

RESEARCH ARTICLE

Strain-Dependent Variations in Stress Coping Behavior Are Mediated by a 5-HT/GABA Interaction within the Prefrontal Corticolimbic System

Diego Andolina, PhD; Dario Maran, MSc; Maria Teresa Viscomi, PhD; Stefano Puglisi-Allegra, PhD

Santa Lucia Foundation, Rome, Italy (Drs Andolina, Viscomi, and Puglisi-Allegra); Dipartimento di Scienze Cliniche Applicate e Biotecnologie, Università degli Studi dell'Aquila, Via Vetoio, L'Aquila, Italy (Dr Andolina); Dipartimento di Psicologia and Centro 'Daniel Bovet,' Sapienza Università di Roma, Rome, Italy (Drs Maran and Puglisi-Allegra).

Correspondence: Diego Andolina, PhD, Dipartimento di Scienze Cliniche Applicate e Biotecnologie Università degli Studi dell'Aquila, Via Vetoio, L'Aquila 67010, Italy (diego.andolina@gmail.com).

Abstract

Background: Serotonin and γ -aminobutyric acid (GABA) transmission is crucial in coping strategies.

Methods: Here, using mice from 2 inbred strains widely exploited in behavioral neurochemistry, we investigated whether serotonin transmission in medial prefrontal cortex and GABA in basolateral amygdala determine strain-dependent liability to stress response and differences in coping.

Results: C57BL/6J mice displayed greater immobility in the forced swimming test, higher serotonin outflow in medial prefrontal cortex, higher GABA outflow in basolateral amygdala induced by stress, and higher serotonin 1A receptor levels in medial prefrontal cortex accompanied by lower GABA_B receptor levels in basolateral amygdala than DBA/2J mice. In assessing whether serotonin in medial prefrontal cortex determines GABA functioning in response to stress and passive coping behavior in C57BL/6J and DBA/2J mice, we observed that selective prefrontal serotonin depletion in C57BL/6J and DBA/2J reduced stress-induced GABA outflow in basolateral amygdala and immobility in the forced swimming test.

Conclusions: These results show that strain-dependent prefrontal corticolimbic serotonin/GABA regulation determines the strain differences in stress-coping behavior in the forced swimming test and point to a role of a specific neuronal system in genetic susceptibility to stress that opens up new prospects for innovative therapies for stress disorders.

Keywords: serotonin, GABA, basolateral amygdala, medial prefrontal cortex, strain

Introduction

Comparative studies on neurotransmitter activity in different brain regions of inbred strains of mice represent a major strategy to investigate neurochemical mechanisms underlying behavioral expression. The genetic stability of inbred strains over the years and through laboratories has allowed much relevant information to be accumulated for several strains commonly used in the field. Moreover, behavioral, pharmacological, physiological, and

biochemical comparisons between inbred strains represent a preliminary stage of more thorough genetic research as quantitative trait loci, or recombinant inbred (available for C57BL/6J [C57] and DBA/2J strains [DBA]) analyses to identify and map genes in the mouse, a species characterized by broad gene homology with humans. Various studies have shown that mice of the C57 and DBA strains differ in their behavior outcomes in the forced

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swimming test (FST). C57 mice show high immobility and DBA mice show low immobility (Cabib and Puglisi-Allegra, 1996; Alcaro et al., 2002; Ventura et al., 2002), an index of passive coping and depression-like phenotype. Pharmacological (Lucki et al., 2001) or neurochemical (Ventura et al., 2002; Calcagno et al., 2007) studies have shown differences in brain neurotransmitters that have been linked to strain differences in passive coping behavior. As recently shown in C57 mice, a background commonly used in molecular approaches, amygdalar γ -aminobutyric acid (GABA) regulation by prefrontal serotonin (5-HT) is crucial in processing stressful experiences and determining passive coping outcomes measured by immobility in the FST (Andolina et al., 2013). Indeed, sustained, stress-induced 5-HT outflow in the medial prefrontal cortex (mpFC) and GABA outflow in the basolateral amygdala (BLA) lead to sustained immobility, whereas prefrontal 5-HT to GABA BLA disconnection leads to low immobility in the FST (Andolina et al., 2013). Moreover, we showed that 5-HT depletion in mpFC determined an increase in c-fos expression in the BLA during the FST, thus pointing to a control of prefrontal 5-HT transmission on neuronal activation and GABAergic transmission in amygdala during stress (Andolina et al., 2013). Genetic variation in cortico-amygdala system is strongly implicated in susceptibility to stress-related disorders such as anxiety and depression as shown by a body of evidence (Drevets et al., 1992; Holmes, 2009; Wellman et al., 2009). DBA mice are characterized by prefrontal 5-HT functioning that differs from that in the C57 strain (Calcagno et al., 2007). In particular, DBA mice present lower 5-HT transporter binding and lower immobility in the FST than C57 (Sugimoto et al., 2008; Popova et al., 2009). Moreover, DBA mice are homozygous for the 1473G allele of mouse TPH-2, linked to low 5-HT synthesis rate, while C57BL/6 mice are homozygous for the 1473C allele (Zhang et al., 2004; Cervo et al., 2005). This allelic variant in DBA causes lower brain 5-HT synthesis than in C57BL/6 mice carrying the "C" allele (Zhang et al., 2004; Cervo et al., 2005). Moreover, differences between C57 and DBA mice have been reported for amygdala functioning that have been linked to strain-dependent difference in stress responsiveness (Dubois et al., 2006; Yang et al., 2008; Mozhui et al., 2010). Thus, these strains are a model of choice for investigating individual differences in the prefrontal-amygdalar system and related serotonin-GABA functioning in stress-induced coping behavior.

Here, we investigated whether prefrontal/amygdala connectivity mediated by 5-HT and GABA transmission is a critical neural substrate determining strain-dependent differences in stress response and passive coping behavior. First, we phenotyped the 2 strains for 5-HT and GABA receptor subtype distribution in mpFC and BLA. Indeed, 5-HT_{1A} and GABA_B receptors are known to be involved in stress response and stress-related behavior (Heisler et al., 1998; Cousins and Seiden, 2000; Cryan and Kaupmann, 2005; Car and Wisniewska, 2006; Frankowska et al., 2007; Shishkina et al., 2012). Then, we assessed the response of prefrontal 5-HT and amygdalar GABA to stress. To this aim, we used intracerebral microdialysis to compare the response with an acute stressor (restraint) of 5-HT in mpFC and GABA in BLA in the 2 strains. Restraint was chosen to evaluate the time-dependent changes induced by stress in mpFC 5-HT and BLA GABA outflow of C57 and DBA mice. Because we found that C57 and DBA mice differ in 5-HT outflow in mpFC and GABA in the BLA in response to stress, we hypothesized that these differences are responsible for passive coping behaviors characterizing the 2 strains. Stress-induced GABA release in the BLA and behavior in the FST were thus compared in C57 and DBA bearing a selective 5-HT depletion in mpFC. To rule out nonspecific effects of prefrontal 5-HT depletion, locomotor activity and anxiety were also assessed.

Methods

Animals

Male mice of the inbred C57BL/6Jlco (C57) and DBA/2Jlco (DBA) strains (Charles River, Italy), 8 to 9 weeks old at the time of experiments, were housed as previously described (Ventura et al., 2002). All experiments were conducted in accordance with Italian national law (Decreto Legislativo no. 116, 1992) governing the use of animals for research.

