

Research report

Chronic cocaine differentially affects diazepam's anxiolytic and anticonvulsant actions Relationship to GABA_A receptor subunit expression[☆]

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Abstract

Benzodiazepines are used to treat the anxiety associated with cocaine withdrawal, as well as cocaine-induced seizures. Since cocaine exposure was shown to affect BZ binding density, abuse liability, subjective hypnotic actions and seizure susceptibility, we assessed whether chronic cocaine alters diazepam's anxiolytic and anticonvulsant actions. Changes in GABA_A receptor subunit protein expression were also assessed as they may relate to BZ activity at the receptor. Male Sprague–Dawley rats were injected with cocaine-HCl (15 mg/kg, i.p.) or saline once daily for 14 days. One day after the last injection, DZP (1 mg/kg i.p.) significantly increased time spent on and entries into open arms of an elevated plus maze in both saline- and cocaine-treated groups, yet the effect was greater in cocaine-treated rats. Eight days after cessation of treatment DZP did not have a significant anxiolytic effect in either group. To assess the effect of cocaine on DZP's anticonvulsant actions, PTZ was infused at a constant rate via the lateral tail vein and clonus onset was recorded in the presence and absence of DZP (5 mg/kg, i.p.). DZP significantly elevated seizure threshold in both groups of rats. Chronic cocaine also had no effect on the β -CCM seizure threshold. Quantitative immunohistochemistry of GABA_A receptor subunit protein demonstrated significant regulation of $\alpha 2$ (–10%) and $\beta 3$ (+9%) subunits in the hippocampal dentate gyrus and CA1 regions, respectively. Small changes in GABAR subunit expression in specific brain areas may relate to DZP's enhanced anxiolytic effectiveness whereas its anticonvulsant actions likely remain intact following cocaine administration. © 2000 Elsevier Science B.V. All rights reserved.

Theme: Neural basis of behavior

Topic: Drugs of abuse: cocaine

Keywords: Anticonvulsant; Anxiety; Cocaine; Benzodiazepine; Elevated plus maze; Pentylentetrazol

1. Introduction

Benzodiazepines (BZ's) are effective anxiolytics and anticonvulsants, and are used in the treatment of cocaine-withdrawal anxiety and cocaine-induced convulsions [30,41]. However, there are multiple lines of evidence suggesting that BZ clinical actions are altered following repeated cocaine use. For example, chronic cocaine expo-

sure differentially regulates the number of both central [13,20] and peripheral [4] BZ binding sites. Chronic cocaine exposure also increases the sedative activity of BZ's in animals [19] and humans [44]. Furthermore, although BZ's are rarely abused when prescribed clinically [15,8] cocaine addiction increases their abuse liability [15].

A goal of the present study was to evaluate whether or not the anxiolytic and/or anticonvulsant properties of diazepam (DZP), a prototype BZ, were altered in saline- vs. cocaine-treated rats. The anxiolytic properties of DZP were compared using an elevated plus maze, which is a standard test for the measurement of baseline and drug-altered anxiety [24,25]. Suppression of pentylentetrazol (PTZ)-induced seizures is a measure of anticonvulsant drug action, particularly those drugs acting at the γ -aminobutyric acid type A (GABA_A) receptor (e.g. [23]). There-

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fore, the ability of DZP to suppress PTZ-induced clonus was compared between experimental groups. Methyl- β -carboline-3-carboxylate (β -CCM), on the other hand, binds at the BZ recognition site and elicits convulsions by negatively regulating GABA_A receptor function [22]. Evaluation of β -CCM seizure threshold following cocaine exposure could lend more specific insights into cocaine's ability to modulate the BZ binding site. The effects of chronic cocaine treatment on GABA_A receptor subunit protein expression were also assessed, as changes in subunit expression may underlie alterations in BZ pharmacology (e.g. [42,49]). The results of the present study may have important implications for the use of these agents in the clinical treatment of the cocaine abuser.

2. Methods

2.1. Cocaine treatment

Male Sprague–Dawley rats (130–140 g) were housed singly on a 12-h light/dark cycle (lights-off at 7 p.m.), and allowed unlimited access to food and water. After a 2-day acclimation period, rats were injected (i.p.) once daily for 14 days with 15 mg/kg cocaine–HCl (COC) or 1 ml/kg 0.9% saline vehicle (CVH). All injections occurred between 9 and 11 am. Behavioral sensitization is commonly used to assess the effectiveness of repeated cocaine injection on the CNS [26]. However, the dose and schedule used in the current study does not reliably induce sensitization in 100% of the animals [26]. However, in the present study, an independent observer was able to differentiate saline- from cocaine-treated groups based on differences in stereotypy and locomotion. To determine the effect of cocaine on the anxiolytic action of DZP, COC and CVH ($n=24$ /group) rats were given DZP ($n=9$ /group), DZP vehicle (DVH; $n=8$ – 9 /group) or no injection ($n=8$ /group) and tested in an elevated plus maze, 1 and 8 days after treatment. To assess the effect of chronic cocaine treatment on the anticonvulsant action of DZP, COC and CVH rats ($n=11$ /group) were administered DZP ($n=5$ /group) or DVH ($n=6$ /group), 1 day following treatment, 30 min prior to infusion of PTZ. A separate group of rats received a single, acute injection of cocaine ($n=12$) or saline ($n=10$) 1 day prior to DZP or DVH ($n=5$ – 6 /group) injection and subsequent PTZ threshold determination. To assess the effect of chronic cocaine on functional BZ binding sites, β -CCM-induced seizure threshold was compared between COC ($n=5$) and CVH ($n=6$) rats 1 day after the end of treatment. Additionally, quantitative immunohistochemical experiments for GABAR subunits were performed on rats treated with cocaine or saline ($n=6$ /group). None of the rats received DZP, PTZ or β -CCM prior to immunohistochemical studies.

2.2. Anxiolytic testing

Anxiolytic testing began at the onset of the light cycle. Rats were moved to a novel environment, adjacent the testing room for ≥ 30 min prior to placement in the plus maze. All testing was performed under constant fluorescent (35 watt) illumination. The elevated plus maze (open arms 50×10 cm; closed arms 50×50×40 cm; elevated 50 cm) gives a reliable and valid measure of anxiety [6,25,25]. It has been effectively used to screen drugs for their anxiolytic properties, without interference due to locomotor effects [24,25]. Rats were given DZP (1 mg/kg, i.p.), vehicle (1 ml/kg, i.p.) or no injection 1 day after cessation of cocaine or saline treatment and 30 min prior to placement at the center of the maze, facing an open arm. The number of entries, and the time spent in open and closed arms were recorded for 5 min by an observer in the same room and later confirmed by videotape. The maze was cleaned with 100% EtOH and a damp cloth between trials. Rats were re-tested in the elevated plus maze 8 days after the 2-week cocaine treatment.

