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Research report

Changes in the glutamate biomarker expression in rats vulnerable or resistant to the rewarding effects of cocaine and their reversal by ceftriaxone

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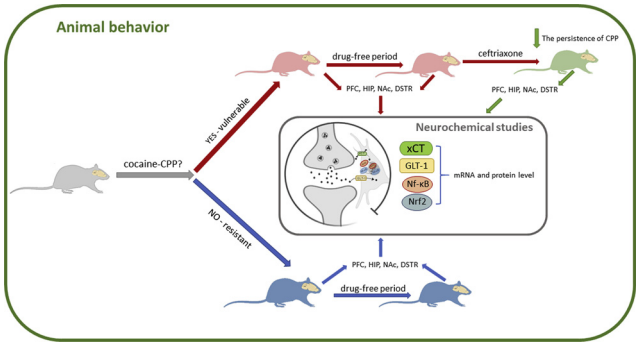
GRAPHICAL ABSTRACT



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ABSTRACT

Literature data show diverse vulnerability to the rewarding effects of cocaine in human as well as in laboratory animals. The molecular mechanisms of these differences have not been discovered yet. While the initial effects of cocaine depend primarily on the dopamine system, numerous studies have shown that adaptation within the glutamatergic system is responsible for the development of addiction.

In this paper, we used the unbiased conditioned place preference (CPP) to identify rats showing a vulnerable or resistant phenotype to the rewarding effects of cocaine. Next, we investigated the expression of membrane glutamate transporter proteins: GLT-1 and xCT in selected brain structures in the above-mentioned groups of rats. Moreover, we determined the nuclear level of NF-κB and Nrf2 to verify whether changes in GLT-1 and xCT expression correlate with NF-κB and Nrf2 levels, respectively. In addition, we determined GLT-1, NF-κB, xCT and Nrf2 mRNA levels to verify the involvement of transcriptional mechanisms. We also analyzed the ability of the β-lactam antibiotic, ceftriaxone, to attenuate the persistence of CPP after a cocaine-free period in animals showing vulnerability to cocaine rewarding effects, and furthermore we determined GLT-1, xCT, NF-κB and Nrf2 protein expression.

Our findings demonstrated molecular and neurochemical differences in the response to cocaine administration that are characteristic of the phenotype vulnerable or resistant to the rewarding effects of cocaine.

Abbreviations: CPP, conditioned place preference; DSTR, dorsal striatum; EAAT, excitatory amino acid transporter; GLT-1, glutamate transporter 1; HIP, hippocampus; ip, intraperitoneally; mRNA, messenger ribonucleic acid; NAc, nucleus accumbens; Nf-κB, nuclear factor kappa B; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PFC, prefrontal cortex; RT-qPCR, real-time quantitative polymerase chain reaction; xc, cystine/glutamate antiporter; xCT, subunit of cystine/glutamate antiporter

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Moreover, repeated administrations of ceftriaxone during cocaine-free perios attenuated CPP persistence and normalized GLT-1 level in the NAc. The results suggest the a lack of NF-κB involvement in the regulation of GLT-1 expression by ceftriaxone in the NAc. Additionally, we are the first to report that ceftriaxone strongly upregulates the GLT-1 in the HIP in a transcriptional mechanism involving the Nf-κB transcription factor. Future experiments may resolve the question concerning whether modulation exclusively of the GLT-1 expression in the HIP may attenuate cocaine-induced place preference or relapse.

1. Introduction

Addiction to psychoactive substances, including cocaine, is a chronic brain disease. Cocaine addiction with drug craving and relapse, develops only in 20% of people who began to abuse it [1,2]. Similar differences in addiction vulnerability can be observed in laboratory animals. Molecular mechanisms underlying these phenotypic differences have not yet been discovered.

While the initial effects of cocaine (feeling pleasure, euphoria) depend primarily on the activation of the dopamine system, numerous studies have shown that adaptation within the glutamatergic system in the brain is responsible for the development of addiction [3–5]. Preclinical reports indicate that acute cocaine administration increases the glutamate level in the nucleus accumbens (NAc) [6–8], ventral tegmental area (VTA) [9–12] and dorsal striatum (DSTR) [11]. On the other hand, a drop in the extracellular glutamate level in the NAc during withdrawal in the home cage was observed [13–15] with a further rise in the NAc [14,16–19] and in the DSTR [11,20] after relapse induced by priming or cue presentation/context.

The level of the extracellular glutamate is regulated by excitatory amino acid transporters 1–5 (EAAT 1–5) and cystine-glutamate antiporter (xc-) [5]. The EAAT subtype, GLT-1 (EAAT2), is especially important because of its ubiquitous expression throughout the brain and responsibility for ˜90% of glutamate uptake [21]. Both in laboratory animals and in humans, GLT-1 is mainly located on the cell membrane of astrocytes [21,22]. GLT-1 removes glutamate from extracellular space into the astrocyte before glutamate can then be transported further from the astrocyte into the extrasynaptic space by the xc-. Under physiological conditions, the xc- transfers the cystine inside the cell in exchange for glutamate in a 1:1 stoichiometric ratio down the concentration gradient. The xc- system is composed of two subunits: the heavy chain 4F2hc and the light chain xCT being a catalytic subunit essentially involved in the functioning of the antiporter [23].

Studies have shown that GLT-1 and xCT expression was downregulated in the NAc during a cocaine-free period (extinction or withdrawal) [24–28] and during reinstatement of cocaine seeking [26,29].

The molecular mechanism of cocaine-induced changes in GLT-1 and xCT expression remains unknown. The regulation of GLT-1 and xCT expression may occur at various levels, among them at the transcriptional level [5,21,30,31], for which NF-κB and Nrf2 seem to be important, respectively. NF-κB has been shown to play a role in the rewarding property of cocaine, because inhibition of its activity blocks the acquisition of a cocaine-induced place preference [32].

We used an unbiased conditioned place preference (CPP), which investigates the conditioned rewarding effects of drugs, to identify the animals vulnerable to cocaine rewarding effects (showing a cocaine-induced place preference) and resistant to cocaine rewarding effects (not showing a cocaine-induced place preference). Furthermore, we investigated the expression of membrane GLT-1 and xCT proteins in the above-mentioned groups. Moreover, we determined the nuclear level of NF-κB and Nrf2 in order to verify whether the changes in GLT-1 and xCT expression correlate with their transcription factors. In addition, we determined GLT-1, NF-κB, xCT and Nrf2 mRNA levels to verify if transcriptional mechanisms are responsible for the likely changes. Changes in the expression of all proteins/mRNAs were examined in the NAc (reward processing), prefrontal cortex (executive control), hippocampus (learning and memory) and DSTR (habit forming learning). All analyzes were performed immediately after the end of the CPP test and after the re-test preceded by drug-free period of 7 days in the home cage to assess the permanence of the observed changes.

We chose a CPP because it is considered to be a valid method for investigating the rewarding effects of drugs and to study a relapse to drug-seeking behavior in laboratory animals [33–39]. Increased glutamatergic transmission was shown to be essential for the cocaine-induced place preference [40] and, as such, changes in the glutamate system induced by cocaine administration may be analyzed using CPP.

Preclinical studies indicate that substances increasing the expression of GLT-1 or/and xCT, like ceftriaxone [26,41] or ampicillin [29,42] attenuate relapse to cocaine or ethanol seeking. In this study, we also analyzed the ability of ceftriaxone to attenuate a context-induced relapse to cocaine-induced place preference after home-cage withdrawal in animals showing vulnerability to cocaine rewarding effects. We extended our research with further determination of GLT-1, xCT, NF-κB and Nrf2 protein expression in these groups of animals.

2. Materials & methods

2.1. Animals

All experiments were performed on 103 male Wistar rats (250–300 g, Charles Rivers). The animals were maintained on a day-night cycle at 22 ± 2 °C with free access to food and water. All experimental protocols were in accordance with the European Directive 2010/63/EU and were approved by the First Local Ethical Committee at the Jagiellonian University in Krakow (Permit No: 147/2013).