Drugs

Chloral hydrate, 5,7-dihydroxytryptamine (5,7-DHT), and desipramine hydrochloride (DMI) were purchased from Sigma-Aldrich (St. Louis, MO). Chloral hydrate (350–450 mg/kg) and DMI were dissolved in saline (0.9% NaCl) and injected intraperitoneally (i.p.) in a volume of 10 mL/kg. The 5,7-DHT was dissolved in saline containing ascorbic acid (0.1%).

Stress Protocols

The restraint apparatus was formed by an adjustable neck-blocking support mounted on a Plexiglas base and movable U-shaped metal piece that could be fixed to the base at the level of the animal's hips, thus preventing the mouse from turning on its back (Cabib and Puglisi-Allegra, 1991). The FST was as previously described (Alcaro et al., 2002; Cabib et al., 2002). Briefly, mice were individually placed in a glass cylinder (diameter 18 cm, height 40 cm) containing 20 cm of fresh tap water maintained at $28 \pm 2^\circ\text{C}$ during a single session lasting 10 minutes and tested only once to assess coping behavior in a new stressful condition. The behavior of the animals was monitored by a video system and scored by a trained observer blind to the animals' treatment.

Elevated Plus Maze

Emotional reactivity was measured by the behavioral responses to the Elevated Plus Maze as previously described (Cabib et al., 2003). Briefly, mice were individually tested in a single 5-minute session. At the beginning of each test, the mouse was placed on the center facing an open arm to initiate the test session. The number of entries and the time (seconds) spent inside each type of arm were recorded. Two measures were considered: the percentage of entries in the open arms (open entries/open closed $\times 100$) and the percentage of time spent in the open arms (time in open/open closed $\times 100$).

Locomotor Activity

Locomotor activity was measured by a single session of open field test. The apparatus was a circular open field, 60 cm in diameter and 20 cm in height. Each mouse was introduced individually for 5 minutes and distance travelled (centimeters) was recorded. The apparatus was cleaned between subjects.

Microdialysis

Animals were anaesthetized with chloral hydrate, mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) equipped with a mouse adapter, and implanted unilaterally with a guide cannula (stainless steel, shaft outer diameter 0.38 mm, Metalant AB, Stockholm, Sweden) in mpFC or BLA. Guide cannula implantation in mpFC and BLA was counterbalanced for left and right

hemisphere. The length of the guide cannula was 1mm (mpFC) and 4.5mm (BLA) for C57 and 1mm (mpFC) and 4.0mm (BLA) for DBA. The guide cannula was fixed with epoxy glue, and dental cement was added for further stabilization. Since the brains of C57 and DBA mice are different in size and weight, different coordinates for probe implantation were used for the 2 backgrounds. The coordinates from bregma (measured according to the atlas of Franklin and Paxinos, 1997 and Mouse Brain Atlases, The Mouse Brain Library, www.nervenet.org/mbl/) were: mpFC: C57 = 2.5 AP, 0.6 L; DBA = 2.0 AP, 0.6 L. BLA: C57 = -1.16 AP, -3.55 L; DBA = -0.9 AP, -3.05 L. The probe (dialysis membrane length: 2 mm for mpFC and 1 mm for BLA; outer diameter 0.24 mm, MAB 4 cuprophane microdialysis probe, Metalant AB) was introduced 24 hours after implantation of the guide cannula. The animals were lightly anaesthetized to facilitate manual insertion of the microdialysis probe into the guide cannula. The day before use, the membranes were tested to verify in vitro 5-HT and GABA recovery. The microdialysis probe was connected to a CMA/100 pump (Carnegie Medicine, Stockholm, Sweden) through PE-20 tubing and an ultra-low torque dual channel liquid swivel (Model 375/D/22QM, Instech Laboratories, Inc., Plymouth Meeting, PA) to allow free movement. Artificial cerebrospinal fluid was pumped through the dialysis probe at a constant flow rate of 2 μ L/min. Experiments were carried out 22 to 24 hours after probe placement as previously described (Pascucci et al., 2009). The mean concentration of the 3 samples collected immediately before treatment (<10% variation) was taken as basal concentration. Twenty microliters of the dialysate samples was analyzed by high-performance liquid chromatography (HPLC). The HPLC analysis of 5-HT concentration in the dialysates was as previously described (Pascucci et al., 2009). GABA concentrations in the dialysates were determined as described by Rea et al. (2005). The detection limit of the assay was 4.2 and 0.1 pg/20L (signal-to-noise ratio 2) for GABA and 5-HT, respectively.

Selective 5-HT Depletion in the mpFC

Animals were i.p. injected with DMI (35 mg/kg) 30 minutes before 5,7-DHT microinjection to protect noradrenergic neurons. Bilateral injection of 5,7-DHT (2.5 μ g/0.2 μ L/4 min for each side) or vehicle was made into the mpFC [coordinates: C57: 2.52 AP; \pm 0.6L; 2.0V; DBA: 2.0 AP; \pm 0.6L; 2.0V with respect to bregma (Franklin and Paxinos, 1997; Mouse Brain Atlases, The Mouse Brain Library, www.nervenet.org/mbl/)] through a stainless-steel cannula (0.15 mm outer diameter, UNIMED, Lausanne, Switzerland) connected to a 1- μ L syringe by a polyethylene tube and driven by a CMA/100 pump (Figure 1). The cannula was left in place for an additional 2 minutes after the end of the infusion. Sham animals (sham) were subjected to the same treatment but received intracerebral vehicle. Note that preliminary experiments showed that naive mice were not significantly different from sham in our testing conditions. Animals were implanted with a guide cannula in the mpFC and the BLA and used for microdialysis experiment 7 days after surgery, as previously described.

5-HT, norepinephrine (NE), and dopamine (DA) tissue levels in the mpFC (prelimbic [PL] or infralimbic, Franklin and Paxinos, 1997; Mouse Brain Atlases, The Mouse Brain Library, www.nervenet.org/mbl/) were assessed to evaluate the amount and extent of 5,7-DHT-induced depletion. Punches were obtained from brain slices (coronal sections) not thicker than 300 μ m (Puglisi-Allegra et al., 2000). Stainless-steel tubes of 0.5 or 1.0 mm inside diameter were used. The coordinates were measured according to the atlas of Franklin and Paxinos (1997) and Mouse Brain Atlases (The Mouse Brain Library, www.nervenet.org/mbl/). Monoamine tissue level analysis was as previously described (Ventura et al., 2007; Andolina et al., 2013).

