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CHRONIC STRESS CHANGES PREPULSE INHIBITION AFTER AMPHETAMINE CHALLENGE: THE ROLE OF THE DOPAMINERGIC SYSTEM

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The goal of this research was to examine the influence of chronic mild stress (CMS) on prepulse inhibition (PPI). We used an amphetamine challenge to study the role of the dopaminergic system in limbic structures. Chronic stress caused a reduction in both sucrose preference and body weight. It was found that the initially strong response to amphetamine in the control rats was weakened after stress on both the behavioural and biochemical levels: improved PPI, decreased dopamine D2 receptor expression in the central nucleus of amygdala (CeA) and nucleus accumbens (NAC), and decreased dopamine and 3-MT (3-methoxytyramine) levels in NAC. We observed that the stress-evoked attenuation of amphetamine-induced stimulation was also paralleled by changes in corticosterone level. These effects were accompanied by a decrease in both glutamate and the glutamate/gamma-aminobutric acid (GABA) ratio in the NAC. The interpretation of these results is that prolonged stress induces compensatory mechanisms in the mesolimbic system which are responsible for psychostimulant (amphetamine) effects.

Key words: *chronic mild stress, prepulse inhibition, amphetamine, dopaminergic system, body weight, sucrose, glutamate, gamma-aminobutric acid, dopamine D2 receptor*

INTRODUCTION

The mesocorticolimbic dopaminergic system has been proven to play a pivotal role in reward-motivated behaviours (1-3). The effects of stress on attention, motivational processes and reward are powerful, including complex interactions between the hypothalamic-pituitary-adrenal (HPA) axis activity and regulation of the dopaminergic system (4-6). Recent research has suggested that prepulse inhibition (PPI) would be a useful tool, as it is believed to reflect the integration of cortical - limbic mechanisms in the control of psychiatric disorders also related to anxiety and fear (7-9). PPI is a procedure for assaying primary unconscious information processing and occurs when a relatively weak sensory event (the prepulse) is presented 30 - 500 ms before a strong, startle-inducing stimulus, thus reducing the magnitude of the startle response. PPI deficits have been associated with multiple neuropsychiatric disorders characterised by inhibitory deficits in sensory, motor, and cognitive function (10-15). It was shown that catechol-O-methyl transferase (COMT) deficient mice presented higher dopamine levels in the striatum and mimicked schizophrenia-related behaviours as reduced prepulse inhibition (16). The circuits of the blink reflex, the startle reaction and PPI share some commonalities with important emotional components controlled by the limbic system (PPI is modulated by both attentional and emotional responses to the prepulse stimulus, and PPI in rats is enhanced by auditory fear conditioning) (14, 17-20). Disruption of PPI in the acoustic startle response in rats has been widely used as an animal model for the sensorimotor gating deficit that is usually found in schizophrenia (17, 21-24). PPI was reported to be regulated by forebrain circuits, including the mesolimbic cortex, nucleus accumbens, ventral pallidum, thalamus, and the pedunculopontine tegmental nucleus (14, 17, 18). The dopaminergic turnover in prefrontal cortex and striatum is important for maintaining proper dopaminergic activity (25). Some works reported on unbalance between cortical and striatal dopaminergic system and its effects on PPI (24, 26-28). The studies have also found strain-related differences in sensitivity to the PPI-disruptive effects of dopamine agonists, with the Wistar strain often used in this model (29, 30). In rodents, disruption of PPI in the startle response is produced by stimulation of D2 dopamine receptors by amphetamine or apomorphine; by activation of serotonergic systems; by serotonin releasing agents or direct agonists at certain serotonin receptors; by blockade of N-methyl-D-aspartate receptors; and by drugs such as phencyclidine (31, 32). For example, MK-801, phencyclidine and apomorphine disrupted PPI, and the median effective dose (ED_{50} value) of the drugs needed to reverse the apomorphine-induced PPI disruption, including typical antipsychotics, were significantly correlated with its affinity for the dopamine D2 receptor (21). PPI inhibition deficits can also be induced by many environmental factors (33-35).

The chronic mild stress (CMS) is a good model of anhedonia, learned helplessness, and depressive-like symptoms

in rodents (36, 37). Chronic stress causes behavioural changes that correspond with cognitive and motivational impairment (38-40). Dopaminergic functioning is to a large extent modified by D2 receptors, which can internalise in response to a stimulus (41, 42). Chronic stress can decrease both dopamine metabolism and D2 receptor expression in the NAC and striatum, changes that are related to reduced behavioural response to motivation (37).

In light of these data, it seems interesting to use PPI to analyse behavioural and neurochemical changes under stress conditions. We used CMS model to study changes in the dopaminergic part of the motivational limbic system. Since high anxiety level is often comorbid with many other psychopathologies, we considered the additional use of a conditioned fear test to analyse the relationship between negative emotional state and PPI. The effect of CMS on sucrose preference and body weight was also studied, followed by an examination of the changes in glutamate and dopamine metabolism, as well as changes in the expression of D2 dopamine receptors in brain structures associated with positive reinforcement the (NAC shell and core), emotional behaviour (CeA, BA), and the HPA (corticosterone level in prefrontal cortex) control.

MATERIALS AND METHODS

Animals

Forty eight male Wistar rats (200 g body weight, 6 weeks old at arrival), purchased from the Centre for Experimental Medicine, Medical University of Bialystok Poland were used. The animals were acclimated and housed under standard laboratory conditions (21 ± 2°C; humidity 45 – 55%; 12 h light/dark cycle, light on at 7 a.m.) with *ad libitum* access to water and rodent chow.

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

Experimental protocol

As shown in *Fig. 1* after 7 days of acclimatisation to the laboratory conditions, the animals undergo the conditioned fear test (CFT) (43). Next, starting from the 13th day, the groups were randomised to unpredictable, chronic mild stress for 5 weeks (stressed rats n = 24 and control rats n = 24). The control rats were handled for 5 min daily. The body weights of all the rats were measured weekly. Once a week, beginning on the 16th day of the experiment (4th day of the CMS), the animals were exposed for 20 h to Two Bottle Sucrose Preference Test (*Fig. 1*). On the 48th and 55th days, the rats underwent the prepulse inhibition test (PPI). Amphetamine (1.5 mg/kg) was administered intraperitoneally 30 min before the PPI test to the following group of animals (control amph, n = 12; stressed amph, n = 12), while the following group of animals were administered saline (control saline, n = 12; stressed saline, n = 12). Between the first and second PPI tests, the stress procedure was pursued. To exclude the effects of acute stress, the rats were rested on the testing days and on the two days preceding the PPI tests. One rat was excluded because of technical problems. Next, 90 min after the second PPI test, the animals were decapitated, and their brains were removed and frozen at –70°C.

