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Stress–restress evokes sustained iNOS activity and altered GABA levels and NMDA receptors in rat hippocampus

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Abstract *Rationale:* Stress-related glucocorticoid and glutamate release have been implicated in hippocampal atrophy evident in patients with post-traumatic stress disorder (PTSD). Glutamatergic mechanisms activate nitric oxide synthase (NOS), while gamma-amino-butyric acid (GABA) may inhibit both glutamatergic and nitrenergic transmission. Animal studies support a role for NOS in stress. *Objectives:* We have studied the role of NOS and glucocorticoids, as well as inhibitory and excitatory transmitters, in a putative animal model of PTSD that emphasizes repeated trauma. *Methods:* Hippocampal NOS activity, *N*-methyl-D-aspartate (NMDA) receptor binding characteristics and GABA levels were studied in Sprague-Dawley rats 21 days after exposure to a stress–restress paradigm, using radiometric analysis, radioligand studies and high-performance liquid chromatography (HPLC) analysis with electrochemical detection, respectively. The NOS isoform involved, and the role of stress-mediated corticosterone release in NOS activation, was verified with the administration of selective iNOS and nNOS inhibitors, aminoguanidine (50 mg/kg/day i.p.) and 7-nitroindazole (12.5 mg/kg/day i.p.), and the steroid synthesis inhibitor, ketoconazole (24 mg/kg/day i.p.), administered for 21 days

prior to and during the stress procedure. *Results:* Stress evoked a sustained increase in NOS activity, but reduced NMDA receptor density and total GABA levels. Aminoguanidine or ketoconazole, but not 7-nitroindazole or saline, blocked stress-induced NOS activation. *Conclusions:* Stress–restress-mediated glucocorticoid release activates iNOS, followed by a reactive downregulation of hippocampal NMDA receptors and dysregulation of inhibitory GABA pathways. The role of NO in neuronal toxicity, and its regulation by glutamate and GABA has important implications in stress-related hippocampal degeneration.

Keywords PTSD · Nitric oxide · NMDA · Glutamate · GABA · Glucocorticoids · Time-dependent sensitization

Introduction

Post-traumatic stress disorder (PTSD) is a debilitating anxiety disorder that may develop after an individual has experienced or witnessed a severe traumatic event. Apart from symptoms of hyperarousal, characteristic features of PTSD include avoidance and amnesic symptoms (American Psychiatric Association 1994). Furthermore, imaging studies in PTSD patients have demonstrated volume reductions in the hippocampus (Elzinga and Bremner 2002) that appear correlated with illness severity and the degree of cognitive deficit (Bremner 1999). Although there is evidence of decreased hippocampal volume predating PTSD (Pitman et al. 2001), the hypothesis that PTSD leads to hippocampal atrophy and associated memory deficits is supported by recent work that, during treatment of PTSD, there is an increase in hippocampal volume (Vermetten et al. 2003).

Associative learning and other behavioral processes mediated by the hippocampus and that play a role in PTSD involve glutamate *N*-methyl-D-aspartate (NMDA) receptors (Heresco-Levy and Javitt 1998). Dysfunctional brain glutamatergic systems, particularly the NMDA receptor, have recently been suggested as an important neurobiological component of PTSD, perhaps contribut-

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ing to hippocampal toxicity (Chambers et al. 1999). Animal studies implicate the hippocampus in emotional processing as well as explicit memory (Brown et al. 1999), where stress-related hippocampal structural changes appear related to the neurotoxic effects of glucocorticoids and the subsequent release of glutamate (McEwen 1999; Sapolsky 2000). In a recent pilot study, Heresco-Levy et al. (2002) reported on the clinical evidence for the efficacy of D-cycloserine, a partial agonist at the glycine regulatory site on the NMDA receptor, in the treatment of PTSD.

The activation of neuronal nitric oxide (NO) synthase (nNOS) and other Ca^{2+} -dependent enzymes accounts for many of the deleterious effects associated with excessive NMDA receptor activation (Almeida et al. 1998), while recent studies have provided evidence that severe forms of stress, such as chronic restraint stress, activate pro-inflammatory pathways in the brain, particularly inducible NOS (iNOS) and cyclooxygenase (Madrigal et al. 2001, 2003). Since iNOS involves a gradual induction over time, resulting in the protracted release of large amounts of NO, this may have direct implications for neuronal degeneration and atrophy evident in neuroimaging studies in patients with PTSD. Furthermore, several lines of evidence indicate that the NMDA–nNOS pathway plays an important role in anxiety-related behaviors (Podhorna and Brown 1999; Masood et al. 2003), while mice lacking a fully functional glutamate NMDA receptor are less sensitive to stress induced by the elevated plus-maze, light–dark box, and forced swimming tests (Miyamoto et al. 2002).

Inhibitory gamma aminobutyric acid (GABA) pathways also have an important regulatory role in the stress response (Shiah and Yatham 1998). Moreover, GABA inhibits glutamatergic transmission via activation of pre-synaptic GABA_B heteroreceptors (Yamada et al. 1999), while it also attenuates stress-induced NO release (Ishizuka et al. 2000). GABA thus has an important role in curbing excessive glutamate–NOS activation.

By definition, PTSD occurs in the aftermath of exposure to trauma, but there is growing awareness of the importance of multiple exposures to trauma in predicting the incidence and severity of this disorder (Brewin 2001; Johnsen et al. 2002; Maes et al. 2001). Stress–restress or time-dependent sensitization (TDS) is an animal model that emphasizes repeated exposure to stress (Yehuda and Antelman 1993; Uys et al. 2003) and that has neuroendocrine overlap with PTSD (Liberzon et al. 1997; Harvey et al. 2003). TDS stress also evokes various behavioral responses resembling that seen in PTSD, including an exaggerated startle response (Kahn and Liberzon 2003), and cognitive deficits (Harvey et al. 2003). Here, we have investigated whether stress–restress, or TDS, can evoke protracted effects on critical markers of NO, glutamate and GABA in the rat hippocampus, important systems involved in neurotoxicity, neuroprotection and memory function. To this end, hippocampal NOS activity, NMDA receptor-binding characteristics and GABA levels were studied in Sprague–Dawley rats 21 days after exposure to

a stress–restress paradigm, using radiometric analysis, radioligand studies and HPLC analysis with electrochemical detection, respectively. Finally, the NOS isoform involved and the role of stress-mediated corticosterone release in NOS activation were verified with the administration of selective iNOS and nNOS inhibitors, aminoguanidine and 7-nitroindazole, and the steroid synthesis inhibitor, ketoconazole.

