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Stress Impairs 5-HT_{2A} Receptor-Mediated Serotonergic Facilitation of GABA Release in Juvenile Rat Basolateral Amygdala

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The occurrence of stress and anxiety disorders has been closely associated with alterations of the amygdala GABAergic system. In these disorders, dysregulation of the serotonergic system, a very important modulator of the amygdala GABAergic system, is also well recognized. The present study, utilizing a learned helplessness stress rat model, was designed to determine whether stress is capable of altering serotonergic modulation of the amygdala GABAergic system. In control rats, administration of 5-HT or α-methyl-5-HT, a 5-HT_2 receptor agonist, to basolateral amygdala (BLA) slices dramatically enhanced frequency and amplitude of spontaneous inhibitory postsynaptic currents (sIPSCs). This effect was blocked by selective 5-HT_{2A} receptor antagonists while a selective 5-HT_{2B} receptor agonist and a selective 5-HT_{2C} receptor agonist were without effect on sIPSCs. Double immunofluorescence labeling demonstrated that the 5-HT_{2A} receptor is primarily localized to parvalbumin-containing BLA interneurons. Thus, serotonin primarily acts via 5-HT_{2A} receptors to facilitate BLA GABAergic inhibition. In stressed rats, the 5-HT_{2A} receptor-mediated facilitative actions were severely impaired. Quantitative RT-PCR and western blot analysis showed that the impairment of 5-HT_{2A} receptor signaling primarily resulted from receptor downregulation. The stress-induced effect appeared to be specific to 5-HT_{2A} receptors because stress had no significant impact on other serotonin receptors, as well as histamine H_3 receptor and α_2 adrenoceptor signaling in the BLA. This severe impairment of 5-HT_{2A} receptor-mediated facilitation of BLA GABAergic inhibition might result in an amygdala circuitry with hyperexcitability, and a lower threshold of activation, and thus be an important mechanism underlying the emergence of stress-associated psychiatric symptoms. *Neuropsychopharmacology* (2009) **34,** 410–423; doi:10.1038/npp.2008.71; published online 4 June 2008

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INTRODUCTION

The basolateral complex of the amygdala (BLA) is a brain region critically involved in associative processes for aversive emotions (LeDoux, 2003). Synaptic transmission within the BLA and its subsequent activity-dependent long-term changes are believed to be essential for the generation of aversive emotional responses and the formation of emotional memories (Cheng *et al*, 2006; Davis *et al*, 1994; LeDoux, 2003). The pathophysiological alterations in synaptic transmission within the BLA, thus, are closely associated with multiple stress and anxiety disorders (Manji *et al*, 2001; Shin *et al*, 2006).

Neuronal circuitries in the BLA are interconnected extensively with GABAergic terminals (Nitecka and Ben-Ari, 1987; Washburn and Moises, 1992). Activity of projection neurons in the BLA is under strong inhibitory control by GABAergic synaptic transmission (Royer *et al*, 1999; Szinyei *et al*, 2000). Manipulation of this neurotransmission within the BLA has a critical influence on behavioral, emotional sequelae resulting from stress (Jasnow and Huhman, 2001; Kim *et al*, 2005; Sanders and Shekhar, 1995; Van Nobelen and Kokkinidis, 2006). Therefore, the occurrence of stress-associated psychiatric syndromes has been closely associated with changes in the GABAergic system in the BLA. For instance, repeated infusion of corticotrophin-releasing factor into the rat BLA, which mimics chronic stress, induces a pronounced reduction in both spontaneous and evoked inhibitory postsynaptic potentials in the amygdala that results in

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anxiety-like symptoms (Rainnie *et al*, 2004). Intensive stress attenuates inhibitory GABAergic control in the BLA (Braga *et al*, 2002; Rodriguez Manzanares *et al*, 2005), which appears to be responsible for behavioral impairments resulting from stress (Minor and Hunter, 2002). In addition, many drugs for mood and anxiety disorders achieve their therapeutic effects at least partially by modifying the GABAergic system in the amygdala (Millan, 2003; Schallek and Schlosser, 1979). Hence, stress-induced alteration of GABAergic transmission in the amygdala may be an important pathophysiological mechanism underlying anxiety and stress disorders, such as posttraumatic stress disorder (PTSD) and depressive illnesses.

The dorsal raphe nucleus serotonergic system is a very important modulator of the GABAergic system in the BLA, and dysregulation of this system has long been recognized in stress and anxiety disorders (Southwick *et al*, 1999; van Praag, 2004). During stressful/emotional experiences, this system is activated and serotonin levels are strongly enhanced in the BLA (Amat *et al*, 1998; Minor and Hunter, 2002). The primary action of this enhanced serotonergic neurotransmission is to reduce amygdala excitability by increasing GABAergic transmission in the BLA (Rainnie, 1999). Such serotonergic modulation in the amygdala is of great importance in normal emotional/stressful signal processing, and dysregulation of the serotonergic system in the amygdala may result in abnormal responses of the amygdala to emotional stimuli and generation of anxiety and depression (Canli *et al*, 2005; Hariri *et al*, 2002). Therefore, malfunctioning in the serotonergic modulation of GABAergic transmission in the amygdala may be an essential mechanism underlying the emergence of symptoms associated with anxiety and stress disorders. Since the symptoms associated with these disorders usually develop in the aftermath of intense or chronic stress, these types of stress might alter serotonergic modulation of the GABAergic transmission in the amygdala.

Thus, the present study investigated whether serotonergic modulation of GABAergic transmission in the BLA was altered by exposure to restraint/tail shock, a stress protocol which could stably induce abnormalities associated with stress-related disorders, such as sustained body weight loss and enhanced acoustic startle responses (ASR) (Servatius *et al*, 1995). As the receptor subtypes involved in serotonergic modulation of GABA release in the BLA were not entirely clear, we first determined which serotonin receptor subtypes mediated the serotonergic modulation of GABA release.

METHODS

Stress Protocol

All animal experiments were performed in accordance with our institutional guidelines after obtaining the approval of the Institutional Animal Care and Use Committee. Male, Sprague–Dawley rat pups (Taconic Farms, Germantown, NY, USA) were received at postnatal day (PND) 17 and housed in a climate-controlled environment on a 12 h light/dark cycle (lights on at 0700 hours). On PND 21, the rats were weaned, assigned numbers, and randomly divided into control and stressed groups. They were housed individually, with food and water supplied *ad libitum*. The 'stressed group' was exposed to stress on PND 22, 23, and 24. The rats were killed and brain slices were prepared on PND 24 and 25. The experiments were performed in a blind manner. The investigators did not know whether they used a control or a stressed rat until the data were analyzed.

Stress exposure consisted of a 2 h per day session of immobilization and tail-shocks for 3 consecutive days. The animals were stressed in the morning (between 0800 and 1200 hours). They were restrained in a plexiglas tube, and 40 electric shocks (1 mA, 3 s duration) were applied at varying intervals (140–180 s). We stressed the rats for 3 consecutive days because it has been previously demonstrated that repeated stress sessions for 3 days is more effective than a single stress session in producing physiological and behavioral abnormalities, such as exaggerated ASR and reduced body weight (Servatius *et al*, 1995).

Slice Preparation

The amygdala slice preparation has been described previously (Li *et al*, 2001). Briefly, the rats ($\sim$25 days old unless otherwise stated) were anesthetized with halothane and then decapitated. The brain was rapidly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) composed of (in mM) 125 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, and 11 glucose, bubbled with 95% O_2/5% CO_2. A block containing the amygdala region was prepared by rostral and caudal coronal cuts, and coronal slices, 400 μm thick, were cut using a Vibratome (series 1000, Technical Products International, St Louis, MO, USA). Slices were kept in a holding chamber containing oxygenated ACSF at room temperature, and experiments started $\geqslant$1 h after slice preparation.

Electrophysiology

For whole-cell recordings, slices were transferred to a submersion-type recording chamber where they were continuously perfused with oxygenated ACSF at a rate of 2 ml per min. All experiments were carried out at room temperature. Tight-seal ($\geqslant$1 GΩ) whole-cell recordings were obtained from the cell body of neurons in the BLA region. Patch electrodes were fabricated from borosilicate glass and had a resistance of 1.5–5.0 MΩ when filled with a solution containing (in mM) Cs-gluconate, 135; $MgCl_2$, 10; $CaCl_2$, 0.1; EGTA, 1; HEPES, 10; *N*-(2,6-dimethylphenylcarbamoylmethyl) triethylammonium chloride (QX 314), 20; NaATP, 2; Na_3GTP, 0.2 and lucifer yellow, 0.4% (pH 7.3, 285–290 mOsm). Neurons were visualized with an upright microscope (Olympus BX51WI) using Nomarski-type differential interference optics through a $\times$40 water immersion objective. Neurons with a pyramidal appearance were selected for recordings, and were voltage clamped using a Multiclamp 700A amplifier (Axon Instruments Inc., Foster City, CA, USA). Access resistance (8–26 MΩ) was regularly monitored during recordings, and cells were rejected if it changed by more than 15% during the experiment. The signals were filtered at 2 kHz, digitized (Digidata 1322A, Axon Instruments Inc.), and stored on a computer using pCLAMP8 software (Axon Instruments Inc.). The

frequency, peak amplitude, 10–90% rise time and the decay time constant of IPSCs were analyzed offline using the Mini Analysis Program (Synaptosoft Inc., Leonia, NJ, USA).