Conditioned fear test (CFT)

The fear-conditioning experiment was performed in experimental cages (36 × 21 × 20 cm, w/l/h) under constant white noise conditions (65 dB) (*Fig. 1*). On the first day, after 5 min of habituation to the training box, the animals underwent a fear-conditioning procedure, with each animal receiving three foot shocks (stimulus: 0.7 mA, 1 s, repeated every 59 s). Conditioned fear was tested on the second day (test day) by re-

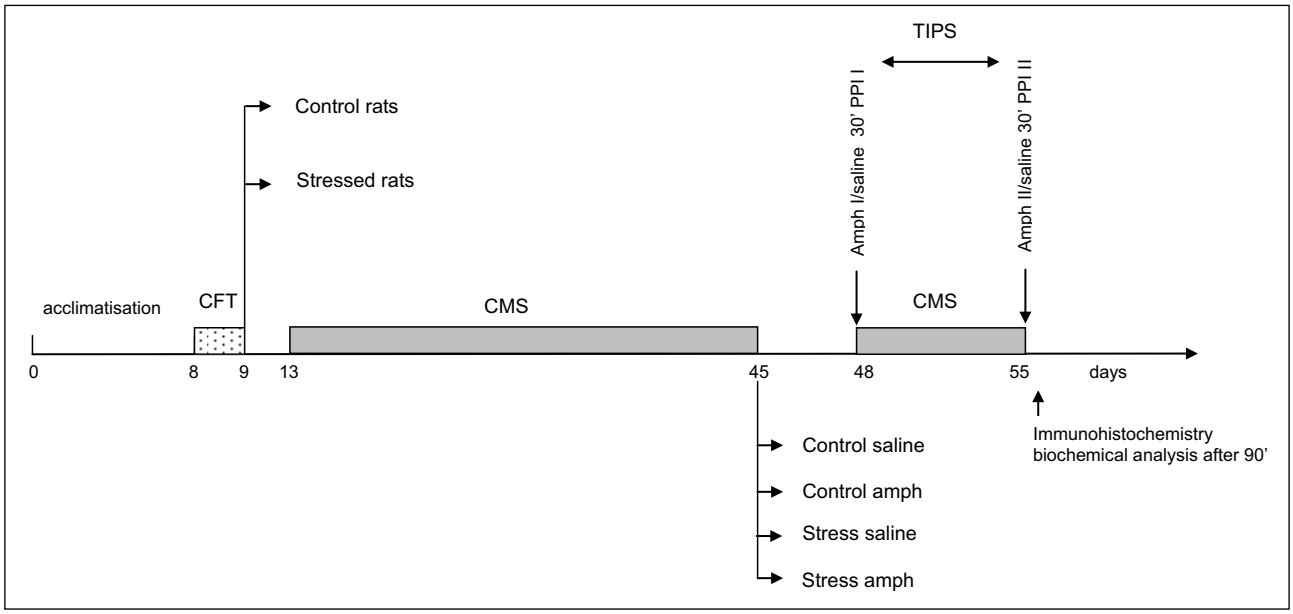

Fig. 1. The diagram of the experiments. CFT - conditioned fear test; CMS - chronic mild stress; Control saline - control saline administered, non-stressed rats, n = 12; Control amph - control amphetamine administered, non-stressed rats, n = 12; Stressed saline - stressed, saline administered rats, n = 12; Stressed amph - stressed, amphetamine administered rats, n = 12. TIPS - two injection protocol of amphetamine. Amph I/ saline - first injection of amphetamine (1.5 mg/kg) or saline; Amph II/ saline - second (challenge) injection of amphetamine (1.5 mg/kg) or saline; PPI I - first prepulse inhibition test; PPI II - second prepulse inhibition test. For more details, see Methods.

exposing the rats to the testing box and recording their freezing responses over 10 min (freezing was measured by infrared photo beams, 10 Hz detection rate, and controlled by the fear conditioning software, TSE, Bad Homburg, Germany).

Chronic mild stress

Chronic mild stress was implemented based on a modified protocol that was validated in our laboratory (*Fig. 1*). The animals were randomly and uninterruptedly exposed to a variety of mild stressors: wet bedding, home cages angled 10 – 40°, restraint (0.5 – 3 h, varying duration), light off during the day, light on during the night, water deprivation (6 – 12 h, varying duration), isolation housing, flashing (light switched on and off alternately in a short period of time). Two of these stressors were used daily for varying lengths and at random occurrences modified by Gouirand and Matuszewicz (44).

Sucrose preference test

The sucrose preference test was used to show anhedonic states in the animals for five consecutive weeks, starting from the first week of the stress procedure. The sucrose preference tests lasted 20 h (12 h at dark period, 8 h at light period). During each test, the animals were singly housed in cages with two bottles (one bottle was filled with 1% sucrose solution, and the second one with water). The animals had free access to food. Sucrose and water consumption were measured by weighing bottles before and after the test. To accustom the rats to drink the 1% sucrose solution, the same bottle was used during the group-housed acclimatisation period (modified by Wislowska-Stanek *et al.*) (45).

Prepulse inhibition test (PPI)

The PPI apparatus consisted of eight startle chambers (SR-LAB, San Diego Instruments, San Diego, CA, USA). Each chamber consisted of a Plexiglas cylinder (8.9 cm diameter × 20 cm long) resting on a Plexiglas frame located in a sound attenuated, ventilated enclosure. Acoustic stimuli and background noise were presented *via* a loudspeaker mounted 24 cm above the animal. For dB measurement we used a power quantity scale *i.e.* a power directly proportional to acustic intensity. We used the A weighting scale in units dBA Sound Pressure Level. Startle responses, which reflect the motion of animals in the cylinder following the acoustic stimulus, were detected by a piezoelectric transducer mounted below the frame. SR-LAB software was used to control the administration of the stimuli and response recording. The chamber light was on, and the background white noise was set at 70 dB during the whole session. The rats were placed individually in the Plexiglas cylinder. Each session lasted 30 min and started with a 5-min acclimatisation period (46). The test session included 3 initial startling stimuli. These stimuli (120 dB, duration: 40 ms) were given during the acclimatisation period with an average inter-trial interval (ITI) of 22.5 s (15 - 30 s). The inter-trial interval was randomised by the SR-LAB software. The initial stimuli were followed by 60 trials of different intensities presented in a random order, with a mean ITI of 22.5 s. The startle responses were measured for 100 ms after the onset of the acoustic stimulus. The interstimulus between prepulse and startle pulse (ISI) was 80 ms. For each type of stimulation, the startle amplitudes were averaged across 10 trials. The PPI session consisted of 10 background trials with a sham stimulus (70 dB, 40 ms), two types (2 × 10) of prepulse trials (PP) that included only 20-ms of PP stimuli (84 dB or 90 dB), 10 pulse trials (P) that included only a pulse (startling) stimulus (120 dB, 40 ms), and two types (2 × 10) of prepulse-and-pulse trials (PP-P) that included a 20-ms PP (84 dB or 90 dB). The magnitude of the PPI was calculated as a percent inhibition of the startle amplitude in the P trial (treated as 100%) according to the formula: [(startle amplitude in P trials - startle amplitude in PP-P trials)/startle amplitude in P trials] × 100%. Startle responses to the three initial stimuli were excluded from the statistical analyses.

Drugs

D-amphetamine sulphate (Sigma-Aldrich, USA) was dissolved in a sterile aqueous 0.9% NaCl solution (Polpharma, Starogard Gdanski, Poland) and was injected intraperitoneally at a dose of 1.5 mg/kg, 30 min before the prepulse inhibition test.

Two-injection protocol of amphetamine

This procedure involved the initiation of sensitisation to the drug with the two-injection protocol of sensitisation (TIPS procedure), which consisted of the administration of a first dose of amphetamine and a second dose of amphetamine 6 days later. We previously used the TIPS procedure to assess 50-kHz USV responses to amphetamine in rats (47). On the 1st day of drug treatment, the rats were amphetamine injected and then submitted to the PPI test 30 min later. Then, after a 6-day break from the injections, the rats were given a 2nd drug injection (challenge) and tested in the PPI test (*Fig. 1*).

Tissue homogenisation

After decapitation, the brains were removed, frozen in dry-ice cooled cyclopenthane, and stored at –70°C. Frozen brains were cut into slices on a cryostat. For corticosterone analysis: the prefrontal cortex (4.7 – 4.2 mm anterior to bregma); for amino acids, dopamine and its metabolites analysis: the nucleus accumbens (1.7 – 0.7 mm anterior to bregma) and the amygdala complex (2.80 to 3.30 mm posterior to bregma), were micropunched according to the rat brain atlas by Paxinos and Watson (48). For amino acids, dopamine and its metabolites analysis each tissue was weighed, placed in a dry ice-cooled polypropylene vial, and homogenized with a polytron-type homogenizer (30 s, 4°C) in a solution containing 15 volumes of perchloric acid (0.2 M) with dihydroxybenzylamine as the internal standard as described previously with minor modifications (49). The obtained supernatants were filtered through 0.45-µm pore filters and then kept at –70°C until analyses.