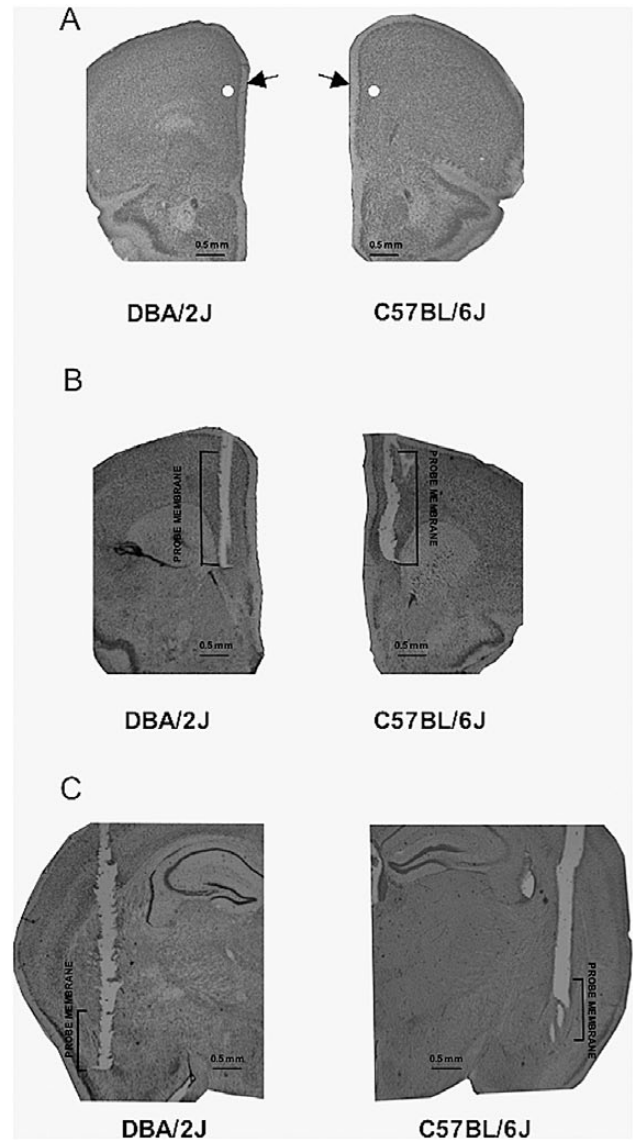


Figure 1. Representative positions of approximate location of 5,7-dihydroxytryptamine (5,7-DHT) point microinjection (white dot) in medial prefrontal cortex (mpFC) in C57BL/6J (C57) and DBA/2J (DBA) (A). The arrow indicates the point of microinjection. Representative positions of the probe in the mpFC (B) and basolateral amygdala (BLA) (C) in DBA mice and C57 mice. The segment of the membrane probe is also shown.

Probe Placement

At the end of the experiment, mice were killed by decapitation. Brains were postfixed in 4% paraformaldehyde, and correct probe placements were checked by visual inspection of the probe tracks on Nissl-stained coronal sections (40 μ m). Only mice with correct probe placement in BLA and mpFC were considered in the results (Figure 1).

Immunohistochemistry

Animals were transcardially perfused with 50 mL of saline followed by 40 mL of 4% paraformaldehyde under anesthesia that was induced by i.p. injections of chloral hydrate. Each brain was immediately removed, postfixed in the same paraformaldehyde solution for 2 hours, and after 3 washes in phosphate-buffered saline (PBS) was transferred to a 30% sucrose solution at 4°C

until it sank. Each brainstem was cut into 4 series of 30- μ m-thick transverse sections using a freezing microtome, and slices were collected in PBS. The following primary antibodies were used: rabbit polyclonal anti-5-HT_{1A} receptor (Immunological Sciences; 1:200), mouse monoclonal anti GABA_BR1 receptor (Immunological Sciences; 1:100), and rabbit polyclonal anti-parvalbumin (Immunological Sciences; 1:200). One series of sections (3 sections, distance approximately C57: 2.71, 2.22, and 1.98 mm; DBA: 2.80, 2.40, and 1.70 from bregma for PL area; C57: -1.28, -1.64, and -2.12 mm, DBA: -1.06, -1.46, and -1.70 from bregma for BLA, according to the above-mentioned atlas (Franklin and Paxinos, 1997; Mouse Brain Atlases, The Mouse Brain Library, www.nervenet.org/mbl/) was incubated overnight with 5-HT_{1A} primary antibody or a cocktail of GABA_BR1 and parvalbumin. All primary antibody solutions were prepared in PBS and 0.3% Triton X-100 and incubated overnight at 4°C. After 3 washes in PBS, sections were incubated 2 hours at room temperature with a cocktail of secondary antibodies, including Cy2-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch, West Grove, PA) and Cy3-conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch, West Grove, PA) and counterstained with NeuroTrace 640/660 fluorescent Nissl (Invitrogen). Sections were examined under a confocal laser scanning microscope (Zeiss, LSM700, Germany) equipped with 4 laser lines: violet diode emitting at 405 nm (for DAPI), argon emitting at 488 nm, and helium/neon emitting at 543 and 633 nm. Quantification of the 5-HT_{1A} receptor in PL area and GABA_B receptor in BLA was performed by densitometric analyses. After background subtraction, 5-HT_{1A} receptor- and GABA_B receptor-associated signals were quantified by manually outlining individual cells and measuring cell-associated fluorescence intensity with the ImageJ software (<http://rsb.info.nih.gov/ij/>). The ratio F/A defines mean fluorescence of individual cells (F) normalized to total cellular surface (A). Quantification was done on 3 sections for PL (C57: 2.71, 2.22, and 1.98 mm; DBA: 2.80, 2.40, and 1.70 from bregma) and on 3 sections for BLA (C57: -1.28, -1.64, and -2.12 mm, DBA: -1.06, -1.46, and -1.70 from bregma) per animal (n=6/group).

Statistics

Immunohistochemistry

C57 vs DBA differences in 5-HT_{1A} in mpFC and GABA_B in BLA expression were evaluated by Student's t test for each brain structure (PL and BLA).

Microdialysis

Data on the effect of restraint stress on 5-HT release in mpFC and GABA release in BLA were statistically analyzed by repeated-measures analysis of variance (ANOVA) with 2 between factors (strain, 2 levels: C57 and DBA; treatment, 2 levels: sham and 5-HT depleted) and one within factor (minutes, 7 levels: 0, 20, 40, 60, 80, 100, and 120 minutes of restraint). Statistical analyses were performed on raw data (concentration of pg/20 μ L). Simple effects were assessed by 1-way ANOVA for each time point. The effects of strain on basal extracellular 5-HT levels in mpFC and GABA levels in BLA and the effect of selective 5-HT depletion in mpFC on basal extracellular 5-HT levels in mpFC and basal extracellular GABA levels were analyzed in each group (C57 sham, C57 5-HT depleted, DBA sham, DBA 5-HT depleted). Duncan's test was used post hoc in this case.

FST

Statistical analyses for the FST were run on the duration (second) of immobility, swimming, and struggling behavior during a 10-minute test. Data were analyzed by 2-way ANOVA (factors: strain, 2 levels: C57 and DBA; treatment, 2 levels: sham and 5-HT depleted).

5-HT Depletion in mpFC

The effects of prefrontal 5-HT depletion on tissue levels of 5-HT, DA, and NE in mpFC (PL or IF) in C57 and DBA mice were analyzed by 2-way ANOVA. The factors were: treatment (2 levels: C57 sham and C57 5-HT depleted) for C57, and 2 levels (DBA sham and DBA 5-HT depleted) for DBA and experiment (2 levels: FST experiment and microdialysis experiments). Statistical analyses were carried out on data from the FST and microdialysis experiments.

Elevated Plus Maze

Statistical analyses for Elevated Plus Maze were run on the percentage of entries in the open arms (open entries/open closed \times 100) and the percentage of time spent in the open arms (time in open/open closed \times 100) during a 5-minute test. Data were analyzed by 2-way ANOVA (factors: strain, 2 levels: C57 and DBA; treatment, 2 levels: sham and 5-HT depleted).

Locomotor Activity

Statistical analyses for the open field test were run as distance travelled (cm) during a 5-minute test. Data were analyzed by 2-way ANOVA (factors: strain, 2 levels: C57 and DBA; treatment, 2 levels: sham and 5-HT depleted).

For experiments (microdialysis, FST, 5-HT depletion, Plus Maze, open field), individual between-group comparisons were carried out when appropriate by a posthoc test (Duncan's multiple-range test).