For field potential recording, the amygdala slice preparation was same as described as above except that young adult rats (35–40 days) were also used in some of the experiments examining the effect of α-methyl-5-HT on field potentials (see 'Results'). Slices were transferred to an interface chamber that was continually superfused with ACSF at a rate of 1–2 ml/min. The temperature of perfusion solution in the chamber was maintained at 32°C through a TC-202A Bipolar temperature controller (Harvard Apparatus Inc., Holliston, MA, USA). Microelectrodes pulled from borosilicate glass had a resistance of 2–5 MΩ when filled with 3 M NaCl. The recording microelectrode tips were positioned in the basolateral region of the amygdala. The sharpened tungsten bipolar stimulating electrodes (World Precision Instruments, Sarasota, FL, USA) were placed in the external capsule (EC) to evoke the synapse response at 0.1 Hz. Field potentials were amplified with a differential amplifier (Warner Instrument Corporation, Hamden, CT, USA). The output was digitized with a Digidata 1200 interface (Axon Instruments, Inc.). On- and offline data acquisition and analysis was carried out using the Whole Cell Electrophysiology Program version 1.7b (John Dempster, University of Strathclyde, Glasgow, UK).

Quantitative Real-Time PCR

The amygdala complex (from ~25 days old rat) was dissected and kept frozen. The frozen tissue from each rat was homogenized, and total RNA was extracted using the RNeasy kit (Qiagen, Germany) following the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed into first-strand cDNA using the RETROscript reverse transcriptase kit and oligo dT primers (Ambion Inc., Austin, TX, USA) according to the manufacturer's protocols. cDNA (1 μl) from the RT reaction was used as the template for quantitative real-time PCR reaction with a final PCR reaction volume of 25 μl, with the 5′ and 3′ gene-specific PCR primer concentrations at 100 nM each. PCR primers were designed using Primer3 software (Whitehead Institute, MIT, Cambridge, MA, USA) according to the coding sequences of each gene (GenBank accession numbers: 5-HT$_{2A}$ receptor, X13971; 5-HT$_{2C}$ receptor, NM_012765; 5-HT$_{1A}$ receptor, J05276; Table 1). Quantification of mRNA expression was performed (in triplicate) using SYBR Green SuperMix (Bio-Rad, Hercules, CA, USA) and a two-step PCR reaction procedure, performed on a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad). To compensate for variations in input RNA amounts and efficiency of reverse transcription, we normalized data for serotonin receptor mRNAs for each sample by reference to the data obtained for β-actin (GenBank accession number: BC063166) determined from the same sample. The mean and SEM were calculated from three replicate amplifications. Each RT–PCR assay was repeated twice.

Differences between the stress and control groups and between the brain regions were examined for statistical significance using one-way ANOVA (with stress or brain region as the main factors) analysis followed by Fisher's *post hoc* test. A difference with p-value less than 0.05 was considered statistically significant.

Western Blot Analysis

The amygdala tissues (from ~25 days old rat) were homogenized and the homogenate then was centrifuged at 980g for 10 min at 4°C. The supernatant was recentrifuged at 15,000g for 15 min at 4°C. The resulting pellet was resuspended in homogenizing buffer. Equal volumes of protein samples (20 μl containing 25 μg protein) were loaded onto 7.5% (w/v) polyacrylamide gel and subsequently transferred to an ECL nitrocellulose membrane (Amersham, Piscataway, NJ, USA). The blots were incubated overnight with primary 5-HT$_{2A}$ receptor antibody (Pharmingen, San Diego, CA, USA) at a dilution of 1 : 500, followed by horseradish-peroxidase-linked secondary antibody (anti-mouse IgG; 1 : 500) for 4 h at room temperature. The membranes were exposed to the ECL film. The specificity of the antibody was checked by competition with a 5-HT$_{2A}$ receptor fusion protein. To normalize the data, β-actin was measured in the same immunoblot using anti-β-actin as the monoclonal primary antibody (1 : 5000 for 2 h) and anti-mouse IgG (1 : 5000 for 2 h) as the secondary antibody. The optical density of each 5-HT$_{2A}$ receptor band was corrected by that of the corresponding β-actin band.

Immunofluorescence Labeling

Five rats (~35 days old) were deeply anesthetized with halothane and transcardially perfused with 100 ml of 0.9% sodium chloride solution at 37°C followed by 200 ml of freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Brains were removed and post-fixed in 4% paraformaldehyde overnight. Serial 40 μm coronal sections with the amygdala, corresponding to a rat brain atlas, were cut from each brain with a Vibratome tissue slicer (series 1000, Technical Products International).

Table 1 5-HT$_{2A}$, 5-HT$_{2C}$, and 5-HT$_{1A}$ Receptor Primer Sequences for PCR Experiments

Gene	Sense primer (5′-3′)	Antisense primer (5′-3′)
5-HT$_{2A}$	ATACCAGCATTGGCCTACAAGT	TAACCATGGAGCAGTCATCAAC
5-HT$_{2C}$	AGCCCAGACCATTTCTAATGAA	TGAGAGTAGTCTGGTTGCAGGA
5-HT$_{1A}$	ATGATGATGATGGTGGTGGTAA	GGCTAGGGTACTGAGTGAATGG

The sections were incubated with the primary antibody rabbit anti-5-HT$_{2A}$ receptor (1 : 100, ImmunoStar Inc., Hudson, WI, USA) alone or in combination of this antibody and mouse anti-parvalbumin (1 : 2000, Sigma Chemical Co., St Louis, MO, USA) overnight at 4°C, rinsed in three changes of PBS (10 min each), and then incubated with Alexa 488-labeled goat anti-mouse IgG (1 : 500; Molecular Probes, Eugene, OR, USA) for 2.5 h and Alexa 568-labeled goat anti-rabbit IgG (1 : 1000; Molecular Probes) for 2.5 h at room temperature. Sections were then rinsed in three changes of PBS (10 min each) and mounted on glass slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA), then examined with a Zeiss LSM 510 confocal laser scanning microscope equipped with an argon–krypton laser. Fluorescence of Alexa 488 (green) and Alexa 568 (red) dyes was analyzed using filter configurations for sequential excitation/imaging by 488 and 568 nm channels. Digital images were adjusted for brightness and contrast using Photoshop software.

Drugs

The compounds from Tocris (Tocris Cookson, Ballwin, MO, USA) include: D-(−)-2-amino-5-phosphonopentanoic acid (D-AP5); 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX); 4-[3-[*tert*-butylamino]-2-hydroxypropoxy]-1*H*-indole-2-carbonitrile hemifumarate (Cyanopindolol hemifumarate); Tropanyl 3,5-dichlorobenzoate (MDL 72,222); 2-methyl-5-hydroxytryptamine hydrochloride; 3-[2-[4-(4-fluorobenzoyl)-1-piperidinyl] ethyl]-2,4[1*H*,3*H*]-quinazolinedione tartrate (Ketanserin tartrate); 3,5-dihydro-5-methyl-*N*-3-pyridinylbenzo[1,2-*b*:4,5-*b*'] dipyrrole-1(2*H*)-carboxamide hydrochloride (SB 206553 hydrochloride); α-phenyl-1-(2-phenylethyl)-4-piperidinemethanol (MDL 11,939); α-methyl-5-(2-thienylmethoxy)-1*H*-indole-3-ethanamine hydrochloride (BW 723C86 hydrochloride); α-methyl-5-hydroxytryptamine maleate; 1,2,3,4,8,9,10,11-octahydro[1,4]diazepino[6,5,4-jk]-carbazole hydrochloride (WAY 629); QX 314 chloride; R-α-methylhistamine dihydrobromide and clonidine. R-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperidinemethanol (MDL 100,907) was kindly donated by Kenner C Rice (NIDDK, National Institutes of Health). Bicuculline and tetrodotoxin (TTX) were purchased from Sigma-Aldrich (St Louis, MO, USA).

RESULTS

Serotonin and α-methyl-5-HT Facilitated GABA Release in the BLA *In Vitro*

We first examined the effects of serotonin on action potential-dependent spontaneous inhibitory postsynaptic currents (sIPSCs) recorded from BLA pyramidal neurons in control rats to confirm whether serotonin could facilitate GABAergic synaptic transmission in the BLA as reported (Rainnie, 1999). The sIPSCs were recorded at a holding potential of −70 mV, and in the presence of D-AP5 (50 μM) and CNQX (10 μM), to block NMDA and AMPA/kainate receptors, respectively. In control rats, the mean frequency of sIPSCs recorded from the soma of BLA pyramidal neurons was 2.8 ± 1.5 Hz ($n = 21$). Bath application of bicuculline (10 μM) eliminated sIPSCs, confirming that they were mediated by GABA$_A$ receptors. Serotonin produced a dose-dependent enhancement in the frequency and amplitude of sIPSCs (data not shown). The effect still remained in the presence of the 5-HT$_3$ and 5-HT$_1$ receptor antagonists MDL 72,222 (20 μM), and cyanopindolol (20 μM), respectively, and could be mimicked by the broad 5-HT$_{2/4}$ receptor agonist α-methyl-5-HT (Figure 1), suggesting involvement of the 5-HT$_2$ and/or 5-HT$_4$ receptors. α-methyl-5-HT, at concentrations of 2, 15, and 100 μM, increased the frequency and amplitude of sIPSCs in a dose-dependent manner (Figure 1c). The concentration of 15 μM appeared to be close to the EC$_{50}$, and therefore it was used in subsequent experiments. Application of 15 μM α-methyl-5-HT increased the frequency of sIPSCs to 612.8 ± 70.9% of the baseline values ($n = 16$, $p < 0.01$; Figure 1). The amplitude of sIPSCs was increased to 172.3 ± 16.6% of the baseline values ($n = 16$, $p < 0.05$). These effects persisted through and even outlasted the application of α-methyl-5-HT, and were completely reversed 2–3 min after removal of the agonist. The response of sIPSCs to agonist application in half of recorded neurons is oscillatory in nature; the effect of the agonist application on frequency and amplitude of sIPSCs periodically waxed and waned.