Materials and methods

Animals

The study protocol was approved and carried out in accordance with the guidelines stipulated by the ethics committee for the use of experimental animals at the North West University. Male Sprague–Dawley rats, weighing 200–250 g, were housed in cages measuring 28 cm (W), 44.5 cm (L), 12.5 cm (H), in a 12-h/12-h light/dark cycle, with free access to food and water. The conditions in the animal center were controlled at $21 \pm 0.5^\circ\text{C}$ and $50 \pm 5\%$ relative humidity. The room was exposed to full-spectrum cold white light, with a light intensity of 350–400 lux provided over a 12-h/12-h light/dark cycle. A positive air pressure was constantly maintained.

Drugs and reagents

All chemicals and reagents were of the purest grade commercially available. Aminoguanidine, GABA, 7-nitroindazole, DL-homoserine and glycine, were purchased from Sigma. [^3H]L-arginine (specific activity 24.6 uCi), and [^3H]dizocilpine were purchased from Amersham. Ketoconazole was a gift from Janssen Pharmaceutica.

Time-dependent sensitization

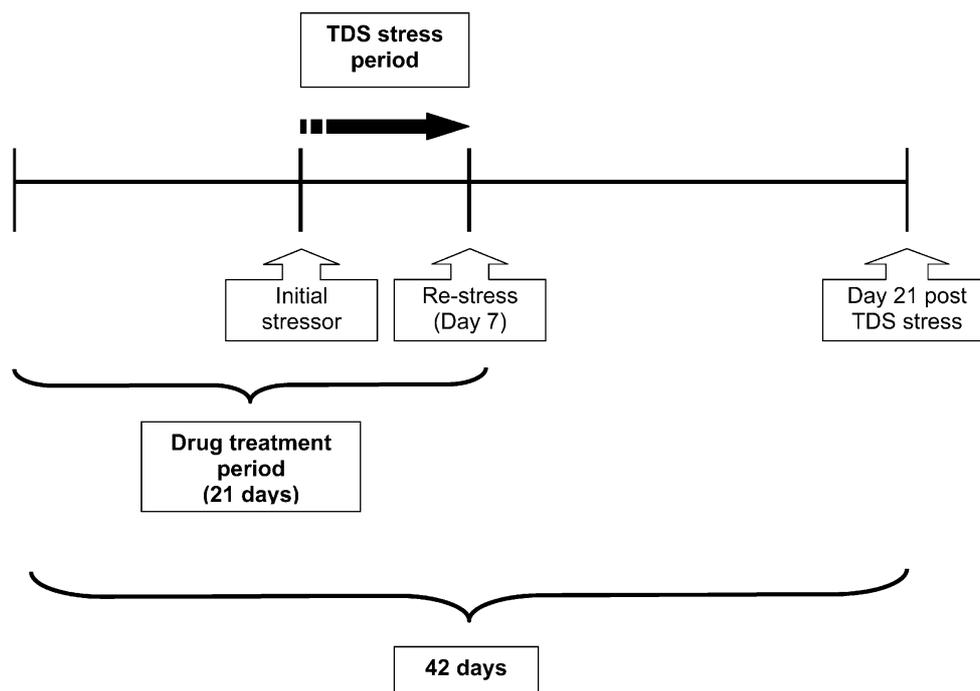
TDS involves exposure of the animal to a severely stressful event followed by a situational reminder of the prior stress. The model was implemented using a procedure described by Harvey et al. (2003).

Briefly, rats were placed in a Perspex restrainer for 2 h with the tail-gate adjusted to keep the rat well contained without impairing circulation to the limbs. Immediately thereafter, the rats were individually placed in 18 cm of ambient water (25°C) in a Perspex swim tank and allowed to swim for 20 min. Following this, each rat was then immediately exposed to 0.8 ml of 4% halothane vapors in a 5-l sealed plastic container until loss of consciousness, and finally dried in a Tempadair drier. The animals were left undisturbed for 7 days (Fig. 1). The rats were then exposed to a “restress” session on day 7 (Fig. 1), consisting of a 20-min swim in 18 cm of ambient water (25°C) in a Perspex swim tank.

Treatment groups

During the first series of experiments, rats were randomly assigned to either an unstressed control group or a TDS group. Unstressed and stressed animals were housed separately, four animals per cage. The TDS group was exposed to the TDS procedure as described above. The control/basal groups of animals were only handled on the day of the stress procedures, but were not subjected to TDS stress. On day 21 post-TDS stress, the TDS animals and control, unstressed animals (basal) were sacrificed by decapitation. In each case, pooled hippocampi (left and right) were split into three sections that were randomly selected from the dissected hippocampal tissue from each group and used for NMDA receptor studies, determination of GABA, and NOS enzyme kinetic analysis, as de-

Fig. 1 Diagrammatic representation of the stress–restress paradigm indicating the period and duration of drug treatment and the conclusion of the study on day 21 post-TDS stress



tailed below. One group of animals were sacrificed immediately after the initial stressors (day 1; Fig. 1) to note the acute effects of these stressors on hippocampal NOS activity, and to confirm whether these responses wane or are sustained 21 days post-TDS stress