Corticosterone analysis

The dissected tissue was weighed, placed in a dry, ice-cooled polypropylene vial, and homogenised with an ultrasonic homogeniser (30 s, 4°C) in protease inhibitor cocktail (Sigma-Aldrich). The homogenate was boiled (2 min), centrifuged (4°C, 11,000 rpm, 20 min) and the obtained supernatants were kept at –20°C. Corticosterone level was measured using a commercial ELISA kit (Enzo Life Sciences) according to the manufacturer's procedure (wave length: 405 nm and 570 nm). The results were converted into tissue mass (ng/g) (modified by Weber *et al.* (50)). Brain corticosterone levels closely reflects the peripheral concentration (51).

High-performance liquid chromatography (HPLC) analysis

1. Amino acids

HPLC analysis of amino acids was performed using a Luna C18 (250 × 5 mm) 5 µm reverse phase column. Compounds were eluted isocratically with mobile phase delivered at 0.7 ml/min using a Shimadzu Class LC-10ADvp pump. Electrochemical detector with a flow-through cell (Intro-Antec

Leyden), linked to Shimadzu Class VP Integrator SCL-10 Avp, was used. A high-density glassy carbon-working electrode (Antec) was operated at +0.84 V. Rheodyne injection valve with a 20 µl sample loops was used manually inject the samples. Preparation of the mobile phase and the derivatising agents were based on the method of Rowley *et al.* (52) with some modifications. The mobile phase consisted of 45 mM disodium phosphate and 0.15 mM EDTA with 24% methanol (v/v) water adjusted to pH 3.9 with 0.2 M citric acid. It was then filtered through 0.45 µm filters and degassed for 15 min. Stock solution (0.01 M) of amino acids standards were prepared in double deionised water and kept at 4°C for five days. To prevent adhesion to glass, amino acids (especially GABA) standards were prepared in polyethylene vials. Working solutions were prepared daily by dilution of the stock solutions. To obtain agents for derivatisation; 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 the pH 10.4 with 5 M sodium hydroxide. The reaction of derivatisation was performed at room temperature. Derivatising agent (20 µ) was reacted with 1 ml of amino acid standard for 8 min in polyethylene vial before injection onto the column. For reaction with samples (20 µl), the volume of derivatising agent was reduced to 0.4 µl to eliminate contamination of chromatogram by exessive reagent, which is electroactive. The concentration of amino acids was calculated as µM.

2. Dopamine and its metabolite

The levels of monoamines and their metabolites were assessed using a modified high-pressure liquid chromatography (HPLC) metod previously described by Kaneda *et al.* (53) with minor modifications (54). The HPLC system consisted of a Shimadzu LC-10AD VP pump and an electrochemical detector with a flow-through cell (Waters 2465). A high density, glassy carbon-working electrode was operated at +800 mV. The sample was injected manually *via* a Rheodyne 7725i injection valve with a 20-µl sample loop. The separation of monoamines and their metabolites was attained on a Phenomenex Luna C 18 (150 mm × 3 mm i.d., 3-µm particle size) with a Phenomenex KJO-4286 precolumn. The column temperature was 32°C. The mobile phase consisted of 64.4 mM disodium phosphate (Na_2HPO_4), 67.8 mM citric acid ($C_6H_8O_7$), 0.054 mM ethylenediaminetetraacetic acid (EDTA), 0.39 mM octane sulphonic acid ($C_8H_{17}NaSO_4$) 2 mM potassium chloride (KCl) and 12% methanol. It was filtered through 0.45-µm filters (Millipore). The flow rate was 0.4 ml/min. The mobile phase was degassed with helium. The chromatograf registration and analysis were performed using the Chr-mod 2007 software. The concentrations of dopamine (DA), 3-metoxytyramine (3-MT) were calculated as ng/g of brain tissue.

Immunocytochemistry

Coronal 20-µm cryostat sections, identified using the rat brain atlas (48), were cut, mounted on silane-coated slides and fixed in methanol for 10 min. The slices from each section were taken for immunostaining D2 receptors expression. After blocking endogenous peroxidase activity and non-specific binding, the tissue samples were incubated with primary rabbit polyclonal antibodies against D2 receptor (1:1000, Abcam) at 4 – 8°C for 24 hours. Then, the staining levels were detected with peroxidase-conjugated anti-rabbit IgG (1:2000, ImmunoJackson Research). The peroxidase reaction was developed with DAB (0.2 mg/ml) and hydrogen peroxide (0.003%) in Tris buffer. Next, the sections were dehydrated by serial immersion in alcohol, immersed in xylene to remove the alcohol, and cover slipped in the histofluid mounting medium. Immunopositive cells were manually counted using an image analysis system (Olympus BX-51 microscope with Camera DP 70, Olympus cellSens software) in the following manner: the examined areas including the nucleus accumbens shell, (NAC shell), and its core, (NAC core); 0.2 mm^2 frame and the central nucleus (CeA) of the amygdala, were sampled using a 0.15 mm^2 frame. The counts were expressed as the number of positive cells per mm^2.

Controls

Western blot analysis performed with the D2 receptor antibodies confirmed specific binding to the D2 receptors. Control studies were performed without primary or secondary antibodies (to detect non-specific binding of antibodies and endogenous peroxidase activity) and yielded negative results.

Statistical analysis

The data are shown as the means + S.E.M. The body weight data and sucrose preference (during CMS) were analysed with a repeated measures ANOVA. The behavioural analysis (prepulse inhibition after CMS) and immunohistochemistry data were analysed with ANOVA. The ANOVA was followed by the Newman-Keuls test. For correlation analysis a Pearson's coefficient was calculated. All the statistical analyses were performed using the Statistica v.12 software.

RESULTS

Behavioural data

1. Body weight

As shown in *Fig. 2*, the repeated measures ANOVA showed significant differences among the groups for the effects of the unpredictable mild stress: stress effect [$F(1,135) = 7.02$, ($P < 0.05$)]; time effect [$F(1,135) = 212.31$, ($P < 0.01$)]; time × stress interaction effect [$F(1,135) = 6.95$, ($P < 0.01$)]. Post hoc tests showed reduced weight gain in the fourth week of mild stress in the stressed compared to the control rats ($P < 0.01$) (*Fig. 2*).

2. Sucrose preference

The repeated measures ANOVA showed significant differences among the groups for the effects of the unpredictable mild stress: stress effect [$F(1,135) = 29.1$, ($P < 0.01$)]; time effect [$F(1,135) = 7.49$, ($P < 0.01$)]; time × stress interaction effect [$F(1,135) = 7.6$, ($P < 0.01$)] (*Fig. 2*). Post hoc tests showed a decrease in sucrose consumption in the second ($P < 0.05$), third ($P < 0.01$) and fourth ($P < 0.01$) weeks in the stressed compared to the control rats.