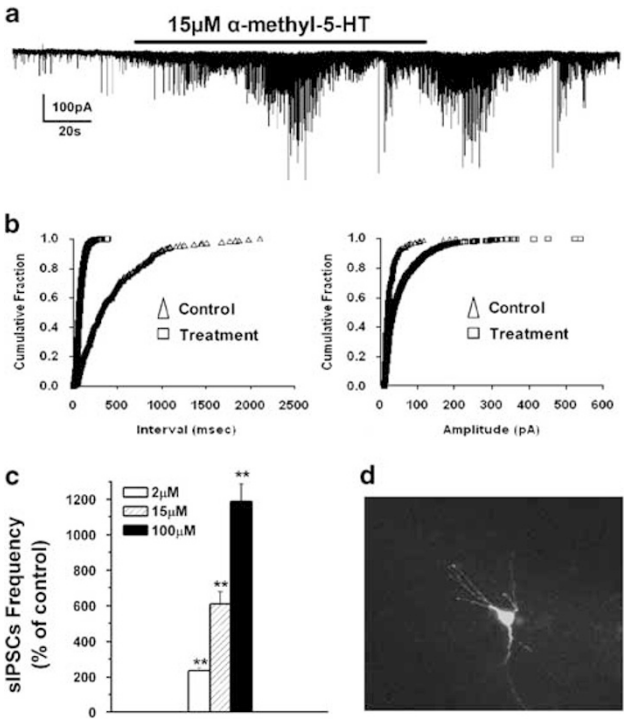

Figure 1 The 5-HT$_{2/4}$ receptor agonist α-methyl-5-HT dose dependently facilitated spontaneous inhibitory postsynaptic currents (sIPSCs) in the basolateral amygdala (BLA). (a) A sample neuron where administration of α-methyl-5-HT (15 μM) dramatically enhances the frequency of sIPSCs and shifts their amplitude distribution toward larger sizes. (b) Cumulative probability plots of interevent intervals and amplitude of sIPSCs (same cell as in the top trace). (c) The bar graph shows the group data of the effects of α-methyl-5-HT on sIPSC frequency ($n = 10$ for each concentration of α-methyl-5-HT, $^{**}p < 0.01$). (d) Photomicrograph of a pyramidal cell showing the typical morphology of the recorded neurons. The cell has been labeled with lucifer yellow. All data are represented as means ± SEM.

The Facilitative Effect of Serotonin was Mediated by 5-HT$_{2A}$ Receptors in the BLA

To identify which 5HT receptor (5-HT$_2$ or 5-HT$_4$) was involved in the effects of serotonin and α-methyl-5-HT on sIPSCs in control rats, we first pretreated the slices with the 5-HT$_{2A/2C}$ receptor antagonist ketanserin (10 μM) for 30 min. In the presence of ketanserin, α-methyl-5-HT (15 μM) no longer induced any change in the frequency and amplitude of sIPSCs, suggesting that the 5-HT$_{2A/2C}$ receptor, rather than the 5-HT$_4$ receptor, was responsible for the facilitative effect. Thus, the frequency of sIPSCs before and after application of α-methyl-5-HT in the presence of ketanserin was 2.84 ± 1.1 and 2.69 ± 0.9 Hz, respectively ($n = 11$, $p > 0.05$; Figure 2e).

We then used the selective 5-HT$_{2A}$ receptor antagonists, MDL 11,939 and 100,907, to further examine which 5-HT$_2$ receptor subtype mediated the facilitative effect. Pretreatment of the slices with MDL 11,939 (1.5 μM) prevented the effects of α-methyl-5-HT. Thus, the frequency of sIPSCs before and after the application was 2.23 ± 0.31 and 2.61 ± 0.55, respectively ($n = 8$, $p > 0.05$; Figures 2a, b, and e). Similarly, pretreatment of the slices with MDL 100,907 (150 nM) prevented the effects of α-methyl-5-HT. The frequency of sIPSCs before and after the application was 2.56 ± 0.30 and 2.67 ± 0.23, respectively ($n = 16$, $p > 0.05$; Figures 2c–e). To exclude the involvement of 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors in the effects of α-methyl-5-HT, we incubated the slices with the 5-HT$_{2B/2C}$ receptor antagonist, SB 206553 (5 μM), for 30 min. The presence of this antagonist did not significantly diminish the effects of α-methyl-5-HT. Thus, in the presence of SB 206553, the application of α-methyl-5-HT increased the frequency of sIPSCs to $598.2 \pm 73\%$ of the baseline values ($n = 6$, $p > 0.05$ compared to α-methyl-5-HT alone; Figure 2e). In addition, the selective 5-HT$_{2B}$ receptor agonist and 5-HT$_{2C}$ receptor agonist had no facilitative effect on sIPSCs. The frequency of sIPSCs after application of the selective 5-HT$_{2B}$ receptor agonist, BW 723C86 (15 μM), and the selective 5-HT$_{2C}$ receptor agonist, WAY 629 (30 μM), was $94 \pm 3.5\%$ ($n = 10$, $p > 0.05$) and $100 \pm 2.5\%$ ($n = 13$, $p > 0.05$) of the baseline values, respectively (data not shown). Taken together, it was the 5-HT$_{2A}$ receptor subtype rather than the 5-HT$_{2B}$ or 5-HT$_{2C}$ receptor subtypes that mediated facilitation of GABAergic synaptic transmission by α-methyl/serotonin in the BLA.

We then investigated the extent to which the 5-HT$_{2A}$ receptor contributed to serotonergic facilitation of GABA release in the BLA, given that the 5-HT$_3$ receptor has also been shown to mediate serotonergic facilitation of GABAergic synaptic transmission in the BLA (Koyama *et al*, 2000; Stein *et al*, 2000). We first examined the effect of 10 μM serotonin on sIPSCs in the presence of the 5-HT$_{2A}$ receptor antagonist MDL 100,907 (150 nM) alone. As shown in Figures 3a, b, and e, serotonin-induced (10 μM) increase of the amplitude of sIPSCs was diminished from $190.8 \pm 12.9\%$ ($n = 14$) to $103.7 \pm 4.4\%$ of the baseline value ($n = 16$, $p < 0.01$) in the presence of 150 nM MDL 100,907. In addition, the serotonin-induced (10 μM) increase of the frequency of sIPSCs was diminished from $460.6 \pm 38.9\%$ ($n = 14$) to $142.3 \pm 14.8\%$ ($n = 16$, $p < 0.01$) of the baseline value in the presence of MDL 100,907. These results indicated that the 5-HT$_{2A}$ receptor antagonist alone could block the majority of serotonergic facilitative effect on sIPSCs in the BLA, suggesting that the 5-HT$_{2A}$ receptor is the primary receptor mediating serotonergic facilitation of

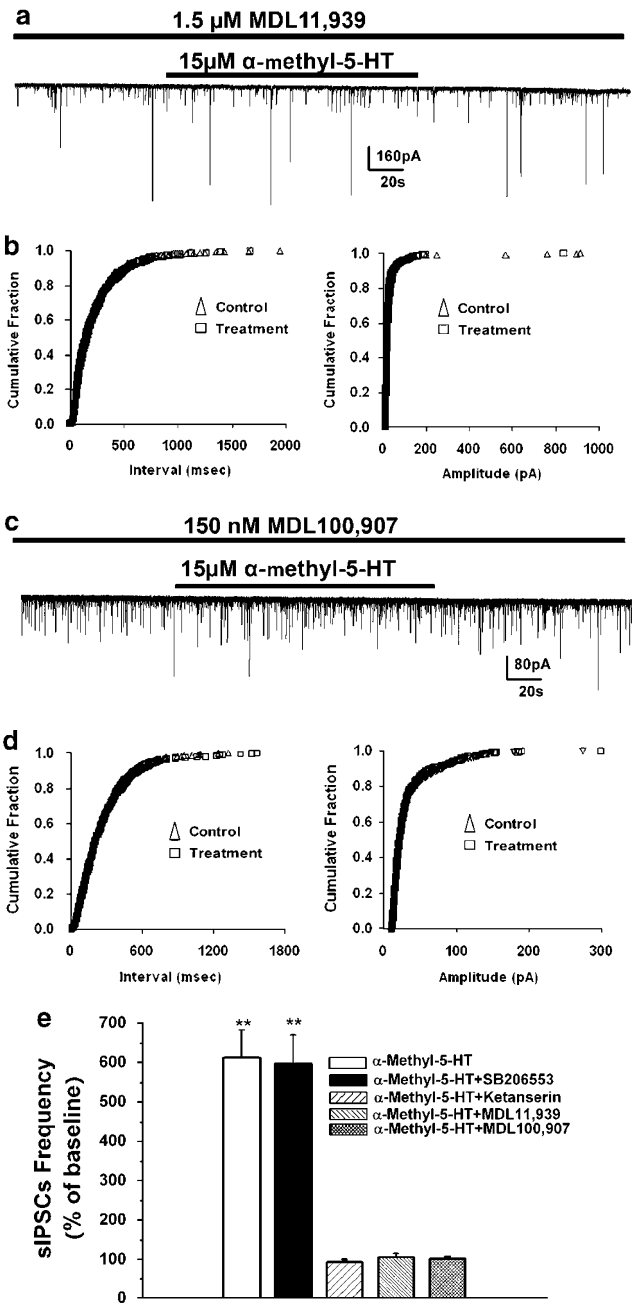

Figure 2 The facilitative effect of α-methyl-5-HT on spontaneous inhibitory postsynaptic currents (sIPSCs) in the presence of different 5-HT$_2$ receptor antagonists. (a) An example of a neuron where α-methyl-5-HT could no longer induce the facilitation of sIPSCs in the presence of 1.5 μM MDL 11,939 (holding potential is −70 mV). (b) Cumulative probability plots of interevent intervals and amplitude of sIPSCs (same cell as in the top trace). (c) An example of a neuron where α-methyl-5-HT could no longer induce the facilitation of sIPSCs in the presence of 150 nM MDL 100,907 (holding potential is −70 mV). (d) Cumulative probability plots of interevent intervals and amplitude of sIPSCs (same cell as in the trace in (c)). (e) Pooled data (mean ± SEM) indicating the effect of α-methyl-5-HT alone ($n = 9$) and the effects of α-methyl-5-HT in the presence of 10 μM ketanserin ($n = 11$) or in the presence of 1.5 μM MDL 11,939 ($n = 8$), or 5 μM SB 206553 ($n = 6$) or 150 nM MDL 100,907 ($n = 16$).