Results

Effect of Strain on 5-HT_{1A} Receptor Expression in mpFC and GABA_B Receptor Expression in BLA

Densitometric analysis in the PL area revealed that C57 mice showed significantly higher levels of 5-HT_{1A} receptor expression than DBA mice ($t=2.53$, $df=10$; $P<.05$) (Figure 2), whereas it showed in the BLA GABA_B receptor that C57 mice had a significantly lower level of GABA_B receptors than DBA mice ($t=3.06$, $df=10$; $P<.05$) (Figure 3).

Effects of 5-HT Depletion in mpFC on Stress-Induced 5-HT Outflow in mpFC and GABA Outflow in BLA in C57 and DBA Mice

First, a significant difference was found in basal extracellular 5-HT levels in mpFC and GABA levels in the BLA of C57 and DBA mice, whereas selective 5-HT depletion of mpFC in C57 and DBA mice did not change basal extracellular 5-HT levels in mpFC and GABA levels in BLA compared with C57 sham and DBA sham, respectively [5-HT: $F_{3,27}=30.08$; $P<.01$; C57 sham (n=8), 0.92 ± 0.073 pg/20 μ L; DBA sham (n=8), 0.36 ± 0.07 pg/20 μ L; C57 5-HT-depl (n=8), 1.08 ± 0.079 pg/20 μ L; DBA 5-HT depl (n=7), 0.33 ± 0.48 pg/20 μ L;

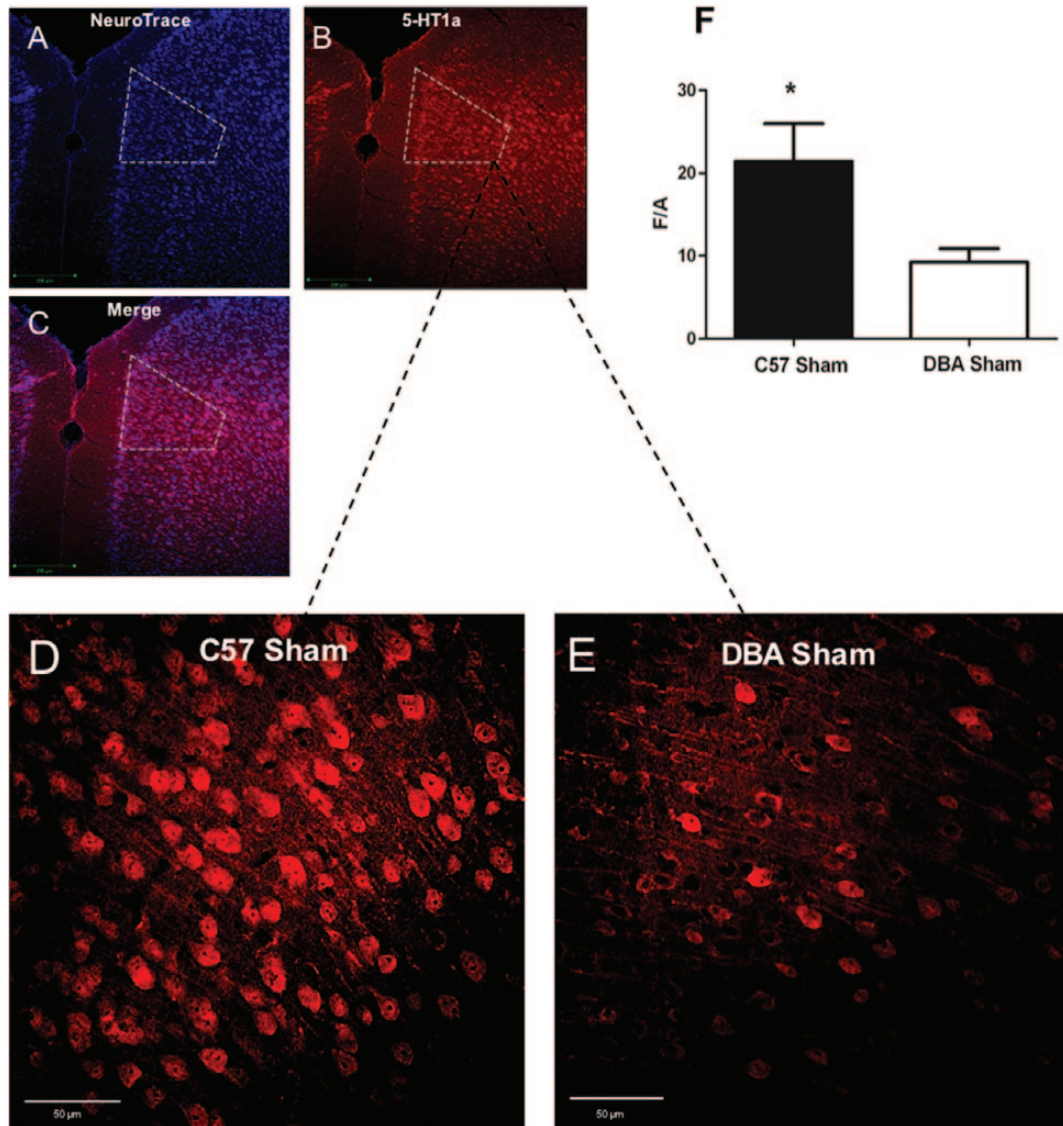


Figure 2. Serotonin (5-HT1A) immunoreactivity in the medial prefrontal cortex (mpFC) prelimbic area (PL) of C57BL/6J (C57) sham and DBA/2J (DBA) sham double-labeled confocal images of NeuroTrace counterstaining (A: blue) and 5-HT1A labeling (B: red) plus merged (C). Panels D and E are higher magnification pictures from PL of C57 sham (D) and DBA sham (E) reacted with 5-HT1A antibody. Histogram of densitometric values (F) of 5-HT1A immunofluorescence expressed as mean fluorescence of individual cells normalized to total cellular surface (F/A). Data are reported as means \pm SD (n=6 animal/group). * $P < .05$; scale bars: A-C=200 μ m; D-E=50 μ m.

GABA: $F_{3,28} = 8.23$; $P < .01$; C57 sham (n=8), 57.9 ± 6.4 pg/20 μ L; DBA sham (n=8), 27.16 ± 4.4 pg/20 μ L; C57 5-HT depl (n=8), 63.87 ± 9.1 pg/20 μ L; DBA 5-HT depl (n=8), 29.44 ± 5.8 pg/20 μ L]. The effects of restraint on 5-HT release in mpFC and GABA release in BLA are shown in Figure 4. Statistical analyses revealed a significant interaction of strain \times treatment \times time for both the 5-HT outflow in mpFC and GABA outflow in BLA (5-HT: $F_{6,19} = 2.01$; $P < .01$; GABA: $F_{6,19} = 1.73$; $P < .05$). Restraint produced a time-dependent increase in 5-HT outflow in mpFC and in GABA outflow in the BLA in both strains, but C57 sham mice displayed significantly higher 5-HT levels in mpFC and higher GABA levels in BLA than DBA sham mice. Selective 5-HT depletion in mpFC of C57 and DBA mice dramatically reduced the increase in 5-HT outflow in mpFC and of GABA in BLA. Note that no significant differences were evident between the left and right sides of the mpFC or BLA probe implanted on 5-HT and GABA outflow, respectively. Prefrontal 5,7-DHT in C57 and DBA mice produced a significant decrease in 5-HT tissue levels in PL and infralimbic, whereas it spared NE and DA (Table 1).