2.3. Seizure threshold testing

For determination of DZP's ability to elevate PTZ seizure threshold, rats were given an injection of DZP (5 mg/kg) or DVH (1 ml/kg) 24 h after cessation of chronic (or acute) cocaine or saline-treatment and 30 min prior to infusion of PTZ. β -CCM seizure threshold testing was performed in the same manner, but no injection was given 30 min prior to anticonvulsant testing. The chemoconvulsants (20 mg/ml PTZ or 0.3 mg/ml β -CCM) were infused via the lateral tail vein at a constant rate (0.57 ml/min). The time to the onset of forelimb clonus (within 2.5 min), was recorded and convulsant threshold was expressed as mg/kg PTZ or β -CCM. Rats were euthanized with sodium pentobarbital (120 mg/kg, i.p.) immediately following the onset of seizure.

2.4. Tissue preparation

Rats were anesthetized (ketamine 80 mg/kg, i.m.), perfused (200 ml 0.1 M PBS, pH 7.4), and decapitated 24 h after final injection, according to IACUC approved techniques. Brains were removed, rapidly frozen in isopentane, cooled in an acetone/dry ice bath and equilibrated in the cryostat (-12°C) for 1 h. Parasagittal sections (20 μm) were thaw-mounted onto glass slides coated with 0.5% gelatin/0.05% chrome–alum and stored at -70°C until immunohistochemical staining.

2.5. Quantitative immunohistochemistry

Details of the staining protocol and antibody specificity are provided elsewhere [5,42]. Briefly, sections were

brought to room temperature under vacuum and post-fixed in 4% paraformaldehyde for 8 min. Sections were blocked for 1 h in 10% normal goat serum, incubated overnight with 1° antibody ($\alpha 2$, 3 $\mu\text{g}/\text{ml}$, $\beta 3$ and $\gamma 2$, 10 $\mu\text{g}/\text{ml}$; W. Seighart), rinsed in PBS, and incubated with biotinylated anti-rabbit IgG F(ab')₂ fragment for 1 h (1:250, v/v, Boehringer Mannheim). The signal was amplified by incubating sections for 1 h with avidin biotin peroxidase complex (1:100), sections were rinsed, then visualized with 0.06% (w/v) diaminobenzidine (DAB, Sigma)/0.02% H₂O₂ (v/v). All tissues were developed in DAB for an equal amount of time, allowing accurate assessments of immunostaining density to be made [5,17]. Slides were then dehydrated in a series of graded ethanols, cleared in xylene, and coverslipped with Permount (Fisher). Immunostaining densities in areas of interest were compared between groups using NIH image software v1.61 [5,17]. Areas measured included those reported to play a role in drug reinforcement or dependence and/or those areas in which GABAR structure and function was regulated by pharmacological manipulations [5,29,46,48]: nucleus accumbens, striatum, olfactory tubercle, superior colliculus and the hippocampal CA1, CA3 (st. oriens, st., pyramidale, st. radiatum and st. lacunosum) and dentate (molecular, granular, and polymorph layers) regions.

2.6. Drugs

DZP (1 and 5 mg/ml) was dissolved in a standard vehicle containing 40% propylene glycol, 10% EtOH, 1.5% benzyl alcohol 0.2 M sodium benzoate, and 0.02 M benzoic acid. PTZ (20 mg/ml) and cocaine (15 mg/ml) were dissolved in 0.9% saline immediately prior to use. The convulsant β -CCM was dissolved (0.3 mg/ml) in a small amount of 1 N HCl and the pH adjusted to 4.0 immediately prior to use.

2.7. Statistical analysis

Elevated plus maze activity between saline and cocaine-treated groups was analyzed by a two-way (group \times time) repeated measures ANOVA, comparing the number of open arm entries, time spent in open arms, and total (open+closed) entries in the presence and absence of DZP. Additionally, a one-way ANOVA was used to compare change scores for the same dependent variables in the elevated plus maze. Post-hoc analyses were performed with an LSD test. A one-way ANOVA with a Tukey–Kramer post hoc-test was used to compare the ability of DZP to elevate PTZ seizure threshold (within group testing), as well as differences due to cocaine treatment (between group testing). A Student's *t*-test was used to compare differences in β -CCM seizure threshold differences between saline- and cocaine-treated groups. For comparison of GABA_A receptor subunit immunostaining

density an ANOVA was followed by a post-hoc analysis using the Fisher's LSD test. The confidence interval was set at 95% for all statistical tests.

3. Results

3.1. Anxiolytic testing

3.1.1. One day after treatment

Open arm activity in an elevated plus maze is sensitive to the anxiolytic effect of DZP [11,24]. There was a significant effect of group (measured 1 day after cessation) on open arm entries $F(3,31)=5.4$, and time spent in open arms $F(3,31)=5.6$. Post-hoc analysis revealed that open arm entries and time spent in open arms increased significantly in both COC and CVH rats given DZP compared to DVH. In addition, DZP had a greater effect to increase open arm entries in COC treated rats compared to controls. A similar yet non-significant trend was detected using open arm time as the dependent variable. Early withdrawal (24 h) from the chronic cocaine did not result in detectable anxiety, i.e. there was no significant difference in open arm exploration between COC and CVH groups given DVH prior to testing (Fig. 1). In fact, cocaine-treated rats exhibited a non-significant increase in open arm exploration without a corresponding increase in total entries, compared to their matched controls. To clarify whether the injection (of DZP or DVH 30 min prior to testing) itself affected cocaine-withdrawn rats, we evaluated an additional group of rats, which did not receive a DZP or DVH injection prior to testing. In rats tested under this latter paradigm, cocaine treatment was again associated with a trend (non-significant) suggestive of less anxiety, manifested as increased open arm entries and open arm time (data not shown).

