were coated with Nafion (5% solution) and calibrated for NE selectivity. Micropipette barrels were filled with neurotransmitter (NE) or phosphate buffered saline (PBS). Electrochemical recording assemblies were stereotaxically lowered into the CA3 region of the dorsal hippocampus according to coordinates retrieved from Franklin and Paxinos (1997). Barrel solutions were applied locally by pressure injection, and *in vivo* high-speed chronoamperometric recordings were made using the FAST-12 system. A cryostat was used to section brains for histological verification of electrode placement accuracy and quantitative autoradiography evaluation of NET density. Quantitative autoradiography was used

to measure [$^3$H]nisoxetine binding to NETs. Sections were dried on a slide warmer and apposed to Kodak Biomax MR film. Autoradiograms were analyzed using the NIH Image program, version 1.47.

*4.2. Participants*

Male C57Bl/6 wild-type mice (20-30g, Jackson Laboratories) were used in all experiments. Animals were housed in groups of four, maintained under a 12:12-hr light cycle (21 ± 1 ºC), and provided food and water *ad libitum*. All animal procedures were approved by the UT Health Science Center at San Antonio (UTHSCSA) Institutional Animal Care and Use Committee (IACUC) and were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize the number of animals used and the amount of discomfort animals endure during experiments.

*4.3. Forced Swim Tests (FST)*

Stress condition animals receive daily 10 minute FST between the hours of 9:00 a.m. and 12:00 noon for 14 consecutive days. Mice were gently lowered into a transparent Plexiglas swim tank (20 cm diameter x 25 cm height) filled with 12 cm of tap water (25 ± 1 ºC). The depth of the water was sufficient in keeping the mice from using their tails to support themselves while in the water. Following the same procedures, Boyce-Rustay et al. (2007) have shown CORT levels remain significantly elevated throughout the 14 day duration signifying the animal does not become habituated to the FST paradigm. After the completion of each FST, the mice were dried and returned to their home cage. To prevent residue odors from unfamiliar animals inducing additional stress, the tank water was refreshed after each FST. *In vivo* electrochemistry or quantitative autoradiography was performed 24 hr after the last FST, or at matched times for the non-stressed controls.

*4.4. Electrochemical recordings*

The Fast-12 system (Quanteon, Nicholasville, KY) was used to make all high-speed chronoamperometric recordings. A stepped (square wave) oxidation potential of +0.55V was applied for 100 ms per second (1000 ms). Each 100 ms pulse was separated by a 900 ms interval where resting potentials were maintained at 0.0V. Electrode voltage was applied in respect to an

Ag/AgCl reference electrode placed rostral to the recording sight. NE oxidation and reduction currents were digitally integrated during the last 80 ms per 100 ms voltage pulse and expressed as a function of time (μM/sec).

*4.4.1. Electrode preparation and in vitro calibration*

All *in vivo* recordings were made with custom fabricated single carbon fiber electrodes (30 μm diameter) cut to extend 150 μm from the pulled glass capillary. For further details on electrode fabrication refer to Perez and Andrews (2005) or Daws and Toney (2006). Prior to *in vitro* calibrations, electrodes were coated with 5% Nafion solution to prevent signal interference from anionic neurotransmitter metabolites present in the ECF once *in vivo*. Coating electrodes entailed swirling the carbon fiber tip in Nafion for 2-3 s then drying the electrode at 200°C for 3 minutes. This process was completed three times to give each electrode a total of three Nafion coats. To calibrate, electrodes were first challenged with ascorbic acid (AA, 250 uM) in 40mL PBS (pH ~ 7.3) to verify Nafion coated electrodes were not sensitive to anions. Electrodes were then exposed to increasing concentrations of NE (1.0 uM – 3.0 uM) to improve electrode sensitivity to NE. Only electrodes with a selectivity ratio for NE over AA greater than 200:1 and liner response of $r^2 \geq 0.9$ were used *in vivo*.

