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Effects of ampicillin, cefazolin and cefoperazone treatments on GLT1 expressions in the mesocorticolimbic system and ethanol intake in alcohol-preferring rats

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Abstract

Chronic ethanol consumption is known to downregulate expression of the major glutamate transporter 1 (GLT-1), which increases extracellular glutamate levels in subregions of the mesocorticolimbic reward pathway. While β-lactam antibiotics were initially identified as potent upregulators of GLT-1 expression, only ceftriaxone has been extensively studied in various drug addiction models. Therefore, in this study, chronic ethanol-drinking adult male alcohol-preferring (P) rats were treated with other β-lactam antibiotics, ampicillin, cefazolin or cefoperazone (100 mg/kg) once daily for five consecutive days to assess their effects on ethanol consumption. The results demonstrated that each compound significantly reduced ethanol intake compared to saline-treated control group. Importantly, each compound significantly upregulated both GLT-1 and pAKT expressions in the nucleus accumbens and prefrontal cortex compared to saline-treated control group. In addition, only cefoperazone significantly inhibited hepatic aldehyde dehydrogenase-2 enzyme activity. Moreover, these β-lactams exerted only a transient effect on sucrose drinking, suggesting specificity for chronically inhibiting ethanol reward in adult male P rats. Cerebrospinal fluid concentrations of ampicillin, cefazolin or cefoperazone have been confirmed using high-performance liquid chromatography. These findings demonstrate that multiple β–lactam antibiotics demonstrate efficacy in reducing alcohol consumption and appear to be potential therapeutic compounds for treating alcohol abuse and/or dependence. In addition,

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Conflict of Interest

The authors declare no conflict of interest. The views expressed in this manuscript are strictly those of the authors and do not necessarily represent those held by the National Institutes of Health (NIH) or NIAAA.

these results suggest that pAKT may be an important player in this effect, possibly through increased transcription of GLT-1.

Keywords

Cefazolin; cefoperazone; ampicillin; glutamate; alcohol intake; GLT-1; EAAT2

Introduction

Chronic exposure to drugs of abuse causes dysregulation of glutamatergic neurotransmission, with alterations to glutamatergic projections from the prefrontal cortex (PFC) to the nucleus accumbens (NAc) implicated (Kalivas, 2009, Kalivas and Volkow, 2011, Quintero, 2013). This projection is part of the mesocorticolimbic reward pathway and has been shown to mediate drug-seeking and relapse behaviors (Kalivas, 2009, Kalivas and Volkow, 2011, Quintero, 2013, Wise and Koob, 2014). Furthermore, recent studies have demonstrated that substance abuse is associated with impaired glutamatergic release, leading to an imbalance in glutamatergic homeostasis within the mesocorticolimbic pathway (Parsegian and See, 2014, Shen et al., 2014).

Ethanol, similar to other drugs of abuse, is known to significantly alter extracellular glutamate levels in the mesocorticolimbic pathway, which is due, at least partially, to impaired clearance of glutamate from the synapse (Melendez et al., 2005, Ding et al., 2013). These elevated NAc glutamate levels following ethanol intake, in turn, appear to promote continued excessive ethanol consumption (Griffin et al., 2014). Consistent with these findings, we have demonstrated significant downregulation of the major glutamate transporter, glutamate transporter 1 (GLT-1, its human homolog is excitatory amino acid transporter 2, EAAT2), in the NAc of alcohol-preferring (P) male rats after five weeks of free-choice ethanol exposure compared to their ethanol-naïve counterparts (Sari and Sreemantula, 2012, Sari et al., 2013).

Importantly, upregulation of GLT-1 expression in the mesocorticolimbic pathway is associated with restored glutamate homeostasis and attenuated drug-seeking behavior (Knackstedt et al., 2010, Rasmussen et al., 2011). While ceftriaxone treatment has yielded promising results in reducing drug abuse in cocaine, ethanol, and methamphetamine exposure animal models (Sari et al., 2009, Sari et al., 2011, Abulseoud et al., 2012, Rao and Sari, 2014), other β-lactam antibiotics, identified earlier as GLT-1 upregulators (Rothstein et al., 2005), have not been evaluated for their *in vivo* efficacy. Therefore, the aim of this study is to evaluate the effect of other FDA approved β-lactam antibiotics – ampicillin (AMP), cefazolin (CZN) or cefoperazone (CPZ) treatments (100 mg/kg/day) – on daily ethanol intake in male P rats following five weeks of free-choice ethanol exposure. Since five weeks of chronic ethanol exposure results in a consistent reduction of GLT-1 expression in the NAc and PFC (Sari and Sreemantula, 2012, Sari et al., 2013), ethanol-naïve animals were not included in this study.

In order to associate the changes in ethanol consumption following these treatments with changes in glutamatergic activity, GLT-1 levels in the NAc and PFC were compared

between the β-lactam-treated and saline-treated groups. To confirm the previously established pharmacological mechanism of GLT-1 upregulation in these brain regions (Wu et al., 2010), phosphorylation of signaling molecule AKT was also measured in the NAc and PFC of treated vs. control groups. Finally, to determine the CNS bioavailability of drug treatments, the cerebrospinal fluids (CSF) from AMP-, CZN-, and CPZ-treated P rats were analyzed by high-performance liquid chromatography (HPLC). Furthermore, we determined the effects of these β-lactam antibiotics on sucrose intake, a consummatory control for ethanol-drinking behavior. In addition, the N-methyltetrazolethiol side chain present in β-lactams is known to exhibit disulfiram-like effects on ethanol metabolism via inhibition of the enzyme, aldehyde dehydrogenase-2 (ALDH2) (Matsubara et al., 1987). Therefore, liver samples collected from AMP-, CZN-, and CPZ-treated P rats were analyzed for ALDH2 activity, an enzyme accountable for 60% of hepatic acetaldehyde metabolism (Weiner, 1987).

Materials and Methods

Animals

Adult male P rats were obtained from the Indiana University School of Medicine, Indianapolis, IN and housed in standard plastic tubs with corn-cob bedding in the Department of Laboratory Animal Resources vivarium at The University of Toledo. All animals had ad lib access to food and water during the study, and the animal vivaria were maintained at a temperature of 21°C on a 12-hour light/dark cycle (0600h/1800h). All of the animal experimental protocols were approved by the Institutional Animal Care and Use Committee of The University of Toledo in accordance with guidelines of the Institutional Animal Care and Use Committee of the National Institutes of Health and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, 1996). P rats, at the age of three months, were single-housed in bedded plastic cages and divided randomly into four different groups: (a) the saline vehicle group that received the saline vehicle (i.p., n=6); (b) the ampicillin group (AMP) that was treated with 100 mg/kg ampicillin (i.p., n=8); (c) the cefazolin group (CZN) that was treated with 100 mg/kg cefazolin (i.p., n=8); and (d) the cefoperazone group (CPZ) that received 100 mg/kg cefoperazone (i.p., n=8). The β-lactams were administered as a solution prepared in 0.9% saline.

Ethanol drinking procedures

At three months of age, all male P rats were given concurrent, free-choice continuous access to two ethanol concentrations, 15% and 30%, v/v, as well as food and water for five weeks. During week 4, ethanol consumption for each animal was evaluated as g/kg of body weight/day. Water consumption and body weight were also measured three times per week. The amount of ethanol and water consumed were determined to the nearest tenth of a gram by subtraction of the measured bottle weights from their previous day's values.

During the two weeks preceding treatment (weeks 4 and 5), all animals were required to meet the criterion of ≥4 g/kg/day of ethanol consumption, a criterion of ethanol consumption based on our previous studies (Sari et al., 2011, Sari and Sreemantula, 2012). The averaged

data collected during weeks 4 and 5 served as baseline values for ethanol consumption, water intake, and animal body weight. During week 6, P rats were injected with either saline vehicle, AMP (100 mg/kg, i.p.), CZN (100 mg/kg, i.p.), or CPZ (100 mg/kg, i.p.) once daily for five consecutive days. Ethanol and water consumption, along with body weight, were measured daily for the rest of the study. Previous studies have shown that five-day treatment with ceftriaxone treatment upregulated GLT-1 expression in the mesocorticolimbic pathway (Miller et al., 2008, Sari et al., 2009, Sari et al., 2011), and hence this treatment period was chosen for the present study. P rats were euthanized 24 h after receiving the last β-lactam or saline injection.

