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A. SKORZEWSKA[1], A. WISLOWSKA-STANEK[2], M. LEHNER[1], D. TURZYNSKA[1],
A. SOBOLEWSKA[1], P. KRZASCIK[2], A. PLAZNIK[1,2]

CORTICOTROPIN RELEASING FACTOR RECEPTOR 1 ANTAGONIST DIFFERENTIALLY INHIBITS FREEZING BEHAVIOR AND CHANGES GAMMA-AMINOBUTYRIC ACID-ERGIC ACTIVITY IN THE AMYGDALA IN LOW- AND HIGH-ANXIETY RATS

[1]Department of Neurochemistry, Institute of Psychiatry and Neurology, Warsaw, Poland;
[2]Department of Experimental and Clinical Pharmacology, Medical University, Warsaw, Poland

The aim of this study was to examine the effects of non-peptide corticotropin-releasing factor receptor 1 (CRF_1) antagonist (antalarmin) administration on rat conditioned fear responses and gamma-aminobutyric acid (GABA)-ergic brain activity (GAD67 expression and GABA concentration) in low-anxiety (LR) and high-anxiety (HR) rats. The animals were divided into the LR and HR groups based on the duration of their conditioned freezing response in the first contextual fear test. After 28 days, the animals were re-subjected to the contextual fear training and test. The rats received an antalarmin injection (10 mg/kg or 20 mg/kg) 80 min before the second exposure to the aversive context. Antalarmin significantly attenuated the conditioned fear response only in the HR rats. The behavioral effect of a lower dose (10 mg/kg) of antalarmin was accompanied by increased GAD67 expression in the prelimbic cortex (PL) and central nucleus of the amygdala (CeA) and an increased GABA concentration in the amygdala. These studies showed that HR rats were more susceptible to the anxiolytic effects of CRF_1 antagonist administration, which were associated with increased GABAergic activity in the medial prefrontal cortex and amygdala. The current data may provide insights into the neurobiological mechanism operating within the mesolimbic CRF-GABA neurotransmitter systems, which may be responsible for individual differences in stress-related diseases. This knowledge can be applied to further elucidate the pathophysiology of anxiety and trauma/stress-related disorders.

Key words: *contextual fear re-conditioning, antalarmin, corticotropin-releasing factor, gamma-aminobutyric acid, prefrontal cortex-amygdala circuitry, stress*

INTRODUCTION

The amygdala and medial prefrontal cortex (mPFC) are structures that are engaged in the modulation of anxiety and fear learning (1-4). Evidence strongly supports a role for the basolateral amygdala (BLA) as a critical structure for the formation and storage of fear memory (3-5). The central nucleus of the amygdala (CeA) is required for the acquisition, consolidation and expression of fear memories in parallel with the BLA (1-3, 6). Corticotropin-releasing factor (CRF) has been shown to play an important role within the amygdala in fear learning processes by acting at the CRF_1 and CRF_2 receptors. Stimulation of the CRF_1 receptors induces hormonal and behavioral stress-like responses (7-9). Mice lacking CRF_1 receptors display reduced anxiety and selective CRF_1 receptor antagonists inhibit the anxiogenic action of CRF (10). The BLA contains a high density of CRF_1 receptors. In contrast, the CeA contains many CRF-expressing neurons but lacks strong CRF receptor expression (3, 11). The infusion of a CRF_1 receptor antagonist (DMP696) into the BLA disrupts contextual fear conditioning (12). Other studies have found that intra-CeA infusion of a non-selective CRF antagonist (alpha-helical CRF_{9-41}) prior to contextual fear conditioning or administration of the CRF antisense oligonucleotide at different time points after contextual fear conditioning is effective at attenuating the acquisition and expression of the fear response (3, 13-15).

The mPFC is recognized as important in mediating learning, attention and emotional behavior (16-18). Rats given mPFC lesions prior to training express a stronger fear reaction than control rats (19). Immunocytochemical studies revealed that CRF was present in the mPFC and was expressed in glutamate decarboxylase-positive interneurons in the cerebral cortex (20-21). In situ hybridization studies showed that CRF_1 receptors were found in large densities in the mPFC. Moreover, CRF injection into the mPFC increased anxiety-like behavior in the elevated plus maze in both acutely and repeatedly stressed animals compared to vehicle (16).

GABA is the main inhibitory neurotransmitter in the central nervous system. Its transmission in the amygdala is particularly important for controlling fear and anxiety levels (22). Clinical evidence suggests that alterations in normal GABA transmission might contribute to the pathophysiology of anxiety disorders in humans. For instance, various studies using nuclear imaging techniques have revealed diminished central GABA and GABA-

A receptor activity in patients suffering from anxiety (panic disorder) and trauma/stress-related disorders (posttraumatic stress disorder) (22). GABA is synthesized from glutamate by the enzyme glutamate decarboxylase (GAD), which exists in two isoforms: GAD67 (thought be involved in GABA synthesis) and GAD65 (controls the synaptic release of GABA) (23-25). Deletion of the GAD67 gene in mice resulted in a > 90% reduction in the basal GABA levels in the brain, whereas GAD65-deleted homozygous mice expressed normal GABA levels (22, 26-27). The preclinical study indicated that alterations in the GAD67 levels might participate in the development and/or expression of symptoms associated with fear and anxiety (22).

The aim of this study was to examine the interaction between the CRF system and the inhibitory neurotransmitter (GABA) in the modulation of amygdala and prefrontal cortex activity in low- and high-anxiety rats. Recently, we studied the central mechanisms that are responsible for individual vulnerability to stressors by employing a model that divided rats into high-anxiety (HR) and low-anxiety (LR) groups (28-31). Our model is based on differences in the expression of a conditioned fear to the context. We did this on purpose, considering that in the clinic there occur different types of anxiety, e.g. panic fear, phobias or trauma (PTSD). Thus our model refers mainly to the post-traumatic stress disorder. Different types of anxiety and trauma/stress-related disorders are treated in a different way using pharmacological or psychotherapeutic methods. This indicates that the neurobiological mechanisms of various types of anxiety are different at the level of cortical and limbic structures, and only at the final point of execution of emotional reactions in the structures like the brainstem and the hypothalamus, they show a similar expression of behavioral and hormonal symptoms of fear. Consequently, both populations of animals should be defined as groups of HR and LR rats, in the model of a conditioned fear. The division of HR and LR rats is validated and justified by the results of many already published reports indicating a different reactivity of HR and LR rats in different models of chronic stress (immobilization stress, chronic corticosterone) (28-31), and their different reactivity to the ligands changing the activity of brain neurotransmitter systems which regulate emotional reactions (GABA, 5-HT, CRF, glutamate, glucocorticoid receptors) (32-34). Moreover, we demonstrated in a recent study that LR rats were more sensitive to re-exposure to fear stimuli and that midazolam pretreatment was associated with the attenuation of brain activity in the amygdala and prefrontal cortex (c-Fos and CRF immunocytochemistry) in this group of animals (35). Based on these data, in the present study, we tested the hypotheses that animals with different vulnerabilities to fear stimuli would be differentially sensitive to the effects of the non-peptide CRF_1 antagonist (antalarmin) on amygdala-prefrontal cortex activity and fear expression and that these effects would be accompanied by changes in the local activity of the GABAergic system.

MATERIALS AND METHODS

Animals

The experiments were performed on 70 male Wistar rats (200 – 220 g body weight), purchased from a licensed breeder (The Center for Experimental Medicine of the Medical University, 24A Sklodowskiej-Curie Str., Bialystok, Poland) and housed under standard laboratory conditions with a 12 h light/dark cycle (lights on at 7 a.m.) at a constant temperature (21 ± 2°C). The rats were group-housed, 4 per cage in the polycarbonate cages (556 × 324 × 195 mm, floor area, 1875 cm^2) containing bedding (Lignocel; Hygienic Animal Bedding; JRS GmbH + Co KG, Germany). Cages were cleaned and the bedding replaced twice a week.

The experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609 EEC). The Local Committee for Animal Care and Use at the Warsaw Medical University, Poland approved all experimental procedures using animals.