2.2. Drugs

Cocaine hydrochloride (Sigma-Aldrich, St. Louis, USA) and ceftriaxone (MIP Pharma, Poland) were dissolved in sterile 0.9% NaCl. Drugs were injected ip in the volume of 1 ml/kg (cocaine) or 2 ml/kg (ceftriaxone). Doses of cocaine and ceftriaxone were selected based on the previous papers showing that 15 mg/kg of cocaine can induce a significant CPP and proving that 200 mg/kg of ceftriaxone can attenuate relapse to cocaine seeking behavior [26,43–46].

2.3. Behavioral studies

2.3.1. Apparatus

The tests were carried out in five identical rectangular apparatuses (79 × 35 × 30 cm) each containing three chambers separated by guillotine doors. Two large end chambers (32 × 30 cm) were separated by a smaller central choice chamber (15 × 30 cm). All chambers differed in the color of the walls and the texture of the floor. One of the end chambers had gray walls and a wire screen floor (0.63 × 0.63 cm squares); the other had a white wall and a stainless steel mesh floor (1.3 × 1.3 cm squares). The central choice chamber (middle chamber) had punched aluminum flooring (0.4 cm diameter holes). It was separated from the two end chambers by gray walls with doorways cut in them that could be closed by removable guillotine doors. All floors were raised by 2 cm to reduce the accumulation of urine and feces.

The whole apparatus was cleaned with Meliseptol ™ after conditioning or testing of each animal to eliminate odors of the previous animal. During conditioning and testing, the room had dim indirect

lighting. Monotonous sounds (65–75 dB) were used to eliminate random sounds. The behavior of the animals and the time spent in each chamber were recorded using a camera integrated with a digital video recorder installed above the CPP box. It has been assumed that a rat is in the chamber if at least a half of its body (without the tail) with the head was inside the chamber. This criterion showed a tendency to increase the residence time of the animal in the middle chamber, but in this way the partial entries into the end large chambers could be avoided.

2.3.2. Conditioned place preference

All animals were acclimatized and habituated to the researcher and apparatus prior to commencing the experiment. The place conditioning procedure consisted of 3 or 5 phases depending on the experiment: the pre-test, the conditioning, CPP test, the drug-free period and the re-test. 1) *The pre-test* (day 1): the pre-test was designed to establish a baseline level of preference for each individual animal. Rats were introduced into the central compartment of the CPP box with free access to all three compartments for 15 min. Only animals that did not show an initial preference for end chambers proceeded to the further part of the experiment (the difference between the times spent in both end chambers was not greater than 80 s, unbiased CPP). The 86 of all animals met these criteria. 2) The *conditioning* (days 2–9): rats were given an injection of cocaine and then were placed in one of the end chambers for 30 min on day 1, 3, 5, 7. On day 2, 4, 6, 8, the animals were injected with saline and then placed in the opposite compartment. A particular outer chamber was assigned to cocaine injections in a randomized manner. In this way, half of the animals were placed in the white compartment after cocaine injection and the other half in the dark gray compartment. 3) *The CPP test* (day 10): the rats were introduced into the central compartment with free access to all three compartments for 15 min. 4) *The drug-free period* (days 11–17): in order to test the consolidation of CPP or a possible anti-relapse effect of ceftriaxone animals were kept in their home cages and received either saline or ceftriaxone injections (once a day). No behavioral test was conducted during this time. 5) *The re-test* (day 18): the rats were introduced into the central compartment with free access to all three compartments for 15 min.

The preference score (CPP score) was expressed by the time spent in the cocaine-paired chamber minus the time spent in the saline-paired chamber.

The locomotor activity was performed as shown previously [47]. Thus, it was expressed as the distance travelled (cm) in the CPP apparatus during the re-test (15 min) in the saline-saline group and the saline-ceftriaxone group.

2.3.3. Experimental protocol

The experimental schedule is illustrated in Fig. 1. Behavioral studies with CPP were divided into two separate experiments:

2.3.3.1. Experiment 1. The experiment 1 is illustrated in Fig. 1A and B. Animals were subjected to CPP pre-test, cocaine conditioning and CPP test. According to the results of CPP test, the animals were divided into two groups: vulnerable and resistant to rewarding effects of cocaine. The vulnerable animals showed preference for the cocaine-paired chamber during CPP test while the resistant animals did not show this preference. Place preference was established if the average difference between the time spent by the animal in the cocaine-paired chamber and in the saline-paired chamber was higher than 100 s during the 15-min CPP test [48].

The animals from this stage of behavioral studies were decapitated in two series: immediately after the end of the CPP test (Fig. 1A) or after a drug-free period of 7 days in home cage and re-test (Fig. 1B). RT-qPCR and Western Blot analysis was further conducted to determine changes in xCT, GLT-1, Nf-κB and Nrf2 mRNA or protein levels.

2.3.3.2. Experiment 2. The experiment 2 is illustrated in Fig. 1C. Animals were subjected to CPP pre-test, cocaine conditioning and CPP test. Further experiment was performed only in animals vulnerable to rewarding effects of cocaine according to the results from the CPP test. They received ceftriaxone injections during drug-free period in their home cage (200 mg/kg for 7 days, 1 in./day) and were tested again for place preference during the re-test. The re-test was used to investigate if ceftriaxone given during drug-free period could attenuate the persistence of cocaine CPP. After the re-test the animals were decapitated and appropriate brain structures were isolated for Western blot analysis. Appropriate control groups were included.

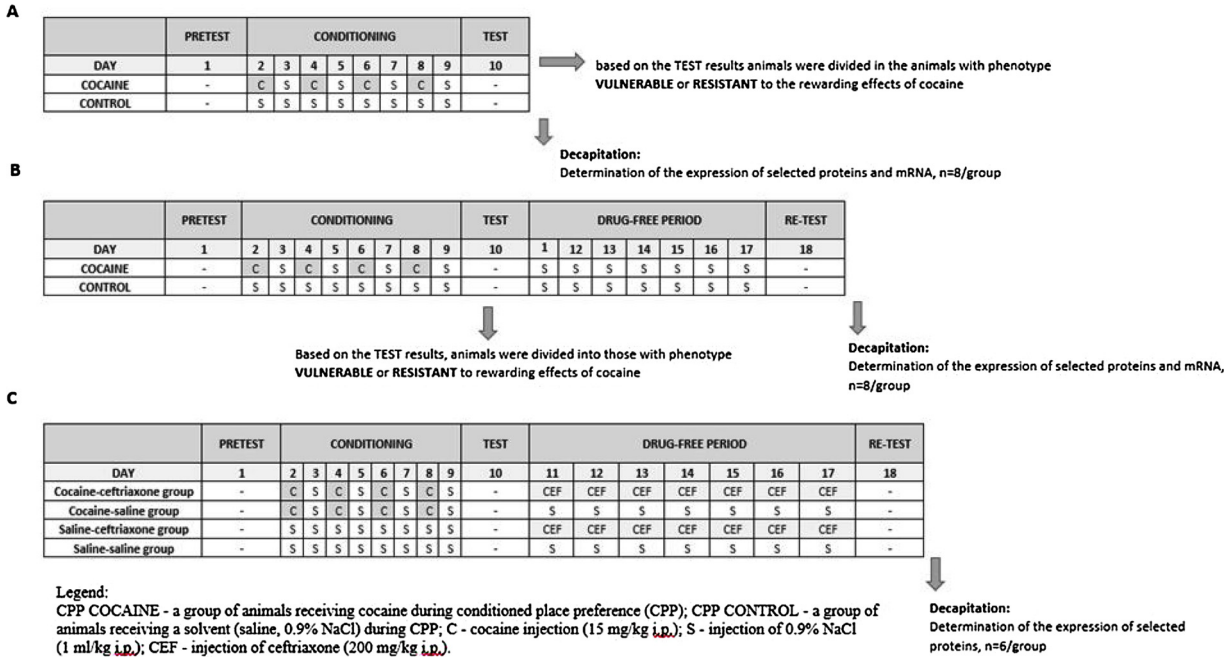

Fig. 1. Experimental design for the behavioral experiments (n = 6–8).