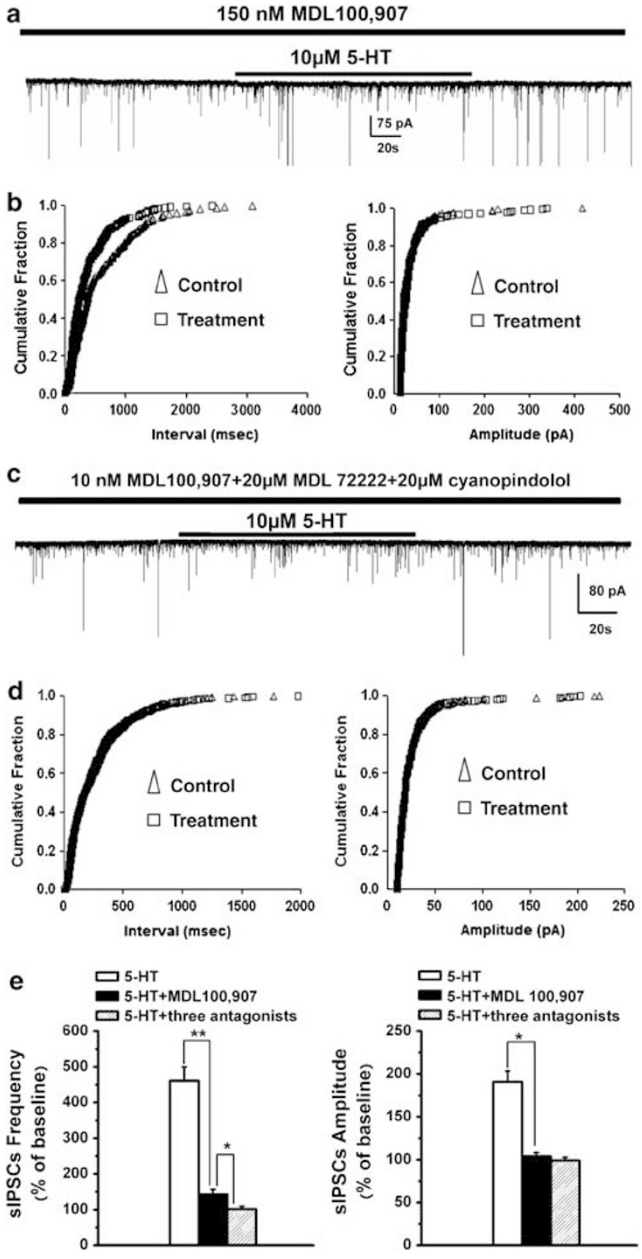

Figure 3 The effect of serotonin on spontaneous inhibitory postsynaptic currents (sIPSCs) in the presence of 5-HT$_2$ receptor and 5-HT$_3$ receptor antagonists. (a) The response of sIPSCs in a sample neuron to serotonin in the presence of 150 nM MDL 100,907 (holding potential is −70 mV). (b) Cumulative probability plots of interevent intervals and amplitude of sIPSCs (same cell as in the top trace). (c) The response of sIPSCs in a sample neuron to serotonin in the presence of 150 nM MDL 100,907, 20 μM MDL 72,222, and 20 μM cyanopindolol (holding potential is −70 mV). (d) Cumulative probability plots of interevent intervals and amplitude of sIPSCs (same cell as in the trace in (c)). (e) Pooled data (mean ± SEM) indicating the effect of serotonin alone ($n = 14$) on frequency and amplitude of sIPSCs and the effects of serotonin on frequency and amplitude of sIPSCs in the presence of 150 nM MDL 100,907 ($n = 16$, $^{**}p < 0.01$) as well as in the presence of 150 nM MDL 100,907, 20 μM MDL 72,222, and 20 μM cyanopindolol ($^{*}p < 0.05$, $n = 6$).

GABA release in the BLA. The residual facilitative effect of serotonin in the presence of MDL 100,907 on the frequency of sIPSCs should be mediated by the 5-HT$_3$ receptor as it could be fully blocked by addition of 5-HT$_3$ receptor antagonist MDL 72,222 (20 μM) and 5-HT$_1$ receptor antagonist cyanopindolol (20 μM, $n = 6$; Figures 3c–e) (Koyama *et al*, 2002).

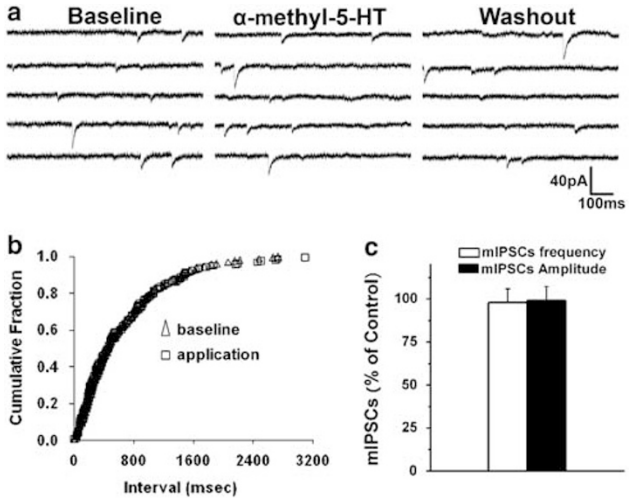

Figure 4 The effect of α-methyl-5-HT on miniature inhibitory postsynaptic currents (mIPSCs) in the basolateral amygdala (BLA). (a) Administration of α-methyl-5-HT (15 μM) did not significantly change the frequency and amplitude of mIPSCs. (b) Cumulative probability plots of interevent intervals of mIPSCs (same cell as in the top trace). (c) Pooled data (mean ± SEM) from eight neurons. The bar graph shows that amplitude and frequency of mIPSCs after application of α-methyl-5-HT were not significantly different from the baseline.

5-HT$_{2A}$ Receptors were Primarily Localized to the Soma and Dendrites of Interneurons in the BLA

Activation of 5-HT$_{2A}$ receptors could enhance GABAergic synaptic transmission through either a presynaptic or a postsynaptic mechanism. To distinguish between these two possibilities, we examined the effects of α-methyl-5-HT in the presence of TTX (1 μM), which blocked action potential-dependent release of GABA. Under this condition, release events should be exclusively composed of single vesicle release events (miniature IPSCs, mIPSCs). As illustrated in Figure 4, in the presence of TTX, the administration of α-methyl-5-HT had no effect on the amplitude of mIPSCs (Figure 4c). Similarly, α-methyl-5-HT also failed to change the frequency of mIPSCs ($n = 8$, $p > 0.05$). These results indicated that α-methyl-5-HT did not enhance GABAergic synaptic transmission via a postsynaptic mechanism. The results also suggested that α-methyl-5-HT may not act on GABAergic terminals, but on somatodendritic sites of GABAergic neurons to facilitate GABA release. Indeed, as illustrated in Figure 5, immunofluorescence staining for 5-HT$_{2A}$ receptors was primarily localized to the soma and dendrites. 5-HT$_{2A}$ receptor signal-positive cell bodies appeared to be interneuron-like and the majority of staining for the 5-HT$_{2A}$ receptor (87.3%) overlapped with that of the interneuron marker parvalbumin (PV; Figure 5).