Experimental protocol

After seven days of acclimatization to the vivarium, the animals (n = 70) were subjected to a contextual fear-conditioning test to assess individual responses to conditioned aversive stimuli (28, 34). The rats were divided into low-anxiety (LR, n = 33) and high-anxiety (HR, n = 34) groups based on the duration of their conditioned freezing in a contextual fear test. Three rats did not meet either criterion. 90 min after exposure to the first aversive context, 6 animals from each group were decapitated (LR_{CFC} - low anxiety animals, n = 6; HR_{CFC} - high-anxiety animals, n = 6) for immunocytochemistry and biochemical analyses. The remaining animals (HR, n = 28 and LR, n = 27) were housed in their home cages for 28 days. Two rats from HR group and two rats from LR group were excluded from the study, because of their bad physical condition. Next, the HR (n = 26) and LR (n = 25) rats were randomly divided into six experimental groups as follows: LR_{Re-CFC} - low anxiety animals pretreated with vehicle solution and conditioned for a second time to the aversive context (n = 9); LR_{Ant10} - low-anxiety rats administered antalarmin at a dose of 10 mg/kg and conditioned for a second time to the aversive context (n = 8); LR_{Ant20} - low-anxiety rats administered antalarmin at a dose of 20 mg/kg and conditioned for a second time to the aversive context (n = 8); HR_{Re-CFC} - high-anxiety animals pretreated with vehicle solution and conditioned for a second time to the aversive context (n = 10); HR_{Ant10} - high-anxiety rats administered antalarmin at a dose of 10 mg/kg and conditioned for a second time to the aversive context (n = 8); and HR_{Ant20} - high-anxiety rats administered antalarmin at a dose of 20 mg/kg and conditioned for a second time to the aversive context (n = 8). Next, the animals were subjected to the contextual fear training and retested. The rats received antalarmin or vehicle injections 80 min before the second contextual fear test. The animals were decapitated ninety minutes after the second exposure to the aversive context (180 min after drug administration) (*Fig. 1*). Their brains were removed, frozen, and stored at –70°C for the immunocytochemistry (GAD67 expression) and biochemistry (GABA concentration) analyses (*Fig. 2*).

Contextual fear-conditioning test

The fear-conditioning experiment was performed using a computerized fear-conditioning system (TSE, Bad Homburg, Germany; FCS 04.11) in a Plexiglas cage (36 × 21 × 20 cm, w × l × h) with a steel foot-shock grid (the 38 floor bars were 0.4 cm in diameter and spaced 0.5 cm apart) under constant white noise (65 dB) and constant illumination (12 V, 10 W halogen lamp, ~150 l×). Freezing behavior was recorded using an infrared photobeam system (10 Hz detection rate) controlled by the fear-conditioning system. The photobeams were spaced 1.3 cm in the direction of the x-axis and 2.5 cm in the direction of the y-axis. This method and equipment have been used in our and other laboratories for years and have been validated pharmacologically using many clinically effective and experimental anxiolytic and anxiogenic agents (36-37).

The total duration of inactivity was calculated by the fear-conditioning system. The total duration was defined as no interruption of any photobeam over a 5-s period; these periods

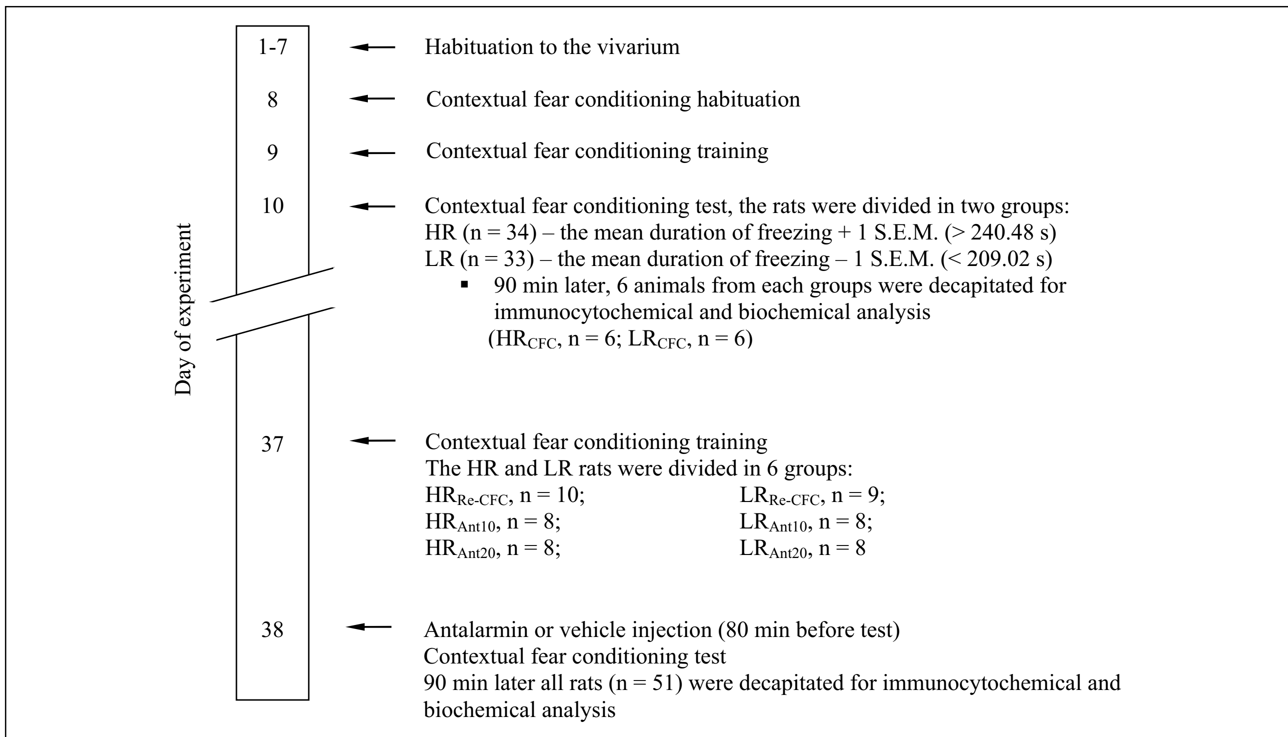

Fig. 1. Scheme of the experiment. HR – high-anxiety rats; LR – low-anxiety rats; HR_{CFC} – high-anxiety animals after the first contextual fear test; LR_{CFC} – low anxiety animals after the first contextual fear test; $HR_{Re\text{-}CFC}$ – high-anxiety animals pretreated with vehicle solution, and conditioned for a second time to the aversive context; $LR_{Re\text{-}CFC}$ – low anxiety animals pretreated with vehicle solution, and conditioned for a second time to the aversive context; HR_{Ant10} – high-anxiety rats administered with antalarmin at a dose of 10 mg/kg, and conditioned for a second time to the aversive context; HR_{Ant20} – high-anxiety rats administered with antalarmin at a dose of 20 mg/kg, and conditioned for a second time to the aversive context; LR_{Ant10} – low-anxiety rats administered with antalarmin at a dose of 10 mg/kg, and conditioned for a second time to the aversive context; LR_{Ant20} – low-anxiety rats administered with antalarmin at a dose of 20 mg/kg, and conditioned for a second time to the aversive context. For more details, see the experimental procedure.