3.1.2. Eight days after treatment

COC and CVH rats were re-tested 8 days after chronic treatment. At this time point, DZP no longer had an anxiolytic effect in COC or CVH rats; i.e. open arm entries and time were not significantly different between groups ($F(3,31)=5.4$ and 5.6, respectively; Fig. 1B). Furthermore, there was no significant difference between COC and CVH rats given diazepam vehicle, suggesting that basal anxiety was not altered 8 days after chronic cocaine treatment. As evident from Fig. 1, there were significant reductions in open arm exploration in the elevated plus maze upon re-testing. However, total entries, a measure of overall activity, significantly decreased in each of the groups. To further assess differences in open arm exploration upon re-testing we performed a one-way ANOVA on the change scores (i.e. day 1 score minus day 8 score) for open arm entries, open arm time, and total entries. There was a significant effect of treatment on the decline in open arm

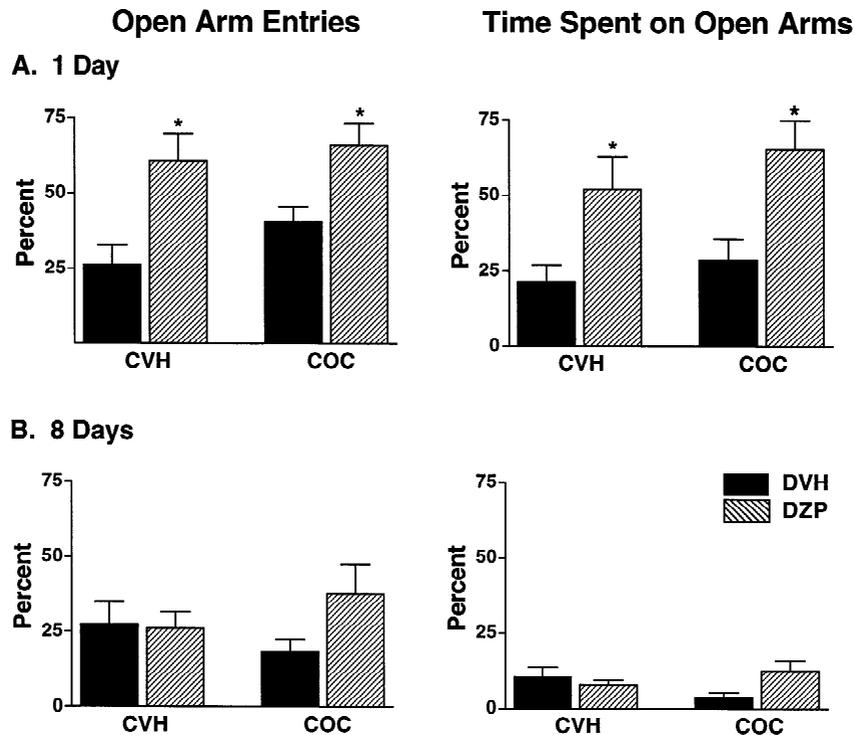


Fig. 1. DZP's anxiolytic effect in rats treated with cocaine (COC) vs. saline (CVH). One day after treatment DZP increased mean open arm entries and mean open arm time (both expressed as percent of total) in control (CVH) and cocaine-treated (COC) rats. Rats were re-tested 8 days after treatment. DZP did not elicit an anxiolytic response as measured by either open arm entries or open arm time in either cocaine- or saline-treated rats ($n=8-9/\text{group}$). Asterisks denote a significant difference ($P \leq 0.05$).

entries and open arm time, but not total entries ($F(3,31)=5.1, 6.2$ and 0.7 , respectively). That is, all rats decreased total entries to the same degree. For both measures of open arm exploration, rats given DZP prior to placement in the plus maze exhibited a significantly larger reduction in open arm entries and time spent on open arms upon re-testing, than did their DVH counterparts, regardless of cocaine- or saline-chronic treatment (see Fig. 2).

3.2. Anticonvulsant testing

3.2.1. PTZ seizure threshold

DZP is well characterized in its ability to significantly elevate PTZ seizure threshold. Furthermore, this action can be altered following prior drug exposure (e.g. chronic BZ treatment) consistent with changes in DZP potency [48]. All rats were tested 24 h after the last cocaine injection and given either DZP or DVH 30 min prior to infusion of PTZ. There was no difference in susceptibility to PTZ seizures (DVH 30 min prior to testing) following a single saline vs. cocaine injection (saline 61.6 ± 6.1 ; cocaine 59.0 ± 3.7 mg/kg). Moreover, acute cocaine had no effect on the ability of DZP to significantly elevate PTZ convulsant threshold (cocaine 97.7 ± 9.9 mg/kg; saline 89.1 ± 8.4 mg/kg). Repeated cocaine treatment per se also had no significant effect on PTZ seizure threshold (Fig. 3). As expected, DZP significantly elevated (+200%) PTZ seizure threshold in

the CVH-treated rats. Chronic cocaine treatment had no effect on the anticonvulsant efficacy of DZP as it also significantly elevated (+190%) PTZ seizure threshold in rats chronically treated with cocaine to a similar degree (Fig. 3).

3.2.2. β -CCM seizure threshold

Chronic cocaine- and saline-treated rats were infused with β -CCM until the onset of clonus was recorded. Cocaine-injected rats had a β -CCM seizure threshold of 0.8 ± 0.1 mg/kg in comparison to rats injected with saline (0.9 ± 0.2 mg/kg; Fig. 4). There was no significant difference between groups ($P=0.5$) due to cocaine treatment (Fig. 4).

3.3. Subunit immunostaining density

Alterations of GABAR subunit protein expression occur in parallel with alterations of BZ pharmacology [38,49] and GABAR function [42]. In the present study we chose GABAR subunit proteins present in high densities in the mesolimbic circuits involved in drug reinforcement [29,46]. We also chose areas in which GABAR subunit protein has been altered in parallel with local functional changes [42]. In the hippocampus, there was no change in $\alpha 2$, $\beta 3$ or $\gamma 2$ subunit immunostaining in CA2 (data not shown) or CA3 regions due to chronic cocaine treatment