NOS pharmacological studies

These studies were designed to confirm the NOS isoform involved in TDS stress and to determine the role of stress-evoked glucocorticoid release on NOS activity post-TDS stress. In these experiments, four TDS groups (aged-matched with the TDS study above) received one daily injection of either the selective iNOS inhibitor, aminoguanidine (AG; 50 mg/kg/day i.p.; Metcalf et al. 2002), the selective nNOS inhibitor, 7-nitroindazole (7-NI; 12.5 mg/kg/day i.p.; Ikeda et al. 1998), the steroid synthesis inhibitor, ketokonazole (KCZ; 24 mg/kg/day i.p.; Cohen et al. 2000) or saline for 21 days prior to and during the stress procedure (Fig. 1). The drugs were administered between 0800 hours and 0900 hours. For 7-NI, a dose of 12.5 mg/kg/day was used to avoid undue toxicity over chronic treatment. Moreover, chronic treatment with 7-NI at a dose as low as 5 mg/kg decreased NOS-related motor dysfunction and motor neuron degeneration (Ikeda et al. 1998). In all cases, drug treatment commenced 14 days prior to TDS stress procedure up to the day of re-stress (day 7; Fig. 1). Animals were then left undisturbed for another 21 days after the TDS procedure (i.e., 42 days after starting drug administration). At this time, the animals were decapitated, and hippocampi dissected for NMDA receptor, GABA and NOS studies, as described below.

Assay methodologies

Assay of hippocampal NOS

Hippocampal NOS was determined immediately after the initial stressors, since changes have been found to follow an acute severe stressor (Madrigal et al. 2001, 2003), and

on day 21 post-TDS stress, since its persistence over time was of particular interest for this study. Brains were rapidly dissected on an ice-cooled dissection slab. The hippocampus was removed, and immediately fixed in liquid nitrogen and stored at -70°C until assay. The tissue was then homogenized in 1 ml homogenizing buffer containing 25 mM Tris, 1 mM EDTA, and 1 mM EGTA (pH 7.2) with a teflon homogenizer (15 strokes). The homogenate was then centrifuged at $3000g$ for 15 min at $2-4^{\circ}\text{C}$. The supernatant was separated from the tissue pellet, and kept on ice until used. Hippocampal NOS activity was determined as previously described (Harvey and Nel 2003). Briefly, 1 M Tris (pH 7.4), 1 mM BH_4 , 4 mM DTT, 0.1 mM FAD, 0.1 mM FMN, 10 mM NADPH, 10 $\mu\text{g}/\mu\text{l}$ calmodulin, 5 μM L- ^3H arginine, and 125 mM CaCl_2 were incubated for 2 min at 37°C . Thereafter, tissue homogenates were added to the reaction cocktail and incubated for 15 min at 37°C . After 15 min, the reaction was stopped by dilution with 500 μl of ice-cold stop-buffer. Reaction mixtures were then separated on 1 ml Dowex 50W \times 8 columns and the eluted L- ^3H citrulline activity measured by scintillation counting (Packard United Technologies, Tri-Carb 4660). Blanks, containing all the reagents except the tissue homogenates, were used to determine the extent of L- ^3H citrulline formation in the absence of NOS activity, and 5 μM L- ^3H arginine alone was used to determine total counts per picomole. NOS kinetic data, V_{max} and K_d , were expressed as $\mu\text{M}[^3\text{H}]\text{-L-citrulline}/\text{mg protein}/\text{min}$, and μM , respectively, and determined using Lineweaver–Burke analysis. Protein was determined using the method of Lowry et al. (1951).

Assay of hippocampal GABA

Hippocampal GABA levels were studied in control animals and TDS animals on day 21 post-stress. Dissected tissues were placed in 1.5-ml eppendorf tubes and immediately immersed in liquid nitrogen (-196°C) and stored at -80°C until analysis. The day before analysis, approximately 10 mg hippocampal extract was placed into separate Pony vials, together with 2 ml ice-cold 0.05N HClO_4 . The vials were then sonicated in a MSE sonicator for 2×12 s at a setting of 20 μm , after which it was centrifuged at 20,000 g for 15 min at 4°C . The resulting supernatant was stored at -80°C until assay the following day.

High-performance liquid chromatography (HPLC) of tissue GABA was measured by means of an electrochemical method described by Harvey et al. (2002), using pre-column derivatization of GABA with fluoraldehyde and homoserine as internal standard. The chromatographic system consisted of a Waters 510 HPLC pump equipped with a Phenomenex KJO-4282 pre-column inlet filter preceding a 5- μm C18 Phenomenex Luna reverse-phase analytical column (75 mm \times 4.6 mm, id) which, in turn, was connected to a Waters M460 electrochemical detector (-0.4 V and $+0.6$ V). The mobile phase consisted of 0.10 M Na_2HPO_4 , 0.13 mM Na_2EDTA and 28% methanol (pH 6). GABA concentrations were calculated from the area under the concentration-time curve (AUC) values and expressed as $\mu\text{g/ml}$ (mean \pm SEM).

Assay of glutamate NMDA receptor binding

NMDA receptor studies were performed in control animals, and on day 21 post-stress. Prior to the radioligand binding assays, the tissues were thawed and extensively washed (centrifuged) to remove endogenous substances (glutamate and glycine) that could interfere with the binding procedures. Tissues were thawed in 50 volumes of ice-cold HTS buffer (5 mM HEPES/4.5 mM Tris buffer (pH 7.8), homogenized with a Brinkman Polytron PT10 homogenizer (setting 6, 20 s) and centrifuged (20,000g, 20 min, 4°C). This pelleted membrane fraction was re-suspended and centrifuged as before, after which it was reconstituted in 50 volumes of HTS buffer.