The fact that selective 5-HT depletion of mpFC does not change basal extracellular 5-HT levels suggests that spared serotonergic afferents develop a compensatory response that leads to an extracellular 5-HT outflow similar to that of sham animals, in agreement with previous studies (Kirby et al., 1995; Hall et al., 1999). Whether this compensatory response depends on increased neurotransmitter synthesis or on other mechanisms remains to be ascertained. However, 5-HT depletion abolished the serotonergic response to stress in the mpFC, possibly indicating that compensatory response does not allow additional increase in 5-HT outflow after stress challenge.

Effects of Prefrontal Cortical 5-HT Depletion on Stress-Coping Behavior in the FST in C57 and DBA Mice

As for immobility, although 2-way ANOVA did not reveal significant interaction strain \times treatment ($F_{1,28} = 0.83$, $P = .36$), analysis

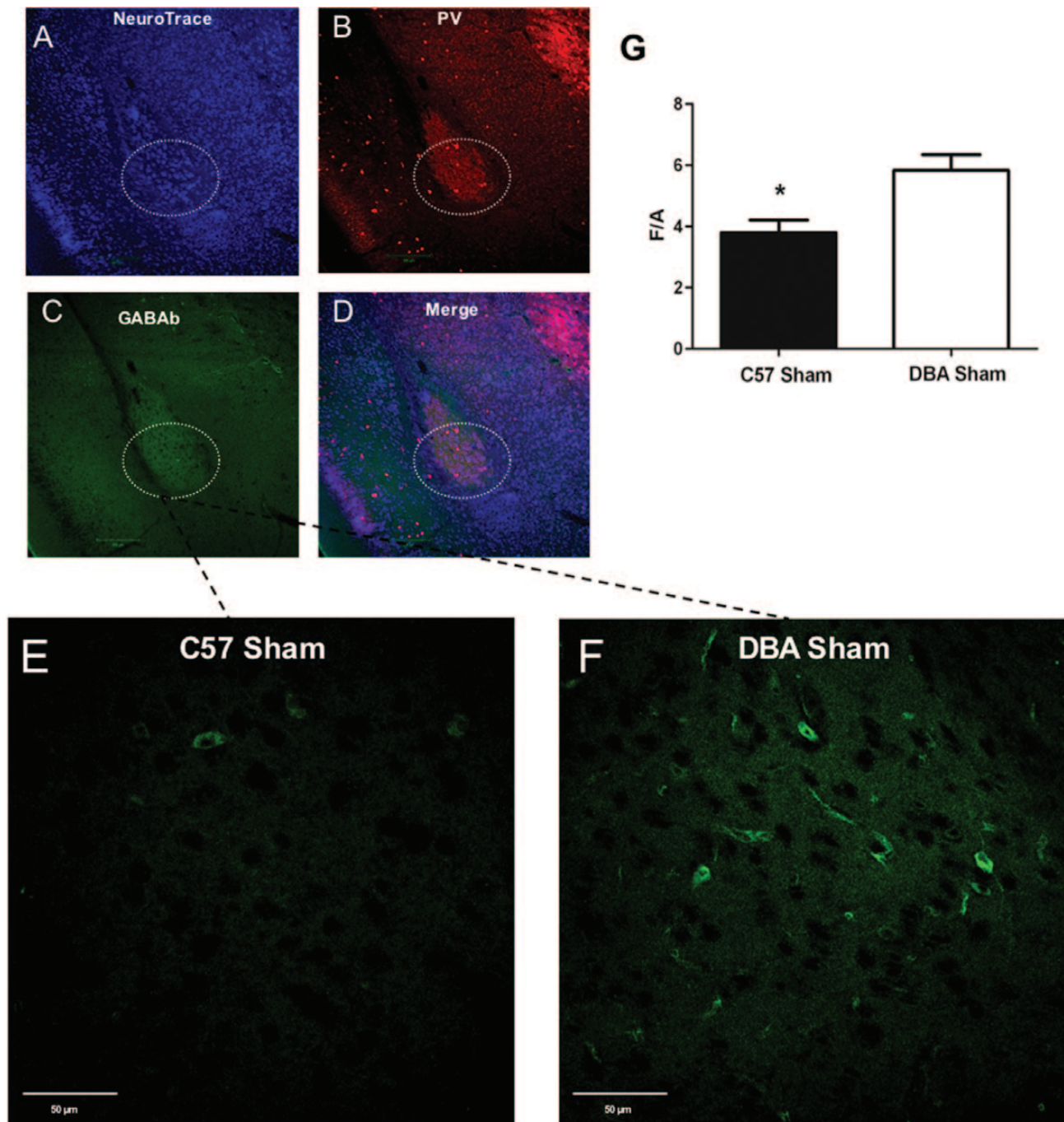


Figure 3. GABAergic immunoreactivity in the basolateral amygdala (BLA) of C57BL/6J (C57) sham and DBA/2J (DBA) sham. Triple-labeled confocal images of NeuroTrace counterstaining (A: blue), and parvalbumin labeling (B: red), GABAergic (C: green) plus merged (D) of BLA. Panels E and F are higher magnification pictures from BLA of C57 (E) and DBA (F) reacted with GABAergic antibody. Histogram of densitometric values (G) of GABAergic immunofluorescence expressed as mean fluorescence of individual cells normalized to total cellular surface (F/A). Data are reported as means \pm SD (n=6 animals/group). * $P < .05$. Scale bars: A-D=200 μ m; E-F=50 μ m.

showed significant effects of strain ($F_{1,28}=31.85$, $P < .001$) and treatment ($F_{1,28}=14.32$, $P < .001$). Duncan's test showed in C57 mice significantly higher levels of immobility than in DBA mice, while in mpFC 5-HT-depleted C57 and DBA mice, significantly lower immobility was evident compared with C57 sham and DBA mice, respectively [C57 sham (n=8), 395.57 ± 17.48 seconds; DBA sham (n=8), 251.40 ± 11.45 seconds; C57 5-HT depl (n=8), 292.27 ± 37.40 seconds; DBA 5-HT depl (n=8), 188.30 ± 9.86] (Figure 5). It should be noted that the decrease in immobility behavior in C57 and DBA mice receiving the selective 5-HT

depletion in mpFC is accompanied by a significant increase in swimming behavior (Table 2).

Effects of Prefrontal Cortical 5-HT Depletion on Elevated Plus Maze in C57 and DBA Mice

As for the elevated plus maze, 2-way ANOVA revealed significant effects of strain (percent time open/tot: $F_{1,28}=32.78$, $P < .001$; percent time closed/tot: $F_{1,28}=25.04$, $P < .001$; percent entries open/tot: $F_{1,28}=43.92$, $P < .001$; entries closed/tot: $F_{1,28}=43.92$, $P < .001$) and no