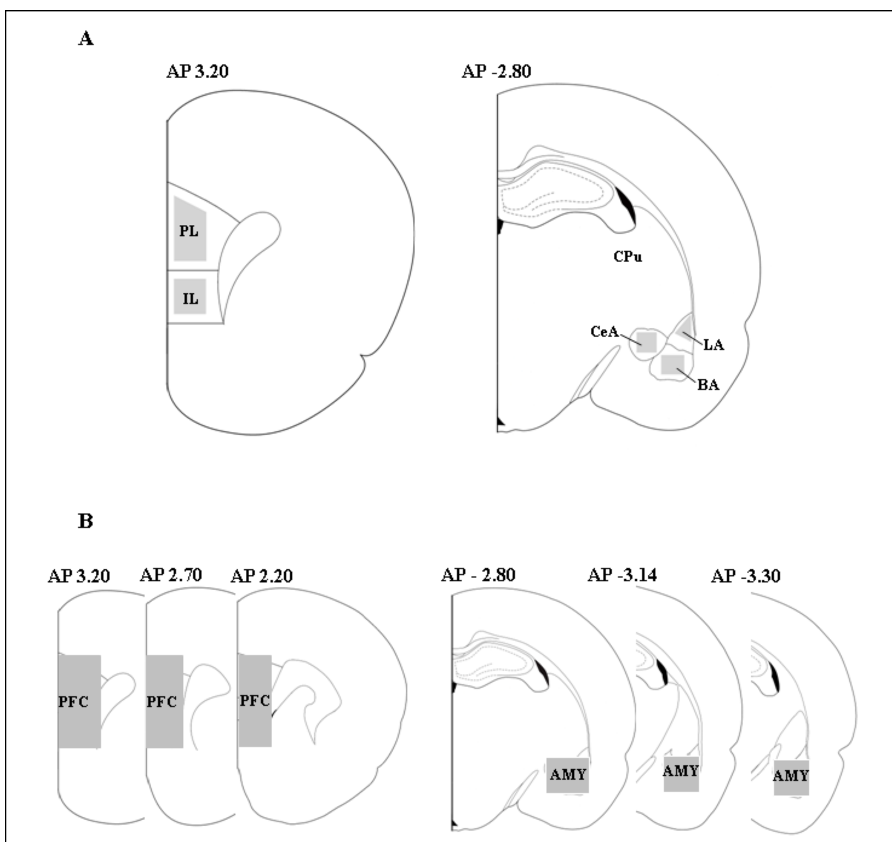

Fig. 2. (A) Schematic view of brain regions analyzed for immunocytochemistry. (B) Schematic view of brain regions used for biochemical study. The number indicates the distance from bregma (mm). AMY – amygdala complex, BA – basal nucleus of the amygdala, CeA – central nucleus of the amygdala, CPU – striatum, IL – infralimbic cortex, LA – lateral nucleus of the amygdala, PFC – prefrontal cortex, PL – prelimbic cortex. The marked areas indicated regions of tissue collections.

were summarized for the entire experimental session to yield the total freezing time. The box was cleaned with 95% ethanol after each trial. The testing was performed from 8.30 to 12.00. The animals were transported from the vivarium to the experimental room in pairs, and 3 hours after the end of the experiment the rats returned to the vivarium. The experiment was performed on three consecutive days in the same testing box and experimental chamber. On the first day, the animals were placed separately for 2 min in a training box without aversive stimulation to adapt to the experimental conditions. On the second day (a training day), the animals were placed for 10 min in the training box. After 5 min of sitting undisturbed in the box, the rat received 4 footshocks (0.7 mA) delivered through the stainless steel floor grid, lasting for 1 s each, with 59 s breaks between stimuli, for final 5 min. The animals were removed from the testing boxes 1 min after the last shock was delivered. On the third experimental day, the freezing behavior of rats was observed for 10 min in the same box. The conditioned response (freezing reaction) was analyzed and recorded by the fear-conditioning system. The absolute duration of inactivity was calculated from the activity plots and expressed as the total time during which the animals were inactive. The computerized method is based on the latency between the photobeam interruption measures obtained during the contextual fear-conditioning tests, which is highly correlated with hand-scored freezing (r values ranged from 0.87 to 0.94) (38-39). The rats were divided into two experimental groups according to the duration of the context-induced freezing responses. The LR group had a total duration of freezing responses at least one S.E.M or more below the mean value (224.75 – 15.73, i.e., < 209.02 s). The HR group had a total duration of freezing responses at least one S.E.M or more above the mean value (224.75 + 15.73, i.e., > 240.48 s). The mean value of freezing for the LR group = 98.18 s and for HR group = 345.88 s. Three rats did not meet either criterion.

Drug treatment

Antalarmin hydrochloride (*N*-butyl-*N*-ethyl-2,5,6-trimethyl-7-(2,4,6-trimethylphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine hydrochloride, Tocris Bioscience, Bristol, United Kingdom) was suspended in a vehicle composed of 10% Tween 80 and distilled water and administered intraperitoneally (i.p.) in a 1 ml/kg injection volume. The injection doses and timing were selected based on previous studies (40-41). For vehicle injection, 10% Tween 80 alone was administered in the same volume.

Immunocytochemistry of GAD67

The immunocytochemical reaction was performed on slide-mounted frozen brain sections. Based on the atlas of Paxinos and Watson (42), coronal 15 μm cryostat slices were cut, mounted on silane-coated slides and fixed in cold methanol for 10 min. In the study, three slices from each section per animal were taken for immunostaining (*Fig. 2A*), and the rest of the tissue samples were used for biochemical analysis of the GABA concentration (see below) (*Fig. 2B*). The specimens were washed twice (2 × 15 min) in 0.01 M PBS solution (pH 7.4), incubated in a 3% hydrogen peroxide (H_2O_2) solution for 30 min to block the activity of endogenous peroxidase, washed twice again (2 × 15 min) in 0.01 M PBS and incubated in a 3% normal horse serum blocking solution. Subsequently, the slide-mounted brain sections were incubated with a rabbit polyclonal antibody directed against GAD67 (1:200, Santa Cruz) at 4 – 8°C for 72 h. Following incubation, the slides were washed in 0.01 M PBS three times (3 × 15 min) and detected with peroxidase-conjugated anti-rabbit IgG (1:1000, ImmunoJackson Research). The peroxidase reaction was developed with DAB (0.2 mg/ml) and hydrogen peroxide (0.003%) in Tris buffer. The sections were dehydrated by serial alcohol rinsing, cleared in xylene, and coverslipped in a histofluid mounting medium. Western blotting analysis confirmed the specific binding of the antibodies.

Cells counts were assessed by light microscopy (Olympus BX-51 light microscope, DP-70 digital camera) at a total magnification of ×100. The number of positive cells was counted with a computerized image analysis system (Olympus CellSens software, Munster, Germany) in the following subregions: AP 3.20: infralimbic cortex (IL) and prelimbic cortex (PL) and AP(–) 2.80: basal nucleus of the amygdala (BA), central nucleus of the amygdala (CeA), and lateral nucleus of the amygdala (LA) (42). The total number of positive cells was manually counted for each region of each rat brain as shown as *Fig. 2A* and expressed as the number of positive nuclei per 1 mm^2. An independent researcher blinded to the groups to which the rats had been assigned performed the analysis.

Biochemical analysis of the GABA concentration

After serial sections were cut for the immunocytochemistry analysis, the anatomical structures of the cortex (bregma 3.20 – 2.20) and the amygdala (bregma –2.80 – –3.30) were micropunched under a dissecting microscope as shown as *Fig. 2B*. Each tissue was weighed (the average weight was 11 mg), placed in a dry ice-cooled polypropylene vial, and homogenized with a polytron-type homogenizer (30 s, 4°C) in a solution containing perchloric acid (0.2 M). The homogenates were centrifuged (26,880 × g at 4°C for 8 min) and the supernatants were filtered through Syringe Driven Filter Units (Millipore) prior to the analysis.

HPLC analysis of GABA was performed using a Luna 5 μm C18(2) 100A (250 × 4.6 mm) reverse phase column according to the previously described procedure (43). The compounds were eluted isocratically with the mobile phase delivered at 0.70 ml/min using a Shimadzu Clas VP LC 10AD pump. An electrochemical detector with a flow-through cell (Intro-AntecLeyden) linked to the Shimadzu Class VP Integrator SCL-10 Avp was used. A high-density glass carbon-working electrode (Antec) was operated at +0.85 V. A rheodyne injection valve with a 20-μl sample loop was used to manually inject the samples. The preparation of the mobile phase and the derivatizing agents were based on the method of Rowley *et al.* (44) with some modifications. The mobile phase consisted of 45 mM disodium phosphate and 0.15 mM ethylenediaminetetraacetic acid (EDTA) with 24% methanol (v/v) in water adjusted to pH 3.9 with 0.2 M citric acid. Then, the mobile phase was filtered through a 0.45 μm filter and degassed for 15 min. A stock solution (0.01 M) of the GABA standard was prepared in double-deionized water and kept at 4°C for five days. The standard was prepared in polyethylene vials to prevent adhesion to the glass. Working solutions were prepared daily by diluting the stock solution. To obtain agents for derivatization, o-phthaldialdehyde (OPA, 22 mg, Fluka) was dissolved in 0.5 ml of 1 M sodium sulfite, 0.5 ml of methanol, and 0.9 ml of sodium tetraborate buffer (0.1 M) adjusted to pH 10.4 with 5 M sodium hydroxide. The derivatization reaction was performed at room temperature. The derivatizing agent (20 μl) was reacted with 1 ml of the GABA standard for 15 min in a polyethylene vial prior to injection onto the column. The GABA concentration was calculated in μM.