NMDA receptor binding was determined using a method previously described by Andersen et al. (1995) using [^3H]-MK801 as the selective radioligand. The in-

cubation mixture for the determination of total binding consisted of 300 μl membrane suspension to which 50 μl [^3H]-MK801 was added. A mixture of 50 μl 10 μM L-glutamate and 50 μl 30 μM glycine was added to activate the receptor ion channel. Finally, 50 μl buffer was added to a final incubation volume of 500 μl . The incubation mixture for the determination of non-specific binding consisted of 300 μl membrane suspension, 50 μl [^3H]-MK801, 50 μl 5 μM MK801 as cold ligand, 50 μl 10 μM L-glutamate and 50 μl 30 μM glycine, in 500 μl final volume. The radioligand was added in ten concentrations ranging from 0.1 nM to 20 nM, mixed and incubated for 1.5 h at room temperature. The reaction was terminated by rapid filtration through Whatman GF/B filters pre-soaked in buffer. The filters were washed twice with 5 ml HTS to dispose of unbound ligand, and placed in polypropylene tubes, with 4 ml scintillant fluid (Ultra gold XR; Packard) added to each tube. Receptor densities (B_{max} ; expressed as nmol/mg protein) and affinity values (K_{d} ; expressed in μM) were calculated using the Combicept program (Packard, Canberra) with results reproduced as saturation curves. Protein was determined according to Lowry et al. (1951).

Analysis of the data

NOS activity was analyzed using a one-way analysis of variance (ANOVA) followed by Dunnetts *t*-test (Statsoft Inc. 2001; Statistica version 6). GABA and NMDA receptor parameters were analyzed using the Students *t*-test. In all cases, data are expressed as mean \pm SEM, with a probability of more than 95% considered statistically significant ($P < 0.05$).

Results

TDS studies alone

Effect of TDS stress on the kinetics of hippocampal NOS (Table 1)

Data obtained from the one-way ANOVA for the V_{max} and K_{m} data, respectively, were $F(2,35)=32.12$; $P < 0.0001$ and $F(2,31)=53.01$; $P < 0.0001$, and revealed significant differences between the basal, acute and day-21 post-stress groups. NOS activity, as determined by rate of re-

Table 1 Kinetic data for hippocampal NOS in control animals (basal), after the acute stressors, after 21 days post-stress (ps), and 21 days post-stress plus various drug treatments, as indicated

Group	V_{max} : mean \pm SEM (μM [^3H]-L-citrulline/mg protein/min)	K_{m} : mean \pm SEM (μM)	<i>N</i>
Basal	6.18 \pm 3.15	3.77 \pm 2.46	15
Acute	12.04 \pm 1.43*	3.54 \pm 2.10	8
21 days ps	16.86 \pm 6.76*	3.16 \pm 1.16	20
21 days ps+AG	4.24 \pm 2.90**	1.20 \pm 1.48	10
21 days ps+7-NI	14.02 \pm 4.36	6.88 \pm 2.59	10
21 days ps+KCZ	3.17 \pm 1.99**	11.32 \pm 3.31**	8

* $P < 0.05$ compared with control (Dunnetts test); ** $P < 0.05$ compared with 21 days ps (Dunnetts test).

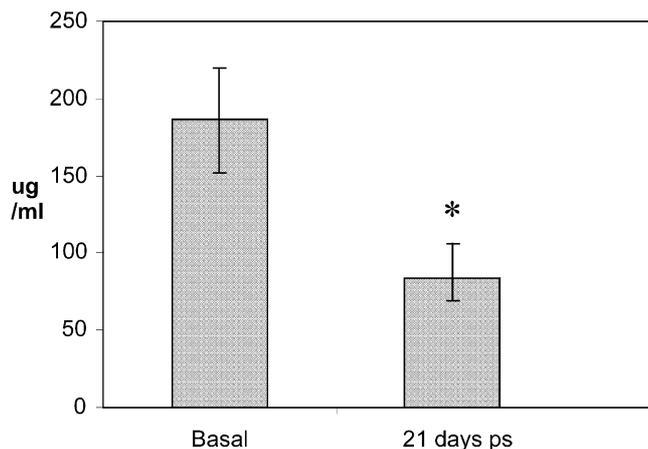


Fig. 2 Total hippocampal GABA levels in control animals (basal; $n=15$) and animals subjected to stress–restress on day 21 post-stress (ps; $n=20$). * $P<0.05$ versus basal (Students t -test)

action (V_{max}), was significantly elevated immediately after the acute stressors (day 1) compared with basal, with enzyme activity still significantly elevated on day 21 post-stress ($P<0.05$; Dunnetts test; Table 1). Enzyme K_m values did not differ significantly among the basal, acute and day-21 post-stress groups (Table 1).

Effect of TDS stress on hippocampal GABA (Fig. 2)

TDS stress was found to significantly lower hippocampal GABA levels on day 21 post-stress compared to basal ($p<0.05$; Students t -test; Fig. 2).

Effect of TDS stress on the affinity and density of hippocampal NMDA receptors (Table 2)

On day 21 post-stress, TDS stress was found to significantly reduce NMDA receptor density compared with basal ($P=0.0002$; Students t -test; Table 2), although receptor affinities remained unaltered (Table 2).

NOS pharmacological studies

Data obtained from the one-way ANOVA revealed significant differences among the drug-treated groups compared with day 21 post-stress [$F(2,35)=32.12$; $P<0.0001$].

Table 2 Hippocampal NMDA receptor binding data, B_{max} and K_d , in control animals (basal) and animals subjected to stress–restress on day 21 post-stress (ps)

Group	B_{max} : mean \pm SEM (nmol/mg protein)	K_d : mean \pm SEM (μ M)	N
Basal	2.06 \pm 1.03	4.05 \pm 3.72	15
21 days ps	1.40 \pm 0.34*	3.55 \pm 3.11	20

* $P<0.05$ versus basal (Students t -test).

Effect of AG on TDS stress-induced effects on hippocampal NOS activity (Table 1)

On day 21 post-stress, chronic treatment with AG blocked TDS stress-induced activation of hippocampal NOS compared with that noted on day 21 post-stress in stressed animals not receiving the drug, bringing it back to basal values ($P<0.05$; Dunnetts test; Table 1). No effects of drug treatment on the K_m values of the enzyme were evident, although there was a slight tendency to decrease NOS K_m (Table 1).