2.4. Isolation of brain structures

All rats were sacrificed through decapitation, and their brains were rapidly removed. Selected brain structures (PFC, HIP, NAc, DSTR) were isolated according to The Rat Brain Atlas (Paxinos and Watson, 1998), immediately frozen on dry ice and stored at −80 °C for Western blot and RT-qPCR analysis.

2.5. Protein extraction and Western blot analysis

Membrane and nuclear fractions were prepared according to Caffino et al. [49]. Briefly, each brain structure was homogenized in a Teflon-glass Potter homogenizer in ice-cold 0.32 M sucrose buffer pH 7.4 containing 1 mM HEPES, 0.1 mM EGTA and 0.1 mM PMSF, in the presence of commercial cocktails of protease (Sigma–Aldrich, USA) and phosphatase (Sigma–Aldrich, USA) inhibitors. The homogenate was centrifuged at 1000 × *g* for 10 min to obtain a pellet (P1) corresponding to the nuclear fraction. The remaining supernatant (S1) was centrifuged again (9000 × *g*, 15 min, 4 °C). The resulting pellet (P2) corresponds to the membrane fraction.

The obtained pellets were dissolved in cold (4 °C) dissolution buffer containing 1 mM HEPES, 0.1 mM DTT (1,4-dithiothreitol), 0.1 mM EGTA, 0.1 mM PMSF, 0.1 mM sodium orthovanadate and protease inhibitors (Sigma–Aldrich, USA). Subsequently, each homogenate was subjected to a short sonication on ice (Ultrasonic Processor UP50H, Hielscher).

Subsequently, using the BCA (bicinchoninic acid) method, total protein concentration was determined using Pierce BCA Protein Assay Kit (ThermoFisher Scientific, USA). All samples were diluted with the dissolution buffer to equilibrate the concentrations. As a result, all samples had the same total protein concentration.

The homogenates were then subjected to thermal denaturation with loading buffer mixed 1:1 (Laemmli buffer 2x concentrated, Sigma Aldrich, USA) at 95 °C for 5 min (thermoblock TS-100C, BioSan). The prepared samples had a final protein concentration of 25 μg/10 μl.

Before the start of the preparation of all samples, appropriate tests were carried out, which confirmed that a concentration of 25 μg/10 μl was sufficient for further steps of determining protein expression by Western blot analysis. The correct separation of fractions (nuclear and membrane) was also validated.

Samples containing 10–20 μg of protein (depending on the protein tested) were run on 4–15% criterion polyacrylamide gradient gels (BioRad, USA) under reducing conditions using a criterion cell (Bio-Rad, USA) and then transferred onto PVDF membranes (Thermo Fisher Scientific, USA) using semi-dry transfer (Trans-Blot® Turbo ™ Transfer System, BioRad, USA).

The blots were blocked with 5% bovine serum (BSA) in TBST and incubated with primary antibodies against the proteins of interest. The following antibodies were used: anti-xCT (1:1000, ab175186, Abcam), anti-GLT-1 (1:4000, ab41621, Abcam), anti-Nrf2 (1:1000, ab89443, Abcam), anti-NF-κB p65 (1:500, sc-372, SantaCruz Biotechnology). The primary and secondary antibodies were diluted with 1% BSA in TBST. Immunocomplexes were visualized by chemiluminescence utilizing the WesternBright Quantum HRP substrate (Advansta Inc., USA) according to the manufacturer's instructions using G:Box (Syngene, Cambridge, UK) and were analyzed using the GeneTools (v. 4.03, Syngene, Cambridge, UK). The results were standardized to total protein staining, which was carried out according to the manufacturer's instruction (Pierce™ Reversible Protein Stain Kit for PVDF Membranes, Thermo Fisher Scientific, USA). All data were expressed as % of control.

2.6. RT-qPCR analysis

The total RNA was extracted from the frozen PFC, HIP, NAc, DSTR using TRIzol reagent (Life Technologies, USA) according to the manufacturer's instruction. Briefly, the brain samples were homogenized using the Bioprep-24 (Allsheng, China) in the presence of zircon beads and TRIzol in a ratio of 1:10. Next, chloroform was added to the homogenate in a ratio of 5:1. After vortexing and incubation, samples were centrifuged for phase separation (12,000 × *g*, 15 min at 4 °C, centrifuge 5702 RH, Eppendorf). The aqueous phase was collected and the RNA was precipitated by adding an equal volume of isopropanol to this phase. The pellet was washed twice with 1 ml of cold 75% ethanol. The air-dried pellet was resuspended in 20 μl of RNase-free water. To remove the trace genomic DNA contamination, RNA was purified with the direct-zol RNA MiniPrep Kit (Zymo Research, USA) according to the manufacturer's protocol. To assess the quality and quantity of the RNA, the samples were analyzed using gel electrophoresis and spectrophotometry (NanoDrop ND-1000, Thermo Scientific, USA) and then stored at −80 °C until further use.

The total RNA was subsequently transcribed into cDNA using the transcriptor high fidelity cDNA synthesis kit (Roche, USA) according to the manufacturer's procedure and using the T100 thermal cycler (Bio-Rad, USA). The cDNA was stored at −80 °C until used. The relative cDNA quantification of GLT-1, xCT (the light chain of xc-), Nf-κB and Nrf2 and a reference gene (hypoxanthine phosphoribosyltransferase, Hprt) was measured using the commercial taqman gene expression assay (Applied Biosystems, USA) (assay ID numbers are listed in Table 1). Real-time PCR was performed using the taqman® gene expression master mix (Life Technologies, USA) according to the manufacturer's procedure. Each reaction was run in duplicate in a 96-well plate with the following thermal conditions: 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. All PCR reactions were performed in a Bio-Rad CFX96 Real Time PCR Detection System, and the data were analyzed using CFX Manager Software 2.1. The relative amount of each mRNA was assessed using the comparative CT method ($2^{-\Delta\Delta Ct}$) and normalization to Hprt mRNA.

2.7. Statistical analysis

Behavioral data were analyzed by a two-way ANOVA for repeated measures followed (if significant) by post hoc Tukey's test to evaluate statistically significant differences between the treatment groups. Molecular data (mRNA and protein expression) were analyzed by a one- or two-way ANOVA and post hoc Tukey's test or t-Student test. A p value < 0.05 was considered as statistically significant.

3. Results

3.1. Behavioral studies

3.1.1. Experiment 1

3.1.1.1. Assessment of cocaine-induced CPP immediately after the CPP test. A two-way ANOVA revealed a cocaine conditioning x phenotype interaction ($F(2,21) = 16.22$, $p < 0.0001$) (Fig. 2A). As a result of cocaine conditioning, some animals showed a strong preference for the chamber paired with cocaine (phenotype vulnerable to rewarding effects of cocaine) during the CPP test compared to the pretest ($p < 0.0001$) (Fig. 2A). Other animals from experimental group did not show cocaine-paired chamber preference despite receiving cocaine injections (phenotype resistant to cocaine rewarding effects).

Table 1
The ID number of primers/probe used in RT-qPCR.