Statistical analysis

The data are shown as the means and standard errors of the mean (S.E.M). To verify the differences between the HR and LR groups in the contextual fear-conditioning test, we used Student's t test. In the first part of the study, we performed the analysis for the first and second contextual fear conditioning test to determine how

fear re-conditioning affected the behavior and local GABA activity in the HR and LR rats. In the second part of the study, we performed the analysis to determine how antalarmin administration modulated the behavioral and biochemical activity after the second contextual fear test in the HR and LR rats. In the analysis of the different brain structures, the number of the analyzed animals may be different from the number of animals in the test group, because for technical reasons some brain sections slices were lost. The data were analyzed by two-way ANOVA followed by the most conservative Tukey's post hoc test. A probability value of $P < 0.05$ was considered significant in this study. The statistical analyses were performed using Stat-Soft Statistica 12.0 for Windows (StatSoft Inc., USA).

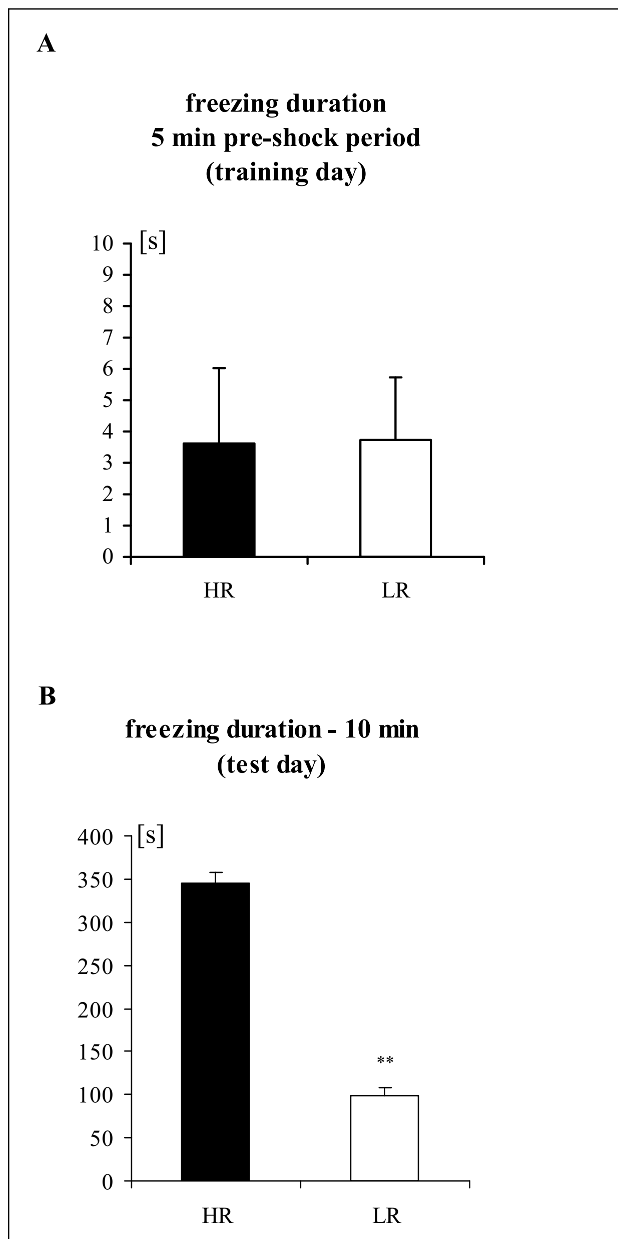

Fig. 3. (A) Freezing duration in the 5 min pre-shock period in the contextual fear test (training day). (B) Freezing duration in the contextual fear test (10 min – test day). The data are shown as the means + S.E.M. HR – high-anxiety rats (n = 34), LR – low-anxiety rats (n = 33). **P < 0.01, differs from HR. For more details, see the experimental procedure.

RESULTS

Contextual fear-conditioning test

Student's t-test did not revealed a significantly differences between LR and HR groups in the 5 min pre-shock period (t = 0.04, df = 65, P > 0.1) (*Fig. 3A*). Student's t-test revealed a significantly weaker freezing response in the LR group compared to the HR group (t = 15.70, df = 65, P < 0.01) (*Fig. 3B*).

The effects of fear re-conditioning

1. Contextual fear-conditioning test

Two-way ANOVA revealed significant differences in the freezing durations between the experimental groups (LR_{CFC}, LR_{Re-CFC}, HR_{CFC}, HR_{Re-CFC}): group effect [F(1,27) = 35.95 (P < 0.01)], fear effect [F(1,27) = 14.48 (P < 0.01)], and group x fear interaction effect [F(1,27) = 4.51 (P < 0.05)]. Tukey's post hoc test revealed a lower freezing duration in the LR_{CFC} group compared with the HR_{CFC} group (P < 0.01) and in the LR_{Re-CFC} group compared with the $HR_{Re–CFC}$ group (P < 0.05). The post hoc test also indicated a much higher freezing duration in the $LR_{Re–CFC}$ group compared with the LR_{CFC} group (P<0.01) (*Fig. 4A*).

2. GAD67 expression

In the IL, two-way ANOVA did not reveal significant differences between the experimental groups (LR_{CFC}, LR_{Re-CFC}, HR_{CFC}, HR_{Re-CFC}): no group effect [F(1,27) = 1.08 (P > 0.1)], no fear effect [F(1,27) = 2.04 (P > 0.1)], and no group × fear interaction effect [F(1,27) = 0.75 (P > 0.1)] (*Fig. 4B*).

In the PL, two-way ANOVA revealed significant differences between groups (LR_{CFC}, LR_{Re-CFC}, HR_{CFC}, HR_{Re-CFC}): group effect [F(1,26) = 4.76 (P < 0.05)], fear effect [F(1,26) = 31.81 (P < 0.01)], but no group × fear interaction effect [F(1,26) = 1.72 (P > 0.1)] (*Fig. 4C*).

In the LA, two-way ANOVA revealed significant differences between groups (LR_{CFC}, LR_{Re-CFC}, HR_{CFC}, HR_{Re-CFC}): fear effect [F(1,27) = 8.17 (P < 0.01)], group × fear interaction effect [F(1,27) = 7.57 (P < 0.05)], and no group effect [F(1,27) = 0.01 (P > 0.1)]. Tukey's post hoc test revealed a decrease in the number of GAD67-positive nuclei in the LR_{Re-CFC} group compared with the LR_{CFC} group (P < 0.01) (*Fig. 4D*).

In the BA, two-way ANOVA revealed significant differences between groups (LR_{CFC}, LR_{Re-CFC}, HR_{CFC}, HR_{Re-CFC}): fear effect [F(1,26) = 52.38 (P < 0.01)] but no group effect [F(1,26) = 0.06 (P > 0.1)] and no group × fear interaction effect [F(1,26) = 0.03 (P > 0.1)] (*Fig. 4E*).

In the CeA, two-way ANOVA revealed significant differences between groups (LR_{CFC}, LR_{Re-CFC}, HR_{CFC}, HR_{Re-CFC}): group effect [F(1,25) = 30.11 (P < 0.01)], fear effect [F(1,25) = 25.12 (P < 0.01)], and group × fear interaction effect [F(1,25) = 7.97 (P < 0.01)]. Tukey's post hoc test revealed lower GAD67 expression in the HR_{CFC} group than in the LR_{CFC} group (P < 0.01) and in the LR_{Re-CFC} group than in the LR_{CFC} group (P < 0.01) (*Fig. 4F*).