Effect of 7-NI on TDS stress-induced effects on hippocampal NOS activity (Table 1)

Chronic treatment with 7-NI failed to modify TDS stress-induced activation of hippocampal NOS compared with that noted on day 21 post-stress in stressed animals not receiving the drug (Table 1). While there was a tendency to increased K_m values in the stressed animals receiving 7-NI, this was not significant (Table 1).

Effect of KCZ on TDS stress-induced effects on hippocampal NOS activity (Table 1)

On day 21 post-stress, chronic treatment with KCZ blocked TDS stress-induced activation of hippocampal NOS compared with that noted on day 21 post-stress in stressed animals not receiving the drug ($P<0.05$; Dunnetts test; Table 1). However, KCZ also exerted a significant increase in the K_m of the enzyme compared with 21 days post-stress, while stress alone did not alter this parameters relative to basal ($P<0.05$; Dunnetts test; Table 1).

Discussion

An increase in the expression of iNOS has been noted to occur in limbic brain regions following restraint stress in rats (Madrigal et al. 2001, 2003). The current study has demonstrated that stress–restress (TDS) similarly evokes an immediate increase in NOS activation. This increase in NOS activity is driven primarily by the iNOS isoform that is sustained for 3 weeks post-stress. Moreover, the latter changes occurred together with significant perturbation of markers of hippocampal glutamate and GABA activity. Attenuating steroid synthesis prior to and during the stress period blocked the increase in NOS activity on day 21 post-stress, suggesting that adrenal steroids are an early mediator of this response. Thus, upregulation of iNOS

plays a pivotal role in TDS stress and has its origin in increased glucocorticoid release during and immediately after emotional trauma.

PTSD develops after exposure to trauma with multiple exposures to trauma apparently making an important contribution to the incidence and severity of the disorder (Brewin 2001; Maes et al. 2001; Johnsen et al. 2002). Moreover, evidence for hippocampal degenerative pathology in PTSD has been found at both anatomical (volume reduction) and behavioral (cognitive deficit) levels (Bremner 1999). Although not all clinical data on the hippocampus in PTSD are consistent, pre-clinical work suggests that structural changes of the hippocampus are mediated by the neurotoxic effect of elevated glucocorticoids following stress and the release of various neurotoxic molecules, particularly glutamate and NO (McEwen 1996, 1999; Almeida et al. 1998; Sapolsky 2000).

Pre-clinical studies have found that corticosterone prolongs NMDA receptor-mediated calcium elevation in the hippocampus (Takahashi et al. 2002), while adrenal steroids regulate neurochemical and structural plasticity of the hippocampus via NMDA receptors (McEwen 1996). In humans and animals, exposure to an acute stressor evokes a rise in glucocorticoids (Yehuda et al. 1990; Elzinga et al. 2003), although evidence for suppressed glucocorticoid release over time, possibly mediated by a gradual hypersensitization of the HPA-axis in the aftermath of the initial stressors, is evident in PTSD (Yehuda et al. 2000), and also in animals subjected to TDS stress (Liberzon et al. 1997; Harvey et al. 2003). The altered state of the NMDA receptor observed on day 21 post-stress in the current study is thus of particular interest.

Long-term attenuation of glucocorticoid release decreases NMDA receptors in the rat hippocampus (Mangat et al. 1998). Moreover, attenuated plasma corticosterone levels after TDS stress has been described in previous studies (Liberzon et al. 1997) and is associated with functional hippocampal deficits in memory performance (Harvey et al. 2003). Since glutamate NMDA pathways in the hippocampus are critical in memory function (Heresco-Levy and Javitt 1998), the reduced NMDA receptor density described in the current study suggests that reduced hippocampal NMDA density may underlie cognitive changes previously observed in this model (Harvey et al. 2003), and which may have their origin in long-term hypocortisolemia evoked by TDS stress.

In agreement with the above hypothesis, Le Greves et al. (1997) have described the downregulation of NMDA receptor expression after acute heat stress. However, these authors have proposed that this response is linked to the upregulation of NOS. Indeed, various studies have demonstrated the regulatory effect of NO on NMDA receptor function (Tanaka et al. 1993; Zanelli et al. 2002), such that reduced NMDA density observed in this study may represent a reactive response of the body to attenuate excessive NMDA activity, with the observed elevated NOS activity instrumental in this response.

The observed increase in NOS activity was blocked with the iNOS inhibitor, AG, but not with the nNOS inhibitor, 7-NI, suggesting a dominant role for iNOS in this response. However, expression of nNOS has also been found to increase after restraint stress (De Oliveira et al. 2000), so that further molecular studies are needed to emphatically exclude a possible contribution from nNOS after stress–restress. Nevertheless, these data are supportive of a prominent role for NOS, particularly the inducible NOS isoform, in the behavioral and neurochemical response to stress–restress. In support of this, neuronal NOS may be more sensitive than iNOS to the autoinhibitory effects of NO on enzyme activity (Griscavage et al. 1995). This suggests that overproduction of NO following stress more likely involves iNOS and not nNOS. Furthermore, the decrease in NMDA receptor density following TDS stress further underlines the involvement of iNOS and not nNOS, since iNOS activation is not regulated by neuronal receptors (McCaslin and Oh 1995).