5-HT$_{2A}$ receptors are generally coupled to Gq/11 protein, which then is further coupled to phospholipase C (PLC). To examine if the 5-HT$_{2A}$ receptor-mediated effect on the BLA interneurons depended on PLC activation, we incubated the slices for at least 30 min with a PLC inhibitor, U73122 (20 μM). We have shown previously that this compound is

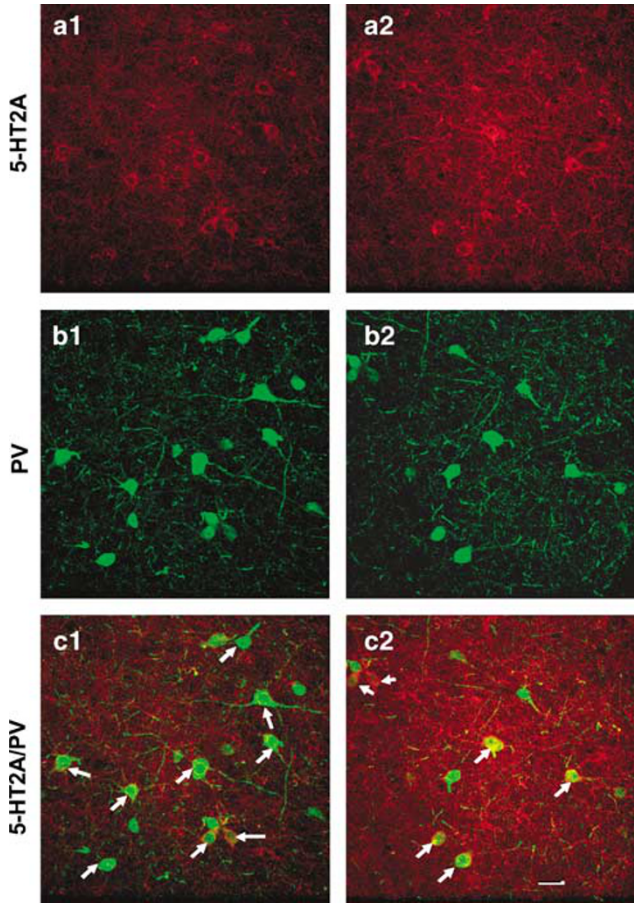

Figure 5 Confocal microscopic images illustrating dual localization of 5-HT$_{2A}$ receptor with parvalbumin (PV) in the basolateral amygdala (BLA). (a1 and a2) The staining for the 5-HT$_{2A}$ receptor in the BLA. The majority of the staining is localized to the soma and dendrites and the shape of signal-positive cells is interneuron-like. (b1 and b2) The staining for interneuron marker PV. (c1 and c2) Colocalization of the 5-HT$_{2A}$ receptor (red) with PV (green) in the BLA. Arrows in c1 and c2 indicate the colocalization of two types of staining overlapped (yellow). Scale bar, 25 μm.

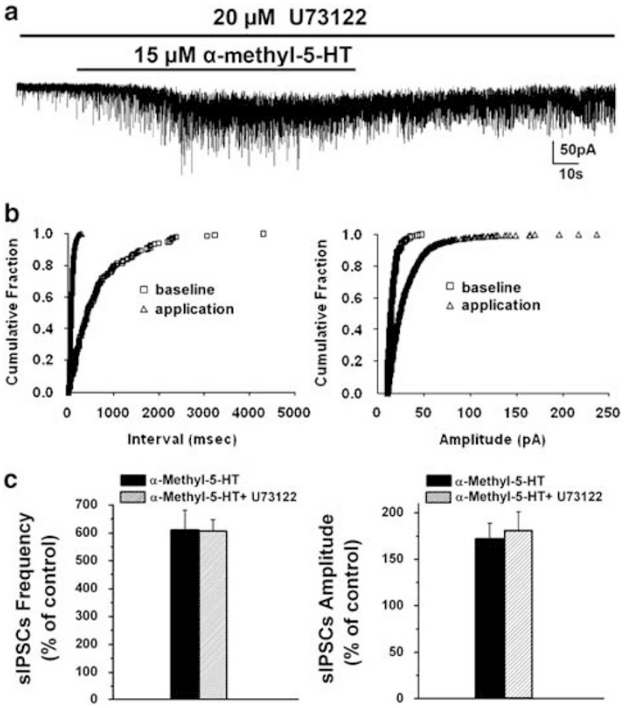

Figure 6 The facilitative effect of α-methyl-5-HT on spontaneous inhibitory postsynaptic currents (sIPSCs) in the presence of phospholipase C (PLC) inhibitor U73122. (a) An example of a neuron where α-methyl-5-HT still induced the comparable facilitation of sIPSCs after pretreatment with 20 μM U73122 for 30 min (holding potential is −70 mV). (b) Cumulative probability plots of interevent intervals and amplitude of sIPSCs (same cell as in the top trace). (c) Pooled data (mean ± SEM) indicating the effect of α-methyl-5-HT alone ($n = 10$), and the effect of α-methyl-5-HT in the presence of 20 μM U73122 ($n = 9$).

able to block the action mediated by another PLC-coupled receptor, α_1 adrenoceptors in the amygdala, indicating that U73122 at our experimental condition is effective in blocking PLC (Braga *et al*, 2004). Surprisingly, the presence of this inhibitor did not significantly change the effect of α-methyl-5-HT (15 μM). The frequency and amplitude of sIPSCs were enhanced to 608.4 ± 41.0 and 169.7 ± 19.4% of the baseline value, respectively, in this condition ($n = 9$, $p > 0.05$; Figure 6), which was not significantly different from the effect of α-methyl-5-HT alone. In addition, we increased the concentration of U73122 to 100 μM, which still did not significantly change the effect of α-methyl-5-HT (15 μM) either. This unexpected result suggested that 5-HT$_{2A}$ receptor-mediated effects might be independent of PLC activation.

Stress Impaired Serotonergic Facilitation of GABAergic Synaptic Transmission in the BLA

To examine if the facilitative effects of 5-HT$_{2A}$ receptor activation on GABAergic transmission in the BLA were subject to change after stress, we randomly assigned rats to control and stress groups. After exposure to the stress protocol for 3 days, the rats were killed for electrophysiological studies. As illustrated in Figure 7a, the parameters of sIPSCs including the frequency, amplitude, rise time, and decay time constant were not significantly changed by exposure to 3-day stress compared to control. The frequencies of sIPSCs in control and stressed animals were 3.03 ± 0.65 and 3.15 ± 0.89 HZ, respectively ($n = 22$, $p > 0.05$). The amplitudes were 33.16 ± 2.10 and 31.70 ± 1.44 pA, respectively ($n = 22$, $p > 0.05$). However, the facilitative effects of α-methyl-5-HT on sIPSCs were impaired by stress. As illustrated by Figures 7c–e, application of α-methyl-5-HT (15 μM) enhanced the frequency of sIPSCs only to 260.7 ± 25.70% of the baseline values in stressed animals, which was significantly different from the effect of α-methyl-5-HT at the same concentration in the control animals (601.0 ± 39.4% of the baseline values; Figures 7b and d). Such a difference became more evident at higher concentrations of α-methyl-5-HT. At 100 μM, α-methyl-5-HT increased the frequency by only 440.5 ± 48.0% in amygdala slices of stressed rats, which appeared to be the maximum effect. In control slices, α-methyl-5-HT at 100 μM enhanced the frequency by 1160.5 ± 99.1%, which still did not reach its maximum effect. Dose–response curves for control and stressed animals showed that the maximum effect of α-methyl-5-HT was markedly decreased by exposure

to stress, indicating that BLA 5-HT$_{2A}$ receptor signaling was severely impaired by exposure of rats to stress (Figure 7e).

To further confirm such stress-induced impairment of 5-HT$_{2A}$ receptor signaling in the BLA, we examined the mRNA and protein levels of the 5-HT$_{2A}$ receptors after stress. As illustrated in Figure 8a, exposure to 3-day stress caused a significant decrease in expression of 5-HT$_{2A}$ receptor mRNA in the BLA and hippocampus compared with control rats (38.5 ± 3.1% of control values for the BLA, $p < 0.01$; 51.2 ± 4.1% of control values for the hippocampus, $p < 0.05$, $n = 7$). To examine the specificity of the stress effect on the 5-HT$_{2A}$ receptor, we also examined the mRNA expression of the 5-HT$_{1A}$ and 5-HT$_{2C}$ receptors, two serotonin receptors that have been shown to have a functional role in the BLA (Chen *et al*, 2003; Cheng *et al*, 1998). As shown in Figure 8a, the levels of 5-HT$_{2C}$ receptor mRNA were not significantly changed by exposure to stress in either the BLA or the hippocampus. 5-HT$_{1A}$ receptor mRNA expression in the BLA was also not significantly changed by stress (Figure 8b).

Representative western blots of 5-HT$_{2A}$ receptors in rat BLA are shown in Figure 8c. The molecular mass of 5-HT$_{2A}$ receptors was 55 kDa. As illustrated by Figure 8c, the protein level of 5-HT$_{2A}$ receptors was significantly decreased in the BLA but not in the hippocampus of stressed rats as compared to control rats. These data indicated that stress downregulated 5-HT$_{2A}$ receptors in the BLA, which was compatible with the electrophysiological data.

Stress Impaired 5-HT$_{2A}$ Receptor-Mediated Suppression of BLA Field Potentials

As the activation of 5-HT$_{2A}$ receptors facilitates GABAergic transmission, the function of these receptors at the network level could be to dampen neuronal excitability and responsiveness. To determine the net effect of 5-HT$_{2A}$ receptor activation on neuronal responsiveness and excitability in the BLA, and whether this effect was altered by stress, we investigated the effects of α-methyl-5-HT on field potential responses in control and stressed rats. We first examined in control rats the effects of α-methyl-5-HT on field potentials in both juvenile (less than 25 days old) and young adult rats (35–40 days old) to see whether the function of 5-HT$_{2A}$ receptor in the BLA is age dependent. The effects of α-methyl-5-HT on the BLA field potentials in juvenile and young adults rats were 30.0 ± 13.8% ($n = 4$) and

