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Acute stress-mediated increases in extracellular glutamate levels in the rat amygdala: differential effects of antidepressant treatment

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Abstract

Depressive illness is associated with changes in amygdalar volume, and stressful life events are known to precipitate depressive episodes in this patient population. Stress affects amygdalar synaptic plasticity and several neurotransmitter systems have been implicated in stress-mediated changes in the brain, including the glutamatergic system. However, the role of the glutamatergic system in stress-mediated plasticity in the amygdala remains to be determined. Accordingly the current study examined the stress modulation of extracellular glutamate levels in the basolateral nucleus (BLA) and the central nucleus (CeA) of the amygdala by *in vivo* microdialysis. Acute stress increased extracellular glutamate levels in the BLA and CeA, although the dynamics of these stress-mediated changes were dramatically different in these amygdalar nuclei. Tetrodotoxin administration reduced basal, and completely eliminated stress-mediated increases in glutamate efflux in the amygdala, demonstrating that stress effects are dependent on local axonal depolarization. Moreover, stress-mediated increases in glutamate efflux in the BLA were inhibited by the antidepressant tianeptine but not by the selective serotonin-reuptake inhibitor fluoxetine. Collectively, these data demonstrate that stress-induced modulation of glutamate neurochemistry reflects a fundamental pathological change that may contribute to the aetiology and progression of depressive illness, and suggest that some antidepressants such as tianeptine may elicit their clinical effects by modulation of glutamatergic neurotransmission.

Introduction

Major depressive illness is one of the most common psychiatric disorders, affecting an estimated 12–15% of the general population (Kessler *et al*., 1994). The symptoms of depression include alterations in mood and perception, as well as physiological changes such as loss of appetite, changes in body weight and disruption in sleep patterns. The aim of antidepressant treatments is the improvement of these depressive symptoms; it should also address all the potential complications resulting from major depression including structural changes in the central nervous system (CNS). For example, volumetric magnetic resonance imaging studies of depressive illness patients have revealed that amygdala volumes may be increased (Bremner *et al*., 2000; Frodl *et al*., 2002) or decreased (Sheline *et al*., 1998; Sheline *et al*., 1999; von Gunten *et al*., 2000) in major depression patients. While these disparities may be related to illness duration and/or therapeutic interventions (Campbell *et al*., 2004), the results demonstrate that the amygdala is a site for neuroanatomical alterations in depressive illness and suggest that the amygdala may exhibit time- and treatment-dependent changes.

In experimental studies, stress leads to morphological remodeling as well as molecular, neurochemical and electrophysiological changes that, it has been suggested, mediate the cognitive deficits observed in depression, anxiety and other mood disorders. Because of the central role of the amygdala in stress responses (Herman & Cullinan., 1997), this region may contribute directly to some of the specific behavioural deficits induced by stress. The amygdala may also be the site of initiation of changes in other brain regions, including the hippocampus and prefrontal cortex. In support of this hypothesis, stress influences the structure and function of the amygdala and clinical investigations have shown that neuroanatomical changes in the amygdala precede those that are observed in the hippocampus of patients with mood disorders, in particular depressive illness patients (McEwen, 2003). Moreover, several recent experimental studies indicate that stress can alter the morphology of the amygdala and also behaviours that are sensitive to amygdalar function (Conrad *et al*., 1999; Vyas *et al*., 2002, 2003; Wood *et al*., 2003; Conrad *et al*., 2004; Vyas & Chattarji, 2004).

As it is proposed that the glutamatergic system plays an important role in eliciting the neurological changes associated with exposure to stress levels of glucocorticoids, and in view of the emerging role for the amygdala in the aetiology, development and progression of recurrent depressive illness, the aim of the current study was to examine acute stress modulation of glutamate efflux in the basolateral (BLA) and the central (CeA) nuclei of the rat amygdala. In addition, as some but not all antidepressant treatments inhibit or reverse the actions of stress in the CNS, we examined the effects of two antidepressants, namely tianeptine and fluoxetine, upon basal and stress-induced changes in glutamate efflux in these amygdalar regions.

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Materials and methods

Animal protocols

Adult male Sprague–Dawley rats (CD strain; Charles River) were provided *ad libitum* access to food and water, in accordance with all guidelines and regulations of The University of South Carolina Animal Care and Use Committee. Animals were maintained in a temperature-controlled room, with a light–dark cycle of 12–12 h (lights on at 07.00 h). Rats were handled daily for 2 weeks prior to surgery.