Sucrose drinking procedures

The effects of 100 mg/kg, i.p., AMP or CPZ or CZN on sucrose (10% w/v; in water) intake as an appetitive control for drinking-motivated behavior was also examined in a separate group of P rats. For this study, in addition to water and food, all male P rats were provided free-choice, continuous access to a 10% sucrose solution. The animals were divided into four groups: (a) a saline vehicle-treated (control) group (i.p., n=6); (b) an AMP group treated with 100 mg/kg/day of AMP (i.p., n=6); (c) a CZN group treated with 100 mg/kg/day of CZN (i.p., n=6); and (d) a CPZ group treated with 100 mg/kg/day of CPZ (i.p., n=5). These drugs were administered as a solution prepared in 0.9% saline. Starting on Day 11 of this study, daily sucrose intake (ml/kg/day), along with water consumption and body weight, were recorded for all P rats through the end of the study. Average sucrose intake data recorded on Day 11 and Day 12 served as the baseline value for data analysis. All groups were treated from Day 13 through Day 16, once daily, with their respective treatments (100 mg/kg/day, i.p.). Daily sucrose intake was monitored during this treatment period (Day 13 to Day 16) to determine the effects of treatment with AMP, CZN, or CPZ on sucrose-motivated drinking behavior by P rats.

Brain tissue harvesting

Twenty-four hours after receiving their last β-lactam or saline injection, all P rats from the ethanol-drinking paradigm were euthanized by CO_2 inhalation followed by rapid decapitation. The brains were immediately dissected, flash-frozen, and stored at −80°C. As described recently, the NAc and PFC were micropunched stereotaxically using a cryostat maintained at −20°C (Rao and Sari, 2014). The stereotaxic coordinates, employed for identification of the NAc and PFC in the rat brain, were in accordance with Paxinos and Watson's Stereotaxic Atlas (Paxinos and Watson, 2007). The NAc was extracted at the appearance of the anterior commissure and surgical blades were used to isolate this area as well as PFC following visualized landmarks. The medial part of the PFC was dissected at the same level of the NAc. These extracted brain regions were then frozen at −70°C for subsequent Western blot procedures.

Western blot protocol for detection of GLT-1, phosphorylated-AKT, and total-AKT

The brain regions collected from P rats (n=6, each group), the NAc and PFC, were analyzed for levels of GLT-1, phosphorylated-AKT (p-AKT), total-AKT (t-AKT), and β-tubulin using our Western blot protocol as described previously (Sari et al., 2011, Rao and Sari, 2014). Briefly, proteins were extracted with lysis buffer (50mM Tris-HCl, 0.15M NaCl,

1mM EDTA, 0.5% NP-40, 1% Triton, 0.1% SDS) supplemented with cocktail protease and phosphatase inhibitors and quantified using Bio-Rad DC protein reagents. Equal amounts of extracted proteins were mixed with Laemmli loading dye, and proteins were separated by electrophoresis. After electrophoretically transferring the proteins onto a PVDF membrane, the membranes were blocked using 3% milk in TBST (0.5 M Tris base, 1.5 M NaCl; pH7.4; 0.1% Tween20) for 30 minutes at room temperature. The membranes were incubated overnight at 4°C with one of the following primary antibodies: guinea pig-anti GLT-1 antibody (1:5000; Millipore), rabbit anti p-AKT (1:500; Cell Signaling Technology), and mouse anti t-AKT (1:1000; Cell Signaling Technology). Mouse anti β-tubulin antibody (1:3000; Covance Inc.) was used as the loading control. The following day, the membranes were washed, blocked with 3% milk solution, and incubated for 90 min at room temperature with their respective secondary horseradish peroxidase (HRP) antibodies: donkey-anti-Guinea pig IgG (1:3000), anti-mouse IgG (1:3000), or anti-rabbit IgG (1:3000). After incubation with HRP Chemiluminescent kit (SuperSignal West Pico, Pierce Inc.), the membranes were exposed to Kodak BioMax MR film (Fisher Inc.), and the films were developed on a SRX-101A machine. Digitized images of immunoreactive proteins were quantified using the MCID system (GE Healthcare Niagara Inc., US). The data are presented as percentage ratios of GLT-1/β-tubulin and p-AKT/t-AKT (each normalized to β-tubulin), relative to ethanol vehicle-treated control levels (control-value = 100%).

Mitochondrial aldehyde dehydrogenase (ALDH2) assay

Following euthanasia, liver samples from four treated groups (n=4, each group) were dissected and stored at −80°C. Liver tissue was homogenized at 4°C in 3 times the volume (w/v) of ice-cold 0.25 M sucrose solution. The homogenate collected was then centrifuged at 700 x g for 10 min at 4°C. The pellet was discarded while the supernatant was further centrifuged at 7200 x g for 20 min at 4°C. The pellet obtained after this centrifugation was washed with 1.5 ml of 0.25 M sucrose solution and centrifuged at 10000 x g for 15 min at 4°C. The final pellet obtained, representing the mitochondrial fraction, was resuspended in 0.5 ml of sucrose solution (containing 1% sodium deoxycholate), aliquoted, and stored at −80 °C. ALDH2 activity for the collected samples was analyzed using the substrate acetaldehyde, as described elsewhere (Karamanakos et al., 2007). Briefly, following the addition of the mitochondrial fraction to the assay mixture (50 μM acetaldehyde, 75 mM sodium pyrophosphate buffer (pH 8.0), 1 mM pyrazole, and 2 μM rotenone), NADH formation was analyzed spectrophotometrically at 340 nm over a 5 min time period. Each liver sample was analyzed three times to obtain an average ALDH2 activity.

Analytical determination of AMP, CZN, and CPZ levels in male P rats' CSF

Approximately one hour after receiving a 100 mg/kg i.p. injection of AMP (n=2), CZN (n=4), or CPZ (n=3), P rats were euthanized and CSF was collected and immediately frozen at −80°C. The time for CSF collection was based on previous report suggesting that peak CSF concentrations of these compounds are approximately 60 mins after drug administration (Griffith, 1974, Clumeck et al., 1978b, Najjar, 1992). Following euthanization by CO_2 exposure, the rats were immediately mounted on a stereotaxic head-holder with the head tilted slightly forward. A midline incision was made behind the occipital bone and the overlying neck muscles cut and removed from the insertions. The

neck muscles were removed with scissors, and cotton tipped swabs were used to abrade the muscle away from the underlying foramen magnum. Once the dural covering over the foramen magnum was visualized, a 1 cc syringe was used to carefully puncture the dura and CSF was slowly withdrawn.

Samples were analyzed using a high-performance liquid chromatography (HPLC) system (Waters Alliance e2695 separation module, Milford, MA) equipped with a 2998 PDA detector and a reverse-phase Kinetex C18 column (250 × 4.6 mm, Phenomenex, USA). AMP was analyzed with a mobile phase composed of 93% phosphate buffer (50 mM, pH 5) and 7% acetonitrile, pumped at a flow rate of 1.2 ml/min. CZN was analyzed with a mobile phase containing 87% citrate buffer (pH 3.6) and 13% acetonitrile, pumped at a flow rate of 1 ml/min. CPZ was analyzed with a mobile phase containing 70% aqueous phase (0.14% glacial acetic acid solution, pH adjusted to 5.85 using 1N NaOH) and 30% methanol. The drug concentration was determined quantitatively by plotting a calibration curve for each drug. A stock solution of 1 mg/ml of AMP, CZN, or CPZ was used in preparing the calibration curve standards in their respective mobile phases. For the calibration curve, each standard concentration was analyzed in triplicate, and the average peak area was plotted against the concentration. One hundred microliters of homogenized CSF samples were mixed with an equal amount of methanol and vortexed for 30 seconds. The mixture was then centrifuged at 5000 rpm for 10 minutes, and the supernatant was analyzed using HPLC.

Statistical analyses

General Linear Model (GLM) repeated measures statistical analyses were conducted using SPSS to determine significant main effects of Day or Day x Treatment interactions (day by treatment) on ethanol, water and sucrose intake, along with animal body weight, for all treated groups. Significant main effects or interactions were followed by one-way ANOVA and Dunnett's t-tests *a priori* multiple comparisons to determine the effects of AMP, CZN, or CPZ treatment on the dependent variable for each test day with respect to the saline-treated group. To determine the effect of treatments on ethanol and sucrose preference, GLM repeated measures followed by one-way ANOVA with LSD post hoc test was conducted. The effect of each β-lactam treatment on protein expression was determined by an independent t-test using GraphPad Prism software. To generate the bar graph representing changes in expression of proteins for β-lactam treated groups, data were obtained by converting western blot band density from saline-treated group (control) to 100%. All statistical analyses were based on a $p \leq 0.05$ level of significance.