Protein	Gene symbol	Assay ID number
GLT-1	Slc1a2	Rn00691548_m1
xCT	Slc7a11	Rn01495123_m1
Nrf2	Nfe2l2	Rn00582415_m1
NF-κB (p65)	RELA	Rn01502266_m1

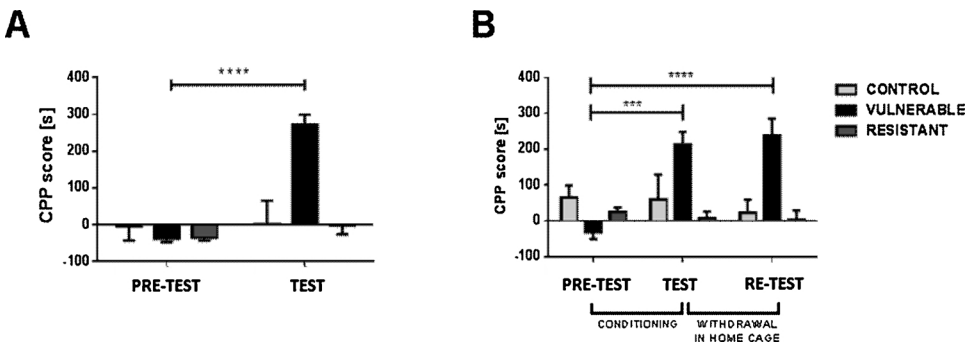

Fig. 2. Cocaine-induced conditioned place preference (CPP) (A) and the re-test of preference (B) in rats. Based on the preference results obtained in the CPP test, the animals were divided into the phenotype VULNERABLE or RESISTANT to rewarding effects of cocaine. The results are expressed as CPP score [s] ± S.E.M. ***p < 0,001 vs. VULNERABLE in PRE-TEST; **** p < 0.0001 vs. VULNERABLE in PRE-TEST. N = 8 rats/group. CPP score - the average difference between the time spent by an animal in a compartment paired with cocaine and in a compartment paired with the solvent. In the case of the control group, the difference relates to the time spent by the animal in both chambers, where each of them was paired with the solvent injection.

3.1.1.2. Assessment of persistence of the cocaine-induced CPP after a drug-free period of 7 days and the re-test. A two-way ANOVA revealed a cocaine conditioning x phenotype interaction ($F(4,42) = 7.308$, $p = 0.0001$) (Fig. 2B). As a result of cocaine conditioning, some animals showed a strong preference for the cocaine-paired chamber (phenotype vulnerable to rewarding effects of cocaine) assessed in the CPP test in comparison with the pretest ($p < 0.0001$). This preference persisted after 7 days of drug-free period, as demonstrated by the re-test ($p < 0.0001$). The remaining animals from the experimental group did not show preference despite receiving cocaine (phenotype resistant to cocaine rewarding effects) and this lack of preferences persisted after a drug-free period of 7 days.

3.1.2. Experiment 2

A two-way repeated measures ANOVA showed an interaction between cocaine conditioning and ceftriaxone ($F(6,40) = 5.736$, $p = 0.0002$) (Fig. 3A). The animals conditioned with cocaine (the cocaine-saline group and cocaine-ceftriaxone group) spent significantly more time in the cocaine-paired chamber in the CPP test as compared to the pretest ($p < 0.0001$ for the cocaine-saline group; $p < 0.001$ for the cocaine-ceftriaxone group).

In the re-test, the animals given saline during a drug-free period (cocaine-saline group) spent significantly more time in the cocaine-paired chamber in comparison to the pre-test phase ($p < 0.001$) while in relation to the CPP test this time did not change (Fig. 3A). This result indicates that the cocaine-conditioned place preference persisted in this group of animals after a drug-free period of 7 days. In contrast, in the group receiving cocaine injections during conditioning and the ceftriaxone injections throughout the drug-free period (the cocaine-ceftriaxone group), the re-test showed that these animals spent significantly less time in the cocaine-paired chamber in comparison with the CPP test ($p < 0.01$). The time spent by the animals of this group in the cocaine-paired chamber during the re-test did not differ significantly as compared to the pretest. Thus, the administration of ceftriaxone (200 mg/kg) during a drug-free period significantly reduced the preference for the cocaine-conditioned chamber measured in the re-test in relation to this preference in the CPP test (Fig. 3A).

The animals from the saline-saline and saline-ceftriaxone group did not show preference for any outer chambers of the CPP box at any stage of the CPP test or the re-test.

A Student's *t*-test did not reveal the effects of repeated administration of ceftriaxone (200 mg/kg ip) on the rats' distance travelled in the CPP apparatus ($t[10] = 1.025$, $p = 0.3297$) (Fig. 3B).

3.2. Molecular studies

To address the question whether the vulnerable or the resistant phenotype is associated with the alternation in the glutamate system, we analyzed the protein and mRNA levels of different components of this system in the PFC, HIP, NAc, DSTR directly upon completion of CPP (the CPP test) (Fig. 4) and after the re-test of preference preceded by a drug-free period of 7 days (Fig. 5).

3.2.1. Changes in genes expression

We analyzed the relative xCT and GLT-1, Nrf2 and Nf-κB mRNA expression. A summary of these results can be found in Figs. 4A and 5A.

3.2.1.1. Experiment 1. Cocaine conditioning changed the relative expression of GLT-1 mRNA in the HIP ($F(2,21) = 4.811$, $p = 0.0190$) and in the NAc ($F(2,21) = 6,869$, $p = 0.0051$) after the CPP test (Fig. 4A) and in the PFC after the re-test preceded by a drug-free period of 7 days ($F(2,21) = 9,153$, $p = 0.0014$) (Fig. 5A). Post-hoc analysis indicated a decrease in GLT-1 mRNA in the HIP only in the animals with the vulnerable phenotype in comparison to the saline-

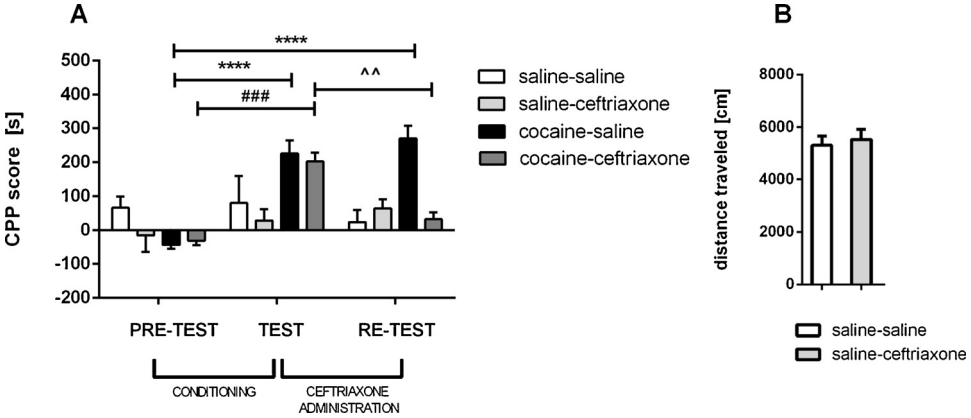

Fig. 3. The effect of ceftriaxone (200 mg/kg ip) administration during cocaine-free period on the re-test of preference (A) and the effect of ceftriaxone on locomotor activity measured at the re-test (B). The results are expressed as CPP score [s] ± S.E.M. ****p < 0.0001 vs. cocaine-saline in PRE-TEST; ### p < 0.001 vs. cocaine-ceftriaxone in PRE-TEST; ^^ p < 0.01 vs. cocaine-ceftriaxone in TEST. N = 6 rats/group. For more explanation see Fig. 2.