Change in Plus Maze Behavior

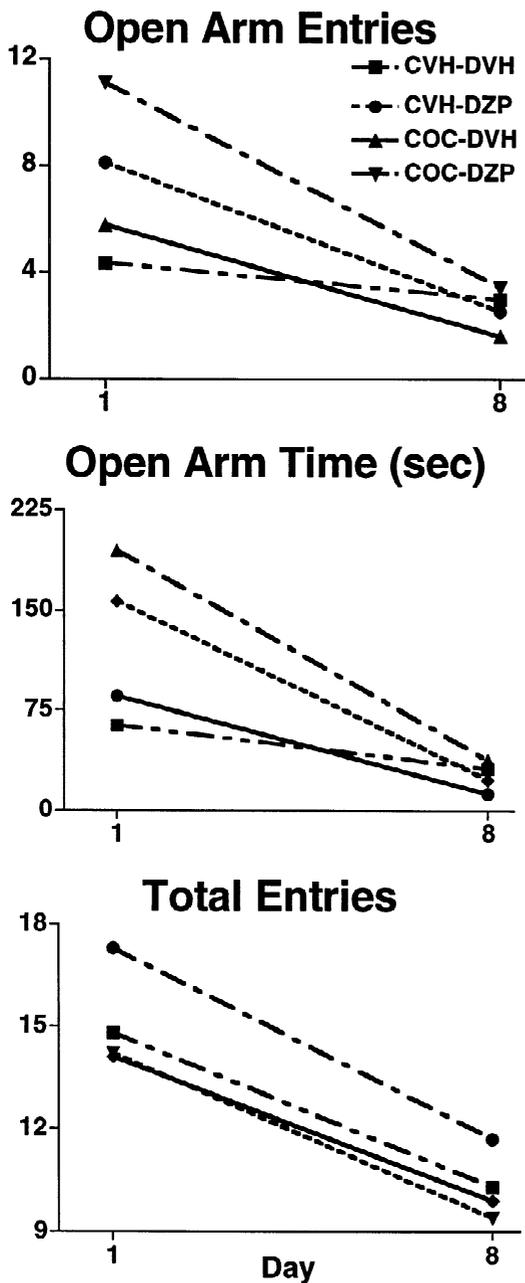


Fig. 2. Change in elevated plus maze behavior upon re-testing. Each of the groups exhibited significantly reduced activity in the elevated plus maze upon re-testing. A one-way ANOVA on the change scores indicated that DZP exposed rats exhibited greater reductions in open arm exploration than their DVH exposed counterparts.

(Table 1). In the CA1 area of hippocampus $\gamma 2$ expression levels were unaltered, but $\beta 3$ subunit protein was significantly increased in the pyramidal cell layer (+9%, Table 1, Fig. 5). In the dentate granule cell layer of the hippocampus $\alpha 2$, but not $\beta 3$ or $\gamma 2$ subunit protein expression was altered (-10% , $P \leq 0.05$, Table 1, Fig. 5). No significant changes in $\alpha 2$, $\beta 3$ or $\gamma 2$ GABAR subunit

PTZ Threshold

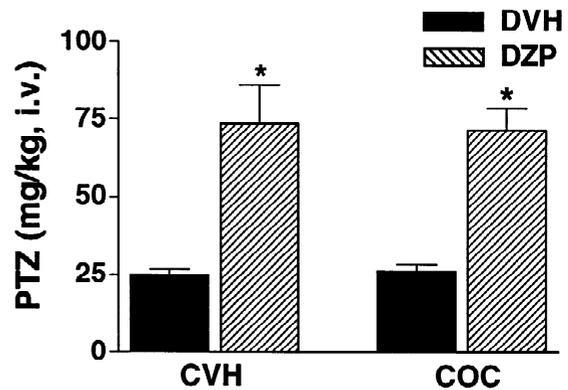


Fig. 3. DZP's anticonvulsant effect in cocaine (COC) vs. saline (CVH) treated rats. Chronic cocaine had no effect on baseline PTZ threshold (CVH–DVH vs. COC–DVH). Moreover, DZP's significant anticonvulsant effect in CVH-treated rats was retained in COC exposed rats ($n=5-6$ /group). Asterisks indicate significance ($P \leq 0.05$).

protein expression were detected in nucleus accumbens, olfactory tubercle, striatum or anterior olfactory nucleus (Table 2).

4. Discussion

The benzodiazepines as a class of drugs are effective anxiolytics, hypnotics, and anticonvulsants [1,16]. They are the most widely prescribed CNS depressants, with selective activity at the inhibitory GABA_A receptor complex. By enhancing the frequency of Cl⁻ channel opening and thus Cl⁻ flux through the GABA_A receptor, BZ's potentiate the inhibitory effect of GABA. Through this mechanism BZs mediate their anxiolytic and anticonvulsant effects.

β -CCM Threshold

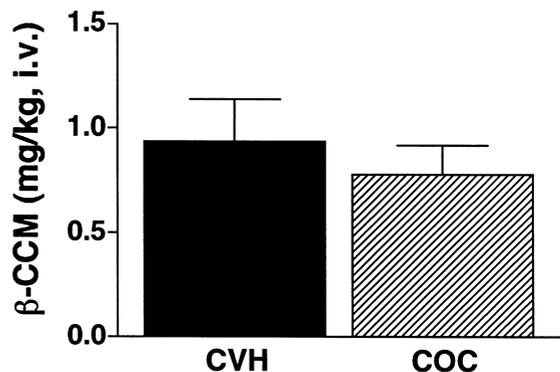


Fig. 4. β -CCM seizure threshold in cocaine (COC) vs. saline (CVH) treated rats. Chronic cocaine treatment did not have a significant effect on convulsions induced by the BZ inverse agonist β -CCM ($n=5-6$ /group). Asterisks indicate significance ($P \leq 0.05$).

Table 1
Effect of 2-week chronic cocaine treatment on $\alpha 2$, $\beta 3$, and $\gamma 2$ subunit antibody immunostaining in the hippocampus^a

Group	DG			CA1				CA3		
	Mol	Grn	PC	SO	SP	SR	SL	SO	SP	SL
$\alpha 2$ subunit										
CVH	44.2±1.8	26.2±4.8								
COC	31.8±2.7	20.5±4.5	Not present in notable amounts							
<i>P</i> -value	0.02*	0.35								
$\beta 3$ subunit										
CVH	56.4±3.0	45.3±3.4	34.5±1.4	46.5±1.0	38.8±1.0	43.0±1.8	47.7±1.9	43.3±1.0	29.0±1.5	42.0±1.6
COC	56.6±1.4	47.6±1.7	36.5±0.9	47.1±1.0	42.7±1.0	42.4±1.2	47.0±0.7	46.0±1.4	32.1±0.4	44.8±1.0
<i>P</i> -value	0.94	0.58	0.26	0.71	0.03*	0.79	0.75	0.17	0.10	0.19
$\gamma 2$ subunit										
CVH	26.7±3.6	25.7±5.1	20.8±3.7	24.8±3.9	23.8±2.5	28.9±3.9	26.6±3.9	27.7±3.6	22.2±3.7	30.4±3.1
COC	29.3±2.6	29.1±2.4	24.0±2.0	27.9±1.7	25.2±2.8	30.8±2.3	30.0±2.4	31.4±2.5	23.5±2.1	32.7±2.5
<i>P</i> -value	0.59	0.56	0.48	0.50	0.73	0.68	0.50	0.43	0.77	0.57

^a CVH: Cocaine vehicle treatment; COC: Chronic cocaine treatment; *n*=4 rats/group; CA1 and CA3: (SO) st. oriens, (SP) st. pyramidale, (SR) st. radiatum, CA1: (SL) st. lacunosum-molecular; CA3 (SL) str. lucidum; DG: (mol) molecular, (Grn) granule, and (PC) polymorph cell layer.