Results

Effects of AMP, CZN, or CPZ treatment on ethanol consumption by male P rats

Figure 1A illustrates the effects of AMP, CZN, or CPZ treatment on daily ethanol consumption (grams/kg/day) in male P rats. Data analysis using GLM repeated measures revealed a significant main effect of Day [$F(1,5)=61.9$, $p<0.001$] and a significant Treatment x Day interaction [$F(3,15)=2.36$, $p<0.01$]. One-way ANOVA followed by two-tailed Dunnett's t-tests demonstrated a significant reduction ($p \leq 0.01$) in ethanol consumption in

animals treated with AMP, CZN, or CPZ, starting on Day 2 and lasting through day 5, compared to the saline-treated P rats.

Effects of AMP, CZN, or CPZ treatment on water intake in male P rats

The effects of AMP, CZN, or CPZ treatment on water intake by P rats were also compared to the saline-treated control animals (Figure 1B). Analysis employing GLM repeated measures showed a significant main effect of Day [$F(1,5)=3.53$, $p<0.01$] but a non-significant Treatment x Day interaction [$F(3,15)=1.45$, $p>0.05$]. Dunnett's t-tests following one-way ANOVA revealed a significant increase in water intake by AMP- -treated P rats on Day 2 and Day 3 ($p \leq 0.05$). CPZ treatment resulted in increased water consumption in P rats on Day 2 and Day 4 compared to saline treated P rats ($p \leq 0.05$). Alternatively, CZN treatment resulted in significantly higher water intake by P rats only on Day 3 ($p \leq 0.001$) compared to saline-treated animals. Importantly, P rats from all β-lactam treated groups had daily average water intake similar to the saline-treated group ($p>0.05$) on Day 5.

Effects of AMP, CZN, or CPZ treatment on body weight of male P rats

Figure 1C summarizes the effects of AMP, CZN, or CPZ treatment on the body weight of male P rats. GLM repeated measures analysis showed a significant main effect of Day [$F(1,5)=18.57$, $p<0.001$] and a non-significant Day x Treatment interaction [$F(3,15)=0.61$, $p>0.05$]. One-way ANOVA did not show any significant effects of AMP, CZN, or CPZ treatments on the body weight of P rats as compared to the control group ($p>0.05$).

Effects of AMP, CZN, or CPZ treatment on ethanol preference in male P rats

To correlate the changes in ethanol intake with change in preference, effects of AMP, CZN, or CPZ on ethanol preference in P rats was examined. Percent ethanol preference was calculated based on daily [ethanol/total fluid intake]*100 in P rats treated with AMP, CZN, and CPZ (Figure 2A). The GLM repeated measures analysis revealed a significant main effect of day [$F(1,5)=3.69$, $p<0.01$] and a non-significant Day x Treatment interaction [$F(3,15)=0.88$, $p>0.05$]. However, LSD post hoc analysis revealed a significant reduction ($p<0.05$) in ethanol preference in AMP treated P rats from Day 2 through Day 5 compared to control animals. CZN treated animals demonstrated reduction in ethanol preference from Day 2 through Day 4 ($p<0.05$). However, CPZ treated P rats demonstrated significant reduction in ethanol preference only on Day 2 ($p<0.05$) compared to saline treated P rats.

Effects of AMP, CZN, or CPZ treatment on sucrose intake in male P rats

Effects of AMP, CZN, or CPZ treatment on sucrose (10%) intake were examined (Figure 1D). The GLM repeated measures analysis revealed a significant main effect of Day [$F(1,4)=4.99$, $p<0.01$] and Day x Treatment interaction [$F(3,12)=2.93$, $p<0.01$]. One-way ANOVA followed by Dunnett's t-test analyses revealed a significant decrease in daily sucrose intake by AMP- and CPZ-treated P rats ($p<0.05$) from Day 13 through Day 15. However, on Day 16, the average sucrose intake was comparable between AMP, CPZ, and saline-treated P rat groups ($p>0.05$). Compared to the saline treated P rats, CZN-treated animals had significantly reduced sucrose intake only on Day 15 ($p<0.05$) of the treatment period. Overall, while sucrose drinking seemed to be affected at the start of treatment with

β-lactams, the drugs did not affect the daily sucrose intake by P rats, compared to saline-treated animals, at the end of the treatment period (Day 16).

Effects of AMP, CZN, or CPZ treatment on sucrose preference in male P rats

Percent sucrose preference was calculated based on daily grams per kg body weight [sucrose/total fluid intake]*100 in P rats treated with AMP, CZN, and CPZ (Figure 2B). The GLM repeated measures analysis revealed a significant main effect of day [$F(1,4)=3.96$, $p<0.01$] and a non-significant Day x Treatment interaction [$F(3,12)=1.08$, $p>0.05$]. LSD post hoc analysis confirmed a non-significant effect ($p>0.05$) of AMP and CZN dosing on sucrose preference during the entire treatment period (Day 13 to Day 16). CPZ treatment, however, was found to reduce sucrose preference in P rats on the last day of treatment (Day 16; $p<0.05$) as compared to saline treated group.

Effects of AMP, CZN, and CPZ treatment on GLT-1 expression in the NAc and PFC

Figure 3A illustrates the effects of AMP, CZN, or CPZ treatment on GLT-1 expression in the NAc. Compared to the saline-treated control group, Western blot analysis demonstrated a significant upregulation of GLT-1 level (normalized to β-tubulin) in the NAc following treatment with each of these three compounds. Independent t-test analyses of the immunoblots revealed increased GLT-1 levels in AMP- ($p<0.005$), CZN- ($p<0.005$), and CPZ- ($p<0.05$) treated groups as compared to the saline-treated group (Figure 3B).

Similarly, five-day dosing of P rats with AMP, CZN, or CPZ was found to significantly increase GLT-1 levels in the PFC with respect to the saline-treated control group (Figure 4A). The GLT-1 level (normalized to β-tubulin), following t-test analyses of immunoblots, was found to be significantly upregulated in the PFC of AMP- ($p<0.05$), CZN- ($p=0.01$), and CPZ- ($p<0.05$) treated P rats (Figure 4B).

Effects of AMP, CZN, or CPZ treatment on p-AKT/t-AKT levels in the NAc and PFC

We further investigated the effects of AMP, CZN, or CPZ treatment on phosphorylation of the signaling molecule AKT, a known regulator of GLT-1 expression (Li et al., 2006), in the NAc and PFC by comparing the ratio of p-AKT/t-AKT (normalized to β-tubulin) levels between treated and control P rats. In the NAc (Figure 5A,B), independent t-test analyses revealed a significant upregulation of AKT phosphorylation following treatment with AMP ($p<0.05$), CZN ($p<0.05$), and CPZ ($p<0.05$).

Similarly, we determined the ratio of p-AKT/t-AKT (normalized to β-tubulin) levels in the PFC. Independent t-test analyses demonstrated significant upregulation of p-AKT in the PFC of the AMP- ($p<0.005$), CZN- ($p<0.05$), and CPZ- ($p<0.05$) treated groups as compared to saline-treated P rats (Figure 6A,B).

Effects of AMP, CZN, or CPZ treatment on hepatic ALDH2 activity

In order to evaluate for any potential peripheral effects of AMP, CZN, or CPZ treatments, we compared the hepatic ALDH2 activity of treated groups with a saline vehicle group (Figure 7). Independent t-test analyses did not reveal any significant differences in the hepatic ALDH2 activity of AMP- and CZN-treated P rats as compared to the control group

(p>0.05). However, compared to the saline- treated group, CPZ treatment was found to significantly inhibit hepatic ALDH2 activity (p=0.011).

Concentration of AMP, CZN, or CPZ in CSF

Following HPLC analysis of the samples, the retention times for AMP (λ_{max} = 220 nm), CZN (λ_{max}= 270 nm), and CPZ (λ_{max} = 254 nm) were found to be 9.4 min, 11.3 min, and 7.2 min, respectively. The limit of detection and limit of quantification of AMP were found to be 0.062 μg/ml and 0.188 μg/ml, respectively. For CZN, the limit of detection and limit of quantification were determined to be 0.073 μg/ml and 0.221 μg/ml, respectively. The limit of detection and limit of quantification for CPZ were 0.105 μg/ml and 0.351 μg/ml, respectively. The intra- and inter-assay precisions of AMP, CZN, and CPZ were satisfactory; the relative standard deviations did not exceed 2%. The CSF concentrations (mean ± SD) of AMP, CZN, and CPZ in P rats were as follows: 0.24±0.09, 0.65±0.035, and 0.54±0.005 μg/ml, respectively.