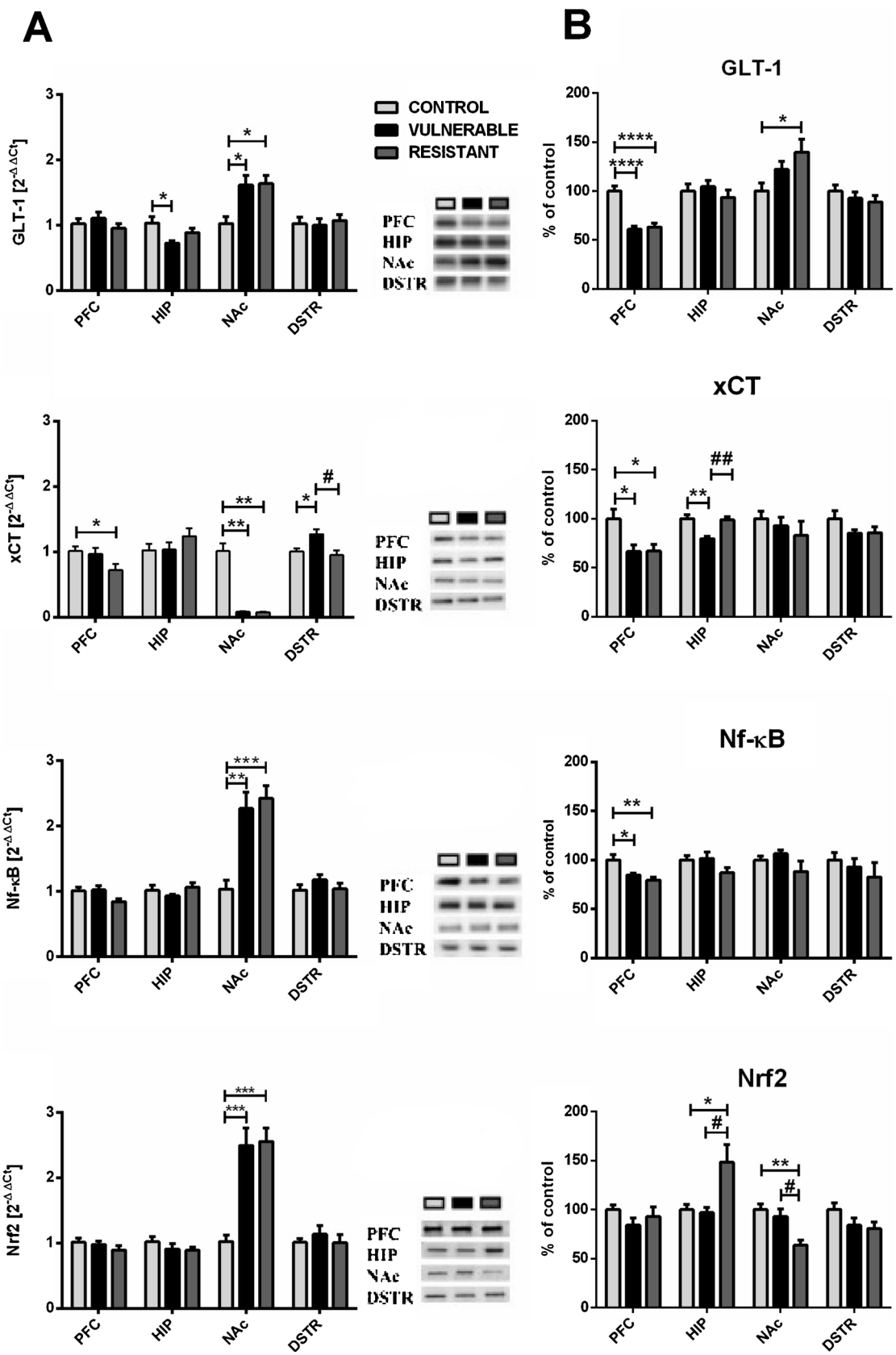

Fig. 4. Changes in GLT-1, xCT, Nf-κB and Nrf2 mRNA (A) and protein (B) levels in brain structures of animals vulnerable and resistant to rewarding effects of cocaine after the CPP test. Data were normalized to the saline-treated animals (% of control). *p < 0.05; **p < 0.01; ***p < 0.001; **** p < 0.0001 vs. CONTROL; #p < 0.05; ##p < 0.01 vs. VULNERABLE (post hoc Tukey's test). N = 8 rats/group. DSTR - dorsal striatum; HIP - hippocampus; NAc - nucleus accumbens; PFC - prefrontal cortex.

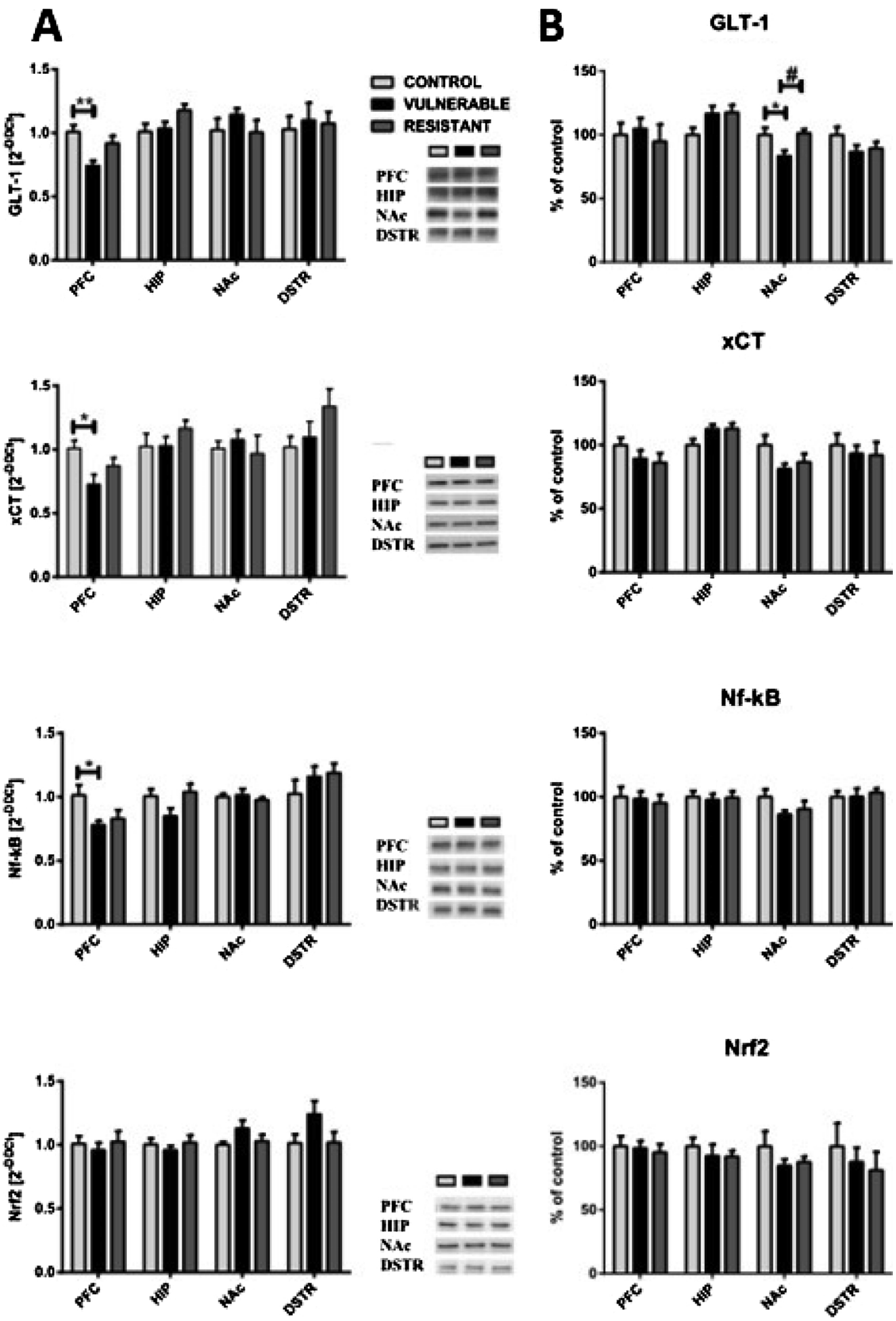

Fig. 5. Changes in GLT-1, xCT, Nf-κB and Nrf2 mRNA (A) and protein (B) levels in brain structures of animals vulnerable and resistant to rewarding effects of cocaine after the re-test. Data were normalized to the saline-treated animals (% of CONTROL). *p < 0.05; **p < 0.01 vs. CONTROL; #p < 0.05 (post hoc Tukey's test). N = 8 rats/group. DSTR - dorsal striatum; HIP - hippocampus; NAc - nucleus accumbens; PFC - prefrontal cortex.

treated control group ($p < 0.05$) and indicated an increase in GLT-1 mRNA in the NAC in the vulnerable ($p < 0.05$) and the resistant phenotype ($p < 0.05$) in comparison to saline-treated control group after the CPP test (Fig. 4A). After a drug-free period of 7 days and the re-test, the relative level of GLT-1 mRNA dropped in the PFC of the animals with the vulnerable phenotype in relation to the saline-treated control animals ($p < 0.01$) (Fig. 5A).