KCZ similarly blocked the effects of stress on NOS. KCZ, which is a well-established pharmacological tool used to inhibit adrenal steroid synthesis, binds to the heme iron of cytochrome *450P* (CYP450) isoforms mediating steroid hydroxylation reactions (Wolff et al. 1993; Yan et al. 2002). Thus, early increases in glucocorticoids immediately after the stressor may be critical for the protracted effects on NOS. However, an interesting observation was the ability of KCZ to increase the K_m of hippocampal NOS, although this parameter was not affected by stress alone. This observation strongly suggests that KCZ may also be affecting NOS directly and independently of released glucocorticoids by binding to a critical site on the enzyme. Indeed, NOS bears striking homology to CYP450 (Bredt et al. 1991), while KCZ binding to the heme structure within NOS has been found to reduce the maximal velocity of the enzyme (Wolff et al. 1993). Moreover, KCZ is a competitive inhibitor of the obligatory NOS cofactor, calmodulin, thereby preventing formation of NO (Wolff et al. 1993). Recent studies also indicate that KCZ inhibits iNOS expression (Baroni et al. 1999). Taken together, these actions represent a means whereby KCZ is able to reverse the increase in NOS activity evoked by stress–restress. Furthermore, the data posit a possible dual mode of action for KCZ in preventing trauma-related sequelae in PTSD, viz. by preventing glucocorticoid release as well as NO-driven neuronal events. Furthermore, explorative studies on the effects of KCZ on stress-evoked NOS activation are required to delineate these effects.

Acute restraint stress promotes the synthesis of NO (De Oliveira et al. 2000; Madrigal et al. 2001, 2003). The current study extends these findings to TDS stress, but also that this increased NOS activity is iNOS mediated which remains sustained for a protracted period after the initial stress. Inducible NOS II is only expressed “on demand” under the influence of pro-inflammatory cytokines, synthesizing NO at markedly higher levels, and over an extended time period, than that of the constitutive forms of the enzyme (NOS I and III). This observation

thus has distinct implications for neurodegenerative complications following severe stress, and particularly the neuropathology of PTSD.

In addition to the described NOS and NMDA receptor changes evoked by stress–restress, a reduction in inhibitory GABA content in the hippocampus was also evident. Indeed, the reduced NMDA density discussed earlier may also represent a reactive response to excitotoxicity under conditions of attenuated GABA levels. NO also inhibits NMDA receptor-mediated GABA release (Moller et al. 1995), thus providing a plausible link between raised NOS activity, reduced NMDA receptor density and attenuated hippocampal GABA levels observed in this study. GABA has an important function in attenuating excessive glutamatergic activity in the brain (Yamada et al. 1999). Moreover, recent evidence also places GABA at a central position in the hypothalamus and in select hippocampal sub-fields that collectively allow it to exert an important regulatory role in the stress axis (Cullinan and Wolfe 2000). Prolonged stress levels of corticosteroids regulate GABA_A receptors in the hippocampus in a complex manner (Orchinik et al. 1995). Further, acute stress, such as forced swimming, evokes GABA release in the hippocampus (Engelmann et al. 2002). While it may be expected that TDS stress would similarly raise GABA levels, the current data suggest that this form of chronic sensitization to stress evokes a decrease in GABA, perhaps contributing to maintenance of the pathogenic process.

The development of PTSD symptoms after repeated emotional trauma suggests the involvement of sensitization (Post and Weiss 1998). The GABA–glutamate data described here are reminiscent of the kindling hypothesis (Post and Weiss 1998). The primary neural substrate of kindling involves glutamate and NMDA receptor activation, while GABA pathways exert essentially a permissive role on the kindling action of glutamate (Post and Weiss 1998). The eventual suppression of GABA levels observed in the TDS model may reflect a gradual diminution of hippocampal GABA in the face of continued stress evoked by an earlier severely traumatic event. This loss of inhibitory GABA control may remove inhibitory actions on glutamate-driven kindling, induced by an increase in glutamate release, and ultimately engendering a reactive downregulation of NMDA receptors, as observed in this study. Of particular interest is stress-evoked GABA release that is potentiated by NO (Engelmann et al. 2002), peroxynitrite (Ohkuma et al. 1995), as well as ischemia, hypoxia, free radicals, hypoglycemia and oxidative stress (Saransaari and Oja 1997). Conversely, GABA_A and GABA_B receptor agonists attenuate stress-induced release of NO (Ishizuka et al. 2000). These interactions may represent an important protective mechanism to curb excessive excitotoxic activity, which may be lost under conditions of stress–restress.

In conclusion, stress–restress evokes a complex neurobiological response in the hippocampus involving neuroexcitatory/neurotoxic pathways as well as neuroinhibitory/neuroprotective responses. In particular, stress–restress

evokes a long-lasting increase in hippocampal NOS activity that is accompanied by a reactive downregulation of hippocampal NMDA receptors and dysregulation of inhibitory GABA pathways. The centrality of iNOS is emphasized by the findings that AG treatment blocks stress-induced NOS activation. Finally, findings with KCZ suggest that the drug may suppress stress-evoked NOS activation by suppressing an initial increase in glucocorticoids immediately after the stress, or by directly suppressing NOS activity. Because of the prominent role of NO in neuronal toxicity and the important regulatory role for glutamate and GABA in this process, this may have important implications in explaining the increasing evidence for stress-related hippocampal degenerative pathology and cognitive deficits seen in patients with PTSD. Future work is needed to determine the relationship between NOS/glutamate/GABA and monoaminergic systems in this model.