Discussion

We report in this study that AMP, CZN, and CPZ treatments (100 mg/kg) attenuated ethanol drinking compared to the saline-treated control group. Importantly, we have demonstrated that the reduction in ethanol intake following treatments with AMP, CZN, and CPZ was associated in part with significant upregulation of the major glutamate transporter, GLT-1, in regions of the mesocorticolimbic reward pathway (the NAc and the PFC). Moreover, AMP, CZN, and CPZ treatment were found to significantly increase the phosphorylation of AKT, a signaling molecule reported to be involved in the cellular pathway of GLT-1 upregulation (Li et al., 2006, Wu et al., 2010), in both the NAc and the PFC. Interestingly, in addition to modulating GLT-1 expression centrally, CPZ treatment was associated with decreased activity of hepatic ALDH2 compared to the saline-treated group.

It is noteworthy that water intake was found to be elevated in rats treated with AMP, CZN and CPZ as compared to saline-treated P rats. Similarly, significant increases in water intake and attenuation of ethanol consumption following treatment with β-lactam compounds have been previously reported by our group (Sari et al., 2011, Sari and Sreemantula, 2012, Rao and Sari, 2014). We postulate that this increase in water intake is a compensatory mechanism in order to maintain fluid balance. The compensatory increase in water intake by P rats, concurrent with decreases in ethanol consumption, might also be compared to the burst of higher water consumption by selectively bred high-alcohol-consuming rats observed during alcohol deprivation intervals (Bell et al., 2008). Moreover, rats treated with these β-lactam compounds showed only a transient reduction in sucrose intake as a control for motivated behavior drinking. These findings parallel, to some extent, previous observations from our laboratory where ceftriaxone and GPI-1046 did not affect 10% sucrose drinking (Sari et al., 2011, Sari and Sreemantula, 2012). Moreover, in comparison with selectively bred alcohol-non-preferring (NP) rats, P rats have significantly higher intake of lower concentrations of sucrose, whereas the intake of higher concentration sucrose solutions (more than 8%) by both strains of rats are comparable (Stewart et al., 1994), supporting the use of a 10% sucrose concentration in the present study. The P rats, compared to NP rats,

have been reported to display heightened impulsivity towards sucrose (10%) intake in a limited session based sucrose consumption paradigm (Perkel et al., 2015). In addition, a time-dependent increase in sucrose (10%) consumption was observed in P rats trained for daily session based sucrose self-administration (Le et al., 2006). Hence, with 24 h free-access to sucrose solution in the present study, the observed amounts of daily sucrose intake is intuitive. Importantly, the saline treated groups at baseline, for instance, displayed no significant difference in the total daily fluid intake in the sucrose and ethanol exposed P rats (data now shown). However, to better estimate the effects of these β-lactams treatments on sucrose intake as consummatory control, testing lower doses of sucrose solution are warranted to be evaluated in future studies.

Importantly, β-lactams tested in this study significantly changed ethanol preference in male P rats during the treatment period. While AMP was effective in reducing preference for ethanol from Day 2 to Day 5 treatment days in P rats, CZN was effective only from Day 2 through Day 4. CPZ was found to be least effective in curbing preference for ethanol in P rats (Day 2 only). In addition, under the tested conditions, AMP and CZN did not change propensity for sucrose solution in male P rats. However, CPZ treatment significantly affected sucrose intake in P rats on the last day of treatment suggesting non-selective suppression of reward responses.

In addition, the chromatographic evaluation of CSF samples from the treated P rats showed distribution of all tested compounds in CSF, strongly suggesting their presence in the CNS, supporting our hypothesis that β-lactam antibiotics exert their adipsogenic effects centrally. The interactions between drugs of abuse and glutamatergic neurotransmission have been studied extensively, and several compounds that modulate glutamatergic signaling have shown promising results for treating drug addiction (for example, (Olive et al., 2012). Thus, drugs of abuse, including ethanol, induce decreased GLT-1 expression within the mesocorticolimbic dopamine system and a hyperglutamatergic state within this reward pathway as well. This glutamatergic imbalance has been shown to promote continued drug dependence as well as relapse to drug-seeking behavior (Sari and Sreemantula, 2012, Fischer et al., 2013, Sari et al., 2013, Abulseoud et al., 2014, Griffin et al., 2014). In addition to reducing ethanol consumption in animal model, GLT-1 mediated decrease in reinstatement of drug seeking behavior has been observed for other drugs of abuse (Knackstedt et al., 2010, Abulseoud et al., 2012, Reissner et al., 2014, Shen et al., 2014). Therefore, reestablishing glutamate homeostasis in the mesocorticolimbic pathway, via normalization of GLT-1 levels, represents an important therapeutic approach for treating drug addiction (For review, see Reissner and Kalivas, 2010). Importantly, restoration of the GLT-1 level in the NAc results in persistent neurochemical changes, as indicated by continued significant attenuation of extracellular glutamate levels in this brain region (Rasmussen et al., 2011).

While several compounds (Zink et al., 2011, Sari and Sreemantula, 2012, Reissner et al., 2014) have exhibited the ability to upregulate GLT-1 levels in brain, the FDA-approved β-lactam, ceftriaxone, has received the most attention (Rothstein et al., 2005, Rasmussen et al., 2011, Sari et al., 2011, Abulseoud et al., 2014). Using adult male P rats and the drinking procedures employed in the present study, we have previously reported the efficacy of

ceftriaxone in attenuating ethanol intake via a GLT-1-dependent mechanism (Sari et al., 2011, Sari et al., 2013). Moreover, we have shown that that daily ceftriaxone dosing upregulated GLT-1 expression and reduced ethanol intake in P rats experiencing ethanol access close to three times longer than that used in the present study (14 weeks) in addition to interfering with relapse-like drinking (Qrunfleh et al., 2013, Alhaddad et al., 2014a, Rao and Sari, 2014). The results from the present study indicate for the first time the ability of other β-lactam antibiotics, AMP, CZN, and CPZ, to attenuate ethanol consumption and concomitantly upregulate GLT-1 expression in the NAc and PFC. Since onset of decrease in ethanol intake is around Day 2 of treatment with these β-lactams, upregulation of GLT-1 expression following short-term treatment would be investigated for these compounds in future studies. In agreement with our previous studies, findings from earlier time points are expected to reveal the AMP-, CZN-, and CPZ-mediated GLT-1 upregulation to be occurring as early as Day 2 of the treatment (Rao et al., 2015).

Furthermore, the changes in expression of GLT-1 following β-lactam treatment were directly associated with increased phosphorylation of AKT in both the NAc and PFC. These observations support previous works indicating that increasing pAKT is one mechanism by which GLT-1 expression is increased (Li et al., 2006, Wu et al., 2010). The results from the present study are in agreement with our recent findings which revealed a prominent association between ceftriaxone- (Rao et al., 2015) and MS-153- induced GLT-1 upregulation and increased phosphorylation of signaling molecule AKT (Alhaddad et al., 2014b) in both NAc and PFC.

Regarding the β-lactam doses used herein, while higher intraperitoneal doses for these antibiotics have been administered in rats (Moxon et al., 1977, Livingston et al., 1988, Walterspiel and Vitulli, 1989), we selected 100 mg/kg based on its use for all three β-lactams in previous rat studies (Najjar, 1992, Tan et al., 1997). In addition, by selecting 100 mg/kg of daily dosing for all compounds, a qualitative comparison of efficacy between these compounds, and previously tested ceftriaxone, for GLT-1 modulatory effects was possible. Also, the CNS bioavailability of AMP, CZN, and CPZ has been studied previously, strengthening our rationale to evaluate the effects of these particular compounds on GLT-1 expression in the mesocorticolimbic pathway (Ishiyama et al., 1970, Clumeck et al., 1978a, Chang et al., 2000, Tsai and Chen, 2000). In addition to documented CNS bioavailability, AMP has the distinct advantage over ceftriaxone as an orally active antibiotic and CPZ, compared to ceftriaxone, has a unique side chain (N-methyltetrazolethiol) in its structure contributing to possible disulfiram-like actions. However, the ability of these compounds to, at other doses, attenuate ethanol consumption by P rats remains to be evaluated. As hypothesized, CPZ treatment induced disulfiram-like actions, owing to the presence of the N-methyltetrazolethiol moiety, by inhibiting the hepatic ALDH2 activity. Apart from modulating the glutamatergic neurotransmission in the NAc and PFC, the inhibition of hepatic ALDH2 activity serves as an additional mechanism of action for CPZ. It is possible that consumption of ethanol following CPZ treatment would have resulted in an accumulation of acetaldehyde, thereby producing aversion to ethanol consumption (Freundt et al., 1990). Importantly, β-lactam compounds without N-methyltetrazolethiol moiety in their structure, AMP and CZN in the present study, were found to have no effect on ALDH2 activity. Therefore, similar to ceftriaxone, the adipsogenic effects on ethanol intake by these

compounds are not dependent upon manipulation of ALDH2 activity but appear to be dependent upon modulation of GLT-1 expression levels. Corroborating the above findings, analytical determination of CSF samples revealed significant concentrations of AMP, CZN, and CPZ levels in the CSFs of male P rats. The CSF concentrations of AMP, CZN, and CPZ were well above the limit of quantification.