The elevated plus maze is a reliable testing paradigm for the detection of anxiety [11,24]. Furthermore, drug-induced increases [7] and decreases [6] in anxiety can be demonstrated, allowing for the screening of anxiolytic agents, including DZP [6,32]. The dose used in the present study (1 mg/kg) is near the EC₅₀ of DZP's anxiolytic effect [24,31]. One day after cessation of chronic cocaine treatment, DZP elicited an anxiolytic response in both the cocaine- and saline-treated rats, evidenced by an increase in both open arm entries and open arm time. DZP had a greater anxiolytic effect in COC treated rats as manifested by a significantly greater increase in open arm entries as compared to control rats. A similar trend was evident when open arm time was compared suggesting that chronic cocaine treatment may indeed enhance the anxiolytic effect of DZP. This finding is consistent with the reported increase in benzodiazepine's sedative actions in human cocaine abusers [44].

Early withdrawal (24 h) from the chronic cocaine did not result in detectable anxiety, i.e., there was no significant difference in open arm exploration between COC and CVH groups given DVH prior to testing (Fig. 1). Although it is generally accepted that withdrawal from chronic cocaine administration elicits anxiety, this anxiety is not consistently detectable with the elevated plus maze [2,50]. The non-significant increase in baseline (COC–DVH) open arm activity of cocaine exposed rats was evident with and without an injection 30 min prior to testing, and at both testing sessions. Nonetheless, behavioral sensitization, to the injection and/or experimenter handling could also have accounted for the increase in plus maze activity.

Pellow et al. [24] reported that rats may be re-tested in the elevated plus maze up to three times, without the interference of learned responses. Eight days after the end of treatment, neither cocaine- nor saline-treated rats exhibited increased open arm exploration when injected with

DZP; i.e. DZP had no anxiolytic effect. As the decrease in open arm exploration was most evident in rats previously exposed to DZP and not DVH, the lack of anxiolytic response to DZP upon re-testing may be a reflection of acquired tolerance. Tolerance to multiple BZ actions, including to their anxiolytic effect has been reported [3,10]. Although normally associated with chronic BZ exposure (e.g. [42]), acute exposure may also result in tolerance [18]. However, it has been reported that un-drugged mice exposed to the plus maze showed a decreased anxiolytic response upon re-testing under the influence of a benzodiazepine [21], indicating that familiarity with the elevated plus maze may affect drug-induced, but not naïve behavior. Thus, the reduced effect of DZP upon re-testing may not be tolerance per se, but contingent upon previous plus maze exposure [21].

One goal of the present study was to characterize the effect of repeated cocaine exposure on the anticonvulsant effectiveness of DZP. Though some contention exists about the usefulness of benzodiazepines in the treatment of cocaine-induced seizures [41,44,47] they are used for convulsions of this type. Although in the present study cocaine was administered to rats at subconvulsive doses, (15 mg/kg, i.p.), it was important to determine whether a single injection could alter PTZ seizure threshold or affect DZP's actions [43]. Consistent with the reportedly brief half-life of cocaine in the rat [36], neither cocaine nor its metabolites had an effect on baseline PTZ seizure threshold, or the ability of DZP to elevate seizure threshold 24 h after acute exposure.

Another important consideration in assessing alterations in DZP's anticonvulsant effect was a potential alteration of baseline PTZ seizure threshold resulting from repeated cocaine exposure. Rats exposed prenatally to cocaine exhibit a decreased PTZ seizure threshold; i.e., enhanced PTZ seizure susceptibility [39], and repeated exposure to