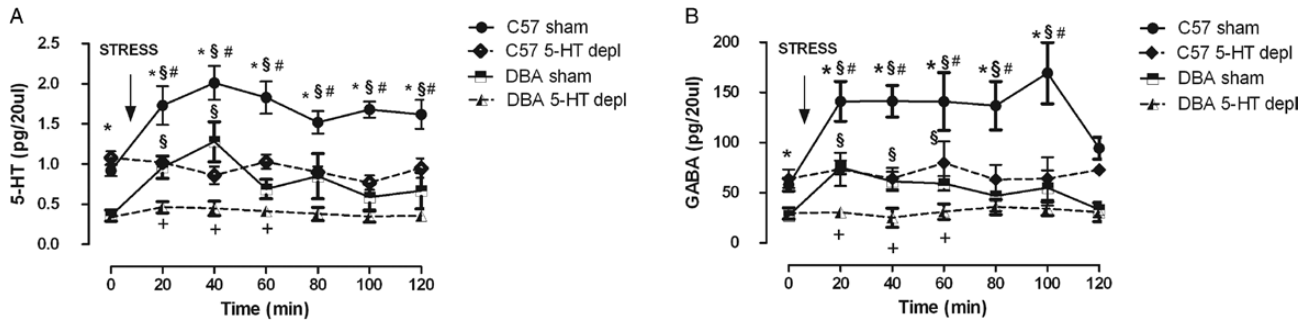


Figure 4. Effects of 120-minute restraint stress on serotonin (5-HT) outflow in the medial prefrontal cortex (mpFC) (A) and GABA outflow in the basolateral amygdala (BLA) (B) of sham C57 and DBA mice and of C57 and DBA mice bearing a selective prefrontal cortical 5-HT depletion in the mpFC (C57 5-HT Depl, DBA 5-HT Depl). Arrows indicate the beginning of the restraint. § $P < .05$ from the basal values. * $P < .05$, C57 sham in comparison with the corresponding time point of DBA sham group. # $P < .05$ C57 sham in comparison with the corresponding time point of C57 5-HT Depl group. + $P < .05$ DBA sham in comparison with the corresponding time point of DBA 5-HT Depl group.

Table 1. Effects of 5,7-DHT Infusion in mpFC on 5-HT, NE, and DA Tissue Levels (ng/g Wet Weight) in mpFC Areas (Prelimbic Cortex and Infralimbic Cortex) of the C57 Sham, C57 5-HT-Depleted, DBA Sham, and DBA 5-HT-Depleted Groups

	Sham	5-HT depleted
Prelimbic cortex (C57)		
5-HT	815 ± 122	147 ± 42**
NE	655 ± 16	644 ± 54
DA	119 ± 61	108 ± 38
Infralimbic cortex (C57)		
5-HT	432 ± 90	161 ± 37*
NE	398 ± 29	405 ± 32
DA	105 ± 26	128 ± 22
Prelimbic cortex (DBA)		
5-HT	808 ± 41	97 ± 19**
NE	945 ± 48	1056 ± 50
DA	95 ± 3	91 ± 4
Infralimbic cortex (DBA)		
5-HT	600 ± 39	91 ± 13**
NE	875 ± 46	1028 ± 70
DA	156 ± 21	177 ± 21

Abbreviations: DA, dopamine; DBA, DBA/2J; 5,7-DHT, 5,7-dihydroxytryptamine; 5-HT, serotonin; mpFC, medial prefrontal cortex; NE, noradrenaline.

* $P < .01$; ** $P < .001$.

significant effects of treatment (percent time open/tot: $F_{1,28} = 1.51$, $P = .22$; percent time closed/tot: $F_{1,28} = 3.34$, $P = .08$; percent entries open/tot: $F_{1,28} = 0.01$, $P = .90$; entries closed/tot: $F_{1,28} = 0.01$, $P = .90$) and strain × treatment interaction (percent time open/tot: $F_{1,28} = 1.78$, $P = .19$; percent time closed/tot: $F_{1,28} = 0.05$, $P = .81$; percent entries open/tot: $F_{1,28} = 0.61$, $P = .43$; entries closed/tot: $F_{1,28} = 0.61$, $P = .43$). Duncan's test showed a significant effect of strain (C57 sham, DBA sham) for the percent of time spent in open or closed arms and for the percent of entries in open or closed arms. Selective 5-HT depletion in C57 and DBA did not produce any significant effect for the percent of time spent in open or closed arms, and for total entries in open or closed arms compared with C57 sham and DBA sham, respectively. The data are reported in Figure 6.

Effects of Prefrontal Cortical 5-HT Depletion on Locomotor Activity in C57 and DBA Mice

As for the locomotor activity analysis on open field, 2-way ANOVA revealed no significant effects of strain ($F_{1,28} = 3.11$, $P = .08$) and treatment ($F_{1,28} = 0.30$, $P = .58$) and interaction ($F_{1,28} = 0.7$, $P = .93$) on distance travelled (cm) (data are reported in Figure 7).

Discussion

Evidence points to a clear-cut strain-dependent role of prefrontal 5-HT and amygdalar GABA transmission in stress response and coping behavior. C57 mice are characterized by higher 5-HT1A receptor levels in mpFC accompanied by lower GABA receptor levels in BLA than in DBA mice. Moreover, C57 mice present higher 5-HT steady levels in mpFC and higher GABA steady levels in BLA than DBA. We have shown that, in mice, a stressful experience such as restraint induces a time-dependent increase of 5-HT output in the mpFC and of GABA in the BLA, in agreement with previous reports (Reznikov et al., 2009; Pascucci et al., 2009; Andolina et al., 2013). These results are the first evidence from an in vivo study showing that acute restraint stress produces different effects on mpFC 5-HT and BLA GABA release in C57 and DBA mice, thus affirming that genetic background causes differences in the response of the prefrontal/amygdala 5-HT/GABA system to stress. Interestingly, the 5-HT and GABA release in mpFC and BLA, respectively, are clearly related to sustained immobility in the FST. In fact, selective prefrontal 5-HT depletion in C57 and DBA mice reduces the amine outflow in the mpFC and GABA release in the BLA in response to restraint stress and leads to a dramatic decrease of immobility in both C57 and DBA mice. These results, in line with previous data (Andolina et al., 2013), support the fact that low serotonergic and GABAergic tone in mpFC and BLA is negatively related to passive coping in a stressful condition such as the FST. A causal relationship between serotonergic and GABAergic tone in mpFC and BLA and immobility in the FST has been previously demonstrated. In fact, we showed that the FST induced a clear-cut time-related increase of GABA output in BLA of mice, whereas selective 5-HT depletion in mpFC caused a dramatic decrease of GABA output throughout (Andolina et al., 2013). Note that the results obtained by the open field test showed that the observed differences in immobility behavior in the FST cannot be ascribed to differences in spontaneous locomotor activity. Moreover, they point to a prefrontal/amygdala system in which 5-HT and GABA are strain-dependently orchestrated to control stress-induced adaptive behavioral outcomes in accordance with other evidence showing a major influence of genetic variation in cortico-amygdala serotonin function and individual differences in stress response and risk for stress-related disease (Holmes, 2009; Homberg, 2012).

In line with previous studies, we report that sham DBA mice displayed higher basal anxiety than sham C57 mice in the elevated plus maze (Vöikar et al., 2005; Mozhui et al., 2010), whereas selective 5-HT depletion in mpFC did not affect anxiety in either

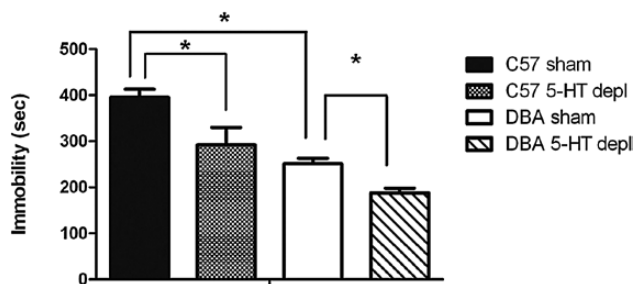


Figure 5. Effects of strain (C57 sham, DBA sham) and of selective prefrontal cortical serotonin (5-HT) depletion in the medial prefrontal cortex (mpFC) of C57 and DBA mice (C57 5-HT Depl; DBA 5-HT Depl) on immobility in the forced swimming test (FST). Results are expressed as mean \pm SE duration (second) of immobility. * $P < .05$. C57 sham in comparison with DBA sham group, C57 5-HT-Depl group and DBA sham group in comparison with DBA 5-HT-Depl group.