Surgery

Under sodium pentobarbital anaesthesia (60 mg/kg, i.p.) all animals received dual microdialysis guide cannula surgery [Bioanalytical Systems, Inc. (BAS), West Lafayette, IN, USA] with placement targeted to the CeA and contralateral BLA. Target coordinates were calculated according to Paxinos & Watson (1998) relative to bregma: for CeA: AP, −2.0; L, ±3.9; DV, −7.0 mm from skull surface; for BLA: AP, −3.1; L, ±5.0; DV, −7.0 mm. These two structures are representative of the two major macrodivisions of the amygdalar complex, the centromedial extended amygdala and corticobasolateral amygdala, respectively. Guide cannulas (with stainless steel stylets to maintain the patency of the cannula) were fixed to the skull with skull screws and dental cement. Animals were allowed 3–4 days recovery following surgery, during which daily handling continued. In addition, rats were placed in the dialysis chambers during the recovery period for 4–6 h per day in order to habituate the rats to this environment.

In vivo *microdialysis*

On the morning of microdialysis, stylets were removed and replaced with concentric microdialysis probes (BAS) with a semipermeable membrane (nominal molecular weight cutoff of 30 kDa) extending 2.0 mm beyond the ventral tip of the guide cannulas. Probes were continuously perfused (2.0 μL/min) with an artificial cerebrospinal fluid (aCSF; pH 6.5) composed of the following (in mM): NaCl, 150; KCl, 3.0; $CaCl_2$, 1.7; $MgCl_2$, 0.9; and D-glucose, 4.9. Collection of dialysates (in 15-min intervals) from both probes began 3 h following probe insertion. After the fourth baseline collection, animals received a single acute i.p. injection of one of the following: saline vehicle, 10 mg/kg tianeptine or 10 mg/kg fluoxetine; this was followed by two additional 15-min collections. Immediately following the sixth collection, animals were subjected to an acute 1-h restraint stress challenge that terminated after the tenth collection. Rats were restrained in flat-bottomed clear plastic rodent restrainers with an adjustable tail piece that held the animal securely. The model was built similarly to those commercially available (Kent Scientific Corporation, CT, USA), with the following modifications: the curved top part was divided in two mobile pieces, one for the body and the other for the head, both attached to the bottom piece through hinges on one side and a lock on the other side. The top piece for the head had an opening that allowed access to the microdialysis guide cannulas while still restraining the movement of the rat head. This design allowed an easy, fast and effective restraining procedure while protecting dialysis tubing and guide cannulas. Four final post-restraint stress collections were made, resulting in a total of 14 collections. Dialysates were assayed for glutamate by HPLC with electrochemical detection, using a precolumn o-phthaldialdehyde/sulfite derivatization procedure (Donzanti & Yamamoto., 1988; Rowley *et al*., 1995; Burrows *et al*., 2000). Quantification was accomplished by comparison to a daily three-point standard curve encompassing the expected range of microdialysate glutamate concentrations. A subset of animals ($n = 4$) received the voltage-gated sodium channel blocker tetrodotoxin (TTX; 10 μM) infused through the dialysis probes, beginning after the fourth baseline collection and continuing until the termination of the dialysis session. These animals were subjected to 1 h restraint stress after the fourth TTX collection, and were then released from the restrainers while dialysate collection (and TTX infusion) continued for an additional hour. At the conclusion of microdialysis, rats were deeply anaesthetized by isoflurane inhalation and transcardially perfused with phosphate-buffered saline followed by 4% paraformaldehyde. The brains were removed, cryoprotected and coronally sectioned (45 μm) on a cryostat. Sections from the rostrocaudal extent of the amygdala were mounted and subjected to an acetylcholinesterase background stain for assessment of probe tract location (See Fig. 1). Data from animals with probe tracts lying outside the target structures were excluded from analysis.

Statistical analysis

Glutamate efflux data were subjected to ANOVA with time as a repeated measure and drug as a between-subjects factor. Bonferroni comparisons were employed to determine the source of significant ($P < 0.05$) main effects or interactions revealed by ANOVA.