The effects of antalarmin pretreatment

1. Contextual fear-conditioning test

Two-way ANOVA revealed significant differences in the freezing duration between the experimental groups (LR_{Re-CFC}, LR_{Ant10}, LR_{Ant20}, HR_{Re-CFC}, HR_{Ant10}, HR_{Ant20}): drug effect [F(1,45) = 11.28 (P < 0.01)], group × drug interaction effect [F(1,45) = 7.64 (P < 0.01)], but no group effect [F(1,45) = 1.30 (P > 0.1)]. Tukey's post hoc test revealed a lower freezing duration in the HR_{Ant10} group

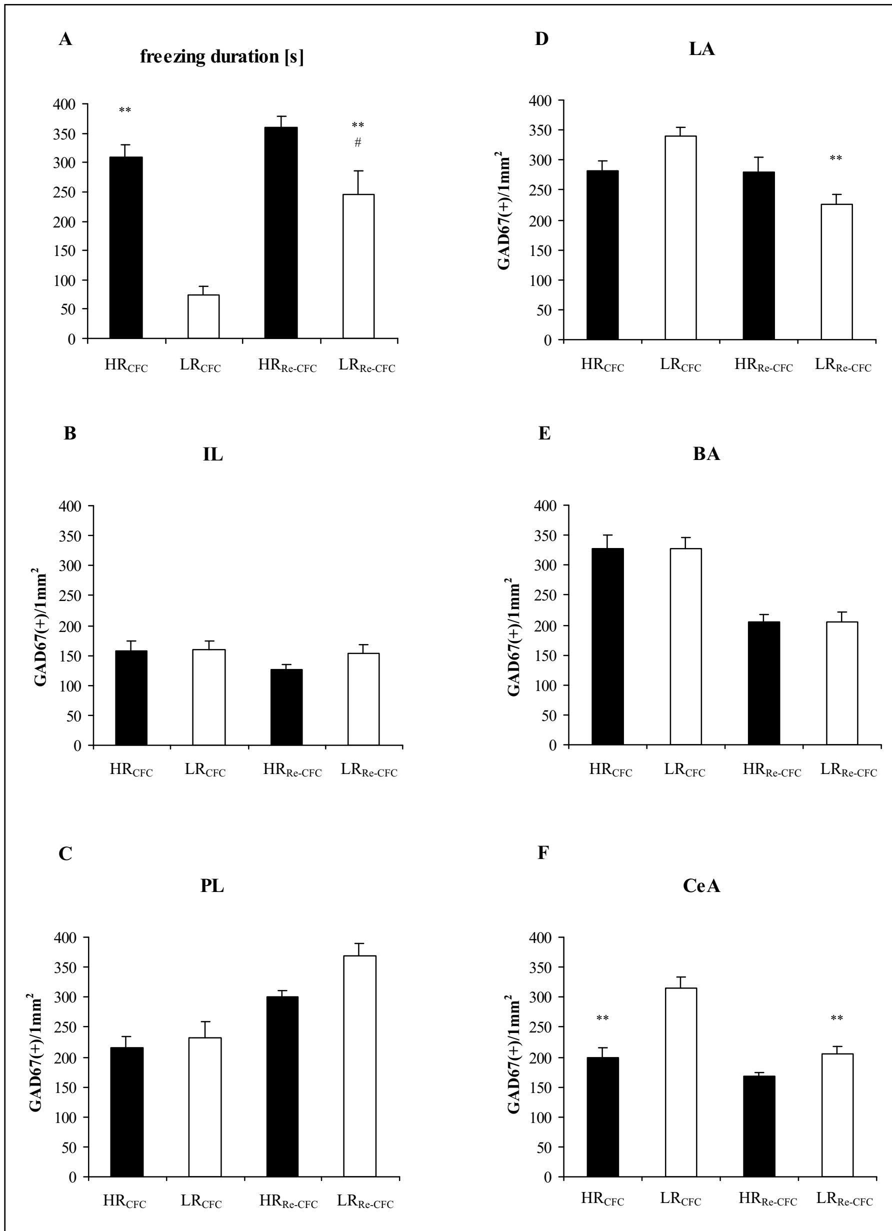

Fig. 4. (A) Freezing duration in the contextual fear test after the first and second contextual fear test. (B-F) GAD67 expression 90 min after the first and second contextual fear test. The data show the number of immunoreactive neurons per 1 mm^2. BA – basal nucleus of the amygdala, CeA – central nucleus of the amygdala, IL – infralimbic cortex, LA – lateral nucleus of the amygdala, PL – prelimbic cortex. HR_{CFC} – high-anxiety animals after the first contextual fear test, n = 6 (A-F); LR_{CFC} – low anxiety animals after the first contextual fear test, n = 6 (A-F); $HR_{Re\text{-}CFC}$ – high-anxiety animals pretreated with vehicle solution, and conditioned for a second time to the aversive context, n = 8 (F), n = 9 (C, E), n = 10 (A, B, D); $LR_{Re\text{-}CFC}$ – low anxiety animals pretreated with vehicle solution, and conditioned for a second time to the aversive context, n = 9 (A-F). The data are shown as the means + S.E.M. **P < 0.01, differs from LR_{CFC}; #P < 0.05, differs from $HR_{Re\text{-}CFC}$. For more details, see the experimental procedure.

compared with the $HR_{Re\text{-}CFC}$ group (P < 0.01), and in the HR_{Ant20} group compared with the $HR_{Re\text{-}CFC}$ group (P < 0.05) (*Fig. 5A*).

2. GAD67 expression

In the IL, two-way ANOVA revealed significant differences between groups ($LR_{Re\text{-}CFC}$, LR_{Ant10}, LR_{Ant20}, $HR_{Re\text{-}CFC}$, HR_{Ant10}, HR_{Ant20}): group effect [F(1,44) = 4.99 (P < 0.05)], but no drug effect [F(1,44) = 1.61 (P > 0.1)], and no group × drug interaction effect [F(1,44) = 0.96 (P > 0.1)] (*Fig. 5B*).

In the PL, two-way ANOVA revealed significant differences between groups ($LR_{Re\text{-}CFC}$, LR_{Ant10}, LR_{Ant20}, $HR_{Re\text{-}CFC}$, HR_{Ant10}, HR_{Ant20}): drug effect [F(1,43) = 3.25 (P < 0.05)], group × drug interaction effect [F(1,43) = 6.09 (P < 0.01)], but no group effect [F(1,43) = 0.02 (P > 0.1)]. Tukey's post hoc revealed higher GAD67 expression in the HR_{Ant10} group compared with the $HR_{Re\text{-}CFC}$ and HR_{Ant20} groups (P < 0.01 and P < 0.05, respectively) (*Fig. 5C*).

In the LA, two-way ANOVA revealed significant differences between groups ($LR_{Re\text{-}CFC}$, LR_{Ant10}, LR_{Ant20}, $HR_{Re\text{-}CFC}$, HR_{Ant10}, HR_{Ant20}): drug effect [F(1,44) = 3.74 (P < 0.05)], but no group effect [F(1,44) = 1.17 (P > 0.1)] and no group × drug interaction effect [F(1,44) = 1.12 (P > 0.1)] (*Fig. 5D*).

In the BA, two-way ANOVA revealed significant differences between groups ($LR_{Re\text{-}CFC}$, LR_{Ant10}, LR_{Ant20}, $HR_{Re\text{-}CFC}$, HR_{Ant10}, HR_{Ant20}): drug effect [F(1,43) = 3.94 (P < 0.05)], but no group effect [F(1,43) = 0.14 (P > 0.1)] and no group × drug interaction effect [F(1,43) = 0.06 (P > 0.1)] (*Fig. 5E*).

In the CeA, two-way ANOVA revealed significant differences between groups ($LR_{Re\text{-}CFC}$, LR_{Ant10}, LR_{Ant20}, $HR_{Re\text{-}CFC}$, HR_{Ant10}, HR_{Ant20}): drug effect [F(1,41) = 7.31 (P < 0.01)], group × drug interaction effect [F(1,41) = 9.93 (P < 0.01)], but no group effect [F(1,41) = 0.83 (P > 0.1)]. Tukey's post hoc test revealed higher GAD67 expression in the HR_{Ant10} group compared with the $HR_{Re\text{-}CFC}$ (P < 0.01), LR_{Ant10} (P < 0.05), and HR_{Ant20} (P < 0.05) groups (*Fig. 5F* and *6*).