Table 2. Level of Swimming and Struggling Behavior in the FST in C57 Sham, C57 5-HT Depleted in mpFC, DBA Sham, and DBA 5-HT depleted in mpFC Groups

	Swimming	Struggling
C57 sham	139.91 \pm 23.34 ^{a,b}	64.51 \pm 10.46
C57 5-HT depleted	221.28 \pm 28.96	86.34 \pm 15.38
DBA sham	257.91 \pm 16.66 ^c	90.69 \pm 16.96
DBA 5-HT depleted	345.8 \pm 15.89	64.79 \pm 6.77

Abbreviations: C57, C57BL/6J; DA, dopamine; DBA, DBA/2J; FST, forced swimming test; 5-HT, serotonin; mpFC, medial prefrontal cortex.

^aC57 sham in comparison with C57 5-HT-depleted group.

^bC57 sham in comparison with DBA sham group.

^cDBA sham in comparison with DBA 5-HT-depleted group.

C57 or DBA mice. These results suggest that a decrease of immobility behavior in the FST in DBA and C57 mice bearing a selective prefrontal 5-HT depletion in the mpFC cannot be ascribed to an alteration of emotional reactivity. Although in this study we evaluated the role of genotype and of the 5-HT prefrontal GABAergic amygdala system in neural and behavioral responses to the first exposure to an aversive experience, the reported differences between C57 and DBA mice could be the basis of differences in fear memory reported in these strains (Waddell et al., 2004). In fact, alteration of fear memory, for example, an impaired fear extinction, is a core symptom of anxiety disorders, such as posttraumatic stress disorder; several studies reported that mpFC 5-HT and BLA GABAergic transmission are involved in this behavioral phenotype (Akirav and Maroun, 2007; Shin and Liberzon, 2010).

Differences in the 5-HT system in C57 and DBA mice have been extensively studied and indicate that these differences underlie strain differences in immobility. For instance, Sugimoto et al. (2008) showed that the amount of 5-HT transporter and 5-HT_{1A} binding in prefrontal cortex was linked to baseline immobility time in C57 and DBA mice (Sugimoto et al., 2008; Popova et al., 2009). In line with these data, our results suggest that differences in prefrontal cortical 5-HT release, which controls BLA GABA release, contribute to strain differences in immobility in these 2 strains. Indeed, selective prefrontal 5-HT depletion in C57 and DBA mice reduces immobility in the FST. Consistent with these data, C57 mice showed a higher level of 5-HT_{1A} receptors in PL cortex compared with DBA mice. Several subtypes of the 5-HT receptor family have been associated with stress response and depressive disorders. However, within this

family, the 5-HT_{1A} receptor has attracted increasing interest in the auxiliary therapy of depression. It has been reported that the 5-HT_{1A} receptor is expressed on pre- and postsynaptic sites. Presynaptic high-density 5-HT_{1A} autoreceptors are located somatodendritically in the raphe nuclei (Albert et al., 1990; Blier and de Montigny, 1990; Ago et al., 2003). Postsynaptic 5-HT_{1A} receptors are widely expressed as heteroreceptors on glutamatergic pyramidal cells and on GABAergic interneurons in the hippocampus, cortical regions, septum, amygdala, and hypothalamus (Pompeiano et al., 1992; Chessell et al., 1993; Wedzony et al., 2007), where their activation leads to decreased firing rates (Sprouse and Aghajanian, 1988; Blier and de Montigny, 1990; Tanaka and North, 1993). Thus, the majority of pyramidal neurons are functionally inhibited by 5-HT in a 5-HT_{1A}-dependent manner (Beique et al., 2004; Zhang and Arsenault, 2005; Goodfellow et al., 2009; Zhong and Yan, 2011), suggesting that 5-HT_{1A} receptors play a dominant role in regulating pyramidal neuron activity. Depressive symptoms and passive coping behavior are associated with prefrontal cortex hypoactivity and changes in 5-HT receptor levels (Hariri et al., 2002; Amat et al., 2005; Canli et al., 2005; Heinz et al., 2005; Pezawas et al., 2005; Firk and Markus, 2007; Maier and Watkins, 2010). Because we found that DBA mice display both lower expression of 5-HT_{1A} in the PL area and lower levels of 5-HT in mpFC mice compared with C57 mice, it is likely that the main consequence of increased extracellular 5-HT in the mpFC of C57 mice is an overinhibition of mpFC neuronal activity through 5-HT_{1A}, leading to an increase in passive-coping behavior in the FST. Thus, our data are consistent with the hypothesis that high 5-HT_{1A} receptor levels in mpFC (PL area) sustain immobility in the FST. Indeed, we found that C57 mice showed both higher levels of 5-HT_{1A} receptor in mpFC and a higher level of immobility in the FST than DBA mice. It is therefore likely that selective 5-HT depletion in mpFC consistently reduces immobility in C57 and DBA mice through consequent reduced stimulation of 5-HT_{1A} receptors. It should be noted that DBA mice (homozygous for the Tph2 allele 1473G) show low immobility in comparison with C57BL/6 mice, which are homozygous for the Tph2 1473C allele (Zhang et al., 2004; Cervo et al., 2005). Moreover, introduction of the 1473G/G single-nucleotide polymorphisms (SNPs) into a C57BL/6N genetic background has been reported to cause a desensitization of 5-HT_{1A} (Berger et al., 2012). In line with this evidence, we found that DBA mice show lower levels of 5-HT_{1A} receptors than C57 mice. The mpFC plays a crucial role in the regulation of stimulus-driven amygdala response, partly through glutamatergic projections to populations of GABAergic neurons within the amygdala (Quirk et al., 2003; Likhtik et al., 2005; Shin et al., 2005). Variability in the structure and function of this corticolimbic circuitry has been associated with individual differences in personality measures, reflecting sensitivity to environmental threat and related risk of psychopathology (Etkin et al., 2004; Pezawas et al., 2005; Shin et al., 2005; Fakra et al., 2009; Holmes, 2009; Homberg, 2012). In particular, human studies have shown that individual differences in prefrontal cortical 5-HT are related to amygdala activity (Fisher et al., 2009, 2011). The GABAergic system is the principal modulator of amygdala and substantial clinical and preclinical evidence implicates a dysfunction of the GABA system in depression (Krystal et al., 2002; Brambilla et al., 2003). Rodent studies have shown that GABA in the amygdala is involved in depression-like behavior in the FST (Ebner et al., 2005; Andolina et al., 2013). For instance, evidence suggests that GABA_B receptor agonist baclofen inhibit central neural circuits and peripheral sympathetic nervous system that are involved in the stress response (Bolser et al., 1995).