Results

Acute stress increased glutamate efflux in the rat amygdala

In vivo microdialysis was performed in rats fitted with guide cannulae directed at BLA and CeA to determine the glutamatergic response to acute restraint stress in the amygdala. Acute restraint stress rapidly increased extracellular glutamate levels in the BLA (Time, $F_{11,77} = 2.762$, $P = 0.005$) and the stress-mediated increases in glutamate efflux in the BLA quickly returned to baseline prior to termination of the stress session (Fig. 2). In the CeA, acute restraint stress elicited a rapid increase in extracellular glutamate levels similar in magnitude to those observed in the BLA (Time, $F_{11,77} = 1.945$; $P = 0.046$). However, in the CeA the rapid and robust increase in glutamate efflux was consistently followed by a significant drop below baseline (i.e. prestress) levels (Fig. 2). Extracellular levels of

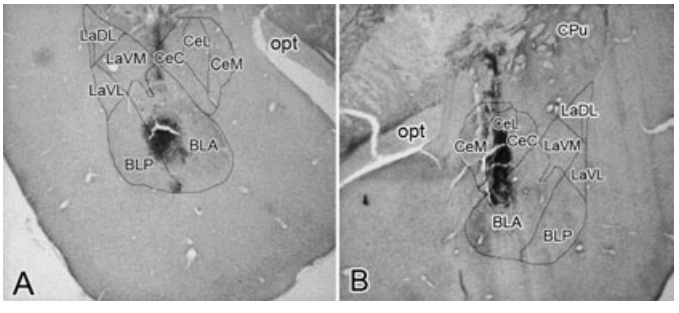


FIG. 1. Two representative photomicrographs of acetycholinesterase-stained sections illustrating probe placement in targeted regions. Note that the central tract of the probe is localized primarily to the BLA (A) and CeA (B) regions. Abbreviations: BLA, basolateral amygdaloid nucleus anterior; BLP, basolateral amygdaloid nucleus posterior; LaVL, lateral amygdaloid nucleus ventrolateral; LaVM, lateral amygdaloid nucleus ventromedial; LaDL, lateral amygdaloid nucleus dorsolateral; CeC, central amygdaloid nucleus capsular; CeL, central amygdaloid nucleus lateral division; CeM, central amygdaloid nucleus medial division; opt, optic tract.

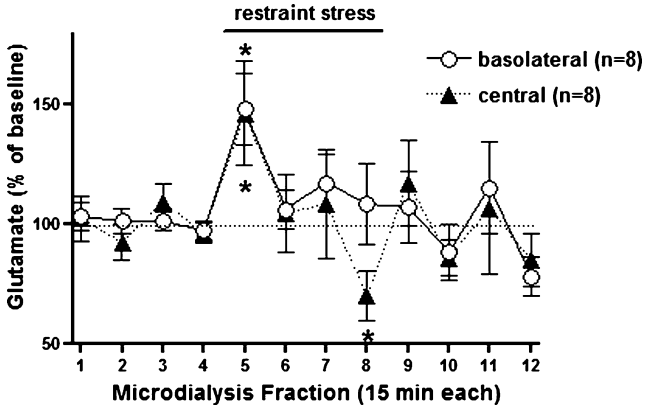


FIG. 2. Acute restraint stress increased glutamate efflux measured simultaneously in the BLA and CeA. After the fourth baseline collection, rats were restrained for 1 h and then released. Glutamate levels in both BLA and CeA rose rapidly with the onset of restraint. However, while glutamate efflux returned to baseline in the BLA, extracellular glutamate levels dropped below prestress baseline levels in the CeA. Data based upon dialysis collections from eight rats per amygdalar nucleus. *$P < 0.05$ vs. baseline.

glutamate quickly returned to baseline upon termination of stress, suggesting that changes in amygdalar glutamate efflux elicited during the restraint stress session were not a handling artifact. Moreover, prior administration of the voltage-gated sodium channel blocker TTX through the dialysis probe completely blocked any stress-related increases in both regions and, in the BLA, reduced glutamate efflux during the stress period to below baseline levels (Fig. 3).