3. GABA concentration

In the cortex, two-way ANOVA revealed significant differences between groups ($LR_{Re\text{-}CFC}$, LR_{Ant10}, LR_{Ant20}, $HR_{Re\text{-}CFC}$,

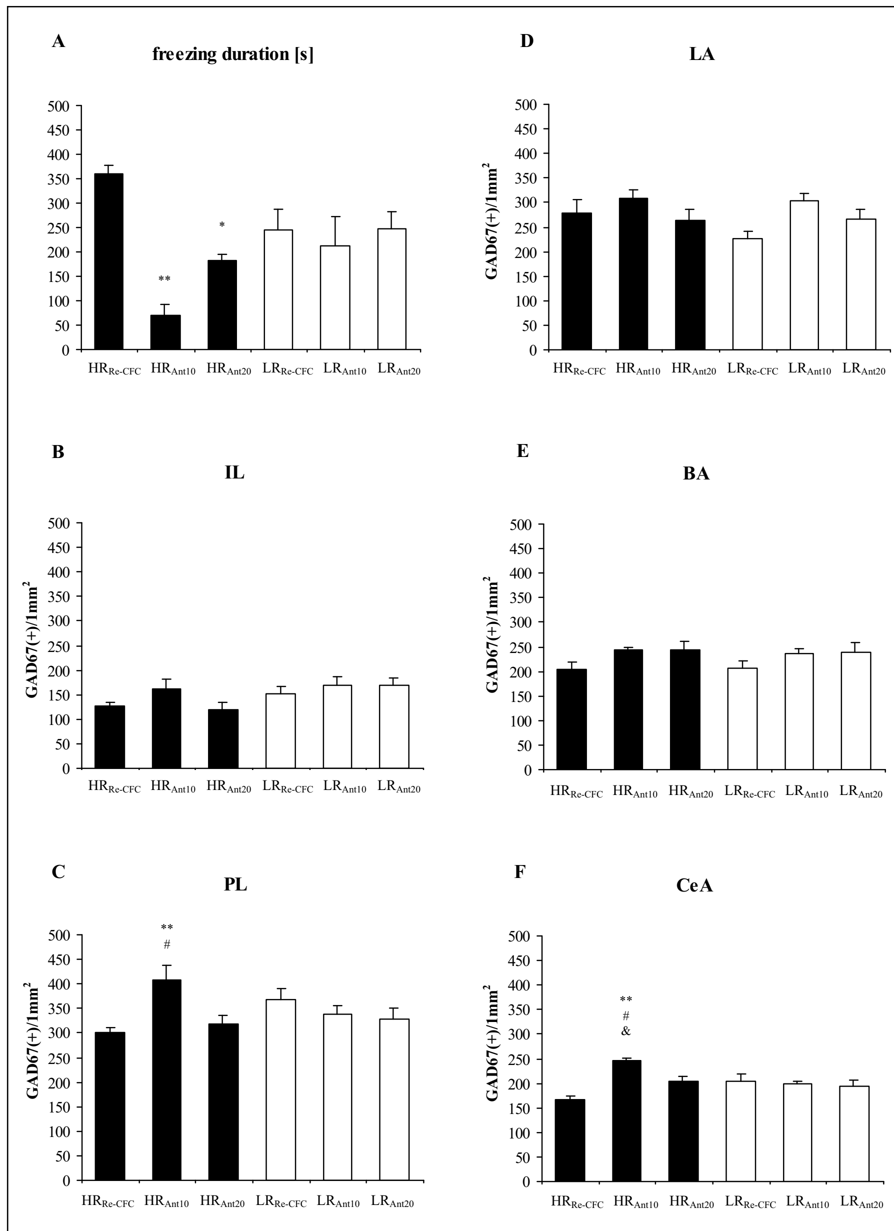

Fig. 5. (A) The influence of antalarmin pretreatment on rat behavioral in the second conditioned fear test. (B-F) GAD67 expression 180 min after antalarmin administration and 90 min after exposure to the conditioning boxes. The data show the number of immunoreactive neurons per 1 mm^2. BA – basal nucleus of the amygdala, CeA – central nucleus of the amygdala, IL – infralimbic cortex, LA – lateral nucleus of the amygdala, PL – prelimbic cortex. $HR_{Re\text{-}CFC}$ – high-anxiety animals pretreated with vehicle solution, and conditioned for a second time to the aversive context, n = 8 (F), n = 9 (C, E), n = 10 (A, B, D); HR_{Ant10} – high-anxiety rats administered with antalarmin at a dose of 10 mg/kg, and conditioned for a second time to the aversive context, n = 8 (A-F); HR_{Ant20} – high-anxiety rats administered with antalarmin at a dose of 20 mg/kg, and conditioned for a second time to the aversive context, n = 8 (A-F); $LR_{Re\text{-}CFC}$ – low anxiety animals pretreated with vehicle solution, and conditioned for a second time to the aversive context, n = 9 (A-F); LR_{Ant10} – low-anxiety rats administered with antalarmin at a dose of 10 mg/kg, and conditioned for a second time to the aversive context, n = 7 (F), n = 8 (A-E); LR_{Ant20} – low-anxiety rats administered with antalarmin at a dose of 20 mg/kg, and conditioned for a second time to the aversive context, n = 7 (B-F), n = 8 (A). The data are shown as the means + S.E.M. *$P < 0.05$, **$P < 0.01$, differs from $HR_{Re\text{-}CFC}$; #$P < 0.05$, differs from HR_{Ant20}; &$P < 0.05$, differs from LR_{Ant10}. For more details, see the experimental procedure.

HR_{Ant10}, HR_{Ant20}): drug effect [$F(1,37) = 15.87$ ($P < 0.01$)] but no group effect [$F(1,37) = 0.01$ ($P > 0.1$)] and no group × drug interaction effect [$F(1,37) = 2.40$ ($P > 0.1$)] (*Fig. 7A*).

In the amygdala, two-way ANOVA revealed significant differences between the experimental groups ($LR_{Re\text{-}CFC}$, LR_{Ant10}, LR_{Ant20}, $HR_{Re\text{-}CFC}$, HR_{Ant10}, HR_{Ant20}): drug effect [$F(1,39) = 6.22$ ($P < 0.01$)], group × drug interaction effect [$F(1,39) = 3.25$ ($P < 0.05$)], but no group effect [$F(1,39) = 2.17$ ($P > 0.1$)]. Tukey's post hoc test revealed a higher GABA concentration in the HR_{Ant10} group compared with the $HR_{Re\text{-}CFC}$ group ($P < 0.01$) (*Fig. 7B*).

DISCUSSION

In the present study, we found that fear re-conditioning increased the freezing duration compared with the first conditioned fear response in the LR group, similar to our previous report (*Fig. 4*) (35). However, the behavioral response of these animals remained lower compared with the HR group. The behavioral changes in the $LR_{Re\text{-}CFC}$ rats were accompanied by decreased GAD67 expression in the LA and CeA compared with the LR_{CFC} group. Pretreatment of the rats with antalarmin (10 mg/kg or 20 mg/kg), prior to the second exposure to the aversive context, decreased the conditioned fear response only in the HR group (*Fig. 5*). The behavioral effect of the lower dose (10 mg/kg) of antalarmin was accompanied by increased the GAD67 expression in the PL and CeA (*Fig. 5*) and GABA concentration in the amygdala, in the HR group (*Fig. 7*).