Changes in the relative xCT mRNA level were observed in the NAc ($F(2,21) = 75.26$, $p < 0.0001$), PFC ($F(2,21) = 5.005$, $p = 0.0167$) and in the DSTR ($F(2,21) = 4.361$, $p = 0.0261$) after the CPP test (Fig. 4A). Post-hoc analysis showed a drop in the xCT mRNA level in the NAc in the animals with the resistant ($p < 0.01$) as well as with the vulnerable phenotype ($p < 0.01$) in relation to the saline-treated control animals. Moreover, the level of xCT mRNA decreased in the PFC in the animals resistant to the rewarding effects of cocaine ($p < 0.05$) in comparison to the saline-treated control group and rose in the DSTR in the group showing the vulnerable phenotype ($p < 0.05$) as compared to the control group and to the animals with the resistant phenotype (Fig. 4A). After a drug-free period of 7 days and the re-test, changes in the relative xCT mRNA level were observed in the PFC ($F(2,21) = 3,947$, $p = 0.0351$), where the level of xCT mRNA decreased in the group with the vulnerable phenotype in comparison to the saline-treated control group ($p < 0.05$) (Fig. 5A).

Cocaine conditioning changed the relative expression of the NF-κB mRNA in the NAc ($F(2,21) = 12.17$, $p = 0.0003$) after the CPP test (Fig. 4A) and in the PFC ($F(2,21) = 3.389$, $p = 0.053$) after a drug-free period of 7 days and the re-test (Fig. 5A). Post hoc analysis showed an increase in the mRNA NF-κB in the NAc in the animals with the vulnerable ($p < 0.01$) as well as with the resistant phenotype ($p < 0.001$) in relation to the saline-treated control group after the CPP test (Fig. 4A). After a drug-free period of 7 days and the re-test, a drop in the NF-κB mRNA level in the PFC in the animals with the vulnerable phenotype in relation to the saline-treated control group was observed ($p < 0.05$) (Fig. 5A).

Cocaine conditioning changed the relative Nrf2 mRNA level in the NAc ($F(2,21) = 14.31$, $p = 0.0001$) after the CPP test (Fig. 4A). Post hoc analysis revealed a rise in the Nrf2 mRNA level in the NAc in the animals with the vulnerable ($p < 0.001$) as well as with the resistant ($p < 0.001$) phenotype in relation to the saline-treated control animals (Fig. 4A). After a drug-free period of 7 days and the re-test, there were no changes in the Nrf2 mRNA level in the all examined brain structures (Fig. 5A).

3.2.2. Changes in proteins expression

3.2.2.1. Experiment 1. A one–way ANOVA showed a significant effect of cocaine conditioning on the GLT-1 expression in the PFC ($2,21) = 27.29$, $p < 0.0001$) and in the NAc ($F(2,21) = 3.810$, $p = 0.0387$) following the CPP test (Fig. 4B) and in the NAc after the re-test preceded by a drug-free period of 7 days ($F(2,21) = 4.818$, $p = 0.019$) (Fig. 5B). The post-hoc analysis indicated a decrease in the GLT-1 expression in the PFC in the animals showing the vulnerable phenotype and in the animals with the resistant phenotype in comparison to the saline-treated control group ($p < 0.0001$) and an increase in GLT-1 level in the NAc in the animals with the resistant phenotype in relation to the saline-treated control ($p < 0.05$) after the CPP test (Fig. 4B). Following a drug-free period of 7 days and the re-test, the level of GLT-1 in the NAc decreased in the group with the vulnerable phenotype in comparison to the saline-treated control animals and to the animals with the resistant phenotype ($p < 0.05$) (Fig. 5B).

Moreover, cocaine conditioning induced changes in the xCT expression in the PFC ($F(2,21) = 6.106$, $p = 0.0081$) and in the HIP ($F(2,21) = 12.82$, $p = 0.0002$) following the CPP test (Fig. 4B). The post-hoc analysis indicated a decrease in the xCT in the PFC after the CPP test in the animals showing the vulnerable phenotype and in the animals with the resistant phenotype in comparison to the saline-treated control group ($p < 0.05$) and a drop in the xCT level in the HIP in the animals showing the vulnerable phenotype ($p < 0.01$) as compared to the control group (Fig. 4B). No significantly important changes in the xCT level were found in the examined brain structures after the re-test preceded by a drug-free period of 7 days (Fig. 5B).

Cocaine conditioning changed the expression of NF-κB (p65) in the PFC ($F(2,21) = 7.967$, $p = 0.0027$), and Nrf2 in the HIP ($F(2,21) = 6.580$, $p = 0.0060$) and the NAc ($F(2,21) = 8.614$,

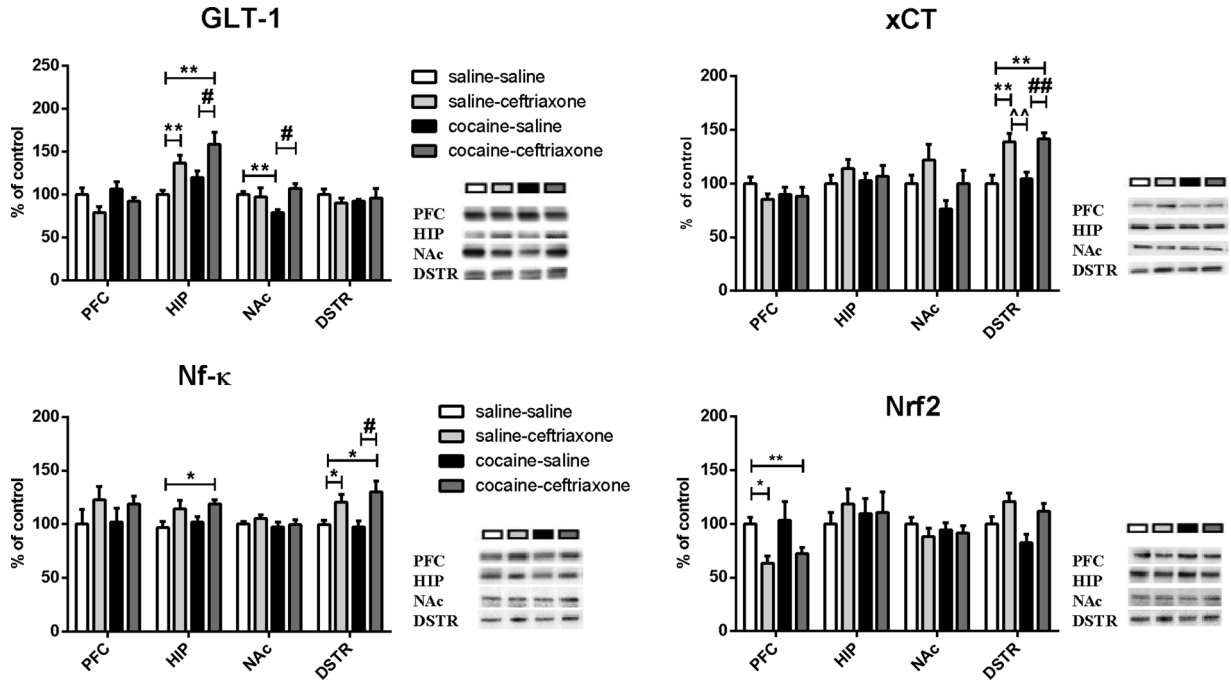

Fig. 6. The effect of ceftriaxone administration during the cocaine-free period on the level of GLT-1, xCT, Nf-κB and Nrf2 in the rat brain structures. Data were normalized to saline-saline animals (% of control). *$p < 0.05$; **$p < 0.01$ vs. saline-saline; # $p < 0.05$, ## $p < 0.01$ vs. cocaine-saline; ^^ $p < 0.01$ vs. saline-ceftriaxone (post hoc Tukey's test). N = 6 rats/group. DSTR - dorsal striatum; HIP - hippocampus; NAc - nucleus accumbens; PFC - prefrontal cortex.

p = 0.0019) measured after the CPP test (Fig. 4B). Post hoc analysis indicated a decrease in the NF-κB expression in the PFC in the animals showing the vulnerable phenotype ($p < 0.05$) and in the animals with the resistant phenotype ($p < 0.01$) after the CPP test in comparison to the saline-treated control group (Fig. 4B). After the CPP test, the level of Nrf2 decreased in the NAc ($p < 0.05$) and increased in the HIP ($p < 0.05$) in the animals showing the resistant phenotype in relation to the saline-treated control group (Fig. 4B).