Tianeptine, but not fluoxetine, inhibited stress-elicited increases in amygdalar glutamate efflux

We examined the ability of two antidepressants, tianeptine and fluoxetine, to modulate basal glutamate efflux and stress-mediated increases in extracellular glutamate levels in the amygdala. Stress-induced increases in extracellular glutamate levels in the BLA (Fig. 4A) or the CeA (Fig. 4B) were not affected by saline injection. Tianeptine administration did not affect basal glutamate efflux in the

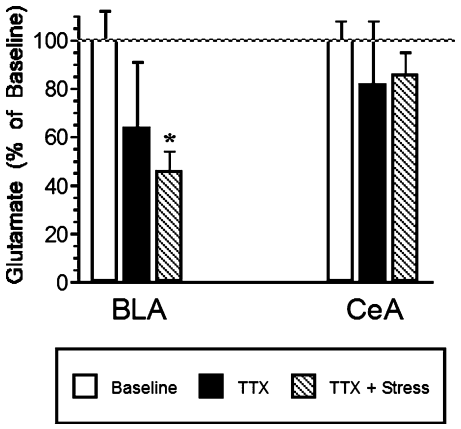
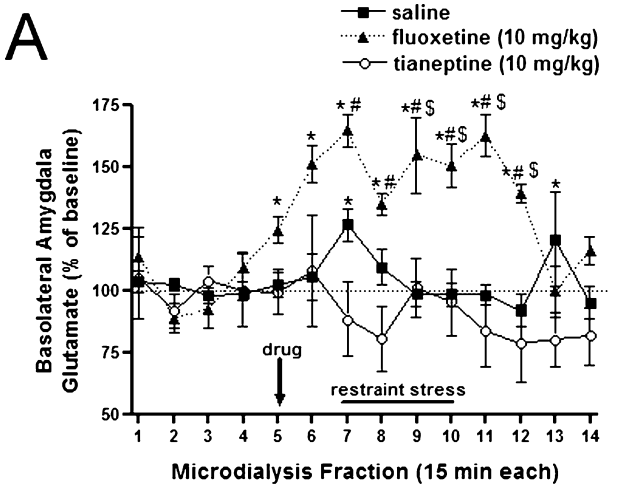


FIG. 3. TTX affected basal and acute stress-elicited glutamate release in the amygdala. Administration of the voltage-gated Na^+-channel blocker TTX (10 μM) reduced basal glutamate efflux in both BLA and CeA and also completely blocked any stress-related increases. Data are shown as normalized area under the curve for the 1-h baseline, 30-min TTX alone and 1-h stress intervals. *$P < 0.05$ vs. baseline.



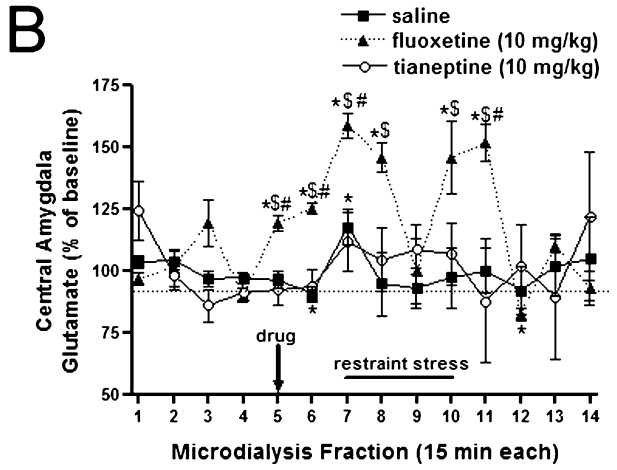


FIG. 4. Tianeptine, but not fluoxetine, attenuated stress-induced glutamate release in amygdala. (A) Administration of tianeptine 30 min prior to the acute restraint stress session inhibited the stress-induced increases in extracellular glutamate levels in the BLA; conversely, fluoxetine treatment increased basal BLA glutamate efflux and did not modulate the increases elicited by stress. (B) Tianeptine did not inhibit stress-mediated increases in extracellular glutamate levels in the CeA. Similar to observations in the BLA, fluoxetine administration increased glutamate efflux in the CeA in the prestress period, increases that were potentiated during stress. Data based upon six rats per amygdalar nucleus for each drug. *$P < 0.05$ vs. baseline; $^{\$}P < 0.05$ vs. saline; $^{\#}P < 0.05$ vs. tianeptine.