The effect of fear re-conditioning on rat behavior and GABAergic system activity in the amygdala of the high- and low-anxiety rats

Fear re-conditioning significantly increased the freezing duration in the LR rats compared with the first conditioned fear response. In the HR rats, the second exposure to the aversive context also increased the freezing duration, albeit not significantly (the mean value of the freezing duration for the HR_{CFC} group = 310.00 s, and for the $HR_{Re\text{-}CFC}$ group = 359.10 s). This effect confirms the result of our previous study that the LR

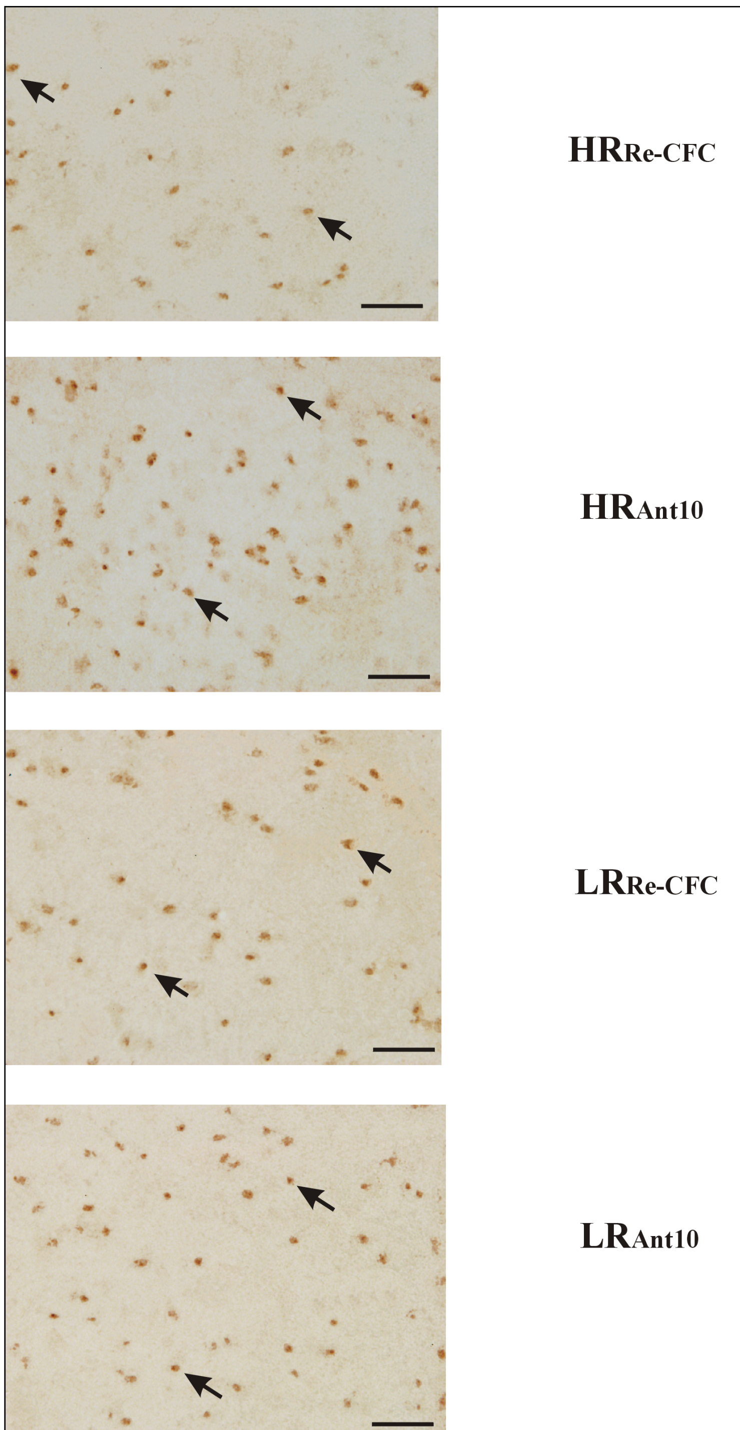

Fig. 6. Photomicrographs showing representative expression of GAD67 in the central nucleus of the amygdala. Slices were photographed with an objective lens at 20 × magnification (total magnification ×200). Scale bar indicates 75 μm. The arrow heads show representative immunopositive cells. $HR_{Re\text{-}CFC}$ – high-anxiety animals pretreated with vehicle solution, and conditioned for a second time to the aversive context (n = 8); HR_{Ant10} – high-anxiety rats administered with antalarmin at a dose of 10 mg/kg, and conditioned for a second time to the aversive context (n = 8); $LR_{Re\text{-}CFC}$ – low anxiety animals pretreated with vehicle solution, and conditioned for a second time to the aversive context (n = 9); LR_{Ant10} – low-anxiety rats administered with antalarmin at a dose of 10 mg/kg, and conditioned for a second time to the aversive context (n = 7).

rats appeared to be more sensitive to re-exposure to the contextual fear stimuli. In this group of animals, the fear re-conditioning increased neuronal activity in the amygdala and decreased neuronal activity in the prefrontal cortex (c-Fos and CRF immunocytochemistry) (35). In the current study, we showed that a second exposure to the aversive context also decreased GABAergic neurotransmission (measured by GAD67 expression) in the LA and CeA in the LR rats.

Our findings are consistent with other data indicating the important role of amygdala GABA innervation in fear memory processing. It was found that, during acquisition and consolidation of fear memory, fear conditioning decreases the extracellular GABA levels in the BLA and reduces the mRNA level of the GABA-synthesizing enzyme GAD67 in the amygdala (2, 45-46). Reduced GAD67 expression during acquisition may be associated with a loss of inhibitory control of the amygdala, thereby contributing to the hyperactivity or prolonged activation of this limbic nucleus (45, 47). Accordingly, in the current study a stronger fear reaction after the second exposure to the aversive context in the LR rats could be due to a decrease in the GABAergic activity (represented by a decrease GAD67 expression) in the amygdala, leading to the disinhibition of amygdala-related processes and enhancement of the fear reaction. This hypothesis is substantiated by our earlier findings that fear re-conditioning induces an increase in the freezing duration that is accompanied by an increase in amygdala activity (measured by c-Fos and CRF expression) and

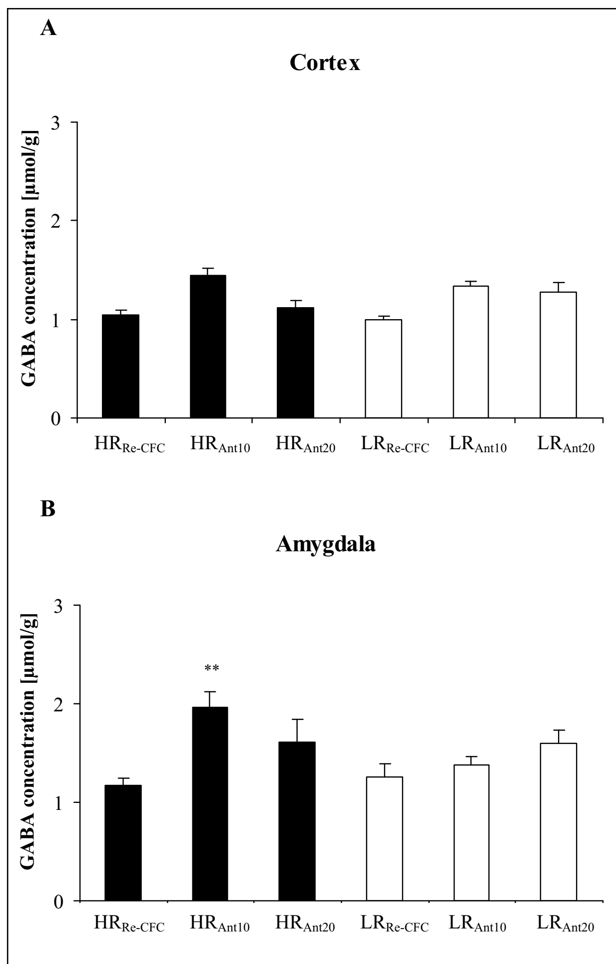

Fig. 7. GABA concentration in the cortex (A) and the amygdala (B) 180 min after antalarmin administration and 90 min after exposure to the conditioning boxes. The data are shown as the means + S.E.M. $HR_{Re\text{-}CFC}$ – high-anxiety animals pretreated with vehicle solution, and conditioned for a second time to the aversive context, n = 8 (A-B); HR_{Ant10} – high-anxiety rats administered with antalarmin at a dose of 10 mg/kg, and conditioned for a second time to the aversive context, n = 7 (A), n = 8 (B); HR_{Ant20} – high-anxiety rats administered with antalarmin at a dose of 20 mg/kg, and conditioned for a second time to the aversive context, n = 7 (A-B); $LR_{Re\text{-}CFC}$ – low anxiety animals pretreated with vehicle solution, and conditioned for a second time to the aversive context, n = 7 (A), n = 8 (B); LR_{Ant10} – low-anxiety rats administered with antalarmin at a dose of 10 mg/kg, and conditioned for a second time to the aversive context, n = 7 (A-B); LR_{Ant20} – low-anxiety rats administered with antalarmin at a dose of 20 mg/kg, and conditioned for a second time to the aversive context, n = 7 (A-B). **$P < 0.01$, differs from $HR_{Re\text{-}CFC}$. For more details, see the experimental procedure.

weaker prefrontal cortex activity (measured by CRF expression) in both the HR and LR groups. However, these effects were significantly stronger in the LR group (35).