In summary, this study has demonstrated, for the first time, the ability of β-lactams, specifically AMP, CZN, and CPZ, to attenuate ethanol consumption in adult male chronic-ethanol-drinking P rats. Moreover, the attenuation of ethanol intake was accompanied by significant upregulation of GLT-1 levels and AKT phosphorylation in the NAc and PFC. Furthermore, these findings indicate that β-lactam- induced reductions of ethanol intake are not dependent upon ALDH2 activity and do not appear to be generalizable to all reinforcers, such that reductions in sucrose intake were transient in nature. The present results provide strong support for the hypothesis that upregulation of GLT-1 levels in central reward pathways is an important target for small molecule development in the treatment of addictive behaviors.

Acknowledgments

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Highlights

- Ampicillin, cefazolin and cefoperazone treatments reduced alcohol intake. > These drugs upregulated both GLT-1 and pAKT levels in the NAc and PFC. > cefoperazone significantly inhibited hepatic ALDH2. > Ampicillin, cefazolin and cefoperazone have been found in CSF.

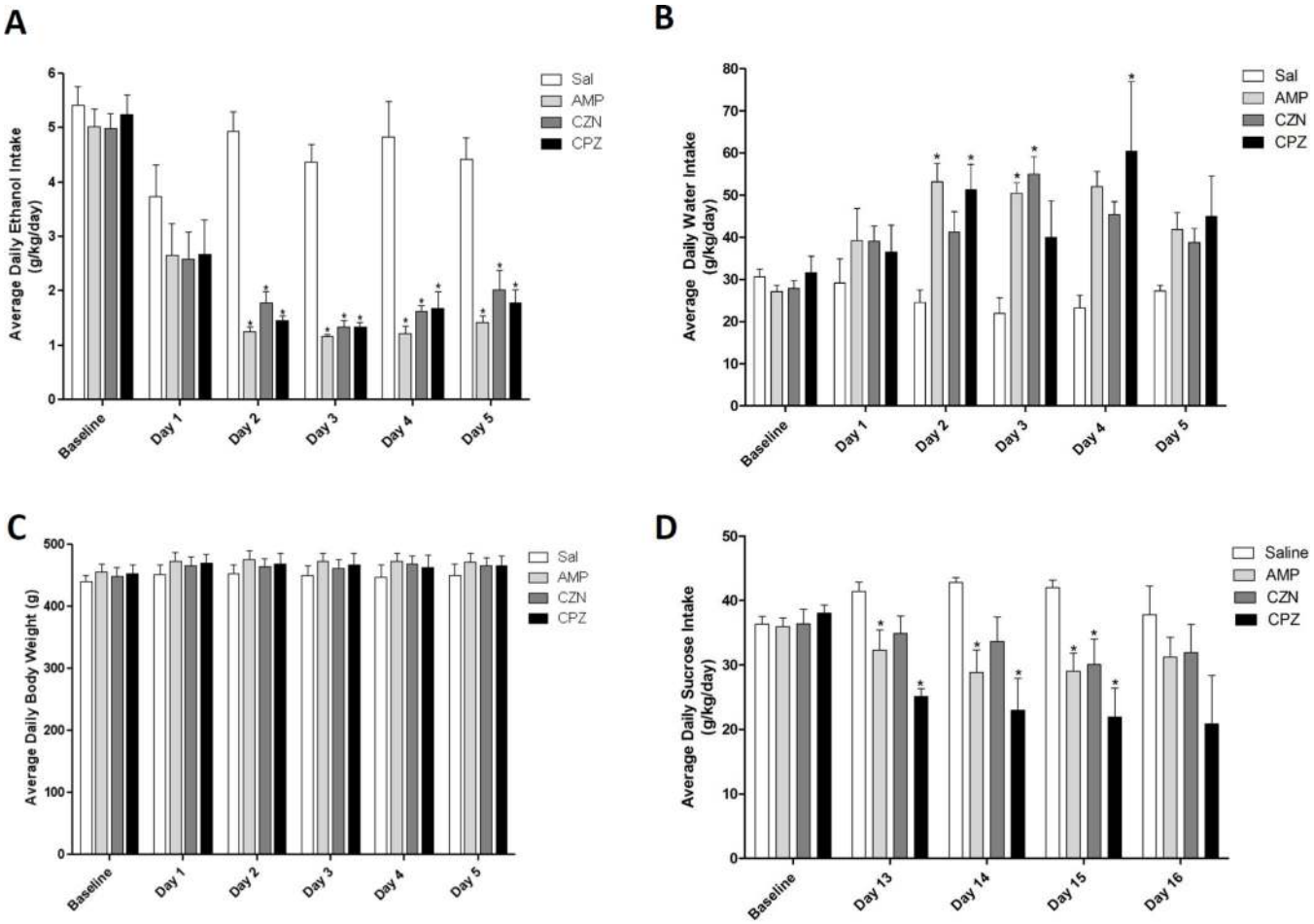

Figure 1.

(A) Average daily ethanol consumption (g/kg/day) in male P rats following five consecutive days of daily treatment with saline vehicle (n=6), or AMP (100 mg/kg; i.p.; n=8), or CZN (100 mg/kg; i.p.; n=8), or CPZ (100 mg/kg; i.p.; n=8). Graph represents ethanol drinking data (mean ± SEM) during the baseline (week 4 and 5) and treatment (Day 1- Day 5) phases of the study. Two-sided Dunnett's t-tests revealed that all three β-lactam treatments resulted in significant reductions in ethanol consumption compared to the saline vehicle-treated control group from Day 2 through Day 5. (B) Average daily water intake (g/kg/day). Graph represents water intake (mean ± SEM) during baseline (week 4 and 5) and treatment (Day 1- Day 5) periods of the study. Two-sided Dunnett's t-tests revealed significant increases in water intake by AMP-(Day 2 and Day 3) and CPZ-treated (Day 2 and Day) P rats, compared to the control group. With respect to saline-treated group, the CZN-treated male P rats consumed significantly greater amounts of water on Day 3. (C) Average body weights (g) of male P rats. Graph represents animal body weight (mean ± SEM) during baseline (week 4 and 5) and treatment (Day 1- Day 5) periods of the study. Two-sided Dunnett's t-test revealed no significant difference in animal body weight following AMP, CZN, or CPZ treatment compared to the saline-treated control group. Data are expressed as mean ± SEM. (*$p \leq 0.05$). (D) Average daily sucrose consumption (g/kg/day) in male P rats following four consecutive days of daily treatment with saline vehicle (n=6), or AMP (100 mg/kg; i.p.;

n=6), or CZN (100 mg/kg; i.p.; n=6), or CPZ (100 mg/kg; i.p.; n=5). Graph represents sucrose drinking data (mean ± SEM) during the baseline (Day 11 and Day 12) and treatment (Day 13 - Day 16) phases of the study. One-way ANOVA followed by Dunnett's t-test analyses revealed a significant decrease in daily sucrose intake by AMP- and CPZ-treated P rats ($p<0.05$) from Day 13 through Day 15. Compared to the saline treated P rats, CZN-treated animals had significantly reduced sucrose intake only on Day 15 ($p<0.05$) of the treatment period. The treatments had no effect on the daily sucrose intake by P rats, compared to saline-treated animals, at the end of the treatment period ($p>0.05$; Day 16).