BLA, but effectively inhibited stress-induced increases in extracellular glutamate levels in the BLA (Fig. 4A). Interestingly, tianeptine did not inhibit stress-induced increases in extracellular glutamate in the CeA, suggesting that tianeptine may be producing region-specific effects in the amygdala (Fig. 4B). We also examined the effects of fluoxetine upon basal and stress-induced glutamate efflux in the amygdala. Fluoxetine administration increased basal glutamate efflux in the BLA and CeA during the prestress period. In addition, these elevated levels of glutamate were maintained (BLA) or increased (CeA) by acute stress in animals treated with fluoxetine (Fig. 4A and B).

Discussion

The results of the current study demonstrate that acute stress increases glutamate efflux in the basolateral nucleus and central nucleus of the

rat amygdala, although the neurochemical dynamics of these changes were distinctly different in these amygdalar subregions. Specifically, in the BLA acute stress elicited rapid and robust increases in extracellular glutamate levels that quickly returned to baseline levels. In the CeA, the initial stress-mediated peak in glutamate efflux was followed by a decline in extracellular glutamate levels observed during the later stages of the stress session. These distinct patterns in stress-mediated changes in glutamate neurochemistry may have important implications for the ability of stress to produce disparate neuroanatomical and plasticity responses in the amygdala. Moreover, the voltage-gated sodium channel blocker TTX completely prevented stress-related increases in both regions. Given that basal efflux reflects a summed correlate of neuronal, metabolic and glial concentrations to the extracellular pool of glutamate, the extrapolation of these data to a presynaptic index of neurotransmission must be made with caution (Timmerman & Westerink., 1997). Nonetheless, even nonexocytotically-derived glutamate may affect neurotransmission and behavioural correlates of glutamatergic function (Baker *et al*., 2002, 2003; Moran *et al*., 2003) and changes in extracellular glutamatergic tone are likely to play a role in the morphological, synaptic and neuroendocrine changes associated with stress in the amygdala and other limbic regions (Gabr *et al*., 1995; Herman & Cullinan, 1997). Acute fluoxetine administration did not inhibit stress-induced increases in glutamate efflux in the rat amygdala. Moreover, fluoxetine administration increased glutamate efflux in the BLA and CeA in the prestress period, which may provide a potential mechanism through which initiation of selective serotonin-reuptake inhibitor treatment elicits anxiety in depressive illness patients (Goldstein & Goodnick., 1998). Conversely, tianeptine effectively inhibited the stress-induced increase in extracellular glutamate levels in the BLA, suggesting that the mechanisms of action of tianeptine may include modulation of glutamatergic tone in the amygdala.

In our antidepressant studies the magnitude of the stress response (saline-treated group; Fig. 4) appeared to be diminished in both the BLA and CeA relative to that observed in noninjected animals (Fig. 2). It is interesting to note that drug or saline injections were previously shown to modulate tail pinch-induced increases in glutamate efflux in the hippocampus and prefrontal cortex when compared to noninjected rats (Bagley & Moghaddam., 1997). The reason for this attenuation is not immediately clear, but may involve recruitment of compensatory mechanisms elicited by the mild stress of the injection itself that subsequently limit the magnitude of the restraint stress effect. Consistent with this hypothesis, *ex vivo* studies demonstrate that acute stress can rapidly increase glutamate uptake in frontal cortex and hippocampus (Gilad *et al*., 1990). In addition to providing a potential mechanism for the diminished response seen in Fig. 4, glutamate reuptake may represent a pharmacological target that could differentiate different classes of antidepressant drugs (Yamamoto & Reagan, 2006). Studies are underway to more clearly determine how alterations in glutamate transport might mediate changes in extracellular amygdalar glutamatergic tone in response to acute or repeated stress. Importantly, however, restraint stress still elicited a significant increase in the control (saline) groups in these studies, thus qualitatively reproducing the basic phenomenon against which antidepressant drug effects could be measured.