*4.4.2. In vivo experimental procedures*

*In vivo* electrochemistry was carried out as described in Montañez et al. (2003) and more details on the methods can be found therein. Mice were anesthetized by an intraperitoneal (i.p.) injection (2 ml/kg body weight) of a chloralose (35 mg/ml) and urethane (350 mg/ml) mixture, intubated to facilitate breathing and placed into a stereotaxic frame. Body temperature was maintained (32 ± 1 ºC) during experiments using a water circulated heating pad. Electrochemical recording assemblies were positioned in the CA3 region of the dorsal hippocampus (AP -1.94 mm, ML +2.0 mm, DV -1.8 to -2.0 mm from the dura; Franklin and Paxinos, 1997) and allowed to stabilize 20-30 minutes before high-speed chronoamperometric recordings were used to measure the rate at which NE cleared from ECF (μM/sec).

Electrochemical recording assemblies were composed of a single carbon fiber electrode previously calibrated for NE sensitivity attached to a single (10-15 μm diameter) or four barrel micropipette (Frederick Haer Corp. Inc.; Bowdoinham, ME). Barrels were filled with NE (200

μM) or PBS (0.1 M), then each fitted with 18 inches of PE tubing (~30 cm) and sealed with cyanoacrylate adhesive. The open end of the PE tubing was attached to a picospritzer, which delivered the pressure needed to inject solutions into the brain. Electrode and micropipette tips were positioned 218 μm apart using a micro-positioner and attached to one another with sticky wax.

To obtain estimates of the maximal velocity (Vmax) for NE clearance and the affinity of NET for NE in vivo ($K_T$), various pmol amounts of NE were pressure ejected into hippocampus to achieve a range of signal amplitudes (0.1-50 μM). A dissection microscope was fitted with an eyepiece reticule to determine how much NE was displaced from the barrel each injection. Every NE amplitude signal produced was allowed to return to baseline before the following injection of NE or PBS was given. The pressure and duration (2-30 pounds per square inch (psi) for 0.04-2 s) used to injected NE was varied in order to randomize NE concentrations administered and prevent order effects from confounding results. If the electrodes became insensitive to NE (20% signal decrease), the recording assembly was removed and a new Nafion coated NE calibrated electrode was attached to the micropipette. The electrochemical recording assembly was then lowered once again into the CA3 region and allowed to stabilize before recording resumed.

*4.5. Histological Procedures*

At the end of each *in vivo* experiment, an electrolytic lesion was made to determine accuracy of electrode placement and the anesthetized mouse was decapitated. The brain was removed, rapidly placed on dry ice then stored at -80°C until sectioned. Brains were allowed to thaw to -15°C prior to sectioning, then sliced 20 μm thick and thaw mounted onto gelatin-coated slides for histological verification. Slides were allowed to dry at room temperature for 24 hrs prior to staining with crystal violet. Only data from brains where the electrolytic lesion was confirmed to be in the CA3 region of the hippocampus was included in the data analysis.

*4.6. Quantitative Autoradiography of [$^3$H] Nisoxetine Binding to NET*

Similar to the histology procedures, non-lesioned brains were removed, sectioned 20 μm thick at -15°C then thaw mounted onto gelatin-coated slides. NET binding sites were then measured using [$^3$H]nisoxetine as described by Tejani-Butt (1992). Brain sliced were incubated

in 1nM at [$^3$H]nisoxetine at 4 ºC for 4 hrs, while non-specific binding was defined by incubating sections with reboxitine (1 μM). Once incubation was complete, sections were washed three times (5 minutes each) in cold buffer, put in ice-cold distilled water and then placed on a slide warmer (60 ºC ) to dry. After completely drying, slides were exposed against a Kodak Biomax MR film (Amersham) for 4 weeks in order to generate autoradiograms. Analysis of NET density was performed using the NIH-IMAGE program, version 1.47.