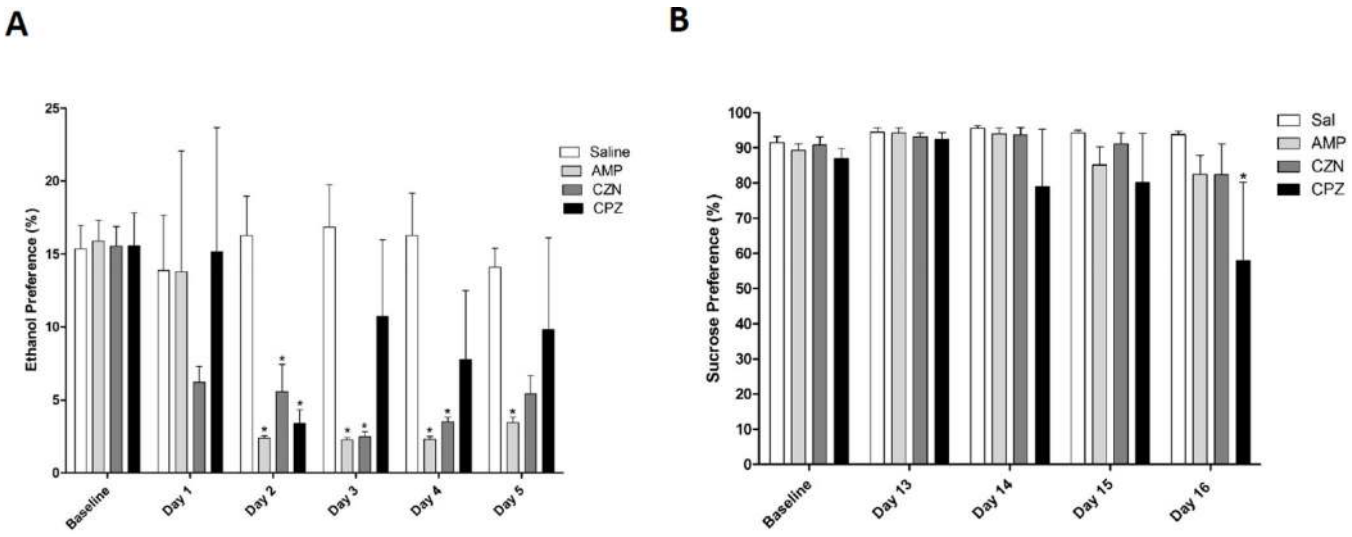

Figure 2.

(A) Ethanol preference (%) in male P rats following five consecutive days of daily treatment with saline vehicle (n=6), or AMP (100 mg/kg; i.p.; n=8), or CZN (100 mg/kg; i.p.; n=8), or CPZ (100 mg/kg; i.p.; n=8). Graph represents ethanol preference data (%) during the baseline (week 4 and 5) and treatment (Day 1- Day 5) phases of the study. One-way ANOVA followed by LSD post hoc test revealed that AMP (Day 2 through Day 5) and CZN treatments (Day 2 to Day 4) resulted in significant reduction in ethanol preference compared to the saline vehicle-treated control group. CPZ treatment had significant effect on ethanol preference on Days 2 only ($p<0.05$). (B) Sucrose preference (%) in male P rats following four consecutive days of daily treatment with saline vehicle (n=6), or AMP (100 mg/kg; i.p.; n=6), or CZN (100 mg/kg; i.p.; n=6), or CPZ (100 mg/kg; i.p.; n=5). Graph represents sucrose preference data (%) during the baseline (Days 11 and 12) and treatment (Day 13 to Day 16) phases of the study. One-way ANOVA followed by LSD post hoc test revealed that AMP and CZN treatments did not affect sucrose preference in P rats compared to the saline vehicle-treated control group during the study ($p>0.05$). CPZ treatment had significant effect on sucrose preference only on Day 16 of the study ($p<0.05$).

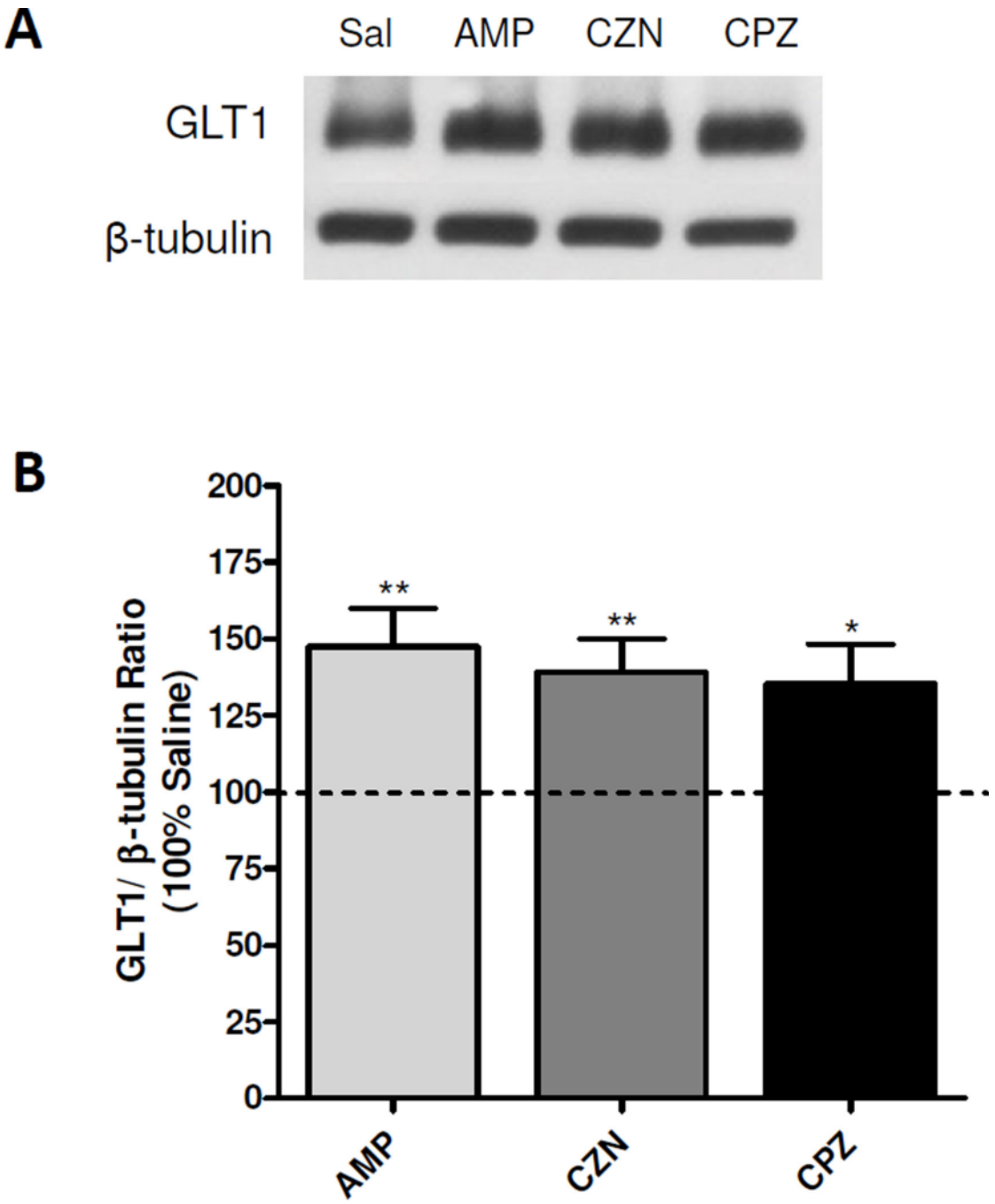

Figure 3.

Effect of five consecutive daily treatments with AMP, CZN, or CPZ (100 mg/kg; i.p., n=6 each) on the expression of GLT-1 in the nucleus accumbens (NAc) of male P rats compared to the saline-treated control group (n=6). (a) Representative immunoblots for GLT-1 and β-tubulin (loading control marker) expression in the NAc following saline, AMP, CZN, or CPZ treatments. (b) Quantitative t-test analysis of the immunoblots revealed a significant increase in expression of GLT-1 in the NAc of AMP-, CZN-, and CPZ-treated groups

compared to corresponding expression in the saline-treated group (100%). Data are expressed as mean ± SEM. (*p ≤0.05; **p ≤0.01).