Stress, glutamatergic function and the amygdala

The amygdalar complex receives glutamatergic afferents from several extrinsic sources, including cortical and thalamic regions (LeDoux *et al*., 1990; Turner & Herkenham., 1991; McDonald *et al*., 1999). An additional source of neuronal glutamate in the amygdala is from intra-amygdalar projections of excitatory amygdalar output neurons that also project to cortical and limbic regions. Intra-amygdala BLA projections, for example, terminate preferentially on the intercalated cell masses and the CeA, which serves as the major mediator of amygdalar influence over hypothalamic and brainstem effector systems (Pitkanen *et al*., 1997). Stress has been shown to impact virtually all of these regions, and stress-related increases in amygdalar glutamate may reflect, in part, a summed neurochemical correlate of activity in these stress-responsive circuits. Accordingly, elevated glutamate levels in this region are likely to represent an important mediator of stress effects on this area and, in turn, may play a key role in facilitating amygdalar activation of the hypothalamic–pituitary–adrenal axis (Gabr *et al*., 1995; Herman & Cullinan, 1997). The current results provide insight into the neurochemical alterations elicited by acute stress in the input (BLA) and output (CeA) regions of the amygdala. While stress elicits a rapid and robust increase in glutamate efflux in the BLA and CeA, these increases are followed by decreases in glutamate levels in the CeA. It is therefore interesting to speculate that the reductions in glutamate efflux observed in the CeA may modulate stress responses that are transmitted from the CeA to hypothalamic and brainstem effector systems.

In addition to the differences in stress-induced neurochemical profiles in amygdalar nuclei, stress also differentially affects the morphology of neurons in the BLA and CeA. For example, stress produces dendritic hypertrophy in the BLA while not affecting dendritic structure in neurons in the CeA (Vyas *et al*., 2002; Vyas *et al*., 2003). Interestingly, tianeptine reportedly inhibits stress-induced dendritic hypertrophy in the BLA (McEwen & Chattarji, 2004) and the current studies demonstrate that tianeptine more selectively inhibits stress-induced increases in extracellular glutamate levels in the BLA than in the CeA. Such results suggest that, in addition to exhibiting different neurochemical and neuroanatomical responses to stressful stimuli, amygdalar subnuclei may respond differently to antidepressant treatments, at least in the case of tianeptine.

Pathology of the glutamatergic system in depressive illness

Clinical studies support a role for the glutamatergic system in the pathology of major depressive illness. For example, plasma and cerebrospinal fluid glutamate levels and glutamate/glutamine ratios are altered in affective disorders (Kim *et al*., 1982; Altamura *et al*., 1993, 1995; Levine *et al*., 2000). More recent proton magnetic resonance spectroscopic analyses have identified decreases in glutamate levels in the anterior cingulate cortex of depressive illness patients (Auer *et al*., 2000; Mirza *et al*., 2004). Conversely, occipital cortex glutamate levels were significantly increased in depressed subjects (Sanacora *et al*., 2004). Decreases in glial cell densities observed in depressive illness patients (Ongur *et al*., 1998; Rajkowska *et al*., 1999; Cotter *et al*., 2001; Hamidi *et al*., 2004) may result in decreased glial glutamate transporter expression, thereby reducing the capacity to regulate synaptic concentrations of glutamate. Indeed, a recent microarray study suggested that glial glutamate transporter expression is decreased in the anterior cingulate cortex and dorsolateral prefrontal cortex of depressive illness patients (Choudary *et al*., 2005). In experimental studies, stress modulates the expression and functional properties of glutamate receptors (Kole *et al*., 2002; McEwen *et al*., 2002) and glutamate transporters (Reagan *et al*., 2004; Wood *et al*., 2004), and also increases extracellular levels of glutamate in the hippocampus (Bagley & Moghaddam, 1997; Lowy *et al*., 1995). The glutamatergic system also participates in stress-induced morphological

changes in the rat hippocampus (Watanabe *et al*., 1992; Magariños & McEwen., 1995). Interestingly, tianeptine effectively inhibits these stress-induced changes in the glutamatergic system, including stress-mediated increases in glutamate efflux described in the current study. Taken together, these observations from the clinical and experimental settings support the hypothesis that modulation of glutamatergic neurochemistry participates in the pathophysiology of major depressive disorders and suggest that modulation of excitatory neurotransmission may provide an exciting new avenue for therapeutic intervention in the treatment of depressive illness.

In summary, these data represent a critical link in understanding stress-induced neurochemical alterations in two functionally distinct amygdalar nuclei and provide insight into the mechanism of action of the antidepressant tianeptine. These findings further implicate the glutamatergic system in the amygdala as critical mediator in stress responsivity and suggest that it may serve as an important therapeutic target for stress-related disorders.

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Abbreviations

BLA, basolateral nucleus of the amygdala; CeA, central nucleus of the amygdala; CNS, central nervous system; TTX, tetrodotoxin.

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