*4.7. Data analysis*

The oxidation currents gathered *in vivo* were converted into micromolar units (μM) of NE concentrations using the calibration factor established *in vitro*. Using these micromolar units the maximal velocity ($V_{max}$) and transporter affinity ($K_t$) values for NE clearance were determined by fitting a One-site binding hyperbola to a plot of NE clearance rates ($T_c$) verses signal amplitudes. Here $T_c$ represents the rate at which the signal slope decays 20-60% from its peak signal amplitude, which is the most linear portion of the curve. NET binding was analyzed using an ANOVA followed by a post-hoc test. All data was presented as the mean and standard error of the mean, and a two-tailed probability of $p<0.05$ was accepted as statistically significant in all tests.

**5. Results**

*5.1. NE kinetics in stressed and non-stressed mice*

NE was locally applied in the CA3 region of the hippocampus to achieve a rage of concentration amplitudes (0.1-50 μM), and NE clearance rates were measured as a function of time (μM/sec). Results show stressed W/T mice clear NE from ECF at a significantly faster rate than their non-stressed W/T counterparts (*p<0.05) (Figure 3). Data was analyzed using a one-site binding hyperbola.

*5.2. NET density in stressed and non-stressed conditions*

Quantitative autoradiographical analysis determined specific NET binding in the presence of [$^3$H]nisoxetine (1nM), while non-specific binding was determined in the presence of reboxetine (1 μM). Results show there is no significant difference in NET density between stressed and non-stressed W/T mice in the CA3 region of the hippocampus (Figure 4) (Baganz et

al., 2006). Furthermore, our results show a 1:1 ratio of NET to SERT density is present in the CA3 region instead of a 1:4 ratio as previously established by Montanez et al. (2003). Data was analyzed using a one-way ANOVA followed by a Tukey's post-hoc test.