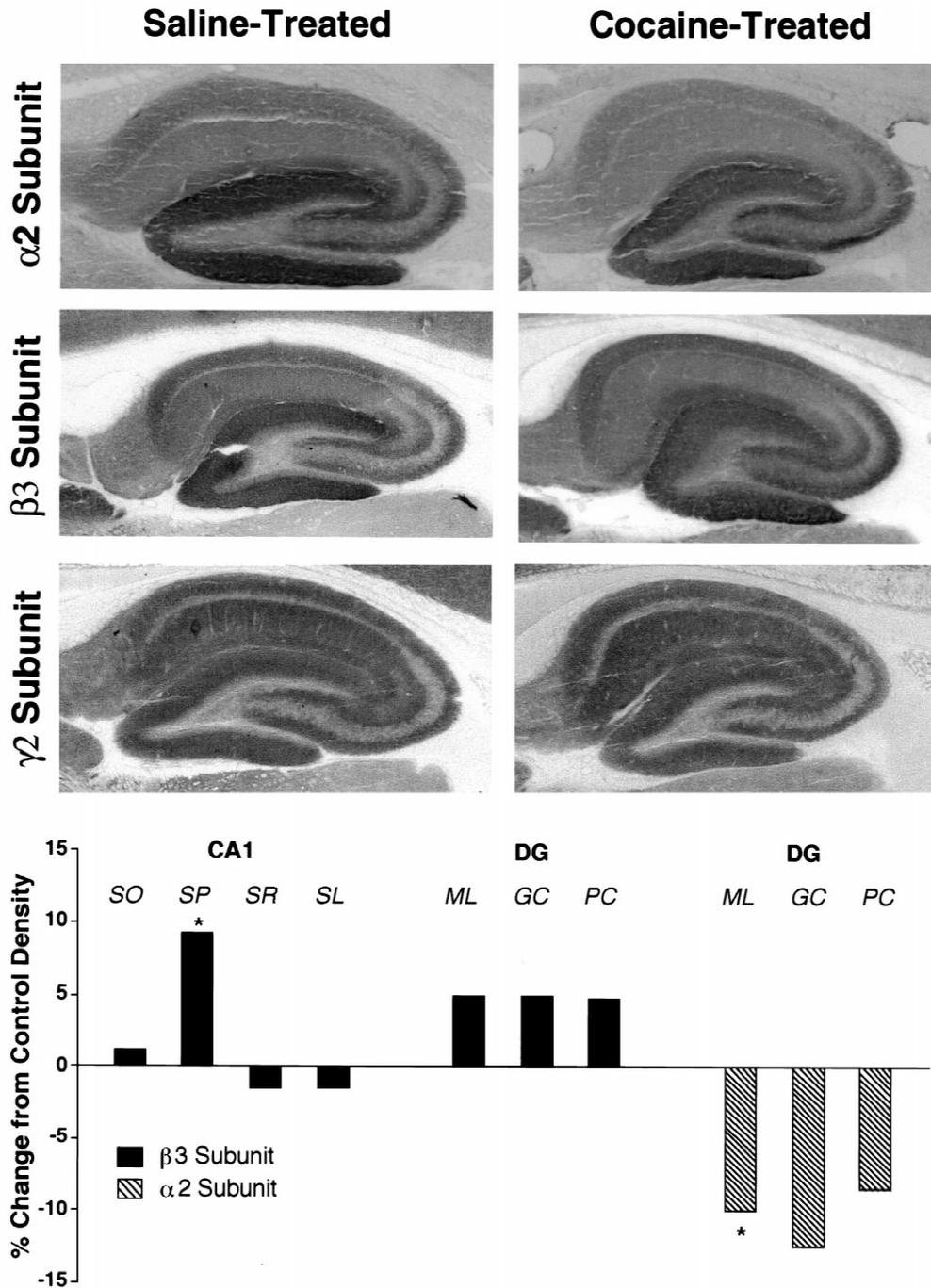


Fig. 5. GABAR subunit protein expression in cocaine (COC) vs. saline (CVH) treated rats. (A) Representative sagittal brain sections of hippocampal GABAR $\alpha 2$, $\beta 3$ and $\gamma 2$ subunit immunostaining. (B) Chronic cocaine treatment resulted in a small, but significant change in $\alpha 2$ and $\beta 3$, but not $\gamma 2$, subunit immunostaining density. Twenty-four hours after treatment cessation $\alpha 2$ subunit staining density was decreased in the molecular layer of the dentate gyrus. GABAR $\beta 3$ subunit immunostaining density was increased in the hippocampal CA1 pyramidal cell layer. There were no significant changes in $\alpha 2$, $\beta 3$ and $\gamma 2$ subunit immunostaining in any other brain areas measured (see text). Asterisks represent significant ($P \leq 0.05$) differences between mean immunostaining densities in control and cocaine-treated rats (mean \pm S.E.M.; $n = 6$ /group).

subconvulsive doses of cocaine can result in pharmacological kindling [28]. Shimosato et al. [35] reported that cocaine-kindled mice cross-sensitize to general anesthetic (lidocaine)-induced seizures. Although using doses much lower than those used to cocaine-kill rats [28], an alteration in cocaine's general anesthetic action could be

manifested as an alteration of PTZ seizure threshold. However, PTZ seizure threshold was unaltered in cocaine-treated rats compared to the saline-injected controls. The lack of effect of chronic cocaine treatment on DZP's anticonvulsant action, despite reported changes in BZ binding and sedative properties [13,44] suggests one of at

Table 2

Effect of 2-week chronic cocaine treatment on $\alpha 2$, $\beta 3$, and $\gamma 2$ subunit antibody immunostaining in various brain regions^a

Group	Acb	OT	Str	AON
$\alpha 2$ subunit				
CVH	28.0±1.0	24.2±2.2	25.9±2.8	18.6±2.5
COC	29.5±2.2	25.8±2.8	24.4±1.8	22.0±2.6
<i>P</i> -value	0.54	0.68	0.70	0.40
$\beta 3$ subunit				
CVH	48.5±2.5	57.1±2.2	45.5±1.7	46.0±3.2
COC	50.3±1.5	62.2±2.5	47.1±0.9	50.3±0.8
<i>P</i> -value	0.57	0.18	0.44	0.24
$\gamma 3$ subunit				
CVH	23.5±2.7	28.0±6.1	24.7±1.7	30.7±4.6
COC	25.8±2.5	26.2±2.6	25.5±2.9	33.9±3.3
<i>P</i> -value	0.56	0.80	0.82	0.59

^a CVH: Cocaine vehicle treatment; COC: Chronic cocaine treatment; *n*=4 rats/group; (Acb) nucleus accumbens; (OT) olfactory tubercle; (Str) striatum, (AON) anterior olfactory nucleus.

least three possibilities: (1) cocaine differentially regulates subpopulations of GABA_A receptors such that those associated with the functional circuit responsible for PTZ seizures remained functionally intact; (2) although DZP's anticonvulsant activity is generally thought to be mediated via a direct interaction with the GABA_A receptor, a different site of action may be responsible for, or capable of maintaining a sufficient anticonvulsant effect; (3) given the length of treatment, the alterations in BZ binding etc. [13] may be accompanied by complimentary changes in excitatory systems such that neuronal homeostasis is maintained. Nevertheless, the functional circuits responsible for PTZ seizure expression retain their DZP sensitivity.

The locus of PTZ's convulsant effect is likely the GABA_A receptor [40,45], and the classical BZ's reliably increase the PTZ seizure threshold [33]. However, PTZ's interaction with other membrane proteins may also play a role in its convulsant effect [9], allowing the possibility that cocaine-induced changes in the interaction of BZ's with the GABA_A receptor could be masked. β -CCM binds at the BZ binding site on the GABA_A receptor, with inverse agonist properties, i.e. having actions opposite to those of typical BZ's [22,37]. β -CCM seizure threshold varies in relation to the number of functional BZ binding sites [49], such that reducing the number of BZ binding sites is associated with and increase in β -CCM seizure threshold. Rats exposed to cocaine did not show an altered β -CCM seizure threshold compared to saline-treated rats. As Goeders [13] reported, chronic cocaine exposure can differentially regulate BZ binding sites as a function of brain region. A modification of BZ receptors outside the neuronal circuits mediating PTZ and β -CCM seizure may have gone undetected. Thus, discrete changes in BZ binding sites may not be detectable with this testing paradigm.

Changes in subunit immunostaining were not detected in a majority of brain regions evaluated: nucleus accumbens, anterior olfactory nucleus, olfactory tubercle, or striatum, despite strong evidence for cocaine-induced alteration of GABAergic systems in these regions [13,14,26,27]. We did find significant up- and down-regulation of hippocampal $\beta 3$ and $\alpha 2$ subunits, respectively. Hippocampal $\beta 3$ subunit protein levels were also reported to be regulated as a function of time after discontinuing chronic BZ treatment [5,42]. Changes in subunit protein expression in the hippocampus associated with chronic cocaine treatment may underlie the reported changes in hippocampal benzodiazepine binding following cocaine exposure and withdrawal [13]. Interestingly, $\alpha 2$ -subunit containing GABARs may in part mediate BZ anxiolytic actions [34]. Further studies will be required to determine whether other GABAR subunit proteins are regulated, and if such alterations may contribute to GABARs with different subunit composition and functional properties. The subunits investigated in the present study are the primary GABAR subunit proteins expressed in these regions of interest [12], however lower levels of $\alpha 5$ and $\beta 2$ are present and thus are also candidates for regulation.