3. Prepulse inhibition (Fig. 3A)

As shown in *Fig. 3A,* after first amphetamine administration the ANOVA revealed significant differences between the groups in the prepulse inhibition: amphetamine effect [$F(1,43) = 4.9$, ($P < 0.05$)]; PPI 84/120, 90/120 effect [$F(1,43) = 24.2$, ($P < 0.01$)]; stress × amphetamine × PPI 84/120, 90/120 interaction effect [$F(1,43) = 7.5$, ($P < 0.01$)]; no stress effect [$F(1,43) = 0.1$, ($P = 0.74$)]; no stress × amphetamine interaction effect [$F(1,43) = 4.0$, ($P = 0.052$)]; no stress × PPI 84/120, 90/120 interaction effect [$F(1,43) = 1.6$, ($P = 0.22$)]; no amphetamine × PPI 84/120, 90/120 interaction effect [$F(1,43) = 0.11$, ($P = 0.73$)]. Post hoc tests showed, in the control saline group, a reduced PPI 84/120 compared to PPI 90/120 ($P < 0.01$) and in stressed amphetamine

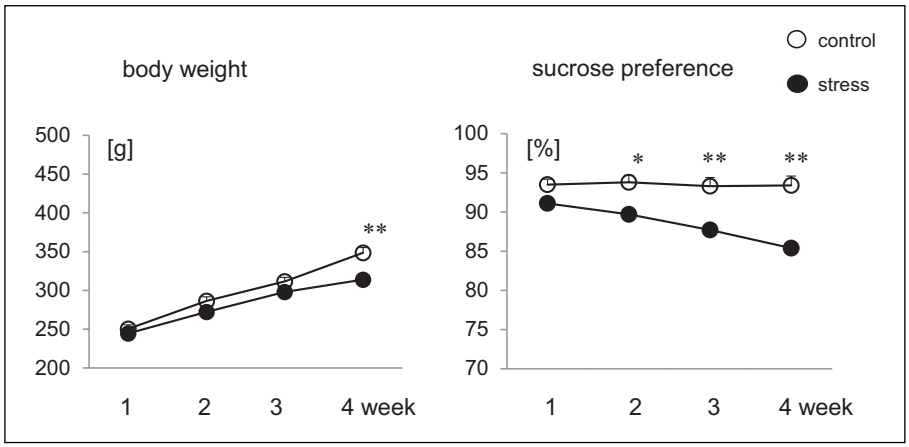

Fig. 2. Changes in body weight and sucrose preference during chronic mild stress. *differs from stressed rats, *P < 0.05; **P < 0.01. Control rats n = 24; stressed rats n = 23. The data are shown as the means ± SEM.

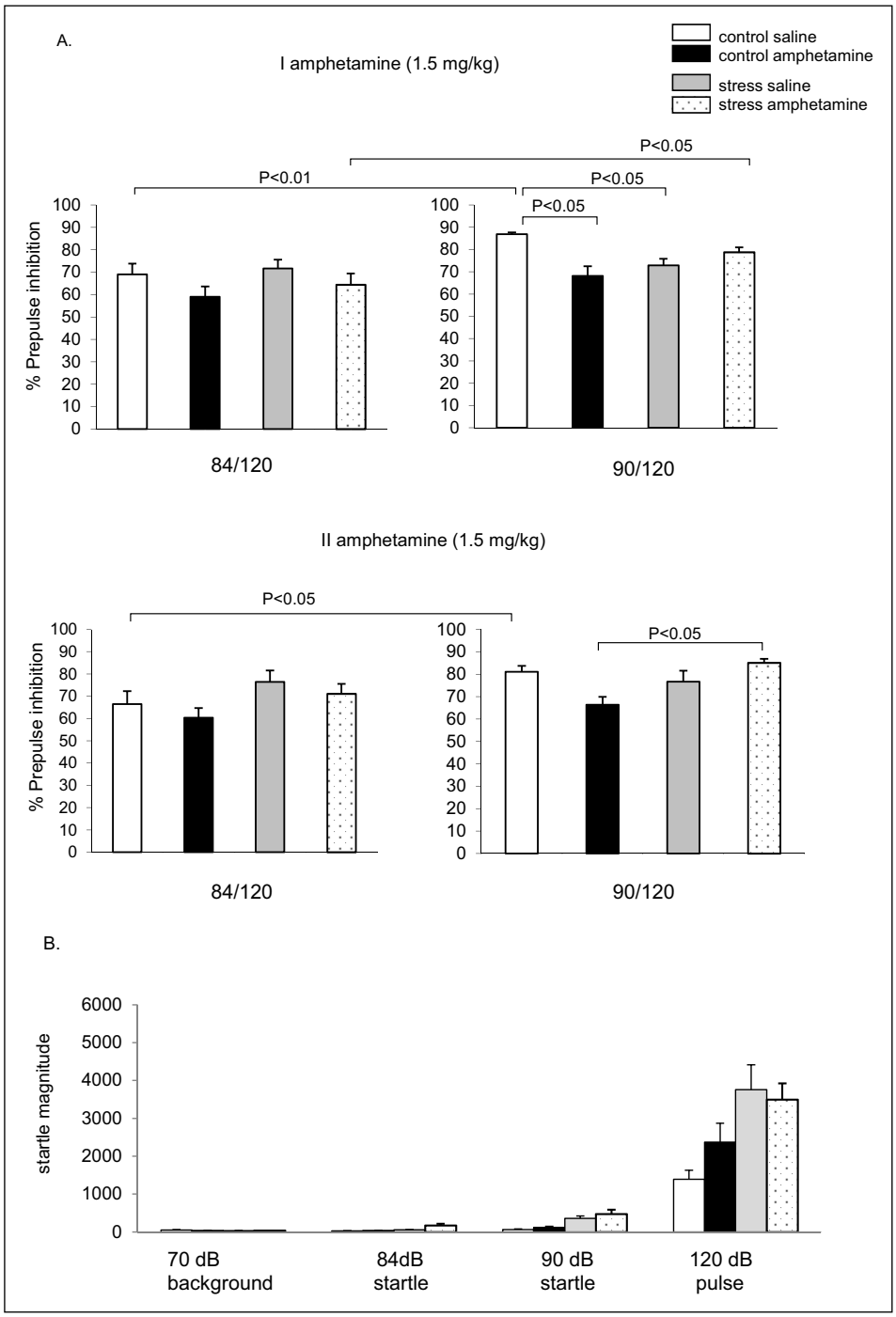

Fig. 3. (*A*) Prepulse inhibition after the first and second injection of amphetamine or saline. (*B*) Startle and Pulse response after second dose of amphetamine. Startle amplitudes in manufacturer's arbitrary units. Control saline rats n = 11; Control amphetamine rats n = 12; Stressed saline rats n = 12; Stressed amphetamine rats n = 12. The data are shown as the mean percentage prepulse inhibition response ± SEM.

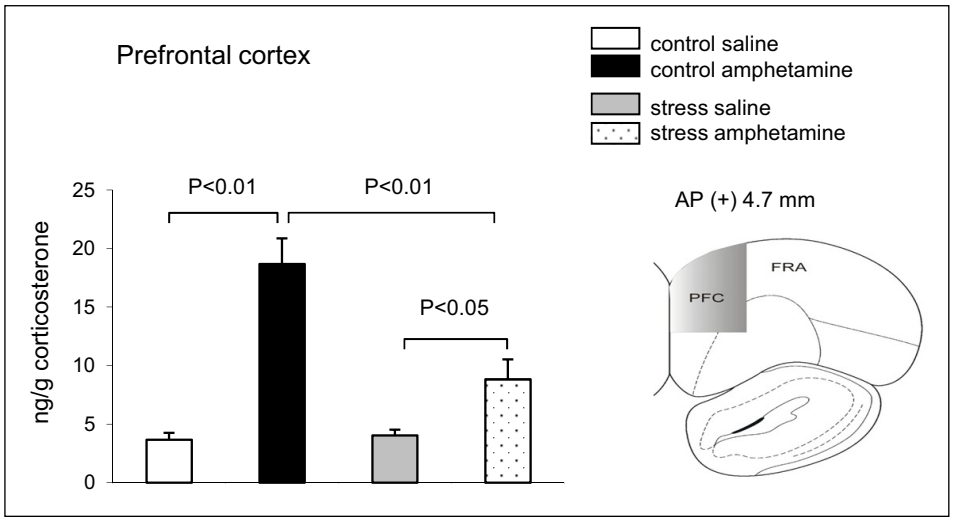

Fig. 4. The level of corticosterone in the prefrontal cortex after the second injection of amphetamine (1.5 mg/kg) or saline. Control saline rats n = 11; Control amphetamine rats n = 12; Stressed saline rats n = 12; Stressed amphetamine rats n = 12. The schematic coronal section adapted from the atlas of Paxinos and Watson (48*)* showing brain structures used in corticosterone level analysis. AP - anterior, posterior from bregma; PFC - prefrontal cortex; FRA - frontal cortex. The data are shown as the means ± SEM.

group PPI 84/120 compared to PPI 90/120 ($P < 0.05$). The reduced PPI 90/120 was shown in control amphetamine group compared to control saline group ($P < 0.05$), and in the control stressed group compared to control saline group ($P < 0.05$) (*Fig. 3A*).