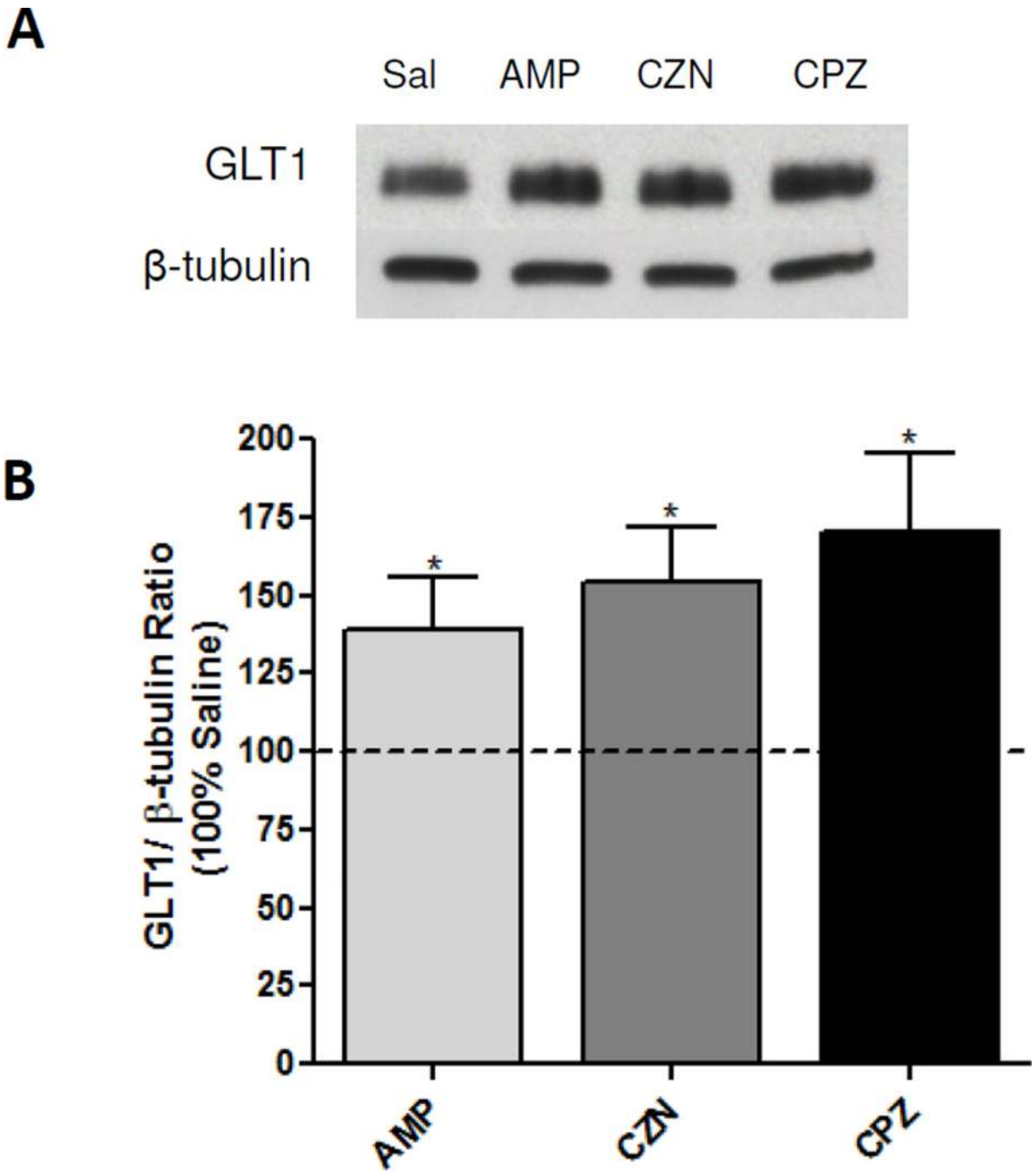

Figure 4.

Effects of five consecutive daily treatments with AMP, CZN, or CPZ (100 mg/kg; i.p., n=6 each) on the expression of GLT-1 in the prefrontal cortex (PFC) of male P rats compared to the saline-treated control group (n=6). (a) Representative immunoblots for GLT-1 and β-tubulin (loading control marker) expression in the PFC following saline, AMP, CZN, or CPZ treatments. (b) Quantitative t-test analysis of the immunoblots revealed a significant increase in GLT-1 expression within the PFCs of AMP-, CZN-, and CPZ-treated groups

compared to corresponding expression in the saline-treated group (100%). Data are expressed as mean ± SEM. (*p ≤ 0.05).

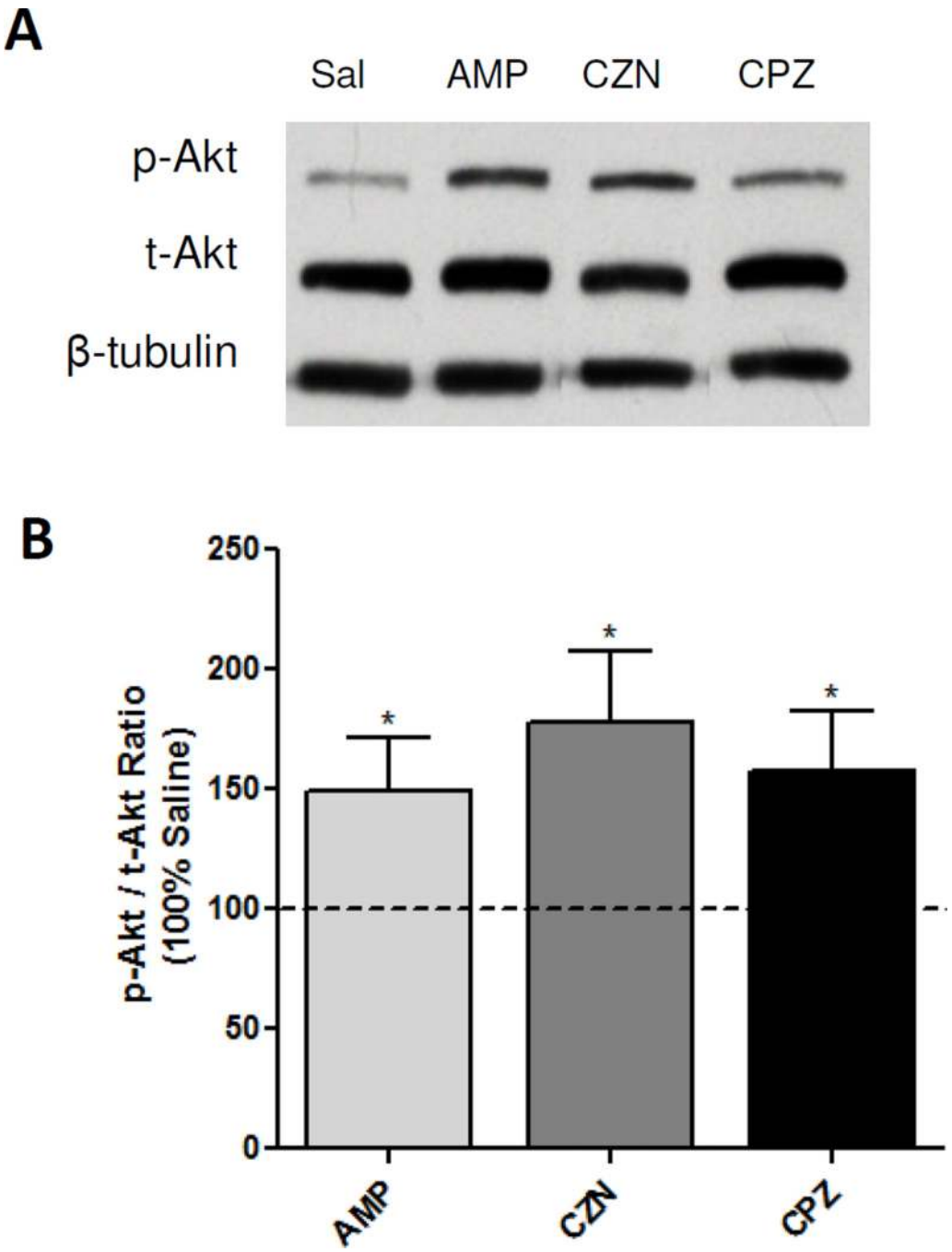

Figure 5.

Effect of five consecutive daily treatments with AMP, CZN, or CPZ (100 mg/kg; i.p., n=6 each) on phosphorylated-AKT/total-AKT(p-AKT/t-AKT) in the nucleus accumbens (NAc) of male P rats compared to the saline-treated control group (n=6). (a) Representative immunoblots for phosphorylated-AKT, total-AKT, and β-tubulin (loading control marker) expression in the NAc following saline, AMP, CZN, or CPZ treatments. (b) Quantitative t-test analysis of the immunoblots revealed a significant increase in p-AKT/t-AKT level in the

NAc of AMP-, CZN-, and CPZ-treated groups compared to corresponding expression in the saline-treated group (100%). Data are expressed as mean ± SEM. (*p ≤0.05).