3.2.2.2. Experiment 2. A two-way ANOVA revealed a significant effect of cocaine x ceftriaxone interaction ($F(1,20) = 5.92$, $p = 0.0245$) on the GLT-1 expression in the NAc (Fig. 6). The expression of GLT-1 in the NAc in the group of animals that were conditioned with cocaine and received ceftriaxone injections during cocaine-free period was higher in relation to the GLT-1 level in the same brain structure in the animals that were conditioned with cocaine and received saline instead of ceftriaxone ($p < 0.05$) (Fig. 6). Moreover, in the group of animals that received saline injections during cocaine-free period the expression of GLT-1 decreased in the NAc in comparison to the animals that received saline throughout the experiment ($t[10] = 3.88$, $p = 0.0031$).

In the group of animals that were conditioned with cocaine and received the ceftriaxone injections during cocaine-free period no changes in the accumbal GLT-1 level were observed. It means that the administration of ceftriaxone during a cocaine-free period prevented the decrease of the GLT-1 expression in this brain structure. A priori comparisons with Student's *t*-test also showed a significant increase in the GLT-1 level in the HIP in the animals which received saline during CPP and the ceftriaxone injections afterwards ($t[10] = 3.659$, $p = 0.0044$) and in the animals that were conditioned with cocaine and received ceftriaxone during a cocaine-free period ($t[10] = 2.377$, $p = 0.0388$) in comparison to the animals that received the saline injections throughout the experiment. A priori comparisons with Student's *t*-test also revealed a significant increase in the GLT-1 level in the HIP in the animals that were conditioned with cocaine and received the ceftriaxone injections during a cocaine-free period in comparison to the animals that were conditioned with cocaine and received the saline injections during a cocaine-free period ($t[10] = 3.903$, $p = 0.0029$) (Fig. 6).

A priori comparisons with Student's *t*-test revealed a rise in the xCT level in the DSTR in the animals which received saline injections during CPP and ceftriaxone afterwards ($t[10] = 3.411$, $p = 0.0066$) as well as in the rats that were conditioned with cocaine and received the ceftriaxone injections during a cocaine-free period ($t[10] = 4.240$, $p = 0.0017$) in comparison to the animals which received saline throughout the experiment. Moreover, a priori comparisons with Student's *t*-test showed that the animals which were conditioned with cocaine and received the ceftriaxone injections during a cocaine-free period displayed a significant increase in the xCT level in the DSTR in relation to the animals that were conditioned with cocaine and received the saline injections during a cocaine-free period ($t[10] = 4.526$, $p = 0.0011$) (Fig. 6). The level of xCT was lower in the DSTR of the animals which were conditioned with cocaine and received saline during a cocaine-free period as compared with the group that received only saline during CPP and ceftriaxone afterwards (Fig. 6).

A priori comparisons with Student's *t*-test revealed an increase in the NF-κB level in the HIP in the animals which were conditioned with cocaine and received the ceftriaxone injections during a cocaine-free period in comparison with the group that received saline throughout the experiment ($t[10] = 2.942$, $p = 0.0147$). Moreover, a priori comparisons with Student's *t*-test showed a rise in the NF-κB expression in the DSTR in the animals which received saline injections during CPP and the ceftriaxone injections afterwards in relation to the group that received saline throughout the experiment ($t[10] = 2.865$, $p = 0.0168$). The group which was conditioned with cocaine and received the ceftriaxone injections during a cocaine-free period displayed a significant increase in the NF-κB expression in the DSTR in comparison with the animals which were conditioned with cocaine and received the saline injections during a cocaine-free period ($t[10] = 2,859$, $p = 0.017$) (Fig. 6).

A priori comparisons with Student's *t*-test revealed a decrease in the Nrf2 level in the PFC in the animals which received the saline injections during CPP and the ceftriaxone injections afterwards ($t[10] = 2.645$, $p = 0.0245$) and in the animals which were conditioned with cocaine and received ceftriaxone injections during a cocaine-free period ($t[10] = 3.343$, $p = 0.0075$) in comparison to the rats that received the saline injections both during CPP and a cocaine-free period (Fig. 6).

4. Discussion

The present study identifies the molecular and neurochemical determinants that distinguish vulnerable versus resistant animals to cocaine rewarding effects. The data obtained in this study suggest that during cocaine-induced conditioned place preference, the glutamatergic signaling was affected mostly in animals vulnerable to the rewarding effects of cocaine. The most important change that differentiates the phenotypes seems to be related to a significant reduction of the GLT-1 expression in the NAc after a re-test of preference a drug-free period of 7 days in animals vulnerable to the rewarding effects of cocaine and a lack of such changes in animals with the resistant phenotype at the same stage of the behavioral test.

Numerous preclinical studies showed an increase in the glutamate level in the NAc during relapse induced by priming, cue presentation or context [14,17–19]. The increased level of glutamate in the NAc during relapse may not only result from its increased release from neurons but also from the impaired removal of this neurotransmitter through the GLT-1. Indeed, in our study, a decrease in the expression of GLT-1 in the NAc in animals with the vulnerable phenotype was observed after drug-free period of 7 days and a re-test of preference. The appearance of GLT-1 changes in our experiment after the re-test in the vulnerable phenotype, and not immediately after the end of the CPP test, suggesting that the extent of the decrease in GLT-1 expression depends on the drug-free period after cocaine administration, as confirmed by Fisher-Smith et al. [27]. Moreover, the hypothesis that the increase in the glutamate level in the NAc during relapse may largely depend on the decreased GLT-1 expression on the cell membrane of astrocytes is supported by the results obtained by Spencer and Kalivas [5]. Interestingly, the expression of the GLT-1 level in the NAc in animals with a resistant phenotype increased after the CPP test and remained at the control level after the re-test. We speculate that the rise in GLT-1 in resistant animals after the CPP test is an adaptive mechanism that aims to efficiently remove the excess glutamate released during exposure to the experimental cage (environment) that was associated with cocaine administration. Moreover, no changes after the re-test suggest that glutamate removal was indeed effective and might underlie the resistance to the rewarding properties of cocaine.

The molecular mechanism of cocaine-induced changes in GLT-1 expression remains unknown. The obtained results suggest that different mechanisms are involved in the regulation of GLT-1 expression at 1) the CPP test and 2) the re-test.

Our results indicate a contribution of transcriptional mechanisms in the regulation of GLT-1 expression in the NAc involving NF-κB only after the CPP test. GLT-1 and NF-κB mRNA both increased at that time point in animals with a vulnerable and resistant phenotype. As the changes in both phenotypes are identical, the pharmacological effect of cocaine is suggested rather than the characteristic feature of a given phenotype. On the other hand, after the re-test, the reduction in the GLT-1 protein expression in NAc in the vulnerable phenotype did not parallel the changes in the NF-kB nuclear protein or mRNA expression. The obtained results exclude genetic mechanisms and suggest the post-transcriptional or post-translational mechanisms in the GLT-1 protein downregulation. This suggestion is supported by the fact that GLT-1 may be subjected to post-translational modifications (e.g. SUMO-

modified GLT-1) [50]. Similar results confirming no GLT-1 mRNA changes in cocaine extinguished rats despite a decrease in protein expression were recently published by LaCrosse et al. [51].