In summary, 2-week chronic cocaine treatment had an apparent effect to increase DZP's anxiolytic, but not its anticonvulsant actions, soon after cessation of cocaine administration. Similarly, chronic cocaine treatment did not change β -CCM seizure threshold. Changes in the hippocampal GABAR subunit proteins, or perhaps limbic areas not evaluated in the present study, may underlie the apparent increase in the anxiolytic actions of DZP. Together, these findings suggest that the clinical effectiveness of BZ's as anxiolytics may be enhanced without compromising their anticonvulsant effects, warranting their continued use in the treatment of the cocaine abuser.

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References

- [1] H. Ashton, Guidelines for the rational use of benzodiazepines, *Drugs* 48 (1994) 2540.
- [2] A.M. Basso, M. Spina, J. Rivier, W. Vale, G.F. Koob, Corticotropin-releasing factor antagonist attenuates the "anxiogenic-like" effect in the defensive burying paradigm but not in the elevated plus-maze following chronic cocaine in rats, *Psychopharmacology* 145 (1999) 21–30.
- [3] C.M. Borghese, N.E. Cordoba, C.H. Laino, O.A. Orsingher, M.C. Rubio, V. Niselman, Lack of tolerance to the anxiolytic effect of diazepam and pentobarbital following chronic administration in

- perinatally undernourished rats, *Brain Res. Bull.* 46 (1998) 237–244.
- [4] S.R. Chesley, A.D. Schatzki, J. DeUrrtia, D.J. Greenblatt, R.I. Shader, L.G. Miller, Cocaine augments peripheral benzodiazepine binding in humans, *J. Clin. Psychiatry* 41 (1990) 404–406.
- [5] S. Chen, X. Huang, X.J. Zeng, W. Sieghart, E.I. Tietz, Benzodiazepine-mediated regulation of $\alpha 1-2$, $\beta 1-3$ and $\gamma 2$ GABA_A receptor subunit proteins in the rat brain hippocampus and cortex, *Neuroscience* 93 (1999) 33–44.
- [6] J.C. Cole, R.J. Rodgers, Ethological comparison of the effects of diazepam and acute/chronic imipramine on the behaviour of mice in the elevated plus-maze, *Pharmacol. Biochem. Behav.* 52 (1995) 473–478.
- [7] B. Costall, M.E. Kelly, R.J. Naylor, E.S. Onaivi, The actions of nicotine and cocaine in a mouse model of anxiety, *Pharmacol. Biochem. Behav.* 33 (1989) 197–203.
- [8] H. deWit, C.E. Johanson, E.G. Uhlenhuth, Reinforcing properties of lorazepam in normal volunteers, *Drug Alcohol Depend.* 13 (1984) 31–41.
- [9] R. Dubberke, L.A. Vasilets, W. Schwarz, Inhibition of the Na⁺, K⁺ pump by the epileptogenic pentylenetetrazole, *Eur. J. Physiol.* 437 (1998) 79–85.
- [10] C. Fernandes, S.E. File, Dizocilpine does not prevent the development of tolerance to the anxiolytic effects of diazepam in rats, *Brain Res.* 815 (1999) 431–434.
- [11] S.E. File, The validation of animal tests of anxiety — Pharmacological implications, *Pol. J. Pharmacol. Pharm.* 36 (1984) 505–512.
- [12] J.M. Fritschy, H. Mohler, GABA_A receptor heterogeneity in the adult rat brain: Differential regional and cellular distribution of seven major subunits, *J. Comp. Neurol.* 359 (1995) 154–194.
- [13] N.E. Goeders, Cocaine differentially affects benzodiazepine receptors in discrete regions of the rat brain: Persistence and potential mechanisms mediating these effects, *J. Pharmacol. Exp. Ther.* 259 (1991) 574–581.
- [14] N.E. Goeders, B.D. Irby, C.C. Shuster, G.F. Guerin, Tolerance and sensitization to the behavioral effects of cocaine in rats: Relationship to benzodiazepine receptors, *Pharm. Biochem. Behav.* 57 (1997) 43–56.
- [15] R.R. Griffiths, E.M. Weerts, Benzodiazepine self-administration in humans and laboratory animals — implications for problems of long-term use and abuse, *Psychopharmacology* 134 (1997) 1–37.
- [16] R.W. Homan, H.C. Rosenberg, Benzodiazepines in the treatment of epilepsy, in: E. Wyllie (Ed.), *Principles and Practice*, Williams and Wilkins, Baltimore, 1997, pp. 865–883.
- [17] X. Huang, S. Chen, E.I. Tietz, Immunocytochemical detection of regional protein changes in rat brain sections using computer-assisted image analysis, *J. Histochem. Cytochem.* 44 (1996) 981–987.
- [18] J.M. Khanna, H. Kalant, A. Chau, G. Shag, Rapid tolerance and cross-tolerance to motor impairment effects of benzodiazepines, barbiturates and ethanol, *Pharmacol. Biochem. Behav.* 59 (1998) 511–519.
- [19] J.W. Lipton, G.D. Ellison, Continuous cocaine induces persisting changes in behavioral responsivity to both scopolamine and diazepam, *Neuropsychopharmacology* 7 (1992) 143–148.
- [20] J.W. Lipton, R.W. Olsen, G.D. Ellison, Length of continuous cocaine exposure determines the persistence of muscarinic and benzodiazepine receptor alterations, *Brain Res.* 676 (1995) 378–385.
- [21] R.G. Lister, The use of a plus-maze to measure anxiety in the mouse, *Psychopharmacology* 92 (1987) 180–185.
- [22] R.L. Macdonald, R.W. Olsen, GABA_A receptor channels, *Ann. Rev. Neurosci.* 17 (1994) 569–602.
- [23] B.S. Meldrum, Identification and preclinical testing of novel antiepileptic compounds, *Epilepsia* 38 (1997) S7–S15.
- [24] S. Pellow, P. Chopin, S.E. File, M. Briley, Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat, *J. Neurosci. Meth.* 14 (1985) 149–167.
- [25] S. Pellow, S.E. File, Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: A novel test of anxiety in the rat, *Pharmacol. Biochem. Behav.* 24 (1986) 525–529.
- [26] J. Peris, Repeated cocaine injections decrease the function of striatal γ -aminobutyric acid_A receptors, *J. Pharmacol. Exp. Ther.* 276 (1996) 1002–1008.
- [27] J. Peris, B. Junh, A. Resnick, P. Walker, O. Malakhova, Y. Bokrand, D. Weilbo, Antisense inhibition of striatal GABA_A receptor proteins decreases GABA-stimulated chloride uptake and increases cocaine sensitivity in rats, *Mol. Brain Res.* 57 (1998) 310–320.
- [28] R.M. Post, S.R. Weiss, A. Pert, Sensitization and kindling effects of chronic cocaine administration, in: Lakoski, Galloway, White (Eds.), *Cocaine: Pharmacology, Physiology, and Clinical Strategies*, CRC Press, USA, 1992, pp. 159–162.
- [29] J.A. Pratt, Psychotropic drug tolerance and dependence: Common underlying mechanisms? in: P. Jenner, J. Pratt (Eds.), *Neuroscience Perspectives: The Biological Bases of Drug Tolerance and Dependence*, Academic Press, London, 1991, pp. 1–25.
- [30] J.R. Richards, R.W. Derlet, D.R. Duncan, Chemical restraint for the agitated patient in the emergency department: Lorazepam versus droperidol, *J. Emergency Med.* 16 (1998) 567–573.
- [31] M. Ramanathan, A.K. Jaiswal, S.K. Bhattacharya, Differential effects of diazepam on anxiety in streptozotocin induced diabetic and non-diabetic rats, *Psychopharmacology* 135 (1998) 361–367.
- [32] J.F. Rosenbaum, Current concepts in psychiatry: The drug treatment of anxiety, *New Eng. J. Med.* 306 (1982) 401–404.
- [33] H.C. Rosenberg, Differential expression of benzodiazepine anticonvulsant cross-tolerance according to time following flurazepam or diazepam treatment, *Pharmacol. Biochem. Behav.* 51 (1995) 63–68.
- [34] U. Rudolph, F. Crestani, D. Benke, I. Brunig, J.A. Benson, J.M. Fritschy, J.R. Martin, H. Bluethmann, H. Mohler, Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes, *Nature* 401 (1999) 796–800.
- [35] K. Shimosato, S. Watanabe, R.J. Marley, T. Saito, One-way cross-sensitization and cross-tolerance to seizure activity from cocaine to lidocaine, *Annals NYAS* 801 (1996) 340–352.
- [36] L. Shuster, Pharmacokinetics, metabolism, and disposition of cocaine, in: J. Lakoski, M. Galloway, F. White (Eds.), *Cocaine: Pharmacology, Physiology, and Clinical Strategies*, CRC Press, USA, 1992, pp. 1–14.
- [37] W. Sieghart, Structure and pharmacology of γ -aminobutyric acid_A receptor subunits, *Pharmacol. Rev.* 47 (1995) 181–234.
- [38] E. Sibille, C. Pavilides, D. Benke, M. Toth, Genetic inactivation of the serotonin_{1A} receptor in mice results in downregulation of major GABA_A receptor α subunits, reduction of GABA_A receptor binding, and benzodiazepine-resistant anxiety, *J. Neurosci.* 20 (2000) 2758–2765.
- [39] A. Snyder-Keller, R.W. Keller Jr., Prenatal cocaine exposure increases susceptibility to drug-induced seizures, *Ann. NY Acad. Sci.* 846 (1998) 419–422.
- [40] R.F. Squires, E. Saderup, J.N. Crawley, P. Skolnick, S.M. Paul, Convulsant potencies of tetrazoles are highly correlated with actions on GABA/benzodiazepine/picrotoxin receptor complexes in brain, *Life Sci.* 35 (1984) 1439–1444.
- [41] J.E. Tarr, M. Macklin, Cocaine, *Ped. Clinics N. Am.* 34 (1987) 319–331.
- [42] E.I. Tietz, X. Zeng, S. Chen, S.M. Lilly, H.R. Rosenberg, P. Kometiani, Antagonist-induced reversal of functional and structural measures of hippocampal benzodiazepine tolerance, *J. Pharmacol. Exp. Ther.* 29 (1999) 932–942.
- [43] I. Ushijima, T. Kobayashi, M. Suetsugi, K. Watanabe, M. Yamada, K. Yamaguchi, Cocaine: Evidence for NMDA-, β -carboline- and dopaminergic-mediated seizures in mice, *Brain Res.* 797 (1998) 347–350.
- [44] N.D. Volkow, G.J. Wang, J.S. Fowler, R. Hitzemann, S.J. Gatley, S.S. Dewey, N. Pappas, Enhanced sensitivity to benzodiazepines in