After second amphetamine administration the ANOVA revealed significant differences between the groups in the prepulse inhibition: stress effect [$F(1,43) = 6.4$, ($P < 0.05$)]; PPI 84/120, 90/120 effect [$F(1,43) = 11.6$, ($P < 0.01$)]; stress × amphetamine × PPI84/120, 90/120 interaction effect [$F(1,43) = 4.7$, ($P < 0.05$)]; no amphetamine effect [$F(1,43) = 1.6$, ($P = 0.2$)]; no stress × amphetamine interaction effect [$F(1,43) = 3.0$, ($P = 0.08$)]; no stress × PPI 84/120, 90/120 interaction effect [$F(1,43) = 0.4$, ($P = 0.53$)]; no amphetamine × 84/120, 90/120 interaction effect [$F(1,43) = 0.24$, ($P = 0.63$)]. Post hoc tests showed, in the control saline groups, a reduced PPI 84/120 compared to PPI 90/120 ($P < 0.05$). The reduced PPI 90/120 was shown in control amphetamine group compared to stress amphetamine group ($P < 0.05$).

4. Startle response and pulse response

Fig. 3B shows that after second amphetamine administration, the ANOVA revealed significant differences between the groups in the startle response: stress effect [$F(1,43) = 20.2$, ($P < 0.01$)]; amphetamine effect [$F(1,43) = 4.45$, ($P < 0.05$)]; startle intensity effect [$F(1,43) = 22.7$ ($P < 0.01$)]; stress × startle intensity interaction effect [$F(1,43) = 14.8$, ($P < 0.01$)]; no stress × amphetamine interaction effect [$F(1,43) = 2.5$, ($P = 0.11$)]; no amphetamine × startle intensity interaction effect [$F(1,43) = 1.69$, ($P = 0.19$)]; no stress × amphetamine × startle intensity interaction effect [$F(1,43) = 1.2$, ($P = 0.3$)].

After second amphetamine administration the ANOVA revealed significant differences between the groups in the 120 dB pulse response: stress effect [$F(1,43) = 16.5$, ($P < 0.01$)]; no amphetamine effect [$F(1,43) = 0.5$, ($P = 0.49$)]; no stress × amphetamine interaction effect [$F(1,43) = 1.43$, ($P = 0.2$)] (*Fig. 3B*).

Biochemical results

1. Corticosterone concentration

As shown in *Fig. 4* the two-way ANOVA revealed significant differences between groups in terms of the corticosterone levels in the prefrontal cortex: stress effect [$F(1,43) = 9.3$, ($P < 0.01$)]; amphetamine effect [$F(1,43) = 45.4$, ($P < 0.01$)], stress × amphetamine interaction effect [$F(1,43) = 12.3$, ($P < 0.01$)]. Post hoc tests showed increased corticosterone concentrations in the control amphetamine compared to the control saline and the stressed amphetamine rats ($P < 0.01$), and in the stressed amphetamine compared to stressed saline rats ($P < 0.05$) (*Fig. 4*).

2. Amino acids

2.1. Glutamate

As shown in *Fig. 5*, the two-way ANOVA revealed significant differences between groups in glutamate level in NAC: stress × amphetamine interaction effect [$F(1,43) = 18.8$ ($P < 0.01$)]; but no stress effect [$F(1,43) = 1.9$, ($P = 0.17$)]; and no amphetamine effect [$F(1,43) = 3.8$, ($P = 0.06$)]. Post hoc analysis showed increased glutamate concentration in the amphetamine control group compared to control and the amphetamine stressed group ($P < 0.01$) (*Fig. 5*).

Two-way ANOVA revealed significant differences between groups in glutamate levels in AMY: amphetamine effect [$F(1,43) = 11.57$, ($P < 0.01$)]; no stress effect [$F(1,43) = 0.3$, ($P = 0.57$)]; stress × amphetamine interaction effect [$F(1,43) = 5.8$ ($P < 0.05$)]. Post hoc analysis showed increased glutamate acid level in the stressed saline group compared to the stress amphetamine group ($P < 0.05$) (*Fig. 6*).

2.2 Gamma-aminobutyric acid

Two-way ANOVA did not revealed any significant differences between groups in GABA level in NAC: no stress effect [$F(1,43) = 0.002$, ($P = 0.97$)]; no amphetamine effect [$F(1,43) = 0.5$ ($P = 0.50$)]; and no stress × amphetamine interaction effect [$F(1,43) = 3.3$, ($P = 0.08$)] (*Fig. 5*).

Two-way ANOVA revealed significant differences between groups in GABA level in the AMY: stress × amphetamine interaction effect [$F(1,43) = 16.97$, ($P < 0.01$)]; but no stress effect [$F(1,43) = 0.75$, ($P = 0.39$)]; and no amphetamine effect [$F(1,43) = 0.5$ ($P = 0.48$)]. Post hoc analysis showed increased GABA levels in the control amphetamine group compared to the control saline ($P < 0.01$) and the stressed amphetamine rats ($P < 0.05$) (*Fig. 6*).

2.3 Glutamate/GABA ratio

Two-way ANOVA revealed significant differences between groups in glutamate acid/GABA ratio in the NAC: amphetamine effect [$F(1,43) = 5.8$, ($P < 0.05$)]; stress × amphetamine