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References

- Almeida A, Heales SJR, Bolanos JP, Medina JM (1998) Glutamate neurotoxicity is associated with nitric oxide-mediated mitochondrial dysfunction and glutathione depletion. *Brain Res* 790:209–216
- American Psychiatric Association (1994) Diagnostic and statistical manual of mental disorders, 4th edn. APA, Washington, DC
- Andersen DL, Tannenberg AEG, Burke CJ, Dodd PR (1995) Development rearrangements of cortical glutamate-NMDA receptor binding sites in late human gestation. *Dev Brain Res* 88:178–185
- Baroni A, Ruocco V, De Paolis P, Cicatiello L, Esumi H, Tufano MA (1999) Ketoconazole inhibits lipopolysaccharide-induced activation of the nitric oxide synthase gene in the murine macrophage cell line J774. *Arch Dermatol Res* 291:54–58
- Bredt DS, Hwang PH, Glatt C, Lowenstein C, Reed RR, Snyder S (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P450 reductase. *Nature* 351:714–718
- Bremner JD (1999) Traumatic memories lost and found. In: Williams LM, Banyard VL (eds) *Trauma and memory*. Sage Publications, California, pp 217–227
- Brewin CR (2001) A cognitive neuroscience account of post traumatic stress disorder and its treatment. *Behav Res Ther* 39:373–393
- Brown ES, Rush AJ, McEwen BS (1999) Hippocampal remodeling and damage by corticosteroids: implications for mood disorders. *Neuropsychopharmacology* 21:474–484
- Chambers RA, Bremner JD, Moghaddam B, Southwick SM, Charney DS, Krystal JH (1999) Glutamate and post-traumatic stress disorder: towards a psychobiology of dissociation. *Semin Clin Neuropsychiatry* 4:274–281
- Cohen H, Benjamin J, Kaplan Z, Kotler M (2000) Administration of high-dose ketoconazole, an inhibitor of steroid synthesis, prevents posttraumatic anxiety in an animal model. *Eur Neuropsychopharmacol* 10:429–435
- Cullinan WE, Wolfe TJ (2000) Chronic stress regulates levels of mRNA transcripts encoding beta subunits of the GABA(A) receptor in the rat stress axis. *Brain Res* 887:118–124

- De Oliviera RM, Aparecida Del Bel E, Mamede-Rosa ML, Padovan CM, Deakin JF, Guimaraes FS (2000) Expression of neuronal nitric oxide synthase mRNA in stress-related brain regions after restraint stress. *Neurosci Lett* 289:123–126
- Elzinga BM, Bremner JD (2002) Are the neural substrates of memory the final common pathway in posttraumatic stress disorder (PTSD)? *J Affect Disord* 70:1–17
- Elzinga BM, Schmahl CG, Vermetten E, van Dyck R, Bremner JD (2003) Higher cortisol levels following exposure to traumatic reminders in abuse-related PTSD. *Neuropsychopharmacology* 28:1656–1665
- Engelmann M, Wolf G, Horn TF (2002) Release patterns of excitatory and inhibitory amino acids within the hypothalamic supraoptic nucleus in response to direct nitric oxide administration during forced swimming in rats. *Neurosci Lett* 324:252–254
- Grisavage JM, Hobbs AJ, Ignarro LJ (1995) Negative modulation of NOS by NO and nitroso compounds. In: Ignarro L, Murad F (eds) *Advances in Pharmacology*, vol 34: nitric oxide: biochemistry, molecular biology, and therapeutic implications. Academic Press, London, pp 215–234
- Harvey BH, Nel A (2003) Role of aging and striatal nitric oxide synthase activity in an animal model of tardive dyskinesia. *Brain Res Bull* 61:407–416
- Harvey BH, Jonker L, Brand L, Heenop M, Stein DJ (2002) NMDA receptor involvement in imipramine withdrawal-associated effects on swim stress, GABA levels and NMDA receptor binding in rat hippocampus. *Life Sci* 71:43–54
- Harvey BH, Naciti C, Brand L, Stein DJ (2003) Endocrine, cognitive and hippocampal-cortical 5HT_{1A}-2A receptor changes evoked by a time-dependent sensitization (TDS) stress model in rats. *Brain Res* 983:97–107
- Heresco-Levy U, Javitt DC (1998) The role of *N*-methyl-D-aspartate (NMDA) receptor-mediated neurotransmission in the pathophysiology and therapeutics of psychiatric syndromes. *Eur Neuropsychopharmacol* 8:141–152
- Heresco-Levy U, Kremer I, Javitt DC, Goichman R, Reshef A, Blanaru M, Cohen T (2002) Pilot-controlled trial of D-cycloserine for the treatment of post-traumatic stress disorder. *Int J Neuropsychopharmacol* 5(4):301–307
- Ikeda K, Iwasaki Y, Kinoshita M (1998) Neuronal nitric oxide synthase inhibitor, 7-nitroindazole, delays motor dysfunction and spinal motorneuron degeneration in the wobbler mouse. *J Neurol Sci* 160:9–15
- Ishizuka Y, Ishida Y, Jin QH, Mitsuyama Y, Kannan H (2000) GABA(A) and GABA(B) receptors modulating basal and footshock-induced nitric oxide releases in rat prefrontal cortex. *Brain Res* 872:266–270
- Johnsen BH, Eid J, Laberg JC, Thayer JF (2002) The effect of sensitization and coping style on posttraumatic stress symptoms and quality of life. *Scand J Psychol* 43:181–188
- Kahn S, Liberzon I (2004) Topiramate attenuates exaggerated acoustic startle in an animal model of PTSD. *Psychopharmacology* 172:225–229
- Le Greves P, Sharma HS, Westman J, Alm P, Nyberg F (1997) Acute heat stress induces edema and nitric oxide synthase up-regulation and down-regulates mRNA levels of the NMDAR1, NMDAR2A and NMDAR2B subunits in the rat hippocampus. *Acta Neurochir* 70:275–278
- Liberzon I, Krstov M, Young EA (1997) Stress-restress: effects on ACTH and fast feedback. *Psychoneuroendocrinology* 22:443–453
- Lowry OH, Rosebrough NJ, Farr AL (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275
- Madrigal JL, Moro MA, Lizasoain I, Lorenzo P, Castrillo A, Bosca L, Leza JC (2001) Inducible nitric oxide synthase expression in brain cortex after acute restraint stress is regulated by nuclear factor kappaB-mediated mechanisms. *J Neurochem* 76:532–538
- Madrigal JL, Moro MA, Lizasoain I, Lorenzo P, Fernandez AP, Rodrigo J, Bosca L, Leza JC (2003) Induction of cyclooxygenase-2 accounts for restraint stress-induced oxidative status in rat brain. *Neuropsychopharmacology* 28:1579–1588
- Maes M, Mylle J, Delmeire L, Janca A (2001) Pre- and post-disaster negative life events in relation to the incidence and severity of post-traumatic stress disorder. *Psychiatry Res* 105:1–12
- Mangat HS, Islam A, Heigenskind C, Mustafa A, Winblad B, Adem A (1998) Long-term adrenalectomy decreases NMDA receptors in rat hippocampus. *Neuroreport* 9:2011–2014
- Masood A, Banerjee B, Vijayan VK, Ray A (2003) Modulation of stress-induced neurobehavioural changes by nitric oxide in rats. *Eur J Pharmacol* 458(1–2):135–139
- McCaslin PP, Oh S (1995) Nitric oxide and glutamate receptors. In: Stone TW (ed) *CNS neurotransmitters and neuromodulators: glutamate*. CRC Press, New York, pp 159–179
- McEwen BS (1996) Gonadal and adrenal steroids regulate neurochemical and structural plasticity of the hippocampus via cellular mechanisms involving NMDA receptors. *Cell Mol Neurobiol* 16:103–116
- McEwen BS (1999) Stress and hippocampal plasticity. *Annu Rev Neurosci* 22:105–122
- Metcalf K, Jungersten L, Lisander B (2002) Effective inhibition of nitric oxide production by aminoguanidine does not reverse hypotension in endotoxemic rats. *Acta Anaesthesiol Scand* 46:17–23
- Miyamoto Y, Yamada K, Noda Y, Mori H, Mishina M, Nabeshima T (2002) Lower sensitivity to stress and altered monoaminergic neuronal function in mice lacking the NMDA receptor epsilon 4 subunit. *J Neurosci* 22:2335–2342
- Moller M, Jones NM, Beart PM (1995) Complex involvement of nitric oxide and cGMP at *N*-methyl-D-aspartic acid receptors regulating gamma-[³H]aminobutyric acid release from striatal slices. *Neurosci Lett* 190:195–198
- Ohkuma S, Katsura M, Chen D-C, Narihara H, Kuriyama K (1995) Removal of hydroxyl radical increases nitric oxide generation-induced [³H] GABA release from mouse cerebral cortical neurons. *Neurosci Lett* 194:101–104
- Orchinik M, Weiland NG, McEwen BS (1995) Chronic exposure to stress levels of corticosterone alters GABA_A receptor subunit mRNA levels in rat hippocampus. *Brain Res Mol Brain Res* 34:29–37
- Pitman RK, Shin LM, Rauch SL (2001) Investigating the pathogenesis of post-traumatic stress disorder with neuroimaging. *J Clin Psychiatry* 62(suppl. 17):47–54
- Podhorna J, Brown RE (1999) Inhibition of nitric oxide synthase reduces ultrasonic vocalizations of rat pups. *Eur J Pharmacology* 382:143–150
- Post RM, Weiss SRB (1998) Sensitization and kindling phenomena in mood, anxiety, and obsessive-compulsive disorders: the role of serotonergic mechanisms in illness progression. *Biol Psychiatry* 44:193–206
- Sapolsky R (2000) Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. *Arch Gen Psychiatry* 57:925–935
- Saransaari P, Oja SS (1997) Enhanced GABA release in cell-damaging conditions in the adult and developing mouse hippocampus. *Int J Dev Neurosci* 15:163–174
- Shiah IS, Yatham LN (1998) GABA function in mood disorders: an update and critical review. *Life Sci* 63:1289–1303
- Takahashi T, Kimoto T, Tanabe N, Hattori TA, Yasumatsu N, Kawato S (2002) Corticosterone acutely prolongs *N*-methyl-D-aspartate receptor-mediated Ca²⁺ elevation in cultured rat hippocampal neurons. *J Neurochem* 83:1441–1451
- Tanaka T, Saito H, Matsuki N (1993) Endogenous nitric oxide inhibits NMDA- and kainate-responses by a negative feedback system in rat hippocampal neurons. *Brain Res* 631:72–76
- Uys J, Stein DJ, Daniels MW, Harvey BH (2003) Animal models of anxiety disorders. *Curr Psychiatry Rep* 5:274–281
- Vermetten E, Vythilingam M, Southwick SM, Charney DS, Bremner JD (2003) Long-term treatment with paroxetine increases verbal declarative memory and hippocampal volume in posttraumatic stress disorder. *Biol Psychiatry* 54:693–702