**Figure 3**

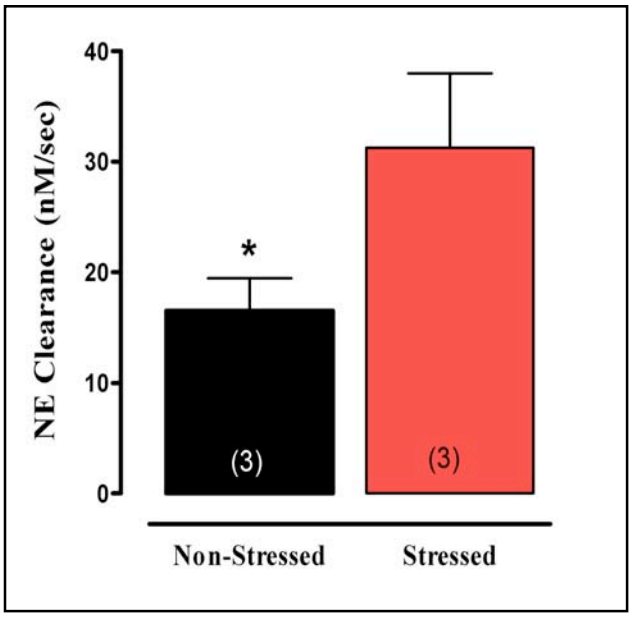


**Figure 4**

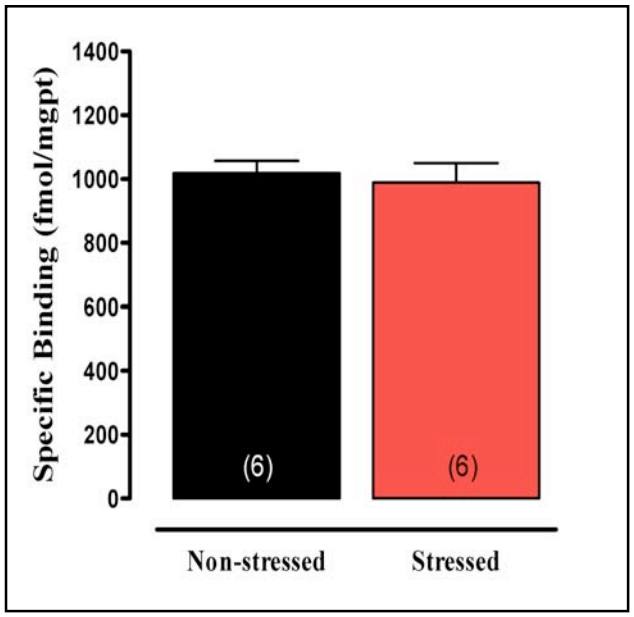


**Figure 1:** Stressed W/T mice cleared NE at a markedly faster rate then non-stressed W/T mice when NE was applied locally to the CA3 region of the hippocampus (* $p<0.05$ compared to stressed). **Figure 2:** NET binding in the CA3 region of stressed and non-stressed mice were labeled by binding of [$^3$H]nisoxetine (1nM). Nonspecific binding was determined in the presence of reboxetine (1 μM).

## 6. Discussion

This study aimed to clarify the pathological effects of chronic stress on serotonergic (5-HT) and noradrenergic (NE) systems, and to yield insight into the poorly understood correlation between chronic stress and mental illness. More specifically, the goal was to investigate how chronic exposure to stress effects NET function and/or expression in the CA3 region of the hippocampus. Considering NETs express a low affinity but high capacity for 5-HT uptake (Shaskan and Snyder, 1970; Daws et al., 1998), it is reasonable to suspect alterations in NE neurotransmission caused by chronic stress may trigger deregulation in 5-HT neurotransmission. Based on this, we hypothesized the reduced 5-HT clearance rates expressed in stressed W/T mice resulted from a downregulation in NET expression and/or function in the CA3 region of hippocampus.

*6.1. Effects of chronic stress on NET function and expression*

Contrary to our hypothesis, we found stressed W/T mice actually clear NE from the ECF at a significantly faster rate than their non-stressed counterparts (Figure 3). This data alone would suggest chronic stress upregulates NET function and expression instead of downregulates as anticipated. Autoradiographical analysis, however, showed NET expression did not differ significantly between stressed and non-stressed conditions (Figure 4). Although his data seems contradictory, it is worth noting autoradiography is a labeling method that quantifies overall protein expression and cannot differentiate between NET activity at the plasma membrane and those inactive within the cell cytoplasm.

Keeping in mind further testing is necessary to confirm the faster NE clearance rates expressed in stressed conditions does in fact result from an increase in plasmalemmal NET, it seems highly probable given previous studies showing stress increases NE activity (Ressler & Nemeroff, 2000). A study conducted by Miner et al. (2003) found 85-90% of NET in the prefrontal cortex (PFC) of naive rats where localized within the cytoplasm of NE axons, meaning the large majority of NET in this area of the brain are not actively clearing NE from the EFC during basal conditions. A follow up study by Miner et al. found the number of plasmalemmal NET expressed in chronically stressed rats was significantly higher (~51%) than their non-stressed counterparts (29%) (2006). In addition, the study showed stressed animals express a significant increase in tyrosine hydroxylase (TH), which is the rate limiting enzyme for NE and dopamine (DA) synthesis. Together this data suggests NET trafficking and NE synthesis are both regulated by an increase in NE cell activity during stressful or high arousal conditions (Miner et al., 2006).

Considering the PFC and hippocampus both influence HPA axis and amygdala activity and are innervated by NE projections from the LC, it is possible an upregulation in plasmallemmal NET expression and NE synthesis also occurs in the hippocampus as a result of chronic stress. In order to further clarify the pathology between chronic stress, monoamine dysfunction, and onset of mental illness it will be imperative for future studies to further investigate NET function in stressed and non-stressed conditions in the CA3 region of the hippocampus.