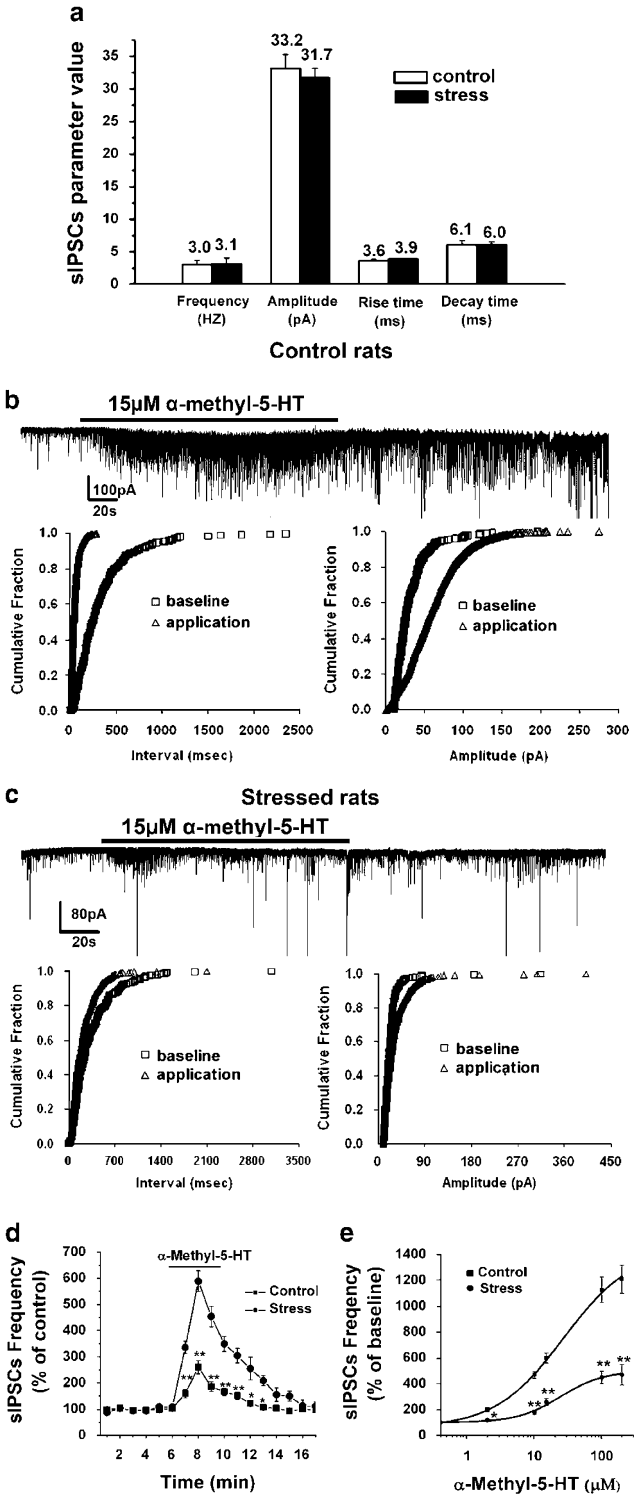

Figure 7 Stress attenuated 5-HT$_{2A}$ receptor-mediated facilitative effect on spontaneous inhibitory postsynaptic currents (sIPSCs) in the basolateral amygdala (BLA). (a) Stress did not significantly affect the frequency, amplitude, rise time, and decay time constant of sIPSCs in the BLA ($n = 23$). (b) Upper trace: effects of α-methyl-5-HT (15 μM) on sIPSCs recorded from a BLA pyramidal cell of a control rat (holding potential is −70 mV). Lower graphs: cumulative probability plots of interevent intervals and amplitude of sIPSCs, in baseline conditions and during α-methyl-5-HT application (same cell as in the top trace). (c) Upper trace: effects of α-methyl-5-HT (15 μM) on sIPSCs recorded from a BLA pyramidal cell of a stressed rat (holding potential is −70 mV); the effects of α-methyl-5-HT (15 μM) are significantly less than in control animals. Lower graphs: cumulative probability plots of interevent intervals and amplitude of sIPSCs in baseline conditions and during α-methyl-5-HT application (same cell as in the top trace). (d) Pooled data (mean ± SEM) indicating the time course of the effects of α-methyl-5-HT (15 μM) in both control and stressed animals. Note that stress significantly attenuated the effects of α-methyl-5-HT (15 μM) at the different time points. ($n = 18$ for stress and $n = 14$ for control, $^*p < 0.05$, $^{**}p < 0.01$). (e) The dose–response relationship of the effects of α-methyl-5-HT in control and stressed animals (0.5 μM, $n = 5$ for control and $n = 4$ for stress; 2 μM, $n = 13$ for control and $n = 20$ for stress; 10 μM, $n = 11$ for control and $n = 8$ for stress; 15 μM, $n = 14$ for control and $n = 18$ for stress; 100 μM, $n = 9$ for control and $n = 10$ for stress; 200 μM, $n = 4$ for control and $n = 5$ for stress; $^*p < 0.05$, $^{**}p < 0.01$).

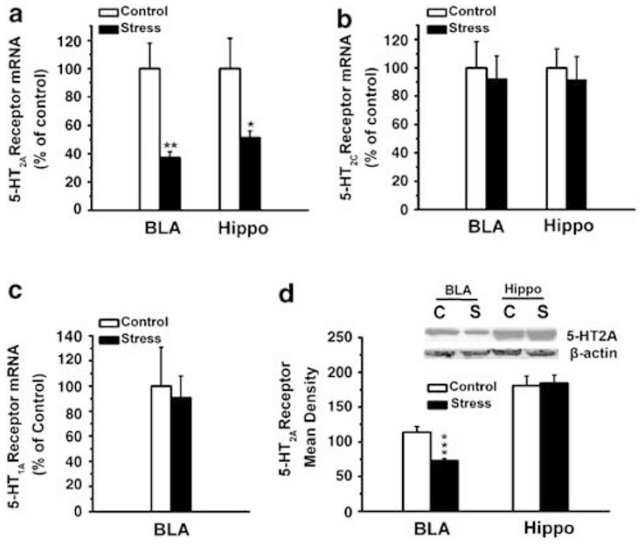

Figure 8 Stress decreased the expression of 5-HT$_{2A}$ receptor mRNA and its proteins in the basolateral amygdala (BLA). (a) Stress decreased 5-HT$_{2A}$ receptor mRNA levels in the both BLA and hippocampus, while 5-HT$_{2C}$ receptor mRNA levels in the two brain regions were not significantly changed by stress ($n=7$, $^{*}p<0.05$, $^{**}p<0.01$). (b) Stress had no significant effect on the levels of 5-HT$_{1A}$ receptor mRNA in the BLA. (c) Stress decreased protein levels of 5-HT$_{2A}$ receptors in the BLA, while protein levels of 5-HT$_{2A}$ receptors in the hippocampus (Hippo) were not significantly changed by stress. β-Actin was used as an internal control. Blot results shown are representative of three separate experiments. $^{***}p<0.001$ (stress *vs* control).

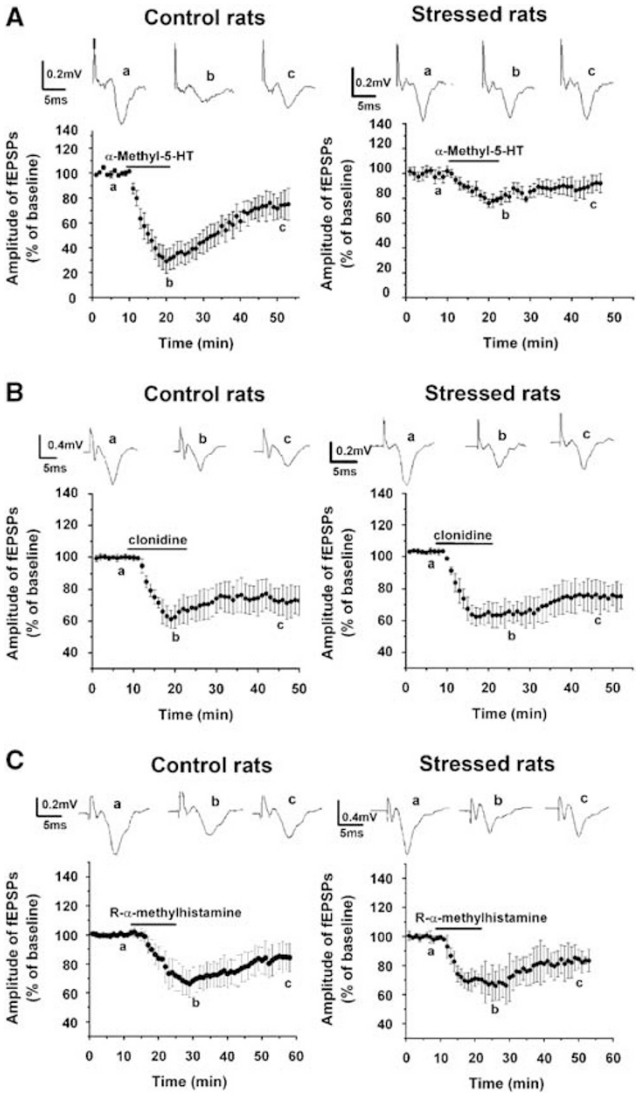

Figure 9 The effect of stress on the 5-HT$_{2A}$ receptor-mediated, α_2 adrenoceptor-mediated and H_3 receptor-mediated inhibitory action on field potentials (fEPSPs) in the basolateral amygdala (BLA). (A) The suppressive effect of α-methyl-5-HT (50 μM) on fEPSPs recorded in the BLA from control (29.1 ± 9.6% of the baseline, $n=9$) and stressed animals (77.8 ± 5.3% of the baseline, $n=11$). (B) The suppressive effect of clonidine (50 μM) on fEPSPs recorded in the BLA from control (60.2 ± 5.7% of the baseline values, $n=12$) and stressed animals (57.6 ± 6.2% of the baseline, $n=13$). (C) The suppressive effect of R-α-methylhistamine (2 μM) on fEPSPs recorded in the BLA from control (66.2 ± 9.3% of the baseline values, $n=13$) and stressed animals (63.4 ± 7.5%, $n=15$).