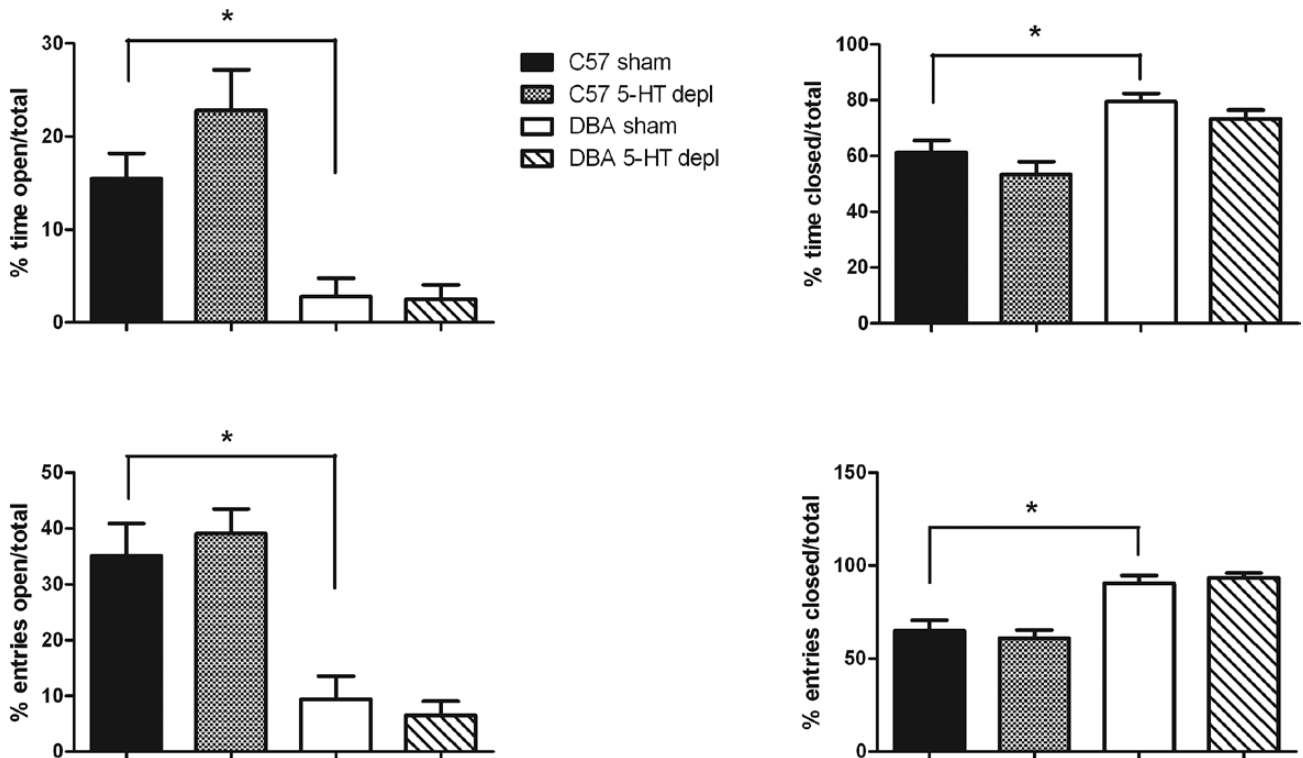


Figure 6. Effects of strain (C57 sham, DBA sham) and of selective prefrontal cortical serotonin (5-HT) depletion in the medial prefrontal cortex (mpFC) of C57 and DBA mice (C57 5-HT Depl; DBA 5-HT Depl) on anxiety behavior in the elevated plus maze. Data are expressed as mean percent time spent or mean percent entries made in the open arm and as mean percent time spent or mean percent entries made in closed arms \pm SE. * $P < .05$.

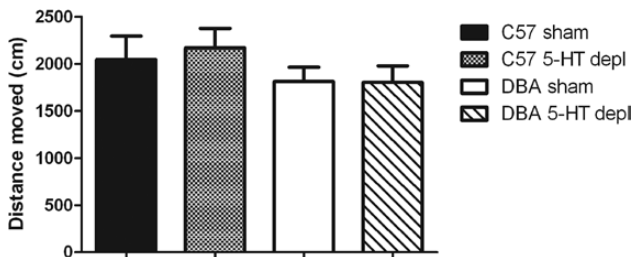


Figure 7. Effects of strain (C57 sham, DBA sham) and of selective prefrontal cortical serotonin (5-HT) depletion in the medial prefrontal cortex (mpFC) of C57 and DBA mice (C57 5-HT Depl; DBA 5-HT Depl) on locomotor activity in the open field. Results are expressed as mean \pm SE distance moved (cm).

Moreover, high levels of GABA_B receptor expression in the BLA have been shown to play an important role in regulating emotional behavior and depression (McDonald et al., 2004; Cryan and Kaupmann, 2005), and there is evidence of baclofen having antidepressant effects in the FST (Cryan and Kaupmann, 2005; Car and Wiśniewska, 2006; Frankowska et al., 2007). In line with this evidence we found that C57 mice show higher levels of immobility behavior in the FST and lower GABA_B expression in BLA than in DBA mice. Interestingly, an antidepressant effect of baclofen could be mediated by a decrease of GABA release. It has been reported that baclofen produces a decrease of GABA release (Rea et al., 2005). In support of this hypothesis, we found that DBA show lower levels of immobility behavior in the FST and lower GABA levels in BLA accompanied by increased expression of GABA_B than C57 mice.

Many studies suggest that prefrontal cortex-amygdala system is highly implicated in stress response and stress-related

disturbances (Siegle et al., 2002; Phillips et al., 2003; Phelps et al., 2004; Akirav and Maroun, 2007; Qi et al., 2008). Moreover, dysfunctions of this neural circuit have been associated with individual differences in risk for psychopathology (Drevets et al., 1992; Pezawas et al., 2005, Holmes, 2009). mpFC is considered to play a critical role in regulation of amygdala-mediated arousal in response to emotionally salient stimuli (Quirk et al., 2003; Likhnik et al., 2005) through 5-HT (LeDoux, 2000; Martín-Ruiz et al., 2001; Fisher et al., 2009, 2011). A growing body of evidence, in particular rising from genetically modified or inbred mice, has provided significant insight into the way genetic variation in the 5-HT system can affect the development and functioning of mpFC-amygdala circuitry (Holmes, 2009). For instance, Wellman et al. (2007) showed that the loss of 5-HTT gene function, leading to a marked increase in extracellular levels of 5-HT in different brain region including frontal cortex (Mathews et al., 2004), compromises the capacity to cope with environmental stress and causes morphological abnormalities in both BLA and mpFC. Our results demonstrate that mpFC controls amygdala to moderate stress response and its intensity through 5-HT transmission in mpFC modulating amygdalar GABA depending on genetic background.

The prefrontal-amygdala system envisaged here involves a parallel stress-induced increase in 5-HT and GABA transmission in the 2 brain regions, the former controlling the latter through neural pathways (circuitry) that can be validly hypothesized (Amat et al., 1998; Rainnie, 1999). Evidence suggests that stress-induced increase of prefrontal cortical 5-HT release overinhibits mpFC glutamatergic neuronal activity through 5-HT_{1A} (for review, see Puig and Gullledge, 2011), thus inhibiting GABA release within the DRN, leading to permissive effects on 5-HT neurons in DRN and producing increased 5-HT

release in BLA. Since 5-HT is a modulator of GABA release in BLA (Rainnie, 1999), increased 5-HT is likely to increase GABA activity in this area and increase passive coping behavior. This hypothesis is supported by data showing that passive coping is related to pronounced 5-HT immunostaining in the BLA (Lehner et al., 2006). It should be noted that various studies suggest a link between the GABAergic and monoaminergic hypotheses of depression (Pilc and Lloyd, 1984; Lloyd et al., 1985; Slattery et al., 2005). Thus, taken together, the data presented herein point to a role of 5-HT and GABA neurotransmission in the prefrontal cortex-amygdala system in stress response and coping outcomes. Moreover, they point to a role of 5-HT and GABAergic neurotransmission in the prefrontal cortex/amygdala system in strain-dependent susceptibility to stress response and stress coping and suggest a way of developing therapeutic approaches for the treatment of depression by combining actions on different neurotransmitter systems, especially 5-HT and GABA.

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Statement of Interest

None.

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