*6.2. Alternative mechanism for 5-HT uptake*

Since there are relatively no DAT present in the CA3 region of the hippocampus (Javitch et al., 1985) and evidence suggests NET are upregulated instead of downregulated as a result of chronic stress (Miner et al., 2006), it is possible another transporter system or unknown mechanism capable of regulating 5-HT neurotransmission in the hippocampus is being compromised by stress hormones. It has recently established the organic cation transporter (i.e. OCT3) is present in the brain and exhibits a low affinity but high capacity for clearing biogenic amines (e.g. 5-HT, NE, DA, and histamine) from the ECF (Grundemann et al., 1998; Koepsell et al., 2003). In addition, OCT is the only transporter system currently known to express sensitivity to CORT, which means CORT can block OCT from clearing biogenic amines from the EFC thus increasing extracellular concentrations of the neurotransmitters (Hayer-Zillgen et al., 2002). Based on these characteristics alone, there is good reason to suspect prolonged occupancy of OCT by CORT (e.g. chronic stress conditions) plays a significant role in the deregulation of monoamines and the progression of mental illness.

Additional evidence supporting OCT involvement in monoamine dysfunction has come to light through recent studies in our lab (manuscript in preparation). Using western blots we found OCT3 expression increases in a manner inversely proportional to genetic reductions in SERT expression in the CA3 region of the hippocampus (OCT3 increased 136% in SERT KO mice). This is finding is of great important when taking into account individuals who are carriers of the *s* allele (express a reduction in SERT) are insensitive to traditional antidepressant treatments (i.e. selective serotonin reuptake inhibitors (SSRI) and selective norepinephrine reuptake inhibitors (SNRI)) (Lee et al. 2004; Rausch, 2005). Along these lines, we also found SERT HET and SERT KO mice express a significantly greater inhibition in histamine and 5-HT clearance *in vivo* when exogenous CORT is pressured injected in the CA3 region of the hippocampus. Although prolonged signaling may not seem a serious threat acutely, it can become neurotoxic or cause receptor desensitization if prolonged. In addition, using immunostaining techniques we found non-stressed W/T mice have a higher OCT3 density in the CA3 region of the hippocampus and in the granular and pyramidal cell layers of the same structure. As previously mentioned, both of these hippocampal regions are target areas for antidepressant activity and express severe atrophy under chronic stress conditions (Blier et al., 1990; Sapolsky and Plotsky, 1990).

Although previous research (Schmitt et al., 2003) and our present finding briefly described above give insight into how increased CORT and OCT inhibition influence 5-HT neurotransmission, little work has been done to better understand the effects of CORT inhibition of OCT on NE neurotransmission. Considering 5-HT and NE systems are highly interconnected thought the central nervous system makes further investigation towards understanding the relation between NET and OCT function in stress and non-stress conditions a necessity to fully understand the pathology between monoamine deregulation and mental illness.

## 7. Conclusion

In order to further elucidate the effects of chronic stress on 5-HT and NE transmission individually and in relation to their regulatory effects on each other, future studies will include administering selective norepinephrine reuptake inhibitor (SNRI) and/or a cocktail mixture (SSRI, selective serotonin reuptake inhibitor and SNRI) to index the functional tone of NETs *in vivo* in stressed and non-stressed conditions. Similarly, in order to gain a better understanding of OCT functional tone and influence on NE and 5-HT neurotransmission *in vivo,* future studies will also include cocktail mixtures of SNRI, SSRI, CORT or decynium-22 (D-22, OCT inhibitor) in stressed and non-stressed conditions. Furthermore, additional OCT density studies will need to be completed to compare if OCT expression is altered in the hippocampus of stressed W/T, SERT HET, and SERT KO mice. In the case OCT is downregulated in stressed conditions, knowing what regions of the hippocampus (e.g. CA3, dentate gyrus etc.) express significant reductions may help clarify the pathology between elevated CORT, reduction in neurogenesis, neuronal cell death and how these alterations promote mental illness. In clarifying how SERT, NET and OCT change in relation to one another as a consequence of chronic stress antidepressant and drug addiction treatments can be improved by creating psychiatric drugs that are more specific, effective and elicit fewer side effects.

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