28.4 ± 9.1% of the baseline, respectively ($n=5$, $p>0.05$). As there was no significant difference in the effect of α-methyl-5-HT on the BLA field potentials in animals of different ages, the results collected from the two groups were pooled together. Thus, in control rats, 50 μM α-methyl-5-HT produced a robust reduction in the peak amplitude of evoked field potentials (29.1 ± 9.6% of the baseline, $n=9$; Figure 9a). This inhibitory effect was abolished when GABA receptors were blocked, confirming that this inhibitory effect on field potentials resulted from facilitation of the BLA GABAergic system. In stressed rats, α-methyl-5-HT at 50 μM produced only a small reduction in the peak amplitude of evoked field potentials (77.8 ± 5.3% of the baseline, $n=11$; Figure 9a), which was significantly different from the effect of α-methyl-5-HT in control rats ($p<0.01$). These results further confirmed that the function of 5-HT$_{2A}$ receptors in the BLA was impaired by stress. To further examine the specificity of this stress effect on 5-HT$_{2A}$ receptor function, we also examined the functions of other aminergic systems in the BLA after stress, including the noradrenergic and histaminergic systems. Both norepinephrine and histamine could suppress amygdala excitability, which is mediated by α_2 adrenoceptors and histamine H_3 receptors, respectively (Jiang *et al*, 2005; DeBock *et al*, 2003). As shown in Figure 9b, the α_2 adrenoceptor agonist clonidine (50 μM) suppressed the field potentials to 57.6 ± 6.2% of the baseline values in stressed rat amygdala slices, which was not significantly different from the effect of clonidine in control animals (60.2 ± 5.7% of the baseline values, $n=12$, $p>0.05$). The protein levels of the α_{2A} adrenoceptors in the BLA were also not significantly changed by stress (133 ± 3.47% and 138 ± 2.58% for control and stress respectively, $n=4$, $p=0.2872$). Stress also had no significant effect on histamine H_3 receptor-mediated action in the BLA. Thus, in stressed animals, an H_3 receptor agonist, R-α-methylhistamine (2 μM) decreased the field potentials in the BLA to 63.4 ± 7.5% of the baseline values, which was not significantly different from that in control animals (66.2 ± 9.3% of the baseline values, $n=13$, $p>0.05$; Figure 9c).

DISCUSSION

This study, for the first time, demonstrated that the 5-HT$_{2A}$ receptor was the primary receptor mediating serotonergic

facilitation of BLA GABA release. Our data also, for the first time, showed that stress induced a specific, severe impairment of the 5-HT$_{2A}$ receptor-mediated facilitation of GABAergic synaptic transmission in the BLA. This signaling impairment might primarily result from 5-HT$_{2A}$ receptor downregulation as both mRNA and protein levels of the 5-HT$_{2A}$ receptor in the BLA were decreased by stress. As a characteristic feature of stress-associated psychiatric disorders, such as PTSD, is the hyperexcitability of the amygdala, these findings may not only reveal a possible mechanism underlying this pathophysiological phenomenon, but may also help to explain why serotonin dysregulation is a crucial link in pathogenesis of these disorders (Brown and Linnoila, 1990; Stanley and Stanley, 1990).

5-HT$_{2A}$ Receptors Mediate Serotonergic Facilitation of GABA Release in the BLA

Although a previous study has shown that the primary action of serotonin in the BLA is to facilitate GABA release (Rainnie, 1999), knowledge of the serotonin receptor subtypes involved in this action has remained inconclusive. We observed that the broad 5-HT$_2$ receptor agonist α-methyl-5-HT strongly facilitated GABAergic synaptic transmission in almost all BLA pyramidal cells (105 of 123, 85%), suggesting the involvement of the 5-HT$_2$ receptor. As α-methyl-5-HT is also a potent agonist for the 5-HT$_4$ receptor (Gerald *et al*, 1995; Xiang *et al*, 2005), involvement of this receptor could not be ruled out. Thus, the 5-HT$_{2A/2C}$ receptor antagonist ketanserin was first used to distinguish between the 5-HT$_2$ and 5-HT$_4$ receptors; it completely blocked the effect of α-methyl-5-HT, indicating no involvement of the 5-HT$_4$ receptor.

There are three subtypes of the 5-HT$_2$ receptor, 5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$. The primary 5-HT$_2$ receptor subtype involved in serotonergic facilitation of GABA release in the BLA is the 5-HT$_{2A}$ receptor. First, the 5-HT$_{2A}$ receptor antagonists, MDL 11,939 and MDL 100,907, completely blocked the effects of α-methyl-5-HT. Second, the 5-HT$_{2B/2C}$ receptor antagonist, SB 206553, could not significantly diminish facilitation of GABA release by α-methyl-5-HT. Furthermore, the selective 5-HT$_{2B}$ receptor agonist, BW 723C86, and the selective 5-HT$_{2C}$ receptor agonist, WAY 629, had no significant effect on GABA release in the BLA. Hence, the 5-HT$_{2A}$ receptor appears to be the primary receptor subtype mediating serotonergic facilitation of GABA release in the BLA.

One major concern is the extent to which the 5-HT$_{2A}$ receptor contributes to serotonergic facilitation of GABA release, given that activation of another serotonin receptor, the 5-HT$_3$ receptor, has also been shown to facilitate GABA release in dissociated BLA neurons (Koyama *et al*, 2000, 2002). To address this concern, we examined the effect of serotonin in the presence of the 5-HT$_{2A}$ receptor antagonist alone. The 5-HT$_{2A}$ receptor antagonist alone blocked ~96% of the effect of serotonin on the amplitude of sIPSCs and ~89% of the effect of serotonin on the frequency of sIPSCs (see Figure 3 for detail), indicating that the 5-HT$_{2A}$ receptor rather than the 5-HT$_3$ receptor played a major role in mediating serotonergic facilitation of GABA release in the BLA. There may be several reasons why the contribution of the 5-HT$_3$ receptor to serotonergic facilitation of GABA release is small under our experimental conditions. First, the 5-HT$_3$ and 5-HT$_1$ receptors could coexist in the same GABAergic terminals, and activation of the 5-HT$_1$ receptor could antagonize 5-HT$_3$ receptor-mediated facilitation of GABA release (Koyama *et al*, 2002). Thus, the effect of 5-HT$_3$ receptor activation may not be evident with the 5-HT$_1$ receptor unantagonized. Second, the 5-HT$_3$ receptor is present only in a subset of GABAergic terminals and the effect of 5-HT$_3$ receptor activation on GABA release is mainly transient in nature and quickly desensitized (Koyama *et al*, 2000, 2002). To see the effect mediated by the 5-HT$_3$ receptor, a fast perfusion system (in which the exchange rate of the perfusion is in the millisecond range) was required to apply the 5HT$_3$ agonist (Koyama *et al*, 2000, 2002). Therefore, when serotonin was being applied using our bath perfusion system in which the exchange rate of bath solution is in the range of 1–2 min, a significant amount of the 5-HT$_3$ receptors may have been desensitized before the agonist reached the effective concentration. This may explain why the effect of the pure 5-HT$_3$ receptor agonist 2-methyl-5-hydroxytryptamine is still not evident when being applied this way (data not shown). Overall, under our experimental conditions, the 5-HT$_{2A}$ receptor is the primary receptor mediating serotonin effects on GABA release in the BLA.

As most of experiments were done in juvenile rats, another question that arose was whether this primary role of the 5-HT$_{2A}$ receptor in mediating serotonergic facilitation of GABA release would still be the case in adult rats. In a young adult rat (35–40 days old), the 5-HT$_{2A}$ receptor agonist still induced comparable, strong inhibition of field potentials in the BLA that were not significantly different from the effect of the 5-HT$_{2A}$ receptor agonist in the juvenile animals. As there is no significant difference between the two different ages, the data have been pooled together into the same Figure 9a. Therefore, the 5-HT$_{2A}$ receptor still appears to be primary in mediating serotonergic facilitation of GABA release in the BLA at least in young adult rats. It will be interesting to see whether it is still the case in fully mature adults (more than 60 days old) in a future study.

5-HT$_{2A}$ Receptors are Localized to Soma and Dendrites of PV-Containing Interneurons

The 5-HT$_{2A}$ receptor could be localized to the somatodendritic area and/or axonal terminals of GABAergic interneurons in the BLA, and activation of the receptors in either area could result in facilitation of GABA release. Nevertheless, as α-methyl-5-HT had no significant effect on mIPSCs frequency, 5-HT$_{2A}$ receptors may not be located on the axon terminals of interneurons, thus suggesting that the 5-HT$_{2A}$ receptors localized in the somatodendritic area of interneurons mediate the facilitative action. Our immunofluorescence data supported this contention because the majority of 5-HT$_{2A}$ receptor immunofluorescence signals were localized to soma and dendrites of PV-containing interneurons. In addition, while this paper was in preparation, a similar immunofluorescence study of BLA 5-HT$_{2A}$ receptors was reported and also supported the same contention (McDonald and Mascagni, 2007). Thus, 5-HT$_{2A}$ receptors in the BLA are primarily somatodendritically localized

and activation of these receptors by serotonin might depolarize BLA interneurons to facilitate GABA release (Rainnie, 1999).