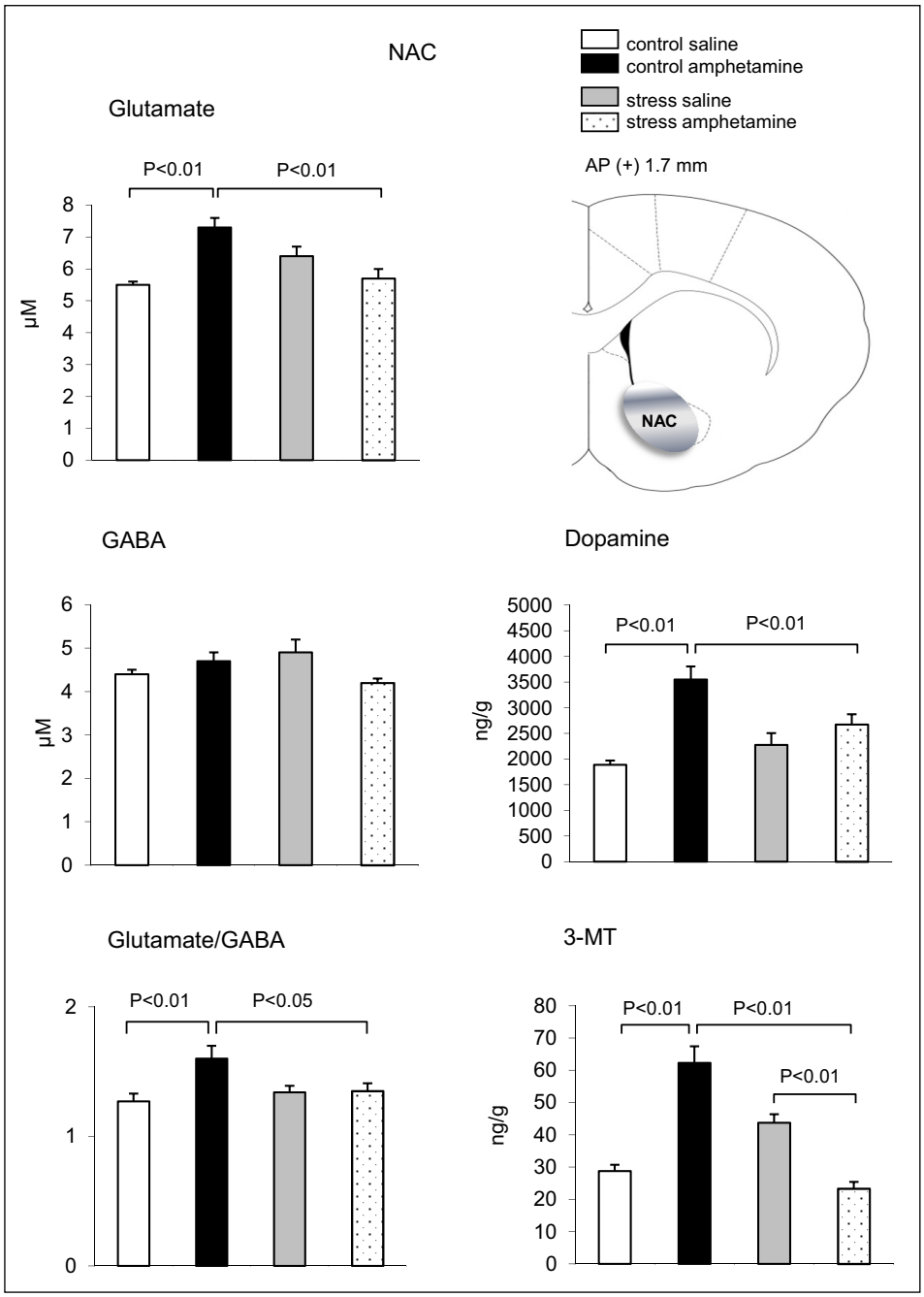

Fig. 5. Concentrations of glutamate, GABA, dopamine, and 3-MT in the nucleus accumbens (NAC) after the second injection of amphetamine (1.5 mg/kg) or saline. Control saline rats n = 11; Control amphetamine rats n = 12; Stressed saline rats n = 12; Stressed amphetamine rats n = 12. The schematic coronal section adapted from the atlas of Paxinos and Watson (48) showing brain structures used in HPLC analysis. AP - anterior, posterior from bregma; 3-MT - 3-methoxytyramine. The data are shown as the means ± SEM.

interaction effect [F(1,43) = 5.2 (P < 0.05)]; but no stress effect [F(1,43) = 1.7, (P = 0.2)]. Post hoc analysis showed increased glutamate acid/GABA ratio in the control amphetamine group compared to the control saline group (P < 0.01) and the stressed amphetamine group (P < 0.05) (*Fig. 5*).

Two-way ANOVA did not revealed any significant differences between groups in glutamate acid/GABA ratio in the AMY: no stress effect [F(1,43) = 3.02, (P = 0.09)]; no amphetamine effect [F(1,43) = 0.3 (P = 0.58)]; and no stress × amphetamine interaction effect [F(1,43) = 0.91, (P = 0.34)] (*Fig. 6*).

3. Dopamine and metabolites

Fig. 5 shows that the two-way ANOVA revealed significant differences between groups in dopamine level in NAC: amphetamine effect [F(1,43) = 24.7 (P < 0.01)]; stress × amphetamine interaction effect [F(1,43) = 9.22, (P < 0.01)]; no stress effect [F(1,43) = 1.38, (P = 0.25)]. Post hoc analysis showed increased dopamine level in the control amphetamine group compared to the control saline and the stressed amphetamine (P < 0.01) groups (*Fig. 5*).

Two-way ANOVA revealed significant differences between groups in 3-MT levels in NAC: stress effect [F(1,43) = 14.1 (P < 0.01)]; amphetamine effect [F(1,43) = 70.6, (P < 0.01)]; stress × amphetamine interaction effect [F(1,43) = 4.2, (P < 0.05)]. Post hoc analysis showed increased 3-MT level in the control amphetamine group compared to the control saline and the stressed amphetamine (P < 0.01) group, and decreased in the stressed amphetamine compared to stressed saline group (P < 0.01) (*Fig. 5*).

Two-way ANOVA did not revealed any significant differences between groups in dopamine level in AMY: no stress effect [F(1,43) = 3.6, (P = 0.06)]; no amphetamine effect [F(1,43) = 0.05, (P = 0.83)]; and no × amphetamine interaction amphetamine effect [F(1,43) = 0.82 (P = 0.37)] (*Fig. 6*).