As mentioned above, literature data indicate that withdrawal from cocaine administration decreases the extracellular glutamate level in the NAc, probably due to a drop in the xCT transporter expression in the astrocytes [28,29,52]. In our studies, a significant decrease in the level of the xCT mRNA in the NAc after the CPP test (48 h after the last cocaine injection) was observed. Interestingly, the drop in the level of the xCT mRNA occurred both in animals with the resistant and vulnerable phenotype which was accompanied by a significant increase in the Nrf2 mRNA. The substantial increase in the Nrf2 mRNA can be explained by a positive feedback mechanism in which a decrease in the xCT mRNA would induce an increase in the Nrf2 mRNA, whose role (in the form of the Nrf2 protein) is to raise the xCT level. These changes normalized after the 7-day drug-free period and the re-test. Surprisingly, in our studies, the changes in the xCT protein level in the NAc in both vulnerable and resistant animals were not observed at any stage of the experiment. These discrepancies can result from the xCT and Nrf2 mRNA translation kinetics.

Recent studies indicate that besides the NAc, the PFC plays a significant role in the cocaine addiction [53,54]. Our studies demonstrated that cocaine-CPP evoked a decrease in the level of xCT and GLT-1 protein in the PFC in rats showing either a vulnerable or resistant phenotype suggesting the pharmacological effect of cocaine. It should be mentioned that in preclinical studies Williams and Stekeete reported an increase in the release of glutamate in PFC following cocaine priming after a drug-free period of 1 and 7 days in animals showing behavioral sensitization [55]. Our results seem to be complementary to data of the above-mentioned authors, indicating neuroplastic changes induced by cocaine administration as a reason for an increase in the glutamate level in the PFC during relapse. Surprisingly, despite the visible behavioral differences between both phenotypes no neuromolecular differences within the chosen glutamatergic markers in the PFC were observed at this stage of the experiment. However, the reduction in the GLT-1, xCT, Nf-κB mRNA levels was seen also after the drug-free period of 7 days and the re-test only in the vulnerable phenotype. Therefore, it can be assumed that in the absence of differences in the expression of the selected glutamatergic system markers after the CPP test, what distinguishes one phenotype from the other in the PFC is the persistence of the observed changes. Moreover, the drop in the GLT-1 and xCT mRNA only in the vulnerable phenotype suggests that relapse to cocaine seeking could involve transcriptionally induced changes in the expression of these proteins. However, due to the duration of the translation process, differences at the protein level have not yet been observed in our experiment. The drop in the GLT-1 level in the PFC was accompanied by a decrease in the Nf-κB level in the nuclear fraction suggesting the role of this transcription factor in the GLT-1 expression regulation at that particular moment. A similar relationship was demonstrated by Muriach et al. [56].

It is well-known that drug addicts develop an association between cocaine-related experiences and environmental stimuli accompanying the use of this psychoactive substance [57]. It is the HIP that is the reward system element, which is itself involved in contextual memory formation and recalling [58], where the experimentally-induced glutamatergic neuron stimulation led to relapse to drug seeking [59]. Our results demonstrated a drop in the xCT level in the HIP of animals with a vulnerable phenotype after the CPP test. Other researchers have shown that removal of xCT from the surface of astrocytes causes an increase in AMPA expression along with a strong enhancement of spontaneous and evoked synaptic transmission [60]. Thus, the expression of cocaine-induced CPP demonstrated in our experiment in response to the cocaine-related context in vulnerable animals accompanied by a decrease in xCT expression in the HIP may be associated with the enhancement of synaptic transmission in this structure. In parallel to the above observations in the vulnerable animals, the GLT-1 mRNA level decreased after the CPP test as was previously confirmed for nicotine-induced CPP in the HIP [61].

Our study revealed almost no changes in the examined parameters in the DSTR, which is the brain structure that participates in the formation of habits and associated motor behaviors due to links with the motor cortex [62]. No neuroadaptation in this structure suggests its insignificant role in CPP induction, as was previously demonstrated by other authors [63].

4.1. The effect of ceftriaxone on the persistence of cocaine-induced CPP and on the expression of some markers of the glutamatergic system

The results of this study proved that ceftriaxone given during a cocaine-free period in the home-cage attenuated the persistence of cocaine-induced CPP while preventing a drop in the GLT-1 expression in the NAc. These results are in agreement with other authors' findings showing that ceftriaxone could restore GLT-1 expression and normalize extracellular glutamate in the NAc after cocaine self-administration + extinction training, and that ceftriaxone attenuated both cue- and cocaine-primed reinstatement in a non-contingent animal addiction model [19,52,64]. It is worth mentioning that our studies revealed that ceftriaxone alone did not increase the GLT-1 level in the NAc which is fully consistent with the results of Knackstedt et al. [26].

Until now, the molecular mechanism of the regulation of GLT-1 and xCT expression by ceftriaxone has not been unequivocally explained. *in vitro* studies demonstrated the engagement of Nf-κB and Nrf2 in the enhancement of GLT-1 and xCT expression [65,66]. The results of our studies did not confirm this relationship as we observed a lack of correlation between the changes in the xCT and GLT-1 expression in the membrane fraction and the alterations in the Nrf2 and NF-κB levels in the nuclear fraction what was in line with previous observations [51,67]. The newest proteomic studies indicate that ceftriaxone binds to complex 1 proteins of the mitochondrial oxidative phosphorylation chain [68]. Thus, this newly discovered ceftriaxone activity can potentially play a significant role in the observed drug effects in this study.

There was also a significant (˜50%) ceftriaxone-dependent increase in the GLT-1 protein level in the rat HIP and this is the first report showing changes in the GLT-1 protein level in the rat HIP. Ceftriaxone simultaneously induced an increase in the Nf-κB expression in the nuclear fraction in the HIP, thus suggesting the transcriptional Nf-κB-dependent mechanism in the GLT-1 level regulation. Based on the present experiments, however, it cannot be concluded what the behavioral consequences of this modulation are. However, referring to previous studies, an increased glutamate uptake (due to the enhanced expression of GLT-1) in the HIP can prevent the activation of postsynaptic mGluR1, revealing a new mechanism, by which GLT-1 locally regulates synaptic plasticity [69]. Interestingly, it was also demonstrated that ceftriaxone induced spatial learning disturbances and distorted the mGluR-dependent long-term synaptic depression in the HIP [70]. These mechanisms might be the mechanisms somehow responsible for the ceftriaxone anti-relapse efficacy in drug addictions.

5. Conclusion

In conclusion, our findings demonstrated differences in response to cocaine administration at the behavioral level manifested as a phenotype vulnerable or resistant to the rewarding effects of the drug. Each phenotype is characterized by specific molecular and neurochemical changes within the glutamatergic system. The most important change distinguishing these phenotypes appears to be related to reduction in the GLT-1 expression in the NAc after the re-test of preference in animals with the phenotype vulnerable to the rewarding effects of cocaine and the lack of such changes in animals with a resistant phenotype, at the same stage of the behavioral test. The results of mRNA analysis for the above biomarkers and transcription factors NF-κB and Nrf2 in some cases confirmed and in other precluded the engagement of transcription

mechanisms in the changes observed at the protein level. Moreover, multiple administrations of ceftriaxone during the cocaine-free period attenuated CPP persistence and normalized the GLT-1 level in the NAc. These results suggest that extracellular glutamate homeostasis was restored and probably contributed to the suppression of CPP persistence. Our results also suggest a lack of the NF-κB involvement in the regulation of the GLT-1 expression by ceftriaxone in the NAc.

We are the first to report that ceftriaxone strongly upregulates the GLT-1 in the HIP in a transcriptional mechanism involving the Nf-κB transcription factor. Theferore, modulation of the GLT-1 expression, not only in the NAc, but also in the HIP may play a role in attenuation of the persistence of CPP by ceftriaxone. Future experiments may resolve the question concerning whether modulation exclusively of the GLT-1 expression in the HIP may attenuate a cocaine-induced place preference or relapse. Moreover, a similar determination in other neuropsychiatric diseases with significant hippocampus involvement in pathogenesis would be equally interesting.

Declarations of interest

None.

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