The interneurons in the BLA are quite heterogeneous, with certain interneurons expressing only PV and others expressing peptides such as somatostatin and CCK (McDonald and Mascagni, 2006). A recent study also identifies a subclass of interneurons along the EC and along the medial border between the BLA and central amygdala, which do not express any types of known interneuron marker including PV (McDonald and Mascagni, 2007; Marowsky *et al*, 2005). As the majority of the 5-HT$_{2A}$ receptor signals overlapped with PV, serotonin might only excite the PV-containing interneurons by the 5-HT$_{2A}$ receptor. Serotonin might exert effects on other subclasses of interneurons by other serotonin receptors such as the 5-HT$_3$ receptor (Mascagni and McDonald, 2007), especially the subclass of interneurons mediating feedforward inhibition from the BLA to the central nucleus (McDonald and Mascagni, 2007; Marowsky *et al*, 2005). In this respect, the strong effect of serotonin on GABAergic transmission mediated by other serotonin receptors, such as the 5-HT$_3$ receptor, might be seen in the central nucleus, but not in the BLA. This might be another reason why the 5-HT$_3$ receptor agonist had a small effect on GABAergic transmission in the BLA, although 5-HT$_3$ receptors have been observed to be highly expressed in the BLA interneurons (Mascagni and McDonald, 2007).

The 5-HT$_{2A}$ receptor is a Gq/11-protein-coupled receptor (Saudou and Hen, 1994). In general, the Gq/11-protein-coupled receptors modulate neuronal excitability by activating the signal pathways that result in inhibition of background potassium channels (Goldstein *et al*, 2001; Talley *et al*, 2003). Although the mechanisms engaged in this receptor-mediated cation channel inhibition have not been clearly established, significant evidence supports the involvement of activation of PLC, the downstream product of the Gq/11 protein in this modulation (Suh *et al*, 2004; Suh and Hille, 2005; Hilgemann *et al*, 2001). However, in our study, blockade of PLC with U73132 did not significantly affect facilitation of GABA release by α-methyl-5-HT. This unexpected result first prompted us to try U73132 derived from different batches and from different manufacturers; however, consistent results were obtained in every experiment using U73132. A plausible explanation at present is that the 5-HT$_{2A}$ receptor-induced enhancement of GABA release does not involve activation of a PLC signaling pathway in BLA interneurons. In fact, emerging evidence indicates that certain Gq-coupled receptors appear to be able to directly modulate ion channel activities without further involving downstream mediators, especially for TASK channels (Boyd *et al*, 2000; Chen *et al*, 2006). However, the cellular mechanisms underlying such explanations require further exploration and we are inclined not to draw conclusions from evidence that was obtained using only pharmacological approaches.

5-HT$_{2A}$ Receptor-Mediated Serotonergic Facilitation of GABA Release Could be Specifically Impaired by Stress

Dysregulation of the serotonergic system is a characteristic pathophysiological change observed in stress-associated psychiatric disorders and this dysregulation is believed to be induced by stress (van Praag, 2004). In this study, repeated restraint/tail-shock stress induced a severe impairment in 5-HT$_{2A}$ receptor-mediated facilitation of GABA release in rat BLA. Such impairment may be due to impaired 5-HT$_{2A}$ receptor signaling, or altered sensitivity and/or a change in the number of GABA receptors. However, as the baseline of the GABAergic activity in the amygdala of stressed rats was not significantly different from that in control animals, stress-induced attenuation of 5-HT$_{2A}$ receptor-mediated GABA release should primarily result from impaired 5-HT$_{2A}$ receptor signaling. Indeed, stress not only decreased levels of 5-HT$_{2A}$ receptor mRNA, but also the levels of expressed receptors, indicating that stress-induced downregulation of the 5-HT$_{2A}$ receptor is the primary cause for such impairment.

Such impairment of 5-HT$_{2A}$ receptor signaling after inescapable stress could occur for several reasons. First, as receptor desensitization and downregulation after chronic agonist stimulation is a common phenomenon for the 5-HT$_{2A}$ receptor, and has been observed in both cell culture (Roth *et al*, 1995) and in *in vivo* systems (Anji *et al*, 2000; Smith *et al*, 1999). The BLA 5-HT$_{2A}$ receptor downregulation after inescapable stress is very likely caused by long-lasting enhanced serotonin levels in the amygdala. As mentioned earlier, inescapable stress can dramatically enhance serotonin levels in the BLA and this enhancement can last at least 40 h after the end of stress (Amat *et al*, 2005). This prolonged stimulation of the 5-HT$_{2A}$ receptor by excessive serotonin would subsequently induce receptor desensitization and downregulation. Alternatively or additionally, the impairment of 5-HT$_{2A}$ receptor signaling may result from mutual interaction between the 5-HT$_{2A}$ receptor and brain-derived neurotrophic factor (BDNF). Inescapable stress could decrease the BDNF levels in the brain possibly due to high levels of brain glucocorticoids (Vaidya *et al*, 1997; Xu *et al*, 2006; Duman and Monteggia, 2006; Greenwood *et al*, 2007), whereas the function of the 5-HT$_{2A}$ receptor appears to depend on BDNF as knockout of BDNF severely impairs 5-HT$_{2A}$ receptor signaling (Rios *et al*, 2006). Thus, decrease of BDNF by inescapable stress may also participate in the occurrence of the impairment of 5-HT$_{2A}$ receptor signaling.

As the stress protocol used in this study is far more potent than most of the stress protocols currently in use, the impairment of 5-HT$_{2A}$ receptor signaling in the BLA could be a nonspecific effect of traumatic stress on G-protein-coupled receptors (GPCRs). However, stress had neither significant effect on mRNA expression of the 5-HT$_{2C}$ and 5-HT$_1$ receptors nor on the electrophysiological function of α_2 adrenoceptors or histamine H$_3$ receptors in the BLA. As it is not feasible to check the effect of stress on every GPCR in the BLA, these negative results at least indicated that it is unlikely that the impairment of 5-HT$_{2A}$ receptor signaling in the BLA is a nonspecific effect of stress. Nevertheless, our previous observations indicate that stress also impairs α_{1A} adrenoceptor-mediated facilitation of GABA release in the BLA (Braga *et al*, 2004), suggesting that stress may specifically alter modulation of the inhibitory GABAergic system by the GPCRs (such as 5-HT$_{2A}$ receptor and α_{1A} adrenoceptor). The modulation of excitatory synaptic transmission by the GPCRs (such as α_2 adrenoceptor, 5-HT$_1$ and 5-HT$_{2C}$

as well as H_3 receptors) appears to be more resistant to the current stress protocol. The reason for such a differential effect of stress on inhibitory *vs* excitatory synaptic transmission is unknown and needs to be determined by future research.

The effect of stress on 5-HT$_{2A}$ receptor signaling in the BLA could be transient or long-lasting. Our preliminary data showed that the mRNA levels of the 5-HT$_{2A}$ receptor in the BLA still remained lower than control 7 days after stress, whereas 5-HT$_{2C}$ receptor mRNA levels in the BLA 7 days after stress remained unchanged. As the turnover of receptor protein levels take a longer time in general to manifest than the level of receptor mRNA, we speculate that 5-HT$_{2A}$ receptor protein levels will remain lower than control values 7 days after stress. These observations suggest that impairment of 5-HT$_{2A}$ receptor signaling in the BLA may persist after cessation of stressful events.

Functional Implications

What are the possible functional implications of a stress-induced impairment of the 5-HT$_{2A}$ receptor-mediated serotonergic facilitation of GABA release in the BLA? It is well recognized that the amygdala is involved in stressful/emotional information processing (LeDoux, 1994, 2000, 2003; Leppanen, 2006). The BLA contains the entry nuclei that funnel and integrate aversive sensory input signals arriving from the thalamus and cortex (LeDoux, 2000, 2003). The alterations of signal processing and the excitability of these nuclei have been closely associated with the emergence of a negative emotional state resulting from severe aversive experiences, such as phobia and anxiety (Stein *et al*, 2007; Phan *et al*, 2006; Kent and Rauch, 2003). Accumulating evidence also indicates that pathophysiological alterations in the neuronal excitability of these amygdala nuclei are characteristic features of certain stress-related psychiatric illnesses, such as PTSD and depressive disorders (Drevets, 2000; Kalia, 2005; Manji *et al*, 2001; Shin *et al*, 2006). Although dysregulation of the serotonergic system in these stress-related psychiatric illnesses has been recognized for over 40 years and may be a critical factor in the pathogenesis of these disorders, the exact role this dysregulation might play in the pathophysiology and symptomology of these disorders still remains unclear. The present findings may reveal an important pathophysiological link between dysregulation of the serotonergic system and pathogenesis of amygdala hyperactivity, a characteristic feature of these disorders. Dysregulation of this system in the amygdala, specifically, with malfunctioning of 5-HT$_{2A}$ receptor-mediated facilitation of GABA release, would result in reduced GABAergic tone and a lower intrinsic threshold of the amygdala circuitry in response to the challenging events (Villarreal and King, 2001; Drevets, 2003). The consequence of such a change will be an amygdala that is hyperresponsive to traumatic reminders and even innocuous stimuli that may underlie the symptoms of these disorders.

Pharmacological prevention and treatment of stress-related psychiatric disorders such as PTSD is a topic of current medical interest (Pitman *et al*, 2002). Most of the research performed thus far has focused on the effectiveness of serotonin reuptake inhibitors in alleviating the symptoms of stress-related symptoms (Albucher and Liberzon, 2002). Although clinical research has shown that these agents are useful in alleviating symptoms and facilitating recovery, their overall efficacy is limited and very often hindered by their serious side effects. The development of more specific pharmacological agents with potentially less significant side effects as a therapeutic strategy aimed at preventing the establishment of stress-related disorders is an important step in the treatment of these illnesses. The present findings suggest that 5-HT$_{2A}$ receptor ligands may be more efficacious pharmacological interventions aimed at regulating neuronal excitability in the amygdala circuitry, and thus treating stress-induced anxiety disorders such as PTSD.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare that except for income received from the primary employer no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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