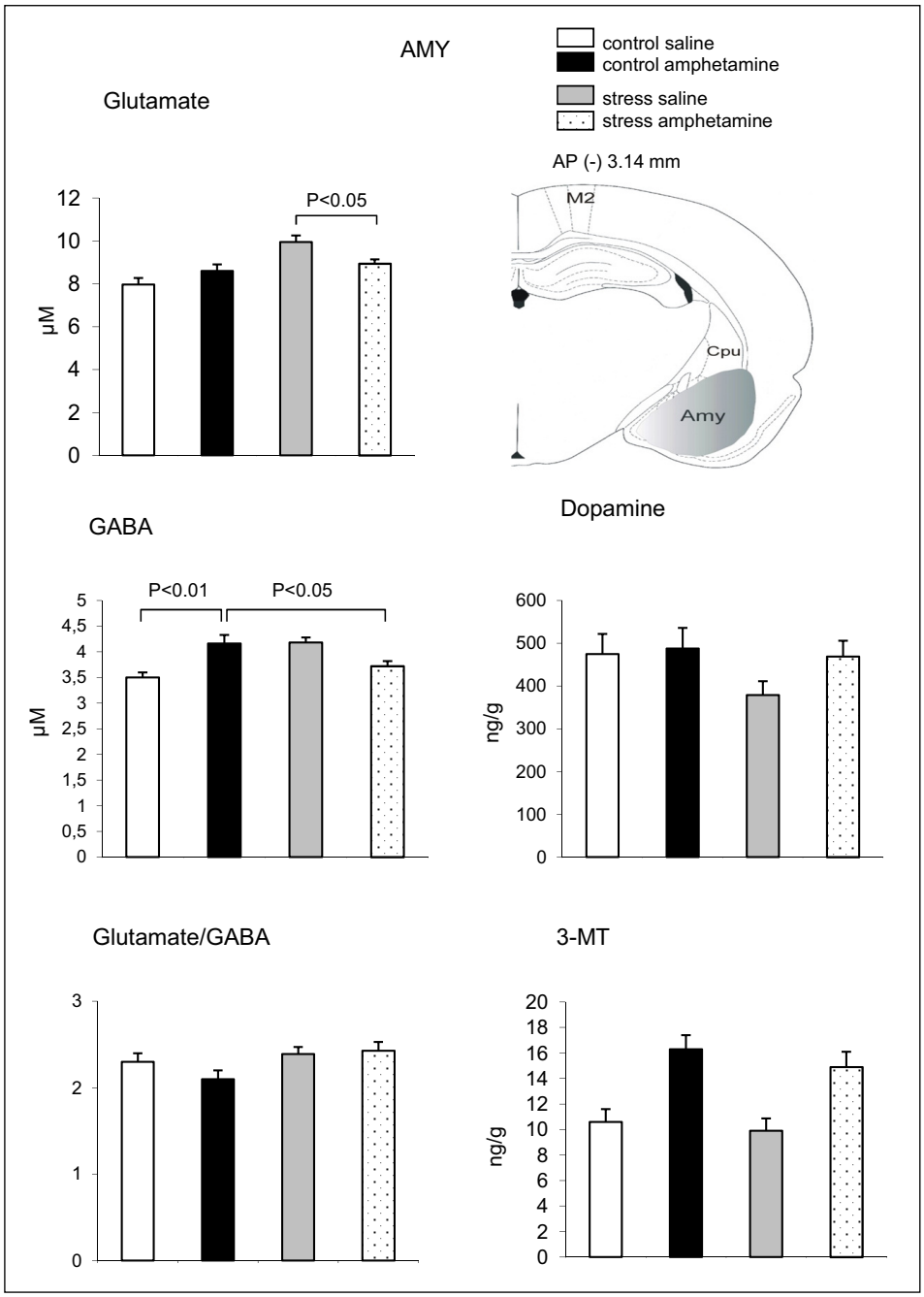

Fig. 6. Concentrations of glutamate, GABA, dopamine, and 3-MT in the amygdala (AMY) after the second injection of amphetamine (1.5 mg/kg) or saline. Control saline rats n = 11; Control amphetamine rats n = 12; Stressed saline rats n = 12; Stressed amphetamine rats n = 12. The schematic coronal section adapted from the atlas of Paxinos and Watson (48) showing brain structures used in HPLC analysis. AP - anterior, posterior from bregma; 3-MT - 3-methoxytyramine, M2 - secondary motor cortex; Cpu - caudate putamen. The data are shown as the means ± SEM.

Two-way ANOVA revealed significant differences between groups in 3-MT level in AMY: amphetamine effect [$F(1,43) = 21.4$, ($P < 0.01$)]; but no stress effect [$F(1,43) = 0.68$ ($P = 0.41$)]; and no stress × amphetamine interaction effect [$F(1,43) = 0.02$, ($P = 0.88$) (*Fig. 6*).

Immunohistochemistry, D2 receptor expression

As shown in *Fig. 7*, the two-way ANOVA revealed significant differences between groups in D2 receptor expression in NAC shell: stress effect [$F(1,43) = 11.6$, ($P < 0.01$)], stress × amphetamine interaction effect [$F(1,43) = 5.7$, ($P < 0.05$)], no amphetamine effect [$F(1,43) = 0.2$, ($P = 0.63$)]. Post hoc showed decreased D_2 expression in the stressed amphetamine compared to the control amphetamine ($P < 0.01$) rats, and the stressed saline rats ($P < 0.05$).

Two-way ANOVA revealed significant differences between groups in D2 receptor expression in NAC core: stress × amphetamine interaction effect [$F(1,43) = 4.8$, ($P < 0.05$)], no stress effect [$F(1,43) = 2.1$, ($P = 0.15$)]; no amphetamine effect [$F(1,43) = 0.1$, ($P = 0.75$)]. Post hoc showed no significant changes.

Two-way ANOVA revealed significant differences between groups in D2 receptor expression in the CEA: stress × amphetamine interaction effect [$F(1,43) = 23.3$, ($P < 0.01$)]; stress effect [$F(1,43) = 15.91$, ($P < 0.01$)]; no amphetamine effect [$F(1,43) = 0.7$, ($P = 0.39$)]. Post hoc showed increased D2 receptor expression in the control amphetamine compared to the control saline ($P < 0.05$) and the stressed amphetamine ($P < 0.01$) groups; in the stressed saline compared to the stressed amphetamine ($P < 0.01$) rats.

Two-way ANOVA did not revealed any significant differences between groups in D2 receptor expression in the BA:

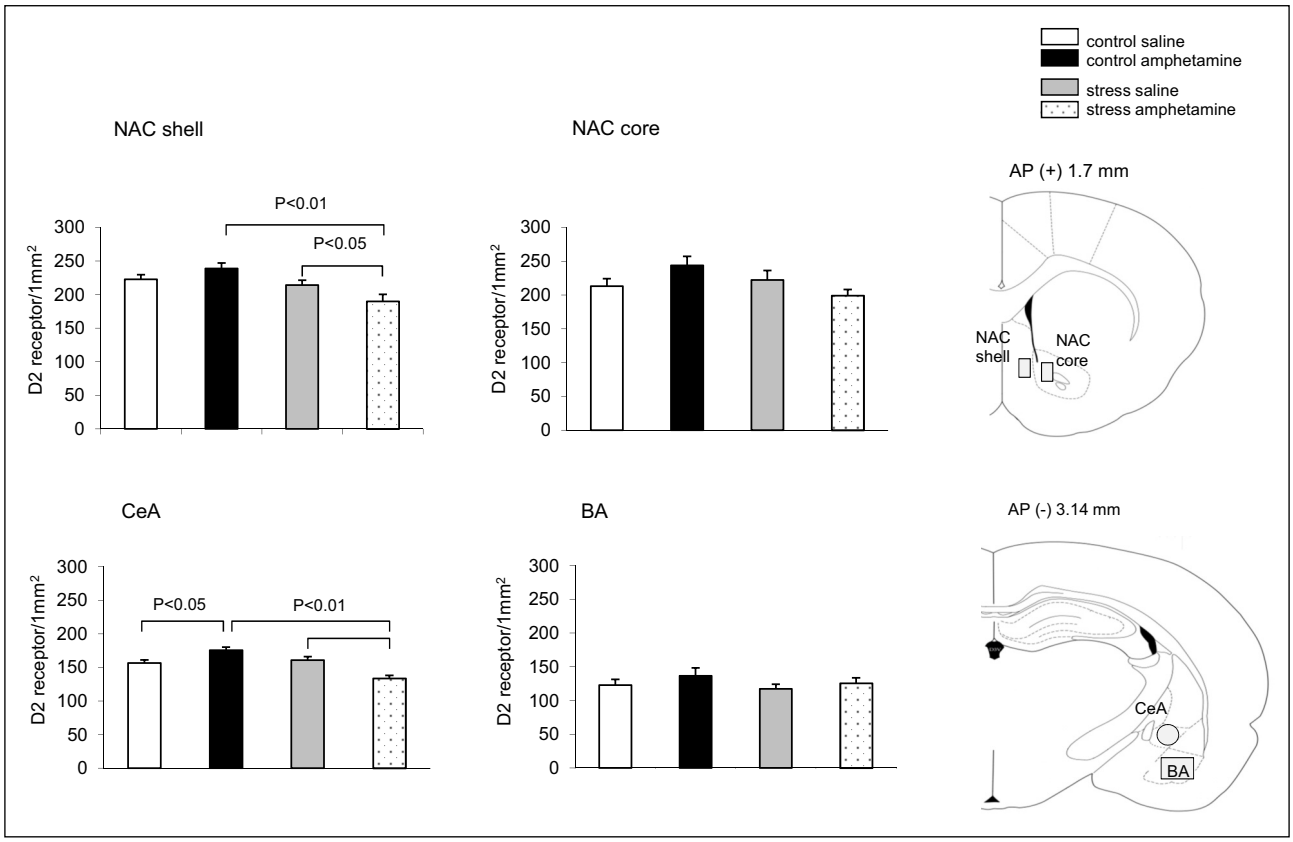

Fig. 7. The D2 receptor immunocytochemistry results after the second injection of amphetamine (1.5 mg/kg) or saline. Control saline rats n = 11; Control amphetamine rats n = 12; Stressed saline rats n = 12; Stressed amphetamine rats n = 12. The schematic coronal section adapted from the atlas of Paxinos and Watson (48) showing brain structures. AP - anterior, posterior from bregma, NAC shell - nucleus accumbens shell; NAC - nucleus accumbens core; CeA - central nucleus of amygdala; BA - basal nucleus of amygdala. The data are shown as the means + SEM.

no stress effect [F(1,43) = 0.8, (P = 0.36)], no amphetamine effect [F(1,43) = 1.5, (P = 0.22)]; no stress × amphetamine interaction effect [F(1,43) = 0.1, (P = 0.75)] (*Fig. 7*).