The effect of antalarmin on the fear response and GABAergic system activity in the amygdala of the high- and low-anxiety rats

The CRF plays an important role in modulating the activity of the brain structures involved in fear learning and fear expression. Exposure to footshock increases CRF expression in the amygdala of male rats (48). This increase may be critical for fear conditioning because reducing the effects of CRF in both the BLA (BA and LA) and the CeA disrupts the consolidation or stabilization of fear memories in male rats (12, 14). Compelling evidence indicates that CRF activation of the CRF_1 receptor is sufficient and in many cases necessary to initiate an anxiety-like response (7-9). The anxiogenic character of the CRF_1 receptor ligands was verified by the consistent anxiolytic effects of peptide or non-peptide CRF_1 receptor antagonists (49). For example, pharmacological blockade at the CRF_1 receptor by the nonpeptide corticotropin-releasing factor antagonist antalarmin produced anxiolytic-like effects in animal models of anxiety, including a blockade of the anxiogenic-like effects of CRF in the elevated plus maze test (10 and 20 mg/kg), impaired induction and expression of conditioned fear (20 mg/kg), and reduced burying behavior in rats (10 and 20 mg/kg) (11, 41, 50, 51). In agreement with these findings, in the present study the pretreatment of rats with antalarmin (10 mg/kg and 20 mg/kg) prior to the second exposure to the aversive context inhibited the conditioned fear response in the HR group. We did not observe any significant inhibitory effects of antalarmin in the LR rats. Similar results were presented by Keck *et al.* (52). In this report, the anxiolytic-like effects of a different non-peptide CRF_1 receptor antagonist (R121919) were found to depend on the level of innate emotionality in the rats. The authors found that R121919 displayed anxiolytic effects in the elevated plus maze only in rats selectively bred for high anxiety-like behavior (HAB rats) and had no anxiolytic effects in rats selectively bred for low anxiety-like behavior (LAB). Similarly, Rotzinger *et al.* (49) suggested that the effects of a CRF_1 receptor antagonist in animal models of anxiety were dependent upon the baseline anxiety state of the animal and the test parameters (49, 52).

In the present study, the behavioral effect of a lower dose of antalarmin was accompanied by increased GAD67 expression in the PL and CeA and increased the GABA concentration in the amygdala in the HR rats. The PL, which is a subregion of the medial prefrontal cortex, seems to be critical for the expression of fear-related behavior (18, 53-54). PL activity increases during and following fear conditioning (17, 55). Additionally, PL has a reciprocal connection with the amygdala, especially with the BA. Subsequently, augmented BA activity mediated through the PL is a necessary condition to activate CeA output neurons, which results in fear responses (53-54). Thus, the antalarmin treatment-related increase in GAD67 activity in the PL of HR rats *via* the enhancement of local GABA synthesis might diminish the activity of this important neuronal loop for the expression of fear.

Pretreatment of the HR rats with antalarmin enhanced GABAergic neurotransmission (shown by the increased GAD67 expression) in the CeA. Similar results were observed in our previous study, where the non-selective CRF receptor antagonist α-helical CRF(9-41) significantly decreased the rat freezing responses and increased the GABA concentration in the CeA during the first 30 min of observation (microdialysis) (56). The CeA is largely GABAergic, receives glutamatergic projections from the LA and expresses a wide variety of neuropeptides (CRF, vasopressin, neuropeptide Y, and oxytocin). The role of all these peptides in regulating of anxiety-related behavior has been suggested (2, 57-58). We can not exclude that antalarmin could disinhibit the activity of other neuropeptide systems found in the CeA by blocking the action of CRF, thereby contributing to the anxiolytic effect. For example, Huber *et al.* (58) demonstrated that oxytocin, which is a neuropeptide with a strong anxiolytic potency, excited a subpopulation of GABAergic neurons in the CeA. When activated by oxytocin, these neurons exerted tonic inhibition by reducing the excitability of CeA neurons (2, 58).

Some data from the literature indicate that CRF has a stimulating effect on GABAergic activity in the amygdala (10, 59). However, these data are limited and significantly differ

methodologically from our study. For example, Nie *et al.* (10) based their conclusions solely on the analysis of electrophysiological changes in IPSC (inhibitory postsynaptic current) amplitudes in CRF_1 and CRF_2 receptor knock-out mice. Roberto *et al.* (59) analyzed the effect of a different CRF_1 antagonist (R121919) on the ethanol-induced release of GABA in the CeA *in vivo*. However, these authors did not observe any effect of this CRF_1 receptor antagonist on the basal release of GABA. The mechanisms underlying the effect of CRF antagonists on the GABA system require further analysis. The possibility that this effect is indirect and secondary to the influence of the CRF_1 receptor antagonist on the equilibrium between the other neurotransmitter systems present in the prefrontal cortex and amygdala also cannot be excluded. As mentioned earlier, CRF is expressed in GAD-positive interneurons in the cerebral cortex (20-21). Another point is that fear conditioned context may stimulate corticosterone secretion, which has actions in the amygdala not only inhibiting GABA release, but also facilitating glutamate release (32, 60-62). The effects of CRF_1 antagonist could be linked to these activities, as shown by a number of other publications (51, 63-65), however, more accurate discussion of this topic is beyond the scope of our work.

The effects of a lower dose (10 mg/kg) of antalarmin were more potent than the effects of the higher dose (20 mg/kg) of the antagonist. Accordingly, Heinrichs *et al.* (66) found that only the lowest dose (1 μg, i.c.v.) of α-helical $CRF_{9\text{-}41}$ tested was effective at blocking the stress-induced decrease in exploration on the elevated plus maze test, whereas higher doses (5 and 25 μg) were ineffective (49, 66). Although there are some important differences between the experimental protocols of Heinrichs *et al.* (66) report and our study (e.g., administration of a different CRF_1 receptor antagonist and a different type of aversive stimulation), these results indicate a non-linear dose response curve of the CRF receptor antagonists effects. It is widely accepted in the pharmacological sciences that a lower dose of a drug is more selective than a higher dose. Therefore, the higher doses of antalarmin could activate other receptors (e.g., alpha-2 and beta-2 adrenergic, kappa opioid, cholecystokinin B, and D2 receptor) and impair the selectivity of the drug action through these interactions (67-71).

It is noteworthy, that the CRF_1 receptors are expressed in numerous extrahypothalamic brain regions including the ventral tegmental area (VTA) a structure that appears important for aversive learning (72-73). The recent study showed that intra-VTA injection of a lentivirus against CRF_1 mRNA did not affect tone-elicited freezing during conditioning but increased freezing duration to the tone even after extinction and reinstatement (72). This study demonstrated that CRF_1 receptors located in the VTA also play an important role in the conditioned fear. Another structures of the brain, which could contribute to the effects of CRF antagonist, may involve the central serotonin system (74), as well as CRF receptors within the periaqueductal gray matter, regulating pain responses (75).

In summary, this study shows that the LR rats appear to be more sensitive to the second exposure to contextual fear stimuli. This phenomenon was accompanied by increased neuronal activity in the amygdala. Furthermore, this study demonstrates that HR rats are more sensitive to the anxiolytic effects of acute antalarmin administration, which are accompanied by changes in CRF-GABA system activity. In this group of animals, antalarmin administration enhanced GABA synthesis and the GABA concentration in the medial prefrontal cortex and amygdala. These results indicate that the fear responses in the HR rats may be regulated by innate and individually variable changes in the activity of the local CRF and GABAergic systems. The current data may help increase our understanding of the neurobiological mechanism controlling the CRF-GABA interaction within the prefrontal cortex-amygdala circuitry, which is responsible for individual differences in reactivity to stressors. This knowledge can be applied to elucidate the pathophysiology of the predisposition to anxiety and trauma/stress-related disorders.

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Author's address: Dr. Anna Skorzewska, Department of Neurochemistry, Institute of Psychiatry and Neurology, 9 Sobieskiego Street, 02-957 Warsaw, Poland
E-mail: skorzews@ipin.edu.pl