- Wolff DJ, Datto GA, Samatovicz (1993) The dual mode of inhibition of calmodulin-dependent nitric oxide synthase by antifungal imidazole agents. *J Biol Chem* 268:9430–9436
- Yamada J, Saitow F, Satake S, Kiyohara T, Konishi S (1999) GABA(B) receptor-mediated presynaptic inhibition of glutamatergic and GABAergic transmission in the basolateral amygdala. *Neuropharmacology* 38:1743–1753
- Yan Z, Rafferty B, Caldwell GW, Masucci JA (2002) Rapidly distinguishing reversible and irreversible CYP450 inhibitors by using fluorometric kinetic analysis. *Eur J Drug Metab Pharmacokinet* 27:281–287
- Yehuda R, Antelman SM (1993) Criteria for evaluating animal models of post-traumatic stress disorder. *Biol Psychiatry* 33:479–486
- Yehuda R, Southwick SM, Nussbaum G, Wahby VS (1990) Low urinary cortisol excretion in patients with posttraumatic stress disorder. *J Nerv Ment Dis* 178:366–369
- Yehuda R, Bierer LM, Schmeidler J, Aferiat DH, Breslau I, Dolan S (2000) Low cortisol and risk for PTSD in adult offspring Holocaust survivors. *Am J Psychiatry* 157:1252–1259
- Zanelli SA, Ashraf QM, Mishra OP (2002) Nitration is a mechanism of regulation of the NMDA receptor function during hypoxia. *Neuroscience* 112:869–877

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