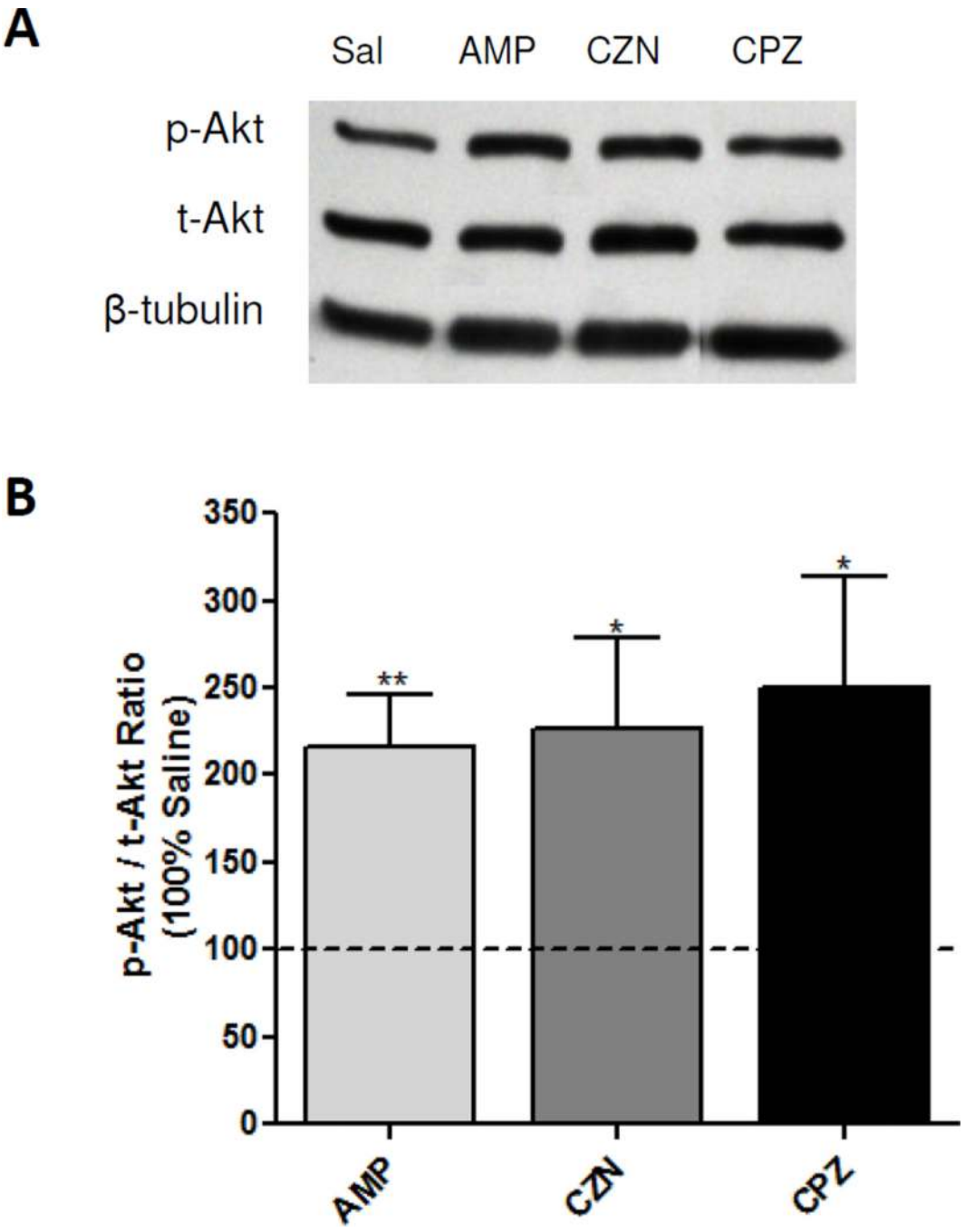

Figure 6.

Effect of five consecutive daily treatments with AMP, CZN, or CPZ (100 mg/kg; i.p., n=6 each) on phosphorylated-AKT/total-AKT (p-AKT/t-AKT) in the prefrontal cortex (PFC) of male P rats compared to the saline-treated control group (n=6). (a) Representative immunoblots for phosphorylated-AKT, total-AKT, and β-tubulin (loading control marker) expression in the PFC following saline, AMP, CZN, or CPZ treatments. (b) Quantitative t-test analysis of the immunoblots revealed a significant increase in p-AKT/t-AKT level in the

PFCs of AMP-, CZN-, and CPZ-treated groups compared to corresponding expression in the saline-treated group (100%). Data are expressed as mean ± SEM. (*p ≤0.05; **p ≤0.01).

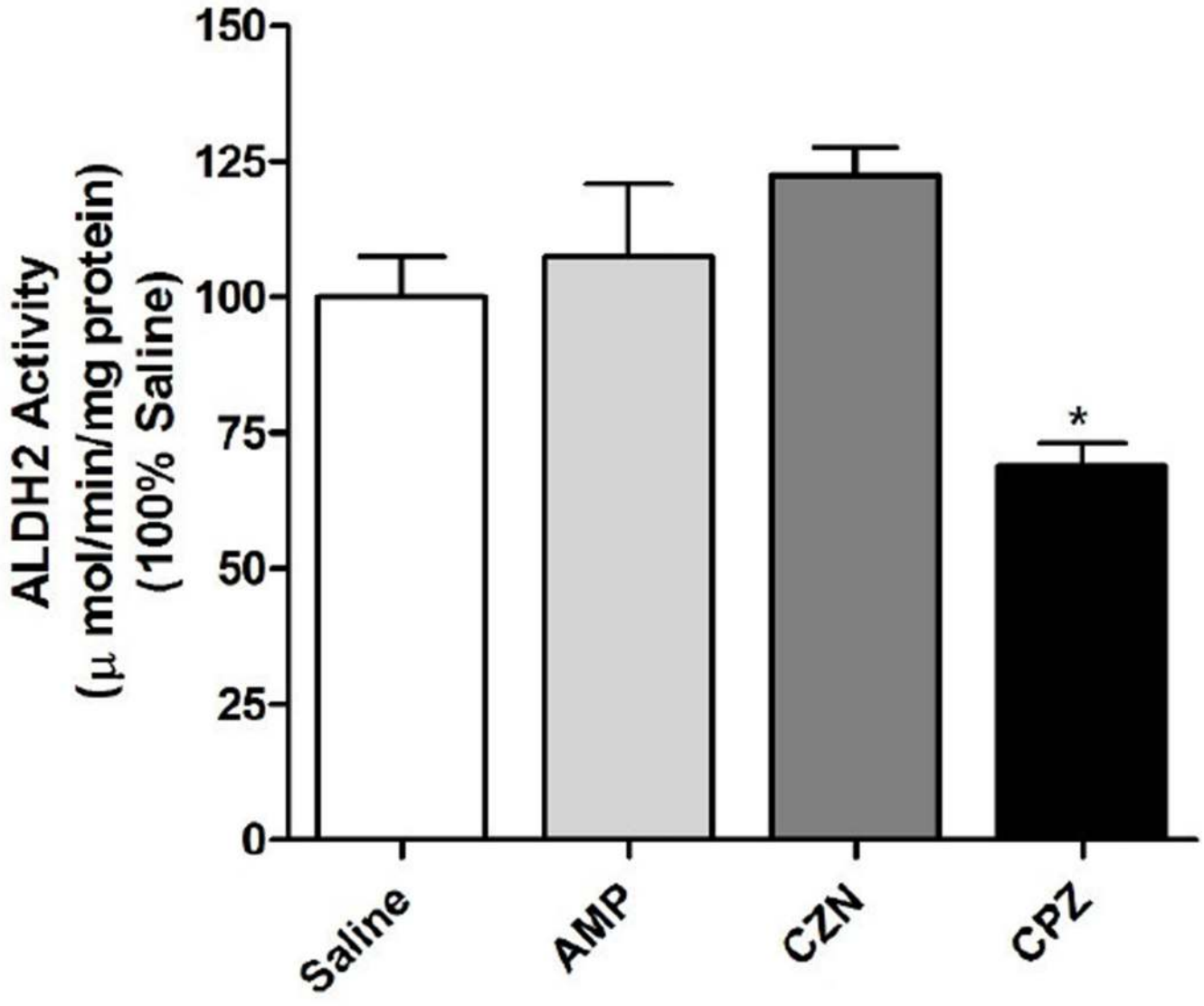

Figure 7.

Effects of five consecutive daily saline, AMP, CZN, or CPZ treatments on hepatic ALDH2 activity (μmol/min/mg protein) in male P rats. Mitochondrial fraction was extracted from liver samples corresponding to saline-, AMP-, CZN-, or CPZ-treated groups of P rats (n=4; each group) and analyzed for activity of ALDH2 enzyme as a percentage of saline group levels (100%). AMP and CZN treatments did not affect the activity of the ALDH2 enzyme when compared to the saline-treated group. However, ALDH2 enzyme activity was significantly inhibited in CPZ-treated P rats as compared to saline-treated control animals. Data are expressed as mean ± SEM (*p ≤0.01).