DISCUSSION

The most important finding of this paper is the relationship between the behavioural and biochemical data of the stressed group. In general, the stressed rats fundamentally differed from the control rats in the limbic dopaminergic system activity. These effects were also accompanied by a reduction in both sucrose preference and body weight. Initially, the strong response to amphetamine in the control condition became weaker after stress (improved PPI, decrease in D2 receptor expression in the CeA and NAC shell and decrease in dopamine and 3-MT levels in NAC). Similar changes in effects of amphetamine on PPI were reported in antipsychotic-medicated schizophrenia patients (55). Amphetamine 'normalised PPI' and this impact was associated with hedonic effects of drug (55). In our study the startle responses and pulse 120 dB were slightly higher after stress, but these results had not effects on PPI. Stress also attenuated the stimulatory effect of amphetamine on corticosterone level. These effects were accompanied by a decrease in glutamate/GABA ratio in NAC and a decrease in GABA levels in the amygdala. The 20 dB over background prepulses appear to elicit startle responses in the active experimental groups, and the relative magnitude of these responses parallels the levels of PPI in these groups. Weaker prepulses (14 dB over background) neither elicit detectable startle nor elicit the stress-amphetamine interaction effects on PPI. These data point to a selective influence of stress and amphetamine on PPI.

One interpretation of these results is that the dopaminergic system has significantly weaker compensatory mechanisms under prolonged stress conditions in the limbic structures. A good example of this is the change in the function of the HPA axis controlled by the dopaminergic system. Attenuation by stress of the stimulatory effect of amphetamine in stressed rats was paralleled by changes in corticosterone levels. It is well known that amphetamine and its analogues stimulate corticosterone release (56, 57). Control rats showed greater excitability in response to amphetamine compared to stressed rats (corticosterone levels in PFC, dopaminergic activity in NAC, dopamine and 3-MT levels). The question arises of what mechanisms may be involved in this phenomenon. To at least partially answer this question, we studied changes in amino acids, dopamine levels, and D2 receptor expression in brain structures that are associated with positive reinforcement (the NAC shell and core) and emotional behaviour (BA and CeA). The NAC is the main target of the dopaminergic projection neurons in the VTA. It is one of the important structures in the integration of neurotransmitter systems that regulate sensorimotor gating (58-60). It has been shown that an infusion of amphetamine or a D2 receptor agonist (quinpirole), resulted in a dose-dependent reduction of PPI (61). The NAC receives modulatory inputs 1) *via* glutamatergic projections from 'limbic' structures such as the prefrontal cortex, the cingulate gyrus and

the the amygdala 2) *via* dopaminergic projections from the VTA (62). Additionally, using photoactivation of glutamatergic fibres, it was shown that parvalbumin-expressing GABAergic-interneurons in the NAC are activated by glutamatergic inputs from VTA neurons (63). Research has suggested that differences in the activity of the dopaminergic system in the amygdala and prefrontal cortex may be one of the biological factors that underlie the response to stress (4, 63). Prolonged stress may result in the impairment of the dopaminergic system in the VTA-NAC *via* amygdalar projection that causes decrease in motivational processes (63). Our results indicate that the control animals expressed strong reactivity to the psychostimulant action, *i.e*., an increase in glutamate, dopamine and 3-MT levels in the NAC, and increase in GABA levels in the amygdala. Under prolonged stress, amphetamine decreased glutamate levels in the amygdala and improved postsynaptic dopamine levels (3-MT is believed to be a marker of synaptic dopaminergic activity) in NAC (64).

There is a large amount of evidence in preclinical models to support the role of the D2 receptor on measures of PPI (42, 61, 65, 66). Abnormal dopamine receptor signalling and dopaminergic function have been implicated in several neuropsychiatric disorders (65, 67-69). For example, dysfunction of dopaminergic transporters (DAT) could be involved in mechanisms of attention deficit hyperactivity disorders (ADHD), schizophrenia and many other psychiatric disorders (70). D2 receptors are highly expressed in the basal ganglia, nucleus accumbens, ventral tegmental area and the substantia nigra, as well as in lower concentrations in the amygdala, hippocampus, thalamus, cerebellum and cerebral cortex (71-74). In the present study, we found very similar changes in the number of D2 receptors in the CeA and NAC shell after stress following amphetamine administration: an initially strong response to amphetamine in the control condition was weakened after stress.

In the amygdala, postsynaptic D2 receptors primarily residing in GABA-ergic neurons modulate limbic system activity (75). It has been reported that signalling through D2 dopamine receptors in the amygdala is important for encoding emotional perceptual properties of the stimuli; the D2 receptors may attenuate the local inhibitory processes in response to a stimulus (76, 77). Disturbances in this regulation *via* D2 receptors in the amygdala are thought to contribute to enhanced neural activity associated with a negative emotional state (77, 78). It is thus probable that in the stressed rats, the decreased D2 expression in the CeA that was observed after amphetamine exposure could promote inhibitory mechanisms in CeA-NAC communication. These inhibitory mechanisms are thought to reduce the impact of further stimulation until processing of the prepulse stimulus is completed (65, 79). This may result in the improvement of PPI in stressed rats after amphetamine.

Although the statistical analysis has not shown any changes in PPI in stressed rats, the correlation analysis suggests an inverse proportional relationship between a strong fear response in CFT and PPI [r (-) 0.78, $P < 0.01$] in the saline stressed rats. Our previous papers showed that the rats that had a stronger conditioned fear also displayed depressive-like symptoms (80, 81). Accordingly, it has been reported that a significant negative correlation between PPI and depression severity occurs in men but not women (82).

The significance of the similar pattern of behavioural (PPI) and biochemical changes in the D2 receptors is not known to the end. Tentatively, it could be suggested that the decrease in the number of D2 receptors in the CeA after stress and amphetamine is directly related to the improvement of the PPI effect. In line with this theory, blocking D2 receptors with neuroleptics is an important mechanism of inhibiting the disrupting action of amphetamine in this test (31, 83). It is also worth noting that low doses of neuroleptics are also used as anxiolytics in humans what links changes in PPI with emotionality observed in our study (correlation data) (84, 85). Moreover, a recent clinical trial shed more light on this issue (77). The investigation was aimed to evaluate the regulation of emotion in subjects who met the DSM-IV criteria for methamphetamine dependence and to test for a relationship between self-reports of difficulty in the regulation of emotion and D2-type dopamine receptor availability in the amygdala. In the study, using self-report psychological scales and positron emission tomography with [(18)F] fallypride to assay D2-type dopamine receptor binding in the amygdala, it was found that the Difficulties in Emotion Regulation Scale score was positively correlated with amygdala D2 receptor binding potential. The authors concluded that the D2-type dopamine receptors in the amygdala contribute to the regulation of emotion in both healthy and methamphetamine-using subjects. Our research supports this conclusion and points to the dopaminergic system in the amygdalar nuclei in regulating the processes responsible for the effects of amphetamine (77, 79). Our results allowed for in-depth analysis of mechanisms dependent on changes in activity of various components of dopaminergic innervation of the NAC - amygdala projection. This can be helpful in understanding the psychopathology behind the comorbidity of drug dependence and emotional disorders.

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