- active cocaine-abusing subjects: A PET study, *Am. J. Psychiatry* 155 (1998) 200–206.
- [45] L.A. Walsh, M. Li, T.J. Zhao, T.H. Chiu, H.C. Rosenberg, Acute pentylentetrazol injection reduces rat GABA_A receptor mRNA levels and GABA stimulation of benzodiazepine binding with no effect on benzodiazepine binding site density, *J. Pharmacol. Exp. Ther.* 289 (1999) 1626–1633.
- [46] F.J. White, M.E. Wolf, Psychomotor Stimulants, in: P. Jenner, J. Pratt (Eds.), *Neuroscience Perspectives: The Biological Bases of Drug Tolerance and Dependence*, Academic Press, London, 1991, pp. 154–190.
- [47] J.M. Witkin, M. Gasior, B. Heifets, F.C. Tortella, Anticonvulsant efficacy of *N*-Methyl-D-Aspartate antagonists against convulsions induced by cocaine, *J. Pharmacol. Exp. Ther.* 289 (1999) 703–711.
- [48] X. Xie, E.I. Tietz, Reduction in potency of selective γ -aminobutyric acid_A agonists and diazepam in CA1 region of in vitro hippocampal slices from chronic flurazepam-treated rats, *J. Pharmacol. Exp. Ther.* 262 (1992) 204–211.
- [49] T. Zhao, H.C. Rosenberg, T.H. Chiu, Treatment with an antisense oligodeoxynucleotide to the GABA_A receptor γ_2 subunit increases convulsive threshold for β -CCM, a benzodiazepine “inverse agonist”, in rats, *Eur. J. Pharmacol.* 306 (1996) 61–66.
- [50] Z. Sarnyai, E. Biro, J. Gardi, M. Vecsernyes, J. Julesz, G. Telegdy, Brain corticotropin-releasing factor mediates “anxiety-like” behavior induced by cocaine withdrawal in rats, *Brain Res.* 675 (1995) 89–97.